Editorial

Fetal RhD typing with free DNA in maternal plasma
Kenneth J. Moise, Jr, MD
Chapel Hill, NC

Editors’ Choice

Fetal RhD genotyping by maternal serum analysis: A two-year experience
Evelyne Gautier, MD, Alexandra Benachi, MD, Yves Giovangrandi, MD,
Pauline Ernault, Martine Olivi, Thierry Gaillon, Jean-Marc Costa
Neuilly and Paris, France

This report demonstrates that a reliable fetal RhD genotype determination can be achieved with 100% accuracy.

Commentary

This study, along with other recent supporting literature, provides support for major changes in obstetric practice. The authors used maternal serum antenatally to accurately determine fetal Rh status using PCR techniques. Results were obtained in over 99% of cases with 100% accuracy. While the authors used these results to avoid unnecessary administration of Rh immune globulin to mothers with Rh negative babies, this test may also replace amniocentesis in Anti D/Rh sensitized mothers in determining whether the fetus is at risk for hemolytic disease.

Prenatally diagnosed Down syndrome: Mothers who continued their pregnancies evaluate their health care providers
Brian G. Skotko, BS
Boston, Mass

Health care providers can improve the ways in which prenatal diagnoses are conveyed, according to mothers who have children with Down syndrome.

Commentary

The report by Skotko is sure to be of interest to our readers. Mothers of infants with prenatally detected Down syndrome were surveyed about their experience with prenatal diagnosis. There is much to be learned from this study of women who chose to continue the pregnancy. A similar survey of women who chose not to continue pregnancy would also be a welcome addition to the literature.
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**A short cervix in women with preterm labor and intact membranes: A risk factor for microbial invasion of the amniotic cavity**  
Ricardo Gomez, Roberto Romero, Jyh Kae Nien, Tinnakorn Chaiworapongs, Luis Medina, Yeon Mee Kim, Bo Hyun Yoon, Mario Carstens, Jimmy Espinoza, Jay D. Iams, Rogelio Gonzalez  
Puente Alto, Chile, Detroit, Mich, Seoul, Korea, and Columbus, Ohio  
Among women presenting with preterm labor and intact membranes, the shorter the sonographic cervical length, the higher the risk for microbial invasion of the amniotic cavity.

**Commentary**  
Patients with symptoms of preterm labor have been know to have approximately 10% likelihood of bacterial invasion of the amniotic cavity. In this study the authors found that in these patients with cervical lengths evaluated by endovaginal sonography, those with a short cervix has a greater than six fold likelihood of infection/colonization. These findings have profound implications from many standpoints including pathophysiologic understanding, making diagnostic choices with patients with preterm contractions, and ultimately tailoring appropriate therapies for these patients.

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**CLINICAL OPINION**

**Controversies and uncertainties: Abdominal versus vaginal surgery for pelvic organ prolapse**  
Linda Brubaker, MD  
Maywood, Ill  
In selecting the optimal route for prolapse repair, the superior anatomic outcomes of abdominal prolapse repair must be weighed against the increased morbidity of this route.

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**REVIEW ARTICLE**

**A systematic review of the association between factor V Leiden or prothrombin gene variant and intrauterine growth restriction**  
Heather E. A. Howley, MSc, Mark Walker, MD, MSc, Marc A. Rodger, MD, MSc  
Ottawa, Ontario, Canada  
Meta-analysis of case control studies suggests that maternal factor V Leiden and PGV increase the risk of intrauterine growth restriction.

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**SELECTIONS FROM THE 25TH ANNUAL MEETING OF THE SOCIETY FOR MATERNAL-FETAL MEDICINE, FEBRUARY 9-12, RENO, NEVADA**

**Editorial to SMFM Section**  
Jay D. Iams, MD

**Proteomic biomarkers that predict the clinical success of rescue cerclage**  
Carl P. Weiner, MD, Keun-Young Lee, MD, Catalin S. Buhimschi, MD, Rob Christner, PhD, Irina A. Buhimschi, MD  
Baltimore, Md, Seoul, Korea, New Haven, Conn, and Fremont, Calif  
Proteomic analysis reveals biomarkers that predict the failure of rescue cerclage for presumed cervical incompetence.
Commentary
Weiner et al used the emerging technique of proteomics to investigate the etiology of mid-trimester cervical effacement. Their findings suggest that the failure of cerclage to prolong pregnancy in some cases is due to intrauterine inflammation or decidual hemorrhage.

Dexamethasone prevents long-lasting learning impairment following a combination of lipopolysaccharide and hypoxia-ischemia in neonatal rats
Tomoaki Ikeda, MD, Kenichi Mishima, PhD, Naoya Aoo, An Xin Liu, Nobuaki Egashira, Katsunori Iwasaki, PhD, Michihira Fujiwara, PhD, Tsuyomu Ikenoue, MD
Miyazaki and Fukuoka, Japan

Dexamethasone treatment is effective in prevention not only of histologic brain damage, but also of learning and memory impairment against a subsequent combination of endotoxin and a hypoxic-ischemic insult.

No phenotype associated with established lipopolysaccharide model for cerebral palsy
Sarah H. Poggi, MD, Jane Park, Laura Toso, MD, Daniel Abebe, Robin Roberson, Jade E. Woodard, Catherine Y. Spong, MD
Bethesda, Md, and Washington, DC

Using an established lipopolysaccharide rat model for cerebral palsy, immunohistochemical evidence for white matter damage was reproduced, but no motor or cognitive phenotype was demonstrated.

Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia
Catalin S. Buhimschi, MD, Errol R. Norwitz, MD, PhD, Edmund Funai, MD, Susan Richman, MD, Seth Guller, PhD, Charles J. Lockwood, MD, Irina A. Buhimschi, MD
New Haven, Conn

VEGF, sFlt-1, and PlGF are present in the urine of pregnant women, and a ratio of sFlt-1-to-PlGF is superior to proteinuria alone in identifying severe preeclampsia.

Absence of association of inherited thrombophilia with unexplained third-trimester intrauterine fetal death
Ron Gonen, MD, Noa Lavi, MD, Dina Attias, MD, Liliana Schliamsner, MD, Zvi Borochowitz, MD, Elias Toubi, MD, Gonen Ohel, MD
Haifa, Israel

Unexplained third-trimester intrauterine fetal death was not found to be associated with inherited thrombophilia or with placental infarcts.

Prospective randomized clinical trial of inpatient cervical ripening with stepwise oral misoprostol vs vaginal misoprostol
Iris Colón, MD, Kaytha Clawson, MD, Kenneth Hunter, DPA, Maurice L. Druzin, MD, M. Mark Taslimi, MD
Stanford, Calif, and Jacksonville, Ala

Stepwise oral misoprostol appears to be as effective as vaginal misoprostol for cervical ripening without increasing side effects, and is associated with a lower cesarean delivery rate.

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Effects of selective and nonselective PGE$_2$ receptor agonists on cervical tensile strength and collagen organization and microstructure in the pregnant rat at term
Helen Feltovich, MD, Huiling Ji, MD, PhD, Jessie W. Janowski, BA, Nicole C. Delance, BS, Colleen C. Moran, BS, Edward K. Chien, MD
Burlington, VT, and Providence, RI

Prostaglandin induced cervical ripening is mediated by the EP4 type of PGE$_2$ receptor, and is associated with measurable changes in collagen organization and microstructure.

GENERAL OBSTETRICS AND GYNECOLOGY: GYNECOLOGY

Recognition and treatment of irritable bowel syndrome among women with chronic pelvic pain
Rachel E. Williams, PhD, Katherine E. Hartmann, MD, PhD, Robert S. Sandler, MD, MPH, William C. Miller, MD, PhD, MPH, Lucy A. Savitz, MBA, PhD, John F. Steege, MD
Chapel Hill, NC

In a pelvic pain clinic, 40% of women with irritable bowel syndrome were not diagnosed and 67% were not recommended treatment for bowel symptoms.

Laparoscopic radiofrequency thermal ablation: A new approach to symptomatic uterine myomas
Valentino Bergamini, MD, Fabio Ghezzi, MD, Antonella Cromi, MD, Gaia Bellini, MD, Giovanni Zanconato, MD, Stefano Scarperi, MD, Massimo Franchi, MD
Verona and Varese, Italy

Laparoscopic radiofrequency thermal ablation appears to be a safe and effective approach for the conservative treatment of uterine fibroids.

The effect of vaginal candidiasis on the shedding of human immunodeficiency virus in cervicovaginal secretions
Arsenio Spinillo, MD, Francesca Zara, MD, Barbara Gardella, MD, Eleonora Preti, MD, Roberta Mainini, MD, Renato Maserati, MD
Pavia, Italy

Symptomatic vulvovaginal candidiasis is associated with an increased rate of detection of cell-associated and cell-free HIV-1 RNA in cervicovaginal secretions of HIV-infected women.

Reliability of health-related quality-of-life measures 1 year after surgical procedures for pelvic floor disorders
Patricia A. Wren, PhD, MPH, Nancy K. Janz, PhD, Linda Brubaker, MD, Mary Pat Fitzgerald, MD, Anne M. Weber, MD, Frankie B. LaPorte, MS, John T. Wei, MD, for the Pelvic Floor Disorders Network
Ann Arbor, Mich, Maywood, Ill, and Bethesda, Md

The reliability and validity of a comprehensive health-related quality-of-life assessment are established in a sample of women 1 year after surgical procedures for pelvic floor disorders.

Pelvic floor morbidity at 3 years after instrumental delivery and cesarean delivery in the second stage of labor and the impact of a subsequent delivery
Rachna Bahl, MRCOG, Bryony Strachan, MD, Deirdre J. Murphy, MD
Bristol and Dundee, UK

Rates of urinary incontinence continue to be higher at 3 years after instrument vaginal delivery compared with cesarean delivery in the second stage of labor.
Pelvic Organ Support Study (POSST): The distribution, clinical definition, and epidemiologic condition of pelvic organ support defects
Steven Swift, MD, Patrick Woodman, DO, Amy O’Boyle, MD, Margie Kahn, MD, Michael Valley, MD, Deirdre Bland, MD, Wei Wang, MSPH, Joe Schaffer, MD Charleston, SC, Tacoma, Wash, Galveston, Tex, Shakopee, Minn, Winston-Salem, NC, and Dallas, Tex

This study describes the distribution of pelvic organ support in a gynecologic clinic population and proposes a definition of clinically significant pelvic organ prolapse.

Comparison of 2-dimensional and 3-dimensional power-Doppler imaging in complex adnexal masses for the prediction of ovarian cancer
Juan Luis Alcázar, MD, Gerardo Castillo, MD Pamplona, Spain

Three-dimensional is not better than 2-dimensional power Doppler for the prediction of ovarian cancer in complex adnexal masses.

Racial differences in the overexpression of epidermal growth factor type II receptor (HER2/neu): A major prognostic indicator in uterine serous papillary cancer
Alessandro D. Santin, MD, Stefania Bellone, PhD, Eric R. Siegel, MS, Michela Palmieri, MS, Maria Thomas, MD, Martin J. Cannon, PhD, Helen H. Kay, MD, Juan J. Roman, MD, Alexander Burnett, MD, Sergio Pecorelli, MD Little Rock, Ark, and Brescia, Italy

HER2/neu oncogene is a major prognostic indicator and novel therapeutic target in uterine serous papillary cancer.

Expression of cyclooxygenase-2 in advanced stage ovarian serous carcinoma: Correlation with tumor cell proliferation, apoptosis, angiogenesis, and survival
Rouba Ali-Fehmi, MD, Robert T. Morris, MD, Sudeshna Bandypadhyay, MD, Mingxin Che, MD, Veronica Schimp, DO, John M. Malone Jr, MD, Adnan R. Munkarah, MD Detroit, Mich

COX-2 overexpression is associated with increased tumor-cell proliferation, vascularity, and shortened survival in women with advanced serous ovarian carcinoma.

GENERAL OBSTETRICS AND GYNECOLOGY: OBSTETRICS

Catastrophizing labor pain compromises later maternity adjustments
Sari Goldstein Ferber, PhD, Michal Granot, DSc, Etan Z. Zimmer, MD Haifa, Israel

The assessment of labor pain intensity and pain catastrophizing before analgesia administration revealed that pain catastrophizing predicted maternity blues and postpartum social functioning.

D. Yvette LaCoursiere, MD, MPH, Lois Bloebaum, BSN, Jeffrey D. Duncan, MS, Michael W. Varner, MD Salt Lake City, Utah

Maternal overweight and obesity are steadily increasing and are associated with adverse outcomes.
Neonatal death and morbidity in vertex-nonvertex second twins according to mode of delivery and birth weight
Qiuying Yang, MD, PhD, Shi Wu Wen, MB, PhD, Yue Chen, MD, PhD, Daniel Krewski, PhD, MHA, Karen Fung Kee Fung, MD, MHPE, Mark Walker, MD, CM, MSc
Ottawa, Ontario, Canada
The risk of neonatal death and morbidity for vertex-nonvertex second twins was increased in cesarean delivery after vaginal delivery of the first twin.

Maternal age and the likelihood of a maternal request for cesarean delivery: A 5-year population-based study
Herng-Ching Lin, PhD, Sudha Xirasagar, MBBS, PhD
Taipei, Taiwan, and Columbia, SC
This study explores the role of maternal age in the request for an elective cesarean delivery in Taiwan.

Second trimester abortion using isosorbide mononitrate in addition to gemeprost compared with gemeprost alone: A double-blind randomized, placebo-controlled multicenter trial
Wolfgang Eppel, MD, Fabio Facchinetti, MD, Ekkehard Schleussner, MD, Federica Piccinini, MD, Cristina Pizzi, MD, Doris M. Gruber, MD, Barbara Schneider, PhD, Walter Tschugguel, MD
Vienna, Austria, Modena, Italy, and Jena, Germany
Second-trimester abortion using isosorbide mononitrate with gemeprost reduces the number of doses required, but is associated with more headache versus gemeprost alone.

Risk factors for neonatal mortality among extremely-low-birth-weight infants
Stephen J. Bacak, MPH, Kesha Baptiste-Roberts, MPH, Erol Amon, MD, Belinda Ireland, MD, Terry Leet, PhD
St Louis, Mo
Neonatal mortality among extremely low-birth-weight infants is associated with severe anomalies, early gestational age, maternal age, mode of delivery, level of hospital care, and other factors.

Birth simulator: Reliability of transvaginal assessment of fetal head station as defined by the American College of Obstetricians and Gynecologists classification
Olivier Dupuis, MD, Ruimark Silveira, MS, Adrien Zentner, MS, André Dittmar, PhD, Pascal Gaucherand, MD, Michel Cucherat, MD, Tanneguy Redarce, PhD, René-Charles Rudigoz, MD
Lyon, France
This prospective study analyzed the reliability of transvaginal assessment of fetal head station as defined by the American College of Obstetricians and Gynecologists classification by using a newly designed birth simulator.

Factors predicting severe perineal trauma during childbirth: Role of forceps delivery routinely combined with mediolateral episiotomy
Gernot Hudelist, MD, Janos Gellè´n, MD, Christian Singer, MD, Ernst Ruecklinger, PhD, Klaus Czerwenka, MD, Othmar Kandolf, MD, Joerg Keckstein, MD
Vienna, Austria
Although the rate of perineal damage in assisted vaginal deliveries is low in this study, forceps with routinely combined mediolateral episiotomy and/or large infants are risk factors for perineal damage.
The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth
Israel Hendler, MD, Robert L. Goldenberg, MD, Brian M. Mercer, MD, Jay D. Iams, MD, Paul J. Meis, MD, Atef H. Moawad, MD, Cora A. MacPherson, PhD, Steve N. Caritis, MD, Menachem Miodovnik, MD, Kate M. Menard, MD, Gary R. Thurnau, MD, Yoram Sorokin, MD
Bethesda, Md

Maternal obesity is associated with a significantly lower risk of spontaneous preterm birth.

Assessment of cervical antibody concentrations fails to enhance the value of cervical length as a predictor of preterm delivery
Rodney K. Edwards, MD, MS, Ronald J. Ferguson, PhD, Jonathan J. Shuster, PhD, Douglas Theriaque, MS, Susan Gentry, MSN, ARNP, Patrick Duff, MD
Gainesville, Fla

Measuring the concentrations of total immunoglobulin A and G in cervical fluid does not enhance the value of cervical length as a predictor of preterm delivery.

Prenatal cardiovascular manifestations in the twin-to-twin transfusion syndrome recipients and the impact of therapeutic amnioreduction
Catherine Barrea, MD, Fawaz Alkazaleh, MD, Greg Ryan, MB, Brian W. McCrindle, MD, Anita Roberts, BSc, Jean-Luc Bigras, MD, Jon Barrett, MD, Gareth P. Seaward, MB, Jeffrey F. Smallhorn, MB, BS, Lisa K. Hornberger, MD
Toronto, Ontario, Canada

The recipient twin in twin-to-twin transfusion syndrome has biventricular hypertrophy with more diastolic than systolic dysfunction, which persists or progresses despite therapeutic amnioreduction.

Tocolytic effect of a Rho-kinase inhibitor in a mouse model of lipopolysaccharide-induced preterm delivery
Masahiro Tahara, MD, Rikako Kawagishi, MD, Kenjiro Sawada, MD, Kenichiro Morishige, MD, Masahiro Sakata, MD, Keiichi Tasaka, MD, Yuji Murata, MD
Osaka, Japan

A Rho-kinase inhibitor significantly reduced the preterm delivery rate in a mouse model, which suggests that Rho-kinase could be a new therapeutic target for preterm labor.

The cost of twin pregnancy: Maternal and neonatal factors
Barbara Luke, ScD, MPH, RD, Morton B. Brown, PhD, Pierre K. Alexandre, PhD, Toyin Kinoshi, MS, Mary Jo O’Sullivan, MD, Dibe Martin, MD, Ruta B. Misiunas, BA, Clark Nugent, MD, Cosmas Van De Ven, MD, Roger B. Newman, MD, Jill G. Mauldin, MD, Frank R. Witter, MD
Miami, Fla, Ann Arbor, Mich, Charleston, SC, and Baltimore, Md

Factors significantly affecting birth charges for twin pregnancies include cesarean delivery, preeclampsia, slowed growth between 20 to 28 weeks, monochorionicity, and birth before 37 weeks of gestation.

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Mehmet R. Genç, MD, PhD, Emre Karaşahin, MD, Andrew B. Onderdonk, PhD, Ann Marie Bongiovanni, AB, Mary L. Delaney, MS, Steven S. Witkin, PhD, the Microbiology and Prematurity Study Group
Boston, Mass, and New York, NY
Inducible 70-kd heat shock protein is associated with bacterial vaginosis and elevated interleukin-1 receptor antagonist concentrations in mid trimester pregnant women.

Aspirin use during early pregnancy and the risk of congenital abnormalities: A population-based case-control study
Bente Nørgård, MD, PhD, Erzsebet Puhó, MSc, Andrew E. Czeizel, MD, PhD, Mette V. Skriver, MSc, Henrik T. Sorensen, MD, PhD
Aarhus, Denmark, and Budapest, Hungary
Recently, a meta-analysis has suggested an increased risk of certain congenital abnormalities after the use of aspirin in pregnancy, but our data could not confirm that.

Dose response of RU486 in a novel rabbit model of noninfectious preterm birth: Comparative efficacy of 3 routes of administration
David Gorenberg, MD, Kay Beharry, BS, Kenji C. Nishihara, BA, Eileen Chang, BS, Joshua Waltzman, Aamir Akmal, MD, Tamerou Asrat, MD
Long Beach and Orange, Calif
The rabbit model of hormonally mediated preterm birth appears to be a useful model for the investigation of the possible mechanisms of preterm labor.

Pregnancy outcome of women exposed to bupropion during pregnancy: A prospective comparative study
Brian Chun-Fai-Chan, MSc, Gideon Koren, MD, Ibrahim Fayez, MD, Sanjog Kalra, BSc, Sharon Voyer-Lavigne, MSc, Andrew Boshier, MD, Saad Shakir, MD, Adrienne Einarson, RN
Toronto, Ontario, Canada, Farmington, Conn, and Southampton, UK

Reference ranges for serial measurements of umbilical artery Doppler indices in the second half of pregnancy
Ganesh Acharya, MD, Tom Wilsgaard, PhD, Gro K. Rosvold Berntsen, MD, PhD, Jan Martin Maltau, MD, PhD, Torvid Kiserud, MD, PhD
Tromsø and Bergen, Norway
New reference ranges for serial umbilical artery Doppler measurements (pulsatility index, resistance index, and systolic:diastolic ratio) are established on the basis of longitudinal data.

Maternal gestational protein-calorie restriction decreases the number of glomeruli and causes glomerular hypertrophy in adult hypertensive rats
Jorge R. Almeida, MD, Carlos A. Mandarim-de-Lacerda, MD, PhD
Rio de Janeiro, RJ, Brazil
Impairment of glomerulogenesis in rats causes hypertension, a decrease of the number of glomeruli, and glomerular hypertrophy in adult offspring from malnourished mothers.
Antihypertensive effects of flutamide in rats that are exposed to a low-protein diet in utero

Pandu R. R. Gangula, PhD, Luckey Reed, BS, Chandrasekhar Yallampalli, DVM, PhD
Galveston, Tex

Flutamide reduces blood pressure in hypertensive female rats.

Placental vascular disease and toll-like receptor 4 gene expression

Xin Wang, PhD, Neil Athayde, MBBS, Brian Trudinger, MD
Sydney, New South Wales, Australia

In placental vascular disease, there is up-regulation of the TLR4 gene in endothelium of the placental villi, which suggests exposure to gram-negative infection.

Aberrant patterns of cellular communication in diabetes-induced embryopathy in rats: II, Apoptotic pathways

E. Albert Reece, MD, PhD, MBA, Xiang-Dong Ma, MD, PhD, Zhiyong Zhao, PhD, Ying-King Wu, MD, PhD, Danny Dhanasekaran, PhD
Little Rock, Ark, and Philadelphia, Pa

Hyperglycemia-induced embryopathy is mechanistically associated with aberrant signaling pathways and apoptosis.

Impaired K\textsubscript{ATP} channel function in the fetoplacental circulation of patients with type 1 diabetes mellitus

Tanya M. Bisseling, MD, Marieke G. Versteegen, MD, Selina van der Wal, MD, Jenny J. H. Copius Peereboom-Stegeman, PhD, Joop M. P. M. Borggreven, Eric A. P. Steegers, PhD, Jeroen A. W. M. van der Laak, PhD, Frans G. M. Russel, PhD, Paul Smits, PhD
Nijmegen and Rotterdam, The Netherlands

Diabetes affects the fetoplacental vascular K\textsubscript{ATP} channel.

LETTERS TO THE EDITORS

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Sébastien Tassy, MD, Guillaume Gorincour, MD
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Miranda A. Farage, PhD, Angela Stadler, PhD
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READER SERVICES

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Fetal RhD typing with free DNA in maternal plasma

Kenneth J. Moise, Jr, MD*

University of North Carolina School of Medicine, Chapel Hill, NC

The finding of free fetal DNA (ffDNA) in the plasma of pregnant women was first reported by Lo et al in 1997. Considerable research has aided our understanding of this phenomenon. The source of the fetal DNA is probably the result of apoptosis of villous trophoblasts, either in situ or after they have entered the maternal circulation. The breakdown of intact fetal cells in the maternal circulation may also contribute to ffDNA. Total cell-free DNA is higher in maternal serum than plasma; however, similar levels of ffDNA are detected in both. ffDNA is demonstrable as early as 32 days of gestation and increases to comprise 3% of the total DNA pool in maternal plasma in the second trimester and increases to 6% in the late third trimester. After delivery by cesarean section, the mean half-life of ffDNA is 16 minutes, with no detectable levels by 2 hours after delivery in virtually all patients. In the first instance, the ffDNA may provide a false-positive result. In this scenario, the fetus would be found to be RhD-positive when in reality it was RhD-negative. This could lead to unnecessary interventions in the case of an alloimmunized pregnancy. In most circumstances, an RhD-positive ffDNA result indicates that the fetus is RhD-positive because maternal plasma (from the RhD-negative patient) should not contain RhD genetic sequences. The one exception to this situation is when an RhD gene that is present in the mother is not available, pregnancies were followed routinely with serial amniocenteses for ΔOD_{450}. In the mid 1980s, fetal blood sampling gained widespread acceptance for the determination of the RhD status of the fetus with the use of serologic testing. Although the procedure was associated with a 1% risk for fetal loss, pregnancies in which the fetus was determined to be RhD-negative were no longer subject to unnecessary multiple amniocenteses. In 1993, Bennett et al reported the use of amniotic fluid DNA analysis to perform fetal RhD typing, thereby eliminating the need for fetal blood sampling. Five years later, preliminary work on RhD typing of the fetus with ffDNA in maternal plasma was described.

In the case of a heterozygous paternal genotype, pregnant women who are alloimmunized to the RhD red cell antigen are at risk to carry an affected fetus in only 50% of cases. Because, in the past, a method for the determination of the RhD type of the fetus was not available, pregnancies were followed routinely with serial amniocenteses for ΔOD_{450}. In the mid 1980s, fetal blood sampling gained widespread acceptance for the determination of the RhD status of the fetus with the use of serologic testing. Although the procedure was associated with a 1% risk for fetal loss, pregnancies in which the fetus was determined to be RhD-negative were no longer subject to unnecessary multiple amniocenteses. In 1993, Bennett et al reported the use of amniotic fluid DNA analysis to perform fetal RhD typing, thereby eliminating the need for fetal blood sampling. Five years later, preliminary work on RhD typing of the fetus with ffDNA in maternal plasma was described.

In this month’s journal, Gautier et al report a large prospective series of 285 patients who were examined at a prenatal diagnostic center in France and who underwent fetal RhD typing with ffDNA in maternal serum. Confirmation of fetal blood type was available in 95% of cases through DNA typing by amniocentesis and/or neonatal serologic testing. In 102 patients who carried an RhD-negative fetus and 170 patients who carried an RhD-positive fetus, Gautier et al noted a diagnostic accuracy of 100%. The authors identify 2 important issues that must be considered in the use of ffDNA for RhD typing of the fetus.

In the first instance, the ffDNA may provide a false-positive result. In this scenario, the fetus would be found to be RhD-positive when in reality it was RhD-negative. This could lead to unnecessary interventions in the case of an alloimmunized pregnancy. In most circumstances, an RhD-positive ffDNA result indicates that the fetus is RhD-positive because maternal plasma (from the RhD-negative patient) should not contain RhD genetic sequences. The one exception to this situation is when an RhD gene that is present in the mother is not available, pregnancies were followed routinely with serial amniocenteses for ΔOD_{450}. In the mid 1980s, fetal blood sampling gained widespread acceptance for the determination of the RhD status of the fetus with the use of serologic testing. Although the procedure was associated with a 1% risk for fetal loss, pregnancies in which the fetus was determined to be RhD-negative were no longer subject to unnecessary multiple amniocenteses. In 1993, Bennett et al reported the use of amniotic fluid DNA analysis to perform fetal RhD typing, thereby eliminating the need for fetal blood sampling. Five years later, preliminary work on RhD typing of the fetus with ffDNA in maternal plasma was described.

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expressed in her phenotype (ie, the patient is determined to be RhD-negative through serologic testing). This has been reported because of the presence of the RhD pseudogene or the RhD-CE-D gene in up to 50% of black pregnant patients and other RhD gene variations that are not expressed in 12% of Japanese individuals. In their study, Gautier et al used primers that targeted exon 10 of the RhD gene. This technique will not detect the abnormal exon 4 of the RhD pseudogene. It is therefore likely that the presence of the RhD pseudogene was the explanation for the usually high levels of RhD sequences that were present in the maternal serum of 2 patients in their series. Because ethnicity cannot be determined reliably for all patients, fIDNA testing should be undertaken with polymerase chain reaction primers that target multiple exons of the RhD gene.

The second concern with the use of fIDNA for fetal RhD typing is the issue of a false-negative diagnosis. This scenario has more grave implications for fetal management because an RhD-positive fetus may be misdiagnosed as being RhD-negative and appropriate prenatal interventions would be omitted. The most likely explanation for this error is the lack of amplification of the fetal DNA in the overwhelming background of maternal DNA in the plasma sample. Gautier et al added tracer mouse DNA to their assay as an internal control to indicate that the DNA amplification was successful. However, this technique does not confirm that fetal DNA was amplified specifically. The authors state in their discussion that they have not encountered a false-negative result to date. Some laboratories use the presence of the SRY gene (on the Y chromosome) to confirm the presence of fetal DNA in cases of a male fetus. This would seem to represent a more reliable control to prove the amplification of fetal DNA. In the case of a female fetus, maternal DNA polymorphisms in leukocytes from theuffy coat of the maternal blood sample can be used to verify the presence of fetal DNA. The finding of unique polymorphisms (inherited by the fetus from the patient’s partner) that were not seen in the maternal white cells indicates that fetal DNA had indeed been amplified and that an RhD-negative result accurately reflects the fetal RhD type. In as many as 4% of cases, however, this type of analysis may not be informative. FIDNA testing should then be repeated on a new maternal sample. Alternatively, amniocentesis can be undertaken to confirm the fetal RhD type before a decision is made on a course of nonsurveillance for the fetus.

Unfortunately, laboratories in the United States are lagging behind our British and European counterparts. FIDNA for fetal RhD typing is not yet available in the United States but is used clinically on a routine basis in countries such as the United Kingdom. The current report by Gautier et al contributes to a growing body of evidence that fIDNA for fetal RhD typing is now ready for prime time.

What is the future for testing with fIDNA in maternal plasma? Clearly, assays will be developed for other red-cell antigens that are involved in severe hemolytic disease of the fetus/newborn, such as c, E and K. Fetal typing for HPA-1 (PlA) and other platelet antigens that are associated with alloimmune thrombocytopenia purpura is plausible. Routine fetal typing in all RhD-negative, unsensitized pregnant women could eliminate the need for antenatal Rhesus immune globulin in cases of an RhD-negative fetus. Such maternal testing that uses automated technology and real-time polymerase chain reaction has been estimated to cost less than one third that of Rhesus immune globulin. Studies of routine screening of all RhD-negative women in pregnancy with fIDNA are ongoing in the Netherlands and France and have been recommended by the National Institute of Clinical Excellence in the United Kingdom. The report by Gautier et al opens the door to other future applications for noninvasive prenatal diagnosis. LaRabbe et al have reported that microarray technology can be applied to fIDNA in amniotic fluid. These investigators found that increased hybridization was present for most markers on chromosome 21 in cases of fetal trisomy 21. The eventual application of such exciting new technology to screen for fetal trisomies with fIDNA in maternal plasma may relegate such invasive techniques as chorion villus biopsy and amniocentesis to the historic annals of obstetrics.

References


Fetal RhD genotyping by maternal serum analysis: A two-year experience

Evelyne Gautier, MD, Alexandra Benachi, MD, Yves Giovangrandi, MD, Pauline Ernault, Martine Olivi, Thierry Gaillon, Jean-Marc Costa

Centre de Diagnostic Prénatal, American Hospital of Paris, Neuilly, France; Maternité, Hôpital Necker-Enfants Malades, Paris, France; and Maternité, Hôpital Notre-Dame de Bon Secours, Paris, France

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Objective: The purpose of this study was to determine the accuracy of the none-invasive prenatal determination of polymerase chain reaction (PCR)-based fetal RhD genotyping.

Study design: A prospective case series was undertaken on all RhD-negative pregnant women presenting for genetic counseling in our prenatal diagnosis center from January 2001 until December 2002. Results were compared with serologic RhD typing of the newborns.

Results: Among the 285 pregnant women who participated in the study, fetal RhD status could be determined for 283 patients. In 2 cases, the RhD-negative phenotype of the mother was not the result of a complete RHD gene deletion, and therefore, the status of the fetus could not be determined. Neither false-negative nor false-positive results were observed.

Conclusion: The present report demonstrates that a reliable fetal RHD genotype determination can be achieved with 100% accuracy. It is therefore possible to consider that such an assay could be systematically proposed to all RhD-negative pregnant women in order to more effectively utilize RhD prophylaxis.

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now modified the strategy of prenatal diagnosis in X-linked disorders, as well as the management of pregnancies at risk for congenital adrenal hyperplasia. The use of cell-free DNA in maternal plasma or serum has also been achieved for noninvasive fetal RhD genotyping by several groups, but most often on a limited number of cases.

More recently, 2 large studies have been conducted on 137 and 893 patients, respectively (most of them being alloimmunized), before implementation of routine clinical practice. In the present publication, we report the results of our 2-year experience in the noninvasive prenatal determination of fetal RhD genotyping routinely proposed in a prenatal diagnosis center.

### Material and methods

#### Patients and samples

Noninvasive prenatal determination by maternal serum analysis was systematically proposed to every RhD-negative pregnant woman presenting for genetic counseling in our prenatal diagnosis center from January 2001 to December 2002. Two hundred and eighty-five women elected to participate; all were of Caucasian origin but none were alloimmunized. After informed consent, 5 mL of blood were collected into Vacutainer SST tubes (Becton Dickinson, Meylan, France) before amniocentesis or CVS if indicated. Anti-D immunoglobulin was not provided to patients whose fetus tested to be RhD-negative. Immediately after clotting, serum was obtained by centrifugation for 10 minutes at 3000g, aliquoted and stored at −80°C until further processing if the assay was not performed on the same day. The mean gestational age was 15.2 weeks, ranging from 8 to 35.

### Real-time PCR for the RHD gene in maternal serum

The procedure was similar to that previously described. Briefly, as tracer for the DNA extraction and amplification steps, a low amount (250 pg) of mouse DNA (Sigma, Grenoble, France) was added to each patient’s sample (400 μL of serum) immediately before DNA extraction. Total DNA was then extracted by the PCR Template Preparation Kit (Roche Biochemicals, Meylan, France), and the adsorbed DNA eluted with 50 μL of elution buffer, 10 μL of which was used per PCR reaction. Amplification was carried out in a LightCycler® instrument (Roche Biochemicals). PCR reactions were set up in a final volume of 20 μL using the Fast DNA Master Hybridization Probes Kit (Roche Biochemicals), with 0.5 μmol/L of each primer, 0.25 μmol/L of each probe (Proligo, France) (Table), 1.25 units of uracil DNA glycosylase (UDG) (Biolabs, Saint-Quentin en Yvelines, France), 4.75 mmol/L of magnesium chloride. After an initial 1-minute incubation at 50°C, a first denaturation step of 8 minutes at 95°C was followed by an amplification performed for 50 cycles of denaturation (95°C, 10 seconds, ramping rate 20°C/second), annealing (56°C, 10 seconds, ramping rate 20°C/second), and extension (72°C, 20 seconds, ramping rate 2°C/second).

Each sample was treated twice for DNA extraction, and the RHD assay was performed in duplicate on each DNA extract. Definitive results were considered only when the 4 PCR reactions were concordant. During each run, sera obtained from patients carrying an RhD-positive or an RhD-negative fetus were used as a positive and a negative control.

The results were compared with those obtained later in pregnancy on amniotic fluid cells and by RHD serology of the newborn.

### Results

Among the 285 pregnant women tested for RHD gene presence in their serum, the status of the fetus could not be determined in 2 cases because the RhD-negative phenotype of the mother was not in relation with a complete RHD gene deletion. These 2 particular cases were easily identified using real-time PCR because the amount of RHD sequences detected in maternal serum was abnormally high (expressed by an unusual crossing point) to be from fetal origin (Figure). Because the maternal DNA represents the major part of DNA isolated from serum, it can be concluded that RHD sequences were present in the maternal genome. This hypothesis was confirmed by analysis of DNA extracted from the maternal leukocytes.

Fetal RhD genotype could be determined from all other 283 maternal sera. In 179 cases, RHD sequences had been detected in maternal serum, and the fetuses...
could therefore be considered as RhD-positive, while the others (n = 104) were RhD negative.

For 11 patients, the result obtained by maternal serum analysis could not be confirmed due to an early pregnancy termination in relation to fetal chromosomal abnormality (3 cases), or because the patients were lost to follow-up (8 cases). The results of the fetal RHD genotyping on maternal serum could be controlled for the other 272 patients either by analysis of amniotic fluids for the presence of RHD gene (n = 209) and/or by serologic study of the newborn (n = 232). Results were in complete concordance, and neither false-negative nor false-positive results were observed; all sera from women carrying an RhD-positive fetus (n = 170) gave positive results for RHD gene detection, while all sera from women carrying an RhD-negative fetus (n = 102) gave negative results. Specificity and sensitivity of the assay were 100% (95% CI: 98-100).

**Comment**

Fetal RHD genotype can be determined with a high level of accuracy by analysis of fetal DNA circulating in maternal plasma and serum. It is therefore possible to consider that such an assay could be included in prenatal care of RhD-negative women.16

The results of a 2-year experience of such a clinical practice are reported herein. In 2 cases, the pregnant women did not carry a complete deletion of the RHD gene and a final answer regarding the fetal RhD status could not be provided. Although a thorough analysis was not undertaken, theses patients were highly suspected of carrying a RHD pseudogene or variant. Except for these 2 pregnant women, the fetal RhD status could be addressed for the other 283 patients who had accepted the test (overall success rate 99.3%). The result was in complete concordance with that obtained either by analysis of amniotic fluid for the presence of RHD gene (n = 209) and/or by serologic study of the newborn (n = 232). Therefore, the present report demonstrates that a reliable fetal RhD genotype determination can be achieved with 100% accuracy. Although the presence of fetal DNA cannot be formally proven in maternal serum in case of RhD-negative fetus, a false-negative result was never observed in our experience. This was the result of the use of a similar procedure successfully used for many years for fetal sex determination7 that includes a careful ultrasonographic control of the date of the pregnancy, the assay never being realized before 8 weeks. On the other hand, a false positive due to the transmission by the father of a silent RHD gene (women carrying such genes being easily detected by our assay) could not be excluded in the future. The patients were systematically informed about this without any medical risk possibility during genetic counseling.

Among the studied women, 104 (36.8%) carried an RhD-negative fetus. Injection of anti-D immune globin was thus avoided, and pregnancy follow-up was therefore made easier, decreasing the patient’s anxiety.

Determination of fetal RHD genotype using a non-invasive approach is an important challenge because it has crucial implication in the clinical management of sensitized RhD-negative pregnant women. Fetal DNA analysis in maternal serum offers the opportunity to achieve this challenge.

Attempts to identify the RhD-negative women who are at risk of sensitization contribute to a more efficient prophylaxis of alloimmunization in obstetrics being targeted specifically at these women. Our data could be considered as part of a novel strategy in order to maintain and increase anti-D supplies, as well as (1) the use of minidose of anti-D in some circumstances (ie, first trimester indications), (2) increased and accurate use of tests to assess feto-maternal hemorrhage, and (3) increase compliance with guidelines on the use of anti-D.

Moreover, while efforts are made to ensure that the blood used for the anti-D preparation is safe and free of human pathogens, it cannot be formally excluded that an infectious agent is present because of lack of availability or sensitivity of tests at present.17

The high accuracy rate achieved in the prediction of fetal D status has resulted in the implementation of a noninvasive fetal RHD genotyping service. Although this test is actually performed by a limited number of specialized laboratories in the world, increasing demand is anticipated in the near future. As a consequence, the actual “made-by-hand” assays must move towards more automated ones (until the availability of commercial tests) in order to ensure robustness and reliability. This major point was recently highlighted by an interlaboratory study.18 Therefore, time has come for an external quality control scheme.

Finally, large-scale studies regarding the economic aspect of such routine offer of fetal RhD genotyping are necessary. A consideration of cost-effectiveness is particularly important because of the limited supply of anti-D.

### Table

<table>
<thead>
<tr>
<th>Characteristic and sequences of primers and probes used in the PCR assay</th>
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<tbody>
<tr>
<td><strong>Gene target</strong></td>
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</tr>
<tr>
<td>Human RHD exon 10</td>
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</tbody>
</table>

- **Table**
  - Characteristics and sequences of primers and probes used in the PCR assay
  - **Gene target**
  - **Primers sequences**
  - **Probes sequences**

<table>
<thead>
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<th><strong>Gene target</strong></th>
<th><strong>Primers sequences</strong></th>
<th><strong>Probes sequences</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human RHD exon 10</td>
<td>5’-GCCTGCAATTGTACGTGAGA-3’</td>
<td>3'FITC-TGACAGCAAAGTCCTCAAATGTCG</td>
</tr>
<tr>
<td></td>
<td>5’-CAAGAGTGCCAGAAAGGA-3’</td>
<td>5'LCRed640-GCAGGCACTGGAGTCAGAAA-3’Ph</td>
</tr>
</tbody>
</table>
available. These economic evaluations must be country-dependent because guidelines for anti-D use, if existing, may vary from one country to the other and from centers where no recommendations exist. Such a study is actually in progress in France.

Attempts to predict other fetal blood groups (ie, Kell, Duffy) by maternal blood analysis is currently under investigation by some groups. However, the accurate, sensitive, and reliable determination of such “single base” modifications are difficult to achieve using the actual methods caused by the presence of a high background of negative maternal DNA.

Acknowledgments

We are indebted to Dr Jocelyn McGinnis for reviewing this manuscript.

References

Objective: This study was undertaken to ask mothers who had children with Down syndrome after receiving a prenatal diagnosis: How was the process and what, if anything, could be improved?

Study design: An 11-page survey was mailed to 2945 persons on the membership lists of 5 Down syndrome parent organizations. The survey gathered both quantitative and qualitative data from yes/no questions, open-ended questions, and a series of statements asking the mothers to rate their level of agreement on a 1-to-7 Likert scale. Qualitative data were analyzed using the Constant Comparative Method of Qualitative Analysis, and quantitative data were summarized using linear regressions, mixed stepwise multiple regressions, and grouped means, 1-way analysis of variance analyses.

Results: Of 1126 surveys received, 141 (12.5%) were from mothers who had received a prenatal diagnosis. Though satisfied with the care that they had received, the majority of respondents expressed frustration with the process. The most common suggestions were that the diagnosis be conveyed in person, that up-to-date printed materials on Down syndrome (DS) be provided, and that mothers be referred to local DS support groups.

Conclusion: Receiving a prenatal diagnosis of DS need not be a negative experience. By implementing suggestions proposed herein by the mothers, health care providers can even make the situation a positive one.

The risk of Down syndrome (DS) can now be assessed and a diagnosis confirmed in fetuses in the first trimester of pregnancy.1 Delivering and receiving a prenatal diagnosis of DS, however, is not an easy experience for either the physician or the mother. Obstetricians often have little direct contact during their training with children who have developmental disabilities.2 Physicians often distance themselves from their own personal beliefs in a commitment to provide balanced information for the new mother. A survey of 499 primary care physicians revealed that 63% reported that they “tried to be as unbiased as possible when delivering a prenatal diagnosis.”3 Thirteen percent reported that they “emphasize” the

**KEY WORDS**
Down syndrome  
Prenatal diagnosis  
Amniocentesis

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negative aspects of DS so that parents would favor a termination, 10% actively “urge” parents to terminate, and 10% indicated that they “emphasize” the positive aspects of DS so that parents favor continuation, and 4% actively “urge” parents to continue the pregnancy.

A study of 10 women who chose to continue the pregnancy after a prenatal diagnosis of DS reported that they “were not supported in arriving at their own fully informed decision because the providers were overtly or covertly advocating from their own point of view.” The study concluded that “negative terminology or accentuation of difficulties was found to be quite unhelpful and resulted in long-term resentment.” The purpose of this current study is to reexamine this issue in a larger cohort with a more robust survey instrument. We asked mothers who had children with prenatally detected DS: How did your health care provider convey the information and what, if anything, could have been better?

Material and methods

Study members

This study was nested in larger cross-cultural epidemiologic research on prenatal and postnatal support for mothers who have children with DS in Spain and the United States. Surveys were distributed exclusively to mothers of children with DS—other family members were not polled—to standardize the perspectives of our respondents and capture the specific sentiments of the mother. A national database of families who have children with DS does not exist. A survey of parents of children with DS was thus performed through organized parent support groups. Surveys were distributed to mothers through 5 DS parent support groups, chosen on the basis of the size and geographic distribution of their membership. Survey packets were sent to members of the Mile High Down Syndrome Association (Colorado), Triangle Down Syndrome Network (North Carolina), Massachusetts Down Syndrome Congress, Down Syndrome Association of Los Angeles (California), and the Down Syndrome Society of Rhode Island. Approximately 8 weeks after the first mailing, research packets were again sent to the support groups and reforwarded to all nonresponders.

Questionnaires

All materials were approved by the Committee on Human Studies at Harvard Medical School, and the confidentiality of participants was strictly maintained. The survey used for this current study is available as an Appendix, 11 pages in length, on the online Journal. The survey was developed from published studies and anecdotal data in the popular literature. Before distribution, the survey was reviewed by a panel of experts in the disability field and was first distributed to 6125 mothers in Spain to validate the questionnaire and sharpen the wording.

The questionnaire gathered both quantitative and qualitative data from yes/no questions, open-ended questions, and a series of statements asking mothers to rate their level of agreement on a 1-to-7 Likert scale with 7 being “strongly agree,” 4 being “neutral,” and 1 being “strongly disagree.” Also gathered was information on the sex and age of the child with DS. As optional measures, mothers were asked to provide their own background characteristics, including ethnicity, religious affiliation, educational level, household income, and number of pregnancies.

Data analyses

As the survey collected both quantitative and qualitative data, a mixed methodology was used to analyze the data. The quantitative data were analyzed with SAS software (SAS Institute, Cary, NC), and the qualitative data were coded and abstracted with the use of the Constant Comparative Method of Qualitative Analysis.

Means and SD were calculated for each survey item on the Likert Scale. A 1-way analysis of variance (ANOVA) was used to assess for potential differences among mothers: (1) those who had received a triple screen and amniocentesis; (2) those who had received an ultrasound and amniocentesis; (3) those who had received a triple screen, ultrasound, and amniocentesis; and (4) those who had only received an amniocentesis. Responses over time were addressed by a linear regression generated for each Likert statement, using the child’s age as the independent variable. For instances where the mother did not complete 1 or both of these measures, the mother’s calculated age at the time her child was born was subtracted from her current age. The standardized βs and R² values from the regressions are reported. To determine the significance of the predicted models, an ANOVA analysis was generated. Reported here are the df, F, and P values for those Likert statements achieving significance at the .05 level.

Maternal reactions to the prenatal diagnosis according to the physician or other health care provider’s behaviors, the printed materials, or any of the mothers’ background characteristics were assessed by mixed stepwise multiple regressions generated for each of the maternal reactions (frightened, anxious, suicidal, optimistic). The independent variables included all of the other Likert scale responses on provider behavior and printed materials. Background characteristics entered into the regression included income, educational level, mother’s age at birth, her child’s age, and parity. Variables were entered at the probability of .05, and the standardized βs and R² values from the regressions are reported here. ANOVAs were also run, and the df, F, and P values for those Likert...
statements that achieved significant at the .05 variables are reported.

Five variables were categorical: maternal ethnicity, religious affiliation, state of residence during pregnancy, whether she received the results of amniocentesis in person, and whether she had received those results with her partner present. For these variables, a grouped means, 1-way ANOVA analysis was performed.

Results

A total of 2945 survey requests were sent, and 1250 responses (42.4%) were received, including 289 from Massachusetts (state response rate: 36.1%), 176 from Colorado (29.3%), 72 from Rhode Island (29.4%), 86 from North Carolina (43.0%), 352 from California (32.0%), and 166 from other states. Of these surveys, 43 were completed by fathers and were excluded. An additional 81 declined to complete the questionnaire; most of these responses were returned from people or groups on the mailing list, but not mothers of an infant with DS, eg, teachers, professional groups, and support organizations. Of the remaining 1126 surveys, 141 (12.5%) were submitted by mothers who had received a prenatal diagnosis of DS from an amniocentesis result (38 from Massachusetts [state response rate: 13.1%], 30 from Colorado [17.0%], 9 from North Carolina [10.5%], 40 from California [11.4%], and 11 from other).

Of the mothers who had an amniocentesis, 85 (60.3%) first had a multiple serum marker test at a mean gestational age of 16.3 weeks (SD = 3.4, n = 72). The majority of the respondents were scared and anxious after receiving the results of the triple screen (Table II) and indicated that their obstetricians had neither explained DS before nor after the test. About half of the mothers already knew something about DS before the triple screen, but nearly all of them thought their obstetricians had failed to provide enough up-to-date printed material on DS. These variables remained consistent over time.

The respondents had amniocentesis performed at a mean gestational age of 19.4 weeks (SD = 5.5, n = 138), 31 (22%) because of questionable ultrasonographic findings, 51 (36%) because of multiple marker test results, 34 (24%) because of ultrasonographic and multiple marker test findings, and 25 (18%) because of advanced maternal age only. One-way ANOVA analyses did not show any statistical differences among the responses for these 4 groups. In regard to the amniocentesis, 26.8% of them had received the results in person, and 71.0% had learned of the diagnosis without their partners present. The majority reported feeling anxious and scared. About half felt rushed or pressured into making a decision about continuing the pregnancy (Table II). Mixed multiple stepwise regressions revealed that the level of a mother's fear could be predicted by her feeling pressured: Scared = 5.75 + 0.13 Pressured.
Table II  Mothers’ reflections on their prenatal support

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triple screen</strong></td>
<td></td>
</tr>
<tr>
<td>Before the triple screen procedure, I already had a good idea about what DS was.</td>
<td>4.0 2.3 85</td>
</tr>
<tr>
<td>Before receiving test results from my triple screen, my physician explained to me what DS was.</td>
<td>2.3 1.7 85</td>
</tr>
<tr>
<td>After receiving test results, my physician explained to me what DS was.</td>
<td>3.7 2.3 83</td>
</tr>
<tr>
<td>After receiving the results, I felt encouraged by my physician to terminate my pregnancy.</td>
<td>3.1 2.3 82</td>
</tr>
<tr>
<td>After receiving the results, I felt encouraged by my physician to continue my pregnancy.</td>
<td>3.5 1.9 82</td>
</tr>
<tr>
<td>After receiving test results, I felt scared.</td>
<td>6.0 1.6 81</td>
</tr>
<tr>
<td>After receiving test results, I felt anxious.</td>
<td>5.8 1.8 80</td>
</tr>
<tr>
<td>After receiving test results, I experienced suicidal thoughts.</td>
<td>1.5 1.2 81</td>
</tr>
<tr>
<td>After receiving test results, I felt positive.</td>
<td>3.1 1.9 81</td>
</tr>
<tr>
<td>After receiving test results, I felt my physician gave me enough up-to-date printed material on DS.</td>
<td>2.4 2.0 79</td>
</tr>
<tr>
<td><strong>Amniocentesis (mothers’ reflections)</strong></td>
<td></td>
</tr>
<tr>
<td>After receiving the results, I felt positive.</td>
<td>3.0 1.8 141</td>
</tr>
<tr>
<td>After receiving the results, I experienced suicidal thoughts.</td>
<td>1.5 1.4 140</td>
</tr>
<tr>
<td>After receiving the results, I felt anxious.</td>
<td>6.1 1.5 140</td>
</tr>
<tr>
<td>After receiving the results, I felt scared.</td>
<td>6.3 1.3 141</td>
</tr>
<tr>
<td>After receiving the results, I felt rushed or pressured into making a decision about the continuation of my pregnancy.</td>
<td>4.0 2.6 137</td>
</tr>
<tr>
<td>I am glad that my physician gave his/her opinion about what he/she would do in my situation.</td>
<td>2.9 1.7 111</td>
</tr>
<tr>
<td>Before the amniocentesis, I already had a good idea about what DS was.</td>
<td>4.2 2.2 141</td>
</tr>
<tr>
<td>I wanted to have an amniocentesis done.</td>
<td>5.2 2.0 141</td>
</tr>
<tr>
<td><strong>Amniocentesis (physician behaviors)</strong></td>
<td></td>
</tr>
<tr>
<td>I felt encouraged by my physician to have an amniocentesis.</td>
<td>6.0 1.3 139</td>
</tr>
<tr>
<td>I felt pressured by my physician to have an amniocentesis.</td>
<td>3.6 2.1 140</td>
</tr>
<tr>
<td>My physician explained the results to me in a manner that I could understand.</td>
<td>5.7 1.6 140</td>
</tr>
<tr>
<td>After receiving test results, my physician encouraged me to terminate my pregnancy.</td>
<td>3.0 2.2 139</td>
</tr>
<tr>
<td>After receiving test results, my physician encouraged me to continue my pregnancy.</td>
<td>3.6 2.0 140</td>
</tr>
<tr>
<td>After receiving the test results, my physician told me about the positive aspects of DS.</td>
<td>3.3 2.1 141</td>
</tr>
<tr>
<td>After receiving the test results, my physician emphasized the positive aspects of DS.</td>
<td>3.2 1.9 138</td>
</tr>
<tr>
<td>After receiving the test results, my physician told me about the negative aspects of DS.</td>
<td>3.8 2.0 140</td>
</tr>
<tr>
<td>After receiving the test results, my physician emphasized the negative aspects of DS.</td>
<td>3.3 2.1 137</td>
</tr>
<tr>
<td>After receiving test results, my physician gave me his/her opinion about what he/she would do in my situation.</td>
<td>2.6 2.0 136</td>
</tr>
<tr>
<td>My physician pitied me.</td>
<td>3.0 2.1 137</td>
</tr>
<tr>
<td>After receiving test results, my physician provided me with enough phone numbers of parents who have a child with DS.</td>
<td>2.4 2.0 139</td>
</tr>
<tr>
<td>After receiving test results, my physician gave me enough up-to-date printed material on DS.</td>
<td>2.7 2.1 139</td>
</tr>
<tr>
<td><strong>Printed materials</strong></td>
<td></td>
</tr>
<tr>
<td>The printed materials that I received provided an equal mix about the positive and negative aspects of DS.</td>
<td>4.4 2.0 98</td>
</tr>
<tr>
<td>The printed materials that I received emphasized the negative aspects of DS.</td>
<td>3.2 1.9 97</td>
</tr>
<tr>
<td>The printed materials that I received emphasized the positive aspects of DS.</td>
<td>4.6 1.9 98</td>
</tr>
<tr>
<td>The printed materials were helpful in understanding DS.</td>
<td>5.5 1.7 97</td>
</tr>
<tr>
<td>The printed materials encouraged me to continue my pregnancy.</td>
<td>4.1 2.0 94</td>
</tr>
<tr>
<td>The printed materials encouraged me to terminate my pregnancy.</td>
<td>2.5 1.7 92</td>
</tr>
<tr>
<td>I liked the printed materials that I received.</td>
<td>4.7 1.9 95</td>
</tr>
<tr>
<td>The printed materials were easy to read and understand.</td>
<td>5.5 1.6 95</td>
</tr>
<tr>
<td><strong>Prenatal testing overall</strong></td>
<td></td>
</tr>
<tr>
<td>My physician was supportive of my decision to continue my pregnancy.</td>
<td>5.0 2.0 140</td>
</tr>
<tr>
<td>My physician tried to change my decision about continuing my pregnancy.</td>
<td>2.5 2.0 138</td>
</tr>
<tr>
<td>The prenatal medical support that I received following my decision to continue my pregnancy was exceptionally good.</td>
<td>5.3 2.0 139</td>
</tr>
<tr>
<td>After I decided to continue my pregnancy, it was a struggle to find adequate prenatal care.</td>
<td>1.7 1.5 139</td>
</tr>
<tr>
<td>After I decided to continue my pregnancy, my physician began giving me parenting tips on how best to raise a child with DS.</td>
<td>2.2 1.7 137</td>
</tr>
</tbody>
</table>

Mothers were asked to rate their level of agreement with the statements on a 1-to-7 Likert scale with 1 being “strongly disagree,” 4 being “neutral,” and 7 being “strongly agree.”
Respondents were asked to use the 1-to-7 Likert scale to assess 19 factors that might have played a role in their decision to continue their pregnancy (such as, religion, partner’s opinion, meeting a person with DS, reading about someone with DS). Six of these items averaged over the neutral mark of “4” (Table III). A mother’s conscience was the primary influence for continuation, with a mother’s religion and her partner’s opinion ranking second and third, respectively.

The mothers who received printed materials from their obstetrician reported that the literature was easy to read and helpful in understanding DS (Table II). A majority of the respondents thought the materials emphasized the positive aspects of DS, and about half thought the materials had encouraged them to continue their pregnancy. Overall, most mothers “liked the printed materials” that they had received. These variables remained consistent over time. Mothers agreed that their obstetricians had been supportive of their decision to continue their pregnancy (Table II). This, however, was not always the case: Supportive physician = 5.60 – 0.11 Child’s age \( (R^2 = 0.04, F[0.05; 1, 128] = 6.90, P < .01) \). From this model, mothers receiving a prenatal diagnosis of DS in 2003 would be predicted to report a satisfaction level of 5.6, whereas those in 1983 would be expected to have a dissatisfaction level of 3.4. Very few thought that their physician had tried to change their decision about continuing the pregnancy, but this, too, has evolved: Change decision = 1.93 + 0.11 Child’s age \( (R^2 = 0.05, F[0.05; 1, 126] = 8.11, P < .01) \). This means that the mothers receiving a prenatal diagnosis in 1983 would be predicted to indicate an agreement level of 4.13, suggesting that their obstetricians did try to influence decisions, at least partially. Few reported that it was difficult to find adequate prenatal support, and most agreed that their prenatal support was good. Most respondents reported that their birthing experience was positive (mean = 5.2, SD = 1.9, n = 137). In contrast, by previous report, mothers who learned about the diagnosis of DS after their child was born labeled their experience as negative (mean = 3.4, SD = 3.1, n = 929).6

Respondents recommended that physicians do the following to improve the process:

1. Results of the triple screen should be clearly explained as a risk assessment, not a “positive” or “negative” result. Many mothers understood the triple screen to be an all-or-nothing diagnostic test, even after their obstetrician had given them the results. The weak sensitivity and positive predictive value of the test should be explained in terms that each mother can understand. In addition, mothers requested that DS be first explained after the screening test rather than waiting for the results of an amniocentesis or chorionic villus sampling (CVS) to begin a discussion.

<table>
<thead>
<tr>
<th>Table III</th>
<th>Top factors that influenced mothers to continue their pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD) N</td>
</tr>
<tr>
<td>My “inner voice”</td>
<td>6.2 (1.6) 134</td>
</tr>
<tr>
<td>My religion</td>
<td>5.8 (1.9) 135</td>
</tr>
<tr>
<td>My husband’s/partner’s opinion</td>
<td>5.6 (2.1) 135</td>
</tr>
<tr>
<td>Material that I found on my own</td>
<td>4.3 (2.3) 134</td>
</tr>
<tr>
<td>Talking to another parent who had a child with DS</td>
<td>4.3 (2.4) 135</td>
</tr>
<tr>
<td>Positive images and stories about persons with DS in printed materials</td>
<td>4.1 (2.2) 136</td>
</tr>
</tbody>
</table>

Mothers were asked to rate their level of agreement with the statements on a 1-to-7 Likert scale with 1 being “strongly disagree,” 4 being “neutral,” and 7 being “strongly agree.”

\( (R^2 = 0.06, F[0.05; 1, 135] = 10.0, P < .01) \). With all other variables held constant, mothers who felt “strongly pressured or rushed into making a decision” would be expected to rate their fear level at 6.66 on a 1-to-7 Likert scale.

For the few mothers who felt positive about the experience, satisfaction was associated with providers who explained the results in an understandable manner that included discussion of the positive aspects of DS and with a maternal educational level: Positive = 2.51 + 0.19 Understand DS + 0.29 Positive aspects – 0.36 Educational degree \( (R^2 = 0.15, F[0.05; 3, 134] = 8.80, P < .001) \). When all other variables are held constant, a college-educated mother who strongly agreed that her obstetrician explained DS and talked about the positive aspects would be expected to have a satisfaction level of 4.43, slightly better than neutral.

About half of the mothers had a “good idea” about DS before the amniocentesis, and nearly all mothers strongly agreed that their birthing experience was positive \( (\text{mean} = 5.2, \text{SD} = 1.9, n = 929) \). The mothers who received printed materials from their obstetrician reported that the literature was easy to read and helpful in understanding DS (Table II). Obstetricians did seem to explain the results in a manner that could be understood; however, mothers who explained the results in an understandable manner, who felt “strongly pressured or rushed into making a decision” and with a maternal educational level: Positive = 2.51 + 0.19 Understand DS + 0.29 Positive aspects – 0.36 Educational degree \( (R^2 = 0.15, F[0.05; 3, 134] = 8.80, P < .001) \) were more likely to report that their physicians had been supportive of their decision to continue their pregnancy (Table II). This, however, was not always the case: Supportive physician = 5.60 – 0.11 Child’s age \( (R^2 = 0.04, F[0.05; 1, 128] = 6.90, P < .01) \). From this model, mothers receiving a prenatal diagnosis of DS in 2003 would be predicted to report a satisfaction level of 5.6, whereas those in 1983 would be expected to have a dissatisfaction level of 3.4. Very few thought that their physician had tried to change their decision about continuing the pregnancy, but this, too, has evolved: Change decision = 1.93 + 0.11 Child’s age \( (R^2 = 0.05, F[0.05; 1, 126] = 8.11, P < .01) \). This means that the mothers receiving a prenatal diagnosis in 1983 would be predicted to indicate an agreement level of 4.13, suggesting that their obstetricians did try to influence decisions, at least partially. Few reported that it was difficult to find adequate prenatal support, and most agreed that their prenatal support was good. Most respondents reported that their birthing experience was positive \( (\text{mean} = 5.2, \text{SD} = 1.9, n = 137) \). In contrast, by previous report, mothers who learned about the diagnosis of DS after their child was born labeled their experience as negative \( (\text{mean} = 3.4, \text{SD} = 3.1, n = 929) \).
2. Results of the amniocentesis or CVS should, whenever possible, be delivered in person, with both parents present. Mothers who had learned of the diagnosis by telephone reported intense resentment for their obstetricians and/or genetic counselors. Ideally, physicians should ask that all persons receiving definitive prenatal testing return in person to hear the results. If a personal visit is not possible, physicians should offer each couple the option of returning or receiving the results over the telephone. If the latter, physicians should note that women who have children with DS wish they had learned the results in person, with their partner present. If the diagnosis is delivered on the telephone, the physician should arrange for a follow-up in person visit as soon as possible.

3. Sensitive language should be used when delivering a diagnosis of DS. Mothers requested that physicians not begin by saying, “I'm sorry,” or “Unfortunately, I have some bad news to share.” In addition, several mothers, including some who had children as recent as 1997, reported that obstetricians had used the word “mongoloid” in describing DS, a term that is reprehensible in today’s society and should not be used by today’s physicians.

4. If obstetricians rely on genetic counselors or other specialists to explain DS, sensitive, accurate, and consistent messages must be conveyed. In 1999, 1 mother reported that her genetic counselors “told my husband and I that our child may not be able to complete school, will have limited cognitive abilities, and may remain a child, emotionally and mentally for life. Her information didn’t include any possibilities of the lowest to highest range of functioning at all.” Another mother wrote, “[the genetic counselor] showed a really pitiful video first of people with DS who were very low tone and lethargic-looking and then proceeded to tell us (in 1999) that our child would never be able to read, write, or count change.”

5. Discuss all reasons for prenatal diagnosis including reassurance, advance awareness before delivery of the diagnosis of DS, adoption, as well as pregnancy termination. Many of the mothers who responded to this survey never planned to terminate the pregnancy and were upset when their physicians provided detailed descriptions of pregnancy terminations without knowing whether they would like those options discussed.


7. Contact with local DS support groups should be offered, if desired. Respondents appreciated providers who gave them the contact information for local DS support groups. One mother reported that after talking to other parents, “I felt 100% better and positive about having my daughter.” Another mentioned, “I regret that I didn’t get involved with any support groups in the beginning. I thought everyone would sit around and cry on each other’s shoulders, and I wasn’t ready for a pity party. I only wish that physicians, nurses, and hospitals were better informed about the wonderful opportunities that are out there to help parents.” The National Down Syndrome Society maintains a directory of all DS support groups at the Web site, http://www.ndss.org/content.cfm?fuseaction=InfoResSrchFrm.

Comment

Only 12.5% of respondents, all mothers of a child with DS, had received a diagnosis prenatally. It is estimated that 1 of every 800 to 1000 live births is to an infant with DS, suggesting that around 5000 new persons with DS are born each year. This means that approximately 625 newborn infants with DS will have been diagnosed prenatally each year. This intimates that (1) the majority of women who have fetuses with DS still find out about the diagnosis postnatally, or (2) a large number of women who receive prenatal diagnoses of DS choose to terminate their pregnancies, or (3) a combination of both circumstances.

This study indicates that women who choose to continue their pregnancy after a prenatal diagnosis of DS do so primarily because of religious or personal reasons. The majority of these mothers approached the amniocentesis or CVS either confident that they would continue the pregnancy, no matter what the results indicate, or undecided, needing to gather more information if the results indicated the fetus had DS. Rarely, did a mother in this study indicate that she was adamant about terminating, only to have her opinion changed after receiving more information from her obstetrician or other sources. Some of the women, however, did feel
rushed into making a decision about the continuation of their pregnancies. This might have stemmed, in part, from the late timing of their amniocenteses.

Mothers who received prenatal care within the last 5 years seemed especially satisfied with the care that they received. In addition, these mothers were generally happier over the birth of their infant with DS than their counterparts who had received the diagnosis postnatally. This difference might stem from the fact that mothers who received a prenatal diagnosis tended to resolve any grief before their child was born. As no therapeutic intervention yet exists to cure DS or ameliorate some of its manifestations in utero, prenatal screening and diagnosing have almost exclusively existed to allow women the option of terminating their pregnancies. Knowing this, health care providers have historically operated under the assumption that if a woman consents to prenatal screening or diagnosing, she must believe that having a child with DS would be an undesired outcome and wish to terminate her pregnancy if such a diagnosis were made prenatally. The results of this study indicate that this is not true for all women. Consequently, health care providers should appreciate that many women consent to prenatal testing with ambivalence or no intent whatsoever to terminate.

As with all retrospective studies, this research is subject to recall bias. Our respondents answered the survey with approximately 4.4 years of hindsight. Their answers could have been based, in part, on information and resources that they would have preferred to receive now that they have become quite knowledgeable about DS. From the clarity in which mothers described their experiences, this does not seem to be the case, suggesting that receiving a prenatal diagnosis of DS is a true flashbulb memory—accurate, complete, and immune to forgetfulness. A previous longitudinal study has also shown that mothers who have 21-year-old children with DS could describe the births of their children with nearly 82% accuracy from their initial accounts. The current study is also subject to selection bias. Only mothers who were members of a DS support group were sampled. As there is no national database of families who have children with DS, the most comprehensive way to sample these mothers is through the support groups. However, our study is limited by the socioeconomic and ethnic composition of these groups, primarily middle- to upper-class college-educated white mothers. The current study does not adequately capture the sentiments of mothers from other ethnic or socioeconomic groups.

Also, this study purposefully focused on mothers who chose to continue their pregnancies. Future research should investigate the sentiments of those mothers who chose to terminate fetuses with DS.

Despite the limitations of this report, the message from the 141 mothers surveyed is a constructive one. Delivering a prenatal diagnosis still remains a challenge for even the most experienced physicians, but the process should no longer be viewed as a gloomy affair. In fact, with the appropriate sensitivity and explanation, obstetricians can make the births of children with DS celebratory experiences for mothers who choose to continue their pregnancies after receiving prenatal diagnoses.

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I sincerely thank Allen Crocker, MD, for this thoughtful mentorship and warm wisdom for this research. I also thank Gary, Sharon, Allison, and Kristin Skotko for assembling the research mailings; Linda Barth, Michelle Schwab, Claudia Lowe, Suzanne Shea, and Marian Chen who distributed the surveys to their respective Down syndrome support groups, and the multiple people who provided excellent feedback on survey drafts: Kerim Munir, MD, Deborah Metzel, PhD, David Helm, PhD, Carola Eisenberg, MD, Edward O’Rourke, MD, Amy Doling, PhD, Elizabeth Pinsky, and Renata Thronson.

References


Supplementary data

Supplementary data associated with this article can be found, in the online version, at www.ajog.org.
A short cervix in women with preterm labor and intact membranes: A risk factor for microbial invasion of the amniotic cavity

Ricardo Gomez,a Roberto Romero,b,* Jyh Kae Nien,b Tinnakorn Chaiworapongsac, Luis Medina,a Yeon Mee Kim,c,d Bo Hyun Yoon,e Mario Carstens,a Jimmy Espinoza,b Jay D. Iams,f Rogelio Gonzaleza

Center for Perinatal Diagnosis and Research (CEDIP), Sótero del Río Hospital, P Universidad Católica de Chile, Puente Alto, Chile,a Perinatology Research Branch, National Institute of Child Health and Human Development, NIH, DHHS, Bethesda, Md, and Detroit, Mich,b Department of Obstetrics and Gynecology, and Pathology,d Wayne State University School of Medicine, Detroit, Mich, Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Korea,e and Department of Obstetrics and Gynecology, The Ohio State University, Columbus, Ohiof

KEY WORDS
Uterine cervix
Ultrasound
Chorioamnionitis
Amniocentesis
Preterm delivery

Objective: The purpose of this study was to determine whether there was a relationship between sonographic cervical length and the presence of culture-proven microbial invasion of the amniotic cavity in women with preterm labor and intact membranes.

Study design: Ultrasonography and amniocentesis were performed in 401 patients admitted with preterm labor (22-35 weeks) and cervical dilatation of ≤3 cm, as assessed by digital examination. Cervical length was determined by transvaginal ultrasound at admission. Outcome variables were the presence of microbial invasion of the amniotic cavity (defined as a positive amniotic fluid culture) and the occurrence of preterm delivery before 35 weeks. Contingency tables, χ² test, receiver-operator characteristic (ROC) curves, and logistic regression were used for statistical analysis.

Results: The prevalence of microbial invasion of the amniotic cavity was 7% (28/401). Spontaneous preterm delivery (≤35 weeks) occurred in 21.4% (82/384) of patients. ROC curve analysis showed a significant relationship between the frequency of microbial invasion of the amniotic cavity and the length of the uterine cervix (area under the curve: 0.77; P < .005). Patients with a cervical length <15 mm had a higher rate of a positive amniotic fluid culture than patients with a cervical length ≥15 mm (26.3% [15/57] vs. 3.8% [13/344], respectively; P < .05). Moreover, patients with a short cervix (defined as <15 mm) were more likely to deliver spontaneously before 35 weeks, 32 weeks, within 7 days, and within 48 hours of admission (P < .05 for all comparisons). Forty percent of patients (161/401) had a cervical length ≥30 mm. These patients had a very low risk of microbial invasion of the amniotic cavity (1.9% [3/161]), spontaneous delivery ≤35 weeks (4.5% [7/154]), ≤32 weeks (2.6% [2/76]), within 7 days (1.9% [3/154]), and within 48 hours (0% [0/154]) of admission.

* Reprints not available from the authors. Please address correspondence to: Roberto Romero, MD, Perinatology Research Branch, NICHD, NIH, DHHS, 3990 John R Boulevard, Detroit, MI 48201.
E-mail: warfielda@mail.nih.gov

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Microbial invasion of the amniotic cavity is present in approximately 10% of patients with preterm labor, and is a risk factor for impending preterm delivery. Clinical chorioamnionitis, neonatal morbidity, bronchopulmonary dysplasia, and cerebral palsy. Maternal clinical symptoms and signs of clinical chorioamnionitis are insensitive predictors for the identification of patients with microbial invasion of the amniotic cavity. Thus, amniocentesis and amniotic fluid analysis have been used for the detection of both intra-amniotic infection and inflammation. We have previously reported that women with microbial invasion of the amniotic cavity present with a greater degree of cervical dilatation than patients with a negative amniotic fluid culture, an observation confirmed by others. Thus, we propose that the likelihood of microbial invasion of the amniotic cavity may also vary as a function of cervical length. This study was conducted to test this hypothesis.

**Study design**

**Study population**

Patients admitted between December 1997 and October 2003 to the Sotero del Rio Hospital with the diagnosis of preterm labor and intact membranes were asked to participate in a prospective cohort study designed to examine the relationship between clinical, biochemical, and biophysical parameters and the risk of preterm delivery, intrauterine infection, and neurologic disabilities. For the purposes of this study, we selected patients in this cohort who met the following criteria: (1) singleton gestation; (2) gestational age between 22 and 35 weeks and a live fetus; (3) cervical dilatation ≤3 cm by digital examination; (4) intact membranes; and (5) signed informed consent approved by the Institutional Review Board of both the Sotero del Rio Hospital and the National Institute of Child Health and Human Development, NIH. Seventy-nine patients in this study were also included in another investigation, exploring the relationship between cervical length, vaginal fetal fibronectin, and preterm delivery.

**Definitions, study procedures, and clinical management**

Preterm labor was diagnosed in the presence of regular uterine contractions of at least 3 in 30 minutes. Betamimetic agents and/or magnesium sulfate were given intravenously for tocolysis. Steroids were administered between 24 and 34 weeks. Endovaginal ultrasonography was performed shortly after admission, around the time of amniocentesis, with a 5-7.5 MHz transvaginal probe. Patients were asked to empty their bladder before endovaginal sonography. Measurements were obtained between contractions by orienting the transducer so that the endocervical canal and internal cervical os were visualized in the same sagittal plane. Three images were obtained, and the one demonstrating the shortest cervical length was used to generate cervical biometric parameters. An amniocentesis was performed transabdominally to assess the microbiologic state of the amniotic cavity. The fluid was transported to the laboratory in a capped plastic syringe and cultured for aerobic and anaerobic bacteria, as well as genital Mycoplasmas. A white blood cell count, glucose concentration, and Gram stain for microorganisms were performed in amniotic fluid. The results of these tests were used for patient management. The standard of care is to use tocolysis in patients without evidence of infection and inflammation of the amniotic cavity. On the other hand, in patients with evidence of intra-amniotic inflammation/infection (as determined by a combination of a positive amniotic fluid Gram stain, white blood cell count >50 cells/mm³, and/or a glucose determination ≤14 mg/dL), the management depended upon gestational age and the results of the test(s). The presumptive diagnosis of microbial invasion of the amniotic cavity/intra-amniotic inflammation was an indication for discontinuation of tocolysis at all gestational ages, as well as administration of parenteral antibiotics until delivery. Steroid administration (betamethasone) was used regardless of the presumptive diagnosis of amniotic fluid inflammation, except in patients who had evidence of fetal lung maturity, as determined by a shake test and/or lamellar body count. Tests such as a lecithin/sphingomyelin ratio and phosphatidyl glycerol determination are not performed at our institution because their cost is prohibitive for our health care system. After the 32nd week of gestation, patients with presumptive microbial invasion of the amniotic cavity/intra-amniotic inflammation who remained pregnant after 48 hours underwent augmentation of labor, if required. Before the 32nd week, management consisted of antibiotic administration without tocolysis. Clinical chorioamnionitis was an indication for augmentation of labor with oxytocin or misoprostol. Clinical chorioamnionitis was defined according to the criteria proposed by Gibbs et al. The
by transvaginal ultrasonography. Patients who were according to the results of cervical length determined performed to assess the examination-to-delivery interval preterm delivery. A Kaplan-Meier survival analysis was relationship between cervical length and spontaneous preterm delivery. A Kaplan-Meier survival analysis was used to explore the relationship between cervical length and spontaneous preterm delivery. 

### Table I  Clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y [mean ± SD])</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>Gestational age at admission (wk [mean ± SD])</td>
<td>31.1 ± 2.8</td>
</tr>
<tr>
<td>Nulliparous (%), n</td>
<td>48.9% (196/401)</td>
</tr>
<tr>
<td>Multiparous (%), n</td>
<td>51.1% (205/401)</td>
</tr>
<tr>
<td>Previous preterm delivery (%), n</td>
<td>14.9% (68/401)</td>
</tr>
<tr>
<td>Delivery ≤ 35 wk (%), n</td>
<td>26.8% (103/384)*</td>
</tr>
<tr>
<td>Delivery ≤ 32 wk (%), n</td>
<td>14.7% (27/184)*</td>
</tr>
<tr>
<td>Delivery within 7 d (%), n</td>
<td>20.3% (78/384)*</td>
</tr>
<tr>
<td>Delivery within 48 h (%), n</td>
<td>11.9% (46/384)*</td>
</tr>
<tr>
<td>Microbial invasion of the amniotic cavity (%), n</td>
<td>7% (28/401)</td>
</tr>
<tr>
<td>Gestational age at delivery (wk [mean ± SD])</td>
<td>36.5 ± 3.3*</td>
</tr>
<tr>
<td>Admission to delivery interval (d [median, range])</td>
<td>34 (0-123)*</td>
</tr>
</tbody>
</table>

* Excludes patients who were lost to follow-up (n = 17).

### Analysis

Outcome variables were the presence of a positive amniotic fluid culture and the occurrence of spontaneous preterm delivery (≤ 35 weeks, ≤ 32 weeks, within 7 days, and within 48 hours of admission). We used these outcomes because previous reports differed in end points, making comparisons among studies difficult. Comparisons between proportions were performed with chi-square or Fisher exact tests. Receiver-operator characteristic (ROC) curves were constructed to describe the relationship between the sensitivity and the false-positive rate (1−specificity) of cervical length in the prediction of microbial invasion of the amniotic cavity and preterm delivery. Diagnostic indices (sensitivity and specificity), as well as positive and negative predictive values for the cervical length, were obtained. Likelihood ratios for positive and negative tests were also calculated. Logistic regression analysis was used to explore the relationship between the occurrence of microbial invasion of the amniotic cavity and various explanatory variables, including the results of the ultrasonographic examination of the uterine cervix. Patients with a condition requiring delivery (abruption, fetal distress, clinical chorioamnionitis, fetal death/malformations, severe maternal conditions) were excluded from the analysis of the relationship between cervical length and spontaneous preterm delivery. A Kaplan-Meier survival analysis was performed to assess the examination-to-delivery interval according to the results of cervical length determined by transvaginal ultrasonography. Patients who were delivered preterm for maternal or fetal indications were included in the analysis with a censored time equal to the examination-to-intervention interval.

### Results

#### Clinical and ultrasonographic characteristics of patient population

Four hundred and one patients met the entry criteria for the study. Forty-six were either lost to follow-up (n = 17) or had an indication for delivery (n = 29) and were, thus, excluded from the analysis of the relationship between cervical length and spontaneous preterm delivery. Table I describes the clinical characteristics of patients. Mean gestational age at admission was 31.1 weeks (± 2.8), while mean gestational age at delivery was 36.5 weeks (± 3.3). The median cervical length was 27 mm (range 0-58.5 mm). Fifty-seven patients (14.2%) had a cervical length < 15 mm, while 161 (40.1%) had a cervical length ≥ 30 mm.

#### Relationship between cervical length and microbial invasion of the amniotic cavity

The prevalence of microbial invasion of the amniotic cavity was 7% (28/401), and the most common microorganism isolated from amniotic fluid was *Ureaplasma urealyticum*. Other microorganisms found were *Mycoplasma hominis*, *Streptococcus viridans*, *Gardnerella vaginalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Candida albicans*, and *Prevotella sp*. ROC curve analysis showed that the curve constructed for cervical length was above the 45-degree angle, indicating a significant relationship with the presence of a positive amniotic fluid culture (area under the curve = 0.77, P < .005, Figure 1). Patients with a cervical length < 15 mm had a higher rate of microbial invasion of the amniotic cavity than those with a cervical length ≥ 15 mm (26.3% [15/57] vs 3.8% [13/344], respectively; P < .05). In contrast, patients with a cervical length ≥ 25 mm (n = 234) and ≥ 30 mm (n = 161) had a low risk of microbial invasion of the amniotic cavity (2.6% [6/234] and 1.9% [3/161], respectively). Importantly, among patients with a gestational age at presentation ≤ 30 weeks, those with a cervical length < 15 mm had a 43% (9/21) risk of microbial invasion of the amniotic cavity, a rate significantly higher than those with a cervical length ≥ 15 mm (3.9% [3/76], P < .05) (see Table II).

A cut-off of 15 mm identified 54% (15/28) of patients with microbial invasion of the amniotic cavity, with a specificity of 89% (331/373) and positive and negative predictive values of 26% (15/57) and 96% (331/344), respectively. The likelihood ratios for a positive and negative test were 4.7 and 0.5, respectively.
Logistic regression analysis was performed to examine the relationship between the occurrence of a positive amniotic fluid culture and different clinical and ultrasonographic variables. Univariate and multivariate analysis demonstrated that cervical length was the strongest predictor of microbial invasion of the amniotic cavity. When other clinical explanatory variables (gestational age at admission, cervical status as assessed by digital examination, frequency of uterine contractions and others) were added individually to the model, only gestational age at admission and cervical length were significantly associated with the presence of microbial invasion of the amniotic cavity (see Table III).

Therefore, in order to estimate the individual risk of microbial invasion of the amniotic cavity in patients presenting with preterm labor and intact membranes, we developed a regression-based table that provides a specific probability according to gestational age at admission and the sonographic length of the uterine cervix (Table IV). The figures presented in the tables represent estimates derived from mathematic modeling and not from empirical observations in each cell.

Relationship between cervical length and spontaneous preterm delivery

The prevalence of spontaneous preterm delivery ≤35 weeks was 21.4% (82/384). Patients with an indication for preterm delivery were excluded from this analysis (see indications for delivery in Table VI). Patients with a cervical length <15 mm were more likely to deliver spontaneously before 35 weeks than those with a cervical length ≥15 mm (66.7% [38/57] vs 13.5% [44/327], respectively; P < .01). Also, patients with a cervical length <15 mm had a higher frequency of delivery ≤32 weeks, within 7 days, and within 48 hours of admission than those with a cervical length ≥15 mm (see Table V).

On the other hand, patients with a cervical length ≥30 mm (n = 154) had a very low risk of spontaneous delivery ≤35 weeks (4.5% [7/154], ≤32 weeks (2.6% [2/76]), delivery within 7 days (1.9% [3/154]), and delivery within 48 hours of admission (0% [0/154]) (see Table V).

A cut-off of 15 mm had a sensitivity and specificity of 46% (38/82) and 94% (283/302) for delivery ≤35 weeks, respectively, with positive and negative predictive values of 67% (38/57) and 87% (283/327), respectively. Likelihood ratios were 7.37 for a positive test and 0.57 for a negative test (similar indices were observed for delivery ≤32 weeks, within 7 days, and within 48 hours of admission) (see Table V).

Analysis of the duration of pregnancy according to cervical length results

A Kaplan-Meier survival analysis was performed to assess the examination-to-delivery interval according to results of the cervical length, as determined by transvaginal ultrasonography. Patients who were lost to follow-up were excluded (n = 17). Patients with an indicated preterm delivery (n = 29) had their admission-to-delivery interval censored. Indications for delivery are described in Table VI. Patients with a cervical length <15 mm had a significantly shorter admission-to-delivery interval than those with an endocervical canal ≥15 mm (median survival 4 days, 95% CI 2-6 days vs median survival 39 days, 95% CI 36-42 days; P < .00001, log rank test, Figure 2, A). Similarly, patients with a cervical length of ≥30 mm had a longer admission-to-delivery interval than those with a cervical length <30 mm (median survival 47 days, 95% CI 41-53 days vs median survival 27 days, 95% CI 22-32 days, respectively; P < .00001, log rank test, Figure 2, B).

Comment

Principal findings of this study:

This study demonstrates that (1) The shorter the cervix at presentation, the higher the likelihood of culture-proven microbial invasion of the amniotic cavity; and (2) Sonographic cervical length was the best parameter to assess the risk of intrauterine infection among obstetrical clinical factors such as maternal temperature, digital examination of the cervix, frequency of uterine contractions, and other historical information which clinicians can elicit from patients (ie, history of preterm birth and vaginal bleeding) in order to assess the risk of
intrauterine infection (data can be provided upon request).

The importance of microbial invasion of the amniotic cavity

Twenty-five percent of all preterm neonates are born to women with microbial invasion of the amniotic cavity, as demonstrated by studies of amniotic fluid retrieved by transabdominal amniocentesis and cultured with standard microbiologic culture techniques.26 Patients with microbial invasion of the amniotic cavity are more likely to develop maternal complications such as clinical chorioamnionitis1 and pulmonary edema while receiving tocolysis,27 as well as deliver a preterm neonate shortly after admission.1,28,29 Moreover, patients with microbial invasion of the amniotic cavity show evidence of histologic chorioamnionitis (a maternal host response) and funisitis, the pathologic hallmark of the fetal inflammatory response syndrome (FIRS).30,31 FIRS is associated with fetal multisystem involvement,32 including the hematologic system (increased number of nucleated red blood cells, leukocytosis, etc),33 adrenal gland hyperactivity,34 cardiac dysfunction,35 and outpouring of matrix degrading enzymes.36 Fetuses with FIRS are at increased risk not only for short-term morbidity,3 but also long-term handicap such as cerebral palsy13,14 and chronic lung disease.8,10 There is now compelling clinical2,37-39 and experimental40-43 evidence that fetal exposure to infection and inflammation is associated with adverse outcome. Thus, it would seem logical that the optimal management of patients with preterm labor would require knowledge of whether or not there is intra-amniotic infection. Yet, some centers continue to treat patients with premature labor without this determination. Reliance on clinical signs of infection (eg, maternal chorioamnionitis) has been demonstrated for over a decade to be insensitive.15-17

The optimal method to determine whether there is infection/inflammation in the amniotic cavity is analysis of amniotic fluid.1,20,21 Thus, it is desirable to identify women at high risk for microbial invasion of the amniotic cavity. Maternal white blood cell count and C-reactive protein determinations have been demonstrated to have limitations, presumably because most cases of amniotic fluid infection and fetal infection/inflammation may not be detected by these markers.16,17

Our study was conducted to determine if sonographic cervical length could assist in identifying women at risk for intra-amniotic infection, and the results indicate that such is the case.

Sonographic cervical length and microbial invasion of the amniotic cavity

The main finding of this study, namely that a short cervix is associated with an increased frequency of microbial invasion of the amniotic cavity, confirms the observations of Rizzo et al.44 They studied 144 patients with preterm labor and intact membranes, and found a relationship between the cervical index (cervical funnel length divided by cervical length) and the rate of positive amniotic fluid culture for microorganisms. Our findings extend these observations by comparing sonographic cervical length with other clinical parameters, and by providing diagnostic indices, predictive values, and likelihood ratios for the identification of microbial invasion of the amniotic cavity. This information was not available in the literature.

It is noteworthy that the frequency of microbial invasion of the amniotic cavity is higher the earlier the gestational age at presentation.145 Indeed, in 1992 we reported that microbial invasion of the amniotic cavity was present in 55.1% (17/33) of patients presenting between 14 to 24 weeks of gestation with a cervical dilatation ≥2 cm, intact membranes, and without active labor.46 Other studies have subsequently confirmed this observation.47 The relationship between cervical length during pregnancy and increased risk of perinatal infection has been the subject of study in the prediction of prematurity. A short cervix has been considered to be an

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<th>Table II</th>
<th>Risk of microbial invasion of the amniotic cavity (MIAC) according to cervical length and gestational age</th>
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<td>Cervical length (mm)</td>
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<td>Prevalence of MIAC</td>
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P<.001 for all categories of gestational age ($\chi^2$ for trend).

| Table III | Relationship between gestational age at admission, cervical length, and microbial invasion of the amniotic cavity, analyzed by logistic regression |
|---------------------------------------------------|
| Odds ratio | 95% CI |
| Gestational age at admission (wk) | 0.87 | 0.76–0.99 |
| Cervical length (mm) | 0.92 | 0.88–0.95 |
independent risk factor for the subsequent development of clinical chorioamnionitis and neonatal sepsis. 48

The most common organism isolated from the amniotic cavity is *Ureaplasma urealyticum*, which has been demonstrated to be associated with a robust host response in amniotic fluid, maternal and fetal compartments in patients presenting with preterm labor or preterm premature rupture of membranes (PROM).49-52 Compared with patients with sterile amniotic fluid, those who had a positive culture for *Ureaplasma urealyticum* in amniotic fluid had a higher amniotic fluid concentration of proinflammatory cytokines, including tumor necrosis factor-alpha, interleukin (IL)-1 beta and IL-6, higher plasma concentration of IL-6 in umbilical cord blood, higher prevalence of histologic chorioamnionitis, higher risk of impending preterm delivery, and adverse perinatal outcome.49-52

In order to assist clinicians in assessing the risk of microbial invasion of the amniotic cavity in patients presenting with preterm labor and intact membranes, we

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Based on a population with preterm labor and intact membranes, cervical dilatation of <3 cm, no clinical chorioamnionitis at admission, and a 7% rate of microbial invasion of the amniotic cavity.
have generated tables which include the calculated probability of microbial invasion according to gestational age at presentation and cervical length, the two factors strongly associated with the likelihood of a positive culture (Table IV). The implications of these findings are straightforward. The shorter the cervix and the earlier the gestational age, the higher the risk of infection. For example, 43% of women with preterm labor and a cervix of less than 15 mm presenting before 30 weeks will have a positive amniotic fluid culture. On the other hand, patients with a long cervix (≥30 mm) presenting with preterm labor late in pregnancy (eg, after 32 weeks) are unlikely to have microbial invasion of the amniotic cavity.

Why is microbial invasion of the amniotic cavity more frequent in patients with a sonographically short cervix? Two possibilities can be considered. First, patients could have microbial invasion of the amniotic cavity as a cause of preterm labor, and a short cervix simply represents recruitment of the cervical component of “the common terminal pathway of parturition” (see below). The other alternative is that a short cervix may predispose to ascending intrauterine infection.

The term “common terminal pathway of parturition” describes the anatomic, biochemical, endocrinologic, and clinical events that occur in both mother and/or fetus in term, as well as preterm labor. The uterine components of this terminal pathway are: (1) increased myometrial contractility; (2) cervical ripening; and (3) membrane decidual activation. Each component can be studied through a different test. For example, a toco-ynamometer has been used to determine the frequency of myometrial contractility, ultrasound to assess cervical ripening, and fetal fibronectin as presumably an early marker of membrane decidual activation.

A short cervix is the imaging equivalent to “an effaced cervix” or “ripened cervix.” It is now known that some women have microbial invasion of the amniotic cavity at the time of mid-trimester amniocentesis. These patients can have a spontaneous abortion or a spontaneous preterm delivery weeks after the amniocentesis. It is likely that “chronic intra-amniotic infection” is the cause of preterm delivery for these patients. The clinical presentation may be either conventional “preterm labor,” which is the synchronous activation of the 2 or 3 components of the terminal pathway (such as increased myometrial contractility and cervical ripening), or asynchronous activation. We use the term asynchronous activation when patients present predominantly with one of the following: preterm contractions, a dilated cervix, or preterm PROM. In these cases, the predominant clinical feature can be linked to a single component of the pathway, although there may be subclinical activation of the others. For example, patients with preterm labor may have a positive fetal fibronectin and, hence, subclinical evidence of membrane decidual activation without preterm PROM. Membrane rupture represents the extreme and generally irreversible form of membrane decidual activation. The patient with chronic intra-amniotic infection can present with preterm labor with intact membranes with a short cervix. The higher frequency of positive amniotic cultures in patients with a short cervix merely reflects the fact that patients with infection have true preterm labor and, thus, recruitment of the cervical component of the pathway.

Yet, other patients have a short cervix for days or weeks. The short cervix may result in loss of the mucus

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<tr>
<th>Table V</th>
<th>Frequency and likelihood ratio of spontaneous preterm delivery according to cervical length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical length (mm)</td>
<td>Delivery ≤35 wk</td>
</tr>
<tr>
<td>%</td>
<td>LR</td>
</tr>
<tr>
<td>&lt;15</td>
<td>66.7% (38/57)</td>
</tr>
<tr>
<td>≥15</td>
<td>13.5% (44/327)</td>
</tr>
<tr>
<td>&lt;30</td>
<td>32.6% (75/230)</td>
</tr>
<tr>
<td>≥30</td>
<td>4.5% (7/154)</td>
</tr>
<tr>
<td>Prevalence</td>
<td>21.4% (82/384)</td>
</tr>
</tbody>
</table>

LR, Likelihood ratio. Prevalence was calculated excluding patients who were lost to follow-up. Indicated preterm delivery was not included in the calculations of spontaneous preterm delivery rate.

<table>
<thead>
<tr>
<th>Table VI</th>
<th>Principal indications for delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indication</td>
<td>n</td>
</tr>
<tr>
<td>Preeclampsia/eclampsia</td>
<td>5</td>
</tr>
<tr>
<td>Clinical chorioamnionitis</td>
<td>4</td>
</tr>
<tr>
<td>Placental abruption</td>
<td>4</td>
</tr>
<tr>
<td>Stillbirth</td>
<td>4</td>
</tr>
<tr>
<td>Preterm PROM (near term, oligohydramnios, or with MIAC)</td>
<td>4</td>
</tr>
<tr>
<td>Abnormal fetal</td>
<td>3</td>
</tr>
<tr>
<td>Heart rate/umbilical Doppler</td>
<td></td>
</tr>
<tr>
<td>Chronic hypertension</td>
<td>2</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>1</td>
</tr>
<tr>
<td>Symptomatic placenta previa</td>
<td>1</td>
</tr>
<tr>
<td>Cord prolapse</td>
<td>1</td>
</tr>
</tbody>
</table>
plug, which is not only a mechanical barrier but also a component of the innate immune system. The cervical mucus has antimicrobial properties, and the cervical epithelium expresses a series of pattern recognition receptors that can sense the presence of microorganisms and orchestrate an immune response. Patients with a short cervix and changes in the microbial ecosystem of the lower genital tract could have ascending intra-amniotic infection. The fact that intra-amniotic infection is a secondary event does not decrease its importance as a mechanism of preterm labor, although we predict that the fetal attack rate for fetal infection and FIRS is different for chronic infections, which may last weeks, and recent infection lasting only hours or days.

Clinical implications

Our observations suggest that it is possible to assess the likelihood of microbial invasion of the amniotic cavity based upon information easily obtained in clinical care: sonographic cervical length and gestational age. Please note that Table IV is also freely accessible on the journal’s web site. This information could be used to assess when to perform an amniocentesis. However, the risk of microbial invasion of the amniotic cavity remains finite even in patients with a long cervix and advanced gestational age.

A clinical challenge is the optimal management of a patient with microbial invasion of the amniotic cavity. The difficulty derives from the risks that clinicians face while balancing the management (ie, immaturity vs prolonged exposure to infection/inflammation) with the constraints to research in vulnerable patients (pregnant women and fetuses).

Is there evidence that treating microbial invasion of the amniotic cavity could be beneficial? There is no randomized clinical trial specifically designed for preterm gestation in which patients with microbial invasion of the amniotic cavity detected by amniocentesis have been randomized to antibiotics or continued exposure of the fetus to infection in utero (placebo or no treatment). However, this should not be interpreted as absence of evidence that treatment of infection may not be beneficial. For example, two retrospective studies concluded that the rate of neonatal sepsis was lower in patients with clinical chorioamnionitis treated with antibiotics before delivery than in neonates treated immediately after birth. The only randomized trial in which patients with clinical chorioamnionitis (preterm and term neonates, but mostly term neonates >34 weeks) were randomized to either immediate intrapartum treatment vs antibiotic treatment of the neonate after clamping of the umbilical cord was stopped by a Data and Safety Monitoring Committee because of a significant excess of neonatal sepsis in the group allocated to delayed treatment. Gibbs et al concluded that treatment with antibiotics should be initiated immediately upon the diagnosis of clinical chorioamnionitis. In addition, there is evidence that parenteral antibiotic administration can cross the placenta. There is also experimental evidence in several species that treatment of intrauterine infection with antibiotics and other
biological response modifiers improves pregnancy outcome.77

It could be argued that the results of uncontrolled studies and those of the randomized clinical trial cited above are not applicable to preterm gestations with microbial invasion of the amniotic cavity because most fetuses were at term rather than preterm. There are, however, problems with this line of reasoning. The preterm fetus and neonate have been widely considered as “an immunocompromised host” in comparison with the term fetus/neonate. This is based upon both clinical studies indicating that the lethality rate of neonatal sepsis is higher in preterm than term neonates,78,79 as well as the examination of the immune response of the preterm neonate.80,81 Therefore, arguing that antibiotics may be beneficial to the term but not the preterm fetuses implies that an immunocompromised host does not benefit from early antibiotic treatment when exposed to microorganisms. This reasoning contradicts a large body of evidence that has informed medical practice of the immunocompromised host. Thus, unless evidence that prolonged exposure to bacteria is harmless to preterm fetuses is presented, withholding antibiotic treatment is contrary to medical practice. Indeed, experimental40,41,43,82 and clinical4,2,37-39 evidence suggests that acute and chronic intrauterine infection could be harmful to the fetus.

The argument could also be made that the results of a trial of clinical chorioamnionitis should not be extrapolated to women with microbial invasion of the amniotic cavity without clinical chorioamnionitis. The difficulty here is that clinical chorioamnionitis is a maternal and not a fetal host response, and it is the fetal inflammatory response that has been implicated in fetal injury.5,10 There is no evidence at this time that the lack of a maternal host response protects the fetus from microbial invasion of the amniotic cavity and the adverse consequences of a fetal inflammatory response.

The optimal experimental approach required to answer the question is to conduct a randomized clinical trial in which amniocenteses are used to identify microbial invasion of the amniotic cavity, with the patients being randomized to either treatment with antibiotics or no treatment (or placebo). This trial would be difficult to undertake as the current regulations state that fetuses should not be exposed to invasive procedures that are not directly beneficial to the individual fetus. It is unclear whether the current framework governing research in fetuses could permit that a group of patients with infection be allocated to no treatment or placebo. It is not difficult to foresee mothers refusing to participate in such a trial after being counseled that antibiotic treatment of term fetuses exposed to infection reduces the rate of neonatal sepsis. Thus, this question may need to be addressed by a cluster randomized trial or another variation of this design.

An unanswered question: The relationship between cervical length and intra-amniotic inflammation

The current study focused on the relationship between microbial invasion of the amniotic cavity and cervical length. The rationale for our interest in intra-amniotic infection is that the detection of microorganisms in amniotic fluid with culture techniques is the gold standard for the diagnosis of intra-amniotic infection. However, there is now accumulating evidence that intra-amniotic inflammation may be as important as microbial invasion detected by standard microbiologic techniques in determining adverse pregnancy and neonatal outcome.83,84 Therefore, future studies need to examine the relationship between cervical length and intra-amniotic inflammation, as well as cervical length and the detection of microbial footprints with sensitive molecular microbiologic techniques.85,86

References


**Supplemental material**

A complete version of this manuscript with an expanded set of references is available online at www.ajog.org.
CLINICAL OPINION

Controversies and uncertainties: Abdominal versus vaginal surgery for pelvic organ prolapse

Linda Brubaker, MD

Division of Female Pelvic Medicine and Reconstructive Surgery, Departments of Obstetrics, Gynecology, and Urology, Loyola University Medical Center, Maywood, Ill

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Pelvic organ prolapse (POP) surgery is common and increasing in the United States. Boyles et al.1 reported that approximately 200,000 women undergo POP surgery annually. Data from the National Hospital Discharge Survey indicate that approximately 22.7 per 10,000 women had some form of POP surgery in 1977.2 These procedures are costly, with annual direct costs of POP surgery recently estimated at $10 to $12 million.3

Despite the impact to the individual woman and the costs to society, the optimal surgical approach to POP is highly variable and understudied. There is a lack of consensus about the indications for POP surgery, the need for concomitant surgery (especially hysterectomy) and the optimal surgical approach. The limited evidence that exists at present suggests that the majority of POP surgery is performed via the vaginal route, with an estimate of approximately 80% using ICD-9 data.1,2,4 Although clinicians are aware of the limitations of ICD-9 coding for POP, it is likely that the majority of clinicians perform POP surgery transvaginally. It is usually based on tradition, training, and the reduced morbidity of the vaginal surgery route.

Vaginal surgery is near and dear to the hearts of all gynecologic surgeons, for this route of access is virtually unique to our field. However, there is growing evidence that suggests that an abdominal route may be preferred, at least in a subset of POP patients. In addition, laparoscopic POP surgery holds promise pending the results of high-quality clinical trials, although it is still in its scientific infancy.

Despite decades of expert advice to individualize the surgery to the patient, gynecologic surgeons have tended to adopt a preferred POP repair and apply it broadly to all POP patients. There is often little differentiation...
between primary and recurrent, or multiply recurrent POP. The risk of POP recurrence is quite high in all series and trials, although specific risk factors for recurrence are not well understood. Olsen et al⁴ reported that nearly 30% of women undergoing one POP procedure have at least 1 more POP surgery. It is also likely that the rate of POP recurrence is significantly underestimated. Therefore, it is clinically important to understand if there is a method by which POP recurrence can be reduced.

**Surgical approach to primary POP**

The current philosophy for a primary surgical approach to POP is to perform a vaginal procedure, virtually always with hysterectomy, occasionally with some form of vaginal cuff suspension and a variety of concomitant procedures (including colporrhaphy or continence procedures). Although POP is very common epidemiologically, it is relatively uncommon for most individual gynecologic surgeons. It may be difficult for an individual gynecologic surgeon to recognize patterns of anatomic and functional outcomes in a clinical setting because the procedures are performed infrequently. This is made even more difficult because patients with unsatisfactory outcomes may not return to the primary surgeon. It is understandable that most gynecologic surgeons believe that POP surgery is effective in their hands. If a single surgeon performs 10 primary prolapse repairs each year (slightly less than 1 a month), and 30% have recurrent POP, but only 1 of those 3 women returns to their surgeon, the primary surgeon may see only 1 recurrence per year. It is certainly understandable that there is a perception that prolapse repairs are highly effective.

To properly address the problem of POP recurrence, as a specialty we must first admit that there is a problem with the efficacy of our primary procedures, as documented by abundant current evidence. There may be several reasons for the insufficient efficacy for primary repairs.

Recurrence may also be related to progression of the underlying disorder that may cause physiologic changes that surpass the otherwise effective repair. Growing evidence of the significant neuromuscular damage that occurs at the time of vaginal delivery emphasizes that surgical compensatory repairs incompletely address underlying pathophysiology. The impact of muscle and neural defects may allow expression of the disorder primarily, but the progression of these pathophysiologic conditions continues and is likely associated with recurrent prolapse, especially in women with profound anatomic or functional changes early in life.

Evidence regarding long-term durability for POP procedures is sorely lacking, and most gynecologic surgeons overestimate the durability of reconstructive surgery. When faced with a 40-year-old woman with a life expectancy of more than 40 more years, it is daunting to assure the patient that her POP will be repaired once and for all. Common patient questions about lifting restrictions and other lifestyle modifications need urgent study, as there is no scientific basis for any current clinical recommendations. Shull⁵ has suggested that POP surgery may have a “life-time” of its own, with recurrence inevitable for many women.

Finally, our surgical approach may be flawed, at least in a subset of women who may be at higher risk for POP recurrence. As with most aspects of medicine and surgery, identification of high-risk individuals should alter the therapeutic approach. To scientifically evaluate these “differential diagnoses” and identify individuals who are at high risk for recurrent prolapse, we must be willing to set aside strongly held traditions and embrace the advances that scientific research can offer. Reconstructive gynecologic surgery is 1 of few areas of surgery that regularly performs procedures that are more than 100 years old! The surgical goals have not changed, however, in that time. We want to optimize function, optimize anatomy, and minimize morbidity. Compared with 100 years ago, we have many new scientific tools, materials, and the ability to perform high-quality clinical trials.

There are 2 randomized controlled trials that compare the vaginal and abdominal routes for the POP surgery.⁶,⁷ These 2 studies have been reported with sufficient detail to allow comparison of surgical routes. It is striking that together these 2 trials report on only 175 women, given the commonality of POP surgery. These 2 trials occurred in different parts of the world, Australia and the United States, with different patient populations and different outcome measures. These research teams are to be congratulated on their efforts in this groundbreaking research because these trials are extremely difficult to design and conduct. Therefore, although each trial has limitations, we can learn a great deal from each of these 2 trials.

Benson et al⁸ compared a sacrocolpopexy-based abdominal repair to a sacrospinous ligament suspensions based vaginal repair (with both arms also having individualized concomitant surgery). Both primary and recurrent POP patients were included. By using an outcome measure of “optimal outcome” defined as no symptoms of POP, the vaginal apex above levator plate, and no vaginal segment beyond the hymen, this group reported that the abdominal approach was superior (58%) to vaginal (29%), odds ratio (OR) 3.44 (95% CI = 1.24-9.69). This study was stopped before full enrollment because of concerns of the study surgeons who believed it was unethical to continue randomization with these preliminary findings. This landmark study has been criticized for the specific surgical combinations,
the mix of primary and recurrent POP in the same study population, and the high rate of vaginal surgery in the abdominal group.

The Maher randomized controlled trial compares sacrocolpopexy-based abdominal repair with sacrospinosus-based vaginal repair. These researchers restricted their study to a posthysterectomy population. The abdominal route had a higher anatomic efficacy, especially for POP-Q point C (the vaginal cuff). Within 1 year, the vaginal group had 8 of 43 (17%) apical recurrences (to or beyond the introitus) compared with 2 of 43 (5%) of the abdominal group. By using an overall outcome that included quality of life, they reported similar outcomes in both groups, and success was reported as similar in the 2 groups (vaginal 94% and abdominal 91%). Interested readers are encouraged to review the details of these 2 important studies.

These studies raise an important issue regarding the optimal outcome. Most gynecologic surgeons would call an apically based POP repair a failure if the vaginal cuff is at or beyond the hymen within 1 year of an apically oriented POP repair. This is particularly true if that anatomic defect was the indication for the procedure. Yet, it is clear that there is a discrepancy between anatomy and function, and an even further discrepancy between patient and physician surgical goals. Given this difficulty, selection of an optimal surgical outcome needs to have an anatomic, functional, and patient-oriented outcome.

These 2 studies agree that the abdominal route is anatomically superior to the vaginal route for POP repair; however, this advantage comes at the costs of increased morbidity and some risk of foreign body complications. These clear disadvantages must be weighed against the suboptimal anatomic results of sacrospinosus-based vaginal repairs. Experienced clinicians know that there are some women whose POP is more effectively treated with one route than another. Patient characteristics, including age, concomitant medical morbidities and other surgical risks, prior and concomitant surgical procedures, and patient preference are all important factors when planning a procedure.

What then is the role of sacrocolpopexy-based procedures for primary POP repairs? Selected young women with severe POP are more likely to experience recurrent POP based on the severity of their underlying pelvic floor disorders, the need for longer surgical durability, and the severity of the POP itself. However, these women also have a prolonged risk of foreign body complications, a risk that may be increased by concomitant hysterectomy. These risks can be avoided with a vaginal route; however, the risk of recurrent POP increases. The reduced short-term morbidity of vaginal surgery is clear. For many women, these benefits offset the risk of prolapse recurrence. For example, the risk of POP recurrence is probably offset by the limited short-term morbidity of vaginal repair surgery for older or frail women with shortened life expectancies.

Most primary prolapse repairs also involve hysterectomy. Expert opinion varies as to whether hysterectomy performed at the time of sacrocolpopexy increases the risk of mesh-related complication. Current ongoing trials may provide some information on the magnitude of these risks. Moreover, the use of hysterectomy at the time of prolapse repair should be questioned and evaluated scientifically. The observed global differences in this practice warrant further research.

The optimal route and techniques for repair of recurrent prolapse has not been studied. Experts agree that it is rare to obtain optimal surgical outcomes by repeating a procedure that was unsuccessful in the primary repair. The surgically altered topography (narrowed caliber, shortened length) may limit surgical choices. Most, but not all, experts consider abdominal repair with an intervening suspensory mesh, often a synthetic material. The selection of the optimal procedure for a given patient strongly depends of her own goals and expectations for her surgical outcomes.

Surgical planning requires a full assessment of the anatomic and functional disorders in the pelvis.

Our gynecologic forefathers were right. Each surgery needs to be individualized to that particular woman. It is our legacy as gynecologic surgeons to use our best surgical skills to relieve the suffering and impact of POP. We must apply the scientific methods that have advanced other areas of women’s health to this field of POP surgery. It is possible, and indeed it is urgent, that we determine the optimal route of surgery for both primary and recurrent POP, the optimal group of reparative procedures, as well as the individual risk factors for POP recurrence. We can honor our traditional of vaginal surgery and perhaps even enhance our outcomes by preoperatively identifying situations associated with a high risk of POP recurrence. This is likely to improve our results with vaginal surgery and indicate when the use of abdominal methods when the risk/benefit ratio is favorable for that individual woman.

It is clear that for anatomic restoration, the abdominal sacrocolpopexy is a gold standard. Further research may challenge or support this opinion. However, because its anatomic superiority comes at a cost of higher short-term morbidity and potential foreign body problems, the surgeon should counsel an individual woman about the risks, benefits, and alternatives of her surgically appropriate options including transvaginal reconstructions. This counseling should be based on the surgeon’s own results, not published literature. In the unlikely event that the surgeon’s own results exceed published results of high-quality trials, it is imperative to advise the patient of published results as well.

It is not in our patient’s best interest to draw a line in the sand and do battle over “vaginal” versus...
“abdominal.” It is in our patient’s best interest for gynecologic surgeons to design and conduct high-quality clinical trials and well-described prospective cohort studies to provide scientific data that can inform our surgical recommendations for future generations. With knowledge of our surgical heritage, it is time to look forward and elevate the level of care for the many women whose lives are affected by pelvic organ prolapse. If we do not—who will?

References

REVIEW ARTICLE

A systematic review of the association between factor V Leiden or prothrombin gene variant and intrauterine growth restriction

Heather E. A. Howley, MSc,a Mark Walker, MD, MSc,b Marc A. Rodger, MD, MScb,*

University of Ottawa, Ottawa, Ontario, Canada,a and Department of Epidemiology and Community Medicine, University of Ottawa, Ottawa Health Research Institute, and The Ottawa Hospital, Ottawa, Ontario, Canadab

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KEY WORDS
Meta-analysis
Factor V Leiden
Prothrombin gene variant
Intrauterine growth restriction

Objective: The purpose of this study was to conduct a systematic review of the literature of studies that examined the association between factor V Leiden and/or prothrombin gene variant and intrauterine growth restriction.

Study design: This systematic review of studies assesses the association between factor V Leiden and/or prothrombin gene variant and intrauterine growth restriction.

Results: Ten case-control studies fulfilled the selection criteria for inclusion in the meta-analysis. There was a significant association between factor V Leiden and intrauterine growth restriction (odds ratio, 2.7; 95% CI, 1.3-5.5) and prothrombin gene variant and intrauterine growth restriction (odds ratio, 2.5; 95% CI, 1.3-5.0). Five cohort studies were identified in the systematic review; 3 studies were prospective (2 full publications), and 2 studies were retrospective (1 full publication). Combining the 2 full publication prospective studies yields a summary relative risk of 0.99 (95% CI, 0.5-1.9).

Conclusion: This meta-analysis of case-control studies suggests that the factor V Leiden and prothrombin gene variant both confer an increased risk of giving birth to an intrauterine growth restricted infant, although this may be driven by small, poor-quality studies that demonstrated extreme associations. Large well-conducted prospective cohort studies are required to determine definitively whether an association between thrombophilia and intrauterine growth restriction is present.

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Intrauterine growth restriction (IUGR), intrauterine growth retardation, fetal growth restriction, and small for gestational age are all exchangeable terms used in the literature to describe low birth weight infants; we will use the term IUGR. Given the difficulty of in utero measurement of growth, a cross-sectional measure of birth weight adjusted for sex and gestational age is used in both clinical and public health practice to diagnose IUGR. Normally, IUGR is defined as infants whose birth weight is below the 10th percentile of birth weight adjusted for sex and gestational age, although other definitions and cut-offs have been used. Kramer et al have found linear increases in infant morbidity in newborn infants who were born with a birth weight below the 10th percentile.

IUGR is associated with significant perinatal morbidity and mortality rates, for example, the risk of...
respiratory failure and 28 day all-cause mortality rate is increased in IUGR infants (<10th percentile birth weight). Surviving IUGR children also have long-term sequelae (developmental delay, poor school performance, lower academic and professional achievement as adults). Furthermore, a number of adult diseases have been associated with IUGR (e.g., hypertension, coronary heart disease, stroke, and type II diabetes). The cause of IUGR is complex. Kramer previously reviewed the epidemiologic studies of the determinants of IUGR and determined the relative contribution of various risk factors for IUGR. He concluded that, in industrial countries, cigarette smoking accounted for approximately 30% to 40% of IUGR, genetically related factors accounted for approximately 20% to 30%, nutritional factors accounted for approximately 10% to 15%, and parity and general maternal morbidity accounted for approximately 5% to 10%.

Thrombophilias are inherited and acquired predispositions to the development of venous thromboembolism. Inherited thrombophilias are autosomal dominant and include factor V Leiden (FVL), prothrombin gene
<table>
<thead>
<tr>
<th>Study</th>
<th>Case definition</th>
<th>Control definition</th>
<th>NOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Franchi et al, 2003 [abstract]</td>
<td>Birth weight &lt;10th percentile and abnormal abdominal circumference</td>
<td>Normal weight neonate</td>
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<td>McCowan et al, 2003</td>
<td>Birth weight &lt;10th percentile for sex and gestational age; no evidence of chromosomal or congenital abnormality; excluding preeclampsia or pregnancy-induced hypertension</td>
<td>Birth weight &gt;10th percentile; normotensive women; matched for ethnicity</td>
<td>6</td>
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<td>Ogunyemi et al, 2002</td>
<td>Adverse pregnancy outcomes, including IUGR; birth weight &lt;10th percentile for gestational age; excluding congenital malformation/infection, chromosomal abnormalities</td>
<td>At least 1 live birth without obstetrical complications; matched to time period; excluding venous thromboembolism</td>
<td>Not done</td>
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<td>Infante-Rivard et al, 2002</td>
<td>Birth weight &lt;10th percentile for sex and gestational age; excluding severe congenital anomalies</td>
<td>Birth weight ≥10th percentile for sex and gestational age; excluding severe congenital anomalies, matched for gestational age, sex, ethnicity, time period</td>
<td>9</td>
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<tr>
<td>Kupferminc et al, 2002</td>
<td>Birth weight &lt;3rd percentile and amniotic fluid index ≤ 3rd percentile for gestational age of 22-26 wks; excluding history of vascular disease, chromosomal anomalies, structural aberrations, cytomegalovirus infections, chronic maternal disease, and maternal drug/alcohol abuse</td>
<td>Healthy women with at least 1 normal pregnancy, matched for age, smoking, ethnicity, time period</td>
<td>8</td>
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<tr>
<td>Verspyck et al, 2002</td>
<td>Birth weight &lt;3rd percentile for sex and gestational age; excluding congenital malformation and infections, chromosomal abnormalities, maternal drug/alcohol abuse, prescribed beta-blockers; matched for time period</td>
<td>Birth weight &gt;10th percentile</td>
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<tr>
<td>Martinelli et al, 2001</td>
<td>Birth weight &lt;10th percentile; excluding malformations, fetal infection, multigestations</td>
<td>Birth weight &gt;10th percentile; healthy, uneventful pregnancy; matched for ethnicity</td>
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<td>Lee et al, 2001 [abstract]</td>
<td>Birth weight &lt;5th percentile for gestational age</td>
<td>Delivered an appropriate for gestational age infant; excluding venous thromboembolism or preeclampsia; matched for race, age, parity</td>
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<td>Kupferminc et al, 2000</td>
<td>Adverse pregnancy outcomes, including IUGR; birth weight &lt;10th percentile; excluding congenital or chromosomal abnormalities; venous thromboembolism</td>
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<td>Kupferminc et al, 1999</td>
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<td>At least 1 normal pregnancy excluding venous thromboembolism; matched for age and geographic origin of parents</td>
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<td>IUGR cases (n/N)</td>
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<td>OR (95% CI)</td>
<td>Included in meta-analysis? (if No, give reason)</td>
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<tr>
<td>FVL fiberglass</td>
<td>PGV absence</td>
<td>FVL/PGV</td>
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<td>8/145 (6%)</td>
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<td>4/97 (4%)</td>
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<td>7/97 (7%)</td>
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<td>15/61 (25%)</td>
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<td>4/23 (17%)</td>
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FVL: 1.9 (0.4-10.0); PGV: 0.2 (0.0-3.8); both:0.8 (0.3-1.8) Yes

FVL: 0.7 (0.2-2.3); PGV: 0.9 (0.3-2.9); both:0.8 (0.4-1.8) Yes

FVL: 1.9 (0.6-6.3); PGV: 4.6 (1.0-20.0); both:3.3 (1.2-8.8) Yes

No (FVL/PGV not given for IUGR cases)
variant (PGV), and deficiencies of protein C, protein S, and antithrombin. FVL and PGV are very common (combined prevalence range, 6%-10%)\textsuperscript{13,14} and contribute to a large portion of the population-attributable risk of venous thrombembolism. If thrombophilias predispose individuals to thrombosis in the slow-flow blood circulation of the deep venous system, then it is reasonable to suggest that thrombophilias would also predispose individuals to thrombosis in the comparably slow-flow blood circulation of the intervillous space of the placenta. Thrombosis in the intervillous circulation results in the occlusive changes seen in pathologic specimens of the placentas\textsuperscript{15,16} from women with thrombophilia and with IUGR infants.\textsuperscript{17}

Numerous retrospective and a few prospective studies have been conducted to examine the association between FVL/PGV and IUGR. The evidence for an association is conflicting. Given that both FVL/PGV and IUGR are common and that IUGR is a disease with enormous individual and societal burdens, we felt it was important to conduct a systematic review of the literature that surrounds this proposed association. Our objectives were to conduct a systematic review of the literature for studies that examined the association between FVL/PGV and IUGR and to perform a meta-analysis of case-control and cohort studies to determine a pooled estimate of the odds ratio (OR) and 95% CI.

**Material and methods**

**Search strategy**

A sensitive search strategy was developed with an information specialist who was experienced in meta-analysis. The search strategy combined medical subject headings and text words, with no restrictions on language, publication type, or publication date (Appendix). The search strategy was used to search Medline and EMBASE in June 2003. The reference lists of review articles that were identified in the search (n = 13) and the reference lists of all articles that were retrieved were hand-searched for further evaluation. In addition, conference proceedings from both the American Society of Hematology (years 1999 to 2002) and the International Society of Thrombosis and Haemostasis (years 1999 to 2003) were hand searched.

**Selection**

A study was considered eligible for further review (ie, the full manuscript was retrieved) if the exposure of interest was FVL and/or PGV and if the outcome included IUGR (or fetal growth restriction or small for gestational age). To be included in the meta-analysis, studies must have used a case-control or cohort design, and IUGR must have been defined based on birth weight (ie, not diagnosed antenatally). The systematic review and identification of eligible studies was performed by 1 reviewer (H.E.A.H.).

**Validity assessment and data extraction**

Two reviewers (H.E.A.H. and M.A.R.) independently performed data extraction. Standardized data collection forms were developed a priori for data extraction. Disagreements were resolved by consensus (that is, if there was not perfect agreement between reviewers then both reviewers together returned to the original manuscript to re-abstract the pertinent data). One reviewer (H.E.A.H.) performed quality assessments using the Newcastle Ottawa Scale (NOS). The NOS assesses the quality of nonrandomized studies on 3 broad perspectives: the selection of the study groups, the comparability of the groups, and the ascertainment of either the exposure or outcome of interest for case-control studies (see http://www.ohri.ca/programs/clinical_epidemiology/oxford.htm for details).

**Study characteristics**

The following information was extracted from each study: primary author, country, study setting, study design, case/control definitions, participant characteristics (eg, demographics and confounders), thrombophilia criteria and details of thrombophilia testing, number of cases/controls, withdrawals, and number lost to follow-up.

**Analysis**

Both FVL and PGV are inherited in an autosomal dominant fashion, with two possible alleles: the mutant allele and the wildtype, or normal, allele. Thus, there are 3 possible genotypes: homozygous mutant, heterozygous mutant, or homozygous wildtype. Both homozygous mutant and heterozygous mutant individuals express the mutant phenotype to varying degrees. It is important to make this distinction, because case control and cohort studies traditionally examine a dichotomous exposure, whereas case control studies of FVL/PGV consider 3 possible exposures. In summarizing the evidence, we (and most authors) dichotomized exposure on the basis of a genetic dominant model: Cases included homozygous mutant or heterozygous mutant women, and control subjects included only homozygous wildtype women.

We conducted the meta-analyses using Comprehensive Meta-Analysis software (version 1.0.23; Biostat, Englewood, NJ). The summary estimate was calculated by the Mantel-Haenszel method, with the random-effects model given the clinical heterogeneity surrounding IUGR. Statistical heterogeneity was assessed by the Q statistic (heterogeneity chi-squared test) and was
considered significant when \( Q \) was greater than the degrees of freedom. The primary outcome was the dichotomous measure of IUGR, and the OR was used for individual and summary point estimates.

For case-control studies, funnel plots and secondary analyses were conducted. Funnel plots were constructed to assess publication bias for each of the thrombophilias that was studied. Secondary analyses were conducted to assess the association among subgroups and included IUGR definition (birth weight percentile) and study quality (NOS score). Only data from cohort studies that were published as full articles provide sufficient quality information to permit meta-analysis. Prospective cohort and retrospective cohort data were considered separately. Data from other cohort studies were reviewed qualitatively. Because of the paucity of adequate studies, funnel plots to assess publication bias were not constructed, and subgroup analyses could not be considered for cohort studies.

**Results**

**Systematic review**

Results of the systematic review are detailed in Figure 1. A total of 111 citations were identified from database searches and hand searching the reference lists of review articles18-30 and retrieved manuscripts; 39 manuscripts (21 case-control studies31-41 and 18 cohort studies52-69) examined the association between FVL/PGV and IUGR and were retrieved for further evaluation. For the systematic review, 11 eligible case-control studies31-41 and 5 eligible cohort studies52-56 were included. A meta-analysis of 10 case-control studies was conducted (1 study was excluded because it provided insufficient details to calculate ORs23). Of the 5 cohort studies that were identified, 2 studies were retrospective45,52 and 3 studies were prospective. Two prospective54,55 and 1 retrospective56 studies have been published, and the 2 prospective studies were meta-analyzed.

**Study characteristics**

**Case-control studies**

Table I lists the study characteristics that were extracted for each eligible case-control study that was identified in the systematic review. There was clinical heterogeneity among studies, with respect to IUGR criteria and definitions. Although most studies defined IUGR as birth weight below the 10th percentile,32,34,36,38,39 some studies considered more severe forms of IUGR with the use of such criteria as a lower birth weight cutoffs,35,37,40,41 preterm delivery,35 abnormal Doppler/ultrasound findings,35 or abnormal head/circumference ratios in affected infants.31 Many populations from different countries are represented. The prevalence of thrombophilia and incidence of IUGR are known to vary among ethnic groups and among the control populations studied; baseline thrombophilia rates varied from 0% to 8%. Finally, IUGR is in itself a heterogeneous outcome with many causes and risk factors; most,31-40 but not all41 studies excluded known causes of IUGR (such as congenital anomalies/infections, chromosomal abnormalities). Most studies attempted to control for, either by design or analysis, ethnicity (a known confounders of IUGR); but only a few studies also controlled for other important confounders such as smoking.34,35 Funnel plots of FVL and PGV (Figure 2) revealed the potential for publication bias among case-control studies.

![Funnel plots to assess publication bias. The precision (as measured by the SE) of each study is plotted against its OR to investigate possible publication bias. Each study included in the meta-analysis is represented by a circle; the diameter is proportional to the precision of the study. The vertical line represents the summary OR that was calculated in the meta-analysis; the diagonal lines represent the SE of the summary estimate. A, FVL; B, PGV.](image-url)
Cohort studies

Table II lists the study characteristics that were extracted for each eligible cohort study that was identified in the systematic review. The retrospective cohort study of Grandone et al \(^{45}\) examined women with a history of recurrent pregnancy loss or gestational hypertension and randomly selected control subjects. The abstract presented by Roque et al \(^{52}\) provided insufficient details to calculate relative risks or to be assured of the quality of this retrospective cohort study. Both of these retrospective studies were underpowered to detect clinically significant associations and did not control for known confounders of IUGR. Of the 3 prospective cohort studies, only 2 studies have been published. \(^{54,55}\) Lindqvist et al \(^{55}\) reported on 2480 pregnant women who attended their first routine antenatal visit. The investigators did not control for known confounders of IUGR (eg, maternal smoking, gestational age, sex, race, maternal weight gain, prepregnancy weight, parity, history of IUGR, maternal comorbidity). Murphy et al \(^{54}\) conducted a prospective cohort study of 593 primigravid women who attended 2 antenatal clinics at National Maternity Hospital in Dublin, Ireland. They examined whether FVL or methylenetetrahydrofolate reductase resulted in an increased risk of pregnancy complications (preeclampsia, miscarriage, IUGR, and thrombosis). The abstract by Salomon et al \(^{56}\) provided insufficient details to assess the association or study quality in this prospective cohort study.

Data synthesis

Case control studies

Table I lists the results of individual case-control studies. The random-effects model was used for all assessments, given the clinically and statistically significant heterogeneity. The results of the meta-analyses are shown in Figure 3; the summary OR and 95% CI is reported. The pooled OR of the association between FVL and IUGR was 2.7 (95% CI, 1.3-5.5) and was 2.5 (95% CI, 1.3-5.0) for PGV and IUGR. All studies but that of Infante-Rivard et al \(^{34}\) provided sufficient information to calculate the combined prevalence of FVL or PGV. When both thrombophilias were considered, the combined summary OR was 3.4 (95% CI, 1.5-8.0; Infante-Rivard’s study was not included).

We conducted post hoc analyses (separately for FVL and PGV) to examine for an association among subgroups. Studies were grouped according to the infant birth weight cut-off for cases; those studies that used \(< 5\) percentile (including \(< 3rd\) percentile) versus those studies that used \(\leq 10th\) percentile (Figure 4). The summary OR was 2-fold greater (FVL, 4.7 vs 2.0; PGV, 4.3 vs 2.0) among studies that used a \(\leq 5th\) cut-
off percentile compared with the 10th percentile. Also, a statistically significant association among IUGR and FVL and PGV was observed for studies that used a 5th percentile cut-off, but not for those that used the 10th percentile cut-off. Many of the initial published studies that demonstrated an extreme association between FVL/PGV and IUGR were very small; the 2 more recent and larger studies (McCowan et al.32 and Infante-Rivard et al.34) failed to demonstrate any association. This is reflected in the funnel plots depicted in Figure 2. Overall study quality was high, because of the robustness of the exposure (thrombophilia) and outcome (birth weight percentile). To examine the potential effect of the study quality, we grouped studies according to their NOS score (Figure 5). Interestingly, the summary OR for studies with a higher NOS score was only one half that of studies of poorer quality (FVL, 2.0 vs 4.7; PGV, 1.8 vs 4.0, respectively). Furthermore, the summary OR for studies with NOS scores > 5 was not statistically significant.

Cohort studies

The results of individual cohort studies are listed in Table II. Grandone’s45 retrospective cohort found a statistically significant association for PGV and IUGR, but not FVL. However, the cohort was highly selected (as detailed earlier). The overall incidence of IUGR was 15% (170/1103) in this study. In the prospective cohort of Lindqvist et al.55 the incidence of IUGR was 3.3% (9/270) among women with FVL, compared with 3.6% (79/2210) among wildtype women, for a relative risk of 0.93 (range, 0.47-1.84). Of the 588 women who were tested for FVL in the prospective cohort of Murphy et al.54 16 women (2.7%) were heterozygous for FVL. Only 9 women (1.5%) delivered an IUGR infant, none of whom had FVL (OR, 0, 0-27.0). The low incidence of IUGR (3.5% and 1.5%) in both of these prospective studies (despite being defined as birth weight < 10th percentile) raises concerns about ascertainment bias or selection bias. All published cohort studies were underpowered to detect clinically significant associations.

Again, given the clinical heterogeneity, the random effects model was used for the meta-analysis of the published prospective cohort studies (Figure 6). Combining these 2 studies results in a relative risk of 0.99 (range, 0.5-1.9). Because of the paucity of studies neither subanalyses nor funnel plots were performed.

Comment

We have demonstrated that, overall, case-control studies suggest that an association between FVL/PGV and IUGR may exist (OR, approximately 2.5), despite recent reports to the contrary.32,34 However our meta-analysis revealed statistically and clinically significant
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Decreases risk  |  Increases risk

### B  FVL only

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Decreases risk  |  Increases risk

### C  PGV only

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Decreases risk  |  Increases risk

**Figure 3** Meta-analysis of the association between IUGR and FVL and/or PGV. Forest plots are depicted. The results of component studies are shown as squares centered on the point estimate (OR) of each study; the horizontal line represents the 95% CI. The diamond represents the summary estimate and its confidence interval. The center of the diamond represents the pooled point estimate, and its horizontal tips represent the confidence interval. Significance is achieved at the set level if the diamond is clear of the line of no effect. A, FVL and/or PGV. B, FVL only. C, PGV only.
A  Birth weight <10th centile, for FVL

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Decreases risk  Increases risk

B  Birth weight <10th centile, for PGV

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Decreases risk  Increases risk

C  Birth weight <5th centile, for FVL

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Decreases risk  Increases risk

D  Birth weight <5th centile, for PGV

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Decreases risk  Increases risk

Figure 4  Subanalyses by birth weight percentile. Forest plots are depicted. A, Birth weight <10th percentile for FVL. B, Birth weight <10th percentile for PGV. C, Birth weight <5th percentile for FVL. D, Birth weight <5th percentile for PGV. NOS, NOS.
heterogeneity surrounding this estimate; thus, this association should be interpreted with caution. The presence of heterogeneity in our study raises the possibility that significant differences between the published case-control studies may exist and that these differences may be the result of biases in some studies. Funnel plots illustrate how small case-control studies demonstrated large ORs, whereas more recent, large, and robust studies do not.

**Figure 5** Subanalyses by NOS score. Forest plots are depicted. **A.** ≤ 5 for FVL; **B.** ≤ 5 for PGV; **C.** > 5 for FVL; **D.** > 5 for PGV.

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demonstrate any association that suggests publication bias. On balance, the cohort data does not support an association; however, the published cohort studies have significant methodologic limitations (including inadequate power, possible ascertainment bias, and the absence of controlling for known confounders) that call into question the veracity of this finding.

In our post hoc analyses, we attempted to examine the association of FVL/PGV and IUGR that was observed for case-control studies. IUGR is a complex disease, with many causes and risk factors. We explored whether differences in IUGR severity, based on birth weight cut-off, demonstrated similar associations. Interestingly, the summary OR was greater and statistically significant when studies with more severe cut-offs were considered. The morbidity and mortality rates that are associated with IUGR increase as birth weight decreases; if an association exists, it is possibly more likely to be observed in extreme cases of IUGR. Studies of high quality are more likely to demonstrate valid associations, whereas studies of poorer quality are more likely to provide associations that are actually outliers. Many initial genetic associations that are found in case-control studies have been difficult to reproduce, possibly because of publication bias where initial publications represent extreme outliers.70,71 Our current findings are consistent with work done by Ioannidis et al. 72 In an examination of the results of 55 meta-analyses of genetic associations, they found that only 16% demonstrated a consistent significant genetic association devoid of evidence of heterogeneity or bias. Furthermore, they observed that large studies and subsequent research demonstrated weak or null associations, compared with the strong associations shown in smaller studies and initial research. Funnel plots support the notion that the strong association between FVL/PGV and IUGR that were observed initially may be due to publication bias. When studies were stratified according to their NOS scores, the association for studies of higher quality was not statistically significant (whereas it was statistically significant for poorer quality studies); the OR of higher quality studies was less than one half that of poorer quality studies. Given the individual and societal impacts of IUGR and the high prevalence of thrombophilias, small ORs translate into significant population risks and are important to detect.

Genetic association studies are less susceptible to some sources of bias (such as recall and ascertainment bias) that are inherent in the case-control design. Nonetheless, biases remain and threaten the legitimacy of case-control estimates of risk. Selection bias remains a potential problem, especially when considering the variety of IUGR definitions and classifications of severity. Participation bias is a threat; because thrombophilia genotyping is not offered routinely in the antenatal clinic, cases and control subjects have to return for testing and may have different response rates (eg, cases might be more motivated to return for testing). Prospective cohort studies are, in general, of a stronger methodologic design and are better suited to assess genetic association. Prospective cohort studies permit the more accurate collection of confounder and outcome data, reduce participation bias (because exposure and outcome status are not known at the time of data collection), examine an unselected population, and permit a direct measure of the incidence of disease in the exposed and unexposed groups. Furthermore, a prospective cohort study is feasible because the outcome under study is not rare (incidence of IUGR, approximately 10%), the outcome occurs over a short time-frame (ie, gestation of 40 weeks), and loss to follow-up is very unlikely.

It was notable that not all of the case-control studies and none of the cohort studies attempted to account for multiple known confounders of IUGR (such as ethnicity and smoking). Given the many known causes and risk factors for IUGR, accurate controlling of confounders is important in the assessment of any association. It is also important that potential confounders be collected accurately; potential recall bias on important confounding factors (eg, smoking) in case-control studies can constitute a significant threat to the validity of the results, whereas a prospective design allows for the collection of confounders before knowing the disease outcome or risk factor status.

In summary, although an overall association among case-control studies was observed between FVL and IUGR and PGV and IUGR, our subanalyses and funnel plots suggest that this may be driven by small, poor-quality studies that demonstrated extreme associations. Studies of higher quality have failed to find any association at all, and data from cohort studies are

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Random Combined (2) | 0.966 | 0.500 | 1.867 | | | | | |

Decreases risk | Increases risk

Figure 6  Meta-analysis of cohort studies that examined the association between IUGR and FVL. Forest plots are depicted.
unconvincing. More research is needed to determine whether this trend towards a null effect will continue. In particular, large prospective cohorts are required to determine definitively whether an association between FVL/PGV and IUGR exists.

Acknowledgments

We thank the information scientists and librarians at The Ottawa Hospital for their expertise in constructing the search strategy and for document retrieval; Dr Dean Fergussen for his insightful review of an early version of this manuscript; and Michelle Willson and Christine Gagne-Rodger for assistance in manuscript preparation and revision.

References

3. Gagne-Rodger for assistance in manuscript preparation.
Appendix: Search strategy

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2. case-control studies/
3. cohort studies/
4. retrospective studies/
5. prospective studies/
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27. activated protein c/ or blood clotting factor 5
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30. or/20-29
31. and/10,19,30
SELECTIONS FROM THE 25TH ANNUAL MEETING OF THE SOCIETY FOR MATERNAL-FETAL MEDICINE, FEBRUARY 9-12, RENO, NEVADA

Editorial to SMFM Section

Jay D. Iams, MD

Associate Editor

This month’s issue includes seven manuscripts that describe research presented at the 2005 meeting of the Society for Maternal-Fetal Medicine in Reno, Nevada, February 10-12. From more than 1000 abstracts submitted, 68 were selected by the Society for oral presentation. Authors of these 68 abstracts were invited to submit their manuscripts through the new “Fast Track” process in which AJOG reviewers agreed to provide rapid reviews in time to allow revision to meet the deadline for inclusion in this issue, the first since the SMFM meeting. The Editors of the Journal would like to thank the authors and reviewers of these papers for their diligence in submitting and reviewing these papers. We hope our readers will enjoy reading the articles, each of which is denoted as an SMFM Oral Presentation in the attribution on the title page. The reviewers are listed on page 760 of this issue, at the end of the article by Feltovich et al.
Proteomic biomarkers that predict the clinical success of rescue cerclage

Carl P. Weiner, MD,a,* Keun-Young Lee, MD,b Catalin S. Buhimschi, MD,c Rob Christner, PhD,d Irina A. Buhimschi, MDc

Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Maryland School of Medicine, Baltimore, Md,a Department of Obstetrics and Gynecology, Kangnam Sacred Heart Hospital, Hallym University, Seoul, Korea,b Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University, New Haven, Conn,c Ciphergen Biosystems, Fremont, Calif.d

Received for publication June 1, 2004; revised September 20, 2004; accepted October 4, 2004

Objective: The origin of incompetent cervix is multifactorial, and the success of rescue cerclage is unpredictable. We tested amniotic fluid from women who were preparing to undergo rescue cerclage for proteomic biomarkers and correlated their presence with clinical outcome.

Study design: Amniocentesis was performed to facilitate rescue cerclage in 37 consecutive women with painless dilation (>2 cm) and no detectable uterine activity for 4 hours (range, 1-24 hours) before cerclage. Thirty-nine consecutive women with a sonographically normal pregnancy and cervix who underwent amniocentesis for chromosomal testing during the same study interval at the same clinical site provided the control samples. A proteomic fingerprint was generated with the discarded sample and the Mass-Restricted score (MR score) for inflammation calculated. Peaks corresponding to free hemoglobin chains were sought as evidence of decidual hemorrhage or intra-amniotic bleeding.

Results: Amniocentesis was performed at 23.5 weeks in cerclage (mean dilation, 4 cm) versus 19.5 weeks in control subjects. Cerclage subjects were delivered at 28.8 weeks; control subjects were delivered at 39.2 weeks. Thirty-two of 37 of cerclage subjects (86%) were delivered prematurely. Ten of 37 of cerclage subjects (27%), but no control subject, had a MR score that was indicative of inflammation ($P < .001$). Hemoglobin peaks were present in 12 of 37 of cerclage subject (32%), but no control subjects. Among cerclage subjects, those with a MR score of 3 to 4 were delivered earlier than those with a MR score of 0 to 2 ($P < .001$). Women with a MR score of 3 to 4 had a shorter latency period (days from amniocentesis to delivery; 3 days) and a shorter percentage of prolongation (1.8%) than women with a MR score of 0 to 2 (35 days; $P < .05; 17.9%$; $P < .05$). Women with hemoglobin had a shorter latency period (6 days) and a shorter percentage of prolongation (3.8%) than women without hemoglobin (38 days; $P < .05; 21.8%$; $P < .05$). Hemoglobin was present in 7 of 10 of the cerclage subjects (70%) with a MR score of 3 to 4. Women with both a MR score of 3 to 4 and hemoglobin had the shortest intervals to delivery.

KEY WORDS
Incompetent cervix
Cervical cerclage
Surface-enhanced laser-desorption ionization
Proteomic biomarker

Oral presentation at the 25th Annual Meeting of the Society for Maternal-Fetal Medicine, February 7-12, 2005, Reno, Nev.

* Reprint requests: Carl P. Weiner, MD, MBA, Department of Physiology and of Obstetrics and Gynecology and Reproductive Science, University of Maryland School of Medicine, 655 W Baltimore St, BRB-11-033, Baltimore, MD 21201-1559.

E-mail: cweiner@umm.edu

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Early mid trimester pregnancy loss that is associated with painless cervical dilation of undetermined cause remains a frustrating challenge. The traditional treatment of incompetent cervix is the placement of a purse string suture close to the internal cervical os. Although typically placed in the early second trimester electively after a pregnancy loss that is consistent with incompetent cervix, some pregnant women are already dilated with either no relevant or confusing clinical histories. In this setting, a cerclage can still be performed in an attempt to “rescue” the pregnancy. Exposure of the amniotic membranes to the vagina increases the risk of failure, and the failure rate of rescue cerclage is higher than after elective placement.

Amniotic fluid microbial invasion occurs in >50% of women with painless cervical dilation of >2 cm between 14 and 24 weeks of gestation. In one retrospective study, Mays et al noted 7 of 18 women (39%) who were considered candidates for rescue cerclage already had indirect markers of inflammation in their amniotic fluid. They declined to operate and attributed their worse outcome to inflammation rather than the lack of surgical procedure. However, a small, randomized trial conducted by Althusius et al concluded rescue cerclage plus indomethacin and bed rest were superior to cerclage plus bed rest alone. Thus, candidate selection for rescue cerclage and the involvement of inflammation remains an issue.

Cerclage failure manifests as either preterm labor with intact membranes or labor preceded by preterm premature rupture of membranes (PPROM). Although it is not unusual for preterm labor to be associated with PPROM, they are usually considered separate entities. At least 4 distinct pathophysiologic pathways are implicated in the triggering of preterm birth: stress, excessive myometrial stretch, decidual hemorrhage, and infection or inflammation.

Decidual hemorrhage and the resulting placental abruption is recognized histologically in up to 60% of spontaneous preterm deliveries, yet is associated with recurrent vaginal bleeding in just 20%. Hemosiderin deposition is common, which demonstrates that the bleeding had occurred at least 24 to 48 hours before delivery. Hemosiderin deposition in the placental basal plate and extraplacental decidua is associated with lower mean birth weight percentiles in deliveries at <32 weeks of gestation.

We hypothesize that preterm labor and incompetent cervix share pathophysiologic elements and that either intra-amniotic inflammation (as reflected in the MR score) or decidual hemorrhage (as identified by the presence of hemoglobin chains), occur in at least some women with incompetent cervix. We further hypothesize that the success of rescue cerclage reflects their absence. We tested these hypotheses in a consecutive series of

**Conclusion:** These findings illustrate 2 pathologic mechanisms that are associated with preterm delivery are also associated with incompetent cervix. Either an intrauterine inflammatory response or decidual hemorrhage predates surgery in one half the women whose condition requires rescue cerclage. The activation of either mechanism predicts cerclage failure.

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women with cervical dilation in the second trimester resulting from incompetent cervix and no clinical evidence of chorioamnionitis who received care at a single institution.

Material and methods

Cervical incompetence was diagnosed in 37 consecutive women with painless dilation (>2 cm) and no detectable uterine activity for a mean of 4 hours (range, 1-24 hours) before cerclage. In each instance, bulging but intact membranes were visualized at the level of the external cervical os. Each woman was afebrile and free of symptoms of chorioamnionitis at the time surgery. An emergency cerclage (McDonald type purse string with 3 mm Mersilene) was performed under general anesthesia after amnioreduction to reduce the intra-amniotic fluid pressure. A balloon device was used to hold the membranes above the cervix during suture placement. A cohort of 39 women with a sonographically normal pregnancy and cervix who underwent amniocentesis for chromosomal testing during the same time interval at the same clinical site provided the control amniotic fluid samples. All samples were centrifuged to remove cellular debris and stored at −80°C. The samples were collected under a research protocol that was approved by the Institutional Review Board at the Hallym University School of Medicine and Kangnam Sacred Heart Hospital, Seoul, Korea; all women provided written informed consent. Each amniocentesis and cerclage was performed by a single investigator (K.L.).

After delivery, the samples were coded by one of the investigators (K.L.) and transported to the remaining investigators without the key code. The samples were imported on dry ice with Centers for Disease Control approval and in accordance to all federal regulations. The proteomic fingerprint was generated; the MR score (0-4) was calculated, and hemoglobin peaks were sought. The results were transmitted back to the investigator; the blinding was broken, and the relationship between the MR score, hemoglobin chains, and clinical outcome of the rescue cerclage was assessed.

Protein profiling protocol for SELDI–TOF mass spectrometry

The technique was described previously. Briefly, 2 μL of 10-fold diluted amniotic fluid in phosphate-buffered saline solution (PBS) was placed on spots of duplicate H4 arrays (8-spot H4 array; Ciphergen Biosystems). Some spots were covered with PBS alone. After a 1-hour incubation in a humidified chamber, the sample was aspirated, and the spots were washed individually with 25% aqueous acetonitrile solution, air-dried, and covered with energy absorbing molecule (EAM) matrix solution of either 1 μL of 20% saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 25% trifluoroacetic acid/50% acetonitrile on one array, or 2 sequential applications of 0.5 μL saturated solution (in 0.5% trifluoroacetic acid/50% acetonitrile) of sinapinic acid (SPA) on the other.

The arrays were read in the ProteinChip Reader (model PBS II; Ciphergen Biosystems) using the ProteinChip software (version 3.0; Ciphergen Biosystems). SELDI tracings were examined in 3 mass ranges: 3000 to 4000 Da (CHCA), 10 to 12.5 kDa (SPA), 14 to 17 kDa (SPA). The data for the MR score peaks (P1-P4; Figure 1) were extracted from the 3000 to 4000 Da (CHCA: P1 and P2) and 10 to 12.5 kDa (SPA: P3 and P4) ranges. The data for the hemoglobin peaks were analyzed in the 14 to 17 kDa region (SPA: P5 and P6; Figure 1).

Calculation of the MR and hemoglobin scores

The presence or absence of a peak was determined objectively from numeric data with an algorithm that defines a peak that was based on the relative difference in signal-to-noise ratio (S/N) compared with the tracings that were obtained from the PBS-covered spots at the corresponding mass value. A peak was present and assigned a value of 1 if its S/N exceeded the average S/N ± 2 SD from PBS spots (n = 5) at the corresponding mass.

The MR score was derived originally by non-hierarchical analyses of SELDI tracings of amniotic fluid from women with and without intra-amniotic inflammation because of bacterial infection and a data-mining algorithm that was based on the sequential application of 5 Boolean logic criteria. In the present study, the peaks that composed the MR score were identified by their conspicuous aspect at or in proximity to their known respective masses: 3377.0 Da and 3448.1 Da on the CHCA tracing (corresponding to neutrophil defensin-2 and –1, respectively) and at 10443.8 Da and 10834.5 Da (corresponding to calgraulnul C and A, respectively).

The reported SwissProt masses of the alpha and beta hemoglobin chains are 15126.4 and 15867.2 Da, respectively. We recognize that there also may be a component in the later peak of the delta (15924.29 dalton) or gamma chains (16,009 dalton) that is not distinguished from the beta chain. Both alpha and beta chains were present in all instances where hemoglobin was detected. Analogous to the MR score, the presence or absence of hemoglobin peaks was based objectively on the S/N ratio of the PBS tracings, and a score (hemoglobin score) of 0 (hemoglobin chains absent) or 1 (hemoglobin chains present) was assigned for their absence or presence, respectively.

Statistical analysis

Because surgical procedures were conducted across a wide range of gestational ages, we sought to remove
Figure 1  Representative protein profiles of amniotic fluid from 5 patients who underwent rescue cerclage (cases 1-5) and one control patient (Crl-6). A, CHCA; B, SPA. The arrowheads indicate the peaks that compose the MR score (P1, neutrophil defensin-2; P2, neutrophil defensin-1; P3, calgranulin C; P4, calgranulin A) or representative hemoglobin (P5 and P6, alpha and beta hemoglobin chains). The x-axis of the tracings represents the molecular weight in daltons (Da) and kilodaltons (kDa); the y-axis represents normalized peak intensity. R notes a reference protein peak that was present in all (R1) or most (R2) fluid samples. PBS was the sample diluent. The S/N ratio from the PBS tracing was used to estimate objectively the presence or absence of a peak in the samples of amniotic fluid.
any positive bias by normalizing the data to reflect the degree a pregnancy was prolonged after operation. First, we calculated the “latency period” interval, defined as the number of days gestation at delivery minus the number of days of gestation at amniocentesis. Second, we calculated the “percentage prolongation of pregnancy” by dividing the latency period by the number of days of gestation at amniocentesis times 100. We recognize that this calculation actually underestimates the benefit of cerclage because the standard gestational age that is based on the last menstrual period includes 2 weeks at the beginning when the pregnancy does not yet exist. Statistical analyses included testing for normality with the Kolmogorov-Smirnov test, the Student t test, the Mann Whitney test, the Kruskal Wallis analysis of variance on ranks followed by Dunn’s tests and survival analysis, as appropriate. Comparisons between proportions were performed with the Fisher’s exact test. A probability value of ≤.05 was assumed to indicate a significant difference among means, medians, or proportions.

Results

The clinical characteristics of the study groups are summarized in Table I. The average gestation at amniocentesis in the rescue cerclage group was 23.5 weeks (95% CI, 22.2-24.8 weeks) compared with 19.5 weeks (95% CI, 18.5-20.5 weeks) in the control group (P < .001, t-test). The median cervical dilation at cerclage was 4 cm (range, 2-9 cm). Thirty-two women (86%) in the rescue cerclage group were delivered preterm: 10 women (27%) were at <24 weeks of gestation, and 22 women (59%) were between 24 and 37 weeks of gestation. Cervical dilation at cerclage was correlated inversely to both outcome measures (latency period: r = −.398; P < .02; percent prolongation of pregnancy: r = −.378; P = .02).

Women in the rescue cerclage group were delivered at an average of 28.8 weeks of gestation (95% CI, 26.7-30.9 weeks), with a median latency period of 21 days (range, 0-148 days). Identifiable postoperative complications that were related to preterm delivery were intraoperative iatrogenic rupture of membranes (2 cases), late abortion (<20 weeks of gestation, but at >12 hours after cerclage; 3 cases), preterm contractions with intact membranes (11 cases), fever (>38°C; 2 cases), spontaneous PROM (with or without preterm contractions, 9 cases) or abruptio placenta (1 case).

In contrast to the cerclage group, all women in the control group were delivered at term, with a mean gestation of 39.2 weeks (95% CI, 38.9-39.6 weeks) and a median latency period of 143 days (range, 56-170 days).

SELDI tracing analysis

Representative SELDI tracings from 5 patients who were treated by rescue cerclage and 1 control patient who underwent genetic amniocentesis are given in Figure 1. The baseline noise level on the respective CHCA and SPA SELDI readings is illustrated by the tracing that included PBS instead of amniotic fluid. The peaks of the MR score (P1-P4) and those corresponding to the hemoglobin chains (P5 and P6) are shown at their expected masses. Two additional, conspicuous peaks were present in the mass regions of interest in most of the tracings (reference peaks, R1 and R2). The R1 peak was present in all fluids that were studied. We previously identified the R1 peak as a fragment of beta-2 microglobulin.23 Its characteristic SELDI peak was used for rapid orientation to the mass axis of the SPA spectra. The R2 peak was less conspicuous than the R1 peak under these experimental conditions and was present on only 34 of the tracings (67.5% of cases and 23% of control subjects). Although these proportions differ (P < .01, Fisher’s exact test), the R2 peak does not fulfill our criteria for a disease biomarker (all biomarker peaks must be absent in control subjects).20,21 Table II shows the average experimental masses of the biomarker peaks, with Boolean indicators of 1 observed in the 76 samples of amniotic fluid that were included in this study.

Ten of 37 rescue cerclage patients (27%), but no control subjects, had an MR score of 3 to 4 (P < .001, Fisher’s exact). Twelve women in cerclage group had hemoglobin peaks (hemoglobin score, 1). The women with hemoglobin present each showed both the alpha and beta peaks (hemoglobin score, 1). No control patient sample had hemoglobin peaks. Seven women had both an MR score of 3 to 4 and hemoglobin score of 1. Thus, slightly >40% (15/37) of the women had an abnormal proteomic profile.

Excluding the 2 women with accidental intraoperative rupture of membranes, women with cerclage with an MR score of 3 to 4 were delivered earlier than women with an MR score of 0 to 2 (P < .05, Mann Whitney). Women with an MR score of 3 to 4 had a shorter latency period (median, 3 days; range, 0-105 days) and a shorter percentage of prolongation (median, 1.8%; range, 0%-62.8%) compared with women with scores of 0 to 2 (latency period, 35 days; range, 0-148 days; P < .04 Mann Whitney; median percentage prolongation, 17.9%; range, 0-116.5%; P < .03, Mann Whitney).

Women with hemoglobin peaks were delivered earlier that women without (latency period: hemoglobin score 0; median, 38 days; range, 0-148 days; vs hemoglobin score 1; median, 6 days; range, 0-100 days; P < .04; percentage of prolongation: hemoglobin score 0; median, 21.8%; range, 0.7%-116.5%; vs hemoglobin score 1; median, 3.8%; range, 0-56.8; P = .03, Mann Whitney). Three of 10 women (30%) with an MR score of 3 to 4 had no hemoglobin score; 5 of 12 women (42%) with a hemoglobin score had an MR <3 (P = no significance). There was no relationship between the
degree of cervical dilation and the presence of hemoglobin.

The combination of the MR score and hemoglobin proved most valuable in the prediction of cerclage success. Women with an MR score <3 and no hemoglobin had a median latency period of 40.5 days (range, 1-148 days) compared with women with either an MR score of 3 to 4 or hemoglobin of 1 (but not both) whose median latency period was 14 days (range, 0-105 days) compared with women with both an MR score of 3 to 4 and hemoglobin of 1 whose median latency period was 3 days (range, 0-43 days). Likewise, the percentage of prolongation among these 3 groups declined stepwise from 23.3% to 9.6% to 1.9% (survival analysis using log rank chi-squared test, 7.69; \( P = .005 \); relative risk, 10.0; 95% CI, 9.6-10.4; Figure 2).

### Table I Patient, amniotic fluid and outcome characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cerclage cases n = 37</th>
<th>Control subjects n = 39</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics at cerclage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)*</td>
<td>31.5 (30.5-32.6)</td>
<td>32.9 (31.5-34.4)</td>
<td>.137( ^{\dagger} )</td>
</tr>
<tr>
<td>Gestational age (wk)*</td>
<td>23.5 (22.2-24.8)</td>
<td>19.5 (18.5-20.5)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
<tr>
<td>Cervical dilatation (cm)*</td>
<td>4 (2-9)</td>
<td>0 (0-0)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
<tr>
<td><strong>Amniotic fluid SELDI analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR score 3 or 4 (n)</td>
<td>10 (27%)</td>
<td>0 (0%)</td>
<td>&lt; .001( ^{\ddagger} )</td>
</tr>
<tr>
<td>Hemoglobin score 1 (n)</td>
<td>12 (32%)</td>
<td>0 (0%)</td>
<td>&lt; .001( ^{\ddagger} )</td>
</tr>
<tr>
<td>MR score 3 or 4 and hemoglobin score 1 (n)</td>
<td>7 (19%)</td>
<td>0 (0%)</td>
<td>&lt; .001( ^{\ddagger} )</td>
</tr>
<tr>
<td><strong>Delivery and outcome measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery (wk)*</td>
<td>28.8 (26.7-30.9)</td>
<td>39.2 (38.9-39.6)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
<tr>
<td>Birth weight (g)*</td>
<td>1190.0 (140.0-3700.0)</td>
<td>3400.0 (2106.0-4100.0)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
<tr>
<td>PPROM (n)</td>
<td>9 (25%)</td>
<td>0 (0%)</td>
<td>&lt; .001( ^{\ddagger} )</td>
</tr>
<tr>
<td>Latency (d)*</td>
<td>21.0 (0.0-148.0)</td>
<td>143.0 (56.0-170.0)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
<tr>
<td>% Prolongation of pregnancy*</td>
<td>14.3 (0.0-116.5)</td>
<td>111.5 (27.6-149.5)</td>
<td>&lt; .001( ^{\dagger} )</td>
</tr>
</tbody>
</table>

* Data presented as mean (95% CI).
\( ^{\ddagger} \) Data presented as median (range).
\( ^{\dagger} \) Student t test.
\( ^{\ddagger} \) Mann-Whitney U test.
\( ^{\cdagger} \) Fisher’s exact test. Two cases with intra-operative PPROM were excluded from the cerclage group when delivery and outcome measures were reported.

### Table II Presence and experimental masses of the biomarker peaks in the 76 patients studied

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>EAM</th>
<th>Observed mass (95% CI)</th>
<th>Peak present (n)</th>
<th>Protein identity</th>
<th>Calculated mass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MR score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>CHCA</td>
<td>3374.9 (3373.9-3375.9)</td>
<td>14</td>
<td>Neutrophil defensin-2</td>
<td>3377.0</td>
</tr>
<tr>
<td>P2</td>
<td>CHCA</td>
<td>3446.0 (3444.9-3447.1)</td>
<td>16</td>
<td>Neutrophil defensin-1</td>
<td>3448.1</td>
</tr>
<tr>
<td>P3</td>
<td>SPA</td>
<td>10441.4 (10437.2-10445.6)</td>
<td>15</td>
<td>Calgranulin C ( \text{S100 A12} )</td>
<td>10443.8</td>
</tr>
<tr>
<td>P4</td>
<td>SPA</td>
<td>10843.64 (10829.8-10857.4)</td>
<td>7</td>
<td>Calgranulin A ( \text{S100 A8} )</td>
<td>10834.5</td>
</tr>
<tr>
<td><strong>Hemoglobin chains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>SPA</td>
<td>15119.81 (15113.7-15125.9)</td>
<td>12</td>
<td>Hemoglobin ( \alpha )-chain</td>
<td>15126.4</td>
</tr>
<tr>
<td>P6</td>
<td>SPA</td>
<td>16134.75 (16125.8-16143.6)</td>
<td>12</td>
<td>Hemoglobin ( \beta ) or ( \delta )-chain or ( \gamma )-chain</td>
<td>15867.2 or 15924.3 or 16009.0</td>
</tr>
</tbody>
</table>

EAM, Energy absorbing molecule (matrix: CHCA - \( \alpha \)-cyano-4-hydroxycinnamic acid; SPA - sinapinic acid). The calculated mass is the mass reported by Swiss-Prot database (www.expasy.ch). All masses are expressed in daltons.

Comment

The causes of preterm delivery are multiple, and the clinical distinction between preterm labor and incompetent cervix often is difficult. Rescue cerclage is performed typically after the unexpected discovery of cervical dilation in the absence of detectable uterine activity before viability. The success of rescue cerclage is relatively low, certainly compared with elective cerclage. It is less clear how valid this comparison is and unknown whether losses occur despite the cerclage or because of it. Likely, the two are not mutually exclusive. We have demonstrated the existence of two pathophysiologic mechanisms that are associated with preterm delivery that are also associated with incompetent cervix, absent detectable contractions. Almost one half
the women had evidence predating the rescue cerclage of either an intrauterine inflammation\textsuperscript{20,21} or presumably decidual hemorrhage.\textsuperscript{10} There is a stepwise worsening of outcome when one or both of these pathways are active. In contrast, the absence of activity along either pathway suggests that the likelihood of success is high, absent an operative complication. If these biomarkers reflect pathways of causation, their presence suggests that cervical incompetence is not necessarily recurrent in subsequent pregnancies.

Neutrophil defensins (alpha-defensins) belong to a family of cationic antimicrobial peptides. These key components of the host-defense mechanism\textsuperscript{24} exert their bactericidal activity by punching pores into bacterial membranes.\textsuperscript{25} Elevated defensin levels are found in patients with sepsis,\textsuperscript{26} meningitis,\textsuperscript{27} and cystic fibrosis.\textsuperscript{28} Calgranulins are members of the S100 calcium-binding proteins that are implicated in Alzheimer's disease, cancer, cardiomyopathy, psoriasis, rheumatoid arthritis, and other inflammatory disorders.\textsuperscript{29} There are many causes of inflammation, and one of the limitations of the present study is that we do not know whether the cause of inflammation in cerclage subjects was bacterial infection. Such a determination might be clinically relevant in the future.

Similar to inflammation, evidence of decidual hemorrhage is present in many women who are delivered after spontaneous preterm labor.\textsuperscript{9,10} It is suggested that the release of thrombin leads to myometrial activation and uterine contractions.\textsuperscript{30,31} In the present series, one quarter of the women with incompetent cervix had hemoglobin in their amniotic fluid before the cerclage. Its uniform absence from the samples of control women strongly suggests that the hemoglobin that was detected was not the result of the amniocentesis, but rather predated it. Further, we have found that the simple addition of blood to amniotic fluid in vitro does not result in hemoglobin being detectable on SELDI, no doubt because of the centrifugation step. Considering the association between thrombin and uterine contraction, it is interesting to note that these women were felt relatively free of uterine activity. The observation that 30\% of women with a high MR score had no hemoglobin and that 42\% of women with hemoglobin had a normal MR score suggests that the two pathologic pathways have separate origins but can intersect as they act additively. How might bleeding be associated with cervical ripening? Neutrophils play an important role in cervical ripening. Thrombin reportedly stimulates matrix metalloproteinase expression in cultured endometrial stromal and decidual cells.\textsuperscript{32} Further, intracerebral hemorrhage is known to cause activation of both macrophages and matrix metalloproteinases in the absence of bacterial infection.\textsuperscript{33}

We cannot exclude the possibility that some unknown bias was introduced by the use of a control group that was younger in gestational age when sampled than the study group. However, the gestational age range overlapped, and both groups that were studied share a similar stage of myometrial quiescence (supported by absent uterine activity) and no clinical evidence of infection. We do not believe it would have been ethical to have enrolled a control group of women who were matched by gestational age for an unindicated invasive procedure.

The ability to identify women at risk women accurately and rapidly before cerclage creates an opportunity to intervene and improve outcome pharmacologically. There are several therapeutic possibilities. Antibiotics
alone have failed to either improve cerclage results or prolong pregnancy in women with preterm labor and intact membranes. They have also failed to prevent delivery and maintain fetal health in animal models of preterm labor that results from inflammation. Anti-inflammatory or antioxidant therapies may provide a pathophysiologically directed therapy. For example, antioxidant treatment with N-acetyl cysteine prevents preterm delivery and rescues the fetuses from inflammation-induced wastage in a model of preterm delivery that resulted from *Escherichia coli* lipopolysaccharide. Treatment with an anti-inflammatory cytokine, interleukin-10, has a similar effect. It may be possible to treat women with evidence of inflammation before operation with a combination of N-acetyl cysteine or other anti-inflammatory agents perhaps coupled with a broad-spectrum antibiotic agent that achieves high concentrations in the conceptus. Such treatment will require the rapid response time provided by SELDI (<1 hour).

In conclusion, the mid trimester loss of an otherwise healthy fetus after cervical dilation, absent detectable uterine activity, shares with preterm labor an association with intrauterine inflammation and presumed decidual hemorrhage. Although the phrase “cervical incompetence” suggests a structural weakness or deficiency, this investigation demonstrates that there is more to cervical incompetence than just physical strength. The simultaneous assessment of a number of distinct biomarkers with a convenient platform such as SELDI allows for the rapid and accurate identification of high-risk women, creating the possibility for new therapeutic interventions to reduce the failure rate of rescue cerclage.

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Dexamethasone prevents long-lasting learning impairment following a combination of lipopolysaccharide and hypoxia-ischemia in neonatal rats

Tomoaki Ikeda, MD, a, * Kenichi Mishima, PhD, b Naoya Aoo, b An Xin Liu, b Nobuaki Egashira, b Katsunori Iwasaki, PhD, b Michihiro Fujiwara, PhD, b Tsuyomu Ikenoue, MD a

Department of Obstetrics and Gynecology, Miyazaki Medical College, University of Miyazaki, Miyazaki, Japan, a and Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan b

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KEY WORDS
Endotoxin
Hypoxia-ischemia
Dexamethasone
Memory
Learning
Behavior

Objective: There are no established therapies for preventing or rescuing perinatal infection or inflammation-induced perinatal brain damage. We administered dexamethasone (DEX), a synthetic corticosteroid anti-inflammatory drug, to neonatal rats in a model of such damage induced by a combination of lipopolysaccharide (LPS) and hypoxia-ischemia (HI), which produces characteristic histologic and behavioral abnormalities.

Study design: Four hours after the injection of LPS (1 mg/kg, i.p.), 7-day-old Wistar rat pups were subjected to unilateral HI for 1 hour according to Levine’s procedure. Injections of 0.5 mg/kg of dexamethasone (DEX-treated group, n = 15) or saline (saline-treated group, n = 15) were given 4 hours before HI. A sham-operated control group received neither LPS nor HI (n = 15). We chose rats of this age because their stage of brain maturation is similar to the human neonate. Over the 7 to 16 weeks after treatment, a choice reaction time (CRT) task was used for assessment of attention processes in each group, an 8-arm radial maze task was used to test short-term memory, and a water maze task was used to test long-term memory. In the CRT task, the reward food was released when the tested animal correctly pressed a lever on the side of an illuminating lamp. The correct and incorrect lever pressings were counted. In the 8-arm radial maze task, rats were allowed to move freely, seeking a reward of food placed at the end of 1 arm. An error was defined as the choice of an arm that had already been visited. In the water maze, rats had to swim to seek a concealed platform as aversive escape motivation. At 19 weeks, the rats were euthanized, the brain was removed, sectioned coronally, and the volume of each part was measured.

Results: The striatum, cortex, and hippocampus showed reductions in volume in the saline-treated group (42.7%, 49.2%, and 34.9% decreases compared with the sham-operated controls, respectively), but this was not observed in the DEX-treated group. All learning and memory processes were impaired with the combination of LPS and HI treatment, but these deficits were almost completely prevented by DEX treatment.
There is a plethora of evidence on the association between intrauterine infection/inflammation and perinatal brain damage. In epidemiologic studies, maternal infection was associated with cerebral palsy in term and preterm birth infants. In clinical observation, periventricular leukomalacia detected by cranial ultrasonography in premature newborn infants was significantly associated with chorioamnionitis. In a bacteriologic report, bacteria were retrieved from the cardiac blood of almost all the autopsy cases that showed white matter damage of the brain. In animal experiments, fetal sheep at midterm gestation that received the bacterial product lipopolysaccharide (LPS) showed characteristic white matter damage. In contrast to this evidence, there have been no strategies developed for protection or rescue of the developing brain against infective and inflammatory insults. The use of antibiotics and optimally timed termination of pregnancy are currently performed as management measures aimed against infection; however, no measures against inflammation have been established for the developing fetal brain.

For such an anti-inflammatory strategy, corticosteroids are likely candidates for investigation. The reason for this is 2-fold. First, corticosteroids are the most investigated and stereotypical drugs for various inflammatory diseases. Second, they are widely used in cases with threatened premature delivery to accelerate fetal lung maturation, and to prevent intraventricular hemorrhage, necrotizing enterocolitis, and mortality in the newborn.

Recently, we developed an animal model of infection and inflammation-induced perinatal brain damage. In this model, 7-day-old neonatal rat brains were sensitized by bacterial endotoxin to subsequent low-grade hypoxic-ischemic (HI) insult, which by themselves cause no or little damage. This combination treatment of neonatal rats with HI and endotoxin induces characteristic long-lasting behavioral impairment associated with extended cerebral damage. This model may not exactly mimic clinical infection and inflammation-induced perinatal brain damage, for example, periventricular leukomalacia. However, relatively constant results for the long-term effects on both histologic and behavioral deficits can be obtained without the demise of the animals in this model.

In the present study, we examined the effect of dexamethasone, a synthetic corticosteroid, on both histologic and behavioral changes for up to 18 weeks in rats that had been exposed to a combination of endotoxin and HI on postnatal day 7.

Material and methods

Animals

This study was approved by the Animal Research Committee of Miyazaki Medical College. Pregnant Wistar rats were purchased from a regional vendor (Japan Charles River, Atsugi, Japan). Seven days after they gave birth, we selected 45 pups. Pups whose body weights were out of the range determined by our laboratory nomogram were excluded. All rats were housed in groups of 4 or 5 per cage from the fourth week after the HI, and were placed under restricted food intake (10 g/day, CE-2, Crea Japan, Tokyo, Japan) for the 8-arm radial maze and CRT tasks. This fasting was done from the 6th to the 15th week after the HI. They had free access to water throughout the experimental period.

Surgery and treatment

On postnatal day 7, littermates were divided into 3 groups: dexamethasone (n = 15) and saline (vehicle)-treated groups (n = 15), and a sham-operated control group (n = 15). In the dexamethasone-treated group, 0.5 mg/kg of dexamethasone (Decadron, Banyu Co, Inc, Tokyo, Japan) dissolved in 0.1 mL of normal saline was injected intraperitoneally (i.p.) 4 hours before induction of HI stress. In the vehicle-treated group, 0.1 mL of normal saline was injected in place of the dexamethasone given to the first group. Both groups received intraperitoneal application of LPS (1 mg/kg, dissolved in 0.1 mL normal saline). The sham-operated control group received 0.1 mL of normal saline, like the vehicle-treated group.

In the dexamethasone and vehicle-treated groups, HI stress was imposed according to a modification of Levine’s method. Briefly, pups were anesthetized with ether, and the left carotid artery was sectioned between double ligatures with 4-0 silk. The pups were allowed to recover for 1 to 2 hours, and then exposed to 1 hour of hypoxia in a plastic container perfused with a mixture of humidified 8% oxygen in 92% nitrogen. The temperature inside the container was kept at 33°C, the usual temperature to which rat pups are exposed.
when huddling with their mother. LPS from the same lot number was used throughout (Escherichia coli, serotype 026:B6, Sigma Chemical Co, St Louis, Mo). The sham-operated control group underwent the surgical procedure, but the ligation was not performed, and hypoxic stress was not induced.

Experimental design and general behavior

The experimental schedule for behavioral and histologic examination was the same as in our previous report (Figure 1). According to the theory of Atkinson and Schiffrin, learning and memory systems entail 3 steps: (1) attention that includes arousal, decision making, and motivation; (2) the establishment of short-term or working memory; and (3) the consolidation of long-term or reference memory. These 3 processes can be evaluated experimentally with 3 different tasks: (1) a CRT task for attention; (2) an 8-arm radial maze task for short-term (working) memory; and (3) a water maze task for long-term (reference) memory. While the detailed procedures for each task have been described elsewhere, we indicate these in brief as follows. The individuals scoring the tasks were blinded as to whether or not the animals received dexamethasone or HI stress.

Eight-arm radial maze task

The 8-arm radial maze task was started in the seventh week after HI stress. The equipment (Neuroscience, Inc, Tokyo, Japan) and protocol were as described previously. For training, each animal was placed in a circular plastic restraining ring on the platform at the middle of the maze. After 1 minute, the ring was removed, and the test animal was allowed to move freely in the maze. The trial continued until the animal had entered all 8 arms, or until 10 minutes had elapsed. The trial was performed daily for 24 days. Performance of the animal in each trial was assessed from 3 parameters: the number of initial correct choices; the number of errors, defined as choosing an arm that had already been visited; and the time taken before all 8 pellets had been eaten.

Choice reaction time (CRT) task

The apparatus (MED Associates, Inc, St Albans, Vt) and protocol were as described previously. In this system, for 1 to 2 weeks before the actual study, rats were trained to press either of 2 levers with a continuous reinforcement schedule at a fixed ratio of 1:1. Trials began with differential reinforcement of another behavior (DRO) period (random, 2-5 seconds) during which the animals had to refrain from pressing either of the 2 levers. During the CRT period (maximum 10 seconds), the time between sample presentation with the cue lamp on and pressing the correct lever was defined as the CRT, and a food pellet reward was provided through the pellet dispenser. With further lever-pressing responses, a house lamp was illuminated and the intertrial interval (ITI; 20 sec) was begun. One trial took approximately 30 seconds, and each test session consisted of 30 trials. One session was performed every day for 30 days. The variables measured were the number of correct responses and the CRT (in seconds) during the correct response, and the numbers of incorrect lever pressings during the DRO and ITI periods.

Water maze task

The apparatus (150 cm in diameter, 45 cm deep, Neuroscience, Inc) and protocol were as described previously. Each rat received 3 trials daily for 5 consecutive days. A trial consisted of placing a rat by hand into the water facing the wall of the pool, at 1 of 3 starting positions, excluding the quadrant containing the
fixed platform. During each block of 3 trials, each rat started at each of the 3 starting positions, but the sequence of the positions was selected at random. At the end of each trial, the rat was returned to its home cage. The intertrial interval time was approximately 1 minute. Performance of the test animal in each trial was assessed by 3 parameters: swimming time, swimming distance, and swimming speed, using a personal computer for behavioral analysis (AXIS-30, Neuroscience, Inc).

Quantitative histologic analysis

After completion of all experiments, animals were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally into 2 mm slices using a rat brain slicer. This was on week 18 following the HI insult. Photographs of the slice were taken immediately after brain retrieval without staining. We then measured the remaining areas (mm$^2$) of striatum, dorsal hippocampus, and cerebral cortex, as shown in the shadowed area of the Table, using NIH Image software (version 1.62, http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Results are expressed as mean ± SEM. Repeated-measures two-way analysis of variance (ANOVA) was applied to each parameter of the 8-arm radial maze task, CRT task, and water maze task, followed by Tukey’s test for post hoc analysis. Tukey’s test after one-way ANOVA was used for quantitative histologic analysis. $P < .05$ was considered statistically significant.

Results

One rat in the vehicle-treated group died after hypoxic stress; therefore, 14 rats were recruited into the behavioral study. All rats survived in the other 2 groups.
There were no significant differences in body weight or general behavior between the 3 groups.

**Eight-arm radial maze**

The 8-arm radial maze testing was started in the seventh week after HI treatment (Figure 2). For the correct choices and running time, there were no significant differences between the 3 groups (data not shown). On the other hand, there were significant differences in the errors between the 3 groups. The repeated measures two-way ANOVA of the errors revealed an effect of group (F[2, 34] = 8.116, P < .01), an effect of block (F[7, 238] = 27.577, P < .001), and an effect of group × block (F[14, 238] = 1.765, P < .05; Figure 2, A). The average number of errors in all blocks was 2.7 ± 0.2, 3.6 ± 0.3, and 2.5 ± 0.1 in the sham-operated, vehicle-treated, and dexamethasone-treated HI + LPS-operated groups, respectively (F[2, 34] = 8.904, P < .001, one-way ANOVA; Figure 2, B). The vehicle-treated group had significantly increased errors in comparison with the sham-operated control group (P < .01, Tukey’s test; Figure 2, B), and dexamethasone-treated group had significantly decreased errors in comparison with the vehicle-treated group (P < .01, Tukey’s test; Figure 2, B).

**CRT task**

The CRT task was started in the tenth week after HI treatment (Figure 3). There were significant differences in the correct response between the 3 groups. The repeated measures two-way ANOVA for the correct responses revealed effect of block (F[9, 378] = 308.531, P < .001), an effect of block (F[7, 238] = 27.577, P < .001), and an effect of group × block (F[14, 238] = 1.765, P < .05; Figure 2, A). The average choice reaction time in all blocks was 1.61 ± 0.02 seconds in the sham-operated group, and 2.02 ± 0.11 seconds and 1.69 ± 0.04 seconds in the vehicle and dexamethasone-treated HI + LPS-operated groups, respectively (F[2, 42] = 9.511, P < .001, one-way ANOVA; Figure 3, D). The vehicle-treated HI + LPS operated groups had significantly increased choice reaction times (P < .01, Tukey’s test; Figure 3, D) and dexamethasone significantly decreased the choice reaction time in comparison with the vehicle-treated HI + LPS operated group (P < .01, Tukey’s test; Figure 3, D).

There were significant differences of the number of lever pressings between the 3 groups. The repeated measures two-way ANOVA of the number of lever pressings revealed an effect of group (F[2, 42] = 12.928, P < .001), an effect of block (F[9, 378] = 88.923, P < .001), and a group × block interaction (F[18, 378] = 1.524, P = .0784; Figure 3, E). The average number of lever pressings in all blocks was 64.2 ± 3.4 in the sham-operated group, and 106.0 ± 6.0 and 84.7 ± 7.3 in the vehicle and dexamethasone-treated HI + LPS operated groups, respectively (F[2, 41] = 14.162, P < .001, one-way ANOVA; Figure 3, E). The vehicle-treated HI + LPS operated groups had a significantly increased number of lever pressings (P < .01, Tukey’s test; Figure 3, F), and dexamethasone significantly decreased the number of lever pressings in comparison with the vehicle-treated HI + LPS operated group (P < .01, Tukey’s test; Figure 3, F).

**Water maze**

The water maze was started in the sixteenth week after the HI treatment (Figure 4). There were no significant differences in swimming speed between the 3 groups (data not shown). There were significant differences of swimming time between the 3 groups. The repeated measures two-way ANOVA for the swimming times revealed an effect of group (F[2, 36] = 4.114, P < .05) and an effect of block (F[4, 144] = 79.402, P < .001). The average swimming time in all blocks was 24.8 ± 2.1 seconds in the sham-operated group, and 31.9 ± 2.7 seconds and 23.9 ± 1.5 seconds in the vehicle-treated and dexamethasone-treated HI + LPS-operated groups, respectively (F[2, 40] = 4.909, P < .05, one-way ANOVA; Figure 3, B). The vehicle-treated HI + LPS-operated group had significantly decreased correct responses (P < .05, Tukey’s test; Figure 3, B), and dexamethasone significantly increased correct responses in comparison with the vehicle-treated HI + LPS operated group (P < .01, Tukey’s test; Figure 3, B).

There were significant differences of choice reaction time between the groups. The repeated measures two-way ANOVA in the choice reaction time revealed an effect of group (F[2, 42] = 9.571, P < .001), an effect of block (F[9, 378] = 176.637, P < .001), and a group × block interaction (F[18, 378] = 1.916, P < .05; Figure 3, C). The average choice reaction time in all blocks was 1.61 ± 0.02 seconds in the sham-operated group, and 2.02 ± 0.11 seconds and 1.69 ± 0.04 seconds in the vehicle and dexamethasone-treated HI + LPS-operated groups, respectively (F[2, 42] = 9.511, P < .001, one-way ANOVA; Figure 4, A). The average swimming length in all blocks was 463.3 ± 38.3 cm in the sham-operated group, and 688.4 ± 53.6 cm and 522.4 ± 31.0 cm in the vehicle and dexamethasone-treated HI + LPS operated groups, respectively (F[2, 35] = 9.855, P < .001, one-way ANOVA; Figure 4, B). The vehicle-treated HI + LPS-operated groups had significantly increased swimming length (P < .01, Tukey’s test; Figure 4, B), and dexamethasone significantly decreased swimming lengths revealed an effect of group (F[2, 36] = 7.755, P < .01) and an effect of block (F[4, 144] = 74.836, P < .001, Tukey’s test). There were significant differences in swimming length between the 3 groups. The repeated measures two-way ANOVA for the swimming lengths revealed an effect of group (F[2, 42] = 9.571, P < .001), an effect of block (F[9, 378] = 176.637, P < .001), and a group × block interaction (F[18, 378] = 1.916, P < .05; Figure 3, C). The average swimming length in all blocks was 1.61 ± 0.02 seconds in the sham-operated group, and 2.02 ± 0.11 seconds and 1.69 ± 0.04 seconds in the vehicle and dexamethasone-treated HI + LPS-operated groups, respectively (F[2, 42] = 9.511, P < .001, one-way ANOVA; Figure 4, A). The average swimming length in all blocks was 463.3 ± 38.3 cm in the sham-operated group, and 688.4 ± 53.6 cm and 522.4 ± 31.0 cm in the vehicle and dexamethasone-treated HI + LPS operated groups, respectively (F[2, 35] = 9.855, P < .001, one-way ANOVA; Figure 4, B). The vehicle-treated HI + LPS-operated groups had significantly increased swimming length (P < .01, Tukey’s test; Figure 4, B), and dexamethasone significantly decreased swimming length in all blocks (P < .001, one-way ANOVA; Figure 4, B). The average swimming length in all blocks was 463.3 ± 38.3 cm in the sham-operated group, and 688.4 ± 53.6 cm and 522.4 ± 31.0 cm in the vehicle and dexamethasone-treated HI + LPS operated groups, respectively (F[2, 35] = 9.855, P < .001, one-way ANOVA; Figure 4, B). The vehicle-treated HI + LPS-operated groups had significantly increased swimming length (P < .01, Tukey’s test; Figure 4, B), and dexamethasone significantly decreased swimming length in all blocks (P < .001, one-way ANOVA; Figure 4, B).
1) Correct response

A) Each block

B) All blocks

2) Choice reaction time of correct response

C) Each block

D) All blocks

3) Lever pressing

E) Each block

F) All blocks

Figure 3 Effect of dexamethasone (DEX) on attention deficits in a CRT task following combination treatment of lipopolysaccharide (LPS) and hypoxia-ischemia (HI). The task was started in week 10 after the treatment, and was performed with 30 trials per day for 30 days. *P < .05; **P < .01 (Tukey’s test).
swimming length in comparison with the vehicle-treated HI + LPS-operated group ($P < .01$, Tukey’s test; Figure 4, B).

Quantitative histologic analysis

As shown in the Table, there were significant differences in all regions (striatum, $F[2, 40] = 14.610$, $P < .01$; hippocampus, $F[2, 40] = 19.433$, $P < .01$; cortex, $F[2, 40] = 17.825$, $P < .01$, one-way ANOVA). Dexamethasone significantly decreased the brain damage in comparison with the vehicle-treated HI + LPS-operated group ($P < .01$, Tukey’s test).

Comment

In the present study, dexamethasone completely prevented decreases in the volumes of the cortex, striatum, and hippocampus at 18 weeks after the combination stress of endotoxin and HI. Full protection was also observed in 3 different learning processes: attention, short-term working memory, and long-term reference memory, which were severely impaired by combination treatment of endotoxin and HI. These results indicate that dexamethasone and perhaps other corticosteroids are potential candidates as therapeutic agents for perinatal infection/inflammation-induced brain damage.

The mechanisms of corticosteroid-induced prevention of endotoxin–HI brain damage are still not clear. In their review article, Abraham et al. indicated that the neuroprotective effects of corticosteroids may be mediated by distinct mechanisms involving (1) enhanced synthesis of neurotrophic factors, (2) enhanced synthesis of lipocortin-1, (3) down-regulation of cyclooxygenase-2, (4) their ability to attenuate lipid peroxidation, and (5) modulation of Ca ion currents. Corticosteroids are known to stimulate the production of neurotrophic factors, such as basic fibroblast growth factor and nerve growth factor, which are vital for the development and survival of certain populations of nerve cells in the brain. Corticosteroids also stimulate the production of lipocortin-1, which is known to inhibit the synthesis of prostaglandins and leukotrienes by inhibiting phospholipase A2, a key enzyme of the arachidonic acid cascade.

Furthermore, corticosteroids down-regulate cyclooxygenase-2 expression and, thus, inducible prostaglandin production independent of phospholipase activity. LPS induces cyclooxygenase-2 and, thus, dexamethasone may block this pathway in cells in the brain. Owing to their lipophilic nature, corticosteroids may accumulate in biological membranes, where they intercalate with lipid molecules, and protect them from oxygen-radial-induced peroxidation.

There are some issues when corticosteroids are used as therapeutic agents in management of infection/inflammation-induced fetal brain damage. First, in the present experiment, dexamethasone was only effective when it was administered before endotoxin-HI stress. In an unpublished study, we injected 0.5 mg/kg of dexamethasone into the peritoneal cavity of 7-day-old rats just after endotoxin-HI stress. Histologic study 7 days after the stress revealed that dexamethasone treatment did not cause any reversal of brain damage compared with saline treatment (data not shown). In the clinical

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**Figure 4** Effect of dexamethasone (DEX) on spatial learning deficits in a water maze following combination treatment of lipopolysaccharide (LPS) and hypoxia-ischemia (HI). The task was started in week 16 after the treatment, and was performed with 3 trials per day for 5 days. **$P < .01$ (Tukey’s test).**
situation, it is often difficult to diagnose the onset of intrauterine infection.

Using fetal monkeys, the signs of clinical infection including uterine contraction, maternal fever, and leukocytosis were always preceded by an increased level of proinflammatory cytokines. These facts indicate that starting an anti-inflammatory strategy using corticosteroids may be too late in clinical practice to prevent infection/inflammation-induced perinatal brain damage, when clinical signs and symptoms are only used. The second issue that we need to consider is the control of intrauterine infections, as it is well known that corticosteroids exacerbate certain infectious complications.

The present model of perinatal infection/inflammation-induced brain damage is valuable because it is the only model in which long-term behavioral and histologic outcomes can be observed. Our short-term (7-day) histologic study showed no abnormalities in a group that received endotoxin alone, and little abnormality in a group that received 1 hour HI stress alone. On the other hand, long-term (18 weeks) histologic study revealed decreased hippocampal volume in the endotoxin-alone group, and a decrease in the volumes of the hippocampus and striatum in the 1 hour HI-alone group. These histologic changes were accompanied by characteristic learning and memory deficits. The endotoxin-alone group showed an attention deficit in the CRT task. The 1 hour HI-alone group showed a long-term memory deficit in the water maze task, in addition to an attention deficit. The combination of endotoxin and HI produced deficits in short-term memory, long-term memory, and attention, which accompanied significant histologic changes in the cortex, striatum, and hippocampus. These abnormalities in 3 different steps of memory observed with the combined endotoxin and HI insults were almost completely prevented by dexamethasone treatment. In our previous study, 0.5 mg/kg of dexamethasone injected on postnatal day 7 did not show any adverse effects on histologic and behavioral changes, including learning and memory ability.

In order to obtain the similar beneficial effect in human pregnancy, it is important to consider both starting time of corticosteroids therapy relative to the onset of infection/inflammation and control of infection. Therefore, precise diagnosis for presence and severity of intrauterine infection/inflammation is mandatory. One possible indication for fetal brain-oriented therapy with corticosteroids is acute chorioamnionitis immediately before or during delivery without signs of fetal hypoxia, because HI stress might not be yet imposed on inflammatory stress in the brain.

It is concluded that dexamethasone treatment is effective in prevention not only of histologic brain damage, but also of learning and memory impairment following subsequent endotoxin-HI insult. This impact of dexamethasone identifies potential therapeutic pathways once the mechanism of dexamethasone’s protection is determined, warranting future clinical investigation.

References

No phenotype associated with established lipopolysaccharide model for cerebral palsy

Sarah H. Poggi, MD,a,b,* Jane Park,a Laura Toso,a,c Daniel Abebe,a Robin Roberson,a Jade E. Woodard,a Catherine Y. Spong, MDa,b

Unit on Perinatal and Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md, aDepartment of Obstetrics and Gynecology, Georgetown University Hospital, Washington, DC, b and National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Md

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Objective: Cerebral palsy (CP) is associated with childhood spasticity, seizures, and paralysis. Oligodendrocyte damage resulting in periventricular leukomalacia (PVL) in the developing brain has been implicated. Animal models of CP have used prenatal hypoxia and infection with histopathology of PVL as the end point. To evaluate whether this histologic end point is associated with a CP phenotype, we reproduced a lipopolysaccharide (LPS) model for PVL,1 and evaluated developmental, behavioral, and motor outcomes.

Study design: On gestational day 15, Fischer 344 rats were intracervically injected with .1 mg/kg LPS (n = 5) or saline (n = 4). After delivery, evaluation for developmental milestones was performed on days 1 to 21 (LPS = 45; control = 30 pups). Males were also tested at 2.5 months using open-field, rotarod, and anxiety tests. On day 21, 2 pups/litter were perfused for immunohistochemistry, and stained with 2 oligodendrocyte antibodies: 2', d'-cyclic nucleotide phosphodiesterase (CNP), and myelin proteolipid protein (PLP) with relative densities of staining assessed using NIH Image software. Statistical analysis included Mann-Whitney U and analysis of variance (ANOVA).

Results: LPS pups demonstrated decreased CNP (P = .04) and PLP (P = .06) staining, replicating the model. There was no difference seen in neonatal weight, righting, negative geotaxis, cliff aversion, rooting, forelimb grasp, audio startle, air righting, eye opening, and activity. Surprisingly, LPS-exposed neonatal rats mastered forelimb placement (P < .01) and surface righting (P = .02) earlier than control rats. There were no differences between adult groups in open field distance traveled (P = .8), open-field locomotion time (P = .6), rotarod (P = .6), or anxiety (P = .7).

Conclusion: Histologic evidence of white matter damage can be replicated using an LPS model for intrauterine inflammation. Significant phenotypic differences consistent with the motor and...
Cerebral palsy (CP) is a debilitating motor disorder resulting from white matter damage to the brain, particularly in the periventricular region.1 The damage can occur in utero, during delivery, or in the first 2 years of life, and does not change with time.2 CP affects between 1 and 3 per 1000 live births worldwide, and it is estimated that approximately 100,000 Americans under 18 years of age have the disorder.2,4 A wide spectrum of motor abnormalities is seen, among the most common symptoms are spasticity, inability to walk, mental impairment, rigidity, and seizures.2,4,5 Presently, there is no cure for the disorder,4 treatment options range from physical and behavioral therapies to drug therapies that target individual symptoms of the disorder.2

It was previously believed that the leading cause for CP was lack of oxygen during birth.4 Presently, however, an increasing body of evidence has demonstrated a link between CP and intrauterine inflammation.6-9 It has been shown that intrauterine inflammation during pregnancy leads to an increased occurrence of periventricular leukomalacia (PVL), or perinatally acquired white matter damage.5,10 PVL is strongly correlated with the ultimate development of CP.11 Studies have shown that PVL can result from lipopolysaccharide (LPS, also known as endotoxin) proinflammatory action.1,10 LPS is the bacterial cell wall of gram-negative bacteria. It is a potent inflammatory agent that initiates components of an inflammatory response.1,10,12

Previous models for PVL include a chemically induced model using the glutamate agonist ibotenate,13 unilateral carotid artery ligation and hypoxia exposure,14,15 bilateral carotid artery ligation,16 and infectious models using live bacteria.17,18 Infectious models may be the most clinically relevant because the predominant clinical association with cerebral palsy is antecedent infection, particularly in preterm neonates. However, models using live bacteria have a high maternal and fetal morbidity/mortality that limits their usefulness. A recently proposed model for CP involves the intracervical injection of LPS initiating an inflammatory response.1 Using this model, treated rat pups demonstrate the histopathologic changes (immunohistochemical staining against oligodendrocytes) associated with PVL.

This promising model is clinically relevant, technically feasible, and demonstrates a standard histopathologic outcome. However, to date, the motor or cognitive phenotype has not been evaluated. Our goals in the present study were (1) to reproduce this model of intrauterine inflammation in rats using LPS as the inflammatory stimulus, as evidenced by white matter damage with immunohistochemical staining against oligodendrocytes at postnatal day (PND) 21, and (2) to evaluate whether this histologic end point is associated with a CP phenotype by evaluating developmental, behavioral, and motor outcomes in neonatal and adult animals.

Material and methods

Intracervical injections

Pregnant Fischer 344 rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind) at embryonic day (E) 13 and acclimatized for 48 hours. Animals received humane animal care in compliance with the National Institutes of Health guidelines for Care and Use of Experimental Animals, and this protocol was approved by National Institute of Child Health and Human Development Animal Care and Use Committee. Rats were kept in a 12-hour light/12-hour dark regimen with food available at all times.

LPS (Escherichia coli serotype 0111:B4) was obtained from Sigma (St Louis, Mo). Before performing the experiments using LPS injections, pilot experiments were performed on other animals using methylene blue dye to finesse technique, and to assure confinement of the dye to the reproductive tract without intraperitoneal extravasation. On E15, the pregnant rats were anesthetized using 4% isoflurane delivered by face mask, and the cervixes were visualized using a pediatric otoscope tip that served as a speculum. We injected 0.1 mg/kg LPS (in 0.1 mL saline) (n = 5) or 0.1 mL saline only for controls (n = 4) intracervically into the cervical stroma as described by Bell.1

Neonatal developmental tests

After delivery, evaluation for developmental milestones was performed on days 1 to 21 (LPS = 45; control = 30 pups). Beginning at 1 day of age, newborn rats were weighed daily and assessed for neonatal developmental milestones, as described by Wu et al.19 In order to reduce the risk of selection bias, all the newborns were tested. Observers blinded to prenatal treatment group assessed (1) surface righting (the times in seconds for pups placed supine to return to prone position with all 4 paws on the ground), (2) negative geotaxis (time in seconds for pups placed head down on a 45° incline to turn 90° and begin to crawl up the slope), (3) cliff
aversion (time in seconds for pups positioned with forepaws and snout over the edge of a shelf to turn and begin to crawl away from the edge), (4) rooting (head turns toward the side of the face being stroked with a cotton swab), (5) forelimb grasp (pups could remain suspended for at least 1 second after grasping a thin rod with their forepaws), (6) audio startle (pups respond with a quick involuntary jump after a small metal object is dropped on a lab bench 10 inches from the pup), (7) air righting (pups released upside down from a height of approximately 60 cm turn right-side up and land on all 4 paws on a bed of shavings), (8) eye opening (open eyes noted), (9) forelimb placement (pup grasps dowel stroked against dorsal surface of paw), (10) open-field activity assessment (time in seconds for pups to move off a circle 13 cm in diameter), and (11) ear twitch (first day that the ear twitches after stimulation with the tip of a cotton swab). This battery of tests provided an assessment of development throughout the neonatal period because the behaviors measured were each expressed at differing periods throughout the first 21 days of life. All timed responses were limited to a maximum of 30 seconds, and a nonresponding animal was scored as 30 seconds for continuous variables and with “no” for categorical data. Responses were evaluated based on the first day the skill was exhibited. All tests were performed by observers working in pairs.

**Adult motor tests**

Young adult male offspring (2.5 months) were tested using open-field activity assessment, the most standard form of behavioral and motor assessment; the animal is placed on a 75 cm × 60 cm × 28 cm open table, and observed for 45 minutes in 1-day trial. Behavior is analyzed with Ethovision (Noldus, Va) video tracking system for locomotion time, total distance traveled, locomotion frequency, and mean velocity recorded. Following open-field testing, the same adult males were tested on the accelerating rotarod. Using the AccuRotor Rotarod, (Accuscan Instruments, Columbus, Ohio), the rat is placed on the cylinder at a slow rotational speed (4 rpm), which is gradually increased to a maximum speed of 60 rpm. Four trials a day were completed, for a total of 5 days, with the latency to fall off the rod as the main outcome. Different animals were also tested for anxiety: animals were allowed to stay in a box (NIMH Instrumentation group, NIH, Bethesda, Md) composed of two compartments, dark and clear (clear side = 26 cm × 26 cm × 30 cm; dark side = 26 cm × 17 cm × 30 cm) for 5 minutes. Time spent in transparent compartment and number of exits to transparent compartment were recorded and analyzed with EthoVision (Noldus, Va).

**Immunohistochemistry**

On postnatal day 21, following neonatal developmental tests, 2 randomly chosen pups/litter (total of 10 LPS and 8 control animals) were anesthetized using 4% isoflurane, transcardially perfused with buffered 4% paraformaldehyde, and the brains immersed in parformaldehyde for 24 hours (10% followed by 20% sucrose). Using a cryostat, 20-µm sections were cut, mounted on slides, and frozen. From each pup, 2 slides from neuroanatomic levels that included the corpus callosum were quenched for endogenous peroxidase for 30 minutes in 4:1 methanol: 3% hydrogen peroxide in phosphate-buffered saline solution (PBS), sections were washed in PBS, and blocked for 30 minutes in 1.5% horse serum in PBS. Slides with adjacent sections were incubated overnight in 4°C in either 1:100 CNP (2', 3'-cyclic nucleotide phosphodiesterase antibody, clone 11-5B) (Sigma, St Louis, Mo), or 1:50 PLP (myelin proteolipid protein antibody clone p1pc1) (Oncogene Research Products, San Diego, Calif), and then incubated for 30 minutes at room temperature with biotinylated secondary antibody (1:400). Antibody localization was performed using avidin-biotin complex method (Vectastain, Vector Laboratories, Burlingame, Calif), followed by dianminobenzidine. Alternate sections were processed with no primary antibody to detect nonspecific staining (Sigma). The relative densities of staining minus the mean background staining in the corpus callosum were assessed using NIH Image software.

**Statistical analysis**

**Neonatal behaviors**

The data for each behavior variable were analyzed using unpaired Mann-Whitney statistics.

**Adult behaviors**

Small open-field data and anxiety test data were analyzed using analysis of variance (ANOVA). Accelerating rotarod data were analyzed using repeated-measures ANOVA. $P < .05$ was considered significant.

**Immunohistochemistry**

Relative immunostaining density was analyzed by ANOVA. $P < .05$ was considered significant.

**Results**

**Immunohistochemistry**

A total of 75 pups delivered at E21, with 7 to 12 pups per litter, and all survived to PND 21. Animals and
mothers appeared healthy. Replicating the model, we observed a decreased intensity of staining for CNP in LPS pups than in control pups (relative intensity $\pm$ SD; LPS 45.507 ± 15.477 vs control 49.967 ± 19.411). Similarly, there is a decreased intensity of staining for PLP in LPS pups than in control pups (relative intensity $\pm$ SD; LPS 68.638 ± 14.198 vs control 72.365 ± 15.514).

Neonatal developmental tests

There was no significant difference in neonatal weight from PND0 through PND21. There was also no significant difference in the first day of performance for open-field activity, air righting, audio startle, cliff aversion, ear twitch, eye opening, forelimb grasp, forelimb placing, negative geotaxis, or rooting (Table I). Surprisingly, neonatal LPS rats showed a significantly earlier first days of performance in surface righting than control animals ($P = .02$) (Table I). Fifty percent of the LPS-exposed animals completed surface righting 2 days before the controls accomplished the task (Table I). Alternatively, 50% of the control animals completed air righting 1 day before the LPS-exposed animals (Table I), although the difference was not significant when the entire group was analyzed ($P = .16$) (Table I).

Adult motor tests

Treatment with LPS did not significantly alter adult motor function as assessed by open-field distance traveled, open-field locomotion time, or rotarod performance (Table III).

Comment

We replicated a model for PVL using LPS with alterations in CNP and PLP staining in the corpora callosa of PND 21 neonates. CNP and PLP are known oligodendrocyte markers, which are differentially expressed in oligodendrocyte development, with CNP seen in the immature oligodendrocyte and PLP in the more mature oligodendrocyte.21 Damage to oligodendrocyte precursors has been implicated in the pathogenesis of white matter damage/periventricular leukomalacia.22 Both CNP and PLP are distributed throughout the cell body and processes, and become incorporated in the myelin sheath. Thus, a change in these markers can be caused by an altered number of oligodendrocytes within the corpus callosum, an altered number of cellular processes of the oligodendrocytes, an altered expression of these 2 proteins, or to a combination of these processes. Despite immunohistochemical findings of white matter damage in the present study, we did not observe alterations in neonatal developmental behaviors. In addition, there was no discernable difference in the motor outcome of young adult animals, an observation of particular relevance in this disease in humans because the diagnosis of CP is typically made in childhood, not infancy.

Previous models for PVL, both hypoxic and inflammatory in basis, have typically demonstrated histopathologic findings of white matter injury without evaluation of long-term phenotypic changes. Intracerebral injection of the glutamate agonist ibotenate in PND 5 rat pups produced characteristic periventricular white matter cysts.13 Many of the treated animals were noted to have seizure activity and apnea, with several deaths noted following the procedure. No neurodevelopmental phenotype or long-term histologic damage was assessed in this model. Following bilateral carotid artery ligation on PND 5, rat pups were sacrificed on PND 7, and white matter changes were demonstrated.16 Though ligated animals seemed to feed less than controls, they were not noted to be significantly smaller or have any seizures; no other phenotypic assessment was performed.

Importantly, in 1 model, a long-term effect of neonatal ischemic-hypoxic brain injury on rat motor ability was noted following unilateral carotid artery ligation at PND 7 with hypoxic chamber exposure.15 Despite grossly normal motor appearance, significant differences were demonstrated in treated animals when assessed by rotarod and apomorphine-induced motor behavior between 3 and 9 weeks of age. Histopathologic findings in the young rat brains revealed gross atrophy of the sensorimotor cortex following neonatal ischemia-hypoxia.

Figure 1 Relative density of anti-2', 3'-cyclic nucleotide phosphodiesterase (CNP) and anti-myelin proteolipid protein (PLP) staining in rat pups at postnatal day (PND) 21 after mothers had been injected with 0.1 mg/kg of LPS or saline at E15. There is a decreased intensity of staining for CNP in LPS pups than in control pups (relative intensity $\pm$ SD; LPS 45.5 ± 15.4 vs control 50.0 ± 19.4, $P = .04$; Figures 1 and 2). Similarly, there is a decreased intensity of staining for PLP in LPS pups than in control pups (relative intensity $\pm$ SD; LPS 68.6 ± 14.2 vs control 72.4 ± 15.5, $P = .06$; Figures 1 and 2).
In Yoon et al’s experiments with an infectious model using intrauterine inoculation of pregnant rabbits with *Escherichia coli*, the primary outcome was histologic evidence of white matter damage, including karyorrhexis, rarefaction, disorganization of the white matter, and immunohistochemical evidence of apoptosis. Similar white matter injury has been observed following both systemic endotoxemia or asphyxia in fetal sheep. In these infectious models no phenotypic evaluation of motor impairment was reported.

In the models using LPS as an inflammatory agent, Bakos et al injected pregnant rats subcutaneously with increasing doses of LPS from E15 through E19, and interestingly they found motor impairment in 3-month-old animals, as well as a deficit in weight gain at the weaning age. This model is unique in evaluating and identifying a long-term motor impairment, but was using LPS as a marker for stress exposure, and the route of administration resulted in a systemic response. This may not model the chronic inflammatory condition purported to underlie the human condition associated with CP, which is presumably local in origin. Furthermore, the evaluation of adult behaviors in this model used female offspring, which may be less reliable due to hormone cycling.

Other infectious and inflammatory models using LPS injection are predominately designed to study preterm delivery. As a consequence of preterm delivery, the pups do not survive and are not candidates for developmental or motor testing. Such models include intrauterine or systemic LPS injection of pregnant murine animals.

Bell et al created an inflammatory model for white matter brain damage through intracervical injection of LPS at E15 of gestation. Low doses of LPS induced apoptosis of the mature and immature oligodendrocytes within the periventricular area without inducing preterm delivery or fetal death.

![Figure 2](image)

**Figure 2** Representative immunohistochemical and Hematoxylin & eosin staining of brain sections at PND 21. Immunohistochemical staining for PLP and CNP shows a decreased density of staining in the LPS-exposed pups relative to those exposed to saline injections. Shown with 40× magnification. H&E staining shown with 25× magnification.
This model, unlike those that rely on direct intra-uterine inoculation (either via laparotomy\textsuperscript{33} or via the cervical canal\textsuperscript{26}) may reproduce a more physiologic, insidious ascending infection with an inflammatory response throughout the uterus. There is the possibility using this technique that fetuses most proximal to the cervix received a larger LPS dose, and were more affected. Our methods allowed for a natural, full-term delivery with no interference with nursing in the first 24 hours. A limitation of this model is the infeasibility of determining birth order/uterine position and implied LPS exposure severity.

### Table I  Summary of neonatal rat behavior test results

<table>
<thead>
<tr>
<th>Test</th>
<th>Start day</th>
<th>Control (n = 33)</th>
<th>LPS (n = 43)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st yes day</td>
<td>1st yes day</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>12</td>
<td>15 (12-18)</td>
<td>15 (12-17)</td>
<td>.89</td>
</tr>
<tr>
<td>Air righting</td>
<td>8</td>
<td>12 (9-16)</td>
<td>13 (10-18)</td>
<td>.16</td>
</tr>
<tr>
<td>Audio-startle</td>
<td>7</td>
<td>12 (11-14)</td>
<td>12 (10-13)</td>
<td>.86</td>
</tr>
<tr>
<td>Cliff aversion</td>
<td>2</td>
<td>12 (9-18)</td>
<td>12 (8-20)</td>
<td>.40</td>
</tr>
<tr>
<td>Ear twitch</td>
<td>7</td>
<td>10 (8-12)</td>
<td>10 (9-12)</td>
<td>.31</td>
</tr>
<tr>
<td>Eye opening</td>
<td>9</td>
<td>16 (16-17)</td>
<td>16 (16-17)</td>
<td>.24</td>
</tr>
<tr>
<td>Forelimb grasp</td>
<td>4</td>
<td>13 (9-19)</td>
<td>13 (8-16)</td>
<td>.50</td>
</tr>
<tr>
<td>Forelimb placing</td>
<td>2</td>
<td>4 (2-10)</td>
<td>4 (2-8)</td>
<td>.74</td>
</tr>
<tr>
<td>Negative geotaxis</td>
<td>2</td>
<td>8 (5-11)</td>
<td>8 (6-12)</td>
<td>.83</td>
</tr>
<tr>
<td>Surface righting</td>
<td>1</td>
<td>9 (2-12)</td>
<td>7 (2-12)</td>
<td>.02</td>
</tr>
<tr>
<td>Rooting</td>
<td>2</td>
<td>8 (2-10)</td>
<td>8 (2-12)</td>
<td>.94</td>
</tr>
</tbody>
</table>

### Table II  Days of observation for neonatal behavioral tests

![Days of observation for neonatal behavioral tests](https://example.com/tableII.png)

### Table III  Summary of adult rat behavior test results

<table>
<thead>
<tr>
<th>Test</th>
<th>Control (n)</th>
<th>LPS (n)</th>
<th>Control Mean ± SD</th>
<th>LPS Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open-field distance (cm)</td>
<td>9</td>
<td>14</td>
<td>3901.8 ± 1495.8</td>
<td>4012.0 ± 934.9</td>
<td>.83</td>
</tr>
<tr>
<td>Open-field locomotion time (s)</td>
<td>9</td>
<td>14</td>
<td>638.3 ± 333.4</td>
<td>694.025 ± 205.8</td>
<td>.62</td>
</tr>
<tr>
<td>Anxiety time in “open field” (s)</td>
<td>6</td>
<td>6</td>
<td>52.5 ± 57.6</td>
<td>42.167 ± 25.2</td>
<td>.70</td>
</tr>
<tr>
<td>Anxiety (number of exits)</td>
<td>6</td>
<td>6</td>
<td>3.0 ± 3.1</td>
<td>2.833 ± 1.9</td>
<td>.91</td>
</tr>
<tr>
<td>Rotarod*</td>
<td>9</td>
<td>14</td>
<td>NA</td>
<td>NA</td>
<td>.69</td>
</tr>
</tbody>
</table>

* Analyzed with ANOVA repeated measures.
Our goals in the current project were to reproduce an inflammation or infection-related model for PVL because of the clinical relevance to human disease. We were hopeful that Bell’s model, in which LPS induced a more moderate histopathologic change (ie, no gross architectural abnormalities, as cysts or gliosis, characteristic of PVL were seen), would generate a high proportion of surviving animals available for phenotyping across development. This expectation was met, but perhaps at the cost of a demonstrable motor deficit in the offspring. It is likely that the dose of LPS allowed for the animal to compensate for the damage with the resultant lack of a developmental or long-term phenotype.

Additional studies varying the dose or timing of the inflammatory exposure are needed to create a model with a phenotype. Importantly, it is critical when evaluating animal models to assess not only biochemical markers for human disease, but also clinically relevant phenotypes.

References


Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia

Catalin S. Buhimschi, MD,* Errol R. Norwitz, MD, PhD, Edmund Funai, MD, Susan Richman, MD, Seth Guller, PhD, Charles J. Lockwood, MD, Irina A. Buhimschi, MD

Department of Obstetrics, Gynecology and Reproductive Science, Yale University, New Haven, Conn

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KEY WORDS
Hypertensive disorder
Kidney
Preeclampsia
Urine
Vascular endothelial growth factor

Objective: Serum levels of soluble fms-like tyrosine kinase 1 (sFlt-1), vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) are altered in women with clinical preeclampsia. We sought to identify whether similar alterations in urinary levels of these proteins cluster hypertensive disorders in pregnancy, and identify women with severe preeclampsia (sPE).

Study design: Free urinary levels of sFlt-1, VEGF, and PlGF were measured by immunoassay in 68 women enrolled prospectively in the following groups: nonpregnant reproductive age (NP-CTR n = 14), healthy pregnant control (P-CTR n = 16), pregnant hypertensive and proteinuric women who did not meet criteria for severe preeclampsia (pHTN n = 21), and women with sPE (n = 17).

Results: There was no difference in gestational age at the time of enrollment among groups (median [range]: sPE: 31 [24-40], pHTN: 34 [16-40], P-CTR: 28 [7-39] wks). Urinary excretion of VEGF was significantly increased in sPE women compared with NP-CTR (P = .023), but did not differ among pregnant groups. Urinary PlGF levels were significantly increased in pregnant compared with nonpregnant women, but were decreased in all hypertensive women compared with healthy P-CTR (P < .001). Urinary sFlt-1 concentrations were significantly increased in women with sPE relative to all other groups (P < .001). PHTN women had higher sFlt-1 urinary output compared with P-CTR group (P = .001). A cutoff > 2.1 in the ratio log [sFlt-1/PlGF] had 88.2% sensitivity and 100% specificity in differentiating women with sPE from normotensive controls. We also described that the log[sFlt-1/PlGF] ratio identified women with sPE better than proteinuria alone (P = .03). Our regression model revealed that uric acid correlated best with log[sFlt-1/PlGF] ratio (r = 0.628; P = .005).

Conclusion: sPE is associated with increased urinary output of the antiangiogenic factor sFlt-1 and a decreased output of PlGF at the time of clinical manifestation, providing a rapid noninvasive screening of hypertensive women based on a sFlt/PlGF ratio. This ratio may be used as representation for severity of the disease, and appears to be superior to random urinary protein measurements.

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Preeclampsia remains a major cause of maternal morbidity and mortality complicating 6% to 8% of all gestations over 20 weeks. This multisystemic pregnancy-associated disease remains the leading cause of maternal and perinatal morbidity and mortality. Yet, despite prodigious research efforts, the etiology and pathogenesis of preeclampsia remains incompletely understood. Vascular endothelial activation followed by vasospasm appears to be the central feature in the pathogenesis of preeclampsia. It is currently believed that the preeclamptic syndrome may be the result of alteration in the expression of modulators of angiogenesis which cause hypertension, proteinuria, endothelial cell activation and increased platelet aggregation. Moreover, this alteration in angiogenic factor expression may precede clinical evidence of the disorder, and disappears with resolution of the disease. Such derangements in angiogenesis may also exert indirect effects on the maternal vasculature.

During human implantation and placentation, vascular angiogenic growth factors are thought to be critical for successful development. Defective placentalization leads to placental ischemia followed by systemic release of cytotoxic products, which damage maternal vascular endothelium. Vascular endothelial cell injury decreases synthesis of vasorelaxing agents, increases responsiveness to vasoconstrictors, impairs synthesis of endogenous anticoagulants, and increases procoagulant synthesis, causing ischemic brain, liver, and kidney dysfunctions. The existence of widespread endothelial cell injury is evidenced by the characteristic morphologic lesion of preeclampsia, glomerular endotheliosis.

It was recently shown that serum levels of soluble fms-like tyrosine kinase 1 (sFlt-1), vascular endothelial growth factor (VEGF), and placental growth factor (PIGF) are altered in women with clinical preeclampsia (gestational proteinuric hypertension), classically defined as hypertension associated with proteinuria. Moreover, increased serum sFlt-1 appears to precede onset of disease symptoms by 5 weeks, while reduced free PIGF levels becomes evident even late in the first trimester. In contrast, VEGF serum concentrations were low throughout pregnancy, and did not accurately predict preeclampsia.

The significance of angiogenic factors VEGF, sFlt-1, and PIGF in regulation of human kidney glomerular vascular physiology deserves increased attention. Exogenous sFlt-1 administered to pregnant rodents lead to hypertension, proteinuria, and glomerular endotheliosis. Similarly, neutralization of physiologic levels of VEGF, a key mitogen survival factor for glomerular vascular endothelium, leads to increased apoptosis, impaired glomerular capillary repair, and severe proteinuria in an animal model of mesangioproliferative nephritis.

Given that preeclampsia is consistently accompanied by both functional and morphologic derangements of the maternal kidney, our study proposes a new paradigm: urinary sFlt-1 and PIGF levels are altered in severely preeclamptic women at the time of disease appearance. We sought to determine whether alterations in urinary levels of VEGF, sFlt-1, and PIGF clustered hypertensive disorders, and identified women with severe preeclampsia (sPE).

Material and methods

Participants and sample collection

We studied samples of urine from 68 women admitted at Yale New Haven Hospital between February and August 2004. Samples were collected under protocols approved by the Human Investigation Committee of Yale University. Written informed consent was obtained from all participants. Gestational age was established based on menstrual date and/or ultrasonographic examination before 20 weeks’ gestation. All women solicited for enrollment in the study agreed to participate. We requested enrollment from pregnant women admitted to Labor and Delivery ward, and to the antepartum High and Low Risk Units. We enrolled patients prospectively based on the availability of one of the investigators (C.S.B.). None of the enrolled patients were excluded from the final analysis.

We enrolled women in the following groups: severe preeclampsia (sPE, n = 17), hypertensive and proteinuric disorders associated with pregnancy that did not meet criteria for severe preeclampsia (pHTN, n = 21), healthy pregnant control (P-CTR, n = 16), and healthy nonpregnant reproductive age women (NP-CTR, n = 14). Preeclampsia was defined according to established criteria as a diastolic blood pressure of at least 140/90 mm Hg and proteinuria of at least 2+ on dipstick testing, each on 2 occasions 4 to 6 hours apart. sPE was defined as HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count), blood pressure >160/110 mm Hg on at least 2 occasions 6 hours apart, >5 g in a 24-hour urinary protein excretion, or persistent +3 proteinuria on dipstick testing. Other elements of the definition included in utero growth restriction (IUGR) <10% percentile according to the US demographics, persistent neurologic symptoms (headache, visual disturbances), epigastric pain, oliguria (less than 500 mL/24 h), serum creatinine >1.0 mg/dL, elevated liver enzymes (greater than 2 times normal), thrombocytopenia (<100,000 cells/μL). Chronic hypertension (crHTN) was defined as a sustained elevation in BP >140/90 mm Hg before pregnancy or before 20 weeks’ gestation. Proteinuria was defined as >300 mg of protein in a 24-hour urine collection. To assess histologic changes induced by hypertensive disorders in placenta, we consulted the
pathology reports generated by a clinical pathologist unaware of the results of our study. Pathology reports were available in 29 out of 38 hypertensive patients, and were abstracted for presence of chorionitis, infarcts with volumes $>3$ mL, evidence of pathologic changes consistent with preeclampsia (decidual vessels without evidence of trophoblast invasion or physiologic conversion), and/or evidence of abruption (hemosiderin deposition and/or intervillous thrombus).

A random urine sample (5-10 mL/sample) was collected antepartum by standard use of sterile containers. At the time of enrollment, all sPE women had a Foley catheter placed to allow for accurate monitoring of urinary output. Sixty percent of sPE women were enrolled following initialization of the magnesium sulfate seizure prophylaxis. Nine women had urine samples collected before and also 2 to 12 hours after initiation of seizure prophylaxis therapy. Samples obtained from pHTN, P-CTR, and NP-CTR women were also collected under sterile conditions (Foley, “straight cath”) or “clean catch” techniques in nonpregnant subjects. Biochemical analyses of urinary samples were conducted in parallel. Following collection, samples were spun at 3000 g at 4°C for 20 minutes, aliquoted, and immediately stored at −80°C until sFlt-1, VEGF, and PIGF levels were measured by specific immunoassays.

**Table I** Characteristics of pregnant women with hypertension and controls at enrollment in the study

<table>
<thead>
<tr>
<th></th>
<th>P-CTR (n = 16)</th>
<th>pHTN (n = 21)</th>
<th>sPE (n = 17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y), mean (95% CI)</td>
<td>26.4 (23.7-29.1)</td>
<td>29.8 (27.2-32.3)</td>
<td>24.4 (21.6-27.2)</td>
<td>.021*</td>
</tr>
<tr>
<td>Gravity, median (range)</td>
<td>2 (1-6)</td>
<td>2 (1-8)</td>
<td>1 (1-7)</td>
<td>.063</td>
</tr>
<tr>
<td>Parity, median (range)</td>
<td>1 (0-3)</td>
<td>0 (0-5)</td>
<td>0 (0-4)</td>
<td>.250</td>
</tr>
<tr>
<td>Maternal weight (kg), mean (95% CI)</td>
<td>81.8 (74.0-89.6)</td>
<td>100 (84.7-115.3)</td>
<td>88.8 (74.8-101.6)</td>
<td>.166*</td>
</tr>
<tr>
<td>Gestational age (wk), median (range)</td>
<td>28.6 (7.0-39.0)</td>
<td>34.4 (16.6-40.4)</td>
<td>31.3 (24-1-40.2)</td>
<td>.304</td>
</tr>
<tr>
<td>Gestational age (wk), median (range) delivery</td>
<td>38.5 (37.4-39.6)</td>
<td>34.4 (32.4-36.6)</td>
<td>32 (29.5-1-34.4)</td>
<td>.003</td>
</tr>
<tr>
<td>Fetal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g), mean (95% CI)</td>
<td>3355 (3093-3617)</td>
<td>2105 (1593-2617)</td>
<td>1622 (1095-2150)</td>
<td>.001*</td>
</tr>
<tr>
<td>Clinical manifestations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mm Hg), mean (95% CI)</td>
<td>105.5 (99.1-111.9)</td>
<td>160.0 (149.7-170.4)</td>
<td>162.4 (155.7-169.2)</td>
<td>&lt; .001*</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg), mean (95% CI)</td>
<td>64.4 (58.9-70.0)</td>
<td>94.6 (89.0-100.3)</td>
<td>101.5 (94.4-108.6)</td>
<td>&lt; .001*</td>
</tr>
<tr>
<td>Neurological symptoms, n (%)</td>
<td>0.0 (0.0)</td>
<td>3.0 (1.3)</td>
<td>7.0 (4.1)</td>
<td>.008</td>
</tr>
</tbody>
</table>

Data was analyzed by one-way ANOVA (*), Kruskal-Wallis ANOVA (†), and χ² (‡).

**Immunoassay procedures**

Enzyme-linked immunosorbent assay (ELISA) assays for human free VEGF, sFlt-1, and PIGF were performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn). Samples were assayed in duplicate in a 96-well plate precoated with a capture antibody directed against free VEGF, sFlt-1, or free PIGF. Incubation protocols were performed followed by washings and reading at 450 nm in accordance with the procedure summary. The minimal detectable concentrations in the assays for VEGF, sFlt-1, and free PIGF were 5, 5, and 7 pg/mL, respectively. The interassay and intra-assay coefficients of variation varied from 3% to 10%. Protein concentrations were measured using a bicinchoninic acid/cupric sulfate reagent (BCA kit, Pierce, Rockford, Ill). We analyzed and normalized our data based on creatinine concentrations determined from the same aliquot by using standard curves derived from known concentrations.

**Statistical analysis**

We subjected all data sets to normality testing using the Kolmogorov-Smirnov method, and report our data as either mean and 95% CI (for normally distributed data), or as median with range (for skewed data). The VEGF, sFlt-1, and PIGF concentrations were presented as arithmetic means, and statistical analysis was completed before (Kruskal-Wallis analysis of variance [ANOVA]) or after (one-way ANOVA) logarithmetic transformation of data. Comparisons between 2 groups were performed using Student t tests or Mann-Whitney rank sum test. Proportions were compared with Fisher exact or chi-square tests. We applied uni- and multivariate analysis with linear regression modeling to identify significant associations between maternal or laboratory characteristics as independent variables, and ratio sFlt/PIGF as dependent variable. A Pearson or Spearman product moment correlation was used to measure colinearity between the selected independent variables, as well as other relevant correlations between dependent and independent variables. We performed receiver operator curve characteristic (ROC) curve analysis using MedCalc (Broekstraat, Belgium) statistical software. We judged $P < .05$ to indicate statistical significance.
Results

Characteristics of women

Out of 68 patients enrolled in this study, 17 met criteria for sPE. By study design, our patients were enrolled prospectively, so that at the time of enrollment we were aware only whether a subject is or is not hypertensive and proteinuric, and whether it meets or does not meet the clinical criteria for sPE. Because at the time of enrollment we could not precisely establish the nature of the hypertensive condition, the pHTN group (n = 21) was heterogeneous, consisting of women with mild preeclampsia alone (n = 9), women with mild pre-eclampsia but previous medical history of crHTN (n = 10), or hypertensive proteinuric nephropathies (n = 2, lupus and nephritic syndrome).

Compared with sPE women the pHTN group was significantly older (Student-Newman–Keuls, \(P = .021\)) (Table I). There was no difference in gestational age (GA) among groups at the time of sampling. Similarly, there was no difference in gravidity, parity, or maternal weight in our cohort. Hypertensive women (sPE and pHTN groups) had significantly higher blood pressure values compared with P-CTR (mean arterial pressure: sPE: 122, pHTN: 115, P-CTR: 77 mm Hg, \(P < .001\)). A higher proportion of sPE women manifested neurologic symptoms (Table I).

Our clinical diagnosis was supported by clinical laboratory and placental histologic changes that occurred in the hypertensive groups (Table II). sPE women had greater degrees of proteinuria when screened with the rapid urinary dipstick test. However, when we analyzed the laboratory 24-hr urinary protein excretion we could not confirm differences between the sPE and pHTN groups. Patients with sPE had higher levels of lactate dehydrogenase (LDH, indicator of intravascular hemolysis), uric acid, and a lower platelet count compared with pHTN women. Histologic evidence of abruption ( hemosiderin deposition or intervillous thrombi) was more common in pregnancies complicated by sPE (\(P = .003\), Fisher’s exact test).

Urinary levels of VEGF, sFlt-1, and PlGF

There was no correlation between urinary levels of VEGF, sFlt-1, PlGF, and GA at the time of sampling (VEGF: \(r = 0.09\), sFlt-1: \(r = 0.02\), PlGF: \(r = -0.03\), \(P > .05\)). Table III presents urinary levels of angiogenic factors (median levels and range, nonlogarithmic format). We found that women with sPE have higher urine levels of VEGF compared with the NP-CTR (Student-Newman–Keuls, \(P = .023\)). Urinary VEGF did not vary significantly among pregnant groups (one-way ANOVA, \(P = .536\)). The concentration of urinary PlGF was significantly increased in healthy pregnant women compared with NP-CTR group (Student-Newman–Keuls, \(P < .001\)). Urinary PlGF levels were significantly decreased among pHTN and sPE women compared with healthy pregnant controls (\(P < .001\)). Finally, we determined that sPE women had significantly higher urinary levels of sFlt-1 compared with

<table>
<thead>
<tr>
<th>Table II</th>
<th>Clinical laboratory characteristics within the hypertensive groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pHTN (n = 21)</td>
</tr>
<tr>
<td>Clinical laboratory tests</td>
<td></td>
</tr>
<tr>
<td>Dipstick proteinuria, median (range)</td>
<td>1.5 (0-4)</td>
</tr>
<tr>
<td>24-h proteinuria (g/dL), median (range)</td>
<td>0.9 (0.1-13.1)</td>
</tr>
<tr>
<td>AST (U/L) median (range)</td>
<td>20.0 (8.0-59.0)</td>
</tr>
<tr>
<td>ALT (U/L) median (range)</td>
<td>14.0 (4.0-32.0)</td>
</tr>
<tr>
<td>Platelets (cells/(\mu)L(\times)10(^{12})) mean (95%CI)</td>
<td>263.3 (221.5-305.1)</td>
</tr>
<tr>
<td>LDH (U/L) median (range)</td>
<td>204.0 (153.0-366.0)</td>
</tr>
<tr>
<td>Uric acid (mg/dL), median (range)</td>
<td>5.8 (5.1-6.4)</td>
</tr>
</tbody>
</table>

Data was analyzed by Mann-Whitney test (*) and Student t-test (\(y\)).

<table>
<thead>
<tr>
<th>Table III</th>
<th>Concentrations of urinary angiogenic factors measured in random void</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP-CTR (n = 14)</td>
</tr>
<tr>
<td>VEGF (pg/mgc) median (range)</td>
<td>93.5 (21.2-258.6)</td>
</tr>
<tr>
<td>PLGF (pg/mgc) median (range)</td>
<td>14.7 (7.3-21.2)</td>
</tr>
<tr>
<td>sFLT (pg/mgc) median (range)</td>
<td>10.5 (0.4-48.1)</td>
</tr>
<tr>
<td>Protein (mg/mgc) median (range)</td>
<td>6.4 (4.3-9.2)</td>
</tr>
<tr>
<td>Creatinine (mg/mL) median (range)</td>
<td>1.3 (0.3-2-5)</td>
</tr>
</tbody>
</table>

Data was analyzed by Kruskal-Wallis ANOVA (*). Values are reported per mg creatinine (mgc).
either pHTN ($P = .016$) and P-CTR ($P < .001$). pHTN women had higher sFlt-1 urinary levels compared with P-CTR group ($P = .001$). There was no significant difference in urinary sFlt-1 levels between P-CTR and NP-CTR healthy controls ($P = .594$). Within the pHTN group there was no significant difference in urinary angiogenic factors between women with mild preeclampsia alone ($n = 9$) and mild preeclamptic women with history of crHTN ($n = 10$) ($\text{VEGF: } P = .729$, $\text{sFlt-1: } P = .091$, $\text{PlGF: } P = .867$).

**Urinary ratio sFlt-1/PlGF**

Given our observation that normal pregnancy is characterized by an increased urinary PlGF excretion, while hypertensive states are characterized by increased sFlt-1 but decreased urinary PlGF, we reasoned that urinary sFlt-to-PlGF (uFP) ratio would be a better indicator of individual urinary homeostasis of angiogenic factors. We computed the following ratio indicator: $\text{uFP} = \log \frac{\text{sFlt}}{\text{PlGF} \times 10}$. We calculated that uFP was significantly elevated in women with sPE compared with P-CTR ($P < .001$) and NP-CTR ($P < .001$). There was no significant difference in uFP ratio between mild preeclamptic women vs mild preeclamptic women with a history of crHTN, ($P = .245$).

We performed an ROC analysis for the uFP, and concluded that a cutoff $>2.1$ had 88.2% sensitivity and 100% specificity in differentiating women with sPE from normotensive controls (area under the curve [95%CI]: 0.974 [0.849-0.994]) (Figure 2, A). The uFP was significantly better than proteinuria alone (dipstick testing and total protein concentration in respective sample) in clustering sPE women from normotensive controls (area under the curve [95%CI]: 0.809 [0.635-0.924], $P = .03$).

We further examined a possible effect of magnesium sulfate infusion by comparing the uFP ratio in a group of 9 women in whom urine samples were available before and after initiation of treatment. We found that uFP (paired $t$ test, $P = .854$) did not change significantly 2 to 12 hours in response to magnesium sulfate seizure prophylaxis.

To investigate possible relationships between uFP ratio and several maternal and clinical laboratory factors, we modeled the uFP ratio as dependent variable against maternal age, gravidity, parity, GA, IUGR, systolic and diastolic blood pressure, proteinuria, neurologic symptoms ($0 =$ none; $1 =$ present), liver function tests (AST, ALT), platelet count, uric acid, serum LDH, and histopathologic evidence of abruption ($0 =$ none; $1 =$ present) as independent variables. When we entered these variables into a multiple linear regression model, we found that uric acid correlated with uFP ($P = .005$ for uric acid).

In univariate analysis, we identified a significant relationship between uFP and maternal serum uric acid (Pearson $r = 0.458$, $P = .003$) (Figure 2, B), and between uFP and delivery by CD (Spearman $r = 0.514$, $P < .001$). Women delivered by cesarean section had significantly elevated uFP ratios compared with women that delivered naturally (average [95%CI] CS: 2.6 [2.4-2.8] vs SVD: 1.8 [1.8-2.3], $P = .007$), suggesting a possible increase grade of disease severity in these women. We further determined that women with an uFP ratio over 2.1 had an increased risk to deliver by cesarean section (OR [95%CI]: 6.57 [1.51-28.53]. Other variables consistent with disease severity correlated significantly with uFP: systolic and diastolic blood pressures, dipstick proteinuria (direct correlation, $P < .001$), gravidity, fetal weight at delivery, evidence of placental abruption, parity, and IUGR (inverse correlation, $P < .05$).

**Comment**

We found that urinary sFlt-1 and PlGF are altered in women with hypertensive disorders during pregnancy. We further observed that a ratio between the two
functionally opposing angiogenic factors, sFlt-1 and PlGF, has high sensitivity and specificity in differentiating women with sPE from normotensive controls. Most importantly, we discovered that the ratios between sFlt-1 and PlGF could discriminate sPE from other forms of proteinuric hypertensive disorders, including mild pre-eclampsia with or without crHTN.

Nevertheless, we identified that uFP ratio performed better than proteinuria in a random urine sample in differentiating women with sPE women form normotensive pregnant controls. Thus, our working hypothesis was accepted.

Angiogenic growth factors (VEGF and PlGF) promote vascular endothelial proliferation and differentiation, cell migration, increase vascular permeability, inhibit apoptosis, mediate endothelium dependent vasodilation, support vascular survival, growth, and proliferation of glomerular and peritubular endothelial cells. In turn, the anti-VEGF angiogenic factor, sFlt-1, regulates biological availability of VEGF by binding the angiogenic factor in the circulation. Excess sFlt-1 plays an important part in promoting glomerular endothelial hypertrophy, apoptosis, cell detachment from the glomerular basement membrane and, thus, hypertension and proteinuria. There are many other critical factors involved in the physiologic regulation of systemic or placental vessel formation, but importantly, the action of these angiogenic molecules must be very carefully orchestrated to form a functional vascular network.

Excess placental sFlt-1 may contribute to endothelial dysfunction, hypertension, and proteinuria in pre-eclampsia. Indeed, administration of exogenous Flt-1 to pregnant rats induced a syndrome characterized by hypertension, proteinuria, and glomerular endotheliosis similar to human preeclampsia. Pregnancies complicated by preeclampsia are associated with elevated circulating sFlt-1, VEGF, and PlGF. Several recently published reports have focused the attention of clinical investigators because increased levels of circulating sFlt-1 and reduced PlGF may predict the development of preeclampsia several weeks in advance. However, the urinary excretion of these angiogenic factors in women with preeclampsia has not been previously studied.

Human kidneys express mRNA for VEGF and its receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) predominantly in glomerular podocytes, distal tubules,
and collecting ducts. However, the function of constitutively expressed angiogenic factors and their receptors in human kidneys remains largely unknown. We theorize that glomerular endotheliosis results from increased exposure to sFlt-1 and reduced exposure to PlGF. Our present findings bring new perspectives. First, podocytes and mesangial cell destruction, as well as loss of glomerular basal membrane integrity, are histologic changes consistent with preeclamptic glomerular endotheliosis. Thus, urinary excretion of angiogenic factors may reflect increased placentental synthesis and their leakage from maternal circulation. This would be consistent with the previous hypothesis that urinary VEGF in glomerulonephritis may be derived from the systemic circulation. Conversely, presence of urinary VEGF, sFlt-1, and PlGF may reflect a local glomerular and tubular cell response to a hypoxic injury similarly with the mechanisms hypothesized to be responsible for secretion of placentental factors into maternal circulation. Further studies are needed to determine whether excretion of urinary angiogenic factors may reflect over-/underexpression of VEGF, sFlt-1, and PlGF, or simply reflect structurally compromised glomeruli.

Irrespective of the underlying mechanism, we have established that levels of two of the urinary circulating angiogenic factors (sFlt-1 and PlGF) are below that previously reported in maternal serum levels. However, the urinary VEGF levels were higher than the reported maternal serum levels, consistent with findings by Roes et al. Most importantly, we found that urinary level of vascular angiogenic factors mirror previously reported systemic maternal changes at the time when severe preeclampsia was already diagnosed. As such, urinary measurements may represent a truly noninvasive way to differentiate sPE from other proteinuric hypertensive disorders (crHTN). Correlations of plasma and urinary levels of different angiogenic factors (ie, VEGF) in diverse glomerular pathologies have been inconsistent.

Several clinical tools have been previously proposed to serve as screening tests for preeclampsia: blood pressure, dipstick proteinuria, cellular fibronectin, 24-hr urine protein collection, the roll-over test, and/or angiotensin challenge test. While most have either poor sensitivity or poor specificity, others are simply impractical as they entail laborious protocols, and their results are not readily available for clinical management decisions. We propose that measurements of the ratio between urinary sFlt-1 and free PlGF (uFP) will serve to differentiate mild PE and crHTN women suffering from superimposed preeclampsia from those with sPE. Our finding that in a random urine sample uFP ratio performed better than proteinuria alone in clustering sPE women from normal controls or other hypertensive disorders is novel and promising because this ratio indicator is independent of nonspecific proteinuria and/or creatinine concentration and, hence, hydration status.

In conclusion, our results demonstrate that sPE is associated with increased urinary levels of sFlt-1 at the time of clinical manifestation. Rapid noninvasive screening of hypertensive women based on uFP ratio may be used to define the severity of the disease, and appears to be superior to urinary protein measurements. Forthcoming studies designed to evaluate urinary VEGF, PlGF, and sFlt-1 excretion throughout pregnancy, and determine whether their levels may predict imminent preeclampsia, are warranted.

References


Absence of association of inherited thrombophilia with unexplained third-trimester intrauterine fetal death

Ron Gonen, MD, a Noa Lavi, MD, e Dina Attias, MD, b Liliana Schliamser, MD, b Zvi Borochowitz, MD, c Elias Toubi, MD, d Gonen Ohel, MD a

Department of Obstetrics and Gynecology, a Division of Hematology, b The Simon Winter Institute of Genetics, c and Division of Clinical Immunology, d Bnai Zion Medical Center, and Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

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Intrauterine fetal death is relatively uncommon, occurring in approximately 5 of 1000 births. The etiologic factors contributing to fetal death are diverse and numerous; however, in at least 12% to 50% of cases the cause remains unknown.1

Thrombophilia has been associated with various gynecologic and obstetric complications, such as recurrent pregnancy loss, severe preeclampsia, HELLP syndrome, abruptio placentae, intrauterine growth restriction, and an increased risk of late intrauterine fetal death.2-9 A suggested common denominator is intervillous or spiral artery thrombosis and inadequate placental perfusion.4,5 However, recently, several studies have challenged the alleged association between thrombophilia and some of these complications.10-12
The purpose of this study was to further investigate the association between thrombophilia and unexplained third-trimester intrauterine fetal death.

Material and methods

The case subjects were women who had delivered a third-trimester unexplained stillbirth in our institution. Bnai Zion Medical Center is a university hospital serving mainly the urban population of the city of Haifa, Israel. The population mix of our service comprises approximately 75% Jews, 21% Moslem and Christian Arabs, and 4% Druze. The annual number of deliveries is approximately 4500. We searched our database for women who delivered a stillbirth at 27 to 42 weeks’ gestation in our institution between 1994 and 2001. The charts of all women with intrauterine fetal death were reviewed and considered for study enrollment. The inclusion criteria were a singleton nonmalformed fetus, without signs of fetal infection or hydrops, and the absence of any known significant maternal risk factors for fetal death, such as hypertension, diabetes, abruptio placentae, chorioamnionitis, multiple gestation, autoimmune diseases, or antiphospholipid syndrome. The control group was composed of volunteers, mainly hospital personnel, group-matched for ethnic origin. Volunteers were eligible for the control group if they had at least 1 uncomplicated pregnancy resulting in the birth of a healthy newborn, and no history of stillbirth, recurrent fetal loss, or thromboembolism. The exclusion criteria for the 2 groups were: a current pregnancy, history of another stillbirth, and no history of gestational diabetes. The exclusion criteria for fetal death, such as hypertension, diabetes, abruptio placentae, chorioamnionitis, multiple gestation, autoimmune diseases, or antiphospholipid syndrome. The control group was composed of volunteers, mainly hospital personnel, group-matched for ethnic origin. Volunteers were eligible for the control group if they had at least 1 uncomplicated pregnancy resulting in the birth of a healthy newborn, and no history of stillbirth, recurrent fetal loss, or thromboembolism. The exclusion criteria for the 2 groups were: a current pregnancy, a pregnancy in the preceding 2 months, current use of oral contraception or any other hormonal treatment, and current use of anticoagulation medication.

After obtaining approval from the institutional review board, eligible case subjects were approached, first by a letter outlining the purpose and the protocol of the study, followed by a telephone interview by 1 of the authors (N.L.). The purpose of the interview was 2-fold: (1) to exclude noneligible subjects; and (2) to schedule an appointment for a face-to-face interview, enrollment, consent, and blood tests.

Laboratory investigation


DNA was extracted from whole blood using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany), according to the manufacturer’s directions. The presence of the G1691A mutation in the factor V gene, the C677T mutation in the MTHFR gene, and the G20210A mutation in the prothrombin gene was determined according to previously described methods.

Protein C was measured by colorimetric assay on the Automated Coagulation Laboratory using Stachrom protein C (Diagnostica Stago, Asniéres, France). Antithrombin was assayed using a chromogenic assay kit (IL Test™ Antithrombin, Instrumentation Laboratory, Lexington, Mass) on the Automated Coagulation Laboratory. Free protein S antigen was measured by enzyme-linked immunosorbent assay (ELISA) (Asserachrom free protein S, Diagnostica Stago). Coagulation factors VIII and XI were determined by the APTT 1-stage assay method on the ACL 9000 instrument. Lupus anticoagulant was detected by 2 tests: the Russell’s viper venom test–DRVVT (LA screen, Gradipore, Frech Forest, NSW, Australia), and phospholipid-rich Russell’s viper venom test (LA confirm, Gradipore) on the ACL instrument. Lupus anticoagulant confirmation value >1.2 was considered abnormal. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes. Plasma anticardiolipin antibodies were assayed with commercial ELISA kits for both immunoglobulin (Ig) G and IgM isotypes.
reasons. The pathology report of 34/37 placentas of case subjects was reviewed. We searched the report for documentation of placental infarcts. Pathology report was not available for any of the control subjects.

Statistical analysis

A sample size of 40 cases and 40 controls was calculated to have at least 80% power to detect 25% difference in the prevalence of thrombophilia between control and case groups at 5% two-tailed significance level. Control prevalence was set at 15% for calculation. This assumption was based on data from a previous study from our country.\(^7\) Thrombophilia prevalence was compared using the chi-square test (Fisher exact test when appropriate), and associations were summarized using odds ratio (OR) along with 95% CI. Analysis was performed with the Statistical Package for the Social Sciences for Windows, Version 11 (SPSS, Chicago, Ill), and SAS software version 9.1 for windows (SAS Institute, Cary, NC).

Results

During the study period there were 128 fetal deaths, a rate of 3.9 per 1000. Sixty-eight subjects were excluded: 17 were less than 27 weeks’ gestation and 51 had 1 or more of the exclusion criteria. Of the 60 eligible subjects approached, 37 agreed to participate in the study. The mean and standard deviation (SD) gestational age and birth weight among case subjects were 33.7 weeks (4.47) and 1958 g (994), respectively, and the mean (SD) maternal age at the time of delivery was 31.1 years (5.8). Forty-six volunteers, matched for ethnic origin, were recruited to the control group. Table I shows patients’ characteristics.

Table II shows the prevalence of inherited thrombophilia among study and control subjects. In the study group, 14 of 37 women (37.8%) had at least 1 inherited thrombophilia, compared with 19 of 46 (41.3%) among control subjects (OR = 0.87; 95%CI 0.32-2.29). There was also no significant difference in the prevalence of combined thrombophilia, which was detected in 3 of 37 study subjects (8.1%) compared with 4 of 46 among control subjects (8.7%). Moreover, there was no significant difference between cases and control subjects with respect to the prevalence of any single inherited thrombophilia. Subanalysis of our results according to ethnic origin revealed that in the Jewish population the prevalence of inherited thrombophilia was 35% among study subjects and 25% among control subjects (OR = 1.6; 95%CI 0.46-5.7), while among non-Jewish subjects, it was 41.2% in the study group and 66.7% in the control subjects (OR = 0.35; 95%CI 0.09-1.4).

The only significant difference between the 2 groups was a higher prevalence, among study subjects, of lupus anticoagulant (OR = 6.1; 95%CI 1.4-36.2) and anti-cardiolipin antibodies (OR = 8.5; 95%CI 1.6-83.2) (Table III). β₂-glycoprotein 1 antibodies were evaluated only in subjects with at least borderline levels of lupus anticoagulant or anti-cardiolipin antibodies. Thus, 22 study subjects and 9 control subjects were tested for the presence of either IgG or IgM β₂-glycoprotein 1 antibodies. In the study group, 11 subjects (50%) tested positive compared with 7 (77.8%) of the control subjects. Placental infarcts were reported in 21 of 34 case placentas (61.7%); however, no significant difference was noted in the prevalence of either inherited or acquired thrombophilia among subjects with or without placental infarcts (Table IV).

Comment

Our study group was composed strictly of otherwise healthy women who delivered, for the first time, a normal-appearing, nonanomalous stillborn fetus, in the third trimester of an uncomplicated pregnancy. Because the proportion of non-Jewish women among the case subjects was higher than their proportion in the population served by our center, and because the

<table>
<thead>
<tr>
<th>Type of thrombophilia</th>
<th>Study group n = 37 (%)</th>
<th>Control group n = 46 (%)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V Leiden, +/+ or +/−</td>
<td>4 (10.8)</td>
<td>7 (15.2)</td>
<td>0.68 (0.13-2.95)</td>
<td>.75</td>
</tr>
<tr>
<td>MTHFR +/+</td>
<td>5 (13.5)</td>
<td>4 (8.7)</td>
<td>1.64 (0.32-8.91)</td>
<td>.50</td>
</tr>
<tr>
<td>Prothrombin G20210A +/+</td>
<td>0</td>
<td>4 (8.7)</td>
<td>0 (0-1.34)</td>
<td>.13</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>3 (8.1)</td>
<td>5 (7.9)</td>
<td>0.72 (0.11-4.04)</td>
<td>.73</td>
</tr>
<tr>
<td>Antithrombin III deficiency</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High levels of factor VIII</td>
<td>2 (5.4)</td>
<td>1 (2.2)</td>
<td>2.57 (0.12-155.0)</td>
<td>.58</td>
</tr>
<tr>
<td>High levels of factor XI</td>
<td>3 (8.1)</td>
<td>4 (8.7)</td>
<td>0.93 (0.13-5.89)</td>
<td>&gt; .9</td>
</tr>
<tr>
<td>At least one thrombophilia</td>
<td>14 (37.8)</td>
<td>19 (41.3)</td>
<td>0.87 (0.32-2.29)</td>
<td>.75</td>
</tr>
<tr>
<td>Combined thrombophilia*</td>
<td>3 (8.1)</td>
<td>4 (8.7)</td>
<td>0.93 (0.13,5.89)</td>
<td>&gt; .9</td>
</tr>
</tbody>
</table>

* In the study group, 3 subjects had 2 thrombophilias. In the control group, 2 subjects had 2 thrombophilias, and 2 subjects had 3 thrombophilias.
prevalence of inherited thrombophilia may differ between ethnic groups, we group matched our control subjects for ethnicity.

We did not find an association between third-trimester unexplained intrauterine fetal death and inherited thrombophilia. Our findings are consistent with the results of a population sample study from Germany\(^1\) and a multicenter study from Austria.\(^2\) In the former study, which included 1768 women who had at least 1 pregnancy with known outcome, women with and without factor V Leiden mutation did not differ with respect to the number of women with stillbirth.\(^1\) In the latter study, the authors did not find an association between fetal death and polymorphism, including factor V Leiden, prothrombin G20210A, and MTHFR C677T.\(^2\) In contrast, 4 other studies reported a significantly higher prevalence of thrombophilia among subjects with stillbirth compared with control subjects,\(^3-7\) with OR ranging between 2.8 (95% CI 1.5-5.3) in the study by Many et al\(^7\) and 5.5 (95%CI 3.4-9.0) in the study by Gris et al.\(^5\) The prevalence of inherited thrombophilia in our study group was 37.8%, which is comparable to the 42.5% reported by Many et al\(^7\) in a cohort of 40 Jewish women with third-trimester stillbirth. A somewhat lower prevalence of inherited thrombophilia was reported by Martinelli et al\(^6\) in Italy, and by Gris et al\(^5\) in France. The group from Italy\(^6\) studied 67 women with unexplained fetal death after 20 weeks of gestation, 29.9% of whom had thrombophilia. In the French study,\(^5\) at least 1 inherited or acquired thrombophilia was diagnosed in 21.1% of subjects with late fetal loss. It should be noted, however, that the thrombophilia workup differed between the various studies.

Apparently there is a salient difference in the prevalence of thrombophilia among control subjects between our study and the aforementioned studies,\(^5-7\) 46% compared with 3.2% to 15%, respectively. It is possible that this difference stems from the different ethnicity of the populations evaluated. Indeed, several studies from the Middle East have reported that the prevalence of factor V Leiden in the Arab population is much higher than in any other ethnic group, ranging from 12% in Jordan\(^17\) to 24% in Israeli Arabs.\(^18\) A possible explanation suggested for the high prevalence of factor V Leiden among Arabs is that this mutation arose from a single founder and originated in the Middle East.\(^18\) Another factor that may have driven the carrier frequency even higher is the generally high consanguinity rate in the Arab population. This factor could also account for a higher prevalence of other hereditary thrombophilias in this population. In the present study, however, we did not find a statistically significant association in the prevalence of thrombophilia between the study and control groups when the analysis was performed separately for Jewish and non-Jewish women. It is possible that because of the small sample size in each of the ethnic subgroups, there was insufficient power to detect such a difference between the groups, even if it did exist.

The only significant difference that we found between the 2 groups was a higher prevalence of antiphospholipid antibodies among case subjects.\(^5,19\) Some authors have proposed placental thrombosis as the underlying mechanism for fetal death in patients with thrombophilia.\(^4,5\) In the present study, placental infarcts were reported in almost two thirds of the placentas of stillborn fetuses; however, no correlation between placental thrombosis and thrombophilia was

<table>
<thead>
<tr>
<th>Table III Prevalence of lupus anticoagulant and anticardiolipin antibodies among study and control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of thrombophilia</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
</tr>
<tr>
<td>Anticardiolipin (IgM and IgG)</td>
</tr>
<tr>
<td>Lupus anticoagulant and/or Anticardiolipin</td>
</tr>
</tbody>
</table>

* The results of anticardiolipin antibodies were available for 36 case subjects.

<table>
<thead>
<tr>
<th>Table IV The association between placental infarcts and thrombophilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of thrombophilia</td>
</tr>
<tr>
<td>At least 1 inherited thrombophilia</td>
</tr>
<tr>
<td>Lupus anticoagulant and/or anticardiolipin antibodies</td>
</tr>
</tbody>
</table>
observed. Similar results were reported by Martinelli et al. In contrast, Many et al and Gris et al described a higher incidence of placent al infarcts in placentas of women with adverse pregnancy outcome and thrombophilia compared with placentas of women with adverse pregnancy outcome without thrombophilia.

One limitation of the present study is that it may be underpowered to show smaller differences between study and control subjects. In our power calculation, the assumption of 25% difference in the prevalence of thrombophilia between the case and control subjects was based on data from Israel, from Many et al who reported a prevalence of thrombophilia of 42.5% and 15% in study and control groups, respectively. Obviously, this assumption may not be correct in other populations, where the difference in prevalence of thrombophilia between case and control groups is lower, and a larger sample size may be required. Nevertheless, in view of the results of this study, it seems unlikely that a larger sample size would have yielded different results.

In summary, no association was found between unexplained third-trimester intrauterine fetal death and inherited thrombophilia, at least, in a mixed population of Jewish and Arab women, a population with a very high prevalence of thrombophilia, but we did find such an association with the presence of antiphospholipid antibodies. Moreover, we did not find an association between thrombophilia and placental infarcts.

Acknowledgments

We thank Vardit Adir, PhD, head of the molecular genetic laboratory at the Simon Winter Institute of Genetics, Ilana Eldor, BA, head of the coagulation laboratory, Ada Tamir, DSc, and Ofra Barnett, DSc, from the department of community medicine and epidemiology for their invaluable help and assistance.

References

Prospective randomized clinical trial of inpatient cervical ripening with stepwise oral misoprostol vs vaginal misoprostol

Iris Colón, MD, Kaytha Clawson, MD, Kenneth Hunter, DPA, Maurice L. Druzin, MD, M. Mark Taslimi, MD*

Division of Maternal Fetal Medicine, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, Calif, and Department of Political Science and Public Administration, Jacksonville State University, Jacksonville, Ala

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Objective: The purpose of this study was to compare the efficacy and safety of stepwise oral misoprostol vs vaginal misoprostol for cervical ripening before induction of labor.

Study design: Two hundred and four women between 32 to 42 weeks of gestation with an unfavorable cervix (Bishop score ≤ 6) and an indication for labor induction were randomized to receive oral or vaginal misoprostol every 4 hours up to 4 doses. The oral misoprostol group received 50 mg initially followed by 100 mg in each subsequent dose. The vaginal group received 25 mg in each dose. The primary outcome was the interval from first misoprostol dose to delivery. Patient satisfaction and side effects were assessed by surveys completed after delivery.

Results: Ninety-three (45.6%) women received oral misoprostol; 111 (54.4%) received vaginal misoprostol. There was no difference in the average interval from the first dose of misoprostol to delivery in the oral (21.1 ± 7.9 hrs) and vaginal (21.5 ± 11.0 hrs, P = NS) misoprostol groups. The incidence of hyperstimulation in the oral group was 2.2% vs 5.4% in the vaginal group, P = NS. Eighteen patients in the oral group (19.4%) and 36 (32.4%) in the vaginal group underwent cesarean section (P < .05). This difference was attributed to better tolerance of more doses of misoprostol by the women in the oral group. There was no difference in side effects (nausea, vomiting, diarrhea, shivering) between groups. Fourteen percent of women in the vaginal group versus 7.5% in the oral group were dissatisfied with the use of misoprostol (P = NS).

Conclusion: Stepwise oral misoprostol (50 mg followed by 100 mg) appears to be as effective as vaginal misoprostol (25 mg) for cervical ripening with a low incidence of hyperstimulation, no increase in side effects, a high rate of patient satisfaction, and is associated with a lower cesarean section rate.

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Induction of labor has merit as a therapeutic option when the benefits of delivery outweigh the risks of continuing the pregnancy. Lack of adequate cervical ripening is a known obstacle to successful labor induction and expeditious delivery. Obstetricians use a variety of agents and methods to ripen the uterine cervix, achieve a shorter induction to delivery interval, and potentially lower the cesarean section rate. Acceptable methods for cervical ripening include synthetic prostaglandin E1 (PGE1) and prostaglandin E2 (PGE2) analogs, continuous oxytocin infusion, and mechanical cervical dilators.

One of the most widely used agents for cervical ripening is misoprostol, a synthetic methyl ester of prostaglandin E1, approved for the prevention of peptic ulcer disease by the FDA, but not approved for obstetric indications. Nevertheless, its use for cervical ripening and labor induction has been extensively studied, and has been accepted as an effective and safe method for these purposes.

Vaginal, as well as oral, misoprostol administration has been used, but the optimal dose of oral misoprostol has not been established. In general, higher doses of oral misoprostol are associated with improved efficacy but higher rates of hyperstimulation and maternal side effects than vaginal misoprostol. Oral misoprostol at 100 µg or 200 µg every 3 to 6 hours has been shown to have the same or improved efficacy as vaginal misoprostol but higher rates of uterine contractile abnormalities. In contrast, titrated low-dose oral misoprostol (20 µg every 2 hours increased to 40 µg) has been demonstrated to be as efficacious as dinoprostone (PGE2 analog) with no difference in hyperstimulation or other side effects.

Oral misoprostol is an attractive option, as it is inexpensive and stable at room temperature, and has the potential for providing increased patient satisfaction because of its noninvasive route of administration. Moreover, the possibility of misplacement is eliminated.

Previous studies have shown rapid absorption of oral misoprostol with peak plasma concentration at 34 ± 17 minutes and a nadir at 120 minutes. In contrast, vaginal misoprostol peaks at 80 ± 27 minutes, and declines slowly.

Based on pharmacokinetics, previously published regimens, and incidence of side effects, we chose a novel dosing regimen of oral misoprostol. We hypothesized that stepwise dosing of oral misoprostol (50 µg followed by 100 µg) would be as effective for cervical ripening as vaginal misoprostol in the ACOG approved dose of 25 µg every 4 hours, without increasing the rates of hyperstimulation, and with the potential for greater patient satisfaction.

Material and methods

We conducted this prospective randomized clinical trial in the Department of Obstetrics and Gynecology at the Labor and Delivery Unit in Lucile Packard Children’s Hospital, Stanford University. This hospital serves as a Northern California tertiary referral center for high-risk pregnancies. Patients were recruited from March 3, 2003 through July 25, 2004. This study was approved by the Institutional Review Board, and written informed consent was obtained from each participant.

Inclusion criteria included pregnancy between 32 and 42 weeks of gestation admitted for induction of labor because of either obstetric or medical complications, Bishop score ≤6, intact or ruptured membranes, cephalic presentation, and a reassuring fetal heart rate (FHR) pattern. Exclusion criteria included non reassuring fetal heart rate pattern, any contraindication to labor and/or vaginal delivery (placenta previa, vasa previa, active genital herpes), uterine scar, suspected placental abruption with abnormal FHR pattern, vaginal bleeding other than “bloody show,” cervical dilation of ≥4 cm, uterine contractions ≥3 in 10 minutes, significant maternal cardiac, renal or hepatic disease, maternal glaucoma, or hypersensitivity to misoprostol or prostaglandin analogs.

Treatment arm allocation was determined by the use of a computer-generated table of random numbers. The randomization assignments were placed into opaque, sealed envelopes. All eligible women were invited to participate and, after obtaining informed written consent, the next envelope in sequence was opened by the patient’s obstetrician to determine the treatment allocation.

Women assigned to the stepwise oral misoprostol arm received 50 µg initially, followed by 100 µg every 4 hours up to 4 doses; those assigned to the vaginal misoprostol arm received 25 µg every 4 hours up to 4 doses. All women had continuous electronic FHR and uterine contraction monitoring. Subsequent doses of misoprostol were withheld if adequate uterine activity (≥3 contractions in 10 minutes) or a Bishop score ≥8 had been achieved, or active labor had begun. If needed, oxytocin was initiated 4 hours after the last misoprostol dose. Amniotomy was used liberally at the discretion of the managing obstetricians.

Uterine contractility patterns and FHR monitoring tracings were evaluated after delivery by the first author, who was blinded to study group assignment. Tachysystole was defined as > 5 contractions in 10 minutes for 2 consecutive 10-minute periods. Hypertonus was defined as a single contraction lasting more than 2 minutes. Hyperstimulation syndrome was defined as tachysystole or hypertonus with non reassuring FHR changes. FHR changes considered to be non reassuring were late decelerations, severe variable decelerations, prolonged decelerations, tachycardia, or reduced FHR variability as defined by the National Institute of Child Health and Human Development Research Planning Workshop.

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The primary outcome was the interval from first misoprostol dose to delivery. The sample size calculation (n = 194) was based on an alpha of .05 and a beta of .20 to detect a 4-hour difference. Secondary outcomes were incidence of tachysystole, hypertonus, and hyperstimulation syndrome, vaginal delivery achieved within 24 hours, the rate of cesarean section, patient satisfaction, and neonatal outcomes. Treatment side effects and patient satisfaction were assessed by surveys completed 24 hours after delivery.

Statistical analyses were performed using \( \chi^2 \) test, Fisher exact test, linear regression, Student's \( t \) test, and analysis of variance (ANOVA) where appropriate. SPSS for Windows, version 12.0 (SPSS Corporation, Chicago, Ill) statistical software was used for all computations.

**Results**

A total of 212 women were enrolled in the study: 8 were excluded because they did not meet inclusion criteria. Of the 204 women included, 93 (45.6%) received oral misoprostol, and 111 (54.4%) received vaginal misoprostol.

**Table I** Baseline demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>28.1 ± 6.7</td>
<td>27.2 ± 6.4</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>38.8 ± 1.9</td>
<td>39.1 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Parity (no.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>56 (60.2%)</td>
<td>85 (76.6%)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>Multiparous ≥1</td>
<td>37 (39.8%)</td>
<td>26 (23.4%)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>23 (24.7%)</td>
<td>25 (22.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>African American</td>
<td>1 (1.1%)</td>
<td>6 (5.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hispanic</td>
<td>52 (55.9%)</td>
<td>59 (53.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Asian</td>
<td>5 (5.4%)</td>
<td>6 (5.4%)</td>
<td></td>
</tr>
<tr>
<td>Indian</td>
<td>4 (4.3%)</td>
<td>5 (4.5%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>8 (8.6%)</td>
<td>10 (9.0%)</td>
<td></td>
</tr>
<tr>
<td>Bishop score ≤2</td>
<td>48 (51.6%)</td>
<td>70 (63.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Intact membranes</td>
<td>89 (95.7%)</td>
<td>104 (93.7%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD or number (%).

**Table II** Indications for induction

<table>
<thead>
<tr>
<th>Induction</th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postdates</td>
<td>30 (32.3%)</td>
<td>46 (37.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>26 (28.0%)</td>
<td>29 (26.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>10 (10.8%)</td>
<td>7 (6.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Oligohydramnios</td>
<td>7 (7.5%)</td>
<td>8 (7.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Others</td>
<td>20 (21.5%)</td>
<td>21 (18.9%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as number (%).

Demographic characteristics are shown in **Table I**. The groups were similar with respect to age, gestational age, ethnicity, and Bishop score ≤2 at entry. There were more nulliparous women in the vaginal group (76.6% vs 60.2%, \( P < .01 \)). The indications for induction are shown in **Table II**, and were similar between both groups. The most common indications were postdates and hypertension.

There was no difference in the average interval from the first dose of misoprostol to delivery in the oral (21.1 ± 7.9 hrs) and vaginal (21.5 ± 11.0 hrs, \( P = \text{NS} \)) misoprostol groups. When looking only at the women who delivered vaginally, there was no difference in the average interval from first dose to vaginal delivery between the oral (19.3 ± 6.7 hrs) and vaginal (18.0 ± 8.3 hrs, \( P = \text{NS} \)) groups. This finding held true after controlling for parity. Among the women who delivered vaginally, there was no difference in the number that delivered within 12 hours and within 24 hours between the 2 groups (**Table III**).

**Table III** Time intervals to delivery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>First dose to delivery (h)</td>
<td>21.1 ± 7.9</td>
<td>21.5 ± 11.0</td>
<td>NS</td>
</tr>
<tr>
<td>First dose to vaginal delivery (h)</td>
<td>19.3 ± 6.7</td>
<td>18.0 ± 8.3</td>
<td>NS</td>
</tr>
<tr>
<td>Vaginal delivery in 12 h</td>
<td>12 (16.0%)</td>
<td>18 (24.0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Vaginal delivery in 24 h</td>
<td>56 (74.7%)</td>
<td>63 (84.0%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD or number (%).

**Table IV** Mode of delivery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All vaginal deliveries</td>
<td>75 (80.6%)</td>
<td>75 (67.6%)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>71 (94.7%)</td>
<td>68 (90.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>Assisted</td>
<td>4 (5.3%)</td>
<td>7 (9.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cesarean sections</td>
<td>18 (19.4%)</td>
<td>36 (32.4%)</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

Data presented as number (%).
group, and in 108 (97.3%) women in the vaginal group (P = NS).

The mode of delivery differed significantly between groups (Table IV). Eighteen patients in the oral group (19.4%), and 36 (32.4%) in the vaginal group underwent cesarean section (P! .05). In a regression model, 21% and 30% of mode of delivery was explained by birth weight quartile and by primiparity, respectively.

The indications for cesarean deliveries are shown in Table V. There were no significant differences in the indications for cesarean delivery between groups. Among cesarean sections that were performed for failure to progress, there were no differences between study groups in the rates of arrest of dilation, arrest of descent, or active labor not achieved.

Other characteristics of the cesarean deliveries are shown in Table V. Fifty of the 54 cesarean sections were in nulliparas. There was no significant difference in the number of nulliparous women per group. The number of women in each study arm that received only 1 dose of misoprostol before cesarean section differed significantly; 11.1% in the oral vs 63.9% in the vaginal arm (P < .01). The majority (69.6%) of the women in the vaginal arm did not receive the second dose because they had achieved adequate uterine activity (≥3 contractions in 10 minutes). Of the 23 patients in the vaginal arm that received only 1 dose of misoprostol, 60.9% underwent cesarean section for failure to progress.

There were no significant differences in the occurrence of tachysystole, hypertonus, or hyperstimulation between groups (Table VI). Two of the women (both in the vaginal group) who experienced hyperstimulation syndrome received tocolysis (terbutaline or nitroglycerin). Of the 8 cases of hyperstimulation syndrome, only 1 woman (vaginal group) needed urgent delivery by cesarean section.

Nonreassuring FHR patterns that needed urgent delivery were noted in 8 (8.6%) of the women in the oral group, and in 18 (16.2%) of the women in the vaginal group. Of these, 5/8 and 12/18 underwent cesarean delivery.

Treatment side effects and delivery complications were similar between the 2 groups (Table VI).

A total of 153 postdelivery surveys were completed. Patient satisfaction scores revealed that 98.5% of the women in the oral group and 98.8% in the vaginal group were satisfied with their total experience at the hospital during their induction of labor. Fourteen percent of women in the vaginal group vs 7.5% in the oral group were dissatisfied with the use of misoprostol (P = NS).

There were no differences in neonatal outcomes (Table VII) except in Apgar scores <7 at 1 minute, which were more frequent in the vaginal group (14.4% vs 4.3%, P < .05). Cord gases were obtained in 59

---

### Table V  Characteristics of cesarean deliveries

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Oral misoprostol (n = 18)</th>
<th>Vaginal misoprostol (n = 36)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failure to progress</td>
<td>13 (72.2%)</td>
<td>21 (58.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>NRFHT</td>
<td>4 (22.2%)</td>
<td>13 (36.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Others</td>
<td>1 (5.6%)</td>
<td>2 (5.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>15 (83.3%)</td>
<td>35 (97.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiparous ≥1</td>
<td>3 (16.7%)</td>
<td>1 (2.8%)</td>
<td>NS</td>
</tr>
<tr>
<td>Doses of misoprostol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (11.1%)</td>
<td>23 (63.9%)</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>≥2</td>
<td>16 (88.9%)</td>
<td>13 (36.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as number (%).

---

### Table VI  Characteristics of labor, maternal outcomes, and side effects

<table>
<thead>
<tr>
<th></th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxytocin augmentation</td>
<td>91 (97.8%)</td>
<td>108 (97.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Epidural anesthesia</td>
<td>79 (84.9%)</td>
<td>95 (85.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>Tachysystole</td>
<td>26 (28.0%)</td>
<td>28 (25.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertonus</td>
<td>4 (4.3%)</td>
<td>5 (4.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hyperstimulation</td>
<td>2 (2.2%)</td>
<td>6 (5.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>Use of tocolysis for hyperstimulation</td>
<td>0</td>
<td>2 (1.8%)</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal outcomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excessive blood loss</td>
<td>11 (11.8%)</td>
<td>18 (16.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Fever</td>
<td>5 (5.4%)</td>
<td>13 (11.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Retained placenta</td>
<td>1 (1.1%)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal side effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 67)</td>
<td>(n = 86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>9 (13.4%)</td>
<td>13 (15.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Vomiting</td>
<td>9 (13.4%)</td>
<td>11 (12.8%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1 (1.5%)</td>
<td>1 (1.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Shivering</td>
<td>9 (13.4%)</td>
<td>11 (12.8%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as number (%).

---

### Table VII  Neonatal outcomes

<table>
<thead>
<tr>
<th></th>
<th>Oral misoprostol (n = 93)</th>
<th>Vaginal misoprostol (n = 111)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>3283 ± 610</td>
<td>3352 ± 547</td>
<td>NS</td>
</tr>
<tr>
<td>Birth weight &gt; 4000 g</td>
<td>9 (9.7%)</td>
<td>14 (12.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>Apgar score &lt;7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>4 (4.3%)</td>
<td>16 (14.4%)</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>5 min</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Meconium passage</td>
<td>9 (9.7%)</td>
<td>11 (9.9%)</td>
<td>NS</td>
</tr>
<tr>
<td>Admission to neonatal intensive care unit</td>
<td>11 (11.8%)</td>
<td>11 (9.9%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD or number (%).
women (28.9%). Only 4 (2 in each group) had arterial pH <7.10.

**Comment**

Multiple trials have shown that misoprostol is an effective agent for cervical ripening and labor induction. Vaginal, as well as oral, misoprostol administration has been used, with 25 μg every 4 hours of vaginal misoprostol widely accepted as the most effective regimen with the least number of complications. The optimal dose of oral misoprostol has not been established.

Our purpose was to study the effectiveness of a novel dosing regimen of oral misoprostol (50 μg followed by 100 μg) compared with the standard regimen of vaginal (25 μg) misoprostol every 4 hours. Our rationale stems from the proven efficacy of oral misoprostol, and the hypothesis that our stepwise regimen would be as effective as vaginal misoprostol without increasing rates of complications.

Our study has demonstrated that stepwise oral misoprostol appears to be as effective as vaginal misoprostol for cervical ripening before labor induction. The average interval from first dose to vaginal delivery was similar between groups, and the same number of women in each study arm achieved vaginal delivery in 24 hours.

There was a low incidence of hyperstimulation in both groups (oral group 2.2% vs vaginal group 5.4%, \(P = \text{NS}\)). This compares favorably to a generally accepted incidence of hyperstimulation of 7% with vaginal administration.

Our study found stepwise oral misoprostol to be well tolerated, with no increase in maternal side effects compared with vaginal misoprostol. Furthermore, the majority of the patients were satisfied with their hospital experience, and few women reported dissatisfaction with the route of administration (vaginal group 14% vs oral group 7.5%, \(P = \text{NS}\)).

Perhaps the most significant finding of our study is the lower cesarean section rate in the women who received the oral regimen. Detailed analysis was performed on the subgroup of women who underwent cesarean delivery. The only characteristic that was found to explain the difference in cesarean section rates was the number of misoprostol doses administered before delivery. The majority of patients in the vaginal arm received only 1 dose of misoprostol for ripening because they were found to be contracting \(\geq 3\) times in 10 minutes when the next dose was due.

Our interpretation is that patients tolerated the initial 50 μg oral misoprostol dose better than the 25 μg vaginal dose. Although the initial vaginal dose provided adequate uterine activity, it may paradoxically have been less effective in its primary goal of cervical ripening, as excess uterine contractions prevented further dosing. It is probable that the initial 50 μg oral dose prepared the cervix and the uterus to tolerate further doses of misoprostol, and this resulted in a higher rate of vaginal delivery. Other hypotheses to explain the lower cesarean section rate in the oral group include a dose-related or bioavailability effect, more effective priming of the myometrium to respond to endogenous/exogenous oxytocin, or simply a random effect of small numbers.

Our protocol might be considered conservative in that it called for discontinuation of misoprostol after \(\geq 3\) uterine contractions in 10 minutes were achieved, regardless of the strength of the contractions. While some patients with very mild contractions might safely benefit from additional misoprostol doses, this is a well-described protocol used in numerous peer-reviewed studies of cervical ripening with misoprostol. Moreover, the cesarean rates observed in both arms of this study compare favorably with those described in previous studies where misoprostol was also used for ripening of an unfavorable cervix. Our study has the limitation of lack of blinding, which may introduce a potential for bias.

In conclusion, we have shown that stepwise oral misoprostol appears to be as effective as vaginal misoprostol for cervical ripening with a low incidence of hyperstimulation, no increase in side effects, high rate of patient satisfaction, and is associated with a lower cesarean section rate.

**References**

Effects of selective and nonselective PGE2 receptor agonists on cervical tensile strength and collagen organization and microstructure in the pregnant rat at term

Helen Feltovich, MD,a,* Huiling Ji, MD, PhD,c Jessie W. Janowski, BA,c Nicole C. Delance, BS,b Colleen C. Moran, BS,a Edward K. Chien, MDc

Department of Obstetrics and Gynecology,a and Department of Pathology,b University of Vermont, Burlington, Vt, and Department of Obstetrics and Gynecology, Brown University, Providence, RIc

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Objective: The purpose of this study was to determine which of the 4 PGE2 receptors (EP1-EP4) is involved in cervical ripening in the rat, and to correlate its activity with changes in tensile strength and collagen microstructure.

Study design: We assessed tensile strength after administration of selective and nonselective PGE2 receptor agonists. Quantification of collagen organization and microstructure was accomplished with polarized light microscopy and transmission electron microscopy.

Results: Selective agonists for EP1-3 did not produce significant differences when compared with each other or control animals. Significant differences in tensile strength, proportion of organized collagen, and microstructure were found between treatment and control animals with the nonselective receptor agonist (PGE2). This was taken as an indirect measure of EP4 activity.

Conclusion: Changes in cervical collagen organization and microstructure are quantifiable and correlate with changes in tensile strength. These data implicate EP4 as the PGE2 receptor involved in producing these changes in the rat cervix.

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The cervix is remarkably dynamic. Its primary functions, to maintain a fetus in utero, and then permit delivery, are diametrically opposed. Precise regulation is required for normal pregnancy and delivery. Physiologic cervical remodeling (ripening) in preparation for delivery is associated with changes in the collagen and proteoglycan composition of the cervical extracellular matrix (ECM), as demonstrated by biochemical studies as well as light, polarized light, and electron microscopy.1-3 These include a decrease in collagen
concentration, despite increase in absolute amount of collagen, increase in collagen fragmentation, and alterations in proteoglycan concentration. Associated with remodeling are changes in ECM protease activation and expression, which appear critical to this process, although their roles are poorly understood.

Investigation of cervical remodeling typically involves cervical application of prostaglandin E2 (PGE2). This is used clinically for prelabor induction ripening because it is thought to simulate the physiologic PGE2 elevation that occurs before normal parturition. PGE2-induced and physiologic ripening share a similar pattern of cervical collagen disruption. Our previous studies have demonstrated that PGE2 ripens the rat cervix at term, but not at mid gestation, and that expression of only the EP4 type of the 4 PGE2 receptors (EP1-4) increases near term. These suggest that term ripening is likely mediated through activation of the EP4 receptor.

This work was undertaken to expand our understanding of cervical ripening. Using polarized light microscopy and transmission electron microscopy, we investigated microstructural changes in cervical collagen after PGE2-induced ripening in order to establish a reproducible, quantifiable assessment technique. By administering agonists selective for PGE2 receptors, we explored our hypothesis that the EP4 receptor mediates the microstructural changes seen in PGE2-induced cervical remodeling.

Material and methods

Cervical ripening with nonselective and selective prostaglandins

Timed pregnant Sprague-Dawley rats obtained from Charles Rivers Laboratories (Montreal, Quebec) were delivered a week before use and handled daily. The day after mating was considered day 1 of gestation in the presence of a vaginal plug; animals typically deliver the evening of day 22 or morning of day 23. Indomethacin (ICN Biomedicals, Aurora, Ohio) was dissolved in ethanol (10 mg/mL). Animals were pretreated with 1 mg of indomethacin subcutaneously in the early morning on day 19 and 20 to inhibit endogenous prostaglandin production. PGE2 receptor agonists were administered approximately 60 minutes after indomethacin on day 20. They (nonselective PGE2 [0.25 mg, Calbiochem, San Diego, Calif], EP2-selective butaprost [2 mg, Cayman Chemical Co, Ann Arbor, Mich], EP3 > EP1-selective sulprofostone [0.2 mg, Cayman Chemical Co], or EP1 > EP3-selective 17-phenyl-trinor-PGE2 [1 mg, Cayman Chemical Co]) were suspended in 70% ethanol, and placed intravaginally with an equivalent volume of chlorohexidine gluconate to assist in vaginal retention. The dose of each agent was based on reported receptor binding affinities. PGE2, a nonselective agonist, was used to evaluate the EP4 receptor because there is no commercially available EP4-selective agonist. Control animals received only vehicle and chlorohexidine gluconate. Cervical tissue was assessed 18 to 24 hours after treatment. Animals in trials of selective agonists were sacrificed on day 21 because significant differences were noted in the initial PGE2 vs control trial only on day 21 (not days 16 or 20). Tissue was harvested as previously described. All procedures were approved by the Institutional Animal Care and Utilization Committee at the University of Vermont.

Cervical creep method of evaluating cervical tensile strength

Cervical tensile strength was determined using the cervical creep method previously described, with 4 animals per treatment group (20 animals total). Immediately after harvest, a cylindrical segment of cervix 5 mm long was suspended in a heated 37°C water bath containing Dulbecco’s modified Eagle’s medium (DMEM, Mediatech, Herndon, VA) bubbled with 95% O2 and 5% CO2. The segment was suspended between 2 horizontal hooks, 1 fixed to the base of the water bath. The rate of cervical distension under a constant 50 g load was determined using a Linear Variable Differential Transformer from TransTek, Inc (Ellington, Ct) connected to the second hook. The analog signal was digitized by a CB-68LP connector block (National Instruments, Austin, Tex) wired to a PCI-6024E Multifunction I/O board (National Instruments). Data were collected and stored using a Pentium PC and Labview 6.1 (National Instruments) software. Cervical creep was calculated from the slope of distance vs time plots.

Quantification of collagen using polarized light microscopy

Freshly excised tissue from 16 animals (4 per treatment group) was rinsed in normal saline, embedded in OCT, frozen in liquid nitrogen-cooled isopentane, and stored at −70°C until sectioning. The ability to make multiple measurements from a single animal minimized the variation within each treatment group, permitting a relatively small sample size. Tissue blocks were sectioned (7-8 µm) on a Microm HM 505 N cryostat (Apopget, Portsmouth, NH) onto Superfrost Plus microscope slides (Fisher Scientific, Philadelphia, Pa). Sections were stained with picrosirius red and analyzed via polarized light microscopy per the Junqueira protocol, which allows identification of individual collagen fibers on the basis of double refraction of light when illuminated. Slides were removed from the −70°C freezer and warmed to room temperature for 15 minutes. They were then fixed in ice cold acetone for 2.5 minutes, air dried for 30 minutes, and hydrated in distilled water for 2 minutes. Slides were stained for 90 minutes at room
temperature with 0.1% Sirius Red F3BA (color index 35780, Pfaltz and Bauer, Waterbury, Ct) in saturated picric acid. They were placed in 0.01N hydrochloric acid for 1 minute, followed by washes in 70% ethanol, 95% ethanol $\times 2$, 100% ethanol $\times 2$, and xylene $\times 3$. After drying, cover slips were applied with Permount solution (Sigma Aldrich, St Louis, Mo).

Collagen birefringence, a measure of fibrils oriented in parallel (organized), was determined using an Olympus BX50 microscope with a polarizer/analyzer combination (Olympus, Melville, NY) and Optronics MagnaFire digital camera (Optronics, Goleta, Calif) at 40$\times$ magnification with a 1280$\times$1024 pixel .tif image capture. All slides were prescreened to determine maximal birefringence by adjusting analyzer rotation, light intensity, and exposure time. Using identical settings, images were captured. Two random images were taken from each quadrant of the cervix (8 images/cervix). The large contrast between background (black-negative) and collagen fibrils (white-positive) that are birefringent (oriented in parallel) permits creation of binary images to express degree of organization as a percent area of total image via Metamorph software (version 6.1, Universal Imaging Corp). Images were screened to determine the appropriate universal threshold to apply to binary images, and proportion of organized collagen was recorded as a percentage of total image area.

**Determination of collagen fiber morphology by transmission electron microscopy**

Four cervices per treatment group were obtained, totaling 8 animals. Fresh tissue was excised and divided into quadrants that were morselized into 1 mm$^3$ blocks. These were fixed for 45 minutes at 4°C in Karnovsky's fixative (2.5% glutaraldehyde, 1.0% paraformaldehyde in 0.1 mol/L Millonig's phosphate buffer [Electron Microscopy Sciences, Hatfield, Pa], pH 7.2). After rinsing in Millonig's buffer, the tissue was postfixed in 1% osmium tetroxide for 1 hour at 4°C. The tissue was rinsed in Millonig's buffer, dehydrated through graded ethanols, cleared in propylene oxide, and embedded in Spurr's epoxy resin. Semithin sections (1 $\mu$m) were cut with glass knives on a Reichert ultracut microtome, stained with methylene blue–azure II, and evaluated for areas of interest. Ultrathin sections (60-80 nm) were cut with a diamond knife, retrieved onto 150 mesh copper grids, contrasted with uranyl acetate (2% in 50% ethanol) and lead citrate, and examined with a JEOL 1210 Transmission Electron Microscope (JEOL USA, Inc, Peabody, Mass) operating at 60 kV.

Blinded investigators randomly captured 4 longitudinal images and 4 cross-sectional images per quadrant (32 images per animal). Magnifications optimized measurement of longitudinal fibril length, as well as cross-sectional fibril diameter and distance between fibrils (12,000$\times$ for longitudinal images and 40,000$\times$ for cross-sectional images). Negatives were developed and scanned on a MicroTek ScanMaker 8700 (MicroTek, Carson, Calif) to digitalize images for analysis. Still blinded, investigators analyzed images with Metamorph software (Universal Imaging Corp). On longitudinal images, the most prominent collagen fiber was identified. The 10 longest fibrils within this fiber were measured, totaling 160 lengths per animal. On cross-sectional images, a fiber was identified and encircled. The diameters of the 10 fibrils closest to the fiber’s midpoint (central fibrils) were measured, as well as the distance between the outer edge of each of these fibrils and that of its next closest neighbor (interfibrillary distance). The interfibrillary distance between each of the 10 fibrils touching or closest to the perimeter of the encircled fiber (peripheral fibrils) and its closest neighbor was then recorded. Per animal, 160 diameters, 160 central interfibrillary distances, and 160 peripheral interfibrillary distances were measured.

**Data analysis**

Data were collected onto an Excel spreadsheet (Microsoft, Redmond, Wash), and analyzed using SSPS software (Jandel, San Rafael, Calif). The data were assessed for normality via the Kolmogorov-Smirnov test, and then analyzed via one-way analysis of variance (ANOVA) for the cervical creep study, and Student t test for the electron microscopy study. For the polarized light study, data were normalized using the arcsine transformation (the untransformed values are presented for ease of interpretation). The data were then analyzed using a one-way ANOVA, and Dunnett’s test was used to compare the treatment groups with the control group.

**Results**

**Cervical ripening with nonselective and selective prostaglandins**

Our previous studies demonstrated a significant difference in cervical creep between control and PGE2-treated animals on day 21 following treatment on day 20, but not earlier, consistent with the peak of EP4 expression in normal term rat gestation. Therefore, to further elucidate the role of the EP4 receptor, we applied agonists selective for the other 3 PGE2 receptors (EP1-EP3) on day 20 to determine their effects on ripening. Cervical tensile strength in animals treated with a selective agonist was not different from that in control animals, but was significantly different for animals treated with the nonselective PGE2 (Figure 1). We did not apply an EP4-selective agonist because none were commercially available or made available. Because the EP1-3 selective agonists did not induce significant cervical change, it is reasonable to...
infer that the EP4 receptor is responsible for the ripening effects observed after application of PGE2.

**Quantification of extracellular matrix collagen using polarized light microscopy**

Tissue sections were obtained perpendicular to the cervical canal near the internal os because collagen fibrils lying in this plane would provide the greatest resistance to cervical dilation. Several random images from each cervical section were captured. Figure 2 depicts typical bright field and polarized light images of treated and control tissue. Birefringence measured by polarized light microscopy is a measure of fibril orientation (organization). Fibrils in unripe cervical tissue are oriented in parallel (well organized). The extent of this organization is detectable by polarized light because fibrils that are fragmented and disoriented are not able to polarize transmitted light. We used image analysis software to create binary images from the polarized light images, and then prescreened all images to determine a grayscale threshold to apply universally. This permitted evaluation of proportion of organized fibrils. A 30% decrease in organized collagen was detected after PGE2 treatment compared with control animals and selective agonists (Table I).

**Figure 1** Cervical creep measurements (cm/sec) on day 21 after administration of PGE2 analogs or vehicle (control) on day 20. Higher cervical creep values correspond to decreased tensile strength. Bars represent means and error bars standard deviations. Sulprostone is selective for EP1 and EP3, 17 phenyl-trinor for EP1, and butaprost for EP2. PGE2 is nonselective (activates EP1-EP4). Endogenous prostaglandin production was inhibited by pretreatment with indomethacin. Asterisk, Statistical significance compared with control, with t test P value < .05.

**Determination of collagen fiber morphology by transmission electron microscopy (TEM)**

TEM was used to characterize and quantify changes in collagen at the microstructural level for 2 reasons: previous studies have only documented subjective and qualitative changes, and our work investigating matrix metalloproteinase activation and cervical ripening suggested that PGE2-induced changes may be more associated with activity of proteoglycans, and not collagenases, as is the classic belief. Both collagen fragmentation and changes in proteoglycan composition have been proposed as mechanisms of cervical remodeling. We have previously demonstrated an increase in activation of a matrix metalloproteinase that acts directly upon proteoglycans and indirectly upon collagen (MMP-3, a stromelysin). Because collagenases cleave the fibril at specific points along its length, shorter fibrils would imply primary activity of collagenases. Because proteoglycans are involved with cross-linking collagen fibrils and fibers, it is reasonable to theorize that increased spacing between fibrils would be associated with direct activity of a protease with proteoglycanase activity. We attempted to identify which activity may be more relevant to PGE2-induced cervical ripening by quantification of microstructural changes in collagen between PGE2-treated and control groups. We chose not to evaluate groups that received EP1-3 selective agonists because no changes in cervical tensile strength or percentage of organized collagen had been detected.

Figure 3 shows typical TEM images with depiction of fibril length, fibril diameter, and interfibrillary distance. Compared with control animals, the average interfibrillary distance among PGE2-treated animals was approximately 10 nm greater at both the center and the periphery, a difference that was statistically significant (Table II). There were no significant differences in fibril diameter or length between groups.
The present studies support our hypothesis that cervical ripening is mediated by activation of EP4, 1 of the 4 types of PGE2 receptors, and suggest that this occurs by promoting microstructural changes in collagen. To our knowledge, these studies are the first to quantify microstructural changes in the cervical extracellular matrix. The changes (decreased concentration of organized collagen and altered fibril orientation) correlate with biomechanical changes in cervical tensile strength (decreased cervical tensile strength). Statistically significant changes occur after administration of PGE2, but not after that of EP1-3 selective agonists. The specific microstructural changes we report implicate a role for enzymes involved in stromal remodeling, such as proteoglycanases, more than enzymes involved in collagen degradation, such as collagenases.

The effects of PGE2 are mediated through activation of 4 different receptors (Ptger-EP1, EP2, EP3, and EP4), which are expressed in a tissue- and cell type-specific manner. Each receptors’ effect is determined by the second messenger system it activates, and by tissue-specific receptor expression patterns. Although the PGE2 receptor responsible for mediating tissue remodeling has not been identified, our studies and those of others implicate EP4. Our previous data demonstrated that PGE2 application on day 20 of gestation, but not earlier, significantly decreases tensile strength, suggesting that the receptor(s) responsible for mediating cervical ripening is/are present in late gestation but not mid gestation. The central role of EP4 in term cervical

**Comment**

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**Table I**

<table>
<thead>
<tr>
<th>% Birefringence (mean ± SEM)</th>
<th>Dunnett’s test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>PGE2 (nonselective)</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Sulprostone (EP1- and 3- selective)</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Butaprost (EP2-selective)</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

% Birefringence, Percent of total tissue area demonstrating birefringence.
ripening is therefore supported by our previous data showing that EP4 expression peaks in late gestation, but not mid gestation, while expression of EP1 and EP3 is lower at term than in early pregnancy, and EP2 expression does not change throughout gestation. In the present study, agonists selective for EP1-3 failed to affect cervical tensile strength, concentration of organized cervical collagen, or collagen microstructure, providing additional evidence for the role of EP4 in mediating cervical ripening at term.

Tissue tensile strength is determined by cellular and extracellular matrix components. Cervical ECM is comprised predominantly of collagen, elastin, proteoglycans, and glycosaminoglycans. Collagen has been considered the major contributor to tensile strength, although recent studies have implicated proteoglycans, as well, via their effects on cross-linking and orienting of collagen. Collagen fibrils aggregate as fibers, which accumulate via proteoglycans to form bundles or sheets. In many tissues, such as bone, cartilage, cornea, and skin, collagen fibril and fiber diameter, length, and orientation are associated with different tensile characteristics, but this had not been explored quantitatively in the uterine cervix until the present study. Reproducible, quantitative assessment of microstructural changes affecting cervical tensile strength is important to understanding the mechanical properties of the cervix. This information could be used for both biochemical and structural modeling in order to clarify the role of the cervix in the 2 disorders of cervical ripening (preterm and postdate delivery).

Biochemical analyses and qualitative imaging studies at the time of cervical ripening have demonstrated collagen fragmentation and an increase in soluble collagen, indicative of collagen degradation. Increased collagenase activity correlates with these observations. However, the present studies appear to contradict the widely held theory that cervical ripening is primarily associated with collagenase activity. Although we did find that a smaller proportion of organized collagen was associated with decreased cervical tensile strength, our quantitative assessment of collagen microstructure in these cases did not demonstrate the increased fragmentation we would expect with increased collagenase activity. Rather, we found increases in the spaces between collagen fibrils. This finding may be reconciled with those of other studies by invoking increased disorganization and disorientation of fibrils via disruption of cross-linkages, rather than increased cleaving of collagen, to account for the decrease in cervical tensile strength. In
addition, it is possible that collagenases may be more important to cervical dilation during labor than cervical ripening before labor. We did not evaluate changes in labor, but others have shown greater increases in collagenase activity at the time of active labor compared with the peri-ripening period.\textsuperscript{22,23} Importantly, however, a recent paper investigating biomechanical properties of the rat cervix during both ripening and labor reported that collagenolysis plays little, if any, part in cervical ripening and dilation. These data are consistent with our microscopy findings.\textsuperscript{24}

The contribution of proteoglycans to tensile strength is less well understood than that of collagen, although they do seem to play a key role in fibrillogenesis and fibril organization, as demonstrated by studies of wound healing, tumor formation, and tissue repair.\textsuperscript{25} The proteoglycan’s core protein binds directly to collagen fibrils, and its glycosaminoglycan side chains are thought to interact with other side chains to regulate fibril spacing and tissue hydration. Matrix metalloproteinases with proteoglycanase activity, such as MMP-3, are believed to disrupt glycosaminoglycan side chain protein interactions.\textsuperscript{26} Disruption of proteoglycans by proteoglycanases would logically encourage dissociation of collagen fibrils, thereby decreasing tensile strength. The associations we have found between MMP-3 expression, decreased cervical tensile strength, and increased interfibrillar distance without increased collagen fragmentation may imply a greater role for proteoglycanases than for collagenases in PGE\textsubscript{2}–induced cervical ripening at term gestation in the rat.

In summary, the above data suggest that, of the 4 PGE\textsubscript{2} receptors in the cervix, EP4 is primarily responsible for mediating cervical ripening at term gestation in the rat, and that changes in collagen microstructure can be linked to activation of this receptor. Further studies currently underway focus on changes in the proteoglycan component of the extracellular matrix, and on elucidating the difference between collagenase and proteoglycanase activity connected with cervical ripening.

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References


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Recognition and treatment of irritable bowel syndrome among women with chronic pelvic pain

Rachel E. Williams, PhD,a,* Katherine E. Hartmann, MD, PhD,a,b Robert S. Sandler, MD, MPH,a,c William C. Miller, MD, PhD, MPH,a,c Lucy A. Savitz, MBA, PhD,d John F. Steege, MD

Department of Epidemiology, School of Public Health,a Departments of Obstetrics and Gynecologyb and Medicine,c School of Medicine, Department of Health Policy and Administration, School of Public Health,d University of North Carolina at Chapel Hill, Chapel Hill, NC

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KEY WORDS
Irritable bowel syndrome
Chronic pelvic pain
Treatment
Epidemiology
Diagnosis

Objective: We sought to describe irritable bowel syndrome (IBS) treatment among women with chronic pelvic pain.

Study design: We performed a cross-sectional study of new chronic pelvic pain patients between 1993 and 2000 (n = 987). IBS was defined by Rome I criteria. IBS treatment was defined as lower gastrointestinal drugs or referral. Analyses were descriptive and multivariable.

Results: IBS occurred in 35% of patients. In the highest quartile of pain, women with IBS were not more likely to have IBS treatment initiated. In the lowest three quarters of pain, women with IBS were 5.08 times more likely to have IBS treatment initiated. IBS was not diagnosed 40% of the time. IBS treatments were not recommended to 67% of patients with IBS. More than 35% of patients were prescribed narcotics.

Conclusion: IBS is not consistently diagnosed and treated even in a pelvic pain clinic. Yet, treatment of IBS may reduce the overall abdominal pain of these patients.

Chronic pelvic pain is a syndrome that is loosely defined by a long duration of pain in the pelvis. It may originate from any organ system or disease and may have multiple contributing factors that usually do not occur in isolation.1 Chronic pelvic pain affects 12% to 25% of women at any point in time,1-3 and 33% to 39% of women during their lifetime,1,4 with a higher prevalence found in health care settings than in the general population.1 Women with chronic pelvic pain use 3 times more medications of any type, have 4 times more nongynecologic operations, are 5 times more likely to have a hysterectomy,5 and have reduced quality of life2,6 compared with women without chronic pelvic pain.

Approximately one third of women with chronic pelvic pain have irritable bowel syndrome (IBS).3 IBS is a functional gastrointestinal disorder characterized by abdominal pain and bowel symptoms such as bloating,
urgency, diarrhea, and constipation. Documentation shows that IBS is associated with gynecologic problems such as endometriosis, dyspareunia, and dysmenorrhea.\textsuperscript{3,7-10} IBS is most prevalent during menstruating years\textsuperscript{11-13} and is exacerbated during menstruation.\textsuperscript{14} Compared with women with only chronic pelvic pain, those with both syndromes are more likely to have screening and diagnostic procedures performed\textsuperscript{3} and are less likely to have improvement after laparoscopy.\textsuperscript{10}

Few studies have been published on women with IBS and chronic pelvic pain.\textsuperscript{3,10,15,16} Both IBS and chronic pelvic pain include pain in the abdomen. Therefore, if IBS is treated and symptoms are reduced, it is possible that pelvic pain may also be reduced. Although treatment for IBS is challenging, substantial improvements in symptoms can be achieved. Lower gastrointestinal drugs can be beneficial, such as fiber, laxatives, stool softeners, anticholinergics, and antispasmodics. In addition, antidepressants can improve abdominal pain and diarrhea, as well as reduce depression\textsuperscript{17} by affecting pain control, gastrointestinal motility, and emotional behavior.\textsuperscript{18}

We sought to determine whether IBS is being recognized and treated among women presenting for chronic pelvic pain. Our objectives were to evaluate whether women with IBS had greater odds of having a treatment plan with a lower gastrointestinal drug or gastroenterology referral compared with women without IBS, whether IBS is being diagnosed correctly when compared with the Rome I criteria, to describe treatment recommendations for women with and without IBS, and to evaluate whether IBS is associated with increasing complexity of treatment.

### Material and methods

The population of this cross-sectional study is comprised of new patients who entered the Chronic Pelvic Pain Clinic at the University of North Carolina (UNC) between June 10, 1993, and December 11, 2000 (n = 987). Typically, women are referred by their gynecologist or primary care physician within UNC Hospitals or from elsewhere in North Carolina. A few patients come from surrounding states or other countries. Most referrals occur because the patient failed previous treatments, requires additional treatment, or needs a surgical procedure. Data collection was limited to women with self-reported pelvic pain lasting 6 months or longer, although it is possible for a patient to be referred for pelvic pain lasting less than 6 months. The Institutional Review Board approved the use of these clinical data for research purposes.

The clinic personnel systematically collected data to aid in patient evaluation and treatment. Patients answered self-administered questionnaires before examination at the clinic, including the Beck Depression Inventory\textsuperscript{19} (a validated questionnaire for measuring level of depression), McGill Pain Questionnaire\textsuperscript{20} (a validated questionnaire for measuring pain level), a clinic-specific general information form and a history of abuse survey. One of the small number of physicians who specialize in chronic pelvic pain recorded diagnostic impressions and physical findings at the initial visit. Prior pathology reports were often available. All these data were available throughout the study period, except history of abuse, which was not surveyed after 1998. We completed abstraction of medical charts for 98\% (n = 970) of the study population to collect information on prior surgeries, current medications, and treatments recommended after evaluation in the pelvic pain clinic (medications, surgeries, and referrals).

We defined IBS by the Rome I criteria that were assessed on the self-administered general information form. IBS is defined by expert established criteria, which have evolved over time, including the Manning criteria, the Rome I criteria, and the Rome II criteria.\textsuperscript{21} Rome I criteria was used because it could be applied across the entire study period and Rome II criteria could not. According to the Rome I criteria, a positive IBS classification includes at least 3 months of continuous or recurrent symptoms of (1) abdominal pain or discomfort that is relieved with defecation, and/or associated with a change in frequency of stool, and/or associated with a change in consistency of stool; and (2) 2 or more of the following, at least one fourth of occasions or days: altered stool frequency (more than 3 bowel movements each day or less than 3 bowel movements each week), altered stool form (lumpy/hard or loose/watery stool), altered stool passage (straining, urgency, or feeling of incomplete evacuation), passage of mucus, and/or bloating or feeling of abdominal distension.\textsuperscript{21} Gynecologists recorded the diagnosis of IBS as definite, probable, possible, or none. These diagnoses were based on the physician’s judgment after asking questions about bowel symptoms and evaluating questionnaire responses. We grouped definite and probable diagnoses together to indicate a positive IBS diagnosis.

We defined the outcome called “IBS treatment” as having a treatment plan with a recommendation for a lower gastrointestinal drug (anticholinergic/antispasmodic, cholinergic, fiber, laxative, and/or stool softener) and/or a referral to a gastroenterologist. First, we evaluated the initiation/continuation of IBS treatment among all women (n = 970) to see if it was associated with IBS status. Then, we assessed the association of IBS with the initiation of IBS treatment among women who were not taking a lower gastrointestinal drug at the start of the initial examination or were not referred from a gastroenterologist (n = 866).

We performed univariate analyses to evaluate outliers for data entry errors and biologic plausibility. We used
bivariate analyses to calculate the prevalence of recommended treatments by IBS status, unadjusted odds ratios (ORs), and 95% CIs.

We also considered types of treatment recommended, which we grouped as 1 type (any pharmaceutical, any surgery, or any referral), 2 types, or all 3 types of treatment recommended. Each level was grouped as continuation/initiation of IBS treatment compared with no recommendation of IBS treatment. We described the relationship of IBS to complexity of treatment with ORs and 95% CIs calculated from bivariate analyses.

We used stratified analyses to identify characteristics that potentially modified the association between IBS and IBS treatment. They were identified by a Breslow-Day test of homogeneity with \( P < .10 \) and stratum-specific ORs with greater than 100% difference or stratum-specific ORs in opposite directions of the null.

We used bivariate analyses to identify characteristics that potentially confounded the relationship between IBS and IBS treatment. They were identified by calculating the magnitude of effect and strength of association of the covariate-IBS association for all women and the covariate-outcome association for women without IBS. Potential confounders were included in the full model if there was a 10% difference between the Mantel-Haenszel adjusted OR and the crude IBS-outcome OR.

The characteristics that we evaluated as potentially modifying or confounding the association between IBS and IBS treatment included demographics, clinical diagnoses, history of abuse, depression, pain, prior abdominal surgeries, and medications being used at initial visit. Clinical diagnoses included muscular back pain, pelvic floor tension myalgia, endometriosis, pyriformis syndrome, vaginismus, pelvic congestion syndrome, myofascial syndrome, adhesions, adenomyosis, fibroids, urethral syndrome, pelvic relaxation problems, and vestibulitis syndrome. History of abuse included any sexual abuse (adult sexual abuse, child sexual abuse, or rape at any age), adult physical abuse, and physical discipline during childhood. Depression was measured with the Beck Depression Inventory. Pain measurements included number of pain sites, duration of pain, and McGill Pain Score. Prior abdominal surgeries included the prior lysis of adhesions, prior ablation/excision of endometriosis, oophorectomy (bilateral, unilateral, none), prior hysterectomy, prior diagnostic surgery (diagnostic laparoscopy or pain mapping), other prior gynecological procedures (vulvar, vaginal, uterine, ovarian, uterine suspension, laser uterosacral nerve ablation [LUNA]), prior nongynecologic abdominal procedures (bladder, bowel, appendectomy, cholecystectomy, hernia), prior surgery by laparoscopy, prior surgery by laparotomy, prior surgery through vagina, total number of prior abdominal procedures (gynecologic, bladder, bowel, appendectomy, cholecystectomy, hernia), and total number of prior surgeries for “this” pain. Medications being taken at start of initial visit included current antidepressant, current antianxiety/SEDATIVE-HYPNOTIC/ Anticonvulsant, current hormone replacement (estrogen, hormone replacement therapy), current progestins (progesterone, norethindrone acetate [Aygestin, Wyeth, Madison, NJ], medroxyprogesterone acetate [Provera, Pfizer Inc, New York, NY]), current Lupron (TAP Pharmaceutical Products Inc, Lake Forest, Ill) current oral contraceptives, current narcotic, current nonsteroidal anti-inflammatory drug, and current upper gastrointestinal drug (antacid, antisecretory, \( H_2 \) blockers).

We used multivariate unconditional logistic regression to determine the estimate of effect between IBS and IBS treatment. We started with a full model of all variables identified as potential modifiers or confounders of the IBS-outcome association and used backward elimination to remove 1 variable at a time by the highest \( P \)-value until the final reduced model was determined. Characteristics that modified the IBS-outcome association were kept in the final model if the likelihood ratio test resulted in a \( P < .10 \). Characteristics that confounded the IBS-outcome association were kept in the final model if they changed the association between IBS and IBS treatment by greater than 10%.

### Results

There were 987 women who entered the pelvic pain clinic between 1993 and 2000 and medical chart abstraction was completed for 98% \((n = 970)\). The largest reason for loss was an incomplete or missing medical record. In addition, some women had missing information for specific questions. Among the women with available information, 78% of the women were younger than 40 years, 81% were white, 53% had a college or graduate education, and 37% had an annual household income of $35,000 or greater. Twenty-nine percent of the patients had pain for 4 years or more, 19% had pain in at least 6 sites, and 20% had at least 3 prior surgeries for “this” pain. Most of the women had at least mild depression defined by the Beck Depression Inventory (64%). Of the women we could assess for abuse, many were physically abused as adults (34%), sexually abused as adults (16%), physically disciplined as children (68%), sexually abused as children (36%), or raped at any age (27%) (Table I).

Thirty-five percent of the women \((n = 336)\) met the Rome I criteria for IBS. Medical records were not available for all the women, hence our analyses of IBS treatment is based on 970 women. Forty percent \((n = 134)\) of the women with IBS by the Rome I criteria did not receive a diagnosis of IBS from the physician. Sixty-seven percent of the women with IBS defined by Rome I criteria \((n = 225)\) did not have a recommendation for continuation or initiation of a lower gastrointestinal...
drug or gastroenterology referral. Fifty-nine percent of the women with an IBS diagnosis from the gynecologists at the clinic (n = 115/282) did not have a recommendation for continuation/initiation of a lower gastrointestinal drug or gastroenterology referral.

In bivariate analyses, women with IBS had greater odds of having a treatment plan that included continuation/initiation of antidepressants (OR = 1.59, 95% CI = 1.22-2.07) anticonvulsant/sedative–hypnotic/anxiolytics (OR = 1.68, 95% CI = 1.15-2.45), hormone replacement (OR = 1.53, 95% CI = 1.07-2.18) upper gastrointestinal drugs (OR = 2.63, 95% CI = 1.72-4.03), lower gastrointestinal drugs (OR = 3.12, 95% CI = 2.22-4.38), or a referral to a gastroenterologist (OR = 2.54, 95% CI = 1.33-4.86). Those with IBS had lower odds of a recommendation to continue/initiate...

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**Table I** Characteristics of women with chronic pelvic pain by IBS status and recommendation for IBS treatment (lower gastrointestinal drug and/or referral to gastroenterologist)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All women (n = 970)</th>
<th>IBS treatment among women with IBS (n = 336)</th>
<th>IBS treatment among women without IBS (n = 634)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>Continuation (n = 72)</td>
<td>Initiation (n = 39)</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30</td>
<td>380 (39.4)</td>
<td>13 (18.1)</td>
<td>12 (30.8)</td>
</tr>
<tr>
<td>30-39</td>
<td>368 (38.1)</td>
<td>30 (41.7)</td>
<td>19 (48.7)</td>
</tr>
<tr>
<td>≥40</td>
<td>217 (22.5)</td>
<td>29 (40.3)</td>
<td>8 (20.5)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>785 (81.5)</td>
<td>64 (88.9)</td>
<td>27 (69.2)</td>
</tr>
<tr>
<td>Black</td>
<td>149 (15.5)</td>
<td>6 (8.3)</td>
<td>8 (20.5)</td>
</tr>
<tr>
<td>Other</td>
<td>29 (3.0)</td>
<td>2 (2.8)</td>
<td>4 (10.3)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;High school graduate or GED</td>
<td>140 (14.5)</td>
<td>8 (11.1)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>High school graduate</td>
<td>307 (31.9)</td>
<td>22 (30.6)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>College</td>
<td>516 (53.6)</td>
<td>42 (58.3)</td>
<td>15 (38.5)</td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;$20,000</td>
<td>298 (32.9)</td>
<td>22 (31.4)</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>≤$20,000-$34,999</td>
<td>246 (27.1)</td>
<td>20 (28.6)</td>
<td>9 (25.7)</td>
</tr>
<tr>
<td>≥$35,000</td>
<td>363 (40.0)</td>
<td>28 (40.0)</td>
<td>13 (37.1)</td>
</tr>
<tr>
<td>Pain characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McGill Pain score ≥43</td>
<td>236 (24.5)</td>
<td>25 (34.7)</td>
<td>10 (25.6)</td>
</tr>
<tr>
<td>Duration of pain ≥4 y</td>
<td>264 (28.5)</td>
<td>22 (31.4)</td>
<td>17 (44.7)</td>
</tr>
<tr>
<td>Number of pain sites ≥6</td>
<td>182 (19.0)</td>
<td>27 (37.5)</td>
<td>9 (23.1)</td>
</tr>
<tr>
<td>Prior surgeries for pain ≥3</td>
<td>193 (20.2)</td>
<td>19 (26.4)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Psychologic factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression (BDI ≥10)</td>
<td>605 (64.4)</td>
<td>58 (80.6)</td>
<td>25 (64.1)</td>
</tr>
<tr>
<td>Adult physical abuse*</td>
<td>214 (34.3)</td>
<td>18 (42.9)</td>
<td>13 (46.4)</td>
</tr>
<tr>
<td>Adult sexual abuse*</td>
<td>101 (16.4)</td>
<td>7 (17.5)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td>Childhood physical discipline*</td>
<td>430 (67.8)</td>
<td>30 (71.4)</td>
<td>22 (75.9)</td>
</tr>
<tr>
<td>Child sexual abuse*</td>
<td>224 (36.0)</td>
<td>20 (50.0)</td>
<td>11 (37.9)</td>
</tr>
<tr>
<td>Rape at any age*</td>
<td>166 (26.6)</td>
<td>18 (42.9)</td>
<td>11 (39.3)</td>
</tr>
</tbody>
</table>
oral contraception use (OR = 0.66, 95% CI = 0.47-0.94). IBS was not associated with recommendations of surgery (OR = 0.82, 95% CI = 0.63-1.07) (Table II).

Thirty-three percent of the women with IBS had an initial treatment plan that included a lower gastrointestinal drug or gastroenterology referral. The odds of continuation/initiation of IBS treatment were 3.10 (95% CI = 2.25-4.27) times greater for women with IBS than those without IBS. Multivariable analyses had no meaningful effect on the estimate.

Women with no prior IBS treatment (not taking a lower gastrointestinal drug at initial visit and not referred from a gastroenterologist) were similar to all women with respect to demographics, diagnoses, medications, and past surgeries. The odds of continuation/initiation of IBS treatment were 3.10 (95% CI = 2.25-4.27) times greater for women with IBS than those without IBS. Multivariable analyses had no meaningful effect on the estimate.

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not recommended continuation or initiation of treatments that may alleviate IBS symptoms. This indicates that we are missing an opportunity to treat symptoms of IBS, another syndrome with chronic pain in the abdominal region. Because we do not know why these syndromes are associated, if we recognize and treat IBS, pelvic pain may also be reduced.

Women with IBS and lower pain severity had higher odds of having IBS treatment recommended compared with those without IBS, yet this was untrue for those with the most pain. This finding suggests that women with IBS and high levels of pain are viewed as having more acute pelvic pain. Hence, the physician may focus on treating the pelvic pain and not the bowel symptoms. In comparison, women in lower pain categories may have treatment recommended for their IBS before initiating other treatments, because they are viewed as less acute.

A cross-sectional study design was appropriate because we studied whether women with IBS had a treatment plan that included specific recommendations. Although a cross-sectional study does not allow us to make judgments about causality, we know that the information collected at initial visit was used to make recommendations for treatment and not vice versa.

We defined IBS treatment as lower gastrointestinal drugs and/or referral to a gastroenterologist. This definition included drugs that would most likely be prescribed only for bowel symptoms and a referral to a specialist who may aid in IBS treatment. Although antidepressants can be prescribed for IBS symptoms, we did not include them in our IBS treatment definition because we could not determine whether they were prescribed for depression, pain, or IBS. Depression is often associated with chronic pelvic pain. In fact, 64% of our population had mild-to-severe depression according to the Beck Depression Inventory. Therefore, it is not surprising that 44% of our population were recommended continuation/initiation of antidepressants. We could not assign indication of antidepressants with confidence.

Gathering information on past drug therapies and referrals is difficult in this population because they are often referred from elsewhere. We did not know if a patient was seeking care from a gastroenterologist, sought care from a gastroenterologist in the past, or previously tried lower gastrointestinal drug therapy that did not reduce symptoms. If this was the situation, these women would be classified as having no recommendation for IBS treatment in our study. This would bias the OR toward the null and reduce the percentage of women with IBS who we classified as having a recommendation for these treatments. Yet, not having information on past treatments does not explain the failure to diagnose IBS. This failure to diagnose IBS may also explain why so many women are not recommended treatments that may alleviate bowel symptoms.

Other factors not abstracted that may be considered for future study emerging from this inquiry include: gravity, method of delivery, family size (number of dependents), care giving burden, and insurance status. Insurance status is important as benefits and copayments vary markedly. Thus, financial and opportunity costs, such as time off from work and family obligations, may inhibit care seeking behavior.

Although our population likely represents the extreme end of the spectrum of women with chronic pelvic pain, our findings highlight the need to understand the treatment of IBS in this population. We had a unique opportunity to use data that were systematically collected for 8 years by 4 gynecologists in a clinic setting with validated questionnaires and consensus-established criteria. These aspects of data collection enhance the quality of our data by reducing potential information bias and improving consistency. In addition, it makes it easier to compare our study with other studies that use these measures.

We found that among women with chronic pelvic pain, those with IBS are often not diagnosed and recommended treatments that may reduce their symptoms. These findings were identified in a clinic that had a small number of highly specialized physicians who actively asked questions about bowel symptoms. The gynecologists in this tertiary pelvic pain clinic did not consistently make an IBS diagnosis and ensure treatment of IBS in women with chronic pelvic pain. In a more general gynecology office, it is likely that diagnoses and treatment of IBS are even worse. IBS and chronic pelvic pain both affect a large proportion of women and are associated with reduced quality of life, increased health care utilization, therefore clinicians need to understand what pain management plans work best for these women. Clinicians could benefit from studies of the effect of IBS on health outcomes among women with chronic pelvic pain and studies of treatment efficacy among women with IBS and chronic pelvic pain.

Acknowledgments

We would like to thank Anne Shortliffe, RN, for managing the pelvic pain clinic data and Michel Ibrahim, MD, PhD, Rebecca Baker, PhD, Susan Hall, PhD, Dionne Law, PhD, Claire Newbern, PhD, Lynne Sampson, MPH, and Marlene Smurzynski, PhD, for critically reviewing this manuscript.

References

Objective: The purpose of this study was to evaluate the feasibility and efficacy of laparoscopic radiofrequency ablation of uterine fibroids.

Study design: Eighteen women with symptomatic intramural uterine myomas underwent radiofrequency ablation under laparoscopic guidance. Postoperative sonographic evaluations of the fibroids size were scheduled at 1, 3, 6, 9, and 12 months. The impact of myoma-related symptoms on quality of life (QOL) was assessed using a validated questionnaire.

Results: The median number of myomas treated per patient was 1 (1-3). The median baseline volume of the dominant myoma was 67.2 cm³ (14.8-332.8). No intraoperative or postoperative complications occurred. The median reductions in myomas volume were 41.5%, 59%, and 77% at 1, 3, and 6-months follow-up evaluation, respectively. No further change in fibroid size was observed at 9 months and 1 year. A significant improvement in the symptoms score and QOL score was observed at 3 and 6 months, follow-up.

Conclusion: In this pilot study, laparoscopic radiofrequency ablation successfully reduced fibroid symptoms and fibroid volume in short-term follow-up. Additional studies are needed before its efficacy and safety can be confirmed.
offers advantages over conventional myomectomy, such as the reduction in postoperative recovery time, less postoperative pain, and shorter hospital stay.\textsuperscript{4} However, intraoperative complications, mainly related to difficulty in achieving hemostasis, are far from avoided with laparoscopy.\textsuperscript{5} Moreover, prolonged operating times and technical concerns may outweigh the potential benefits of a minimal access surgery.

In the last decade, alternative options for the conservative surgical treatment of uterine fibroids have been introduced, including uterine artery embolization,\textsuperscript{6} cryomyolysis,\textsuperscript{7-9} and laser photocoagulation.\textsuperscript{10-14} Preliminary results of these minimally invasive procedures, aimed at reducing patient morbidity and further hastening postoperative recovery, seem encouraging.

Radiofrequency (RF) thermal ablation has become a widespread modality to achieve the local control of tumors, particularly in patients with primary or metastatic liver disease who are not candidates for resectional therapy.\textsuperscript{15,16} RF heating has never been used as a therapeutic option for the shrinkage of uterine myomas.

In the present study, we report our early experience in a group of patients undergoing laparoscopic RF thermal ablation of symptomatic uterine myomas, with emphasis on the safety and efficacy of this new procedure.

**Material and methods**

Premenopausal women over 40 years presenting with symptomatic intramural uterine myomas were considered eligible for the study. All patients had completed childbearing and declined hysterectomy. Presenting symptoms were menorrhagia or pelvic pain/pressure not responsive to medical therapy including progestin, oral contraceptives, and anti-inflammatory drugs. Patients previously treated with gonadotrophin-releasing hormone agonists were excluded. The presence of more than 3 uterine fibroids, a history of gynecologic malignancy within the past 5 years, a recent pelvic inflammatory disease, an abnormal coagulation screen, current pregnancy, or breastfeeding were considered as exclusion criteria.

All patients were extensively counselled on the potential risks and benefits of the procedure, and on the possible alternative surgical treatments. The local Institutional Review Board of the University of Verona approved the study, and all participants provided written informed consent before study entry.

Preoperative evaluation included an accurate transvaginal ultrasound assessment of the number, size, and location of the myomas. Fibroid volume was estimated according to the following formula: $\text{volume} = \frac{4}{3} \pi r^3$, where $r$ is the mean radius of the fibroid calculated from the measurements of the longitudinal, transverse, and antero-posterior diameter of the lesion. Sonographic evaluations were repeated at 1, 3, 6, 9, and 12 months postoperatively. When more than 1 myoma was treated in a single patient, only the characteristics of the dominant myoma were considered for statistical analysis.

The impact of symptoms on health-related quality of life in the study population was assessed using the Uterine Fibroids Symptom and Quality of Life (UFS-QOL) questionnaire.\textsuperscript{17} The questionnaire consists of 8 questions addressing both the frequency and the severity of symptoms, and 29 questions on health-related QOL. The following health related quality of life items are addressed: fatigue, self-image, mood disturbances-psychoologic distress, fear of embarrassment, interference with daily activities, relationships with family and friends, and sexual function. Two distinct scores were calculated for symptom severity and quality of life. Higher symptoms scores are indicative of greater symptom severity, while higher quality of life scores mean a better health-related quality of life. Women were asked to complete the UFS-QOL questionnaire at baseline and at 3, 6, 9, and 12 months after treatment.

**Equipment**

The RF delivery system (Rita Medical System model 1500, Mountain View, Calif) consisted of a RF generator operating at 460 KHz, with maximum power of 250 watts, and a temperature range from 15 to 125 degrees centigrade. The generator displays the temperature of the needle tip, tissue impedance characteristics, and procedure time. The system is connected through a flexible cable to a 25 cm long, 14-gauge needle, with an exposed tip (primary electrode) and 7 extendible prongs (secondary electrodes) at the distal end (Figure 1). The prongs are designed to bracket the target tissue when they are deployed laterally with a manual movement, in order to produce a spherical area of coagulative necrosis, with a maximum diameter of 5 cm. The secondary electrodes can be extracted partially or completely, according to the maximum diameter of the lesion. Four of the 7 have a thermocouple on their tips, allowing a real-time...
monitoring of the temperatures of the surrounding tissue. The RF generator produces a voltage between the active RF electrode and the dispersive electrode, a large area grounding pad in contact with the patient’s skin.

**Procedure**

The RF ablation of uterine myomas was performed under general anesthesia. Prophylactic antibiotics were not used. A 10 mm trocar was inserted through an umbilical incision, and the RF needle was inserted percutaneously and placed within the target fibroid under laparoscopic video guidance. The depth of needle insertion was determined on the basis of preoperative ultrasound. When the deployable tines of the needle are fully extended, the device mimics the configuration of a “Christmas tree.” Therefore, the tip of the central prong was placed about 1 cm beyond the center of the fibroid, so that the peripheral electrodes were localized where the cross-sectional area of the myoma is largest. A scale on the needle surface makes the placement easier.

The target temperature for the RF ablation was 100°C. The RF generator automatically adjusts the power to maintain the selected temperature. The time required to obtain a given volume of coagulation is a function of both temperature and tissue impedance. The duration of ablative sessions was decided according to the manufacturer’s recommendations: the complete ablation of a 3 cm large myoma takes approximately 5 minutes. Multiple overlapping ablation cycles have been performed for fibroids with a mean diameter larger than 5 cm. The RF device may also be used to coagulate the needle track after the procedure is completed.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif). The Wilcoxon matched pairs test was used to compare the volumes and the percentage of volume reduction of the treated myomas, as well as the UFS-QOL scores. The statistical significance was considered to be achieved when \( P < 0.05 \).

**Results**

During the study period, 18 patients underwent laparoscopic RF ablation of uterine fibroids. The median (range) age of the patients was 44.3 years (40-50). The median number of fibroids treated per patient was 1 (1-3). The median baseline diameter and volume of the dominant myoma were 5 cm (3-8.6) and 67.2 cm\(^3\) (14.8-332.8), respectively. The mean diameter of the dominant fibroid was less than 4 cm in 4 cases, 4 to 6 cm in 11 cases, and larger than 6 cm in 3 cases. The location of the dominant myoma was posterior in 9 (50%) cases, anterior in 4 (22%), and fundal in 5 (28%). The median (range) baseline volume of the dominant myoma was 67.2 cm\(^3\) (14.8-332.8). The primary presenting symptom was menorrhagia in 15 (83.3%) cases, and pelvic pressure or pain in 3 cases (16.7%). The median preoperative symptom score was 43.7 (12.5-90.6), while the median health-related quality-of-life score was 66.7 (35-93.9).

The operative time ranged from 20 to 40 minutes (median 25). Seven (38.9%) of the dominant fibroids were treated with a single pass. No intraoperative or postoperative complications occurred during or after the RF procedure. The RF needle track ablation program completely avoided the occurrence of blood loss in all cases. Only 2 patients complained of mild abdominal pain, not requiring analgesic drugs. All patients were observed overnight and discharged from hospital on the first postoperative day.

**Figure 2** Changes in the volume of the myoma during the follow-up period after radiofrequency ablation.
The median follow-up time was 10 months (3-12). The median fibroid volume and the median reduction of the volume during the follow-up period are shown in Table I. When analysis was restricted only to women who completed the 1-year follow-up period (n = 9), at 9 and 12 months neither a significant further decrease in size, nor a regrowth of myomas, was observed compared with the 6-month postoperative evaluation. Figure 2 displays the volume changes of the dominant myomas after the RF ablation.

The changes in symptoms score and in health-related quality-of-life score are shown in Table II. Seven out of 9 (77.8%) women who completed the 1-year follow-up period were totally symptom-free.

Comment

The results of this pilot study suggest that RF ablation may represent a safe, well-tolerated, and effective alternative to conventional surgery for the treatment of symptomatic uterine myomas in selected groups of patients.

The widespread use of thermoablative procedures in gynecology has been limited by concerns of the ability to create coagulative necrosis in a controlled fashion, with minimum damage to normal surrounding tissues. Myolysis as a treatment option for uterine fibroids was first introduced in the late 1980s as an hysteroscopic technique and, subsequently, as a variation on the technique of laparoscopic myomectomy, in which fibroid tissue was coagulated rather than removed. The first series, where myomas ablation was performed with Nd:YAG laser, have clearly shown the efficacy of this technique in achieving fibroids shrinkage. However, concerns have arisen because of the extremely high incidence of adhesion formation detected at secondlook laparoscopy. Since then, several new techniques for fibroids myolysis have been sought in an attempt to find minimally invasive approaches to uterine myomas, less technically demanding, and less time consuming than laparoscopic myomectomy, while minimizing adhesion formation. A number of case series have been published supporting the feasibility of myomas ablation either by thermotherapy (bipolar or monopolar coagulation, diode laser) or by cryotherapy. Although each method has been proven to be effective, most of them have some limitations related to high costs, difficult application and monitoring, and inability to consistently avoid nontarget tissue.

RF energy is an alternating current with a frequency between 10 and 900 kHz. At these frequencies, the heat generated by the electrical current is dissipated into the area that is close to the electrode-tissue interface. Tissues are heated not by conduction of heat directly, but by agitation of tissue ions at a high frequency. Although several potential mechanisms for cellular injury caused by RF energy have been postulated, the predominant mechanism is thermal damage caused by frictional heating. Once temperature is above 50°C, cell membranes melt and fuse, proteins denature, and irreversible cell death occurs. Pathologic studies in patients with unresectable hepatic tumors have demonstrated that RF lesions behave like aseptic necrosis, with a clearly demarcated rim from surrounding tissue.

Current multiprong RF devices are designed to produce a large spherical volume of necrosis at every insertion, decreasing the total number of needle passes. Conversely, when laser fibers or monopolar/bipolar needles are employed for fibroids myolysis, because of the narrow area of tissue destruction created by a single straight needle, multiple passes or simultaneous insertion of multiple needles are necessary to treat the entire lesion. It has been postulated that the presence of multiple holes on the myoma serosa could increase the risks of postoperative adhesion formation. Further
investigations, including second-look data, are needed to assess whether RF ablation might reduce post-myolysis adhesions.

The ability of RF electrodes used in the present study to create a large spherical area (up to a diameter of 5 cm) of tissue ablation resembles the ice ball around the probe in the cryoablation systems. Radiofrequency energy seems at least as effective as cryomyolysis in achieving myoma shrinkage. Our results are in keeping with the findings of Zupi et al., who recently reported a series of 20 women undergoing laparoscopic cryomyolysis for symptomatic uterine myomas. These authors reported a mean fibroid volume reduction of 56.9 ± 12.3% at 6 months’ follow-up, with resolution or significant improvement of myoma-related symptoms in 95% of cases. Because the choice of the optimal treatment for fibroids should take into account efficacy, complication rate, and cost-effectiveness ratio, RF thermoablation seems to offer some advantages. First, the electrical generators required for RF ablation are considerably cheaper compared with cryosurgery equipments. Second, the cryoprobe’s diameter are usually larger (>2 mm) than the 14-gauge needle used for RF thermotherapy, increasing the risks of bleeding from the puncture site. Third, the needle track ablation system allows a quasi-bloodless procedure. Finally, the need of 1 or more freeze/thaw cycles per myoma during cryotherapy may result in a longer operative time.

Two case series described magnetic resonance-guided percutaneous ablation of uterine myomas with cryotherapy and laser fibers. Although a percutaneous approach is a minimally invasive procedure, performable under local anesthesia as an ambulatory surgery, preliminary results showed a high incidence of major perioperative complications, suggesting that a direct visual monitoring is advisable during ablative procedures.

A controversial issue about uterine fibroid myolysis is whether tissue local destruction without surgical repair might increase the risks of suboptimal healing and, ultimately, the chance of uterine rupture during pregnancy. Because the safety for women whose aim is future pregnancy has not been established, an appropriate selection of patient candidates for ablative procedures is mandatory. Currently, laparoscopic myolysis should be restricted to patients in the late reproductive years, when myoma-related symptoms are more frequently reported.

In this population of women, an increasing trend toward seeking alternatives to hysterectomy has been observed in the last years.

In conclusion, our preliminary results suggest that laparoscopic RF ablation is a promising new approach for the conservative treatment of uterine fibroids. It is a low-cost and low-tech procedure, with an effectiveness and an impact on myoma-related symptoms comparable to those of other minimally invasive innovations. The major limitation of this study is the short follow-up time, which does not allow to evaluate the mid- and long-term recurrence rate, and to draw definite conclusions about the effectiveness of RF myolysis. This pilot study can serve as a stimulus for further investigations aimed at comparing this technique with other surgical approaches, as well as with uterine artery embolization, which has been proven as a valid alternative to surgical therapy.

References

The effect of vaginal candidiasis on the shedding of human immunodeficiency virus in cervicovaginal secretions

Arsenio Spinillo, MD,* Francesca Zara, MD, Barbara Gardella, MD, Eleonora Preti, MD, Roberta Mainini, MD, Renato Maserati, MD

Departments of Obstetrics and Gynecology, Microbiology and Infectious Disease, University of Pavia, IRCCS Policlinico S Matteo, Pavia, Italy

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KEY WORDS
HIV infection
Vulvovaginal candidiasis
HIV shedding
Cervicovaginal secretions

Objective: The purpose of this study was to evaluate the influence of symptomatic vulvovaginal candidiasis on the shedding of HIV-1 in cervicovaginal secretions of HIV-1–infected women.

Study design: We obtained paired blood and cervicovaginal lavage samples from 66 HIV-infected women with symptomatic vulvovaginal candidiasis, and 249 HIV-infected control patients without genital infection. HIV-1 RNA in plasma, proviral HIV-1 DNA, HIV-1 RNA transcripts, and cell-free HIV-1 RNA in cervicovaginal secretions were quantitatively evaluated by competitive polymerase chain reaction (cPCR) and reverse transcriptase PCR (cRT-PCR). We used logistic regression on ordered data to assess the influence of vulvovaginal candidiasis on the HIV-1 load in cervicovaginal secretions adjusting for potential confounders.

Results: Overall, the amount of HIV-1 RNA in plasma was significantly correlated with HIV-1 DNA (Spearman rank 0.153 ± 0.059, \( P = .006 \)), HIV-1 RNA transcripts (Spearman rank 0.169 ± 0.058, \( P = .003 \)), and cell free HIV-1 RNA (Spearman rank 0.185 ± 0.059, \( P = .001 \)) load in cervicovaginal secretion. Forty-eight out of 182 (26.4%) patients who tested negative for HIV-1 RNA in plasma were positive for HIV-DNA in their cervicovaginal secretions. In logistic regression analysis vulvovaginal candidiasis was significantly associated with increasing loads of HIV-1 RNA transcripts (Odds ratio [OR] 1.97, 95% CI 1.09-3.57, \( P = .025 \)) and cell free HIV-1 RNA (OR 2.03, 95% CI 1.10-3.73, \( P = .02 \)) in cervicovaginal secretions.

Conclusion: In HIV-infected women, vulvovaginal candidiasis is associated with an increased number of copies of cell-associated and cell-free HIV-1 RNA in cervicovaginal secretions.

Several factors regulating the rate of genital HIV shedding are capable of influencing sexual acquisition and vertical transmission of HIV.1 Among local factors, genital ulcer disease associated with several sexually transmitted diseases is associated with an increased risk of HIV sexual transmission.2 Vaginal infections such as bacterial vaginosis and Trichomonas vaginalis are associated with an increased risk of HIV-1 RNA detection in cervicovaginal secretions.3 A study from Kenya4 showed that the vaginal detection of proviral HIV-1 DNA is increased in the presence of vulvovaginal candidiasis. In

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* Reprint requests: Arsenio Spinillo, MD, Department of Obstetrics and Gynecology, University of Pavia, IRCCS Policlinico S Matteo, Pavia, Italy.

E-mail: spinillo@smatteo.pv.it

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a previous smaller study,\(^5\) we found that patients with either vaginal *Candida* colonization or infection had a slightly higher prevalence of cell-free HIV-1 RNA in cervicovaginal secretions compared with control patients with negative vaginal cultures. Although a direct relationship between vaginal infection and increased HIV infectivity has not been definitively established, the evaluation of factors regulating the dynamics of HIV shedding or HIV replicative life cycle in genital tract could be important in prevention programs. In this study, we sought to evaluate whether symptomatic vulvovaginal candidiasis is associated with an increased rate of proviral HIV-1 DNA, HIV-1 RNA transcripts (cell-associated), and cell-free HIV-1 RNA shedding in cervicovaginal secretions. The simultaneous detection of proviral HIV-1 DNA and both cell-associated and cell-free RNA in cervicovaginal secretion is necessary to evaluate both the passage of HIV-1–infected cells from plasma to vaginal fluid as transudate, but also the presence of local active HIV replication.

**Material and methods**

**Design and population of the study**

This study is part of a larger research project, started in 1998, on the role of clinical and microbiological factors affecting the presence of HIV in cervicovaginal secretions of HIV-seropositive women. The research project was approved by the Institutional Review Board of the University of Pavia, and by the Scientific Committee of our Hospital (IRCCS Policlinico S Matteo, Pavia, Italy).

This study was planned as a prospective observational case-control investigation. Cases comprised all known HIV-seropositive women with symptomatic vulvovaginal candidiasis attending our vaginitis clinic during the period 1999 to 2003. During this period, out of 121 HIV-seropositive women with symptoms of vaginitis (vaginal pruritus, burning, abnormal vaginal discharge), 71 had a diagnosis of *Candida*, 38 of bacterial vaginosis, 5 were infected by *Trichomonas vaginalis*, and the remaining 7 had a noninfectious cause of vaginal irritation. Five out of the 71 *Candida* patients were excluded from the study because cervicovaginal sample was contaminated by blood, leaving 66 patients suitable for analysis. Control patients were recruited among known HIV-seropositive patients without symptoms of vaginitis attending our department in the same period as the cases for periodic screening of lower genital tract neoplasia. Out of 293 women, 44 (15%) were excluded from the study either because they were colonized by *Candida* (20 patients) or had bacterial vaginosis (24 patients). Thus, the final population under study comprised 66 cases with vulvovaginal candidiasis and 249 control patients. A detailed questionnaire with information on demographic, sexual, and clinical data was administered to each participant. Clinical HIV disease was staged using the Centers for Disease Control and Prevention (CDC) classification system.\(^6\)

**Samples collection and laboratory analyses**

Peripheral blood samples for estimating CD4+ lymphocyte cell counts and quantifying HIV-1 RNA in plasma were collected from all subjects during the visits. After careful speculum examination, vaginal specimens were collected with sterile cotton swabs to diagnose infection by *Candida*, trichomonas, or bacterial vaginosis. To evaluate *Candida* infection, vaginal specimens were inoculated on Sabouraud’s dextrose agar containing gentamicin 40 mg/mL. Cultures were incubated at 30°C for 48 to 72 hours, and then examined for growth. Isolated strains were identified using the germ tube test auxanogram (API 20 C Aux, API system, Montalieu-Vercieu, France). The diagnosis of bacterial vaginosis was made according to the criteria of Amsel et al.\(^7\)

Cervicovaginal secretions for HIV-related nucleic acids were collected by gently rotating a Dacron swab within the posterior fornix, and by lavage after insertion of 10 mL of RPMI-1640 medium into the vagina, followed by aspiration of the suspension after allowing 1 minute for pooling. Swabs were used to detect cell-free HIV-1 RNA, whereas HIV-1 DNA and intracellular HIV-1 RNA transcripts were detected from lavage samples. Upon arrival in the laboratory, and again afterwards, centrifugation samples were examined under the microscope to confirm absence of red blood cells and spermatozoa. The presence of blood contamination was further checked by using a routine screening test for hemoglobin detection (reactive strips, multistic-10 visual, Bayer, Milan, Italy). A detailed description of methods used to detect and quantify HIV-related nucleic acids from blood and cervicovaginal secretions has been reported elsewhere.\(^8\) The following substrates were analyzed using quantitative polymerase chain reaction (PCR) and reverse transcription PCR: 1) genomic HIV-1 RNA from plasma and cell-free cervicovaginal secretions; 2) virus-specific unspliced HIV-1 RNA transcripts from cervicovaginal cells; and 3) proviral HIV-1 DNA from nuclei of cervicovaginal cells. Quantitative analysis of specific RNA and DNA sequences was first done by PCR using the SK 426/431 pair of primers.\(^9\) RNA samples were reverse transcribed using 100 U of Moloney murine leukemia virus reverse transcriptase (RT, Gibco Life Technologies, Paisley, Scotland), 20 pmol of the SK 431 primer, 0.2 mmol/L deoxynucleotide triphosphate, and 20 U of Rnasin (Gibco Life Technologies). DNA was subsequently amplified using 50 pmol of primers SK 462 and SK 431, 1.5 U of taq-DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif). Quantification of HIV-1 DNA and RNA was done...
with a competitive PCR and reverse transcription PCR as described by Menzo et al. Using this method, 2 similar RNA or DNA templates were reverse transcribed or amplified. The primer set (SK38/Sk39) was specific for a highly conserved gag fragment of HIV-1 genome, and the method has been useful in the quantitative analysis of HIV-1 genomic RNA from plasma, unspliced RNA transcripts, and proviral DNA from peripheral blood mononuclear cells. For cervicovaginal samples, quantitative results were expressed as HIV-1 RNA cell-free copy number per mL of cervicovaginal secretion. Swabs were incubated in a predetermine quantity of transport medium (1 mL), so it was possible to know the exact amount of cervicovaginal secretion present in each sample (usually 200-300 μL). Under our experimental conditions, the lower limit of detection of the assay was 2 DNA or RNA copies/105 cells, 20 RNA copies/mL of cervicovaginal secretions, and 50 RNA copies/mL of plasma.

**Statistical analysis**

We used the Mann-Whitney U test, χ² test, and chi-square for trend to compare continuous, nominal, and ordered categorical data, respectively. A nonparametric approach (Spearman rank correlation coefficient) was used to analyze the association between levels of HIV-1 nucleic acids. Univariate associations between nominal variables were assessed using ORs and 95% CIs. To evaluate the associations between levels of HIV-1 load in cervicovaginal secretion and Candida vaginal infection adjusting for potential confounders, we used logistic regression on ordered categorical variables. In logit models, ordered categorical variables (cervicovaginal HIV loads) were inserted as ordered terms with 4 levels of detection, whereas explanatory variables (Candida infection, CD4+ cell counts, plasma HIV-1 load) were inserted as categorical variables. All the analyses were carried out using STATA 8.0 statistical software (Stata Corporation, Lakeway Drive, College Station, Tex).

**Results**

The mean age was 32.1 ± 4.4 (SD) years among patients with Candida infection, and 31.9 ± 4.1 in the control patients (P = .7). The main demographic characteristics of the patients in the study are reported in Table I. There were no significant differences between the 2 groups studied. Candida albicans and C glabrata were responsible for 86.4% (57/66) and 9.1% (6/66) of vaginal infections. In the remaining 3 cases, other Candida species were isolated (C parapsilosis, C krusei, and S cerevisiae).

HIV-1 RNA was detected in the blood of 39.4% (26/66) of the patients with vaginal candidiasis and in 43% (107/249) of the control patients (P = .3). Twenty-four patients (36.4%) with vaginal infection and 77 control patients (30.9%) (P = .4) were at stage C of HIV disease (AIDS-defining conditions). In addition, at the time of the study, 80.3% (53/66) of patients with Candida, and 85.5% (213/249, P = .3) of the control patients were receiving stable highly active antiretroviral therapy (HAART). Overall, the amount of HIV-1 RNA in plasma was directly correlated with HIV-1 DNA (Spearman rank 0.153 ± 0.059, P = .006), HIV-1 RNA transcripts (Spearman rank 0.169 ± 0.058, P = .003), and cell free HIV-1 RNA (Spearman rank 0.185 ± 0.059, P = .001) viral load in cervicovaginal secretions, and inversely correlated with CD4+ cell counts (Spearman rank -0.21 ± 0.09, P = .02). However, 26.4% (48/182) of the patients who tested negative for HIV-1 RNA in plasma were positive for HIV-DNA in their cervicovaginal secretions. The corresponding figures for HIV-1 RNA transcripts and cell-free HIV-1 RNA were 19.2% (35/
than in control patients (352 cells/mm$^3$, range 9-1150 counts was slightly lower among patients with Candida CD4 (yes, no), presence of plasma HIV-1 RNA (yes, no), P = .16), although this difference was of borderline statistical significance. The prevalence of CD4+ cell counts was slightly lower among patients with Candida than in control patients (352 cells/mm$^3$, range 9-1150 compared with 400 cells/mm$^3$, range 0-1569, P = .1), although this difference was of borderline statistical significance. The prevalence of CD4+ cell counts <200 mm$^3$ was 19.7% (13/66) in subjects with Candida infection and 12.9% (32/249) in the control patients (P = .053).

Table II reports the results of HIV-related nucleic acids detection in plasma and cervicovaginal secretions according to the presence of Candida infection. HIV-1 RNA transcripts and cell-free HIV-1 RNA loads were significantly higher in patients with vulvovaginal candidiasis compared with control patients. The median CD4+ cell counts was 19.7% (13/66) in subjects with Candida infection than in control patients (352 cells/mm$^3$, range 9-1150 compared with 400 cells/mm$^3$, range 0-1569, P = .1), although this difference was of borderline statistical significance. The prevalence of CD4+ cell counts <200 mm$^3$ was 19.7% (13/66) in subjects with Candida infection and 12.9% (32/249) in the control patients (P = .053).

In order to perform reliable univariate and multivariate analyses of ordered data, the cervicovaginal HIV-1 viral load, as evaluated by the number of copies of virus-related nucleic acids, was stratified in 4 levels. The first level included the negative cases, and the other 3 were the tertiles of viral load among positive cases. Univariate analysis of trend showed that HIV-1 RNA load in cervicovaginal secretions both in the form of HIV-1 RNA transcripts and as cell-free HIV-1 RNA was higher among patients with Candida infection than in control patients.

To evaluate the effect of vaginal infection on the amount of HIV-related nucleic acids in cervicovaginal secretions adjusting for potential confounders, we used logistic regression on ordered data. HIV-1 loads in cervicovaginal secretions were entered in logistic model as dependent variables with 4 levels of detection. Explanatory variables were Candida vaginal infection (yes, no), presence of plasma HIV-1 RNA (yes, no), CD4+ cell counts <200 mm$^3$ (yes, no), and HAART (yes, no). The results of the analysis are reported in Table III. After adjustment for potential confounders, patients with symptomatic vulvovaginal candidiasis had significantly higher numbers of HIV-1 RNA transcripts and cell free HIV-1 RNA copies in cervicovaginal secretions than did control patients without vaginal infection.

**Comment**

The rates of HIV-1 related nucleic acids detection in cervicovaginal secretions in this study are similar to those found in other investigations. According to other studies, the presence of HIV-1 RNA in plasma is the best predictor of the presence of HIV-related nucleic acids in cervicovaginal secretions. However, we confirm that almost 20% of patients with detectable HIV-related nucleic acids in cervicovaginal secretions are negative for the presence of HIV-1 RNA in plasma. This finding suggests that the factors regulating HIV replication in the genital tract could be different from those modulating their presence in blood.

In our study, the presence of symptomatic vulvovaginal candidiasis was associated with an increased load of HIV-related nucleic acids in cervicovaginal secretions. Potential limitations or biases of this investigation could be related to the number of patients and to the type of population studied. In particular, symptomatic vulvovaginal candidiasis is more frequent and persistent among severely immunosuppressed patients. The severity of immunosuppression, in turn, is associated with factors predicting the presence of HIV-related nucleic acids in cervicovaginal secretions, such as the amount of

<table>
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<tr>
<th>Table II</th>
<th>HIV-1 load in plasma and cervicovaginal secretions among HIV-infected women with vulvovaginal candidiasis and HIV-infected control patients without vaginal infection</th>
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<td></td>
<td>Vulvovaginal candidiasis (n = 66)</td>
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<td><strong>Plasma</strong></td>
<td>HIV-1 RNA (copies/mL)</td>
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<td><strong>Cervico-vaginal secretions</strong></td>
<td>HIV-1 DNA (copies/10$^5$ cells)</td>
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<td>HIV-1 RNA transcripts (copies/10$^5$ cells)</td>
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<td>HIV-1 RNA cell-free (copies/mL)</td>
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<td><strong>Cervico-vaginal secretions</strong></td>
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* Mann-Whitney U test.  
† χ² for trend.
HIV-1 RNA in blood. Furthermore, using different definitions of Candida vaginal infection, Duerr et al suggest that high plasma viral load could be directly associated with an increased rate of vulvovaginal candidiasis. We sought to overcome these limitations by using multivariable methods; after adjustment for potential confounders, Candida vaginal infection was still associated with a higher HIV-1 viral load in cervicovaginal secretions.

In a previous smaller study of HIV-seropositive patients, we found that the detection rate of cell-free HIV-1 RNA was slightly higher among the 25 women with either vaginal Candida colonization or infection than among control patients without vaginal infection. In the present study we decided to restrict the analysis only to patients with symptomatic vulvovaginal candidiasis, exclusive patients with Candida colonization. This approach was chosen to increase the power of the study because patients with symptomatic vaginitis are more likely to have increased rates of HIV genital shedding. In fact, previous studies on the relationship between genital ulcer disease or cervicovaginal inflammation and HIV have suggested that the presence of a local vaginal inflammatory response could be a key factor in HIV genital shedding. In particular, Wright et al found that shedding of HIV-1 into genital secretions could be increased as much as 10,000-fold in HIV-infected women with cervical inflammation.

The relationship between vulvovaginal candidiasis and proviral HIV-1 DNA genital shedding has been previously investigated by Mostad et al. These authors found that vaginal yeast infection was associated with a 2-fold increased likelihood of detection of HIV-1 infected cells on vaginal swabs. In the study by Kovacs et al, the presence of vaginal Candida, as diagnosed by potassium hydroxide examination, was associated with a significantly increased risk of cell-free HIV-1 genital shedding in the univariate study; this result was not, however, confirmed in multivariate analysis. One of the potential limitations of the study by Kovacs et al was that cell-free HIV-1 RNA was used as the only marker of genital shedding. According to other authors, genital infections could result in an increase of both cell-associated and cell-free HIV-1 genital shedding. In our investigation we focused on the presence of HIV-1 RNA transcripts (cell-associated) and cell-free HIV-1 RNA shedding. The 2 types of HIV-1 RNA detected could be markers of viral passage from plasma to vaginal fluid (cell-free HIV-1 RNA), but also of viral replication (HIV-1 RNA transcripts). Our results suggest that both shedding and replication of HIV in genital tract could be increased in patients with symptomatic vulvovaginal candidiasis.

The biological reasons for these results could be related to the immunologic and inflammatory responses elicited by Candida infection on vaginal mucosa. Symptomatic Candida vulvovaginitis is associated with an increased local recruitment of CD4 receptor-bearing lymphocytes in vaginal mucosa, which could influence local viral replication. In addition, erythema and extravasation of vaginal fluid, associated with mucosal inflammation caused by Candida infection, could facilitate the passage of cell-free HIV in vaginal fluid. Wang et al found that the treatment of vulvovaginal candidiasis was associated with a 3-fold reduction in the prevalence of HIV-1 DNA in cervicovaginal secretions. This effect was more marked among patients with evidence of local inflammation such as vulvar erythema.

The potential implications of increased shedding of HIV among patients with vulvovaginal candidiasis could be relevant. Given the high prevalence and persistence of vulvovaginal candidiasis among HIV-seropositive women, even a modest increase in HIV genital shedding could result in a significant increase in the risk of HIV transmission. With the possible exception of genital ulcer disease, the effect of other vaginal infections on the rate of HIV sexual or vertical transmission has been not adequately assessed. However, until definitive data become available, it appears that adequate treatment and prophylaxis of vulvovagi-
nal candidiasis among HIV-infected women is advisable not only to cure associated symptoms but also to reduce the potential risk of HIV infectivity.

References

Reliability of health-related quality-of-life measures 1 year after surgical procedures for pelvic floor disorders

Patricia A. Wren, PhD, MPH,a Nancy K. Janz, PhD,a Linda Brubaker, MD,d Mary Pat Fitzgerald, MD,d Anne M. Weber, MD,e Frankie B. LaPorte, MS,b John T. Wei, MD,c,* for the Pelvic Floor Disorders Networke

Objective: The purpose of this study was to assess the reliability and validity of condition-specific health-related quality-of-life measures in women who are treated surgically for pelvic organ prolapse and urinary incontinence.

Study design: The study used the cross-sectional telephone interview–based administration of a health-related quality-of-life measure, with a 2-week follow-up interview for test-retest reliability.

Results: Initial and follow-up interviews were completed by 88 women (mean age, 65.7 ± 11.6 years) approximately 1 year after surgical procedures. Condition-specific measures demonstrated acceptable reliability with test-retest correlation coefficients that approached or exceeded 0.6 and Cronbach’s alpha that exceeded 0.8 in most domains. Validity was demonstrated with significant correlations of the urinary domains of the Pelvic Floor Distress Inventory and Pelvic Floor Impact Questionnaire, with continence defined by the medical, epidemiologic, and social aspects of aging and Hunskaar severity measures (all \( P < .001 \)).

Conclusion: The condition-specific health-related quality-of-life assessment is reliable and valid in women after surgical procedures for pelvic floor disorders. These findings support the inclusion of condition-specific health-related quality-of-life measures in clinical trials for women with pelvic floor disorders.

A large and growing body of literature has established the relevance of the evaluation of health-related quality of life (HRQOL) and functional status as important adjuncts to standard clinical outcomes.1,2 Quality-of-life questionnaires, functional health status surveys, and symptom measures all offer important information about the way pelvic floor disorders and their treatments affect women’s lives. Because anatomic and physiologic measures do not always reflect patients’
experiences of their conditions, Patrick et al\(^3\) advocated for the inclusion of quality-of-life measures in clinical trials to complement clinical findings. Until recently, there were few condition-specific instruments for pelvic floor disorders. Growing interest in the measurement of urinary-, sexual-, and bowel-related HRQOL for pelvic floor conditions has spawned the development of several new measures.\(^3\). Preliminary studies have suggested that these instruments are valuable; however, before widespread application, further psychometric evaluation of these measures among women with pelvic floor disorders is necessary.

The Pelvic Floor Disorders Network (PFDN) was established by the National Institute of Child Health and Human Development to conduct research that would lead to improved clinical care and quality of life for women with pelvic floor disorders.\(^8\) This study was undertaken to assess more fully the reliability and validity of selected HRQOL measures for use in PFDN-sponsored research studies with the use of a telephone-based interview. Condition-specific measures that included the medical, epidemiologic, and social aspects of aging (MESA) scale,\(^9\) Hunskaar severity measure,\(^10\) pelvic floor distress inventory (PFDI),\(^5\) pelvic floor impact questionnaire (PFIQ),\(^6\) and the pelvic organ prolapse/urinary incontinence sexual function questionnaire (PISQ),\(^7\) general health status (short-form health survey [SF-36]),\(^11\) and life orientation test-revised (LOT-R) were evaluated.\(^12\)

**Material and methods**

**Clinical data collection**

A convenience sample of women who underwent operation for either pelvic organ prolapse or urinary incontinence between September 2000 and March 2002 was identified from clinics at the University of Michigan (Ann Arbor, Mich) and Loyola University Medical Center (Maywood, Ill). Permission to contact patients was obtained from the surgeon, and Institutional Review Board approvals were obtained from both centers.

Relevant demographic and clinical information that included age, race, hysterectomy status, menopausal status, and date and details of the surgical procedures was abstracted from the medical records.\(^13\) Patients usually were seen 2 or 3 times in the first year after the operation. Postoperative visits included an assessment of symptoms and physical findings. Clinically, vaginal support was measured with the validated pelvic organ prolapse quantification (POP-Q) system,\(^14\) and urinary control that was measured according to the physician’s medical chart was characterized as either “continent” or “incontinent.” Data collected during the postoperative clinic visit closest to the date of the telephone HRQOL survey were used for the purpose of these analyses.

**Quality-of-life data collection**

Telephone-based interviews were conducted by the University of Michigan Quality of Life Interviewing Center, which used a team of trained interviewers. None of the interviews was conducted by the treating physician. After completion of the first telephone interview, a second interview was conducted by the same team of interviewers (but not necessarily the same interviewer) 2 weeks later to examine test-retest reliability. Telephone appointments for the interviews were established, and a telephone script that incorporated the HRQOL measures was followed. This included reading each item to the subject followed by the allowable responses. On average, the interviews took 38 minutes to complete.

The HRQOL protocol included condition-specific and generic measures that were designed to operationalize a broad conceptual framework of quality of life.\(^3\) These measures were selected by the PFDN steering committee after consideration of each measure’s usefulness, published reliability and validity, and relevance to the PFDN objectives. Two measures of urinary incontinence were included. The MESA questionnaire (15 items) classifies patients as being continent or as having symptoms of stress, urge, and mixed urinary incontinence.\(^9\) The patient was placed into 1 of the 4 categories on the basis of her response to the stress and urge incontinence items on the MESA measure: (1) pure stress incontinence, if the patient endorsed (answered affirmatively) only stress items; (2) pure urge incontinence, if the patient endorsed only urge items; (3) mixed urinary incontinence, if she endorsed both stress and urge items; and (4) continent, if the patient endorsed any incontinence items as “never” or “rarely.” The Hunskaar severity measure assesses the frequency and amount of urine leakage on scale of 0 to 4, with higher scores indicating more severe incontinence.\(^10\) The Hunskaar measure is scored by multiplication of the response values from 2 items: How often do you experience urinary leakage? How much urine do you lose each time? A raw score that ranges from 0 to 12 is then rescaled into 5 possible incontinence severity levels: continent (0), slight (1-2), moderate (3-6), severe (7-9), and very severe (10-12) incontinence. The PFDI and the PFIQ are companion measures of pelvic organ prolapse, urinary, and colorectal-anal problems for which higher scores indicate greater dysfunction.\(^6\) The PFDI is a 46-item symptom measure that yields 3 subscales: urinary distress inventory (UDI; range, 0-300), pelvic organ prolapse distress inventory (POPDI; range, 0-300), and colo-rectal-anal distress inventory (range, 0-400). The PFIQ is a functional status measure that probes 31 activities of daily living and the extent to which they are affected by bladder, bowel, and/or pelvic symptoms. Three subscales can be derived, and scores range from 0 to 300: urinary incontinence impact questionnaire...
(UIQ), pelvic organ prolapse impact questionnaire (POPIQ), colorectal-anal impact questionnaire. Lower scores for both the PFDI and PFQI domains indicate better health status. For women who had been sexually active within the past 3 months, a 12-item short-form version of the PISQ (range, 0-48) was administered. This measure includes questions that are related to sexual function (such as frequency and orgasmic capabilities and pain, incontinence, and avoidance of sexual intercourse). A lower score on the PISQ indicates better sexual function.

In terms of generic HRQOL, a single validated health utility item was included to capture respondents' perceptions of their current state of health on a scale from 0 to 100 (0 represents death; 100 represents perfect health). The SF-36 (version 2) was used to provide a comprehensive generic assessment of functional status with 8 subscales. The 10-item LOT-R is a separate measure that assesses generalized expectancies for positive versus negative outcomes, on which higher scores indicate greater optimism.

Validity was assessed by 2 approaches. First, the difference in mean HRQOL scores was compared with the use of the Wilcoxon rank sum test (or chi-squared test for categoric data) according to the postsurgical clinical assessments of urinary continence status (continent vs incontinent) and the POP-Q stage (less than stage II vs stage II or more). Second, the relationships between generic and condition-specific HRQOL scores were examined with the Pearson correlation. All analyses were performed with SAS software (SAS Institute, Cary, NC); a significance level of .05 was used for hypothesis testing.

**Results**

**Study sample**

Ninety-two women consented to participate, and 88 women completed both the baseline and follow-up interviews, for a participation rate of 95.6%. The average age for the 88 participants was 65.7 ± 11.6 years; 98% of the women were white. Although most women were currently married (62%), only 40 women (46%) reported being currently sexually active. Two subjects did not have clinical data available and were included solely for test-retest reliability assessments. Of the remaining 86 women, 83 women (97%) had a chief complaint of prolapse; 2 women (2%) had a chief complaint of urinary incontinence, and 1 woman (1%) did not have a chief complaint recorded. Most women (83%) had at least 1 previous prolapse repair, and 81% of them had undergone hysterectomy. Ninety percent were postmenopausal. The following preoperative pelvic organ prolapse stages were represented: POP-Q stage I, 1%; POP-Q stage II, 11%; POP-Q stage III, 73%; and POP-Q stage IV, 15%. Despite the fact that most subjects were being seen for prolapse, approximately one half of the women (53%) had preoperative documentation of urinary incontinence by the treating physician based on their history and physical examinations. Thirty-four of the women (40%) underwent sacrocolpopexy as their primary procedure.

**Clinical and HRQOL outcomes**

The mean time between surgery and the quality-of-life interview was 50 weeks ± 22 weeks; the mean clinical follow-up duration after surgery was 8 weeks ± 10 weeks. Among the 61 women who had POP-Q documented at follow-up examination, most of the women had minimal pelvic organ prolapse (POP-Q stage 0, 18%; POP-Q stage I, 39%; POP-Q stage II, 39%; and POP-Q stage III-IV, 4%). Among the 59 women who had continence that was documented after surgery, 19% were characterized as “incontinent” by physician assessment. Postoperative HRQOL scores (from the initial interview) are summarized in Table I. The number of
respondents do not always total 86 because of incomplete data for some scales. Twenty-four percent of women were continent on the MESA, although another 38%, 26%, and 12% were categorized as having mixed, stress, and urge urinary incontinence, respectively. The Hunskaar severity score offered a different assessment, in that 50% of the sample was considered continent or slightly incontinent, 33% had moderate incontinence, and 17% had severe or very severe incontinence. The agreement between the physician’s rating and the MESA and Hunskaar definitions of continence were poor (kappa, 0.13) and fair (kappa, 0.23), respectively. However, the agreement between MESA and the Hunskaar definitions was modest (kappa, 0.49).

**Test-retest reliability**

A primary aim of the present study was to establish the psychometric properties of the HRQOL measures that were used in PFDN-sponsored research on women with prolapse and incontinence. Most of the measures (PFDI, PFIQ, SF-36, utility item, satisfaction items, and LOT-R) demonstrated good test-retest reliability with correlation coefficients ≥0.6 or kappa scores that approached ≥0.6 (Table I). With the MESA instrument scored on 4 levels (continent, stress, urge, or mixed urinary incontinence), there was fair test-retest reliability (kappa, 0.39); however, when one reduces the MESA to 2 levels (continent vs incontinent), then the agreement is good (kappa, 0.63). More specifically, when the MESA is scored with 4 levels, 49 of 55 women (89.1%) who were classified as having stress incontinence symptoms (either stress or mixed incontinence) at the first testing were categorized similarly at the second interview. Four of 21 women (19%) who were continent at the first interview had stress symptoms on retesting, and 4 of 10 women (40%) who had been classified as urge incontinent were reclassified as being stress.

**Table I  Assessment of HRQOL 1 year after pelvic surgery for urinary incontinence and prolapse**

<table>
<thead>
<tr>
<th>Measure</th>
<th>N*</th>
<th>Median</th>
<th>25th Percentile</th>
<th>75th Percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Continuous measure (range of scores)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFDI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDI (0-300)</td>
<td>85</td>
<td>30.5</td>
<td>11.4</td>
<td>62.3</td>
</tr>
<tr>
<td>POPDI (0-300)</td>
<td>86</td>
<td>28.6</td>
<td>8.3</td>
<td>54.8</td>
</tr>
<tr>
<td>Colo-rectal-anal distress inventory (0-400)</td>
<td>86</td>
<td>37.5</td>
<td>12.5</td>
<td>81.7</td>
</tr>
<tr>
<td>PFIQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary incontinence impact questionnaire (0-300)</td>
<td>86</td>
<td>115.8</td>
<td>104.2</td>
<td>150.8</td>
</tr>
<tr>
<td>POPIQ (0-300)</td>
<td>86</td>
<td>100.0</td>
<td>100.0</td>
<td>104.2</td>
</tr>
<tr>
<td>Colo-rectal-anal impact questionnaire (0-300)</td>
<td>86</td>
<td>100.0</td>
<td>100.0</td>
<td>118.5</td>
</tr>
<tr>
<td>PISQ short form: Sexually active subjects only (0-48)</td>
<td>40</td>
<td>34.5</td>
<td>29.7</td>
<td>37.0</td>
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<tr>
<td><strong>Short-form 36 (0-100)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bodily pain</td>
<td>86</td>
<td>72.0</td>
<td>51.0</td>
<td>100.0</td>
</tr>
<tr>
<td>General health</td>
<td>86</td>
<td>72.0</td>
<td>52.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Mental health</td>
<td>86</td>
<td>80.0</td>
<td>70.0</td>
<td>90.0</td>
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<tr>
<td>Physical functioning</td>
<td>85</td>
<td>80.0</td>
<td>60.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Role emotional</td>
<td>86</td>
<td>100.0</td>
<td>83.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Role physical</td>
<td>86</td>
<td>81.3</td>
<td>62.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Social functioning</td>
<td>86</td>
<td>100.0</td>
<td>75.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Vitality</td>
<td>86</td>
<td>37.5</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Health utility item (0-100)</td>
<td>86</td>
<td>80.0</td>
<td>75.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Life orientation test (1-5)</td>
<td>43</td>
<td>3.8</td>
<td>3.3</td>
<td>4.2</td>
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<tr>
<td><strong>Categoric measure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MESA questionnaire</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continent</td>
<td>21 (24%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress incontinence</td>
<td>33 (38%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urge incontinence</td>
<td>22 (26%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed incontinence</td>
<td>10 (12%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunskaar severity index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continent/slightly incontinent</td>
<td>43 (50%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate/very severely incontinent</td>
<td>43 (50%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Satisfaction with care: Very/somewhat satisfied</td>
<td>68 (89%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Success of treatment: Very/moderately successful</td>
<td>66 (77%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of improvement: Much/a little better</td>
<td>72 (95%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The number of respondents do not always total to 86 because of incomplete or missing data for some scales.
incontinent on retesting. Similarly, the Hunskaar measure with 2 levels (continent/slightly incontinent vs moderate-to-severe incontinence) also yielded good agreement (kappa, 0.73). Test-retest reliability of the satisfaction items ranged from fair to good (Table II).

### Internal consistency reliability

Most of the summary scores demonstrated robust internal consistency (Cronbach’s alpha, $\geq 0.6$ for each), with the exception of the PISQ for sexually active women for which Cronbach’s alpha was 0.36 (Table II). Internal consistency was not calculated for the Hunskaar instrument, which consisted of 2 items: the utility question, which consisted of 1 item, and the satisfaction items, which are not scored as a scale. Similarly, internal consistency could not be assessed for the MESA continence and mixed urinary incontinence categories.

### Validity

Correlation between HRQOL domain scores from each HRQOL summary score and HRQOL scores of other instruments were examined by a comparison of the PFDI, PFIQ, and PISQ scores with the MESA and Hunskaar scores (Table III). As expected, the Hunskaar severity scale was correlated to the PFDI-UDI ($P < .001$) and the PFIQ-UIQ ($P < .001$) scales, such that women with moderate-to-severe incontinence on the Hunskaar scale reported greater symptoms on the PFDI-UDI and greater impairment on the PFIQ-UIQ (Table III). Similarly, women with incontinence that was based on the MESA continence scale reported greater symptoms on the PFDI-UDI (Table III).

Further validity was demonstrated by an examination of the relationships between condition-specific and generic HRQOL (Table IV). Greater pelvic floor symptoms, as measured by the PFDI, and greater impairment, as measured by the PFIQ, were correlated negatively and significantly to the utility item and most of the SF-36 subscales. A strong association was seen between incontinence characterization with the MESA and Hunskaar questionnaires and self-rated success with treatment (Table IV). The weak associations between the PISQ subscales and generic HRQOL measures also supports the validity because these instruments were designed to measure different outcomes (Table IV).

Comparisons between the physician’s clinical diagnosis and the PFDI, PFIQ, Hunskaar incontinence measure, and the MESA incontinence categories were also undertaken. Generally, there was weak agreement between the clinical assessments of incontinence and the POP-Q score and self-reported HRQOL (Table V).

### Comment

The inclusion of HRQOL assessments in clinical trials characterizes patients’ experiences of their conditions and treatments in everyday life that are not measured during traditional physical examinations and physio-

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**Table II** Test-retest reliability and internal consistency of HRQOL measures among women 1 year after pelvic surgical procedures for urinary incontinence and prolapse

<table>
<thead>
<tr>
<th></th>
<th>Test-retest reliability coefficient*</th>
<th>Cronbach’s alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFDI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDI (0-300)</td>
<td>0.78</td>
<td>0.95</td>
</tr>
<tr>
<td>POPDI (0-300)</td>
<td>0.85</td>
<td>0.94</td>
</tr>
<tr>
<td>Colo-rectal-anal distress inventory (0-400)</td>
<td>0.83</td>
<td>0.92</td>
</tr>
<tr>
<td>PFIQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary incontinence impact questionnaire (0-300)</td>
<td>0.89</td>
<td>0.93</td>
</tr>
<tr>
<td>POPIQ (0-300)</td>
<td>0.88</td>
<td>0.88</td>
</tr>
<tr>
<td>Colo-rectal-anal impact questionnaire (0-300)</td>
<td>0.92</td>
<td>0.95</td>
</tr>
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<td>PISQ short form: Sexually active subjects only (0-48)</td>
<td>0.79</td>
<td>0.36</td>
</tr>
<tr>
<td>Short-form 36 (0-100)</td>
<td>0.69</td>
<td>0.87</td>
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<tr>
<td>Bodily pain</td>
<td>0.83</td>
<td>0.82</td>
</tr>
<tr>
<td>General health</td>
<td>0.85</td>
<td>0.84</td>
</tr>
<tr>
<td>Mental health</td>
<td>0.73</td>
<td>0.89</td>
</tr>
<tr>
<td>Physical functioning</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>Role emotional</td>
<td>0.83</td>
<td>0.86</td>
</tr>
<tr>
<td>Vitality</td>
<td>0.70</td>
<td>0.89</td>
</tr>
<tr>
<td>Role physical</td>
<td>0.74</td>
<td>0.82</td>
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<tr>
<td>Social functioning</td>
<td>0.59</td>
<td>N/A</td>
</tr>
<tr>
<td>Health utility item</td>
<td>0.76</td>
<td>0.58</td>
</tr>
<tr>
<td>MESA questionnaire</td>
<td>0.39</td>
<td>N/A</td>
</tr>
<tr>
<td>Continent</td>
<td>0.63</td>
<td>N/A</td>
</tr>
<tr>
<td>Stress incontinence</td>
<td>0.24</td>
<td>0.82</td>
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<tr>
<td>Urge incontinence</td>
<td>0.38</td>
<td>0.85</td>
</tr>
<tr>
<td>Mixed incontinence</td>
<td>0.33</td>
<td>N/A</td>
</tr>
<tr>
<td>Hunskaar severity index</td>
<td>0.73</td>
<td>N/A</td>
</tr>
<tr>
<td>Satisfaction items</td>
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<tr>
<td>Satisfaction with care</td>
<td>0.44</td>
<td>N/A</td>
</tr>
<tr>
<td>Success of treatment</td>
<td>0.73</td>
<td>N/A</td>
</tr>
<tr>
<td>Amount of improvement</td>
<td>0.42</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A represents domains for which there were too few items to assess internal consistency.

* For continuous variables, the intraclass correlation coefficient was calculated by analysis of variance methods; for categoric variables, kappa was used.

1 This shortened version of PISQ instrument was designed to be correlated with the full version of the instrument; therefore, the low internal consistency observed here should not be interpreted as poor reliability.
logic measures. Accordingly, when surgical interventions for pelvic floor disorders performed to treat patient symptoms are evaluated, HRQOL measures should be incorporated as primary and secondary study end points. It is essential that the psychometric properties of the HRQOL measures be evaluated critically in the target population before use so that the results of the clinical trial can be interpreted in a meaningful way. To date, relatively few studies have examined postoperative outcomes using HRQOL measures after surgery for pelvic floor disorders. In the current study, women after surgery reported few pelvic floor symptoms that were consistent with the improvements in pelvic organ prolapse and urinary incontinence reported by their physicians. By and large, the ranges of scores for many of the measures were narrow, likely because of homogeneity of the sample, but poor sensitivity of these measures cannot be excluded.

The prevalence and severity of urinary incontinence symptoms in this sample was dependent on the measure used. With the Hunkskaar measure, one half of the women were considered continent or slightly incontinent; with the MESA measure, however, 24% of women would have been categorized as continent. This is in contrast with the physician’s assessment of urinary continence as documented in the medical chart at 81%. This dramatic variation in the reported rate of urinary continence is consistent with previous studies in which operational definitions of urinary incontinence have a clinically and statistically significant impact on the reporting of urinary incontinence outcomes. Despite demonstrated reliability and validity for any 1 particular HRQOL instrument, it is difficult to compare study results that use different measurement tools. Moreover, this argues for the “standardization” of measures for urinary incontinence as suggested by Corcos et al.

Condition-specific HRQOL measures are useful for a better understanding of the unique concerns of women with pelvic organ prolapse. Concurrently administering generic measures allows investigators to compare the HRQOL of women with prolapse and/or incontinence to that of women with other chronic illnesses. We found that the SF-36 health utility item and satisfaction items can provide complementary information to generic HRQOL. This sample of women reported fairly high levels of function across all but 1 subscale of the SF-36 (vitality); they also rated their present health high on the health utility measure. At the same time, more than one half of the women reported that their surgery was very successful, and 78% reported being “much better” relative to their preoperative condition.

The primary goal of this study was to examine the reliability, validity, and usefulness of a battery of HRQOL measures in women with pelvic floor disorders. In the current study, we found that test-retest reliability and internal consistency overall were quite good, except for the MESA, when using 4 levels and the satisfaction with care and satisfaction with improvement. These limitations should be kept in mind when measures are being selected and also when the reported results are being interpreted. Further modification of the satisfaction items, given their suboptimal reliability, will be necessary before further use. The low value for internal consistency of the short-form version of PISQ was unexpected, given the reliabilities that were reported for the full measure. However, this shortened version of the PISQ instrument was constructed by the selection of items that correlate with the full version of the

<table>
<thead>
<tr>
<th>Quality-of-life measure</th>
<th>MESA</th>
<th>Hunskaar severity index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Continent (n = 21)</td>
<td>Incontinent (n = 65)</td>
</tr>
<tr>
<td>PFDI†</td>
<td>8.9</td>
<td>38.6</td>
</tr>
<tr>
<td>POPDI (0-300)</td>
<td>22.6</td>
<td>32.7</td>
</tr>
<tr>
<td>Colo-rectal-anal distress inventory (0-400)</td>
<td>25.0</td>
<td>38.3</td>
</tr>
<tr>
<td>PISQ (0-48)</td>
<td>35.0</td>
<td>33.5</td>
</tr>
<tr>
<td>PFIQ</td>
<td>33 (7)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Urinary incontinence impact questionnaire</td>
<td>POPIQ 81 (17)</td>
<td>62 (40)</td>
</tr>
<tr>
<td>Colo-rectal-anal impact questionnaire</td>
<td>PFIQ 76 (16)</td>
<td>46 (30)</td>
</tr>
</tbody>
</table>

* Wilcoxon rank-sum test compared MESA strata (continent vs incontinent).
† Wilcoxon rank-sum test that compared Hunkskaar strata (continent/slightly incontinent vs moderately/severely incontinent).
‡ Median score.
§ Percentage at minimum (no. at minimum).
Moreover, Cronbach’s alpha statistic is dependent on the number of items in a measure; therefore, the lower internal consistency that was observed here should not be interpreted as poor reliability (Dr Rebecca Rogers, oral communication, April 1, 2004).

In this study, we validated the HRQOL measures by examining correlations with related measures and also with clinical end points. Strong correlation was observed between the MESA, Hunskaar, and PFDI measures. Moreover, the PFDI and PFIQ correlated significantly with the SF-36, which indicated that pelvic floor disorders have a broad, multifaceted impact on women’s overall health. The weak associations between patients’ perceptions of their prolapse after operation and incontinence (ie, PFDI, PFIQ, MESA, and Hunskaar measures) and the clinicians’ assessments (ie, POP-Q and physician determination of urinary incontinence) may be attributed to the lower postoperative prevalence of prolapse and incontinence relative to untreated populations and to the fact that the HRQOL and clinical assessments did not occur contemporaneously. Others have specifically examined the agreement between physician and patient assessments of urinary incontinence and have found stronger correlations. In this study, however, agreement was modest and supported the contention that patient self-reports are important complementary sources of information. The lack of strong correlations between patient- and provider-derived measures in our study may also be evidence that these HRQOL measures actually capture different aspects of pelvic floor disorders and should not be administered therefore in lieu of clinical assessments. Studies that compare physician and patient self-reported outcomes after pelvic surgery have consistently demonstrated a striking difference in the assessment of this end point. An alternative explanation for the differences may be that physicians determined that minimal incontinence was clinically insignificant.

This study was limited by the use of a small convenience sample that was almost entirely white. Further evaluations of HRQOL should be broadened to include Hispanic, black, and other minority women. Another limitation was that the clinical postoperative data was not collected simultaneously with the HRQOL telephone interviews and was collected retrospectively; hence, the definitions of continence were based only on the surgeons’ annotations, without benefit of uniform objective measures such as cough stress tests. Moreover, the distribution of pelvic organ prolapse severities in this study population was somewhat restricted, given their history of surgical repair; this may well explain why the PFDI and PFIQ failed to be associated with POP-Q stage. In this cross-sectional study, we were not able to examine responsiveness of the measures to interventions nor on the correlation between self-administered and

### Table IV: Correlation between condition-specific and generic HRQOL measures

<table>
<thead>
<tr>
<th>Measure</th>
<th>Utility item</th>
<th>LOT-R</th>
<th>Physical functioning</th>
<th>Role physical</th>
<th>Bodily pain</th>
<th>General health</th>
<th>Vitality</th>
<th>Social functioning</th>
<th>Role emotional</th>
<th>Mental health</th>
<th>Satisfaction with care</th>
<th>Success of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFDI-UDI</td>
<td>-0.28*</td>
<td>-0.28*</td>
<td>-0.37</td>
<td>-0.23</td>
<td>-0.39</td>
<td>0.27</td>
<td>-0.30</td>
<td>-0.42</td>
<td>-0.31</td>
<td>0.27</td>
<td>-0.28</td>
<td>0.41</td>
</tr>
<tr>
<td>PFDI-POPIQ</td>
<td>-0.34*</td>
<td>-0.32</td>
<td>-0.35</td>
<td>-0.54</td>
<td>-0.32</td>
<td>-0.44</td>
<td>0.44</td>
<td>-0.50</td>
<td>-0.61</td>
<td>-0.43</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>PFDI-Colo-rectal-anal</td>
<td>-0.39</td>
<td>-0.31</td>
<td>-0.46</td>
<td>-0.34</td>
<td>-0.47</td>
<td>0.47</td>
<td>-0.39</td>
<td>-0.43</td>
<td>-0.32</td>
<td>-0.28</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>distress inventory</td>
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</tr>
<tr>
<td>PFIQ-Urinary incontinence</td>
<td>-0.40</td>
<td>-0.34</td>
<td>-0.40</td>
<td>-0.24</td>
<td>-0.42</td>
<td>0.34*</td>
<td>-0.42</td>
<td>-0.50</td>
<td>-0.37</td>
<td>-0.37</td>
<td>0.27</td>
<td></td>
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<tr>
<td>impact questionnaire</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFIQ-POPIQ</td>
<td>-0.28*</td>
<td>-0.33</td>
<td>-0.40</td>
<td>-0.26</td>
<td>-0.43</td>
<td>0.35</td>
<td>-0.51</td>
<td>-0.43</td>
<td>-0.36</td>
<td>-0.36</td>
<td>0.34</td>
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</tr>
<tr>
<td>PFIQ-Colo-rectal-anal</td>
<td>-0.34*</td>
<td>-0.23</td>
<td>-0.34</td>
<td>-0.25</td>
<td>-0.41</td>
<td>0.30*</td>
<td>-0.43</td>
<td>-0.36</td>
<td>-0.30</td>
<td>0.23</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>impact questionnaire</td>
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<td></td>
</tr>
<tr>
<td>PISQ</td>
<td>0.40*</td>
<td></td>
<td>-0.37</td>
<td>-0.25</td>
<td>0.25</td>
<td>-0.22</td>
<td>-0.22</td>
<td>0.26</td>
<td>0.37</td>
<td>-0.23</td>
<td>0.37</td>
<td></td>
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<tr>
<td>MESA: Stress</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MESA: Mixed</td>
<td>-0.25</td>
<td>0.25</td>
<td>-0.22</td>
<td>-0.22</td>
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</tr>
<tr>
<td>Hunskaar</td>
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<td></td>
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</tr>
<tr>
<td>severity index</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

Only statistically significant correlations are presented.

* $P < .01$.

1 $P < .001$.

2 $P < .05$. 
telephone-administered questionnaires; therefore, subsequent work on HRQOL measures in pelvic floor disorders should examine their ability to detect significant changes over time and how the use of telephone administration affects HRQOL scores.

Taken together, these data support the premise that condition-specific measures have appropriate reliability and validity when administered to women with pelvic floor disorders. The generic HRQOL measures likewise demonstrated solid psychometric properties in this population. Further evaluation and refinement of these measures in more diverse populations over a longer period of time is necessary to optimize their usefulness in clinical studies. It is important to recognize that no single measure can capture the entire scope of pelvic floor symptoms or impairment; therefore, the use of HRQOL measures as an adjunct to clinical end points offers the most promise for clinicians and researchers to better understand the impact of pelvic floor disorders on women’s daily lives. Future areas of study should consider associations between patient demographics or patient expectations and HRQOL and the development of validated satisfaction measures.

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References

Pelvic floor morbidity at 3 years after instrumental delivery and cesarean delivery in the second stage of labor and the impact of a subsequent delivery

Rachna Bahl, MRCOG, Bryony Strachan, MD, Deirdre J. Murphy, MD

St Michael’s Hospital, Bristol, UK, and Ninewells Hospital and Medical School, University of Dundee, UK

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KEY WORDS
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Urinary incontinence
Instrumental delivery
Emergency cesarean delivery
Cohort study

Objective: To compare pelvic floor symptoms at three years following instrumental delivery and cesarean section in the second stage of labor and to assess the impact of a subsequent delivery.

Study design: We conducted a prospective cohort study of 393 women with term, singleton, cephalic pregnancies who required instrumental vaginal delivery in theatre or cesarean section at full dilatation between February 1999 and February 2000. 283 women (72%) returned postal questionnaires at three years.

Results: Urinary incontinence at three years post delivery was greater in the instrumental delivery group as compared to the cesarean section group (10.5% vs 2.0%), OR 5.37 (95% CI, 1.7, 27.9). There were no significant differences in ano-rectal or sexual symptoms between the two groups. Pelvic floor symptoms were similar for women delivered by cesarean section after a failed trial of instrumental delivery compared to immediate cesarean section. A subsequent delivery did not increase the risk of pelvic floor symptoms at three years in either group.

Conclusion: An increased risk of urinary incontinence persists up to three years following instrumental vaginal delivery compared to cesarean section in the second stage of labor. However, pelvic floor symptoms are not exacerbated by a subsequent delivery.

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The choice between a potentially difficult instrumental delivery and cesarean delivery in the second stage of labor is a fine balance between maternal and neonatal morbidity. Although elective cesarean delivery has become safer for the mother and baby, cesarean deliveries that are performed in the second stage of labor have considerable morbidity. There are concerns about the potential for neonatal trauma and maternal pelvic floor injury after instrumental delivery. This has been reflected in a sharp fall in the number of obstetricians who are prepared to offer mid-cavity or rotational instrumental vaginal delivery and raises further issues regarding rising cesarean delivery rates. At term, 4% of women in a UK setting, with otherwise uncomplicated pregnancies, required instrumental delivery or cesarean delivery in the second stage of labor. When
difficulties arise in labor, women and their obstetricians should be able to make an informed decision based on a balance between maternal and neonatal morbidity and the implications for the future.

Vacuum and forceps deliveries have been associated with bladder and bowel complication rates of up to 50% at 5 years and pose a particular risk of injury to the anal sphincter mechanism in primiparous vaginal delivery. Women with transient fecal incontinence or occult anal-sphincter injury after their first vaginal delivery are at high risk of fecal incontinence after a second vaginal delivery.

We previously identified a cohort of 393 women who experienced either an instrumental vaginal delivery or a cesarean delivery in the second stage of labor and have followed them prospectively. The women reported high levels of urinary, anorectal, and sexual dysfunction at 1 year, with an increased risk of morbidity after instrumental delivery. Further follow-up examination is required to evaluate the progression of these symptoms and the impact of a further pregnancy and delivery.

Therefore, we now report the 3-year follow-up evaluation of this cohort. The aims of the study were 3-fold. First, we examined whether the increased risk of pelvic floor symptoms at 1 year after instrumental vaginal delivery, when compared with cesarean delivery, persisted at 3 years. Second, we examined whether there was a difference in pelvic floor symptoms after cesarean delivery after a failed trial of instrumental delivery compared with immediate cesarean delivery. Third, we examined whether pelvic floor symptoms at 3 years had been exacerbated by a subsequent delivery.

### Material and methods

The original cohort study was conducted at 2 urban university teaching hospitals in the south west of England. These units have a combined total of >10,000 deliveries annually and serve a geographic area with a stable population. Additional patients are received occasionally from a midwifery-led unit and from planned home births, because all operative deliveries for the area are performed in 1 of the 2 hospitals. All women who were fully dilated and underwent cesarean delivery or instrumental vaginal delivery in an operating room were eligible for study recruitment. The decision to conduct an instrumental vaginal delivery in an operating room was made if a rotational mid-cavity delivery was to be undertaken or if mild relative cephalopelvic disproportion was anticipated. The delivery was conducted in an operat-

### Table I  Maternal and neonatal factors in relation to instrument vaginal delivery and cesarean delivery

<table>
<thead>
<tr>
<th>Factor</th>
<th>Original cohort Instrument delivery (n)*</th>
<th>Cesarean delivery (n)</th>
<th>Odds ratio (95% CI)</th>
<th>Cohort at 3 years after delivery Instrument delivery (n)</th>
<th>Cesarean delivery (n)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primiparous</td>
<td>144 (78.0%)</td>
<td>165 (78.9%)</td>
<td>0.96 (0.59, 1.56)</td>
<td>103 (77.4%)</td>
<td>114 (76.0%)</td>
<td>1.08 (0.62, 1.88)</td>
</tr>
<tr>
<td>Maternal age &gt; 35 y</td>
<td>25 (13.6%)</td>
<td>19 (9.1%)</td>
<td>1.72 (0.91, 3.26)</td>
<td>17 (12.8%)</td>
<td>16 (10.7%)</td>
<td>1.22 (0.59, 2.53)</td>
</tr>
<tr>
<td>Non-white</td>
<td>13 (7.1%)</td>
<td>10 (5.0%)</td>
<td>1.51 (0.65, 3.54)</td>
<td>6 (4.5%)</td>
<td>10 (6.7%)</td>
<td>0.66 (0.23, 1.87)</td>
</tr>
<tr>
<td>Social class I and II</td>
<td>84 (45%)</td>
<td>90 (44%)</td>
<td>1.11 (0.75, 1.66)</td>
<td>64 (48.1%)</td>
<td>64 (42.7%)</td>
<td>1.24 (0.77, 1.99)</td>
</tr>
<tr>
<td>Body mass index &gt; 30</td>
<td>13 (7.1%)</td>
<td>31 (14.8%)</td>
<td>0.44 (0.22, 0.86)</td>
<td>11 (8.3%)</td>
<td>23 (15.3%)</td>
<td>0.50 (0.23, 1.06)</td>
</tr>
<tr>
<td>Previous cesarean delivery</td>
<td>11 (6.0%)</td>
<td>15 (7.2%)</td>
<td>0.82 (0.37, 1.84)</td>
<td>8 (6.0%)</td>
<td>12 (8.0%)</td>
<td>0.73 (0.29, 1.83)</td>
</tr>
<tr>
<td>Previous difficult delivery</td>
<td>9 (4.9%)</td>
<td>12 (5.7%)</td>
<td>0.84 (0.35, 2.05)</td>
<td>7 (5.3%)</td>
<td>10 (6.7%)</td>
<td>0.77 (0.28, 2.10)</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>7 (3.8%)</td>
<td>19 (9.1%)</td>
<td>0.39 (0.16, 0.95)</td>
<td>4 (3.4%)</td>
<td>15 (10.0%)</td>
<td>0.27 (0.09, 0.86)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1 (0.5%)</td>
<td>5 (2.4%)</td>
<td>0.22 (0.03, 1.93)</td>
<td>1 (0.8%)</td>
<td>2 (1.3%)</td>
<td>0.56 (0.05, 6.25)</td>
</tr>
<tr>
<td>Second stage &gt; 3 hr</td>
<td>18 (9.7%)</td>
<td>24 (11.4%)</td>
<td>1.11 (0.56, 2.21)</td>
<td>14 (10.5%)</td>
<td>16 (10.7%)</td>
<td>1.00 (0.45, 2.13)</td>
</tr>
<tr>
<td>Fetal malposition**</td>
<td>97 (52.7%)</td>
<td>149 (71.3%)</td>
<td>0.45 (0.30, 0.68)</td>
<td>71 (53.4%)</td>
<td>110 (73.3%)</td>
<td>0.42 (0.25, 0.69)</td>
</tr>
<tr>
<td>Infant birth weight &gt; 4.0 kg</td>
<td>27 (14.7%)</td>
<td>56 (26.8%)</td>
<td>0.47 (0.28, 0.78)</td>
<td>18 (13.5%)</td>
<td>37 (24.7%)</td>
<td>0.47 (0.26, 0.89)</td>
</tr>
<tr>
<td>PPH &gt; 1000 mL</td>
<td>6 (3.3%)</td>
<td>20 (9.6%)</td>
<td>0.31 (0.12, 0.79)</td>
<td>3 (2.3%)</td>
<td>16 (10.7%)</td>
<td>0.19 (0.05, 0.67)</td>
</tr>
<tr>
<td>Hospital stay &gt; 5 d</td>
<td>10 (5.5%)</td>
<td>33 (15.9%)</td>
<td>0.31 (0.15, 0.64)</td>
<td>9 (6.8%)</td>
<td>24 (16.0%)</td>
<td>0.36 (0.16, 0.80)</td>
</tr>
<tr>
<td>Further pregnancy at 3 yr</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>73 (54.8%)</td>
<td>67 (44.6%)</td>
<td>1.60 (0.99, 2.58)</td>
</tr>
</tbody>
</table>

* N = 184.  
† N = 209.  
‡ N = 133.  
§ N = 150.  
□ p < .05.  
* Shoulder dystocia, rotational instrument delivery, third-degree tear, or postpartum hemorrhage.  
† Blood pressure > 140/90 mm Hg on at least 2 occasions, and proteinuria > 0.3 g in a 24-hour collection.  
** Occipitoposterior or occipitotransverse.
ing room to allow rapid recourse to cesarean delivery if necessary. Inclusion criteria were women at \( R \geq 37 \) completed weeks of gestation with a live, singleton, cephalic pregnancy.

The study recruitment period was between February 1999 and February 2000. Women who fulfilled the inclusion criteria were identified from delivery suite records within 24 hours of delivery and were approached personally by researchers before hospital discharge.

Initial research involved the completion of a detailed dataset with the use of hospital records and an interview with the mother that focused on labor and delivery and her views about future pregnancies. Early maternal and neonatal morbidity until hospital discharge was recorded prospectively and is described elsewhere.\(^1\) Further information regarding maternal morbidity, infant feeding, and views on future pregnancies was gathered by postal questionnaires that were sent to each woman at 6 weeks and 1 year after the delivery. A further questionnaire was sent at 3 years that requested information about lower urinary tract, ano-rectal, and sexual symptoms. Women were asked to describe current symptoms, whether the symptoms were experienced occasionally or more than occasionally, and to indicate the severity. The questionnaire was based on previously validated questionnaires that addressed postnatal pelvic floor symptoms.\(^8,10,11\) Pelvic floor symptoms reported to occur more than occasionally (frequent symptoms) or at any time (any symptoms) were taken as positive responses for the main analysis. However, any reporting of the relevant symptom was considered a positive response for the subgroup analyses. Comparisons were reported for pain on intercourse of moderate or severe intensity. Information was requested about further pregnancies and the mode of delivery in the subsequent pregnancy. This information was validated against the maternity database record for each individual woman. Nonresponders were sent reminders and, if these were unsuccessful, were contacted by telephone. Each woman in the study gave written consent, and the local ethics committees granted ethical approval. We did not have consent to establish the pregnancy outcome of the nonresponders.

Statistical analysis

Univariable comparisons were made among the maternal, labor, and postnatal characteristics of the 2 groups. This was performed for both the original cohort and the responders at 3 years to determine whether there was any obvious nonresponder bias and to ascertain potential confounding factors. The instrumental delivery

### Table II  Pelvic floor symptoms at 3 years after instrument vaginal delivery and cesarean delivery

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Any symptoms*</th>
<th>Frequent symptoms†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Instrument delivery (n)</td>
<td>Cesarean delivery (n)</td>
</tr>
<tr>
<td>Lower urinary tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary leakage</td>
<td>61 (45.8%)</td>
<td>44 (29.9%)</td>
</tr>
<tr>
<td>Difficulty holding urine</td>
<td>47 (35.3%)</td>
<td>36 (24%)</td>
</tr>
<tr>
<td>Frequency</td>
<td>40 (30.1%)</td>
<td>33 (22.3%)</td>
</tr>
<tr>
<td>Anorectal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain on defecation</td>
<td>29 (21.8%)</td>
<td>32 (21.3%)</td>
</tr>
<tr>
<td>Constipation</td>
<td>47 (35.3%)</td>
<td>52 (34.6%)</td>
</tr>
<tr>
<td>Hemorrhoids</td>
<td>68 (52.4%)</td>
<td>61 (40.6%)</td>
</tr>
<tr>
<td>Flatus incontinence</td>
<td>38 (28.5%)</td>
<td>40 (26.6%)</td>
</tr>
<tr>
<td>Fecal incontinence</td>
<td>9 (7.1%)</td>
<td>8 (5.3%)</td>
</tr>
<tr>
<td>Sexual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain on intercourse</td>
<td>37 (28.5%)</td>
<td>41 (27.3%)</td>
</tr>
<tr>
<td>Pain that prevented intercourse</td>
<td>21 (15.7%)</td>
<td>18 (12%)</td>
</tr>
</tbody>
</table>

* A comparison between women who reported either “occasional” or “more than occasional” symptoms versus no symptoms.
† A comparison between women who reported “more than occasional symptoms” versus only “occasional” or no symptoms.
†† N = 133.
‡‡ N = 150.
** Adjusted for age, parity, body mass index, and birth weight > 4.0 kg.
* N = 133.
‡‡ N = 150.
** P < .05.
†† The denominators refer to women who were sexually active.
group was considered to be the reference group because this route of delivery would be considered primarily for most women, and the cesarean delivery group was the comparison group. The 2 groups were compared for pelvic floor symptoms at 3 years, and subgroup analyses addressed whether the cesarean delivery was immediate and the impact of a subsequent delivery. Univariable analyses were performed using logistic regression, followed by multivariable analyses that were adjusted for potential confounding factors. Statistical significance was defined a priori as a probability value of \( <0.05 \); factors that fit this criterion and for which there was biologically plausible potential for confounding were explored in the models. Maternal age, parity, body mass index of \( >30 \text{ kg/m}^2 \), and infant birth weight of \( >4 \text{ kg} \) were included in the final models. Results are reported as unadjusted and adjusted odds ratios with 95% CI.

The SPSS statistical package software (version 11.0; SPSS Inc, Chicago, Ill) was used for data analysis.

### Results

Two hundred eighty-three women from the original cohort of 393 women (72%) who underwent operative delivery returned postal questionnaires at 3 years. The follow-up rate at 1 year had been 80%. Follow-up rates were similar for women who experienced instrumental vaginal delivery or cesarean delivery (72.2% and 71.7%, respectively, at 3 years). In the cesarean delivery group, 72 women (48%) had a failed attempt at instrumental delivery, and 78 women (52%) underwent immediate cesarean delivery. In the instrumental delivery group, 55 women (41%) had a vacuum delivery; 43 women (32%) had a forceps delivery, and both instruments were used in 35 women (26%). A total of 101 women (76%) had a right mediolateral episiotomy. The proportion of women in each mode of delivery at 3 years was similar to the original cohort. The demographic, obstetric, and neonatal factors of the responders were similar to the profile of the original cohort (Table I). This suggests that the available study sample at follow up were representative of the original cohort. The duration of the second stage of labor was similar for each group, but both mothers and infants were physically larger in the cesarean delivery group. A total of 140 women had experienced a subsequent pregnancy at 3 years: 73 of 133 women (55%) in the instrumental delivery group and 67 of 150 women (45%) in the cesarean delivery group (Table I).

At 3 years, the increased risk of urinary incontinence after instrumental vaginal delivery compared with cesarean delivery had persisted (10.5% vs 2.0%; odds ratio, 5.37 [95% CI, 1.73, 27.90]; Table II). There were no significant differences in anorectal symptoms and symptoms of dyspareunia; however, both groups reported high rates of flatus incontinence since the index pregnancy. There were 9 women who had sustained a third-degree perineal tear at the time of instrumental vaginal delivery. Within this group, 4 women (44%) reported incontinence of flatus, and 2 women (22%) reported fecal incontinence. There were no significant differences in pelvic floor symptoms within the cesarean delivery group on comparison of women who had a cesarean delivery after a failed attempt at instrumental vaginal delivery and those who had an immediate cesarean delivery (Table III).

Of the 140 women who went on to have a subsequent pregnancy, 106 women (76%) had ongoing pregnancies

| Table III | Pelvic floor symptoms at 3 years in relation to the type of cesarean delivery |
|---|---|---|
| Symptom | Cesarean delivery (n) | | |
| | After failed instrument delivery (n = 72) | Immediate (n = 78) | Adjusted odds ratio (95% CI)* |
| Lower urinary tract\(^1\) | | | |
| Urinary leakage | 16 (22.2%) | 28 (35.9%) | 0.90 (0.53, 1.36) |
| Difficulty holding urine | 18 (25.0%) | 18 (23.1%) | 1.03 (0.95, 1.12) |
| Frequency | 18 (25.0%) | 15 (19.3%) | 1.23 (0.56, 2.78) |
| Anorectal\(^1\) | | | |
| Pain on defecation | 16 (22.2%) | 16 (20.5%) | 0.92 (0.84, 1.01) |
| Constipation | 28 (38.8%) | 24 (30.7%) | 0.97 (0.90, 1.04) |
| Hemorrhoids | 25 (34.7%) | 36 (50.0%) | 0.58 (0.27, 1.13) |
| Flatus incontinence | 19 (26.3%) | 21 (26.9%) | 0.97 (0.89, 1.05) |
| Fecal incontinence | 4 (5.5%) | 4 (6.0%) | 1.10 (0.89, 1.19) |
| Sexual\(^1\) | | | |
| Pain on intercourse | 21/70 (30.0%) | 20/73 (27.3%) | 0.95 (0.88, 1.03) |
| Pain that prevented intercourse | 11/70 (15.7%) | 7/73 (9.5%) | 1.01 (0.90, 1.12) |

* Adjusted for age, parity, body mass index, and birth weight >4.0 kg.

\(^1\) Refers to women who reported occasional or more than occasional episodes of the relevant symptom.

\(^1\) The denominators refer to women who were sexually active.
that resulted in a term delivery. There was a high rate of subsequent vaginal delivery after a previous instrumental vaginal delivery (42/54; 78%) and a high rate of cesarean delivery after a previous cesarean delivery (37/54; 69%). On comparing the pelvic floor symptoms at 3 years within each of the original delivery groups, there were no significant differences in relation to having had a further delivery (Table IV). Of note the rates of urinary and anal incontinence were no worse after a subsequent delivery in either group.

**Comment**

Women who undergo instrumental vaginal delivery are at an increased risk of urinary incontinence at 3 years when compared with women who undergo cesarean delivery in the second stage of labor. We previously reported increased rates of lower urinary tract symptoms after instrumental delivery at 6 weeks and 1 year after the delivery. These symptoms appear to persist. The overall rates of urinary incontinence are similar to those reported in other studies. However, we did not find significant differences in the rates of urinary urgency or frequency according to the mode of delivery. This is consistent with the recent observation that vaginal delivery may be a risk factor for stress incontinence only. Although there was an increased risk of urinary incontinence after instrumental vaginal delivery, the rate among women who were delivered by cesarean delivery was also high. This was consistent with what we observed at 1 year after the index delivery and supports the hypothesis that pelvic floor protection is not provided by a cesarean delivery late in labor, which possibly reflects neuronal damage and injury to the pelvic floor support structures. The additional risk of urinary incontinence after instrumental delivery is likely to relate to the procedure itself because the length of the second stage of labor was similar for both groups and because both mothers and infants were heavier in the cesarean delivery group. Previous research has reported no additional maternal or neonatal risk in the performance of a trial of instrumental vaginal delivery compared with an immediate cesarean delivery as long as immediate recourse to cesarean delivery is available. Our data support this, because we found no increased risk of pelvic floor symptoms among women who had a cesarean delivery after a failed attempt at instrumental delivery compared with women who were delivered by immediate cesarean delivery.

There was a high prevalence of flatus incontinence at 3 years, but the mode of delivery did not influence comparative rates of anorectal or sexual symptoms. It was also reassuring to observe that there were no differences in pelvic floor symptoms at 3 years in relation to having had a subsequent delivery, which suggests that the main insult to the pelvic floor was at the initial operative delivery with little evidence of deterioration. Our results appear to be at variance with previous studies, which suggests that a further vaginal delivery may contribute to deterioration in anal incontinence. However, the women in this study who

| Table IV Pelvic floor symptoms at 3 years in relation to having had a subsequent term delivery by 3 years |
|-------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Symptom                                         | Instrument delivery                              | Cesarean delivery                                |
|                                                 | Term delivery at 3 y*                           | No further delivery                              | Adjusted odds ratio (95% CI) |
|                                                 | to 3 y                                          | No further delivery                              | Adjusted odds ratio (95% CI) |
| Lower urinary tract                             |                                                  |                                                  |                                  |
| Urinary leakage                                 | 24 (44.4%)                                      | 37 (46.8%)                                      | 0.87 (0.42, 1.83)               |
| Difficulty holding urine                        | 17 (31.4%)                                      | 30 (37.9%)                                      | 0.71 (0.33, 1.52)               |
| Frequency                                        | 17 (31.4%)                                      | 23 (29.1%)                                      | 0.88 (0.39, 1.98)               |
| Anorectal                                       |                                                  |                                                  |                                  |
| Pain on defecation                              | 12 (22.2%)                                      | 17 (21.5%)                                      | 1.03 (0.43, 2.47)               |
| Constipation                                    | 16 (29.6%)                                      | 31 (39.2%)                                      | 0.63 (0.29, 1.35)               |
| Hemorrhoids                                     | 29 (53.7%)                                      | 39 (49.3%)                                      | 1.12 (0.53, 2.40)               |
| Flatus incontinence                             | 16 (29.6%)                                      | 22 (27.8%)                                      | 0.69 (0.29, 1.60)               |
| Fecal incontinence                              | 3 (5.5%)                                        | 8 (10.1%)                                       | 0.33 (0.07, 1.40)               |
| Sexual                                          |                                                  |                                                  |                                  |
| Pain on intercourse                             | 15 (27.7%)                                      | 22 (27.8%)                                      | 0.92 (0.41, 2.08)               |
| Pain that prevented intercourse                 | 9 (16.6%)                                       | 12 (15.1%)                                      | 1.07 (0.39, 2.94)               |
| * N = 54.                                      | 1 N = 79.                                       | 2 N = 79.                                       |                                  |
| 1 Adjusted for age, parity, body mass index and birth weight > 4.0 kg. | 3 N = 54.                                      | 4 N = 96.                                       |                                  |
| Refers to women who reported occasional or more than occasional episodes of the relevant symptom. |
had sustained third-degree perineal tears reported high rates of anal incontinence. Although this study was designed to address the impact of delivery on pelvic floor symptoms, the sample size was underpowered to address less prevalent symptoms (such as fecal incontinence) within subgroup analyses. Further large-scale prospective studies are required to evaluate the impact of successive vaginal deliveries on the long-term risk of anal incontinence compared with successive cesarean deliveries among women who have reached the second stage of labor.

To the best of our knowledge, this is the first prospective cohort study that has compared the pelvic floor symptoms between women who had an instrumental vaginal delivery in the operating room and women who had a cesarean delivery in the second stage of labor. The main strength of the study was the high follow-up rate at 3 years and that the responders closely reflected the original cohort. The potential for loss to follow-up bias was therefore reduced. This was a questionnaire-based study, and we relied on women’s recall and willingness to disclose information that may be considered sensitive. However, comparisons between the 2 groups remain rigorous, and any recall bias is likely to apply equally to either group.

Rates of urinary incontinence continue to be higher at 3 years after instrumental vaginal delivery, compared with cesarean delivery, in the second stage of labor. Pelvic floor symptoms do not appear to be exacerbated by a subsequent delivery, and there still appears to be a place for a careful trial of instrumental vaginal delivery in the operating room. Further research is required to determine the optimal approach to a subsequent delivery for women who have experienced a third- or fourth-degree tear as a complication of an instrumental vaginal delivery.

Acknowledgments

We thank the women who were involved in this study for their long-term participation and enthusiasm and Rachel Liebling, Lisa Verity, Rebecca Swingler, and Roshni Patel for recruiting women to the study and for collecting early morbidity data.
Pelvic Organ Support Study (POSST): The distribution, clinical definition, and epidemiologic condition of pelvic organ support defects

Steven Swift, MD, Patrick Woodman, DO, Amy O’Boyle, MD, Margie Kahn, MD, Michael Valley, MD, Deirdre Bland, MD, Wei Wang, MSPH, Joe Schaffer, MD

Department of Obstetrics and Gynecology, Division of Benign Gynecology, Medical University of South Carolina, Charleston, SC, Department of Obstetrics and Gynecology, Division of Urogynecology and Pelvic Reconstructive Surgery, Madigan Army Medical Center, Tacoma, Wash, Department of Obstetrics and Gynecology, Division of Gynecology, and Department of Urology, Division of Urology, University of Texas Medical Branch-Galveston, Galveston, Tex, Department of Obstetrics and Gynecology, Health System Minnesota, Shakopee, Minn, Department of Obstetrics and Gynecology, Section on Gynecology, Bowman Gray Medical Center, Winston-Salem, NC, Department of Biometry and Epidemiology, Medical University of South Carolina, Charleston, SC, Department of Obstetrics and Gynecology, Division of Urogynecology and Reconstructive Pelvic Surgery, University of Texas Southwestern Medical Center, Dallas, Tex

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Objective: The purpose of this study was to describe the distribution of pelvic organ support in a gynecologic clinic population to define the clinical disease state of pelvic organ prolapse and to analyze its epidemiologic condition.

Study design: This was a multicenter observational study. Subjects who were seen at outpatient gynecology clinics who required an annual gynecologic examination underwent a pelvic organ prolapse quantification examination and completed a prolapse symptom questionnaire. Receiver operator characteristic curves were used to define pelvic organ prolapse with the use of symptoms and pelvic organ prolapse quantification examination measures. Standard age-adjusted univariate and multivariate logistic regression analysis were used to evaluate various relationships.

Results: The population consisted of 1004 women who were aged 18 to 83 years. The prevalence of pelvic organ prolapse quantification stages was 24% (stage 0), 38% (stage 1), 35% (stage 2), and 2% (stage 3). The definition of pelvic organ prolapse that was determined by the receiver operator characteristic curve was the leading edge of their vaginal wall that was 0.5 cm above the hymenal remnants. Multivariate analysis revealed age, Hispanic race, increasing body mass index, and the increasing weight of the vaginally delivered fetus as risk factors for pelvic organ prolapse, as defined in this population.
Pelvic organ prolapse is a poorly understood condition that affects potentially millions of women worldwide. Currently, it is the most common non-cancer indication for hysterectomy in menopausal women in the United States. Despite the apparent prevalence of this condition, there is little information regarding its epidemiologic mechanism and natural history. The dearth of knowledge extends to the lack of a clinically useful or scientifically validated definition of the condition, which was highlighted at a recent terminology conference assembled by the National Institutes of Health.

This lack of a good definition stems in part from a poor understanding of what represents normal versus abnormal pelvic organ support in women. There have been several recent reports that described the distribution of pelvic organ support in various populations and that attempted to establish the normal distribution; however, these studies either failed to define pelvic organ support with the use of validated quantification systems or failed to evaluate diverse racial, geographic, or socioeconomic populations. Therefore, the normal distribution of pelvic organ support in a general population still has not been described adequately. Another area of concern in the definition of pelvic organ prolapse involves the nature of the disease. Pelvic organ prolapse is a disease with little, if any, significant morbidity (except in its most severe forms) and essentially no deaths. It is a disease that primarily affects quality of life. Therefore, any attempt to define clinically significant pelvic organ prolapse should include an assessment of the subject’s symptoms and their relation to various levels of support.

This study was designed to describe the distribution of pelvic organ support in a general US gynecologic clinic population and to define clinically relevant pelvic organ prolapse, with the use of a combination of physical examination findings and symptom questionnaire responses. In addition, data were collected on a variety of proposed causative factors to determine their impact on the prevalence of pelvic support defects.

**Material and methods**

This was a multicenter, cross-sectional, observational study. Six centers around the United States that served diverse patient populations participated. The centers included 2 centers in Texas and 1 center each in Washington, North Carolina, South Carolina, and Minnesota. These 6 centers were chosen through self-selection as part of a call for multicenter research sponsored by the American Urogynecologic Society. The study was approved by the Institutional Review Board at each center, and each subject provided written informed consent. The study population included women ≥18 years of age who went to 1 of the 6 outpatient gynecology clinics for routine gynecologic health care. Routine gynecologic health care was defined as patients who required a Papanicolaou test and/or their annual pelvic examination as part of their visit. Pregnant patients and patients who were seen within 6 weeks after delivery were excluded.

Subjects were recruited if they were seeking an annual gynecologic examination. They could report various gynecologic problems but had to identify the need for an annual examination as part of their reason for coming to the clinic. This requirement was established to better define our study population as a general gynecology clinic population and to remove the selection bias of subjects who were being seen for any specific gynecologic disease. After written informed consent was obtained, the subjects filled out a 17-question questionnaire (Figure 1). The questionnaire included 7 symptoms that were felt to be specific to pelvic organ prolapse at the time the study was designed. Subjects were able to respond “yes,” “no,” or “sometimes.” “Yes” or “sometimes” responses were followed-up with an additional question that pertained to symptom bother. The patient could only answer “yes” or “no” to the follow-up bother question. There were another 4 questions about constipation, and the 4 final questions queried job description, race, household income, and smoking history. A Spanish version of the questionnaire was available for Spanish-speaking women. The Spanish questionnaire was developed and validated by experienced translators at 1 of the Texas institutions.

After this, the subjects underwent a pelvic organ prolapse quantification (POPQ) examination in the dorsal lithotomy position by a clinician who was familiar with the POPQ examination technique. To ensure consistency about the POPQ examinations across all sites, an examination manual was created and agreed on by the local investigators. The senior author (S.E.S.) traveled to each of the sites (except the Minnesota site) and performed several POPQ examinations with the local investigators to confirm technique. After visiting several of the sites, the senior author noted excellent interexaminer reliability in the performance of the POPQ examination that was consistent with the literature. The budget did not allow for a visit to the Minnesota...
1. Do you have a sense of something falling out of your vagina?
   Yes____(1)  No____(2)  Sometimes____(3)
   1a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

2. Can you feel with your hand or see something bulging out of your vagina?
   Yes____(1)  No____(2)  Sometimes____(3)
   2a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

3. Do you have uncontrollable loss of urine?
   Yes____(1)  No____(2)  Sometimes____(3)
   3a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

4. Do you have uncontrollable loss of stool or gas from your rectum?
   Yes____(1)  No____(2)  Sometimes____(3)
   4a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

5. Do you have to put fingers in your vagina or push up on your bottom to have a bowel movement?
   Yes____(1)  No____(2)  Sometimes____(3)
   5a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

6. Do you have to put fingers in your vagina or push up on your bottom to empty your bladder?
   Yes____(1)  No____(2)  Sometimes____(3)
   6a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

7. Do you have a heaviness or fullness in the vagina, lower abdomen, or pelvis that increases as the day goes on?
   Yes____(1)  No____(2)  Sometimes____(3)
   7a). If yes or sometimes, does it bother you?
      Yes____(1)  No____(2)

8. Have any of these symptoms interfered with your daily activities?
   Yes____(1)  No____(2)  Sometimes____(3)

9. Have any of these symptoms interfered with sexual activity?
   Yes____(1)  No____(2)  Sometimes____(3)

Figure 1  The 17 questions of the symptom questionnaire that was given to all the subjects who participated in this study.
Bowel Habits
Answer each question as best you can

10). Do you have to strain to empty your bowels at least 25% of the time?
   Yes____(1)  No____(2)

11). Do you have lumpy or hard stools at least 25% of the time?
   Yes____(1)  No____(2)

12). Do you feel you do not completely empty your bowels at least 25% of the time?
   Yes____(1)  No____(2)

13). On average do you have less then 2 bowel movements per week?
   Yes____(1)  No____(2)

14). Employment  service___(1)

    fabricators,
    laborers___(2)
    housewife or
    homemaker___(3)
    professional,
    managerial___(4)
    technical, sales, clerical
    ____ (5)
    other ___(6)

15). Household income

    0-10,000$___(1)  10,001-35,000___(2)
    35,001- 60,000___(3)
    60,001-
    120,000___(4)
    more than
    120,0001___(5)

16). Race  White or Caucasian___(1)

    Black or African American___(2)
    Asian or Pacific Islander ____(3)
    Hispanic____(4)
    Filipino____(5)
    American Indian____(6)
    Indian___(7)
    Other____(8)

17). Smoking history

    never____(1)
    ever, but quit____(2)
    currently 1/2 pack per day____(3)
    currently, 1 pack per day ____(4)
    more than 1 pack per day ___(5)

Figure 1  (Continued).
site, but because of the previously mentioned findings, it was felt that this site could provide accurate data without compromising the integrity of the study.

The results of the questionnaire were withheld from the examining physician until after the examination. For this study, POPQ measures were made in 0.5-cm increments. The 9 POPQ points were recorded. Subjects were then assigned a POPQ stage, as previously described. All points, except the total vaginal length, were recorded with the subject performing maximal Valsalva effort or cough.

A 1-page data collection form was completed and included the patient’s demographic information, medical and surgical history, and the results of the pelvic examination. It also included data regarding obstetric history to include gravidity, parity, number of vaginal deliveries, and the weight of the largest infant delivered vaginally. The data regarding surgical history were specific to pelvic surgical procedures and included total abdominal or vaginal hysterectomy and the number of anti-incontinence and prolapse procedures. Data were entered and maintained at a central location in a write-and-password-protected database with a double-entry validation.

To define the disease state of pelvic organ prolapse, we used the results of the POPQ examination and the responses to the 7 questions about symptoms of pelvic organ prolapse. Receiver operator curve (ROC) analyses were used to identify a reasonable cutoff point that was based on the subject’s vaginal wall leading edge. We constructed curves for each of the 7 questions regarding symptoms of prolapse, using “yes” or “sometimes” as a positive response and evaluating the responses for each vaginal wall leading edge level value between −3 cm and +3 cm (no subjects in this study had prolapse beyond +3 cm). A second set of 7 curves was also constructed that were based on responses to “bother-some” questions. In each case, we first calculated sensitivity and specificity for all available vaginal wall leading edge values between −3 cm and +3 cm. Sensitivity and specificity were calculated in the following manner: sensitivity = % (no. of correctly predicted “prolapse”/no. of “true prolapse”); specificity = % (no. of correctly predicted “non-prolapse”/no. of “true non-prolapse”). We developed criterion standards for each ROC as 1-sensitivity + 1-specificity. The lowest criterion standard for any question was then used to classify subjects as having pelvic organ prolapse.

To determine the contribution of the various proposed causes, for pelvic organ prolapse, age-adjusted univariate logistic regression analysis was used to calculate relative risks as odds ratios with 95% CIs. We used a vaginal wall leading edge value of ≥ −0.5 cm as the definition of pelvic organ prolapse (this was determined after analysis of the ROCs). The risk factors that were evaluated included age, body mass index, gravidity, parity, number of vaginal deliveries, weight of largest vaginally delivered infant, previous hysterectomy (total abdominal hysterectomy or total vaginal hysterectomy vs no hysterectomy), previous prolapse surgery (ever vs never), constipation (reported as a positive response to any of the constipation question), menopausal status (before vs after), hormone replacement therapy (ever vs never), any chronic illness (hypertension, diabetes mellitus, asthma, or emphysema vs none), employment (laborer/housewife vs technical/service/professional), household income (≤ $35K vs $35-120K vs $120K), race (white vs black vs Hispanic vs others), and smoking history (ever vs currently vs never). A stepwise multivariate regression analysis was then used with the variables detected by univariate analysis as possibly having an influence on the prevalence of pelvic organ prolapse. Statistical significance was considered if the probability value was < .05.

Data analysis was performed with SAS statistical software (version 8.2; SAS Institute Inc, Cary, NC). Statistical analyses involved summary and descriptive statistics. Continuous demographic variables were summarized by either medians or means, standard deviations, or minimum and maximum. Qualitative demographic and other patient characteristics were summarized by counts and percentages. Paired t-test was used to compare the difference between the anterior and posterior apical vaginal segments and the anterior and apical vaginal segments.

Results

One thousand four women participated in the study over an 18-month period from September 1999 through March 2002. The centers served various populations: 2 centers served a primarily private gynecologic practice; 1 center combined private practice and house-staff resident clinic populations; 1 center served mainly resident house-staff clinic populations; 1 center served a military population (which included active duty and military dependents), and 1 center served as a gynecology clinic for indigent Hispanic patients. All of the centers contributed significant numbers of subjects that ranged from 110 to 220 subjects enrolled.

The mean age of the population was 42.7 ± 13.9 (± SD) years (range, 18-83 years). The median gravidity was 3 (range, 0-16), and the median parity was 2 (range, 0-13). Seventeen percent of subjects had undergone an abdominal or vaginal hysterectomy. Fifty-eight percent of subjects were premenopausal; 40% of subjects were postmenopausal, and the menopausal status of 2% of subjects was unknown. Racial distribution, as self-reported, was 43% white, 24% black, 29% Hispanic, 2% Asian, and 2% other. The following distribution of household income was self-reported: 37.6%, 0 to
The following data represents the distribution of pelvic organ support by POPQ stages for the population: stage 0, 24%; stage 1, 38%; stage 2, 35%; and stage 3, 2%. There were no subjects in our population with POPQ stage 4 pelvic organ prolapse. In addition to the POPQ stage, the distribution of the leading edge of the vaginal walls was documented (Figure 2). The structure or POPQ point that made up the leading edge was determined by taking the greatest value between points Aa, Ba, C, D, Ap, and Bp within each subject.

To evaluate which vaginal segments had the greatest degree of relaxation, we determined the mean value for each of the nine POPQ measurements (Table I). We took the greatest value between Aa and Ba (−1.7 cm) to represent the anterior vaginal wall and did a similar calculation with points Ap and Bp (−2.2 cm) to represent the posterior vaginal wall and with points C and D (−6.0 cm) to represent the vaginal apex. Paired t-test analysis revealed that the anterior vaginal segment was statistically significantly greater then the posterior vaginal segment (P < .001) or apical segment (P < .001).

Ninety-eight percent of the subjects completed the questionnaire regarding symptoms of pelvic organ prolapse. The number of positive responses per subject and number of positive bothersome responses per subject was determined by the POPQ stage. There was a trend of increasing number of positive responses per subject and positive bothersome responses per subject with increasing stage (Figure 3). However, a clear cut-off point, to allow for a definition of the disease state of pelvic organ prolapse, with the stage was not apparent with this evaluation. Therefore, we developed ROCs for each of the 7 symptoms and their follow-up bother questions. The criterion standard to select a cut-off for pelvic organ prolapse was evaluated. This ROC analysis revealed that all of the symptom and bothersome questions had ideal criterion standards for the various leading edge values of between −2.5 cm and +3.0 cm (Table II). The reason involved very poor and discordant sensitivities. In addition, when we evaluated the c value for each of the ROCs, the values remained between 0.51 and 0.64, which suggested poor ability of the question to properly classify the subjects as having prolapse. This was true for all of the questions, except for the question regarding a vaginal bulge and the follow-up bothersome question that had c values of 0.72 and 0.75, respectively (Table II). This suggests that the question regarding a bothersome vaginal bulge had a 75% chance of correctly classifying a subject with prolapse. The lowest criterion standard for both the symptom of a bulge question and the follow-up bothersome question was −0.5 cm. Therefore, we defined the disease state of pelvic organ prolapse as the leading edge of the prolapse being at −0.5 cm for the rest of our evaluation. This meant that 218 subjects (22%) were defined as having pelvic organ prolapse.

Using the definition of pelvic organ prolapse noted earlier, we performed age-adjusted univariate logistic regression analysis on various factors to define odd ratios for the development of this disease (Table III). Multivariate analysis was performed on those factors that were identified in univariate analysis as potentially affecting pelvic organ prolapse (Table IV).

Comment

The current state of research regarding pelvic support defects has been hampered by the lack of a sound definition of the disease of pelvic organ prolapse. Until the disease can be defined, it cannot be recognized; until it can be recognized, little progress can made into describing its epidemiologic condition. This study was designed to document the distribution of pelvic organ support in a geographically and racially diverse group of women, to develop a meaningful definition of pelvic organ prolapse with the use of physical findings in combination with common symptoms, and once a definition of prolapse was established, to evaluate several proposed etiologic factors.

The data generated from this study on the distribution of pelvic organ support in women are consistent with previously published reports. The distribution follows a bell-shaped curve with most subjects having POPQ stage 1 and 2 pelvic organ support and only 7% of the subjects having the leading edge of the prolapse at or beyond the hymenal remnants (Figures 2). The previous reports regarding the distribution of pelvic organ support demonstrated that between 1% and 3% of subjects had pelvic organ support defects, with the vagina prolapsing to or beyond the vagina opening. The population that shows slightly more prolapse beyond the hymen. This may stem from the lack of racial or geographic diversity in previous reports. In particular, most of these reports had an overwhelming predominance of white subjects. The strength of the data that were generated in this report involves the inclusion of large numbers of minority women, the wide geographic distribution of the data collection centers, and the use of a well-studied POPQ.

One aim of this study was to describe a population that accurately reflects the general US population. However, despite efforts to recruit a representative population, this report still does not accurately reflect the US population. According to the census bureau, the distribution of race across the United States for the year 2000 was 71.2% white, 12.3% black, 12.5% Hispanic, 3.8% Asian or Pacific Islander, and 0.9% Native American. Our study population was more heavily
weighted toward Hispanic (29%) and black (24%) patients, with only 43% white and 2% Asian patients. In addition, according to the 2000 census, 12.4% of families in the United States live below the poverty line, somewhere between $10,000 and $13,000 per year. This study population had >35% earning <$10,000 per year. It can be seen that, in our study population, minorities and patients who are living in poverty are over represented. In addition, only subjects who were being seen for gynecologic health care examinations were selected for participation, which is not necessarily representative of the general population. Therefore, it is difficult to state that our study population accurately reflects the general population of women in the United States. Obtaining data from a truly random population that represents the US population would be difficult, if not impossible. Randomly selecting subjects from the various population centers and requesting that they receive a gynecologic examination would be fraught with disappointing results. In planning this project, the selection bias in examining subjects who were attending gynecologic visits was discussed, but no viable alternative could be found to approximate the general population. This group was chosen as a population that reflects those subjects who come into contact with the gynecologic health care providers and would be the population being screened for pelvic support disorders. Also, these subjects were already undergoing a pelvic examination, so there would be very little additional discomfort involved in their office visit. This group likely

Table I  The mean values (±SD) of the 9 POPQ points

<table>
<thead>
<tr>
<th>POPQ point</th>
<th>Mean value (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa</td>
<td>−1.7 ± 1.1</td>
</tr>
<tr>
<td>Ba</td>
<td>−1.7 ± 1.1</td>
</tr>
<tr>
<td>C</td>
<td>−6.0 ± 1.8</td>
</tr>
<tr>
<td>D</td>
<td>−8.6 ± 1.8</td>
</tr>
<tr>
<td>Ap</td>
<td>−2.3 ± 1.0</td>
</tr>
<tr>
<td>Bp</td>
<td>−2.2 ± 1.1</td>
</tr>
<tr>
<td>Gh</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>Pb</td>
<td>−2.2 ± 0.9</td>
</tr>
<tr>
<td>Tvl</td>
<td>9.6 ± 1.5</td>
</tr>
</tbody>
</table>

Figure 2  The cumulative percent of subjects. The percentage of individuals; the leading edge at any of the points is listed. Note that 95% of subjects have the leading edge of their prolapse at or above 0 cm (or the hymenal remnants).
under represents the true prevalence of more severe forms of pelvic organ prolapse but is probably an accurate reflection of the relative distribution of POPQ stage 0, 1, and 2 pelvic organ support.

Understanding the variation in pelvic organ support and how the degree of support relates to symptoms is crucial for defining the condition or disease state of pelvic organ prolapse. The current definitions of pelvic organ prolapse are based more on expert opinion than on data and are often vague or too inclusive. The American College of Obstetrics and Gynecology defines it as the protrusions of the pelvic organs into or out of the vaginal canal. This could be interpreted to include all patients with POPQ stage 1 or greater prolapse. Another recent attempt to define pelvic organ prolapse occurred at a terminology conference for researchers in the study of pelvic floor disorders that was convened by the National Institutes of Health. They acknowledged that there was not enough current information to properly define pelvic organ prolapse, but they proposed a definition for pelvic organ prolapse as the decent of any vaginal segment to within 1 cm of the hymen or lower. Although this is a very specific definition that includes all patients with POPQ stage 2 or greater prolapse, it may be too inclusive and would encompass almost 40% of the subjects in this report. The members of the National Institutes of Health conference also pointed out that, because pelvic organ prolapse is a condition that primarily affects quality of life, any definition of the disease should include some evaluation of symptoms.

The problem with our current definitions of pelvic organ prolapse is that they do not properly reflect the nature of the disease of pelvic organ prolapse. In a recent article that compared various techniques for the correction of anterior vaginal wall defects, the authors used POPQ stage 0 or 1 as a definition of anatomic cure and a questionnaire to determine the subjective improvement. The anatomic cure rates ranged from 30% to 46% for the various procedures that were studied; despite this, according to the questionnaires, almost all of the subjects were satisfied with the results. The authors concluded that their anatomic definition of cure may have been too stringent; the data from this study agree with this conclusion. Almost 40% of routine gynecology patients have POPQ stage 2 pelvic

Figure 3 The solid line represents the number of positive responses per subject to the 7 questions about pelvic organ prolapse symptoms within each POPQ stage. The dashed line represents the number of positive responses per subject about the bothersome nature of the symptom. Note that subjects with POPQ stage 3 prolapse had, on average, almost 2 symptoms that were associated with pelvic organ prolapse and at least 1 bothersome symptom.
organ support, and in this report many subjects with stage 2 examinations were considered "normal," so it is not surprising that some subjects who attained this level after operation were satisfied with their outcome.

In the population that was studied, 7% of the subjects had the leading edge of the vaginal wall at or beyond the hymenal remnants (some POPQ stage 2 and all stage 3; Figure 2). Previous research would suggest that, once the leading edge of the vaginal wall extends beyond the introitus or region of the hymenal remnants, a subject is more likely to experience symptoms that are attributed to their prolapsing pelvic organs.15,16 Those subjects with POPQ stage 3 prolapse have obvious prolapse and would expect to be symptomatic. However, the graph in Figure 3 does not demonstrate an obvious cut-off point beyond which subjects can be described clearly as having the disease of symptomatic pelvic organ prolapse. Therefore, to further evaluate the relationship between symptoms and leading edge of vaginal support, we developed ROCs for each of the 14 questions regarding either symptoms or bothersome symptoms that are attributed commonly to pelvic support defects. This was done to determine whether there was a best-fit model for defining pelvic organ prolapse with the symptoms in combination with physical examination findings. Using the criterion standards for both the symptom and bothersome questions, we came up with various cut-off values in defining pelvic organ prolapse for each question (Table I). When this project was initiated, there were very little data on what symptoms were related to pelvic organ support defects. The authors developed the questionnaire that was used

Table II The results of the ROC analysis for each of the 7 symptom questions and the follow-up bothersome question (c values and criterion standards)

<table>
<thead>
<tr>
<th>Symptom question/follow-up question</th>
<th>C-value for question</th>
<th>Criterion standard for question (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense of something falling out/does it bother</td>
<td>0.59/0.61</td>
<td>−1.0/−1.0</td>
</tr>
<tr>
<td>Feel or see something bulging out/does it bother</td>
<td>0.72/0.75</td>
<td>−0.5/−0.5</td>
</tr>
<tr>
<td>Uncontrollable loss of urine/does it bother</td>
<td>0.60/0.60</td>
<td>−1.0/−1.0</td>
</tr>
<tr>
<td>Uncontrollable loss of gas or stool/does it bother</td>
<td>0.52/0.51</td>
<td>+3/0</td>
</tr>
<tr>
<td>Splint or digitate to have a bowel movement/does it bother</td>
<td>0.57/0.57</td>
<td>−1.0/−1.5</td>
</tr>
<tr>
<td>Splint or digitate to empty bladder/does it bother</td>
<td>0.56/0.64</td>
<td>−0.5/0</td>
</tr>
<tr>
<td>Heaviness or fullness in the vagina, lower abdomen, or pelvis/does it bother</td>
<td>0.58/0.60</td>
<td>−1.0/−2.5</td>
</tr>
</tbody>
</table>

Table III Univariate logistic regression analysis for the various risk factors for pelvic organ prolapse, with risk factors that are adjusted for age

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Total Prolapse Odds ratio (n)</th>
<th>(95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>998 217</td>
<td>1.04 (1.03-1.05)</td>
</tr>
<tr>
<td>10</td>
<td>1.46 (1.30-1.64)</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>283 25</td>
<td>1.00</td>
</tr>
<tr>
<td>25-30</td>
<td>286 73</td>
<td>3.13 (1.91-5.15)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>374 105</td>
<td>3.52 (2.19-5.66)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>424 41</td>
<td>1.00</td>
</tr>
<tr>
<td>Black</td>
<td>242 47</td>
<td>2.64 (1.66-4.20)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>289 124</td>
<td>6.29 (4.20-9.41)</td>
</tr>
<tr>
<td>Other</td>
<td>31 2</td>
<td>0.77 (0.17-3.36)</td>
</tr>
<tr>
<td>Gravidaity</td>
<td>988 217</td>
<td>1.26 (1.17-1.35)</td>
</tr>
<tr>
<td>Parity</td>
<td>989 216</td>
<td>1.39 (1.27-1.52)</td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>977 213</td>
<td>1.39 (1.27-1.51)</td>
</tr>
<tr>
<td>Weight of vaginally delivered infant (oz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>937 198</td>
<td>1.12 (1.08-1.17)</td>
</tr>
<tr>
<td>20</td>
<td>1.26 (1.17-1.36)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.42 (1.27-1.59)</td>
<td></td>
</tr>
<tr>
<td>Hysterectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No total abdominal or total vaginal</td>
<td>825 166</td>
<td>1.00</td>
</tr>
<tr>
<td>Prolapse surgical procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>173 51</td>
<td>1.10 (0.74-1.62)</td>
</tr>
<tr>
<td>Ever</td>
<td>966 203</td>
<td>1.00</td>
</tr>
<tr>
<td>Ever</td>
<td>32 14</td>
<td>1.81 (0.86-3.78)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>399 123</td>
<td>1.00</td>
</tr>
<tr>
<td>Before</td>
<td>573 80</td>
<td>0.62 (0.36-1.07)</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>463 97</td>
<td>1.00</td>
</tr>
<tr>
<td>Ever</td>
<td>229 53</td>
<td>0.59 (0.39-0.90)</td>
</tr>
<tr>
<td>Constipation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>46 14</td>
<td>1.00</td>
</tr>
<tr>
<td>Yes</td>
<td>942 201</td>
<td>0.73 (0.38-1.42)</td>
</tr>
<tr>
<td>Chronic illness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>727 139</td>
<td>1.00</td>
</tr>
<tr>
<td>Any</td>
<td>267 77</td>
<td>1.18 (0.65-2.13)</td>
</tr>
<tr>
<td>Constipation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>46 6</td>
<td>1.00</td>
</tr>
<tr>
<td>Yes</td>
<td>948 49</td>
<td>0.47 (0.18-1.22)</td>
</tr>
<tr>
<td>Employment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonlabor</td>
<td>430 105</td>
<td>1.00</td>
</tr>
<tr>
<td>Labor</td>
<td>526 161</td>
<td>3.01 (2.10-4.30)</td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (&gt;120K)</td>
<td>21 5</td>
<td>1.00</td>
</tr>
<tr>
<td>Medium (35-120K)</td>
<td>263 19</td>
<td>0.26 (0.08-0.79)</td>
</tr>
<tr>
<td>Low (&lt;35K)</td>
<td>641 182</td>
<td>1.39 (0.49-3.93)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>651 161</td>
<td>1.00</td>
</tr>
<tr>
<td>Ever</td>
<td>187 29</td>
<td>0.50 (0.32-0.78)</td>
</tr>
<tr>
<td>Currently</td>
<td>143 23</td>
<td>0.71 (0.43-1.15)</td>
</tr>
</tbody>
</table>

* N = 1004.
† N = 218.
After a definition of pelvic organ prolapse was established, an age-adjusted univariate analysis was performed on various suspected causes to determine their relative contribution (Table III). The causes, which were identified by univariate analysis as risk factors, largely agree with several previous publications and included increasing age, body mass index, gravidity, parity, number of vaginal deliveries, and weight of vaginally delivered infants. In addition, Hispanic and black race and employment that involved manual labor appeared to increase the risk of prolapse. Increasing age as a risk factor for pelvic organ prolapse is intuitive and is identified consistently as a cause, regardless of the definition that is used to define pelvic organ prolapse, and explains our decision for an age-adjusted analysis. 3,6,8,19-21 Pregnancy is another commonly cited risk factor, and the data in this report, again, are consistent with the literature, which shows a 20% to 40% increase with either gravidity, parity, vaginal delivery, and increasing weight of a vaginally delivered infant. 3,8,18,22,23 Interestingly, vaginal delivery did not increase the odds ratio over parity alone, which would suggest that it is the term pregnancy that increases the risk of pelvic organ prolapse and not the delivery method. It does appear that the increasing weight of the vaginally delivered infant is associated with pelvic organ prolapse. We did not record the weight of the largest infant who was carried by the subject; therefore, we cannot state whether large infants are associated with prolapse or only large infants who are delivered vaginally.

There is very little literature on the impact of job description and the risk of prolapse. One previous report on nursing assistants found that women who functioned in an employment that was associated with a lot of lifting were more likely to undergo surgical procedures to correct prolapse. These data would confirm that report, subjects who self-reported themselves as housewife/ laborers had a significantly increased risk of prolapse over nonlaborers. However, this did not remain statistically significant in the multivariate analysis. Therefore, black race may not increase the odds ratio over parity alone, which would suggest that it is the term pregnancy that increases the risk of pelvic organ prolapse and not the delivery method. It does appear that the increasing weight of the vaginally delivered infant is associated with pelvic organ prolapse. We did not record the weight of the largest infant who was carried by the subject; therefore, we cannot state whether large infants are associated with prolapse or only large infants who are delivered vaginally.

by reviewing textbooks that uniformly listed the symptoms that were queried in our questionnaire. Multiple subsequent studies have demonstrated that the symptoms of urinary and fecal incontinence, splinting or reducing the bulge to void or evacuate, and the sense of pelvic pain are not correlated with increasing pelvic organ prolapse. 17,18 The only bothersome symptom that appears to be related consistently to worsening pelvic organ prolapse is a vaginal bulge that can be seen or felt. 19,20 Therefore, it is not surprising that this was the only symptom that demonstrated any ability to classify pelvic organ prolapse and was the symptom on which we relied to distinguish pelvic organ prolapse from normal in this study. The leading edge of the vagina at or beyond −0.5 cm may not be the most clinically useful definition of pelvic organ prolapse, but it is consistent with other reports that demonstrate that, as the vaginal wall approaches the hymen, subjects tend to report more bothersome symptoms. The original POPQ document describes measuring the various points at 1-cm intervals; if you round up the results of this study, then 0 cm would be the most clinically useful cut-off for the definition of pelvic organ prolapse that may be more clinically useful. 11

<table>
<thead>
<tr>
<th>Table IV</th>
<th>A multivariate logistic regression analysis of those factors that had a significant relationship by univariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>Age (per 10-y)</td>
<td>1.38 (1.09-1.75)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>&lt;25  1.00  25-30  2.51 (1.18-5.35)  &gt;30  2.56 (1.23-5.35)</td>
</tr>
<tr>
<td>Race</td>
<td>White  1.00  Black  1.20 (0.44-3.26)  Hispanic  4.29 (1.80-10.2)  Other  2.40 (0.47-12.1)</td>
</tr>
<tr>
<td>Parity (per 1 unit)</td>
<td>1.11 (0.71-1.73)</td>
</tr>
<tr>
<td>Gravidity (per 1 unit)</td>
<td>0.93 (0.74-1.16)</td>
</tr>
<tr>
<td>NVD (per 1 unit)</td>
<td>1.13 (0.89-1.44)</td>
</tr>
<tr>
<td>Weight of vaginally delivered infant (per 10 oz)</td>
<td>1.11 (1.04-1.19)</td>
</tr>
<tr>
<td>Hormone replacement therapy ever</td>
<td>0.98 (0.57-1.69)</td>
</tr>
<tr>
<td>Employment labor related income</td>
<td>High (&lt;120K)  1.00  Medium (35-120K)  0.21 (0.06-0.74)  Low (&lt;35K)  0.24 (0.06-0.93)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Never  1.00  Ever  1.20 (0.60-2.41)  Currently  0.90 (0.33-2.46)  Chronic illness (any)  1.05 (0.53-2.09)</td>
</tr>
</tbody>
</table>
cigarette smokers, and hormone replacement therapy users. These data are difficult to comment on because there is very little in the literature; however, in other studies, it was noted that cigarette smoking was protective against the development of prolapse.\(^{30}\) This protective effect disappeared in the multivariate analysis; it remains questionable whether smoking should be considered in a strategy to prevent the development of pelvic organ prolapse. Like smoking history, the effects of hormone replacement therapy disappeared in the multivariate analysis, and likewise it would be difficult to recommend hormone replacement therapy to prevent prolapse.

To date, this report represents a description of the most geographically and racially diverse population of women studied for pelvic organ support. It used 6 centers from around the country that examined women from various geographic, racial, and socioeconomic backgrounds with a validated POPQ system. The most interesting finding in these data is the suggestion that POPQ stages 0, 1, and some stage 2 are “normal” degrees of support. Our data suggest that subjects with POPQ stage 2 support (particularly those subjects with the leading edge at \(-1\) cm) represent a variant of normal. The questionnaire that was used to define symptomatic pelvic organ prolapse in this study was not a validated tool (at the initiation of this study and throughout the data collection, no validated pelvic organ prolapse quality-of-life tool was available); therefore, these data on defining clinically significant pelvic organ prolapse can be questioned. However, the relationship between symptoms and physical examination findings suggests that the hymen is an appropriate dividing line in the definition of pelvic organ prolapse. Whether this point should be just proximal to the hymen or at the hymen is still a subject that is open to debate. This definition deserves more study and confirmation in various populations with a validated quality of life questionnaire before it can be advocated as a definition of pelvic organ prolapse.

**References**


Comparison of 2-dimensional and 3-dimensional power-Doppler imaging in complex adnexal masses for the prediction of ovarian cancer

Juan Luis Alcázar, MD, Gerardo Castillo, MD

Department of Obstetrics and Gynecology, Clínica Universitaria de Navarra, University of Navarra, School of Medicine, Pamplona, Spain

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KEY WORDS
Adnexal mass
Power Doppler
Ultrasound scanning
Ovarian cancer

Objective: The purpose of this study was to compare 2-dimensional and 3-dimensional power-Doppler imaging diagnostic performance for the prediction of ovarian cancer in complex adnexal masses.

Study design: Sixty-nine complex adnexal masses in 60 women (mean age, 48.4 years [range, 17-82 years]) were evaluated by 2-dimensional and 3-dimensional power-Doppler imaging for differentiating benign from malignant masses. Complex adnexal mass was defined in the presence of at least 1 of the following features: solid areas, thick papillary projections, thick septa, or purely solid echogenicity. One examiner performed 2-dimensional power-Doppler imaging, and a second examiner performed 3-dimensional power-Doppler imaging. All masses were removed surgically, and definitive diagnosis was obtained. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated for both techniques.

Results: Forty-five tumors (65.2%) were proved to be malignant, and 24 tumors (34.8%) were proved to be benign. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for 2-dimensional power-Doppler imaging were 97.8%, 87.5%, 93.6%, 95.5%, and 94.2%, respectively. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for 3-dimensional power-Doppler imaging were 97.8%, 79.2%, 89.9%, 95%, and 91.3 % respectively. There were no statistical differences in sensitivity and specificity (McNemar test: P = .250)

Conclusion: Three-dimensional power-Doppler imaging did not have a better diagnostic performance than 2-dimensional power-Doppler imaging for the discrimination of benign from malignant complex adnexal masses.

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Transvaginal ultrasound scanning has been useful to detect ovarian cancer. However, its main limitation for differentiating benign from malignant adnexal masses is its relative high false-positive rate, because many benign tumors show a complex or questionable appearance.

Transvaginal color Doppler imaging allows tumor vascularization assessment. Although initial studies that applied color and spectral pulsed Doppler imaging were encouraging, further studies challenged these
previous results. However, a recent meta-analysis has shown that the use of color Doppler imaging may contribute to the increase in the specificity of conventional B-mode ultrasound scanning.

Some studies that used multivariate analysis of different sonographic and Doppler parameters have demonstrated that the most important sonographic features for the prediction of ovarian malignancy are morphologic appearance and tumor blood flow location.

Recently, a variation of conventional color Doppler imaging, termed power-Doppler imaging, has been introduced in clinical practice. It is based on the amplitude rather than the frequency shift.

A multicenter European study has demonstrated that the diagnostic performance of power Doppler imaging for discriminating benign from malignant adnexal tumors is superior to that of conventional color Doppler ultrasound scanning.

More recently, 3-dimensional power-Doppler imaging (3DPD) has become available. Several studies have shown that 3DPD may improve the diagnostic accuracy for ovarian cancer prediction. However, all these studies lack of proper statistical analysis or do not compare 2DPD with 3DPD.

The objective of this study was to prospectively compare the use of 2DPD and 3DPD for the discrimination of benign from malignant adnexal masses with complex or questionable appearance on B-mode transvaginal ultrasoundography.

Material and methods

From January 2002 to April 2004, the 346 patients who had received a diagnosis of an adnexal mass were evaluated and treated at our institution. Of these 346 patients, 60 women with complex adnexal masses were evaluated by 2DPD and 3DPD. A group of these patients (14, 25.4%) were included in a previous study about 3-dimensional ultrasound scanning. Patients who had complex adnexal mass and 3D-ultrasonography availability were selected.

As transvaginal power Doppler sonography is of routine use at our institution for the evaluation of adnexal masses; no institutional review board permission was needed. However, all patients gave verbal informed consent after the nature of the study was explained fully.

Patient mean age was 48.4 ± 16.4 years (range, 17-82 years). Thirty-two women (53%) were premenopausal, and 28 women (47%) were postmenopausal.

Nine patients had bilateral tumors; 69 masses were evaluated.

All cases were evaluated by conventional transvaginal ultrasonography with Kretz SonoAce SA-9900 (Kretztechnik, Zipf, Austria) or Voluson 730-Pro (GE, Milwaukee, Minn) systems with a mechanical 5- to 9-MHz 3-dimensional endovaginal probe with 5-MHz power-Doppler capability. Transabdominal ultrasound scanning (3.5-5 MHz) was also performed in large tumors. On B-mode ultrasound scanning, the definition of complex adnexal mass was based on the presence of at least 1 of the following parameters: thick wall (>3 mm), thick septum (>3 mm), thick papillary projections (>3 mm), solid areas or purely solid echogenicity (Figure 1).

Morphology in which the echo features were highly characteristic of a given pathologic condition (such as simple cyst, cystic teratoma, or endometrioma) were not included. Any multiloculated or uniloculated complex or solid mass, the echo texture of which was not suggestive of benign histologic condition, was categorized as malignant.

Figure 1 Transvaginal B-mode sonogram shows a complex adnexal mass with solid components and thick septa. Histologic evaluation revealed a primary ovarian carcinoma.

Figure 2 2DPD of the complex adnexal mass with solid areas (shown in Figure 1). Abundant blood flow is shown within solid areas.
Thereafter, the 2-dimensional power-Doppler gate was activated to obtain blood flow mapping from the tumor. Power-Doppler settings were set to achieve maximum sensitivity for the detection of low-velocity blood flow without noise (frequency, 5 MHz; power-Doppler gain, 20 [range, 1-30]; dynamic range, 20-40 dB; edge, 1; persistence, 2; color map, 1; gate, 2; filter: 3). Spectral pulsed Doppler analysis was done, but the data were not used in this study.

A malignancy was suspected when blood flow was detected within a papillary projection, solid area, or central area of solid tumors (Figure 2). Immediately after 2DPD ultrasound scanning was performed, the 3-dimensional volume box was activated and adjusted, and the entire lesion was scanned. In some adnexal masses, because of the large size, >1 volume box of different areas of interest were obtained and analyzed. Once a 3-dimensional volume reading was obtained, it was stored on a hard disk (Sonoview; Kretztechnik). Volume acquisition time lasted from 2 to 6 seconds, depending on the size of the volume box.

Malignancy was suspected in the presence of penetrating vessels within papillary projections, solid areas, or central areas of a solid tumor (Figures 3 and 4).

The same examiner (J.L.A.) performed all 2DPD and 3DPD examinations. This examiner has 12 years of experience on gynecologic ultrasound scanning. However, this examiner only interpreted 2DPD sonograms. A second examiner (G.C.) analyzed 3DPD sonograms by reviewing the 3DPD volume boxes that had been stored. This examiner was blinded to the 2DPD findings from the first examiner. The second examiner has 2 years of experience on gynecologic ultrasound scanning and has completed a 6-month training period on 3D ultrasound scanning before this study.

All patients underwent surgical procedures, and a definitive histologic diagnosis was obtained. Tumors were classified according to World Health Organization criteria. Primary ovarian carcinomas were staged surgically,
According to International Federation of Gynecology and Obstetrics criteria.

Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated for 2DPD and 3DPD techniques. Sensitivity and specificity were compared by the McNemar test. The Fleiss $\kappa$ index was used to assess the agreement between both examiners. A probability value of $<.05$ was considered to be statistically significant. All tests were performed with SPSS software (version 11.0 for Windows; SPSS Inc, Chicago, Ill).

**Results**

After surgical removal, 45 tumors (65.2%) were proved to be malignant, and 24 tumors (34.8%) were proved to be benign (Table 1). On B-mode ultrasound scan, 13 tumors (19%) had a unilocular complex appearance; 35 tumors (51%) were multilocular complex masses, and 21 according to International Federation of Gynecology and Obstetrics criteria.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>24</td>
<td>34.8</td>
</tr>
<tr>
<td>Mature teratoma</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>Mucinous cystadenoma</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>Cystadenofibroma</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>Tubo-ovarian abscess</td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>Serous cystadenoma</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>Endometrioma</td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>Luteal cyst</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>Hemorrhagic cyst</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>Pelvic paraganglioma</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Malignant</td>
<td>45</td>
<td>65.2</td>
</tr>
<tr>
<td>Primary ovarian carcinoma*</td>
<td>30</td>
<td>43.4</td>
</tr>
<tr>
<td>LMP tumor(^1)</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>Metastatic tumor</td>
<td>11</td>
<td>15.9</td>
</tr>
</tbody>
</table>

* LMP, Low malignant potential.
\(^1\) All stage Ia.

---

**Figure 4** 3DPD of a multiloculated complex adnexal mass with thick septa. 3DPD shows a linear vessel arrangement with septations and simple branching pattern. This case was considered initially as malignant on 2DPD and was definitively stated as benign after 3DPD. Histologic evaluation revealed a mucinous ovarian cystadenoma.
tumors (30%) were solid tumors. Blood flow with 2DPD
and 3DPD was detected in 66 tumors (95.7%). In 2 benign
 tumors (ovarian endometriomas) and in 1 malignant
tumor (a stage IIIb papillary serous ovarian carcinoma),
no flow could be detected. After 2DPD examination, 46
masses (67%) were considered to be malignant, and 23
masses (33%) were considered to be benign. After 3DPD,
49 masses (71%) were considered to be malignant, and 20
masses (29%) were considered to be benign.

The sensitivity, specificity, positive predictive value,
negative predictive value, and accuracy for 2DPD and
3DPD are shown in Table II. No differences in
sensitivity and specificity were found (McNemar test:
P = .25).

With 2DPD, 1 malignant tumor was considered to be
benign (a stage IIIb papillary serous ovarian carcinoma
without detected blood flow). Three benign tumors were
considered to be malignant (1 mucinous cystadenoma
with thick septa with solid areas and blood flow within
solid area, 1 cystadenofibroma with a solid area and
blood flow within the solid area, and 1 struma ovarii
with multilocular complex appearance and blood flow
within the solid areas).

With 3DPD, 1 malignant tumor was considered as
benign (the same case than for 2DPD), and 5 benign
tumors were considered to be malignant (1 cystadenofib-
roma, 1 mucinous cystadenoma, and 1 mature terato-
toma with struma ovarii [same cases as for 2DPD], and
2 additional cystadenomas).

Agreement between both examiners for the classifi-
cation of adnexal masses was high (κ = 0.90)

Comment

Morphologic evaluation of adnexal masses with pelvic
ultrasound scanning has been shown to be useful for the
prediction of ovarian cancer. Most ovarian cancers
show a complex sonographic appearance with solid
excrescence, irregular borders, and thick septations
and/or papillary projections.1 However, it is also known
that many benign tumors may exhibit similar features,
which limits the usefulness of this technique because of
a relative high false-positive rate.2,3

Color Doppler ultrasound imaging may provide addi-
tional information (such as tumor vascularization fea-
tures). This technique was introduced in early 1990s by
analysis of tumor blood flow location and blood flow
velocity waveforms with spectral pulsed Doppler imag-
ing. The use of spectral pulsed Doppler imaging to
calculate several velocimetric indexes was encouraging
initially.5,6 However, further studies have shown that
the use of such velocimetric indexes as the unique criterion for
the differentiation of benign from malignant adnexal
tumors is unreliable and has no application in clinical
practice.7,8,19

In fact, some studies that used multivariate analysis of
different sonographic and Doppler parameters have dem-
onstrated that the most important sonographic features
for the prediction of ovarian malignancy are morphologic
appearance and tumor blood flow location.10,11

In the mid 1990s, a variation of conventional color
Doppler imaging, termed power-Doppler imaging, was
introduced in clinical practice.12 This technique is based
in the amplitude rather than in the frequency shift. It has
some advantages over conventional color Doppler
imaging such as sensitivity for flow detection and
vascular architecture depiction.12

The results of a multicenter European study have
 demonstrated that power-Doppler imaging has a higher
diagnostic performance than conventional color Dop-
pler imaging for the differentiation of benign from
malignant adnexal masses, by classification of the
tumors on the basis of their sonomorphologic appear-
ance and tumor blood flow location.13

With the advent of 3-dimensional ultrasound scan-
ing, 3DPD has become available for clinical practice.
Few previous studies have addressed the use of 3DPD in
adnexal masses.

Kurjak et al14,15 in 2 different studies of 120 and 90
adnexal masses, respectively, concluded that 3DPD was
superior to conventional color Doppler imaging by
increasing the sensitivity. However, they did not compare
specifically 3DPD and 2DPD. In fact, they compared
a scoring system that included some morphologic features
and 3DPD evaluation of tumor vessels characteristics
(such as vessels arrangement and branching pattern) with
another scoring system that included the same morpho-
logic features with pulsed Doppler velocimetric param-
eters (resistance index [RI], ≤0.42 or > 0.42). They did not
include tumor vessel location in these scoring systems.
Kupesic and Kurjak20 found that the use of sonographic
contrast agents might increase the performance of 3DPD.

Cohen et al16 evaluated the role of 3DPD in a series of
71 complex adnexal masses on 2-dimensional transvaginal
ultrasound scanning. They did not use 2-dimensional conventional color Doppler imaging nor 2DPD. In their approach, they combined 2-dimensional and 3-dimensional morphologic features with 3DPD evaluation of blood flow tumor location, considering a tumor to be malignant in the presence of complex morphologic pattern and central (in papillary projections and/or septations) blood flow location. They concluded that the addition of 3DPD improved the specificity of 2-dimensional transvaginal ultrasound scanning (75% vs 54%), without decreasing the sensitivity. These results are not surprising and can be achieved by using a simpler technique, such as color Doppler imaging.\(^\text{21}\)

To the best of our knowledge, our study is the first that compare 3DPD with 2DPD for the prediction of ovarian cancer in complex adnexal masses. We focused in complex adnexal masses because these masses are the main source of false-positive findings for conventional 2-dimensional transvesical sonography. We agree with other authors that the use of a Doppler technique is not useful in non–complex masses or in adnexal masses with features that are highly characteristic of benign lesions (such as endometrioma or mature teratoma).\(^\text{22}\)

We have found that the diagnostic performance of 3DPD is not statistically better than that of 2DPD, which gives both techniques similar sensitivity and specificity. However, we are aware that our study has a potential weakness because the series is relatively small and its statistical power may be low.

Despite this potential bias, we could conclude that the diagnostic performance of 3DPD is not superior to 2DPD when it is applied to complex adnexal masses and when it is performed by experienced hands. For this reason and because of 3-dimensional technique in probably more expensive and more time-consuming and has a longer learning curve than 2-dimensional ultrasound scanning, we agree with Guerriero et al\(^\text{23}\) that 2DPD should be preferred to 3DPD.

References

Objective: A difference in survival rates between black and white patients with cancer of the corpus uteri is well established. This study was conducted to determine whether the overexpression of HER2/neu oncogene is associated with poor outcome in uterine serous papillary endometrial cancer, which is a highly aggressive variant of endometrial cancer, and whether a racial difference in the frequency of HER2/neu overexpression may contribute to the disparity in endometrial cancer survival.

Study design: Immunohistochemical evaluation was used to examine HER2/neu expression in paraffin blocks from 27 women with stage IA to IV uterine serous papillary endometrial cancer. Univariable analysis was performed and followed by multivariable analysis with Cox’s proportional hazard model to evaluate whether HER2/neu expression was associated with poor outcome in uterine serous papillary endometrial cancer.

Results: Black patients tended to be younger ($P = .02$) and have higher HER2/neu expression than white patients (trend $P = .02$). Seven of 10 black patients (70%) showed heavy (3+) expression, compared with 4 of 17 white patients (24%; $P = .04$). The association of heavy HER2/neu expression with race persisted after age was controlled through stratification ($P = .05$). Earlier deaths from uterine serous papillary endometrial cancer were seen among heavy HER2/neu expressers ($P = .02$), black patients ($P = .04$), and patients $\leq$65 years old ($P = .04$). However, multivariate Cox regression showed that short survival was associated significantly with heavy HER2/neu expression ($P = .02$) but not with age ($P = .07$) or race ($P = .35$), which indicates that HER2/neu expression accounted for much of the race disparity in survival in this patient population.

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* Reprint requests: Alessandro D. Santin, MD, University of Arkansas for Medical Sciences, Division of Gynecologic Oncology, 4301 W Markham St, Slot 518, Little Rock, AR 72205-7199.

E-mail: santinalessandro@uams.edu

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Cancer of the uterine corpus represents the most prevalent gynecologic tumor in women, with an estimated 40,100 cases and 6800 deaths in the United States in 2003. Two subtypes of endometrial carcinoma, namely type I and type II tumors, have been described, on the basis of both clinical and histopathologic variables. Type I endometrial cancers, which account for most of cases (ie, approximately 80%), are usually well differentiated and endometroid in histologic condition. These neoplasms are diagnosed frequently in younger women and are associated with a history of hyperestrogenism as the main risk factor and typically have a favorable prognosis with appropriate therapy. In contrast, type II endometrial cancers are poorly differentiated tumors, often with serous papillary or clear cell histologic condition. Although type II tumors account for only a minority of endometrial cancers, approximately 50% of all relapses occur in this group of patients.

In the last few years, several reports, including population-based data from the National Cancer Institute (NCI), have consistently demonstrated that, although the incidence of endometrial cancer in black women is lower than in white women, a striking racial disparity exists in endometrial cancer survival rates in the United States, with black women having up to 30% worse survival rate than white women. Although a black/white disparity in survival has been reported for other malignancies, the disparity that is described for endometrial cancer is greater than the disparity that is seen in any other human cancer. In an attempt to explain racial disparity in cancer survival rates, several correlates have been identified by the NCI black-white endometrial cancer study. At the time of diagnosis, a higher number of black patients had stage III or stage IV disease compared with white patients. In addition, black women were diagnosed with a 2- to 3-fold higher incidence of aggressive type II tumors, such as uterine serous papillary carcinoma (USPC) and clear cell tumors. However, when survival analyses were adjusted to black and white women by stage and by type II endometrial tumors, differences in survival rates still occurred. These findings are similar to the results of the NCI black-white breast cancer study, in which the 2-fold higher risk of death in black patients could not be accounted for by sociodemographic factors. More importantly, these studies suggest that it is likely that a different distribution of more aggressive biologic factors in the tumors that develop in black women may underlie the racial disparity in survival rates.

USPC represents the most aggressive histologic subtype of endometrial cancer, constituting up to 10% of all endometrial tumors. Unlike the histologically similar high-grade ovarian cancer, USPC is a chemoresistant disease from onset, with responses to combined cisplatin-based chemotherapy in the order of 20% and of short duration. USPC has a propensity for early intraabdominal and lymphatic spread, even at presentation, and is characterized by a highly aggressive biologic behavior. Recently, our group has discovered a striking overexpression of the transmembrane epidermal growth factor type II receptor HER2/neu, (score 2+ and 3+) in 80% (8/10 occurrences) of the USPCs that were tested. Because HER2/neu overexpression has been suggested previously to represent a major prognostic factor in endometrial cancer, and in breast and ovarian tumors, we examined whether HER2/neu overexpression is correlated with poor survival outcome in patients with USPC. In addition, we analyzed whether differences in HER2/neu expression may exist between black and white women harboring USPC. Our results show for the first time that HER2/neu overexpression is correlated with a poor survival outcome in patients with USPC and that a striking higher frequency of HER2/neu overexpression is seen in black patients when compared with white patients.

**Material and methods**

**Patient population**

Paraffin blocks of endometrial adenocarcinomas were retrieved for 27 women (17 white and 10 black) who underwent treatment for International Federation of Gynecology and Obstetrics stage IA to IV serous papillary endometrial adenocarcinoma at the University of Arkansas for Medical Sciences between 1997 and 2004. Study records were reviewed according to institutional review board guidelines. The patient characteristics are described in Table I. A total abdominal hysterectomy with bilateral salpingo-oophorectomy, pelvic washings, and a pelvic lymphadenectomy was performed in all
patients. No patient received chemotherapy or radiation before the operation. Among the 27 USPC cases, 22 cases were pure forms, and 5 cases were admixed with endometrioid or clear cell histologic condition (mixed USPC). Complete clinicopathologic information and survival data were abstracted from the hospital records.

**HER2/neu immunostaining of formalin-fixed tumor tissue**

Study blocks were selected after histopathologic review by a surgical pathologist who was blinded to the patients’ race, outcome, and other nonhistologic covariates. In several patients, both primary and metastatic sites were evaluated for HER2/neu expression. Briefly, immunohistochemical stains were performed on 4 μm-thick sections of formalin-fixed, paraffin-embedded tissue. After pretreatment with 10 mmol/L citrate buffer at pH 6.0 with a steamer, the sections were incubated with anti-HER2/neu monoclonal antibody (Dako Corp, Carpinteria, Calif), both at 1:2000 dilution. Antigen-bound primary antibodies were detected with standard avidin-biotin immunoperoxidase complex (Dako Corp). HER2/neu intensity of immunohistochemical staining was scored as 0 (negative = no staining is observed, or membrane staining is <10% of the tumor cells), 1+ (light staining = a faint partial membrane staining is detected in >10% of the tumor cells), 2+ (moderate staining = a weak to moderate membrane staining is observed in >10% of the tumor cells), or 3+ (heavy staining = a strong complete membrane staining is observed in >10% of the tumor cells).

**Statistics**

The trends with race and immunohistochemical staining were summarized as mean scores and assessed with the Cochran-Armitage test for trend. Immunohistochemical staining for HER2/neu expression was dichotomized as none-to-moderate (0/1+/2+) versus heavy (3+). Age was dichotomized as old if >65 years or young if ≤65 years; this dichotomization coincided with the overall study median age of 66 years. The Fisher exact test was used to assess patient characteristics for race imbalance and also to examine the race disparity in dichotomized HER2/neu expression. Cochran-Mantel-Haenszel (CMH) analysis was used to examine the race disparity in dichotomized expression; age was controlled through stratification. In CMH analysis, the common odds ratio across strata was the Mantel-Haenszel estimate. The CMH test was used to assess the statistical significance of the common odds ratio; the Breslow-Day test was used to assess the strata for evidence of odds ratio in homogeneity. Disease-related survival was defined as the time from diagnosis to death that was related to cancer progression, with right-censoring at last follow-up or at death not related to USPC. The log-rank test was used to assess nonparametrically the impact of HER2/neu, race, and age on disease-related survival. The relationship among these 3 variables was explored further through univariate and multivariate Cox regression, with particular attention paid to the effect of race on survival when in a multivariate model with HER2/neu.

**Table I  Patient characteristics**

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Black patients (n/10)</th>
<th>White patients (n/17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;65 Years old</td>
<td>2 (20%)</td>
<td>12 (71%)</td>
<td>.0183*</td>
</tr>
<tr>
<td>USPC pure-form</td>
<td>9 (90%)</td>
<td>13 (76%)</td>
<td>.6210*</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (20%)</td>
<td>2 (12%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5 (50%)</td>
<td>7 (42%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3 (30%)</td>
<td>7 (42%)</td>
<td></td>
</tr>
<tr>
<td>Mean stage score</td>
<td>2.90</td>
<td>3.12</td>
<td>.5847†</td>
</tr>
<tr>
<td>Whole-pelvis radiation</td>
<td>9 (90%)</td>
<td>14 (82%)</td>
<td>1.0000*</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>8 (80%)</td>
<td>9 (53%)</td>
<td>.2305*</td>
</tr>
</tbody>
</table>

* The Fisher exact test.  
† Cochran-Armitage trend test.

**Table II  HER2/neu relationship with race**

<table>
<thead>
<tr>
<th>HER2/neu expression</th>
<th>Black patients (n/10)</th>
<th>White patients (n/17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining intensity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (None)</td>
<td>1 (10%)</td>
<td>4 (24%)</td>
<td></td>
</tr>
<tr>
<td>1+ (Light)</td>
<td>0</td>
<td>5 (29%)</td>
<td></td>
</tr>
<tr>
<td>2+ (Moderate)</td>
<td>2 (20%)</td>
<td>4 (24%)</td>
<td></td>
</tr>
<tr>
<td>3+ (Heavy)</td>
<td>7 (70%)</td>
<td>4 (24%)</td>
<td></td>
</tr>
<tr>
<td>Mean score</td>
<td>2.50+</td>
<td>1.47+</td>
<td>.0241*</td>
</tr>
<tr>
<td>Dichotomized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-moderate</td>
<td>3 (30%)</td>
<td>13 (76%)</td>
<td></td>
</tr>
<tr>
<td>Heavy (3+)</td>
<td>7 (70%)</td>
<td>4 (24%)</td>
<td>.0402†</td>
</tr>
</tbody>
</table>

* Cochran-Armitage trend test.  
† The Fisher exact test.

**Results**

**Patient characteristics**

Twenty-seven patients satisfied study inclusion criteria; 10 women (37%) were black, and 17 women (63%) were white. The median age of the patients in the study was 66 years (interquartile range, 62-75 years). The breakdown by surgical stage was I (4 patients), II (1 patient), III (12 patients), and IV (10 patients). Twelve deaths (8 within 2 years of diagnosis) have occurred among these patients. Two patients died of causes other than USPC (ie, cardiovascular accidents), and 10 deaths were USPC.
related. Among living patients, the length of the follow-up period had a median of 33 months (interquartile range, 10-48 months). Table I shows the distribution of patient characteristics by race. Twelve of 17 white patients (71%), but only 2 of 10 black patients (20%), were at or above the study’s median age of 66 years ($P = .02$). The races did not differ appreciably in percentage of pure versus mixed USPC (90% black vs 76% white; $P = .62$) or in mean stage scores (2.90 black vs 3.12 white; trend $P = .58$). The high mean scores for stage are consistent with the discovery that 80% of black patients and 82% of white patients had advanced-stage (III/IV) disease at the time of the staging laparotomy. For this reason, most patients received adjuvant therapy in the form of whole-pelvis radiation (90% black vs 82% white; $P = 1.00$) and adjuvant chemotherapy (80% black vs 53% white; $P = .23$).

### Race association with HER2/neu expression in USPC

Moderate-to-heavy expression of HER2/neu protein was noted in 17 of 27 USPC samples (63%) that were evaluated, with 6 samples (22%) showing moderate staining (2+) and 11 samples (41%) showing heavy staining (3+) for HER2/neu. In all cases in which HER2/neu expression was evaluated in both the primary tumor and a metastatic site (ie, omentum and/or pelvic lymph nodes, 9 cases), the intensity of staining was the same when the 2 sites were compared (data not shown). Next, we compared the overexpression of HER2/neu protein between black and white patients whose condition harbored USPC; Table II shows the results. We found a statistically significant difference in staining intensity in samples from black patients that manifested itself as a >1-unit race disparity in mean intensity scores (2.50+ black vs 1.47+ white; trend $P = .02$). Indeed, we found that 90% of black women (9/10) had moderate to heavy staining for HER2/neu expression versus 48% of the white women (8/17; $P < .02$). When we dichotomized HER2/neu staining intensity as heavy (3+) versus none-to-moderate (0/1+/2+), 7 black samples (70%) showed heavy staining compared with 4 white samples (24%; $P = .04$). The prevalence by age of heavy staining was 7 of 13 in the younger (<65 years) patients (54%) compared with 4 of 14 in the older (>65 years) patients (29%; $P = .25$). CMH analysis was then used to study the race association with heavy staining; age was controlled through stratification. In the younger patients, 6 of 8 black patients (75%) versus 1 of 5 white patients (20%) had heavy HER2/neu expression; in the older patients, 1 of 2 black patients (50%) versus 3 of 12 white patients (25%) had heavy HER2/neu expression. The common odds ratio for race versus expression across age groups was 6.76, which favored heavy expression in black patients ($P = .05$).

### Survival and HER2/neu expression in USPC

Next, we evaluated the disease-related survival rate of patients with USPC in relation to HER2/neu expression. We found 9 disease-related deaths among the 11 patients with heavy expression, but only 1 disease-related death among the 16 patients with none-to-moderate expression. The Kaplan-Meier curves of Figure 1 show that heavy HER2/neu expressers have dramatically shorter survival time from diagnosis to disease-related death than do patients with none-to-moderate expression ($P = .002$). Among the latter patients, disease-related survival held steady at 91% from month 10 to year 4 but fell to 11% by the fourth year among heavy HER2/neu expressers. Figure 2 displays the corresponding Kaplan-Meier curves for black patients versus white patients. A clear disparity is shown ($P = .04$), with disease-related survival dropping by year 4 to 23% for black patients and 63% for white patients. Kaplan-Meier analysis for age (not shown) disclosed a higher disease-related mortality rate for younger patients than for patients >65 years old ($P = .04$). Four-year survival dropped to 29% for younger patients and 59% for older patients. To assess the
strength and independence of HER2/neu as a prognostic determinant to explain racial disparity in USPC outcomes, multivariate Cox regression analysis was used to study the simultaneous effect of HER2/neu expression, age, and race on survival. Table III shows multivariate Cox-regression models and univariate Cox-regression results for comparison. Under univariate analysis, heavy HER2/neu expression was significantly prognostic for short survival (hazard ratio, 12.43; \( P = .02 \)), and black and younger age were marginally prognostic for short survival (hazard ratios, 3.73 and 4.50, respectively; \( P = .06 \) for both). When these 3 factors were combined in a trivariate Cox-regression model, heavy expression retained its prognostic significance (hazard ratio, 28.00; \( P = .02 \)), although younger age and race became prognostically insignificant (Table III). In our statistical analysis we also derived bivariate Cox-regression models from the trivariate model by dropping either black or younger age. In bivariate model 1 (HER2/neu and race), heavy expression was prognostically significant for short survival (hazard ratio, 10.30; \( P = .03 \)), but black was not (hazard ratio, 2.67; \( P = .21 \)). In bivariate model 2 (HER2/neu and age), heavy expression was significantly prognostic (hazard ratio, 14.29; \( P = .02 \)), and younger age was marginally prognostic (hazard ratio, 5.50; \( P = .06 \)) for short survival. Therefore, the bivariate models are consistent with the trivariate model with respect to their results; together, these results indicate that heavy HER2/neu expression accounted for much of the shorter survival seen among black patients with USPC in this study.

**Comment**

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and maintenance of the malignant phenotype. The human HER2/neu (c-erbB2) gene product, like the epidermal growth factor receptor, is a transmembrane receptor protein that plays an important role in coordinating the complex ErbB signaling network that is responsible for regulating cell growth and differentiation. In breast, endometrial, and ovarian cancer, several studies have reported that overexpression of this gene is associated with resistance to treatment and poor survival, which suggests that tumors that overexpress HER2/neu may manifest a more aggressive biologic behavior.

In this report, we have analyzed whether overexpression of the HER2/neu oncogene is associated with poor outcome in uterine serous papillary endometrial carcinoma, a relatively rare but highly aggressive variant of endometrial cancer. In addition, we evaluated whether a racial difference in the frequency of HER2/neu overexpression may contribute to the consistently observed racial disparity in endometrial cancer survival rates between black and white women. The absence of a racial disparity in diagnosis and treatment, coupled with the worse survival of black patients (even when compared with white patients with the same initial stage of disease) indeed suggests that underlying biologic differences may contribute to the poor survival of black patients.

In our patients with USPC, all of whom were surgically staged by a gynecologic oncologist, most conditions were found to harbor advanced-stage disease. Consequently, adjuvant therapy in the form of radiation therapy and chemotherapy was administered to most black and white patients with USPC, without significant differences between the 2 groups. Our results show, for the first time, that HER2/neu overexpression is correlated with a poor survival outcome in patients with USPC, regardless of their race. Strikingly however, a significantly higher number of black women were found to harbor USPC with strong HER2/neu expression when compared with white women. In this regard, in our series, the percentage of tumors that show strong HER2/neu expression in the black population was almost 3-fold higher compared with that identified in the white population. Of interest, USPC developed in black women at a significantly younger age compared with white women.

At this time it remains poorly understood why the black population more frequently experiences USPC with the dominant molecular pathways characterized by the aberrant HER2/neu overexpression. In light of our results, however, it is very likely that the significantly higher frequency of these biologically more aggressive tumors in black women may at least partially explain the consistently demonstrated racial disparity in endometrial cancer survival rate. Consistent with this view, previous reports in patients with endometrial cancer found p53
gene overexpression to be associated with poor survival rates in both black and white patients, but with p53 overexpression found to occur more than twice as frequently in black patients.\textsuperscript{21,22} However, these studies also showed that, among cancers with p53 overexpression, survival of black patients was still worse than that of white patients, which suggests that p53 status was not the sole or most important determinant of the racial disparity in survival.\textsuperscript{21,22} Importantly, when in our study multivariate Cox regression analysis was used to evaluate the simultaneous effect of HER2/neu expression, age, and race on USPC patient survival, HER2/neu overexpression remained the only independent variable that was correlated with survival. These results strongly support the hypothesis that the higher frequency of HER2/neu overexpression found in black patients with USPC may contribute greatly to the racial disparity in survival.

In conclusion, we found a significantly higher frequency of HER2/neu overexpression in black women who were diagnosed with USPC, and we have shown that HER2/neu may represent a crucial molecular-genetic prognostic factor that contributes to the racial disparity in survival. Nevertheless, high overexpression of HER2/neu provides support for the proposal that trastuzumab (Herceptin; Genentech, Inc, San Francisco, Calif), a humanized anti-HER2/neu antibody that is showing great promise for the treatment of patients with metastatic breast cancer whose condition overexpresses HER2/neu protein may be a novel, potentially highly effective therapy against USPC.\textsuperscript{23} Consistent with this view, high sensitivity of USPC cells to natural killer cell-mediated antibody-dependent cytotoxicity triggered by anti-HER2/neu-specific antibody in vitro\textsuperscript{13} and clinical responses in vivo\textsuperscript{24} have recently been reported with the use of Herceptin in patients with USPC. The future design and implementation of clinical trials will ultimately determine the validity of this approach.

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Expression of cyclooxygenase-2 in advanced stage ovarian serous carcinoma: Correlation with tumor cell proliferation, apoptosis, angiogenesis, and survival

Rouba Ali-Fehmi, MD, a Robert T. Morris, MD, b Sudeshna Bandyopadhyay, MD, a Mingxin Che, MD, a Veronica Schimp, DO, b John M. Malone Jr, MD, b Adnan R. Munkarah, MD b, *

Departments of Pathology a and Obstetrics and Gynecology, Division of Gynecologic Oncology, b Wayne State University School of Medicine, Detroit, Mich

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KEY WORDS
Cyclo-oxygenase–2
Ovarian carcinoma
Proliferation
Angiogenesis

Objective: Cyclo-oxygenase–2 seems to be involved at various steps in the processes of tumor progression. The objective of this study was to examine the relationship between cyclo-oxygenase–2 expression and tumor proliferation, apoptosis and angiogenesis in patients with advanced stage high-grade ovarian carcinoma.

Study design: Specimens from 118 patients with high-grade and advanced stage (III, IV) serous ovarian carcinoma were evaluated by immunohistochemistry for cyclo-oxygenase–2, Ki-67, vascular endothelial growth factor, and bcl-2 expression. Tumor microvessel density was assessed with CD34 immunostaining. We investigated the relationships between cyclo-oxygenase–2 expression and clinicopathologic characteristics, tumor angiogenesis (tumor microvessel density and vascular endothelial growth factor expression), and tumor proliferation and apoptosis. The effect of cyclooxygenase-2 expression on patient survival was determined.

Results: There was a significant positive correlation between cyclo-oxygenase–2 expression in tumor cells and markers of tumor proliferation and angiogenesis. In univariate survival analysis, high cyclo-oxygenase–2 and high Ki-67 expression showed a significant impact of on patient survival (P < .001). In multivariate regression analysis, only Ki-67 expression retained its significance as an independent poor prognostic factor (death hazard ratio, 2.0; 95% CI, 1.2-3.3; P < .001).

Conclusion: Expression of cyclooxygenase-2 correlates with tumor proliferation and tumor angiogenesis but not with apoptotic markers (bcl-2 expression) in high-grade, advanced-stage serous ovarian carcinoma.

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Epidemiologic studies indicate that the use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of malignancies in the digestive tract. 1 The best known target of NSAIDs is the
cyclo-oxygenase enzyme (COX), also referred to as prostaglandin endoperoxide synthase, a key regulatory enzyme in the prostaglandin/eicosanoid synthetic pathway. Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is expressed constitutively, whereas expression of COX-2 is not detectable in most healthy tissues but can be induced in response to cell activation by proinflammatory cytokines, growth factors, and tumor promoters. Evidence from in vitro and in vivo studies suggests an important role for prostaglandins and their synthesizing enzyme COX-2 in carcinogenesis. COX-2 overexpression has been described in various malignancies including those of the colon, stomach, head and neck, endometrium, and cervix. Exposure of various cancer cell lines to prostaglandins has been shown to induce COX-2 expression and increase cellular proliferation. Transfection of benign intestinal epithelial cells with COX-2-expressing vectors results in malignant transformation. On the other hand, treatment of cancer cells with COX-2 inhibitors reduces cellular proliferation and induces apoptosis. Tumor growth and progression necessitate not only cellular proliferation, but also the creation of new vasculature to supply cells with nutrients and oxygen. In vitro models for angiogenesis have shown that COX-2 expression plays a role in new vessel formation and that COX-2 inhibitors can reverse this effect. There are emerging laboratory data that COX-2 expression may play a role in tumor angiogenesis.

The aim of this study was to investigate the relationship between COX-2 expression and molecular markers of proliferation, angiogenesis, and apoptosis in advanced stage, high-grade serous ovarian carcinomas. Markers selected for study were (1) Ki-67 expression to determine the tumor proliferative activity, (2) vascular endothelial growth factor (VEGF) expression and CD34 staining to evaluate tumor angiogenic activity and microvessel count, and (3) bcl-2 expression to study the level of apoptotic activity.

### Material and methods

Patients who were diagnosed with epithelial ovarian cancer between 1993 and 1999 were identified according to the database files of the Division of Gynecologic Oncology and the Department of Pathology at Wayne State University. Patients with advanced-stage high-grade serous carcinoma who underwent primary surgery without previous chemotherapy were included in this study. From a retrospective review of medical records, the patient’s demographic and surgical data were collected. Survival data were retrieved with the SEER database and the institution computerized clinical information system. Surgical staging was determined with the criteria that are recommended by the International Federation of Gynecology and Obstetrics (FIGO). Histologic type and grade were determined by 2 of the authors (R.A-F., M.C.), who used the World Health Organization criteria. After each case had been evaluated, 2 to 3 representative paraffin blocks were selected for study.

Immunohistochemical staining with antibodies to COX-2, CD34, bcl-2, and Ki-67 was performed; as our study was progressing, we introduced the use of micro tissue array for immunohistochemical and used it for VEGF with the same ovarian cancer tissue blocks that had been selected previously for the other stains. Sections were deparaffinized and subjected to immunohistochemical staining, with standard streptavidin-biotin-peroxidase techniques, with diaminobenzidine as the chromogen. In brief, 4- to 5-μm thick sections were antigen retrieved by steam treatment in a citrate buffer, quenched for 10 minutes with 3% hydrogen peroxide, preincubated with blocking serum at 1:20 in 2% bovine serum albumin/phosphate buffered saline solution (PBS) for 15 minutes at room temperature. After incubation with the primary antibodies, slides were rinsed with PBS, and the secondary antibody was applied at 1:500 in PBS for 30 minutes at room temperature. After rinses with PBS for 30 seconds, slides were incubated with streptavidin/peroxidase at 1:500 in PBS for 30 minutes at room temperature, then rinsed with PBS and incubated for 15 minutes in 0.06% diaminobenzidine and counter-stained with Harris modified hematoxylin (Fisher Healthcare, Hanover Park, Ill). The following antibodies were used for immunohistochemical staining: Bcl-2 (clone:124, Dako Corp, Carpinteria, Calif), COX-2 (clone: H62, 1:100 dilution, incubation overnight; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), CD34 (clone: QBEnd/10, 1:20 dilution, incubation for 45 minutes; BioGenex Laboratories, San Ramon, Calif), Ki-67 (clone: MM1, 1:100 dilution, incubation for 2 hours; Vector Laboratories, Burlingame, Calif), VEGF (polyclonal, 1:100 dilution, incubation for 2 hours; Santa Cruz Biotechnology, Inc).

### Table I Patients’ characteristics and COX-2 expression

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>N</th>
<th>COX-2 expression (n)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 Y</td>
<td>47</td>
<td>Low: 14 (29.8%) High: 33 (70.2%)</td>
<td>.84</td>
</tr>
<tr>
<td>≥60 Y</td>
<td>71</td>
<td>Low: 23 (32.4%) High: 48 (67.6%)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>89</td>
<td>Low: 28 (31.5%) High: 61 (68.5%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>29</td>
<td>Low: 9 (31%) High: 21 (69%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>97</td>
<td>Low: 32 (33%) High: 65 (67%)</td>
<td>.60</td>
</tr>
<tr>
<td>IV</td>
<td>21</td>
<td>Low: 5 (23.8%) High: 16 (76.2%)</td>
<td></td>
</tr>
</tbody>
</table>
Two pathologists (R.A-F., M.C.) individually evaluated the slides blindly under a transmission light microscope. The concordance rate was 95% between the 2 pathologists. In case of disagreement, the slides were reviewed simultaneously by the 2 pathologists seated at a multiheaded microscope with a resolution of the difference in opinion.

For COX-2 and VEGF assessment, the staining intensity and the percentage of tumor cells that were stained were analyzed. Staining intensity was scored as 0 (negative), 1+ (weak), 2+ (medium), and 3+ (strong). A combined score that was based on the staining intensity and the percentage of cells stained was used to assign a final score. Low expression was defined as intensity 0, 1, 2, or 3 and <10% cells staining or intensity 0, 1 and <50% cells staining; high expression was defined as intensity 2, 3, and >10% of cells staining or intensity 1, 2, 3, and >50% of cells staining.

Tumor vascularity was also assessed with CD34 immunostaining. Any single endothelial cell or cluster of endothelial cells was counted as a single microvessel. The 3 most hypervascular areas were selected under low

Figure 1  A, Immunohistochemical staining for COX-2 in a representative section of high-grade ovarian serous carcinoma is shown. High COX-2 expression was mainly in the tumor areas. No significant stain is seen in the stroma (X 200). B, Immunohistochemical staining for CD34 in a section of high-grade ovarian serous carcinoma, large numbers of discrete blood vessels were stained for CD34 (X200). C, Immunohistochemistry staining for Ki-67 showing strong positivity in high-grade ovarian serous carcinoma (×100). D, Immunohistochemistry staining for VEGF showing strong positivity in high-grade ovarian serous carcinoma (×200).
magnification for microvessel density (MVD) assessment. The mean value for the 3 fields was recorded as the MVD for each tumor and expressed as the number of vessels per high-power field (×200). For statistical analysis, the cases were split into 2 groups based on the median of the vessel counts (MVD 60) in the study population.

To determine Ki-67 expression, nuclei from at least 1000 tumor cells were counted from the tumor fields, and the labeling index was calculated as the percentage of labeled nuclei of the total number of tumor cells that were counted. Tumors with a Ki-67 labeling index above the median value were considered as the high proliferative index group, and those indices that were equal to or less than the median value were considered to be the low proliferative index group for statistical evaluation.

The expression of bcl-2 was assessed on the basis of the presence of cytoplasmic staining; the scoring was assigned on the basis of the percentage of positive tumor cells, with a zero score assigned for tumors with no cytoplasmic staining in any cells, a score of 1 with <5% of cells staining positive, a score of 2 with 6% to 30%, and a score of 3 with >30% of cells staining positive. For statistical analysis, tumors with a score of 0 or 1 were considered to be bcl-2 negative, and tumors with a score 2 or 3 were considered to be bcl-2 positive.

Statistics

Statistical analyses were performed with the SPSS for Windows software (version 10.07; SPSS Inc, Chicago, Ill). The correlation between COX-2 expression and the other prognostic variables was assessed with the Fisher’s exact test. The Spearman’s test was performed to evaluate the relationship between COX-2 expression versus Ki-67, VEGF, and MVD. Survival times were estimated in days from the date of diagnosis to the date of death or last follow-up. Survival analysis was computed with the Kaplan-Meier method and comparisons by the various prognostic variables were made with the log-rank test. Nine variables were analyzed including age, race, stage, MVD, and expression of COX-2, Ki-67, VEGF and bcl-2. Statistical significance was defined as a probability value <.05.

Results

One hundred eighteen patients met our study inclusion criteria, and survival data were available on 117 patients. The mean age of the patients was 62 years (range, 38-89 years). The mean follow-up time was 606 days (range, 9-2141 days). The patients’ characteristics are detailed in Table I.

COX-2 expression was low in 31% (37/118 patients) and high in 68% (81/118 patients). No significant COX-2 staining was observed in the stromal compartment (Figure 1). There was no significant correlation between COX-2 expression and patient age, race, or FIGO stage (Table I).

Similarly, there was no association between COX-2 and bcl-2 expression in tumor cells. On the other hand, a significant association was noted between COX-2 expression in tumor cells and (1) VEGF expression, (2) Ki-67 expression, (3) and tumor MVD ($r = .48; P < .001$; $r = .25; P < .006$; $r = .54; P < .001$). This link was confirmed with a Fisher’s exact test, in which tumors with high COX-2 expression had a significantly higher expression of VEGF, Ki-67, and a higher MVD ($P < .001$, <.001, and <.014 respectively; Table II). In addition, a higher VEGF expression by tumor cells was strongly associated with higher tumor MVD ($r = .61; P < .001$).

In univariate survival analysis, high COX-2 and high Ki-67 expression showed a significant impact of on
patient survival. Mean survival in patients with tumors that exhibited low COX-2 expression was 890 days, compared with 660 days in tumors with high expression ($P < .005$; Figure 2). Mean survival in patients with tumors that exhibited low Ki-67 expression was 1168 days, compared with 749 days in those tumors with high expression ($P < .001$; Figure 3). A multivariate regression analysis was conducted to determine the impact of the various prognostic variables that were evaluated on survival. Only Ki-67 expression retained its significance as an independent poor prognostic factor (death hazard ratio, 2.0; 95% CI, 1.2-3.3; $P < .001$).

**Comment**

The differential expression of various molecular parameters in solid tumors has been reported to have a significant impact on the prognosis of patients. It has been suggested that the influence of such expression on survival is related to the biologic role of the molecular marker that is studied and its influence on tumor growth and progression. The COX-2 enzyme seems to be involved in the process of carcinogenesis through a number of mechanisms, which include increased proliferation, reduced apoptosis, and stimulation of angiogenesis and metastases. Ki-67, a proliferation-related nuclear protein, has its gene located on chromosome 10. Increased Ki-67 expression has been noted in the last period of the S-phase and to a higher level in G1-G2 and in mitosis; it is not detectable in G0. As such, Ki-67 has a low level of expression in resting cells and high level in proliferating cells and has been used as a measure of tumor proliferative activity. In a study of serous ovarian tumors, Garzetti et al$^{10}$ found that Ki-67 expression was significantly higher in adenocarcinomas, compared with adenomas and borderline tumors. In addition, the proliferative index, which was reflected by the percentage of positively staining cells, correlated positively with FIGO surgical stage and had a significant impact on disease-free survival.

In view of the COX-2 effect on cellular proliferation, investigators have looked at the potential association between COX-2 and Ki-67 expression in various tumors. Studies of breast, renal cell, pancreas, pituitary, and esophageal carcinomas have reported a significant positive association between COX-2 expression and tumor proliferative index.$^{11-15}$ In contrast to this, Shono et al$^{16}$ found no association between COX-2 and Ki-67 expression in gliomas. Similarly, a study of sporadic colorectal carcinoma reported no correlation between COX-2 and Ki-67 expression and survival.$^{17}$

In the current study, a strong positive correlation between Ki-67 and COX-2 expression was found. In addition, each of the 2 markers was a strong predictor of survival on univariate survival analysis; however, only Ki-67 expression retained its significance as an independent prognostic factor, with the use of multivariate regression analysis.

Several reports have demonstrated a significant correlation between COX-2 expression and tumor vascularity. In a report that included a cohort of patients with colorectal carcinoma, Masunaga et al$^{3}$ reported a significant association between COX-2 expression and tumor MVD, as determined by CD34 immunostaining. Similar observations have been made in other malignancies including gastric adenocarcinoma$^{4}$ and squamous carcinoma of the head and neck.$^{5}$ On the other hand, in a study of 57 epithelial ovarian neoplasms, Matsumoto et al$^{18}$ did not find any significant correlation between COX-2 expression and tumor MVD. Recently, we have reported that high COX-2 expression correlated significantly with high microvessel density in patients with advanced stage, high-grade ovarian carcinoma.$^{19}$

Angiogenesis is a complex process that is regulated by a number of cellular pathways. VEGF, a heparin-binding glycoprotein, is a critical regulator of the angiogenesis process during embryogenesis. It also seems to play an important role in cancer neoangiogenesis. In fact, VEGF is expressed constitutively in a large number of malignancies that includes ovarian carcinomas.$^{20}$ In head and neck squamous cell carcinoma, Gallo et al$^{21}$ demonstrated a close correlation between COX-2 expression, VEGF expression, and tumor angiogenesis. In addition, COX-2 overexpression and higher tumor vascularization predicted a shorter survival in these patients. The same strong correlation between COX-2 and VEGF expression has been found in pancreatic, colon, breast, and endometrial carcinoma.$^{22-25}$ In the study by Matsumoto et al,$^{18}$ a significant association was noted between VEGF and COX-2 expression in tumor cells. The data in the present study demonstrate a strong correlation between COX-2 and VEGF expression, which is a finding that is in line with data published for other tumors. High expression of both markers was associated with higher tumor MVD.
This is also in keeping with recent preclinical data from our laboratory in which epithelial ovarian cancer cells exhibited a dose-dependent increase in COX-2 and VEGF expression when treated with increasing doses of prostaglandin E2. There was a strong positive correlation in the messenger RNA levels of both proteins in response to such treatment (data in press).

Experimental data have shown that the anti-apoptotic activity of COX-2 might be mediated through a bcl-2 pathway. Treatment of epithelial ovarian cancer cells with prostaglandin E2 resulted in an increase in COX-2 and VEGF expression when treated with increasing doses of prostaglandin E2. There was a strong positive correlation between COX-2 and bcl-2 expression has been reported in gastric tumors and squamous cell carcinoma of the esophagus. In contrast, other studies on human gliomas and colon carcinomas have shown no correlation between COX-2 expression and bcl-2 expression. In our study, no statistically significant correlation was detected between COX-2 expression and bcl-2 immunostaining.

The current study has limitations that include the retrospective design, which is prone to selection bias, and the fact that the use of immunohistochemistry to evaluate protein levels does not always reflect the structure or functionality of the protein. Nonetheless, the strength of our study lies in the fact that it includes a large cohort of patients with a uniform tumor type, histologic grade, and stage. In addition, our knowledge, this is the first study that demonstrates a strong association between COX-2 expression and neoangiogenesis in epithelial ovarian cancer with the use of both MVD and VEGF expression. The study also analyzes the interaction between COX-2 and tumor cell proliferation, which is a factor that has been shown to impact patient prognosis.

In conclusion, COX-2 expression correlates significantly with tumor cell proliferation and angiogenesis and is associated with poor patient survival in high-grade advanced-stage serous ovarian carcinoma. These findings must be confirmed in prospective studies. In addition to their prognostic significance, a better understanding of the biologic mechanism of these molecular changes may help to identify new targets for ovarian cancer therapy.

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References


Catastrophizing labor pain compromises later maternity adjustments

Sari Goldstein Ferber, PhD,* Michal Granot, DSc, Etan Z. Zimmer, MD

Faculty of Health and Welfare Studies, University of Haifa, Haifa, Israel; Department of Obstetrics and Gynecology, Rambam Medical Center and Technion-Israel Institute of Technology, Faculty of Medicine, Haifa, Israel

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Objective: The purpose of this study was to evaluate the impact of labor pain intensity and labor pain catastrophizing on maternity blues and postpartum social functioning.

Study design: Pain intensity and pain catastrophizing were assessed in 89 women in active labor before the administration of analgesia. Both these measures were assessed again retrospectively 2 days after delivery in 82 women who had a spontaneous vaginal delivery. Women also filled out the Edinburgh Postnatal Depression Scale. Six weeks later women completed the social functioning domain of the short form SF36 health survey.

Results: Pain catastrophizing during labor significantly predicted both maternity blues \( (P = .001) \) and postpartum social functioning \( (P = .001) \) when being controlled for maternal age and education, parity, type of analgesia, and labor pain intensity. Low level of education and younger age also contributed to the prediction of maternity blues and social functioning.

Conclusion: Labor pain catastrophizing rather than labor pain intensity predicts postpartum maternal adjustments.

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Childbirth is a significant event in maternal life. It includes the physiologic components of pregnancy, labor, and delivery and the emotional and social components that are attributed to the changes in the family structure and the maternal role. Mothers are concerned about self-esteem, body image, and sexuality; they overcome various levels of distress and endure a decline in social activity.

Maternity blues, the depressed mood in mothers during the first days after the delivery, have been acknowledged as a cross-cultural phenomenon. The possible involvement of changes in cortisol, gonadal steroids, thyroid hormones, prolactin, and melatonin has been suggested.\(^1,2\) Some studies claim that the phenomenon is short-lived and declines within a few days.\(^3\) Other investigators have shown that the maternity blues phenomenon is associated with a higher incidence of postpartum depression.\(^4-8\) However, maternity blues is a distinguished phenomenon with high prevalence close to the delivery experience that is compared with postpartum depression, which occurs later in the postpartum period, even after a few months, with lower prevalence and more augmented symptoms of psychopathologic condition and child neglect.\(^3,5,6\) In large samples, the prevalence of postnatal depression in
Israel was 9.9% to 22.3% on the second day after the delivery and 5.2% to 12.4% at 6 to 12 weeks after the delivery. In the United States, 27% of the women experienced maternity blues sometime during the postpartum year. It seems therefore that the early detection of women who are at greater risk of experiencing postpartum blues may be of value to enable early intervention.

Pain is a complex phenomenon that includes physiologic, cognitive, cultural, social, and emotional aspects. Pain catastrophizing is defined as an exaggerated negative orientation to painful stimuli. Thus, pain catastrophizing is an important cognitive and emotional factor in pain experience. Pain catastrophizing includes 3 components: (1) rumination (processes of focusing on the pain), (2) magnification (tendency to exaggerate the negative consequences of the pain), and (3) helplessness (the extent of helplessness experienced during the pain).

Because labor involves acute pain and mood changes, the extent of pain catastrophizing may play a role in critical domains of maternity: the maternity blues and the ability to resume full social functioning and adjust to new duties. Social ability has been reported repeatedly as negatively correlated with postpartum depression and is compromised in the immediate postpartum period. Therefore, the aim of the present study was to determine whether catastrophizing of labor pain is associated with the level of maternity blues and social functioning in the short-term postpartum period.

### Material and methods

#### Study population

The study was conducted at a public university hospital without private patients where medical care is provided by residents who are supervised by senior physicians. It was a controlled environment in which women have no choices other than analgesia/anesthesia. There were no activities that were related to support in labor, except for a midwife general practice. The study was approved by the institutional review board, in accordance with the Helsinki Declaration. Eighty-two women at 37 to 42 weeks of gestation who were in active labor were assigned randomly to this study. All the women who agreed to participate provided written informed consent. The refusal rate was 12%, most of whom refused on the basis of general resistance to research and collection of private details. Of 89 women who agreed to participate and consent, 4 women who had an emergency cesarean delivery and 3 women who had instrumental delivery were excluded from the study. The statistical analysis was performed for all participants who had spontaneous vaginal delivery. There was no induction of labor augmentation. The demographic and obstetric data of the study population are presented in Table I.

#### Study design

Women were first assessed during the active phase of labor. During an interval between contractions they were asked to report the level of perceived pain with the Visual Analog Scale (VAS1). The Pain Catastrophizing Scale (PCS1) was also filled out by the investigator, with the use of the women’s answers. The questionnaires were completed before the women received analgesic treatment. The type of analgesia that was used during labor was recorded from the medical files after the completion of labor. Two days after delivery, women were asked to assess retrospectively, with the use of a VAS measure, the pain intensity of the entire labor process (VAS2). They were also requested to complete the PCS regarding the labor experience again (PCS2), this time by themselves, and to fill out the Edinburgh Postnatal Depression Scale (EPDS). At 6 weeks after delivery, they were visited at home, where they completed the social functioning domain in the short form of the SF36 survey.

#### Material

**PCS**

This questionnaire includes 13 items that assess the 3 components of pain catastrophizing: rumination (4 items), magnification (3 items), and helplessness (6.3 items). Each domain was calculated separately, and the total score of the PCS was computed as a summation of the 3 domains, in accordance with Sullivan et al in their initial study of the PCS validation. The PCS has been studied extensively since 1995 in healthy and sick populations, which included an association with situations of experimental pain. In line with the above, the scores of >24 in the PCS scale are considered as reflecting a tendency of catastrophizing. The Hebrew validation of this scale in a sample of 80 healthy subjects revealed that the scale is in high association with experimental pain; the reliability scores for the 3 factors of the scale were as follows: rumination ($\alpha = .926$), helplessness ($\alpha = .921$), and magnification ($\alpha = .654$).
EPDS
This questionnaire aims to detect mothers’ suffering from postnatal depression. It consists of 10 items that are not categorized into factors. The items describe symptoms of depression from low to high severity. There are 4 possible responses to each of the 10 items. A score of ≥14 points indicates depressed mood with a risk for severe long-term depressive symptoms.

VAS
This 10-cm scale is used for the assessment of pain intensity. It has 2 anchors: Zero represents “no pain at all,” and 10 represents “the worst imaginable pain.”

Social functioning
This measure is 1 domain of the SF36 survey, which is a measure of general mental and physical health status that provides scores on 8 areas of functioning and well being and 2 broad areas of subjective well being, namely mental and physical health. The SF36 survey is acceptable to patients, is internally consistent, and is a valid measure of the health status in a wide range of patients. The domain of social functioning was chosen as the preferred measure for this study because social adjustment is one of the main difficulties of the women after delivery and is considerably impaired in postpartum depression states. The use of 1 domain of the SF36 survey separately is valid because this survey has no total score, only domain scores.

Statistical analysis
Data were analyzed with SPSS software (SPSS, Chicago, Ill). Pearson correlation coefficients were used to test the association between PCS1, PCS2, VAS1, and VAS2. Hierarchic regression models were conducted to reveal the contribution of the PCS to predict maternity blues and social functioning.

Results
The mean maternal age was 29.56 ± 4.97 years (range, 21-42 years), and mean number of previous labors was 1.42 ± 1.42 (range, 0-6). The mean birth weight of the newborn infants was 3291.2 ± 504.9 g (range, 1800-4500 g). The infants were born at 39.43 ± 1.69 weeks of gestation (range, 38-42 weeks of gestation), on average. Table I shows more demographic characteristics of the entire sample.

Pain and catastrophizing scores
Forty-seven mothers (57.32%) received epidural analgesia during labor, and 35 mothers (42.68%) received pethidine or entonox. There was a positive correlation between pain perception during labor (VAS1) and labor pain intensity recalled 2 days after labor (VAS2; r = 0.262; P < .01). VAS1 was higher than VAS2: 7.1 ± 2.6 vs 6.2 ± 2 (P = .014). There was also a positive correlation between PCS1 and PCS2 (r = 0.829; P < .0001). PCS1 was significantly higher than PCS2 (25.5 ± 11.9 vs 20.9 ± 12.7; P < .0001). VAS1 was correlated positively with PCS1 (r = 0.299; P < .0034) and with PCS2 (r = 0.353; P < .0006). VAS2 also was correlated positively with PCS1 (r = 0.373; P < .0003) and with PCS2 (r = 0.356; P < .0006). There was also a positive correlation between PCS1 and PCS2 (r = 0.829; P < .0001). In this sample, the score of 9 in the PCS is a significant predictor of severe maternity blues on postpartum day 2 (β = .410; t = 4.016; P < .0001).

Prediction of maternity blues and postpartum psychosocial adjustment
The level of EPDS 2 days after labor was 19.29 ± 6.06 (range, 9-32), and the level of social functioning at 6 weeks was 73.78 ± 24.50 (range, 0-100). Thirty-five women experienced maternity blues on postpartum day 2 (EPDS, ≥14), and 32 women revealed lower social adjustment at 6 weeks compared with the mean value of the measure. Thirty-one women scored above the cut-off point of catastrophizing in the PCS1 or PCS2, which would be predictive of maternity blues. Twenty-five of the women experienced maternity blues (EPDS, ≥14) on the postpartum day 2, and 10 of the women still showed signs of depression at 6 weeks after delivery. Six mothers did not have any signs of maternity blues or social decreased functioning. Table II shows the correlations between the

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* P < .001.
† P < .01.
‡ P < .05.
variables that predict maternity blues and social functioning. Maternity blues on the postpartum day 2 and social functioning at 6 weeks after delivery were predicted by PCS1 after the results were controlled for maternal age and education, parity (primiparae/multiparae), type of analgesia, and mean VAS that was calculated as an average of VAS1 and VAS2 (Table III).

Further analysis regarding the contribution of the 3 components of PCS1 after the results were controlled for the same variables revealed that rumination ($\beta = .339$; significance of the model $R$ square, 0.227; $F(1,75)$, 3.664; $P = .003$), helplessness ($\beta = .344$; significance of the model $R$ square, 0.214; $F(1,75)$, 3.399; $P = .005$), and magnification ($\beta = .246$; significance of the model $R$ square, 0.190; $F(1,75)$, 2.940; $P = .012$) were significant predictors of maternity blues, whereas only helplessness ($\beta = -.30$; significance of the model $R$ square, 0.310; $F(1,75)$, 5.604; $P < .001$)) was significant in the prediction of social functioning at 6 weeks.

### Comment

This study demonstrates that higher levels of labor pain catastrophizing are associated with a decrease in postpartum maternal adjustment. The PCS that was measured during active labor was found to be a significant predictor for maternity blues and social functioning at 6 weeks. The correlation between the VAS scores and PCS levels is in agreement with previous studies that evaluated these relationships in various healthy, sick, and experimental pain conditions. The finding that VAS scores did not predict later maternal adjustment points to the possibility that it is not the pain intensity alone, but rather the emotional and cognitive factors, attributed to the pain experience are relevant to the future emotional adjustment of the women after childbirth. The results also show that younger and less educated women are at higher risk. Taken together, young age, relatively low level of education, and the tendency to catastrophize pain may be useful in screening women who may need help in adapting to changes after delivery.

The early detection of maternal emotional adjustment is of importance because it may have a negative impact on mother-infant attachment and reduce optimal emotional development of the newborn infant. Earlier studies suggested that the mode and process of labor may be a risk factor for the development of postpartum depression. More recent studies did not find such a correlation. In the present study, we also found that, in women who undergo vaginal delivery, the level of intensity of pain did not predict maternal blues and later social adjustment. However, pain catastrophizing was a significant predictor of both maternal blues and social functioning.

An analysis of the different components of the PCS revealed that rumination and helplessness were the best predictors of maternity blues. Wisner et al studied women with postpartum depression and reported that helplessness and obsessive thoughts (which are similar to the rumination domain) characterized these mothers. Similarly, the role of reduced effectiveness of coping resources and symptoms of obsessive thoughts and obsessive-compulsive disorder were found to be associated with the clinical nature of postpartum affective disorders. It seems therefore that pain catastrophizing contributes to the occurrence of maternity blues in a similar way that postpartum depression does, by blocking spontaneous mood and adjustment processes. It is suggested that the meaning of the catastrophizing that is attributed to the pain may be comprised of cognitive components, which in turn inhibit spontaneous mechanisms of emotional and mood adjustment.

Social support and good social relations are the best predictors of well-being in the early postpartum period and are usually lower in cases of postpartum depression. Pain catastrophizing mothers were found in this study to have diminished social ability and activity; in other words, this catastrophizing tendency in coping with a painful event was shown to predict low resourcefulness in the social perspective after delivery. According to Sullivan et al, pain catastrophizers tend to impose demands on their social partners and use the pain as an

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*R-square total, $0.251$; $F(1,75)$, 4.195 ($P = .001$); *R-square total, 0.289; $F(1,75)$, 5.083 ($P = .001$).

* $P < .05$.
† $P < .01$.
‡ $P < .001$.
attention seeker, yet they obtain only distant responses from their partners, which leaves them without real support and with greater pain experience. This could be a more generalized social behavior beyond pain experience; therefore, it is possible that mothers who catastrophize the labor pain are not effective in getting support and resuming social relations after delivery. Thus, PCS is suggested to be a reliable predictor of social functioning, which is one of the most important facets in the prevention of postpartum depression.

Little is known about the stability of pain catastrophizing over time in regard to specific noxious events. This study is the first to address this issue prospectively in the context of labor and delivery and its impact on motherhood. We found a high correlation between PCS1 and PCS2, but only PCS1 was a significant predictor of postpartum adjustment, which may suggest that, although pain catastrophizing is a personal tendency, the timing of the measurement is important, and assessment during the actual painful event is the best predictor of long-term mood and social adjustment after delivery.

Part of the women received preparation for delivery, but this variable did not turn out to significantly predict or to be in association at all with maternity blues or later social adjustment; thus, it was not included in our regression analyses. Further study should address the issues of specific personal expectation for analgesia versus nonmedicated delivery and the association between the role of the midwife and pain perception. Also, more research must be done to enable generalization of the results and conclusions. Nevertheless, the incidence of maternity blues in the group of women who were studied (30%) is akin to the incidence in the United States and is higher than the published incidence in Israel, which is a result that emphasizes the importance of the PCS tool. Although PCS1 was filled in with the help of a research assistant and PCS2 was filled in by the mothers without such help, the high significant correlation between PCS1 and PCS2 shows that the PCS is reliable beyond these 2 ways of administration that are used to adjust to maternity circumstances and inconvenience (such as contractions) and that the personal judgment of the mothers is kept and revealed with or without help in writing. Thus, both ways of filling in the PCS are appropriate for 2 reasons. First, support from the research assistants potentially could be seen by the mothers as a therapeutic call for reflection on the pain during the completion of the PCS1. Second, the independent filling in of the PCS2 could be experienced by the mothers as reflecting respect and as a symbol of returning to regular functioning. Rigid methods that could try to match the ways of filling in exactly might have resulted in negative attitudes of the mothers.

In summary, because the subjective perception of delivery pain had not been assessed from the angle of catastrophizing, the current study used the PCS with pregnant and laboring women for the first time to reveal that the PCS use involves a safe, appropriate, valid, and reliable administration of this important measure. Most of the current research on labor pain focuses on the different methods of reducing pain intensity. Our study suggests that, in addition to pain reduction, the aspect of pain catastrophizing should also be addressed before analgesia is given. The process of filling in the PCS is short and may have a therapeutic effect of helping the mother to reflect on her pain, thus decreasing her suffering. The score of \( \geq 24 \) on the PCS should alert care providers. It is possible that early assessment with the PCS, which is a simple tool, may be of clinical value for the identification of women with the potential risk for later impaired adjustment.

References


D. Yvette LaCoursiere, MD, MPH,a,* Lois Bloebaum, BSN,b Jeffrey D. Duncan, MS,b Michael W. Varner, MDc

Department of Obstetrics and Gynecology, University of Utah Health Sciences Center,a Utah Department of Health,b Division of Maternal Fetal Medicine,c University of Utah Health Sciences Center, Salt Lake City, Utah

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KEY WORDS
Maternal obesity
Trends
Cesarean section

Objective: This study aims to identify recent population-based trends in maternal overweight and obesity and adverse outcomes.


Results: Prepregnancy overweight and obesity increased from 25.1% in 1991 to 35.2% in 2001, a 40.2% increase (prevalence ratio [PR] 1.40 [1.37-1.43]), whereas maternal obesity at delivery rose 36.2% from 28.7% to 39.1% (PR 1.36 [1.33-1.39]). The attributable fraction of cesarean delivery in overweight and obese women was 0.388 (0.369-0.407). Statewide, among all women having a cesarean delivery in 2001, 1 in 7 is attributable to overweight and obesity.

Conclusion: This is the first state-wide analysis of maternal obesity trends demonstrating a significant increase in maternal overweight and obesity. Overweight and obese women are at increased risk of cesarean delivery, preeclampsia, eclampsia, dystocia, and macrosomia, risks that increase as the body mass index rises.

More than 127 million American adults are overweight (body mass index [BMI] >25), 60 million are obese (BMI >30), and 9 million are severely obese (BMI >40).1 In 1999 through 2000, 62% of women were overweight, 34% were obese, and 6% were severely obese, a significant increase when compared with the National Health and Nutrition Examination Survey (NHANES) data from 1988 to 1994.2,3 In Utah, 40% of women of all ages are overweight or obese.4 Despite the increasing prevalence of overweight and obesity in women in the United States,2,5,6 there has been less attention paid to the trends among pregnant women. National studies that identify trends in BMIs, including the NHANES and the Behavioral Risk Factor Surveillance System (BRFSS) systematically exclude pregnant women from their analyses.2,5

The 2 existing analyses of temporal trends in maternal obesity among American women report an increase in maternal obesity as defined as weight greater than 200 lb over the past 2 decades. These studies are limited to institutionally based samples with high proportions of African American women.7,8 Research suggests that
African American women have higher BMIs, and thus these data may overestimate the prevalence of maternal obesity in other populations.\textsuperscript{2,7}

There are no temporally based analyses that use maternal BMI to define maternal obesity. An analysis that uses BMI greater than 25 in lieu of absolute weight greater than 200 lb will include women previously excluded from trend studies. These women previously excluded may in fact have increased risks.

Studies have shown that obese pregnant women are at increased risk for adverse pregnancy outcomes including diabetes, preeclampsia, preterm labor, induction, cesarean delivery, anesthetic complications, postpartum hemorrhage, maternal infection, macrosomia, fetal anomalies, intrauterine fetal demise, and early neonatal death.\textsuperscript{7–17} As obesity prevalence increases, the absolute number of patients affected by weight-related adverse pregnancy outcomes will also increase. The objectives of this study are to identify recent population-based trends in maternal overweight and obesity in Utah, both preconceptionally and at delivery, to describe the correlates associated with maternal overweight and obesity, and to estimate the impact on overall disease burden.

\section*{Material and methods}

\subsection*{Data source}

Institutional Review Board approval was obtained. Birth certificate information was collected and compiled into a computerized database by the Utah Department of Health (UDOH), Office of Vital Statistics. This solitary database contains demographic, antepartum, delivery, and postpartum information. All live singleton births between January 1991 and December 2001 were included in the primary analysis. The risk factor and outcome data were completed by the care provider and abstracted by a birth certificate specialist. The computerized birth certificate data undergo routine quality assessment by the UDOH. Each hospital contributing to the database is reviewed at least annually. A random sample of patients is selected. The medical record is compared with the information submitted on the birth record. UDOH data have not been published. Published data have revealed moderate-to-good concordance between the medical record and birth certificates on the following variables: race ($\kappa = 0.868$), nulliparity ($\kappa = 0.969$), gestational diabetes ($\kappa = 0.545$), pregnancy-induced hypertension ($\kappa = 0.404$), gestational age ($\kappa = 0.726$), birth weight ($\kappa = 0.976$), and delivery type ($\kappa = 0.963$).\textsuperscript{18} Events with low prevalence were less likely to yield reliable results. Secondary to this finding, we have attempted to limit our analyses to include variables that have shown moderate-to-good correlation in the literature.

\subsection*{BMI and overweight obesity}

We determined the annual prevalence of maternal overweight and obesity using BMI (kg/m$^2$). BMI was calculated by using height and weight data that were collected during postpartum hospitalization. Prepregnancy BMI was calculated by using self-reported height and prepregnancy weight, whereas delivery BMI was calculated by adding the self-reported prepregnancy weight to the pregnancy weight gain.

With the use of prepregnancy and delivery BMI, each woman was stratified into a BMI category according to the International Obesity Task Force classification: underweight less than 19, normal weight 19 to 24.9, overweight 25 to 29.9, class I obesity 30 to 34.9, class II obesity 35 to 39.9, and class III obesity greater than 40.\textsuperscript{19} For analyses of prepregnancy BMI, we defined BMI 19 to 24.9 as normal when compared with those who have a BMI of greater than 25. However, the reference “normal” range for analyses of BMI at delivery was 19 to 29.9 (normal and overweight). This acknowledges that a normal weight woman who gains up to the recommended (Institute of Medicine) amount during pregnancy will have a BMI of up to 29.9.\textsuperscript{20}

\subsection*{Data analysis and statistical methods}

Data analysis was performed with SPSS 12.0 (SPSS, Inc, Chicago, Ill). Extreme values were evaluated and erroneous weight and height data were excluded listwise. Trends in the prepregnancy and delivery BMI distributions were examined with the use of a percentile comparison plot. The slope of the line representing the aggregate overweight and/or obese was fit using the method of least squares. The trend analysis by year was performed with the $\chi^2$ test for trend. The $\chi^2$ analysis was used to compare the proportion of overweight and obese women in the first and last year of the study. An a priori list of independent and dependent variables were selected.

For analyses of outcomes, only the first pregnancy for each woman delivering in the interval was included in the analyses to avoid the effect of repeat measures. For multiple logistic regression models, only data after 1997 were included, because UDOH began discriminating preexisting from gestational diabetes only after this date. Multiple logistic regression was used for analysis of BMI and outcomes. Ordinal scaled data were coded by using dummy variables. The variables were entered in block. Maternal age, parity, gestational age, and weight gain were included as continuous variables. Definitions of the variables include the following: parity was the number of prior deliveries over 20 weeks’ gestation, weight gain was included as number of pounds gained from preconception to delivery, preeclampsia was determined by provider documentation of disease that includes elevated blood pressure and proteinuria,
eclampsia was similarly determined by provider documentation of maternal seizure, and dystocia was characterized by labor progress that deviated from Friedman’s curve. Statistical hypotheses were tested with 2-tailed 95% CIs. There was similar cigarette use in the population and thus cigarette smoking was not included in the model (8.3% vs 9.5%, ns).

The relative risk (RR) is needed to calculate the attributable fraction (AF) of disease. Multiple regression models yield adjusted odds ratios (OR). Thus, to calculate the AF of cesarean delivery we needed to convert the adjusted OR to adjusted RR. Currently, there is debate in the epidemiology literature regarding the most appropriate methods for estimating the RR from an OR. Adjusted RR of cesarean delivery was estimated by using 2 methods, the Zhang and Yu conversion and a modified Poisson regression.21,22 The attributable fraction in the exposed was then calculated.23 This fraction was applied to the percentage of women overweight and obese in 2001. In a similar fashion, we calculated the annual AF of preeclampsia across the study period.

Results

Study population

The trend analyses included 495,051 deliveries of liveborn singleton infants in Utah between 1991 and 2001. Of the half million deliveries recorded in the database, 229,483 women had their first delivery in the interval and these women were included in the outcome analyses; of these, 168,051 were nulliparous. For multiple logistic regression models, data from 1997 to 2001 were included because before 1997 gestational diabetes was not recorded, therefore 93,294 women were included in the analysis. The cohort from 1997 through 2001 was included in its entirety to avoid selection bias.

Height and weight data were available on 93.3% of women before pregnancy and 85.4% of women at delivery. The groups with missing data were similar overall to those with BMI data (specifically, maternal age, parity, gestational age, race, diabetes, hypertension, cesarean delivery, and macrosomia); however, women with missing data were less educated ($P < .0001$).

The percentage of women with prepregnancy overweight and obesity increased from 25.1% in 1991 to 35.2% in 2001, representing a 40.2% increase (prevalence ratio [PR] 1.40; 95% CI 1.37-1.40) (Figure 1). Average prepregnancy weight for all women increased 8.1 lb between 1991 and 2001, from 138.1 ± 30.0 to 146.2 ± 34.3. During the same period, maternal obesity at delivery increased 36.2% from 28.7% to 39.1% (PR 1.36; 95% CI 1.33-1.39). Figure 1 demonstrates the increasing rates of maternal BMI from 1991 through 2001. The proportion of overweight and obesity across all categories increased steadily throughout the interval. The coefficient of the slope of maternal overweight and
obesity prepregnancy was 0.95 and obesity at delivery was 1.02. Both of these trends were statistically significant.

The maternal characteristics of obese women at delivery are described in Table I. Although all the BMI categories show an increase, there has been an approximate 2-fold increase in class III obesity in just 10 years ($P < .0001$). In 2001, 2.7% of women before pregnancy and nearly 5% of women at delivery had a BMI greater than 40.

There was an increase in the prevalence of obesity at delivery across all race, age, education, and parity groups. Similar to the general population, obesity was associated with nonwhite race, age, parity, and less education.$^{9,24}$ In 2001, 61% of Native Americans, almost 50% of women older than 40 years, and 50% of grand multiparous women were obese at delivery.

Among nulliparous women, different weight gain patterns had varying effects on cesarean delivery rates. Women who were overweight or greater before pregnancy and class I obesity or greater at delivery, conferred the greatest risk for primary cesarean delivery (26.3%). This risk was decreased in women who were not overweight before pregnancy and became obese during their pregnancy (22.0%) and was further attenuated in women who were overweight or obese before pregnancy who ultimately fell within normal range at delivery (16.8%). However, all these rates were increased compared with nulliparous women who were normal weight before pregnancy and at delivery (13.3%). In nulliparous women without comorbid conditions, primary cesarean delivery rates rose with increasing prepregnancy BMI from 11.4% (underweight) to 42.6% (class III) ($P < .0001$).

Among nulliparous and multiparous women, pre-existing and gestational diabetes and chronic hypertension (not including preeclampsia and pregnancy-induced hypertension) increased with rising BMI (data not shown). The proportion of cesarean delivery in class III obesity is 40.3% in women without risk factors, 43.8% with chronic hypertension, 49.2% in preexisting or gestational diabetics, and 58.8% with both hypertension and preexisting or gestational diabetes. After controlling for age, race/ethnicity, parity, gestational age, weight gain, diabetes, and hypertension, there was a statistically significant association between maternal overweight and obesity and cesarean delivery, pre-eclampsia, eclampsia, dystocia, and birth weight greater than 4000 g (Table II). We did not find an association between anesthesia complications or preterm labor. When using normal weight women as the reference group, a multiple regression model showed a stepwise increase in adjusted ORs of cesarean delivery as BMI increased (Table III). The model correctly predicted 82.7% of cesarean deliveries. The effect of maternal BMI on cesarean delivery was independent of neonatal macrosomia. When macrosomia (>4 kg birth weight) was added into the model, the effect on the adjusted

| Table I  | Characteristics of women with self-reported BMI > 30 at the time of delivery, Utah 1991 and 2001
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>1991 No.</td>
</tr>
<tr>
<td>Overall</td>
<td>9933/34,552</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>8565</td>
</tr>
<tr>
<td>Black</td>
<td>66</td>
</tr>
<tr>
<td>Hispanic</td>
<td>704</td>
</tr>
<tr>
<td>Native American</td>
<td>339</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
</tr>
<tr>
<td>12-19</td>
<td>839</td>
</tr>
<tr>
<td>20-29</td>
<td>5845</td>
</tr>
<tr>
<td>30-39</td>
<td>3069</td>
</tr>
<tr>
<td>40-49</td>
<td>179</td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>&lt;High school</td>
<td>1374</td>
</tr>
<tr>
<td>High school</td>
<td>3791</td>
</tr>
<tr>
<td>&lt;4-y college</td>
<td>3298</td>
</tr>
<tr>
<td>≥4-y college</td>
<td>1391</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>3184</td>
</tr>
<tr>
<td>Multiparous</td>
<td>6061</td>
</tr>
<tr>
<td>Grand multiparous (h/o ≥ 5 deliveries)</td>
<td>677</td>
</tr>
</tbody>
</table>
ORs of BMI category decreased minimally (underweight 0.82, overweight 1.51, class I obesity 2.24, class II obesity 3.27, and class III obesity 4.20).

The adjusted RR that was calculated varied insignificantly between methods: the model of Zhang and Yu yielded an adjusted RR of 1.664 (1.614-1.713) and the modified Poisson estimate was 1.633 (1.584-1.686). The most conservative estimate of RR yielded an attributable fraction of cesarean delivery in 2001 in the overweight and obese of 0.388 (0.369-0.407).

The AF of preeclampsia secondary to prepregnancy BMI 25 or greater did not change across time, but the incidence of preeclampsia increased from 1991 to 2001 (Figure 2).

Comment

Despite the increased awareness of obesity over the past decade, statewide prepregnancy overweight and obesity increased 40% in Utah, with a similar rise in obesity at delivery. A 2-fold increase in severe obesity has yielded a 5% proportion of women with a BMI of more than 40 at delivery. This increase in obesity has significant public health implications in pregnant women. In this large population-based study, we identified several adverse outcomes associated with increased BMI even when controlling for hypertension and diabetes, including cesarean delivery, preeclampsia, eclampsia, and fetal macrosomia. The odds of cesarean delivery increases stepwise with increasingly heavier strata of BMI.

National trends

Nonpregnant women aged 20 to 39 years experienced a 37.9% increase in obesity (BMI >30) between NHANES III (1988-94) and NHANES data from 1999 to 2000 to 28.4%. In our analysis, the prevalence of preconceptional obesity (BMI >30) is less than those reported in this national sample of reproductive age women. We did, however, see temporal increases of similar magnitudes. In contrast, the prevalence of preconceptional severe obesity (BMI >40), which increased from 1% to 2% over the decade, was similar to national data of nonpregnant women. Strum et al saw a similar pattern when analyzing the BRFSS data from 1986 to 2000.

The lower overall rates of preconceptional obesity in our population have several possible explanations. The population in Utah may be less obese than the general population, in part secondary to the white predominance in the state. This is supported in part by work by Mokdad et al. Although their data from the BRFSS include all adults of both genders, Utah ranked 33rd in the nation with respect to obesity, with 18.5% of adults having a BMI greater than 30.

In Utah, we have fewer women with preconceptional BMI greater than 30 but similar rates of BMI greater than 40 compared with previously reported national data on nonpregnant women. Although this may reflect differences in the weight distribution in Utah compared with the nation, it may also reflect the differences in data collection between studies. The birth records depend on self-reported height and weight data. These data tend to inflate height and diminish weight, thus the bias exists to misclassify women to leaner strata. Our prevalence rates are consistent with the data of Freedman et al that were also obtained by self-report and are somewhat attenuated compared with the results obtained from Flegal et al that used actual BMI measurement. This observation supports the possibility that self-reported measurements downgrade the BMI strata to which women are assigned and may in part be responsible for the difference in obesity prevalence between our findings and that reported in a national sample of nonpregnant women of reproductive age.

Table II: Adjusted ORs for various pregnancy outcomes for preconceptional overweight and obese women in Utah

<table>
<thead>
<tr>
<th>Outcome</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preeclampsia</td>
<td>2.49 (2.35-2.64)</td>
</tr>
<tr>
<td>Eclampsia</td>
<td>1.42 (1.14-1.77)</td>
</tr>
<tr>
<td>Dystocia</td>
<td>1.92 (1.82-2.03)</td>
</tr>
<tr>
<td>BW &gt;4000 g</td>
<td>1.68 (1.58-1.79)</td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td>1.90 (1.82-1.98)</td>
</tr>
<tr>
<td>PT delivery</td>
<td>0.98 (0.92-1.04)</td>
</tr>
<tr>
<td>Anesthetic comp.</td>
<td>1.48 (0.85-2.58)</td>
</tr>
</tbody>
</table>

Controlling for maternal age, race, parity, gestational age (except for PT delivery), weight gain, diabetes, and hypertension. PT, Preterm.

Table III: Multiple logistic regression model for risk of cesarean delivery by maternal BMI

<table>
<thead>
<tr>
<th>Prepregnancy BMI</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal weight (19-24.9)</td>
<td>Reference</td>
</tr>
<tr>
<td>Underweight (&lt;19)</td>
<td>0.81 (0.76-0.86)</td>
</tr>
<tr>
<td>Overweight (25-29.9)</td>
<td>1.55 (1.48-1.63)</td>
</tr>
<tr>
<td>Class I obesity (30-34.9)</td>
<td>2.28 (2.13-2.45)</td>
</tr>
<tr>
<td>Class II obesity (35-39.9)</td>
<td>3.37 (3.04-3.73)</td>
</tr>
<tr>
<td>Class III obesity (&gt;40)</td>
<td>4.52 (3.93-5.20)</td>
</tr>
</tbody>
</table>

Controlling for maternal age, race, parity, gestational age, weight gain, diabetes, hypertension, and macrosomia.
who were obese was significantly larger. Most of the data from Alabama were evaluated by using absolute weights. However, of those subjects for whom they were able to calculate BMI, they described an increase from 25% to 36% in BMI 30 or greater at first prenatal visit. This is 3 times greater than our population and may be explained by the fact that their maternal sample consisted of nearly 70% black women. The mean gestational age at first prenatal visit of the women in this study, at first prenatal visit was 13 to 15 weeks and may slightly raise the BMI compared with pre pregnancy.

In an urban population in Ohio, 28% of women were more than 200 lbs at delivery. For average height women (5 ft 4 in), this is near a BMI of 34. Assuming that 28% of urban Ohio women have a BMI 34 or greater, this would represent a 2-fold greater prevalence than seen in Utah. Both of these studies were derived from single institutions in large cities in Alabama and Ohio. Both states have a higher prevalence of obesity; Alabama ranked second in the country in the percentage of obese adults and Ohio 15th.

**Outcomes**

These data suggest that even moderate increases in body mass increase a woman’s risk of an adverse outcome even when controlling for diabetes and hypertension. Our data confirm previously reported associations between obesity and cesarean delivery, preeclampsia, eclampsia, and macrosomia. The importance of calculating adverse outcomes in a population-based longitudinal study is the ability to estimate the overall disease burden related to the exposure. Although 1 study has previously calculated the AF of cesarean delivery caused by obesity as 11.6%, this estimate was based on a racially skewed sample and did not control for diabetes and hypertension. We compared 2 methods.
for estimating the RR, the Zhang and Yu conversion and the modified Poisson regression of Zou. With our model, there were minimal differences between the 2 estimates. In 2001, more than 35% of women were preconceptionally overweight or obese. Given an attributable fraction of 0.38 for cesarean delivery in the overweight and obese, nearly 1 in 7 cesarean deliveries of singleton infants in our 2001 population was attributable to overweight and obesity. This result was obtained with conservative estimates, controlling for comorbid conditions, in a state that may be less obese than the nation as a whole. Although obesity is not the indication for delivery per se, it contributes to factors that increase the need for surgical intervention. Further research is urgently needed not only to confirm this observation but to explain the mechanism(s) responsible for the association and to develop clinical investigations (above and beyond preconceptional weight loss) to improve the likelihood of safe vaginal delivery for obese women.

The incidence of preeclampsia appears to be increasing among nulliparous women delivering singletons. The AF of preeclampsia caused by BMI ≥ 25 or greater has not increased. Some of the excess cases could have resulted from a shift in maternal BMI. However, results from our study should be evaluated with caution given the suboptimal correlation between preeclampsia as documented in the medical record compared to the birth record. Additional prospective studies of preeclampsia trends may better describe this phenomenon.

In summary, this is the first statewide population-based analysis of pregnancy overweight and obesity trends. Not surprisingly, the epidemic of obesity spills into the pregnant population. Unlike previous trend studies in the obstetric literature, our dataset reported weight and height data and thus permitted calculation of BMI. It also includes overweight women who were previously excluded from other trend studies. There was an increase in overweight and obese women over the 12-year study period and even overweight women experienced increased adverse outcomes in proportion to their increasing BMI. We controlled for diabetes and hypertension to try to evaluate the impact of BMI itself. This may attenuate the ORs for cesarean delivery associated with overweight and obesity. Unlike other studies of outcomes, we included multiparous women to better characterize the overall trends and controlled for parity in the analyses. The study is limited by the self-reported data and may thus underestimate BMI. Also, the women who have missing data were less educated than those who reported height and weight. Although this represented 6% to 14% of the population and lower education was associated with increasing BMI, the absence of these women could reduce the trends and associations. All of these study limitations would impart a diminutive effect and underestimate the magnitude of the problem. However, an overweight and obesity problem even of the scale presented here warrants urgent attention.

References

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Neonatal death and morbidity in vertex-nonvertex second twins according to mode of delivery and birth weight

Qiuying Yang, MD, PhD,a,b,c,* Shi Wu Wen, MB, PhD,a,b Yue Chen, MD, PhD,d Daniel Krewski, PhD, MHA,c,d Karen Fung Kee Fung, MD, MHPE,a Mark Walker, MD, CM, MSc,a,b

OMNI Research Group, Division of Maternal-Fetal Medicine, University of Ottawa, Faculty of Medicine, Ottawa, Ontario, Canada,a Ottawa Health Research Institute, Ottawa, Ontario, Canada,b McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health,c and Department of Epidemiology and Community Medicine, University of Ottawa, Ottawa, Ontario, Canada,d

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KEY WORDS
Twins
Nonvertex presentation
Mode of delivery
Neonatal death
Neonatal morbidity
Asphyxia-related neonatal death

Objective: The purpose of this study was to assess the risk of neonatal death and morbidity in vertex-nonvertex second twins according to the mode of delivery and birth weight.

Study design: Data from a retrospective cohort study that was based on all twin births in the United States (1995-1997) were used.

Results: A total of 15,185 vertex-nonvertex second twins were classified into 3 groups: (1) both twins were delivered by cesarean delivery (37.7%), (2) both twins were delivered vaginally (46.8%), and (3) the second twin was delivered by cesarean delivery after vaginal delivery of the first twin (15.5%). The risk of asphyxia-related neonatal deaths and morbidity was increased in the group in which both twins were delivered vaginally and the group in which both twins were delivered by cesarean delivery. The increase in neonatal death in the group in which both twins were delivered vaginally was stronger in the birth weight of <1500 g. In contrast, in the group in which both twins were delivered vaginally and the group in which the second twin was delivered by cesarean delivery after the first twin was delivered vaginally, the increase in neonatal morbidity was greater in the group in which the birth weight was 1500 to 4000 g.

Conclusion: The risk of neonatal death and morbidity in second-born twins is higher in the group in which both twins were delivered vaginally and the group in which the second twin was delivered by cesarean delivery after the first twin was delivered vaginally compared with the group in which both twins were delivered by cesarean delivery.

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Vertex-nonvertex presentation occurs in approximately 35% to 40% of twin births.1-3 The optimal mode of delivery for the second twin with a nonvertex presentation is controversial.4-6 A recent study by Rydhstroem7 indicated that twins in breech presentation delivery vaginally experienced a worse prognosis than
twin(s) delivered abdominally. However, Rydhstroem did not evaluate outcomes for the first twins and second twins separately. In contrast, several observational studies have found that vaginal delivery of non-vertex-presenting second twins is safe, with no significant differences in neonatal morbidity, as compared with delivery by cesarean delivery. However, in some of these studies, emergent cesarean delivery was not distinguished from planned cesarean delivery.

There are 3 modes of delivery options for twins: (1) both twins are delivered by cesarean delivery; (2) both twins are delivered vaginally, and (3) the first twin is delivered vaginally followed by cesarean delivery for the second twin. The third mode of delivery occurred in approximately 9.5% of second twins. The second twins with emergent cesarean delivery had less favorable neonatal outcomes, as compared with second twins with vaginal delivery and planned cesarean delivery. However, these recent studies did not further examine neonatal outcomes in relation to the presentation of second twins. Although there is a consensus regarding neonatal outcomes in vertex-nonvertex second twins according to mode of delivery. Because both the Society of Obstetric and Gynecology Canada (SOGC) and the American College of Obstetricians and Gynecologists have different management recommendations for infants with birth weight <1500 g and birth weight in the range of 1500 to 4000 g, we further stratify our subjects by these 2 categories of birth weight.

**Material and methods**

We performed an analysis based on data from a population-based retrospective cohort study of all twin births in the United States for the period of 1995 through 1997, using the matched multiple birth file created by the Centers for Disease Control and Prevention. This database included sociodemographic information of the parents; maternal life-style factors such as smoking and alcohol consumption during pregnancy; obstetric history; complications associated with pregnancy, labor and delivery; birth weight, and gestational age.

The analysis was restricted to vertex-nonvertex second twins with live births. Live births with gestational age <24 weeks or with birth weight <500 g were excluded because of concerns about the viability of infants who are born prematurely or with low birth weight. The study subjects were divided into 3 groups by mode of delivery for first twin: second twins delivered by cesarean delivery after vaginal delivery of the first twin (V-C), both twins delivered vaginally (V-V), and both twins delivered by cesarean delivery (C-C). We derived a new variable that represents birth weight discordance within the same pair of twins, based on the second twin being 25% lighter or 25% heavier than the first twin.

The purpose of this study was to compare neonatal outcomes in vertex-nonvertex second twins according to mode of delivery. Because both the Society of Obstetric and Gynecology Canada (SOGC) and the American College of Obstetricians and Gynecologists have different management recommendations for infants with birth weight <1500 g and birth weight in the range of 1500 to 4000 g, we further stratify our subjects by these 2 categories of birth weight.
Table I  Maternal and fetal characteristics for the vertex-nonvertex second twin according to mode of delivery: United States, 1995-1997

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C-C group</th>
<th></th>
<th>V-V group</th>
<th></th>
<th>V-C group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Percent</td>
<td>N</td>
<td>Percent</td>
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</tr>
<tr>
<td>--------------------------------</td>
<td>-----------</td>
<td>---------</td>
<td>-----------</td>
<td>---------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>Maternal age (y)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>379</td>
<td>6.62</td>
<td>529</td>
<td>7.44</td>
<td>186</td>
<td>7.92</td>
</tr>
<tr>
<td>20-29</td>
<td>2541</td>
<td>44.40</td>
<td>3422</td>
<td>48.11</td>
<td>1146</td>
<td>48.79</td>
</tr>
<tr>
<td>30-34</td>
<td>1726</td>
<td>30.16</td>
<td>1992</td>
<td>8.01</td>
<td>630</td>
<td>26.82</td>
</tr>
<tr>
<td>≥35</td>
<td>1077</td>
<td>18.82</td>
<td>1170</td>
<td>16.45</td>
<td>387</td>
<td>16.48</td>
</tr>
<tr>
<td>Maternal race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>4675</td>
<td>81.69</td>
<td>5679</td>
<td>79.84</td>
<td>1763</td>
<td>75.05</td>
</tr>
<tr>
<td>Non-white</td>
<td>1048</td>
<td>18.31</td>
<td>1434</td>
<td>20.16</td>
<td>586</td>
<td>24.95</td>
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<tr>
<td>Marital status</td>
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* The State of California did not send data on smoking.
second twins (37.7%) in the C-C group, 7113 second
twins (46.8%) in the V-V group, and 2349 second
twins (15.5%) in the V-C group. In the vertex-nonvertex
second twins, the emergency cesarean delivery rate for
the second twin after vaginal delivery of the first twin was
24.8%.

Table I gives the distribution of maternal and infant
characteristics for the 3 study groups. The proportions
of non-white mothers and unmarried mothers were
higher in the V-C group than in the C-C group, as
were the proportions of infants with cord prolapse and
fetal distress. Differences in maternal and fetal charac-
teristics between the V-V and C-C groups tended to be
less pronounced. Maternal complication occurred more
frequently in the C-C group than in both the V-V and
V-C groups (Table I).

In vertex-nonvertex second twins, all-cause neonatal
mortality rate and non-congenital anomaly-related neo-
natal mortality rate was higher in the V-V group than in
the C-C group (Table II). Seizure also occurred more
frequently in the V-V group than in the C-C group. The
risks of asphyxia-related death, newborn injury, low
Apgar score, and mechanical ventilation use were in-
creased in both the V-V group and the V-C group
relative to the C-C group.

Among second vertex-nonvertex twins with birth
weight <1500 g, increased risks were noted in the V-V
group for all causes of neonatal death, non-congenital
anomaly-related neonatal death, asphyxia-related deaths,
and seizure compared with the C-C group (Table III).
Elevated risks of a low Apgar score were observed in both
the V-V group and V-C group. Among vertex-nonvertex
second twins with birth weight in the range 1500 to 4000 g,
the risks of newborn infant injury, low Apgar score, and
mechanical ventilation use were elevated in both the V-V
group and the V-C group (Table IV). An increased risk of
seizure was noted in the V-V group along with an elevated
risk of asphyxia-related death in the V-C group.

<table>
<thead>
<tr>
<th>Type of neonatal outcome and mode of delivery</th>
<th>No. of outcomes (%)</th>
<th>Adjusted odds ratio (95% CI)*</th>
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<tbody>
<tr>
<td>Total neonatal deaths</td>
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<td>57 (1.00)</td>
<td>Reference</td>
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<td>V-V group</td>
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<td>2.17 (1.53, 3.10)</td>
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<td>1.01 (0.60, 1.66)</td>
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<td>Noncongenital anomaly–related deaths</td>
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<td>Reference</td>
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<td>Reference</td>
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<td>4.44 (0.82, 33.08)</td>
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* Odds ratios (95% CI) were adjusted for maternal age, race, marital status, cigarette smoking during pregnancy, parity, medical complications (diabetes mellitus, pregnancy-associated hypertension, placenta abruption or placenta previa, and abnormal labor), fetal sex, gestational age, birth weight, cord prolapse, fetal distress, and birth weight discordance with first twin.
There was a notable difference in the effect of the mode of delivery on neonatal mortality and morbidity rates between infants with a birth weight of <1500 g and infants with a birth weight in the range 1500 to 4000 g. The increase in the neonatal death rate in the V-V group was stronger in infants with a birth weight of <1500 g as compared with infants with a birth weight in the range of 1500 to 4000 g (Tables III and IV). In contrast, the increase in neonatal morbidity that was observed in the V-V and V-C groups was greater among infants with a birth weight between 1500 and 4000 g than among infants with a birth weight of <1500 g.

Comment

Of the 15,185 vertex-nonvertex second-born twins who were included in the present study, 5723 of the births (37.7%) involved cesarean delivery for both twins, 7113 of the births (46.84%) involved vaginal delivery of both twins, and 2349 of the births (15.47%) involved cesarean delivery of the second twin after the first twin was delivered vaginally. The risk of asphyxia-related neonatal death and neonatal morbidity was increased in the both the V-C and V-V groups, as compared with the C-C group. The V-V–related increase in neonatal death was stronger in infants with a birth weight <1500 g than in infants with a birth weight in the range of 1500 to 4000 g. In contrast, the increase in neonatal morbidity that was observed in the V-V and V-C groups was greater among infants with a birth weight between 1500 and 4000 g than among infants with a birth weight of <1500 g.

Several previous studies have reported rates of cesarean delivery for the second twin after vaginal delivery of the first twin that ranged from 0.33% to 26.8%. These studies have several limitations, including small sample sizes and a lack of representatives of the general population. The cesarean delivery rate for the second twin after vaginal delivery of the first twin among vertex-nonvertex second twins in the present study was 24.8%, much higher than the 9.5% for all presentation second twins in the same study population. Persad et al also

Table III Comparison of neonatal outcomes in vertex-nonvertex second twins according to mode of delivery (birth weight 500-1499 g), United States, 1995-1997

<table>
<thead>
<tr>
<th>Type of neonatal outcome and mode of delivery</th>
<th>No. of outcomes (%)</th>
<th>Adjusted odds ratio (95% CI)*</th>
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* Odds ratios (95% confidence interval) were adjusted for maternal age, race, marital status, cigarette smoking during pregnancy, parity, medical complications (diabetes mellitus, pregnancy-associated hypertension, placenta abruption or placenta previa, and abnormal labor), fetal sex, gestational age, birth weight, cord prolapse, fetal distress, and birth weight discordance with first twin.
found that vaginal-cesarean delivery rates were higher among nonvertex second twins.

Our results are consistent with those from a recent singleton term breech trial, which found that planned cesarean delivery was associated with reduced neonatal morbidity as compared with planned vaginal birth.29 The present results are also compatible with previous results from our research group, which indicated that second twins with emergent cesarean delivery experienced less favorable neonatal outcomes than did second twins with vaginal delivery or planned cesarean delivery.15,16 The odds ratio for neonatal death in breech vaginal delivery versus cesarean delivery was 1.47 (95% CI, 0.99-2.17) in Swedish study with the use of the population-based registry data during the period 1991 to 1997.7 However, in this study, results were presented only for first twins and second twins combined.

The optimal mode of delivery for vertex-nonvertex second twin remains controversial. Most previous studies found that vaginal delivery of non-cephalic-presenting second twins was safe, with no significant differences in neonatal morbidity in comparison with delivery by cesarean delivery.8-12 Conclusions derived from observations on neonatal outcomes of the nonvertex second twin depend largely on which groups are compared. Most studies compared nonvertex second twins who were born vaginally to those second twins who were born by cesarean delivery. However, in these studies, emergent cesarean delivery was not examined separately from planned cesarean delivery.30-34 Some studies compared all vaginally born second twins, regardless of presentation, with their first-born siblings,35,36 whereas others restricted their comparisons of nonvertex second twins to their first-born siblings.33,37

The uncertainty about the optimal mode of delivery for the vertex-nonvertex second twin also exists within different categories of birth weight. Several studies have emphasized the fetal safety of vaginal delivery relative to the abdominal route for infants with birth weights of >1500 g or >2000g.10-12,30,38 which leads to suggestions that routine cesarean delivery is not justified for non-vertex second twins who are expected to weigh

<table>
<thead>
<tr>
<th>Type of neonatal outcome and mode of delivery</th>
<th>No. of outcomes (%)</th>
<th>Adjusted odds ratio (95% CI)*</th>
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<tbody>
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<tr>
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<tr>
<td>C-C group</td>
<td>4 (0.08)</td>
<td>Reference</td>
</tr>
<tr>
<td>V-V group</td>
<td>10 (0.15)</td>
<td>2.31 (0.76, 8.53)</td>
</tr>
<tr>
<td>V-C group</td>
<td>4 (0.20)</td>
<td>3.18 (0.72, 13.97)</td>
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<tr>
<td>Asphyxia-related deaths</td>
<td></td>
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<td>C-C group</td>
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<td>Reference</td>
</tr>
<tr>
<td>V-V group</td>
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<td>4 (0.20)</td>
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</tr>
<tr>
<td>Newborn infant injury</td>
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<td></td>
</tr>
<tr>
<td>C-C group</td>
<td>1 (0.02)</td>
<td>Reference</td>
</tr>
<tr>
<td>V-V group</td>
<td>64 (0.97)</td>
<td>50.88 (11.22, 899.21)</td>
</tr>
<tr>
<td>V-C group</td>
<td>6 (0.30)</td>
<td>14.02 (2.34, 266.89)</td>
</tr>
<tr>
<td>Low Apgar score (&lt;7 at 5 minutes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C group</td>
<td>74 (1.47)</td>
<td>Reference</td>
</tr>
<tr>
<td>V-V group</td>
<td>241 (3.66)</td>
<td>2.69 (2.07, 3.54)</td>
</tr>
<tr>
<td>V-C group</td>
<td>141 (6.95)</td>
<td>4.13 (3.07, 5.61)</td>
</tr>
<tr>
<td>Ventilation use</td>
<td></td>
<td></td>
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<tr>
<td>C-C group</td>
<td>422 (8.40)</td>
<td>Reference</td>
</tr>
<tr>
<td>V-V group</td>
<td>736 (11.19)</td>
<td>1.42 (1.25, 1.62)</td>
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<td>V-C group</td>
<td>243 (11.98)</td>
<td>1.32 (1.11, 1.58)</td>
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<tr>
<td>Occurrence of seizure</td>
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<tr>
<td>C-C group</td>
<td>2 (0.04)</td>
<td>Reference</td>
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<tr>
<td>V-V group</td>
<td>10 (0.15)</td>
<td>3.84 (1.00, 25.11)</td>
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<tr>
<td>V-C group</td>
<td>4 (0.20)</td>
<td>4.57 (0.83, 34.21)</td>
</tr>
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</table>

* Odds ratios (95% confidence interval) were adjusted for maternal age, race, marital status, cigarette smoking during pregnancy, parity, medical complications (diabetes mellitus, pregnancy-associated hypertension, placenta abruption or placenta previa, and abnormal labor), fetal sex, gestational age, birth weight, cord prolapse, fetal distress, and birth weight discordance with first twin.
Currently being conducted by Barrett et al. The only randomized controlled study that has been conducted to date found no statistically significant difference in fetal outcomes among infants who are born after week 35 of gestation. On the other hand, Barrett et al. found that breech-extracted second twins with birth weight <1500 g had lower Apgar scores and increased neonatal morbidity when compared with their first-born siblings. This difference was not observed among twins who were delivered by cesarean delivery. However, direct comparisons between second twins who were delivered vaginally and by cesarean delivery were not made.

Chervenak et al. examined 76 breech-extracted second twins, of whom 16 infants weighed <1500 g. Although delivery mode was not associated with neonatal outcome, they recommended routine cesarean delivery for non-vertex second twins with birth weights <1500 g. In the present study, vaginal delivery was associated with increased risks of asphyxia-related neonatal death and morbidity in vertex-nonvertex second twins only for a birth weight of 2500 to 4000 g, not for a birth weight of 500 to 1499 g. This finding challenges the results of the aforementioned studies and Society of Obstetric and Gynecology Canada and American College of Obstetricians and Gynecologists recommendations in favor of vaginal delivery for nonvertex second twins who weigh 1500 to 4000 g, provided that the clinical criteria for vaginal breech delivery are met. Unfortunately, the observational nature of our study makes it difficult to draw a firm conclusion about the optimal mode of delivery for a vertex-nonvertex second twin. The randomized controlled twin birth trial currently being conducted by Barrett et al. may help to resolve this issue in the future. We found that the risk of newborn infant injury was higher in twins in the V-V group and the V-C group as compared with the C-C group in a birth weight of 2500 to 4000 g. However, no significant different in the risk of injury in the V-V group and the V-C group as compared with the C-C group after adjustment for covariates in a birth weight of 500 to 1499 g. This means that mature twins who were born vaginally were associated with birth trauma, although premature twins who were born vaginally were not associated with birth trauma.

A number of limitations of the present study need to be acknowledged. Our study is based on birth certificate data, which may underestimate certain complications of pregnancy. Possible errors in coding of the cause of death may be random. For example, the incidence of asphyxia-related death in the present study was lower than that reported in other studies, for unknown reasons. The causes of death in twins may be different, and premature birth may account for a large proportion of deaths among them. If asphyxia-related neonatal death in our data was under-coded, an under-estimation of the effect of emergent cesarean delivery for the section twin on neonatal death may occur. The lack of information on chorionicity further limited our ability to explore the cause of neonatal death. Although we have excluded cases with apparent coding errors in birth order, misclassification of this variable is still possible. Although intrapartum fetal death may be a more informative outcome measure, the present data do not distinguish between intrapartum and antepartum fetal deaths. There are probably heterogeneous neonatal outcomes between second twins who are delivered by breech delivery and those who are delivered by external version. Unfortunately, we have no further information on breech delivery or external version. We also have no information on twins delivered by a physician or hospital. Different physicians may have chosen different modes of delivery, although the situations of the fetuses were similar. This is a limitation of our study.

MacMahon and Collins note that the magnitude of the observed effect is important in interpreting the results of observational studies. Despite the acknowledged limitations of the present study, the high relative risk that was observed here suggests that the apparent protective effect of cesarean delivery may be real. Given the high rate of emergency cesarean delivery rate (about one quarter) in the second twin after vaginal delivery of the first twin among vertex-nonvertex second twins, routine cesarean delivery in vertex-nonvertex twins might be necessary, regardless of birth weight.

References
Maternal age and the likelihood of a maternal request for cesarean delivery: A 5-year population-based study

Herng-Ching Lin, PhD,a,* Sudha Xirasagar, MBBS, PhDb

Taipei Medical University, School of Health Care Administration, Taipei, Taiwana; Arnold School of Public Health, University of South Carolina, Columbiab

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KEY WORDS
Maternal age
Maternal request
Cesarean delivery

Objective: The purpose of this study was to examine associations between maternal age and maternal request cesarean deliveries.

Study design: Five-year population-based data from Taiwan (1997-2001) that covered 904,657 singleton deliveries without a clinical indication for cesarean delivery that were judged by the attending physician were subjected to multiple logistic regression, year-wise, to examine the association of maternal age with request cesarean delivery, adjusted for health care institutional characteristics.

Results: Request cesarean delivery rates steadily increased over the study period within each age group, disproportionately so among the 34+ age group. Women aged <25 years were less likely than women aged 25 to 34 years (reference group) to request a cesarean delivery (odds ratio range, 0.67-0.88) and women aged 34+ were more likely than the reference group to have a request cesarean delivery (odds ratio range, 1.96-2.01), adjusted for health care institutional characteristics.

Conclusion: Population-based data confirms the expectancy that request cesarean delivery propensity increases with maternal age.

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Cesarean delivery rates have been a major concern of health policy makers in many developed and developing countries. For example, CSs account for 40% of all live births in Chile,1 about 36% in Brazil,1 32.3% in Taiwan,2 23.5% in the United States,3 and 22.4% in Italy.4 These rates far exceed the World Health Organization’s recommended rate of 15% of all deliveries.5

Many authors aver that unnecessary cesarean delivery increases maternal morbidity and death risk and contributes to unnecessary consumption of medical resources.6,7

Previous empiric studies have suggested that reduction in cesarean delivery rates is not associated with any increase in morbidity/mortality rates and therefore may be cost-effective, without entailing any loss of health benefits.8,9 Many efforts have been made to identify the factors that contribute to cesarean delivery. Researchers have documented the role of clinical factors (previous cesarean delivery, dystocia, fetal distress, breech presentation, and malpresentation) and nonclinical factors...
(socioeconomic status, race, maternal age, institutional characteristics, physician practice styles, and other characteristics) in cesarean delivery.\textsuperscript{10,11}

In recent years, the justification for request cesarean delivery in the absence of clinical indications has been debated intensely from the clinical, ethical, and legal perspectives.\textsuperscript{12} Proponents of patient choice believe that maternal choice should be paramount in the decision about the mode of delivery. With increasing advocacy for patient rights, request cesarean delivery has become more common in many countries. Irvine and Shaw\textsuperscript{13} reported that maternal request accounted for 24.9\% of all elective cesarean deliveries that were performed at Watford General Hospital in the United Kingdom. In Italy, request cesarean delivery increased from 3.6\% of all deliveries in 1997 to 9\% in 2000 after ratification of a bill on the rights of pregnant women.\textsuperscript{14} In Norway, maternal request accounted for 7.6\% of cesarean deliveries in 1999.\textsuperscript{15} According to 61.9\% of obstetricians in United Kingdom's North Thames Region, maternal request was a major factor in high cesarean delivery rates.\textsuperscript{16}

Although many authors have proposed several reasons for women's requests for elective cesarean deliveries,\textsuperscript{17,18} the role of maternal sociodemographic characteristics remains unclear because of methodologic limitations.\textsuperscript{17} However, the exploration of these relationships becomes increasingly important because patient-driven elective cesarean delivery increases relative to physician-driven cesarean delivery. In particular, the identification of the age groups with higher propensity for a request cesarean delivery can help policy makers to better target research and policy interventions.

This study used 5-year population-based data to examine this issue, to avoid the pitfall of chance findings in any 1 year. The database, which covered every delivery in Taiwan, presents a unique opportunity to explore maternal choice of delivery mode systematically as it relates to age. Possible confounding factors such as health care institutional characteristics are also accounted for.

Material and methods

Data sources

This study used data on all singleton deliveries in Taiwan that were vaginal or by a cesarean delivery done at maternal request (by implication, judged by the provider as clinically suitable for vaginal delivery) between 1997 and 2001 from the National Health Insurance (NHI) Research Database. The database covers all medical benefit claims for Taiwan's population of >20 million people who are covered with comprehensive health benefits and low co-payment rates under the government-sponsored NHI. Claims are reimbursed on the basis of the NHI's diagnosis-related-group (DRG) classification code recorded on each claim. Both cesarean delivery and vaginal delivery are reimbursed at fixed payment rates, regardless of length of stay or resource use. Medically necessary cesarean delivery (defined as cesarean delivery performed at the physician's initiative) coded 0371A is reimbursed at a fixed rate, twice the rate of vaginal delivery. Cesarean delivery performed at maternal request coded 0373B is considered by the Bureau of NHI (BNHI) as medically unnecessary and is reimbursed at the same rate as vaginal delivery, the balance to be recovered by providers from the patients. In addition to the DRG payment code, each claim has 1 principal diagnosis and up to 4 secondary diagnoses, as per International Classification of Disease,(ICD-9CM) codes, 1 principal operative procedure code, and up to four secondary operative codes.

Study group: Inclusion and exclusion criteria

The study objective was to examine the role of maternal age in women’s choice of cesarean delivery in the absence of medical need, judged by the attending physician. Therefore, we selected only singleton vaginal deliveries (DRG code 0373A), and singleton cesarean delivery deliveries that were DRG-coded 0373B, implying that these cases were judged clinically eligible for vaginal delivery, but provided cesarean delivery at the mother’s request. Our study population excludes multiple gestation cases and cases coded 0371A, those women who underwent cesarean delivery based on the physician’s decision to perform a cesarean delivery. The data from the latter group in the year 2000 formed the study population for an earlier study on the role of institutional factors in cesarean delivery and were controlled for clinical indications and comorbidities.\textsuperscript{2}

Figure 1, A, illustrates the inclusion and exclusion criteria, with data from the year 2000, and shows the clinical characteristics of included and excluded cases. (All singleton deliveries that were excluded in the present study had been included in the study population of the earlier study.\textsuperscript{2}) The Appendix shows the distribution of the 929 women in our study group with some comorbidity (we identify conditions that had at least 15 cases). The rest of our study population had no secondary diagnosis. Figure 1, B, presents the clinical characteristics of the excluded singleton deliveries of the year 2000, showing that almost all delivery cases with major obstetric complications were in the group that received medically necessary cesarean delivery and therefore out of the ambit of this (maternal request cesarean delivery) study.

Reimbursement at the cesarean delivery rate is a function of coding the case as medically necessary cesarean delivery (0371A), which is cross-checked by the BNHI through regular audits, with the use of a random sample of records from each hospital. Therefore, it is in
the provider’s interest to ensure documentation of any secondary diagnosis that clinically justifies cesarean delivery. The BNHI imposes high fines (100 times the reimbursement rate) and censures for undue deviations from the admissible norms and supports a patient grievance mechanism. Patients in Taiwan are free to choose any provider; therefore, providers have every incentive to guard against negligent, fraudulent, or

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**Figure 1**  A. Distribution of all deliveries in 2000 by clinical characteristics and delivery mode. Current study includes 183,964 (vaginal delivery cases) + 5685 (request CS without clinical indication for CS), total 189,649 cases. B. Clinical characteristics of physician-decided CS cases in 2000- CS coded DRG 0371A, reimbursed at full CS rates (study population covered in Lin and Xirasagar, 2004).

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* Unequivocal indications for CS = breech presentation, dystocia and fetal distress cases, singly or in combination, indisputable indications for a CS decision.
** Complications which justify a CS decision, subject to clinical judgment of the attending physician
*** Incidental secondary diagnosis with no obstetric relevance as per current state of art. Conditions included are shown in Appendix 2
**** No secondary diagnosis recorded in the claim. CS without any secondary diagnosis can attract BNHI censure or claim denial hospital presents such CS claims with significant frequency.
*****Pelvic floor/birth canal injuries.
erroneous coding. We believe that our inclusion criteria that are shown in Figure 1, A, effectively selected only those women who were eligible for vaginal delivery for the study and therefore eligible for a study of maternal choice of delivery mode.

In-patient claims of all patients who were admitted to hospitals or obstetric and gynecology clinics between January 1, 1997, and December 31, 2001, for delivery were screened for DRG codes 0373B, elective cesarean delivery per maternal request, and 0373A, vaginal delivery. As illustrated in Figure, by definition, the study population excluded all those who were provided a request cesarean delivery but had a significant obstetric diagnosis that could have justified medically necessary cesarean delivery as per current state-of-art and Anderson and Lomas19 hierarchy of obstetric diagnoses (previous cesarean delivery, breech presentation, dystocia, and fetal distress; total = 221 cases).

Descriptive, bivariate, and multiple logistic regression analyses in SAS software (SAS Institute Inc, Cary, NC) were used. Bivariate analyses were used to examine the crude associations between delivery mode versus maternal age, hospital level, ownership, location, and teaching status. For logistic regression analyses, the dichotomous dependent, variable, cesarean delivery at maternal request of 1, vaginal delivery of 0, was used. The independent variable of interest was maternal age classified into 3 categories: <25 years, 25 to 34 years, and >34 years. We also controlled for health care institutional characteristics, namely, ownership (public, private for-profit, and private not-for-profit), geographic location (northern, central, southern, and eastern Taiwan), and hospital level (medical centers with >500 beds, regional hospitals with 250-499 beds, district hospitals with 20-249 beds, and obstetrics/gynecology clinics with <10 beds). All medical centers and regional hospitals are teaching hospitals, and all clinics are nonteaching institutions. A probability level of <.05 was chosen for statistical significance.

Results

Table I shows the distribution of study subjects by age and characteristics of the health care institution and geographic location from 1997 to 2001. There is a steady upward trend of maternal request cesarean delivery rates, which increased from 2.0% in 1997 to 2.6% in 1998, 2.5% in 1999, 3.0% in 2000, and 3.5% in 2001. As

| Table I  Distribution of women without a clinical indication for cesarean delivery |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Variable        | 1997 (n = 207,405) | 1998 (n = 167,633) | 1999 (n = 177,272) | 2000 (n = 189,649) | 2001 (n = 162,698) |
| Delivery mode   |                 |                 |                 |                 |                 |
| Maternally requested cesarean delivery | 4073 (2.0%) | 4276 (2.6%) | 4451 (2.5%) | 5685 (3.0%) | 5734 (3.5%) |
| Vaginal delivery | 203,332 (98.0%) | 163,357 (97.5%) | 172,821 (97.5%) | 183,964 (97.0%) | 156,964 (96.5%) |
| Maternal age    |                 |                 |                 |                 |                 |
| <25 Y           | 54,720 (26.4%) | 46,194 (27.6%) | 45,545 (25.7%) | 49,735 (26.2%) | 45,639 (28.1%) |
| 25-34 Y         | 138,709 (66.9%) | 108,239 (64.6%) | 118,271 (66.7%) | 124,623 (65.7%) | 103,437 (63.6%) |
| >34 Y           | 13,976 (6.7%) | 13,200 (7.8%) | 13,456 (7.6%) | 15,291 (8.1%) | 13,622 (8.4%) |
| Institutional level |                 |                 |                 |                 |                 |
| Medical center  | 33,220 (16.0%) | 26,177 (15.6%) | 28,307 (16.0%) | 29,967 (15.8%) | 25,344 (15.6%) |
| Regional hospital | 42,970 (20.7%) | 36,018 (21.5%) | 38,840 (21.9%) | 41,561 (21.9%) | 37,635 (23.1%) |
| District hospital | 57,264 (27.6%) | 46,712 (27.9%) | 48,409 (27.3%) | 51,941 (27.4%) | 44,892 (27.6%) |
| Obstetric/gynecology clinic | 73,951 (35.7%) | 58,726 (35.0%) | 61,716 (34.8%) | 66,180 (34.9%) | 54,827 (33.7%) |
| Institutional ownership |                 |                 |                 |                 |                 |
| Public          | 24,018 (11.5%) | 17,882 (10.7%) | 18,823 (10.6%) | 19,448 (10.3%) | 17,474 (10.7%) |
| For-profit      | 121,229 (58.5%) | 99,510 (59.4%) | 105,822 (59.7%) | 114,947 (60.6%) | 98,295 (60.4%) |
| Not-for-profit  | 62,158 (30.0%) | 50,241 (29.9%) | 52,627 (29.7%) | 55,254 (29.1%) | 46,929 (28.9%) |
| Geographic location |                 |                 |                 |                 |                 |
| Northern        | 90,633 (43.7%) | 73,948 (44.1%) | 78,344 (44.2%) | 84,369 (44.5%) | 71,995 (44.3%) |
| Central         | 56,076 (27.0%) | 45,392 (27.1%) | 48,467 (27.3%) | 50,778 (26.8%) | 44,555 (27.4%) |
| Southern        | 55,207 (26.6%) | 43,869 (26.2%) | 45,927 (25.9%) | 49,859 (26.3%) | 41,855 (25.7%) |
| Eastern         | 5489 (2.7%) | 4424 (2.6%) | 4534 (2.6%) | 4643 (2.5%) | 4293 (2.6%) |
| Institutional teaching status |                 |                 |                 |                 |                 |
| Yes             | 98,507 (47.5%) | 78,918 (47.1%) | 83,738 (47.2%) | 89,234 (47.1%) | 78,036 (48.0%) |
| No              | 108,898 (52.5%) | 88,715 (52.9%) | 93,534 (52.8%) | 100,415 (52.9%) | 84,662 (52.0%) |
expected, most women every year were in the age group of 25 to 34 years. There is an increasing proportion of women aged 0-34 years with time, possibly representing an underlying trend of increasing childbirth at older ages.

Table II summarizes the distribution of delivery mode by age group. Request cesarean delivery rates progressively increased with maternal age (all $P < .001$; Pearson’s chi-squared test) in every study year. In addition, request cesarean delivery rates in each age group consistently increased from 1997 to 2001. For example, increasing percentages of women aged $<25$ years requested a cesarean delivery, 1.7%, 2.1%, 2.1%, 2.3%, and 2.4% during 1997, 1998, 1999, 2000, and 2001, respectively. The unadjusted odds of maternally requested cesarean delivery for the $<25$ year age group relative to the 25 to 34 age group were 0.91, 0.86, 0.84, 0.76, and 0.67, respectively, for 1997, 1998, 1999, 2000, and 2001, which indicates consistently declining odds of requested cesarean delivery among $<25$ year aged mothers relative to the 25 to 34 year age group, across the study period (data not given).

Figure 2 shows the adjusted odds of the likelihood of maternal request for cesarean delivery by maternal age. The adjusted odds ratio (controlled for institutional factors and geographic location) substantiate the findings of the unadjusted odds ratios, show a similar downward trend for the $<25$ year age group relative to the 25-34 age group (odds ratio, 0.83, 0.81, 0.82, 0.77, and 0.67 respectively). Older women aged $>34$ years showed higher odds (twice as likely) relative to the 25 to 34 year age group. Higher odds for older women are observed consistently for every year during the study period, after adjustment for hospital level, ownership, and geographic location. Overall, the adjusted odds of a maternal request for cesarean delivery significantly increased with maternal age and across the study period.

Because Figure 1, A, shows that 16.5% of request cesarean delivery (939/5685 women) had some medical or obstetric comorbidity, we examined the effect of comorbidities on the odds ratios for the year 2000, by including a dummy variable coded 1 if the patient had a comorbidity (all 939 request cesarean delivery cases with comorbidity and vaginal delivery cases with comorbidity), or zero for no comorbidity. The revised logistic regression model with this control variable added showed the adjusted odds of request cesarean delivery for the $<25$ year age group as 0.77 (95% CI, 0.72-0.82), and for $>34$ year age group as 2.00 (95% CI, 1.85-2.16). These odds are identical to the model without the secondary diagnoses (Figure 2). This indicates that secondary obstetric or medical diagnoses in patients whose condition is judged suitable for vaginal delivery do not change the age-request cesarean delivery relationship.

**Comment**

This study used 5-year, population-based data to explore the relationship between maternal age and maternal request cesarean delivery. Consistently, in every study year, increasing maternal age is associated with increasing odds of request cesarean delivery, with the highest odds for the $>34$ year age group and lowest for mothers $<25$ years old, after adjustment for health care institutional characteristics. Our finding is consistent with some authors’ speculation that older women’s preferences could explain partly their high cesarean delivery rate.20,21 We also find increasing request cesarean delivery likelihood among all age groups with time and that, relative to women aged 25 to 34 years, the youngest age group ($<25$ years) were progressively less likely to have a request cesarean delivery. This suggests...
disproportionately increasing propensity of the 25 to 34 year age group to request cesarean delivery, relative to the time trend increase that has been observed among the <25 year age group. Although many studies are available on the reasons for maternal cesarean delivery preferences, we are not aware of any published population-based study on request cesarean delivery and maternal age.

Anecdotal studies from the United Kingdom, Finland, Sweden, Singapore, and Australia document several reasons for maternal cesarean delivery preference in the absence of clinical need (such as safety of self or the baby, previous negative birth experience [which includes neonatal morbidity or death]), traumatic childhood experiences, fear of perineal damage, a fear of childbirth, physical stamina, the ability to schedule delivery in advance, late childbearing, protection from pelvic floor damage, refusal or reluctance to undergo labor pain, information from the doctor, and social convenience.17,18,22,23 A similar study by Huang et al24 in Taiwan found that 45.1% of request cesarean delivery mothers did so primarily for astrologic reasons and that, relative to Western studies, fewer percentages did so to have a scheduled delivery, to avoid delivery pain, to avoid inconvenience to the husband and family members because of unscheduled vaginal delivery, to avoid sexual dysfunction subsequent to vaginal delivery, because of husband/relatives’ cesarean delivery preference, or to time the cesarean delivery with vacation time.

Because we controlled for institutional factors and the study sample excluded women who had any clinical indication for cesarean delivery, these odds ratios closely reflect maternal choice patterns, despite being clinically suitable for vaginal delivery. A major reason for cesarean delivery preference among older women could be related to safety. Gamble and Creedy,17 on the basis of a comprehensive literature review, documented the widespread belief among women that adverse fetal and maternal outcomes increase with maternal age. Several authors have documented that delayed childbearing beyond 34 years is associated with increasing risk of adverse pregnancy outcomes.25 Therefore, older women, especially primigravid women, may request a cesarean delivery to circumvent adverse outcomes, which runs counter to the many empiric studies that show that clinically un-indicated cesarean delivery increases maternal mortality rates and is relatively disadvantageous to infants.7,26 Specifically, Goer’ presented a comprehensive literature review on maternal and fetal risks of vaginal versus cesarean delivery under various obstetric and medical conditions. Goer also accounted for the negative impact of specific poor obstetric practices during vaginal delivery that may have caused much of the maternal and fetal morbidity, thus, questioning the validity of contentions that vaginal delivery produces inferior outcomes relative to cesarean delivery.

Physician preferences may also influence cesarean delivery preferences among older women through the information they communicate or imply.25 In 1 survey, 98% of cesarean delivery patients said they agreed to their physician’s recommendation for cesarean delivery.28 Among Israeli obstetricians, 79% indicated that they preferred cesarean delivery for 40-year-old primigravid women, despite the lack of clinical indications.23 Possibly, many physicians in Taiwan may recommend cesarean delivery for older women by citing safety reasons, thus influencing “maternal choice.” However, our speculation runs counter to a research study from the United Kingdom, which suggests that request cesarean delivery was largely patient-driven rather than physician-led.17 In-depth surveys are needed to clarify this issue.

Two of our study findings have policy implications. First, higher rates of request cesarean delivery among older pregnant women, even after an adjustment for clinical indications suggest the need for in-depth research on maternal and fetal outcomes, as well as cost and care implications. Although many of the major cesarean delivery risks surface in subsequent pregnancies, which are less likely among older women anyway, cesarean delivery preferences among older mothers must be investigated, in view of the documented adverse fetal and maternal morbidity and mortality rates with cesarean delivery7 and the associated (possibly unnecessary) health care costs being added to the increasingly unbearable health care costs in most developed and middle income countries. Such research can enable informed policy approaches to educate physicians and the public about the pros and cons of elective cesarean delivery.

Together with clinical outcomes and cost, the social and personal reasons for cesarean delivery preferences must be researched with the use of well-designed surveys that cover recent parturients and prepregnant women. This will enable accurate assessments of its physician-led
and patient-driven components. Such research has international significance, given the burgeoning health care costs in almost all countries, and points out that this issue is critical for policy makers internationally to adopt an informed policy stand.

Our second finding is the consistently rising trend of cesarean delivery preference among women of all age groups. This suggests that request cesarean delivery (whether patient-driven or physician-led) is likely to increase further unless the reasons for this trend are identified and reversed, if cesarean delivery turns out to be an inferior option relative to a comprehensive portfolio of maternal and infant outcomes. Longitudinal panel studies of large samples that cover all clinical (maternal and infant) consultations after delivery are needed to settle the issue unequivocally.

The policy implications in the Taiwan context also have several pointers for international policymakers. In 2001, 3.5% of women in Taiwan without a clinical indication for cesarean delivery, as judged by their physician, opted for cesarean delivery, which amounted to 7.6% of total cesarean deliveries (5734/75,304 cesarean deliveries), which is comparable to Norway’s 7.6% and lower than Italy’s 9%.

This raises questions about the avoidable direct and indirect costs of medically unnecessary cesarean delivery. Although the government does not bear the direct costs of request cesarean delivery (request cesarean delivery patients have to bear the cost difference between vaginal delivery and cesarean delivery), it remains liable for the indirect costs because of infant morbidity and delayed maternal morbidity after cesarean delivery. Another issue is that the NHI reimbursement policy may be concealing an optimum gestational age. Second, given the BNHI’s audit procedures and censure mechanisms, such omissions appear unlikely. However, despite the safeguards, some degree of coding error in an administrative dataset cannot be ruled out.

This study is based on data from the NHI Research Database that is provided by the BNHI, Department of Health, Taiwan, and managed by the National Health Research Institutes. The interpretations and conclusions contained herein do not represent those of the BNHI, Department of Health, or the National Health Research Institutes.

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**Appendix**

Distribution of complications among maternal request cesarean delivery cases with medical/obstetric secondary diagnosis (total 939/5685 cases request cesarean delivery cases)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>ICD-9-CM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premature rupture of membranes</td>
<td>658.1</td>
<td>135 (2.37%)</td>
</tr>
<tr>
<td>Other specified indications for care or intervention related to labor</td>
<td>659.8</td>
<td>111 (1.95%)</td>
</tr>
<tr>
<td>and delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early onset of labor</td>
<td>644.2</td>
<td>73 (1.28%)</td>
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<tr>
<td>Excessive fetal growth</td>
<td>656.6</td>
<td>59 (1.03%)</td>
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<td>44 (0.77%)</td>
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<td>Delayed delivery after spontaneous or unspecified rupture of membranes</td>
<td>658.2</td>
<td>34 (0.60%)</td>
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<tr>
<td>Anemia</td>
<td>648.2</td>
<td>31 (0.54%)</td>
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<tr>
<td>Transient hypertensive</td>
<td>642.3</td>
<td>28 (0.49%)</td>
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<td>of pregnancy</td>
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<td>15 (0.26%)</td>
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<td></td>
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<tr>
<td>Others*</td>
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<td>4.5%</td>
</tr>
</tbody>
</table>

* Includes categories with <15 cases.
Second trimester abortion using isosorbide mononitrate in addition to gemeprost compared with gemeprost alone: A double-blind randomized, placebo-controlled multicenter trial

Wolfgang Eppel, MD,a Fabio Facchinetti, MD,b Ekkehard Schleussner, MD,c Federica Piccinini, MD,b Cristina Pizzi, MD,b Doris M. Gruber, MD,a Barbara Schneider, PhD,d Walter Tschugguel, MDa,*

Departments of Obstetrics and Gynecology, a and Medical Statistics, d Medical University of Vienna, Vienna, Austria, Mother-Infant Department, University of Modena and Reggio Emilia, Modena, Italy, b and the Department of Obstetrics and Gynecology Friedrich Schiller University of Jena, Jena, Germany c

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Objective: We aimed to determine whether second-trimester abortion using isosorbide mononitrate (IMN) in addition to gemeprost is more effective and reduces side effects compared with gemeprost alone.

Study design: Eighty women who were age 13 to 23 weeks' gestation were randomly assigned to receive per vaginam either IMN 40 mg (group 1, 40 women) or placebo (group 2, 40 women) in addition to gemeprost 1 mg up to 3 times daily 3 hours apart for 2 days. Analysis of variance, a χ² test, and a multivariate analysis were performed.

Results: Of the 72 women analyzed, 68% (group 1) and 38% (group 2) underwent abortion within day 1 (P < .05). However, group 1 was associated with more headache (18% of women) 3 hours after induction compared to group 2 (0% of women, P = .038).

Conclusion: IMN in addition to gemeprost is effective for second-trimester abortion, but is associated with more headache compared with gemeprost alone.

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Second-trimester abortion for medical reasons is frequently performed by administration of prostaglandins or their analogs to soften the cervix uteri.1-3 Gemeprost is the only prostaglandin currently licensed for cervical softening in the second trimester in Austria, Italy, and Germany; however, its use is associated with several adverse effects such as abdominal pain, nausea, vomiting, diarrhea, and vaginal bleeding.4 The ideal agent for cervical softening should be clinically effective, with a low incidence of side effects, and easy to administer.

* Reprint requests: Walter Tschugguel, MD, Department of Obstetrics and Gynecology, Division of Gynecological Endocrinology and Reproductive Medicine, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria.
E-mail: walter.tschugguel@meduniwien.ac.at

KEY WORDS
Gemeprost
Isosorbide mononitrate
Second trimester
Abortion

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Several lines of evidence indicate that nitric oxide (NO) is the ideal cervical softening agent. Administration of the NO donors isosorbide mononitrate (IMN), glyceryl trinitrate, as well as sodium nitroprusside to the posterior vaginal fornix were reported to reduce the cervical resistance before abortion in patients undergoing termination of pregnancy during the first trimester (12 weeks). Pretreatment with 40 or 80 mg IMN to soften the cervix before first-trimester surgical termination of pregnancy resulted in a greater amount of symptom-free patients than gemeprost (60%-70% vs 14%, respectively; \( P < .005 \)).

NO donors have been shown to stimulate prostaglandin production in the human cervix after vaginal administration. Even if NO did not stimulate prostaglandin production, the cervical softening effects of NO donors and prostaglandins would still be additive. If this hypothesis is correct, this would allow the use of a lower number of interventions needed to affect cervical softening if a NO donor is given in combination with a prostaglandin compared with a prostaglandin given alone. In the current study we examined the effects of 40 mg of IMN administered intravaginally with gemeprost compared with gemeprost alone for second-trimester abortion. For that purpose, we had to consider carefully how often and in which dose IMN should be administered vaginally in combination with gemeprost. Gemeprost administered vaginally reaches maximum plasma levels after 2 to 3 hours, with detectable levels for at least 6 to 8 hours, which justifies routine administration every 3 hours up to a maximum of 3 times a day.

### Material and methods

This double-blind, randomized, placebo-controlled multicenter study was carried out at the Medical University of Vienna, Vienna, Austria, the University of Modena and Reggio Emilia, Modena, Italy, and the University of Jena, Jena, Germany. Before the initiation of the study, approval was granted by the human ethics committees of the 3 participating universities.

A total number of 80 white Austrian (n = 20), Italian (n = 40), and German (n = 20) women scheduled for second-trimester abortion were included in the study (Table I). Inclusion criteria were nulliparity, gestational age between 12 weeks + 0 days and 22 weeks + 6 days confirmed by transvaginal ultrasonography, singleton pregnancy, good general health, and age between 19 and 35 years. Exclusion criteria included cervicitis, vaginitis, diabetes mellitus, chronic obstructive pulmonary disease, simultaneous intake of anti-inflammatory drugs, or...
women who were unable to understand the information sheet. None of the consecutive patients eligible had to be excluded for any of these latter reasons.

All women who participated in the study were fully informed of the nature and scope as well as of the potential risks of the study 1 or 2 days before the first dose of suppositories was administered. Basal assessments included drawing of intravenous blood to measure NOx serum levels as described12 as well as automatic recording of maternal pulse rate and blood pressure. The development of adverse symptoms was recorded by using a symptom questionnaire. The symptom questionnaire involved a structured series of questions regarding the recognized side effects of both agents (ie, lower abdominal pain, nausea, vomiting, diarrhea, headache, and vaginal bleeding). The questions were read from a script by 1 investigator before medication was given. Women were asked to respond “yes” or “no” to the questions, and their responses were recorded.

The women were randomly assigned to either of 2 treatment groups: group 1, 40 mg of the NO donor IMN (Sifa, Shannon, Ireland) formulated as a 40 mg vaginal suppository in combination with 1 mg gemeprost (a 1 mg vaginal suppository, Organon, Oss, The Netherlands), a prostaglandin E1 analogue, or group 2, 1 placebo suppository of similar design as the IMN suppository, in combination with gemeprost 1 mg, in the morning. Randomization was performed by means of numbered, sealed envelopes prepared with random numbered tablets. Each envelope contained 6 smaller sealed envelopes consecutively numbered 1 to 6, with both a verum or placebo suppository in addition to gemeprost. The envelopes were opened and treatment administered in the morning by 1 investigator in each center (W.E., F.F., and E.S.). Each treatment consisting of 2 suppositories (verum or placebo in addition to gemeprost) was administered by the same investigator to the posterior vaginal fornix. Treatment was administered every 3 hours up to a maximum of 3 applications in the first 24 hours and the next 3 applications in the next 24 hours, ie, a maximum of 6 applications within 48 hours. Neither the participating women nor the investigator were aware of the agent administered in addition to gemeprost. All assessments were performed immediately before treatment administration. Maternal pulse rate, blood pressure, and the need for analgesic drugs were recorded at baseline and 3, 6, 24, 27, and 30 hours. Intravenous blood samples to measure NOx serum levels were drawn at baseline and 6 hours. Moreover, adverse symptoms were recorded by using the above mentioned symptom questionnaire. We advised women to discontinue treatment if they had severe abdominal cramps or severe headache develop. Both assessments and procedures for all patients were performed by the same experienced investigators in each center (W.E., F.F., and E.S.) who were blind to the treatment given.

After the abortion, exploration and evacuation of the uterus were performed under general or local anesthesia. The patients were usually discharged 24 hours after abortion. In the case that abortion did not occur within 48 hours, patients were provided with state of the art treatment, consisting of vaginal administration of gemeprost 1 mg 3 times a day 3 hours apart after a treatment-free day. The primary end points were the number of doses required and the onset of new symptoms before abortion.

At the end of the study, the treatment allocation for each study number was revealed and the data were analyzed.

### Statistical analysis

Our main a-priori hypothesis was that the doses of tablets required before successful abortion might be lower in the treatment group compared with the control.
A sample size of 40 patients per group was estimated, assuming significance of 5%, power of 80%, and a dropout rate of 10% to show a reduction of 50% in the number of doses required before abortion. This calculation was based on historical observations indicating that approximately 75% of patients required 5 or more treatments at the Department of Obstetrics and Gynecology at the Medical University of Vienna.

During the trial, the dropout rate was 10% (8 patients). Three patients were excluded because they retrospectively appeared to be multiparous. Five other cases dropped out immediately after randomization after the first treatment application because of protocol violations. All of these latter patients, however, continued with the termination process, except that they received routine treatment consisting of gemeprost alone instead of study medication. Statistical analysis was, thus, performed on 72 cases, 38 of them randomly assigned to group 1 and 34 randomly assigned to group 2. One-way analysis of variance was used for group comparisons in the case of continuous variables. A \( \chi^2 \) test was used to compare distribution of categorical variables (follow-up times: 3, 6, 24, 27, and 30 hours in both groups). Linear correlations were calculated with the least squares method. A multivariate analysis was also performed by using the main outcome as a dependent variable.

Results

Baseline characteristics were comparable at enrollment between women in group 1 (IMN + gemeprost) and group 2 (placebo + gemeprost) for age, gestational age, the indications for abortion as well as for NOx serum levels (Table I).

The characteristics and the outcome of the abortion process are shown in Tables II and III. The distribution of the number of treatments before successful abortion was significantly different among groups, with fewer treatments required in group 1 compared with group 2 (Table III). Accordingly, a post-hoc analysis found women in group 1 to more frequently abort within day 1 by using 3 or less than 3 interventions (26/38 or 68%) compared with women in group 2 (13/34 or 38%; \( \chi^2 \) test, \( P < .05 \)). However, the mean induction-abortion time was not significantly different among groups, even after doing a survival analysis of patients still in labor after treatment (\( \chi^2 = 1.67, P = .197 \)) (Figure). Data were additionally analyzed after exclusion of patients with anhydramnios, with 32 cases remaining in group 1 and 33 cases remaining in group 2. The proportion of women who underwent abortion within day 1 remained to be higher in group 1 (21/32 or 65.7%) compared with group 2 (13/33 or 39.2%) (\( P < .04 \)), demonstrating that anhydramnios did not affect differences observed between groups. Moreover, the induction-abortion time still followed the tendency to be lower in group 1 (18.3 hours) compared with group 2 (32.1 hours) without reaching statistical significance.

Number of treatments and induction-abortion times did not differ among the 3 centers participating to the study.

Sixty-four women (89%) in both groups had undergone abortion by 2 days after starting with treatment with no difference between both groups in the induction-abortion interval, and the remainder 11% (5 cases in group 1 vs 3 cases in group 2, NS) by the next 5 days using a routine treatment starting on day 4 after a treatment-free day demonstrating no difference in the induction-abortion interval between both groups.

Because a few patients underwent abortion and others refused blood sampling before administration of the third tablet (ie, 6 hours after first administration), the number of serum samples for NOx measurement before the administration of the third tablet was restricted to 18 and 15 within group 1 and 2, respectively. We found that group 1, but not group 2 treatment increased NOx serum levels measured before the third course of tablet administrations (18.3 ± 9.5 μmol/L).
Side effects recorded for both treatment allocations at different times are reported in Table IV. Significantly more subjects complained of mild or moderate headache within group 1 compared with group 2 (38% vs 0%, respectively; \( P = .0008 \)), 3 hours after the beginning of treatment. The incidences of all other side effects were similar in both groups. Because no severe abdominal cramps or headache occurred, none of the patients discontinued treatment for any of these reasons. The proportion of patients receiving intramuscular administered opiates or paracetamol per os for treatment of mild-to-moderate abdominal cramps was 8 of 38 (21%) patients in group 1 and 9 of 34 (26.4%) patients in group 2. No changes occurred in the heart rate and blood pressure of the patients during any treatment (not shown), and no cervical or uterine rupture was seen. All patients were discharged 24 hours after uterine evacuation with no difference between groups (not shown).

Comment

Our data show that vaginally administered IMN given in combination with gemeprost reduces the number of doses required before successful abortion compared with the prostaglandin alone. A recently published report, however, shows that vaginal application of IMN appeared to be no more effective than placebo when given 12 hours before second-trimester termination of pregnancy using misoprostol.13 Our data, however, differ from that latter report insofar as we used IMN and the prostaglandin at the same time and not 12 hours apart. Interestingly, however, Ledingham et al14 failed to show any benefit of combining vaginally administered IMN with misoprostol over misoprostol alone for first-trimester pregnancy termination, suggesting a different effectivity of IMN dependent on gestational age.

Furthermore, we hypothesized that combining IMN with gemeprost might reduce the side effects associated with gemeprost because the smooth muscle relaxant properties of IMN may result in a reduced incidence of the side effects attributable to prostaglandin associated gastrointestinal and myometrial contractions.15 In our study, we have found that the addition of IMN does not reduce the incidence of side effects associated with gemeprost. Moreover, the side effects of these 2 agents appear to be additive, with combined therapy resulting in a greater proportion of patients with an episode of headache 3 hours after induction in addition to their gastrointestinal complaints. All other complaints did not change after addition of the NO donor to the prostaglandin, supporting the hypothesis that the NO donor could act via cervical smooth muscle relaxation rather than via stimulation of cervical prostaglandin production, which might, alternatively, have resulted in increased pain or gastrointestinal complaints, which, indeed was not the case here. Our finding demonstrating more headache after vaginal IMN administration is lent support by similar results obtained from a first-trimester trial.14 Our finding that serum NOx levels after IMN with gemeprost exceeded those after gemeprost alone strongly suggests that headache is most likely caused by IMN treatment. Side effects were recorded by 3 of the authors of that study who are familiar with NO donors and therefore know the side effects of this drug, which is of course headache, ie, one could now argue that the study results may be biased by that fact. Interestingly, however, headache was mainly found in the first 2 time intervals and very low rates of headache in both groups thereafter, suggesting that subjects receiving IMN could have adjusted in some way to the effects of IMN, making it unlikely for the investigators to get a feeling of whether they administered IMN or placebo together with gemeprost at the end of each treatment course.

No significant changes in maternal heart rate or systemic blood pressure were detected here, similar to the findings obtained from within first-trimester trials.10,14 Our finding of increased serum NOx levels after IMN with gemeprost being associated with headache, but not with measurable changes in maternal heart rate or blood pressure strongly suggests that although being vaginally administered, IMN might be able to cause subtle changes in systemic maternal vascular hemodynamics.

Our data suggest that vaginally administered IMN given in combination with gemeprost is a more effective method for second-trimester abortion, but causes more headache than gemeprost monotherapy in primigravida women. The ideal regimen with optimal doses and frequency of administration of combined therapy awaits further studies.

We thank Dr Hugh-Bloch, a pharmacist, for preparation of the verum and placebo suppositories used in this study and the women who participated in the study.

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Risk factors for neonatal mortality among extremely-low-birth-weight infants

Stephen J. Bacak, MPH, Kesha Baptiste-Roberts, MPH, Erol Amon, MD, Belinda Ireland, MD, Terry Leet, PhD

Department of Community Health, Saint Louis University School of Public Health, and Department of Obstetrics, Gynecology, and Women's Health, Saint Louis University School of Medicine, St Louis, Mo

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KEY WORDS
Extremely low birth weight
Neonatal mortality
Risk factors

Objective: The purpose of this study was to examine characteristics associated with neonatal mortality among extremely low-birth-weight infants (≤1000 g).

Study design: A population-based, case-control study using linked Missouri birth and death certificates from 1989 to 1997 was conducted. Cases (n = 835) were defined as extremely low-birth-weight infants that died within 28 days of birth. Controls (n = 907) were randomly selected from extremely low-birth-weight infants that were alive at 1 year and were frequency matched to subjects by birth year and birth weight.

Results: Infants born with severe congenital anomalies and at the youngest gestational ages were at greatest risk for neonatal mortality. Other significant risk factors included maternal age (<18 and >34 years), vaginal delivery, nontertiary hospital care, malpresentation, male gender, and small for gestational age. Black race and preeclampsia were protective against early death.

Conclusions: The risk of neonatal mortality among extremely low-birth-weight infants was associated with several maternal, infant, and obstetric factors, some of which may be preventable.

In 2002 extremely low-birth-weight (ELBW, ≤1000 g) infants accounted for less than 1% of all births in the United States. Although numerically small, ELBW infants represent nearly 50% of all perinatal mortality. Data on risk factors for neonatal mortality are limited for ELBW infants. Recently Shankaran et al compared risk factors for mortality among ELBW infants who died within the first 12 hours after birth with those who died after 12 hours using a population-based cohort study. However, most of the previous studies have been observational or have reported data from single perinatal centers. The objective of the present study was to examine maternal, infant, and obstetric characteristics associated with an increased risk for neonatal mortality among ELBW infants using a population-based, case-control study design.

Material and methods

We conducted a population-based, case-control study of neonatal mortality for all single live-born ELBW babies delivered among Missouri residents from 1989 to 1997. Linked birth and death certificates were used to identify cases and controls and to ensure Missouri residency of

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the mother. We defined cases as single live-born ELBW infants who died within 28 days of birth. Prior studies have suggested that 90% of ELBW mortality occurs within the first 28 days of life and that the majority of mortality occurs within the first 72 hours after birth. Only 8% of ELBW infants died between days 28 and 365 and were excluded from our study.

Controls were randomly selected from single live-born ELBW infants who lived for at least 1 year. Although multiple births represent an increasing proportion of ELBW infants, we excluded them from our study population because they do not represent independent events. Controls were frequency matched to the cases by birth year and birth weight categories (350 to 499, 500 to 599, 600 to 699, 700 to 799, 800 to 899, and 900 to 99 g). A total of 2728 ELBW births were identified during this period. Infants were also excluded from the study population if their clinical gestational age estimate was less than 23 weeks or greater than 32 weeks or they had missing data for key variables of interest (n = 986). Twenty-three weeks is often considered the lower end of fetal viability. Thirty-two weeks was selected as the upper limit because of the small number of ELBW infants born at or beyond this gestational age in our sample. The final study population included 1742 infants, comprising 835 cases and 907 controls.

All potential risk factors for neonatal mortality occurring in more than 10 cases in our study population were grouped into 1 of 3 categories: maternal, medical/obstetric, and newborn characteristics (yes/no response unless otherwise indicated). Maternal characteristics included age (<18, 18 to 34, >34 years), race (black, other), education (less than high school, high school or beyond), and tobacco use. Medical/obstetric characteristics included mode of delivery (vaginal, cesarean section), level of hospital care (levels I and II, III) at birth, diabetes, hypertension, preeclampsia (includes eclampsia), incompetent cervix, previous preterm birth, renal disease, placental abruption, premature rupture of membranes, precipitous labor, and malpresentation (includes breech malpresentation). Infant characteristics included gender, clinical gestational age at birth (23 to 24, 25 to 26, and 27 to 32 weeks), respiratory distress syndrome, severe congenital anomalies (includes anencephalus, renal agenesis, spina bifida, meningocoele, hydrocephalus, microcephalus, other central nervous system malformations, other circulatory and respiratory anomalies), and small for gestational age (SGA, defined as ≤10th percentile of fetal growth).

We performed univariate analysis to calculate the crude odds ratio (OR), comparing cases and controls for each potential risk factor. All statistically significant (P < .05) variables from the univariate analysis and potential clinically relevant and previously cited risk factors were entered into a multivariate logistic regression model. The adjusted OR and 95% confidence interval (CI) were calculated for each risk factor. Diagnostic tests for collinearity, outliers, and interaction were conducted. All statistical analyses were performed using SPSS software version 10.0 (SPSS, Inc, Chicago, Ill).

This study was reviewed by the Saint Louis University Institutional Review Board and was classified as exempt under 45 CFR 46.101(b) from the US Department of Health and Human Services regulations for the protection of human subjects.

### Results

The maternal characteristics associated with neonatal mortality among ELBW infants are shown in Table 1. Infants born to women younger than 18 years (adjusted OR 1.4, 95% CI 1.0 to 2.1) and to women older than 34 years (adjusted OR 1.6, 95% CI 1.0 to 2.5) were more...
likely to die than infants born to mothers 18 to 34 years of age. Black infants were 30% more likely to survive than infants of another race (adjusted OR 0.7, 95% CI 0.5 to 0.9).

Several medical and obstetric characteristics were significantly associated with neonatal mortality (Table II). Malpresentation of the infant (adjusted OR 1.6, 95% CI 1.2 to 2.1), birth in a level I or II hospital (adjusted OR 1.5, 95% CI 1.1 to 2.0), and infants whose mothers experienced placental abruption (adjusted OR 1.5, 95% CI 1.0 to 2.2) were most likely to die during the neonatal period. Vaginally delivered ELBW infants (adjusted OR 1.4, 95% CI 1.0 to 1.9) were also at a higher risk of neonatal mortality. Infants born to mothers diagnosed with preeclampsia (adjusted OR 0.6, 95% CI 0.4 to 1.0) were protected against neonatal mortality.

Infants born with any severe congenital anomalies conferred the highest risk for neonatal mortality (adjusted OR 4.9, 95% CI 2.2 to 10.7) among all risk factors (Table III). Infants born at 23 to 24 weeks’ gestation (adjusted OR 4.3, 95% CI 2.9 to 6.4) were 4 times more likely to die than those delivered at older gestational ages. Other infant characteristics significantly associated with neonatal mortality were male gender (adjusted OR 1.7, 95% CI 1.4 to 2.3) and infants born SGA (adjusted OR 1.6, 95% CI 1.1 to 2.2). Because severe congenital anomalies may be underreported on

<table>
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<tr>
<th>Table II</th>
<th>Medical and obstetric characteristics for neonatal mortality among extremely low-birth-weight infants, 1989-1997</th>
</tr>
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<tbody>
<tr>
<td>Characteristics</td>
<td>Cases (n = 835)</td>
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<td>Mode of delivery</td>
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<td>Vaginal</td>
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<td>Malpresentation</td>
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* OR, Odds ratio.

* Adjusted odds ratio (adjusted for all covariates in Tables I, II, and III by logistic regression analysis).

<table>
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<tr>
<th>Table III</th>
<th>Newborn characteristics for neonatal mortality among extremely low-birth-weight infants, 1989-1997</th>
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<td>Female</td>
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<td>496</td>
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<td>25-26</td>
<td>217</td>
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<tr>
<td>27-32</td>
<td>122</td>
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<tr>
<td>Respiratory distress syndrome</td>
<td>180</td>
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<tr>
<td>Severe congenital anomalies</td>
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<tr>
<td>Small for gestational age</td>
<td>149</td>
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* OR, Odds ratio.

* Adjusted odds ratio (adjusted for all covariates in Tables I, II, and III by logistic regression analysis).
the birth certificate, we reanalyzed our data excluding this variable from our logistic model and found no difference in any of the risk estimates shown in Tables I through III.

Comment

We identified several maternal, infant, and obstetric risk factors associated with neonatal mortality among ELBW infants. Infants born with severe congenital anomalies were at highest risk for neonatal death. Although not specific for early death among ELBW infants, our finding is consistent with Druschel et al., who reported that infants born with congenital malformations have a 6-fold increase for mortality than the general population. As expected, infants born at a lower gestational age were at much higher risk for neonatal mortality. Gestational age is often considered the foremost predictor of neonatal survival, with most clinicians acknowledging 23 to 24 weeks' gestation as the lowest end of viability. Infant survival at 23 weeks is estimated between 10% and 30% and increases to 25% to 50% survival for infants at 24 weeks.

In our study, 36% of infants between 10% and 30% and increases to 25% to 50% of infants born 23 to 24 weeks lived at least 1 year after birth. Gestational age and congenital anomalies are also closely associated with a physician’s willingness to provide aggressive intervention. Often physicians decide a priori threshold values for estimated fetal weight, birth weight, and nature of specific congenital anomalies for which they are unwilling to aggressively intervene. These decisions balance heightened concerns for maternal morbidity and mortality and parental autonomy, in contrast to potential improvement in newborn outcomes.

We did not identify another study that examined maternal age <18 years as an independent risk factor for neonatal mortality. Shankaran et al. reported mothers of infants who died within the first 12 hours of life were younger than mothers whose infants survived but presented only a mean age of 25.7 years. Younger mothers may not be completely biologically developed and thus more likely to experience preterm labor and delivery. However, this does not explain why ELBW infants born to younger mothers would be more likely to die within the first month of life. Additionally, there is no clear explanation for the increased risk of neonatal mortality born to women older than 34 years. This relationship should be further examined because the percentage of women older than 35 years giving birth continues to increase.

Our finding that male ELBW infants have a higher risk of neonatal mortality than females was also similar to the findings of Shankaran et al (OR 1.7, 95% CI 1.2 to 2.3) and consistent with several other studies. This phenomenon, known as the male disadvantage, has been recognized for decades; however, the biological mechanisms are not well understood. Recently Stevenson et al. found that relative differences persist between males and females, with males having a higher mortality rate, lower Apgar scores, and a higher risk for most adverse neonatal outcomes. Tyson et al. found that SGA infants were 50% more likely to die early. This suggests that even the youngest infants have a better chance of survival if their weights are appropriate for gestational age.

Prior population-based studies have found a significant association between vaginal delivery and neonatal mortality. Redman and Gonik reported significantly lower mortality rates in 22- and 23-week infants delivered by cesarean section, compared with vaginal delivery. The risk of neonatal mortality may not actually be due to mode of delivery but rather related to physicians’ unwillingness to intervene aggressively on behalf of the fetus, such as classic cesarean section, antenatal corticosteroid use, gestational age, and several other covariates. Although SGA is a function of both gestational age and birth weight, we included it in our regression model because it is a unique variable. When adjusted for potential confounders, including gestational age, SGA infants were 50% more likely to die early. This suggests that even the youngest infants have a better chance of survival if their weights are appropriate for gestational age.

Yeast et al. reported that ELBW infants born in level I and II centers were 3 to 5 times more likely to die than infants born in level III facilities. However, the authors adjusted only for birth weight, race, and number of infants born, which may explain the higher risk estimates, compared with our results. Level III hospitals have a greater frequency of high-risk births and are equipped with more services, such as maternal-fetal medicine specialists, neonatologists, and intensive care units that promote the chance of survival of these infants. Finally, neonatal mortality was higher among infants whose mothers had a placental abruption during

...
delivery. Perinatal mortality in births with placental abruption is approximately 12% in the United States. 24

Our finding that black infants were more likely to survive than infants of another race agrees with Shankaran et al.4 It has been previously suggested that African Americans have a higher survival rate among low-birth-weight populations; however, recent evidence indicates that this advantage is declining, even at the lower end of perinatal viability. 25 The biological mechanisms for race disparities are not completely understood. Factors such as access to health care and responsiveness to medical treatments may be affecting the racial gap in neonatal mortality. The protective association of preeclampsia has been found in several other population-based and single-center studies.4,5,7 One possible explanation for this finding may be the increased use of seizure prophylaxis, including magnesium sulfate, which has been shown in some studies to decrease the risk of adverse birth outcomes. 5

The major limitation to our study was the inability to determine why the infant was delivered prematurely. Furthermore, we do not know what level of intervention was offered to keep the infant alive. We are, however, fairly certain that our population does not include late elective terminations. Legal abortions are available up to 20 weeks in Missouri and generally do not occur unless they are associated with lethal congenital anomalies. The reliability of birth and death certificate data varies considerably for specific covariates. Maternal demographics, pregnancy outcomes, and some congenital anomalies tend to have a higher sensitivity (reporting of a condition on both the birth certificate and hospital record) than other risk factors and comorbidities.26 Access to information not included on the birth certificate (eg, medical records) may have provided us with important clinical information, such as surfactant use, intubation, and resuscitation. Neonatal transfer after birth may also have had an impact on survival; however, we did not include this variable in our model because 60% of our population was missing information. A final limitation was that our study was restricted to Missouri births. However, comparisons can be made to a population with similar demographics as our study population.

Despite the limitations, our study had several strengths. We were able to control more carefully for the confounding effects of birth weight on neonatal mortality by frequency matching on 100-g intervals of birth weight. The mean birth weight of the cases and controls were 664 g (SD = 146 g) and 655 g (SD = 152 g), respectively. Although cases were slightly younger than controls, mean gestational age of 24.7 weeks (SD = 1.9), compared with a mean of 25.6 (SD = 2.1) for controls, this was not significant. Frequency matching on birth year also accounted for the possibility that cases and controls may have received different medical interventions. Finally, our study was one of the few population-based studies that have identified risk factors for neonatal mortality, specifically among ELBW infants.

Neonatal mortality continues to be a significant public health burden in the United States. Over the last 2 decades, improvements in perinatal and neonatal care have led to great success in the survival of the smallest infants. Despite these advances, it is important that we continue to identify characteristics associated with early death as well as develop a better understanding of the etiology of preterm births. Our findings add to the current knowledge of risk factors associated with neonatal mortality among ELBW infants. Clinicians must be aware of the wide range of risk factors, some of which may be preventable (eg, mode of delivery, level of hospital care at birth), in counseling mothers and choosing appropriate medical interventions to ensure the survival of these infants.

Acknowledgments

We thank Garland Land, Joseph Stockbauer, and Janice Bakewell from the Missouri Department of Health and Senior Services for their helpful comments and for providing the data for this study.

References


Birth simulator: Reliability of transvaginal assessment of fetal head station as defined by the American College of Obstetricians and Gynecologists classification

Olivier Dupuis, MD,a,b,* Ruimark Silveira, MS,b Adrien Zentner, MS,b André Dittmar, PhD,b Pascal Gaucherand, MD,c Michel Cucherat, MD,d Tanneguy Redarce, PhD,b René-Charles Rudigoz, MDb

Unite´deGynécologieObstétrique,Hôpitalde laCroixRousse, Lyon, France,a INSA, Villeurbanne, France,b Unite´deGynécologieObstétrique,HôpitalE.Herriot, Lyon, France,c andService de Biostatistique, Hospices Civils de Lyon, Lyon, France d

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KEY WORDS
Birth simulation
Competency
Engagement

Objective: This study was undertaken to investigate the reliability of transvaginal assessment of fetal head station by using a newly designed birth simulator.

Study design: This prospective study involved 32 residents and 25 attending physicians. Each operator was given all 11 possible fetal stations in random order. A fetal head mannequin was placed in 1 of the 11 American College of Obstetricians and Gynecologists (ACOG) stations (−5 to +5) in a birth simulator equipped with real-time miniaturized sensor. The operator then determined head position clinically using the ACOG classification. Head position was described as: (1) “engaged” or “nonengaged” (engagement code); (2) “high,” “mid,” “low,” or “outlet” (group code); and (3) according to the 11 ACOG ischial spine stations (numerical code). Errors were defined as differences between the stations given by the sensor and by the operator. We determined the error rates for the 3 codes.

Results: “Numerical” errors occurred in 50% to 88% of cases for residents and in 36% to 80% of cases for attending physicians, depending on the position. The mean “group” error was 30% (95% CI 25%-35%) for residents and 34% (95% CI 27%-41%) for attending physicians. In most cases (87.5% for residents and 66.8% for attending physicians) of misdiagnosis of “high” station, the “mid” station was retained. Residents and attending physicians made an average of 12% of “engagement” errors, equally distributed between false diagnosis of engagement and non-engagement.

Conclusion: Our results show that transvaginal assessment of fetal head station is poorly reliable, meaning clinical training should be promoted. The choice not to perform vaginal delivery when the fetus is in the “mid” position strongly decreases the risk of applying instruments on an undiagnosed “high” station. Conversely, obstetricians who perform only “low” operative vaginal delivery may unnecessarily increase the risk of applying instruments on an undiagnosed “high” station.
In 1988, the American College of Obstetricians and Gynecologists (ACOG) implemented a new classification system that divided the birth canal into 11 stations according to the position of the fetal head relative to the ischial spines (−5 to +5).1 For clinical purposes, the 11 stations have been divided into 4 groups: “high” (−5, −4, −3, −2, −1), “mid” (0, +1), “low” (+2, +3), and “outlet” (+4, +5).2 There is some debate about the risk associated with “mid” pelvic operative vaginal deliveries, and only 64% of North American residency programs offer training in such deliveries.3 Only 41% of ACOG fellows claim to perform “mid” pelvic deliveries,4 whereas 86% claim to perform “low” and “outlet” forceps deliveries.4 These 4 groups can be pooled according to a third classification system that differentiates between nonengaged fetal head (“high” group) and engaged fetal head (“mid,” “low,” and “outlet” groups).

Little is known about the accuracy of clinical transvaginal assessment of head position. One study compared abdominal and transvaginal clinical assessment1 and another compared clinical and ultrasound assessment,5 but neither of these studies used a gold standard, meaning that they are subject to significant bias.

We designed a birth simulator with a fetal head equipped with a location sensor. This miniaturized, real-time tracking sensor served as a gold standard and allowed us to assess the reliability of the clinical diagnosis. We used this birth simulator to assess the value of clinical transvaginal examination by a resident or an attending physician.

Material and methods

This prospective, randomized study was performed between July 2003 and January 2004. Residents and attending physicians were recruited from 6 university maternity hospitals. We used a newly designed mechanical birth simulator that consisted of 4 parts: a fetal mannequin representing a term newborn head, a maternal mannequin, an interface pressure system, and a location system (Figure). The head was modeled on the skull of a dissected term fetus. Cranial computed tomographic images of this skull were recorded with Amira Software (TGS, Inc, San Diego, Calif). Data were exported to a fast prototyping machine with the use of a Stratasys FDM 1600 device (PADT, Inc, Tempe, Ariz) used to produce an acrylonitrile butadiene styrene (ABS) head. This head was then covered with a 5-mm thick layer of natural latex rubber. The head was equipped with a commercially available, real-time, miniaturized, tracking sensor with 6 df. This sensor can record position to within 1.8 mm and orientation to within 0.5 degrees. A computer program was developed to allow us to visualize on a PC screen and in real time the head station and location. The fetal head was attached to a pneumatic actuator via a spherical link. The maternal mannequin consisted of an anatomically correct pelvic model (Simulaids, Inc, Woodstock, NY). An interface pressure system made of an elastomer balloon inflated to 400 millibar, mimicked the pelvic muscles. The location system consisted of a pneumatic actuator (Festo AG, Inc, Esslingen, Germany), supported on a mechanical system called the “movable mechanical stair” (MMS). The actuator made it possible to move the fetal head along the x-axis from −5 to +5 cm relative to the ischial spines. The MMS was designed to locate the fetal head along the y- and z-axes. The actuator can be set in 5 positions along the z-axis (vertical axis): OA, (ROA LOA), (ROT LOT), (ROP LOP), and OP. This device could also be moved into 1 of 5 positions along the y-axis (horizontal axis): (OA OP), (LOA LOP), (ROA ROP), ROT, and LOT. Combining the 2 systems made it possible to place the fetal head in the OA, ROA, LOA, ROT, LOT, ROP, LOP, and OP locations.

The following definitions were used:

Station was defined as the position of the fetal head relative to the ischial spines according to the ACOG classification.1

High, mid, low, and outlet groups were defined according to the ACOG classification.2,7

Head engagement was defined as the descent of the biparietal plane of the fetal head to a level below that of the pelvic inlet.8

Test “reliability” was defined as the degree to which the test results were consistent.9

Three codes were used to describe the fetal head station. The “numerical” code referred to the 11 ischial spine stations according to the ACOG classification (−5 to +5). The “group” code referred to the high, mid, low, or outlet groups. Finally, the “engagement” code referred either to an “engaged” or a “nonengaged” station. The group and engagement codes were derived from the numerical code using the ACOG classification. Errors were defined as any difference between the station...
(numerical, group, or engagement) given by the operator and the station given by the sensor (e.g., –4 wrongly diagnosed as –2 or high diagnosed as mid, or nonengaged diagnosed as engaged). The rate of errors was analyzed by using the “group” and the “engagement” code. Operators were either residents or attending physicians. Location refers to the fetal head location: OA, ROA, LOA, ROT, LOT, ROP, LOP, and OP.

The following experimental protocol was used. Immediately before the experiment, each operator was allowed to examine the maternal mannequin to palpate the ischial spine, sacrum, coccyx, and fetal head. Each operator was given all 11 possible fetal stations in random order and therefore performed 11 clinical transvaginal examinations. To avoid any bias linked to the fetal head location, a second randomization was performed, giving the location that should be used for every station. The fetal head station and location were changed according to the randomization table between each experiment. For this, 1 of the authors (O.D. or R.S.) moved the fetal head along the x-axis using the actuator and along the y- and z-axes using the MMS. The position was maintained by using a mechanical screw. The real-time sensor allowed the authors to ensure that the fetal head had reached the exact station and location on the PC screen. The operator was asked to give the fetal head station using the numerical code. Operators were blinded to the results; the computer screen was turned away from the operator and no results were given during the study period.

The primary outcome analyzed was the rate of error between the real fetal head station given by the real-time miniaturized tracking sensor (gold standard) and the clinical fetal head station given by the operator using the numerical code. Secondary outcomes were rates of error expressed by using the group and engagement codes. Statistical analysis was stratified by operator to take into account the fact that 11 measurements were performed by each operator. The intraoperator error rates were calculated for each operator and thus were averaged.

Figure The birth simulator. It includes 4 parts: a fetal mannequin representing a term newborn head, a maternal mannequin, an interface pressure system, and a location system.
The group error rate by operator and the engagement error rate by operator were calculated for the 11 error occasions given to each operator. Power analysis, with a prerequisite of a 95% CI around an estimated fraction of error of no more than 20%, indicated that at least 25 subjects were required for each subgroup. Normal CIs around all observed rates were calculated and compared with t tests. P values of less than .05 were considered statistically significant. Statistical packages that were used included Excel 2001 (Microsoft Office 2001, Microsoft Corporation, Redmond, Wash) and the R statistical package (version 1.8.1, R Development Core Team, [2003]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org).

### Results

Thirty-two residents and 25 attending physicians from 6 university-affiliated maternity hospitals participated in this study. Residents had an average of 2.2 years of experience (range 0.5-5). Attending physicians had an average of 9.4 years of experience (range 4-21).

Numerical errors occurred in 50% to 88% of cases in the resident group and in 36% to 80% of cases in the attending physician group depending on the head station (Table I). Residents made an average of 3.3 group errors (95% CI 2.8-3.9), corresponding to an error rate of 30% (95% CI 25-35). None of the residents made zero group errors (Table II). Attending physicians made an average of 3.8 group errors (95% CI 3.0-4.5), corresponding to an error rate of 34% (95% CI 27-41).

Undiagnosed “high” stations accounted for 22.4% of the errors made by residents (Table IV) and 15.9% of the errors made by attending physicians (Table III).

The “mid” when really “high” type of error accounted for 87.5% and 66.8% of the misdiagnosed “high” stations for residents and attending physicians, respectively. Undiagnosed “mid” stations accounted for 30.8% and 27.7% of the resident and attending physician errors, respectively (Table III). Operator seniority did not significantly modify the proportion of correct diagnoses.

Residents made an average of 1.3 engagement errors (95% CI 1.0-1.74), corresponding to an error rate of 12% (95% CI 9%-16%) (Table IV). Attending physicians made an average of 1.3 engagement errors (95% CI 0.89-1.7), corresponding to an error rate of 12% (95% CI 8%-15%) (Table IV).

False diagnosis of engagement and false diagnosis of nonengagement accounted for approximately half of the errors for both residents and attending physicians. No significant difference was found between residents and attending physicians.

### Comment

Over the past decade, medical simulators have been developed in many specialties, including abdominal surgery, anesthesiology, pediatrics, and urology. In the United States, gynecology teaching associates (GTAs) provide hands-on training. GTAs have been trained to recognize adequate technical skills and can provide students with feedback when their cervix, fundus, or ovaries are being examined. Nevertheless, such training cannot mimic pathologic situations and is not available worldwide. Pugh and Youngblood recently reported the use of a female pelvis equipped with electronic sensors. They found that this simulator allowed an objective and reliable assessment of physical examination skills. Gonik et al were the first to report the use of a physical obstetric model, including a rotational maternal pelvis, an aluminum fetal “skeloton,” a tactile sensing glove, and a microcomputer-based data acquisition system. This simulator included fingertip sensors as well as brachial plexus and neck extension sensors. It objectively demonstrated that McRoberts positioning reduces shoulder extraction.

### Table I

<table>
<thead>
<tr>
<th>Actual position</th>
<th>Error rate (%)</th>
<th>95% CI</th>
<th>Error rate (%)</th>
<th>95% CI</th>
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<tr>
<td>-5</td>
<td>50</td>
<td>34-66</td>
<td>36</td>
<td>19-57</td>
</tr>
<tr>
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<td>-2</td>
<td>88</td>
<td>70-96</td>
<td>68</td>
<td>46-84</td>
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<td>-1</td>
<td>66</td>
<td>47-81</td>
<td>76</td>
<td>54-90</td>
</tr>
<tr>
<td>0</td>
<td>72</td>
<td>53-86</td>
<td>72</td>
<td>50-87</td>
</tr>
<tr>
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<td>81</td>
<td>63-92</td>
<td>76</td>
<td>54-90</td>
</tr>
<tr>
<td>+2</td>
<td>69</td>
<td>50-83</td>
<td>68</td>
<td>46-84</td>
</tr>
<tr>
<td>+3</td>
<td>63</td>
<td>44-78</td>
<td>76</td>
<td>54-90</td>
</tr>
<tr>
<td>+4</td>
<td>53</td>
<td>35-70</td>
<td>72</td>
<td>50-87</td>
</tr>
<tr>
<td>+5</td>
<td>56</td>
<td>38-73</td>
<td>68</td>
<td>46-84</td>
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</table>

### Table II

<table>
<thead>
<tr>
<th>No. of errors per operator (% for the 11 error occasions)</th>
<th>Residents</th>
<th>Attending physicians</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of operators (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (0.0)</td>
<td>0 (0)</td>
<td>2 (8.0)</td>
</tr>
<tr>
<td>1 (9.1)</td>
<td>5 (15.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2 (18.2)</td>
<td>3 (9.4)</td>
<td>4 (16.0)</td>
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<td>3 (27.3)</td>
<td>10 (31.3)</td>
<td>7 (28.0)</td>
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<td>4 (36.4)</td>
<td>7 (21.9)</td>
<td>2 (8.0)</td>
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<td>5 (45.5)</td>
<td>5 (15.6)</td>
<td>4 (16.0)</td>
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<tr>
<td>6 (54.5)</td>
<td>1 (3.1)</td>
<td>5 (20.0)</td>
</tr>
<tr>
<td>7 (63.6)</td>
<td>1 (3.1)</td>
<td>4 (0.0)</td>
</tr>
<tr>
<td>8-11 (72.7-100)</td>
<td>0 (0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>32 (100.0)</td>
<td>25 (100.0)</td>
</tr>
</tbody>
</table>
forces, as well as the incidence of brachial plexus stretching and clavicle fracture. The birthing simulators that are currently commercially available do not include an interface system between the maternal bony pelvis and the fetal mannequin. Furthermore, they do not make it possible to assess fetal head station or location.

There is currently a training problem in the field of obstetrics. In the United States, the use of forceps decreased by 22% between 1985 and 1992. With a training program that has 3000 deliveries annually, 12 residents per year, and a transverse arrest incidence of 0.79% to 2.93%, each resident would only perform between 2 and 7 operative vaginal deliveries in 1 year. As the frequency of operative vaginal delivery is decreasing, it is becoming increasingly difficult to provide adequate training. This might explain why 36% of North American residency programs no longer provided training in midpelvic delivery procedures.

Several classification systems have been proposed to classify the difficulties encountered during operative vaginal deliveries. In 1952, Dennen described a 4-level classification system for forceps delivery. A “low midforceps” category was created to subdivide the broad range of midforceps delivery. In 1965, the ACOG adopted a 3-level classification system (ie, high, mid, and low forceps). In 1988 a new system was created, based on the hypothesis that the difficulty of the operation and its inherent risk depend on the station from which delivery is initiated and the need to perform rotations. Maternal risk factors, such as the risk of third- and fourth-degree perineal tears, as well as fetal injuries, have been reported to be correlated with the station from which delivery is initiated. These studies led to the traditional belief that “high” operative vaginal deliveries should not be performed, whereas “mid” ones are potentially traumatic to the fetus and “low” and “outlet” ones safe.

The reliability of clinical transvaginal diagnosis of fetal head station has not been studied in detail. Sherer recently compared transvaginal digital examination and transabdominal ultrasound determinations for the diagnosis of engagement. The ultrasound method described should allow an “objective” engagement diagnosis. To locate the ultrasound transducer correctly it is necessary to locate the sacral promontory via clinical examination. The reliability of this clinical step has not been studied and cannot be considered as a gold standard.

Our study shows that the accuracy of clinical transvaginal examination is poor. With the exception of the “–5” level for attending physicians (error rate = 36%), numerical error rates were always above 50%. For residents and attending physicians, the error rates were lowest at the two ends of the scale. This can be explained by the fact that errors can occur only on one side at the ends of the scale (–5 and +5), whereas errors occurring at any other station are two sided. It can be argued that not every “numerical” error is clinically relevant. Indeed, numerical errors that do not change the group classification are clinically relevant (e.g. –2 falsely diagnosed as –1). This is why we also studied the results using the “group” code. The “group” code helps the clinician to decide whether or not to perform an

<table>
<thead>
<tr>
<th>Table III</th>
<th>Type of group error (%) for residents and attending physicians</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group code</td>
<td>High</td>
</tr>
<tr>
<td>Real position (sensor value)</td>
<td></td>
</tr>
<tr>
<td>Resident results</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>—</td>
</tr>
<tr>
<td>Mid</td>
<td>—</td>
</tr>
<tr>
<td>Low</td>
<td>2 (1.9%)</td>
</tr>
<tr>
<td>Outlet</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Attending physician results</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>—</td>
</tr>
<tr>
<td>Mid</td>
<td>—</td>
</tr>
<tr>
<td>Low</td>
<td>2 (2.1%)</td>
</tr>
<tr>
<td>Outlet</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Results that used the engagement code. Number of errors (and error rate) per operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of errors per operator (for the 11 error occasions)</td>
<td>Residents</td>
</tr>
<tr>
<td>No. of operators (%)</td>
<td>No. of operators (%)</td>
</tr>
<tr>
<td>0 (0.0%)</td>
<td>7 (21.9)</td>
</tr>
<tr>
<td>1 (9.1%)</td>
<td>14 (43.8)</td>
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<td>2 (18.2%)</td>
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<tr>
<td>3 (27.3%)</td>
<td>5 (15.6)</td>
</tr>
<tr>
<td>4 (36.4%)</td>
<td>1 (3.1)</td>
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<tr>
<td>5-11 (45.4-100)</td>
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<tr>
<td>Total</td>
<td>32 (100.0)</td>
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</table>
operative vaginal delivery. Use of the group code showed that group errors are not rare occurring in an average of 30% of cases for residents and in 34% of cases for attending physicians. Two of the 25 attending physicians made no group errors, whereas no residents made zero group errors. This might be explained by a learning curve in clinical skills.

In the case of instrumental deliveries, group errors are potentially dangerous when the operator states that the fetal head is below its real station, especially when the operator fails to identify a “high” station. These dangerous group errors accounted for 22.4% of the errors made by residents and for 15.95% of the errors made by the attending physicians. Interestingly, false diagnosis of “mid” station (“mid” when really “high”) accounted for 87.5% of these errors in the resident group (19.6% of 22.4%) and for 68% (10.65% of 15.95%) of these errors in the attending physician group. In other words, choosing not to perform “mid” instrumental deliveries would decrease the number of potentially dangerous situations considerably for most operators. Therefore, the statement of Knight et al5 that “clinical surveys of the outcome of midpelvic deliveries which have been performed by clinicians...must be considered to possibly include cases of unrecognized high forceps” is true. Such errors could explain the difference between the so-called “easy” mid operative vaginal deliveries that are “real mid operative vaginal deliveries” and “difficult” mid operative vaginal deliveries that are in reality “high operative vaginal deliveries.”

Obstetricians reading studies reporting neonatal and maternal morbidity after “mid” operative vaginal delivery should remember that some of those deliveries are actually “high” operative vaginal deliveries. Some group errors were of the “low” when really “mid” type, meaning that an operator who decides to perform only “low” instrumental deliveries will also perform unrecognized “mid” deliveries. For residents, such errors represent 14% of all errors. This explains why we believe that all residency programs should still provide training in mid operative vaginal deliveries.

Engagement is 1 of the prerequisites for an operative vaginal delivery. The traditional cutoff is the zero station. Expression of our results with the use of the engagement code showed that errors occurred in 12% of cases. In other words, should the delivery be indicated, unnecessary cesarean sections (“not engaged” if “engaged”) or dangerous operative vaginal deliveries (“engaged” if “not engaged”) will be performed in 12% of cases. For residents and attending physicians half of all errors are of the former type and half of the latter type. Knight et al5 compared the value of abdominal and vaginal examinations for the diagnosis of engagement. The 104 consecutive women were divided into 1 of 3 groups according to the results of abdominal and vaginal examinations. Engagement by abdominal examination was defined as the presence of no more than one fifth of the fetal head palpable above the pelvic brim. In 15.3% of cases, the head was found to be engaged using vaginal examination and not engaged using abdominal palpation. Unfortunately, this study compared 2 subjective types of examination (abdominal vs vaginal examination) and did not use a gold standard. It was therefore impossible to know whether errors occurred in the “abdominal” or “vaginal” group.

We are aware that our birth simulator does not mimic every clinical situation. The fetal head mannequin used does not mimic molding or caput succedaneum. These are both classical clinical pitfalls; therefore, even more errors may occur in a real situation than in this “simulation” setting.

Our data suggest that birth simulators equipped with spatial location sensor might help to assess the reliability of traditional clinical parameters. Clinical transvaginal assessment of fetal head station is not fully reliable. Clinical training on a birth simulator should be promoted and resident programs should still provide training in mid operative vaginal deliveries.

Acknowledgments

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References


Factors predicting severe perineal trauma during childbirth: Role of forceps delivery routinely combined with mediolateral episiotomy

Gernot Hudelist, MD,a,* Janos Gelle’n, MD,a Christian Singer, MD,b Ernst Ruecklinger, PhD,b Klaus Czerwenka, MD,c Othmar Kandolf, MD,a Joerg Keckstein, MDa

Department of Obstetrics and Gynecology,a Provencial Hospital Villach, Carinthia, Austria; Department of Obstetrics and Gynecology,b Division of Special Gynecology, and Department of Gynecopathology,c University of Vienna Medical Center, Vienna, Austria

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KEY WORDS
Risk factors
Perineal trauma
Forceps delivery

Objective: Anal sphincter injury and its sequelae are a recognized complication of vaginal childbirth. The aim of the present study was to identify risk factors for third- and fourth-degree perineal tears in patients undergoing either spontaneous or vaginal-assisted delivery by forceps routinely combined with mediolateral episiotomy.

Study Design: We retrospectively reviewed 5377 vaginal deliveries based on the analysis of the obstetric database and patient records of our department during a 5-year period from 1999 to 2003. Cases and control subjects were chosen randomly and patients’ records were reviewed for the following variables: maternal age, parity, gestational age, tobacco use, gestational diabetes or pregnancy-induced hypertension, use of peridural anesthesia, duration of first and second stages of labor, use of mediolateral episiotomy, forceps combined with mediolateral episiotomy, induction of labor, infant head diameter, shoulder circumference, and birth weight.

Results: Of 5044 spontaneous vaginal deliveries 32 (0.6%) and of 333 assisted vaginal deliveries 14 (4.2%) patients sustained a perineal defect involving the external sphincter. An univariate analysis of these 46 cases and 155 randomly selected control subjects showed that low parity (P = .003; Mann-Whitney U test), prolonged first and second stages of labor (P = .001, P = .001), high birth weight (P = .031), episiotomy (P = .004; Fisher exact test), and forceps delivery (P = .002) increased the risk for sphincter damage. In multivariate regression models, only high birth weight (P = .004; odds ratio [OR] 1.68, 1.18-2.41, 95% confidence interval [CI]), and forceps delivery combined with mediolateral episiotomy, (P < .001; OR 5.62, 2.16-14.62, 95% CI) proved to be independent risk factors. There was a statistical significant interaction of birth weight and head circumference (P = .012; OR 0.99, 0.98-0.99, 95% CI). Although the use of episiotomy conferred an increased risk toward a higher likelihood of severe perineal trauma, it did not reach statistical significance (P = .06; OR 2.15, 0.97-4.76, 95% CI).
Damage of the anal sphincter resulting in a third- or fourth-degree perineal tear is a relatively rare but severe complication of vaginal delivery. Although primary surgical repair of the muscle performed immediately after delivery has been associated with a favorable outcome in various studies,1-3 long-term sequelae such as perineal pain, fecal incontinence, urgency, or sexual dysfunction may occur years after. Although anorectal complaint does not appear to be a rare event in patients undergoing vaginal birth with up to 10% of all women suffering from defecatory symptoms postpartum and between 13% and 20% experiencing flatus incontinence,4-6 several follow-up studies on the outcome of perineal trauma involving the external sphincter indicate that fecal incontinence may develop in 29% to 57% of patients.4,7,8,9 In addition, patients sustaining third- or fourth-degree perineal tears are at a higher risk for the development of infection and rectovaginal fistulae.9 As a result, the number of women requesting cesarean section is constantly growing in western European countries, thereby causing controversy between obstetricians on how to reduce maternal intrapartum and postpartum complications to provide optimal care of the childbearing patient. It has therefore been the subject of several studies to identify potential risk factors associated with the development of perineal lacerations during vaginal delivery. Identified maternal and delivery variables reported in previous works include parity,10-12 maternal age,13 race,14 use of episiotomy,5,11,15-17 birth weight,12,13 assisted vaginal delivery,10,12,13,17,18 and induction of labor.17 However, delivery details such as the instrument used for assisted vaginal delivery and/or the use and type of episiotomy greatly varies between European and American study groups. American obstetricians use more forceps and fewer vacuum extractions and prefer midline episiotomy over mediolateral episiotomy, whereas the instrument of choice in most European countries is the vacuum extractor and mediolateral episiotomies are more widespread. Our clinic has adopted a procedure that lies somewhat between the 2 schools in that we combine the use of a mediolateral episiotomy with forceps delivery. Interestingly, there are only limited data on operative deliveries by forceps and combined mediolateral episiotomy.17,19-21 The aim of the present work was to further elucidate variables potentially influencing the occurrence of perineal lacerations including the external sphincter to identify a subset of patients with a higher risk for severe perineal trauma and its complications.

Material and methods

Setting

The present study was a retrospective case-control analysis of obstetric variables using a 1:3 ratio of cases and control subjects. Women included in this study delivered their children (between January 1999 and December 2003) at the Department of Obstetrics and Gynecology at the Provincial Hospital Villach, Carinthia, Austria; a referral, midsize medical center with 1300 deliveries per year. Cases and control subjects were drawn from all vaginal deliveries of >24 weeks’ gestation.

Study population

During a period of 59 months, there were 5377 women undergoing vaginal delivery. Patients with multiple pregnancies (n = 113), stillbirths (n = 24), unavailability of patient records (n = 1), breech deliveries (n = 7), caesarian sections (n = 1095), and vacuum deliveries (n = 8) because of the small number of samples within the study period compared with the use of forceps were excluded from the analysis. After strict application of inclusion and exclusion criteria, data were divided into 2 groups: 1 group (cases) including all patients (n = 46) with laceration of the perineum greater than second degree. To avoid duplication in the case of women who attended the hospital more than once for delivery, patient data were de-duplicated before sampling. A tear greater than second degree was defined as any tear causing any visible interruption of the external anal sphincter, causing exposure or damage to the muscle (partial or complete third degree laceration) with/without involvement of the anal mucosa and/or torn anal epithelium (fourth degree). One case was excluded from further analysis because of incomplete values on crucial predictors in the patient record leaving 46 cases for further investigation. The second group (controls) was selected randomly on the basis of a blinded protocol from patient records of women undergoing uninstrumented or vaginal-operative deliveries. Therefore, the first 3 patients admitted subsequently to the delivery unit after

Conclusions: In consistence with previous reports, women who are vaginally delivered of a large infant are at a high risk for sphincter damage. Although the rate of these complications was surprisingly low in vaginally assisted childbirth, the use of forceps, even if routinely combined with mediolateral episiotomy, should be minimized whenever possible.

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the identified case (≥third-degree perineal tear) were used as controls.

Definition and collection of patient data

Maternal characteristics and delivery details were transcribed from patients’ records to a computerized data system. Information on maternal variables included age, prepregnancy body mass index, prevalence of gestational diabetes or pregnancy-induced hypertension (yes/no), tobacco use (yes/no), parity and gestational age. Delivery characteristics included data on induction of labor (yes/no), augmentation of labor with oxytocin or artificial rupture of membranes (yes/no), use of peridural anesthesia (yes/no), duration of first and second stages of labor (minutes), mode of delivery (uninstrumented, use of forceps), performance of episiotomy (yes/no), fetal weight (g), and head and shoulder circumference (cm).

Statistical analysis

SSPS version 10 (Statistical Package for Social Sciences; SSPS Inc., Chicago, IL) was used for statistical analysis of patient data. Distribution of maternal and obstetrical predictor variables was compared with the use of Mann-Whitney U test (continuous predictors) and Fisher exact test (categorical predictors). A P value less than .05 was considered statistically significant. Multivariate logistic regression analysis was performed to evaluate the influence of potential risk factors on severe perineal lacerations. Odds ratios and 95% confidence intervals were estimated to describe the prognostic strengths of variables potentially influencing the occurrence of third- or fourth-degree perineal tears considered in the logistic regression model. A power analysis resulted in a statistical power of 84.4% and was calculated for detection of a difference of 20% (own experience) using 46 cases and 155 controls.

Results

In 5377 vaginal deliveries that were reviewed during the study period, the incidence of third- and fourth-degree lacerations of the perineum was 0.9% (47 of 5377 patients). Three hundred thirty-three of 5377 (6.2%) women in the underlying patient population delivered their children with the aid of forceps and in 14 of the 333 (4.2%) vaginal-operative births severe perineal tears occurred. Of 5044 spontaneous vaginal deliveries, 32 patients (0.6%) sustained a 3rd- or 4th-degree perineal tear. Median maternal age in the sample group was 29.0 (26.0-31.3) years and 29.0 (25.0-33.0) years in controls (not significant, P > .05). Results of univariate analysis of maternal characteristics and delivery details of cases and controls are listed in Tables I and II. As shown, there were no significant differences in the age, gestational age, body mass index, use of tobacco, prevalence of pregnancy-induced hypertension, or gestational diabetes mellitus. Women with greater than third-degree tearing were more likely to be nulliparous than the controls (P = .003). Furthermore, duration of first- and second-stage labor (P = .011, P = .001), delayed second-stage labor (P = .022), incidence of episiotomy (P = .004) and/or forceps delivery (P = .002), and higher birth weight (P = .031) were significantly associated with the occurrence of third- and fourth-degree perineal tears between the 2 groups (Table II). Other intrapartum and postpartum details, such as the use of peridural anesthesia, labor induction, or augmentation via artificial rupture of membranes or oxytocin use and head or shoulder circumference of the newborn were comparable between cases and controls. A comparison of rates of (1) spontaneous deliveries without episiotomy, (2) spontaneous deliveries with episiotomy, and (3) episiotomy routinely combined with forceps in cases and controls is depicted in the Figure. Table III shows the results of a multivariate logistic regression model. Only 2 of the 17 predictor variables were significantly related to the likelihood of having a severe perineal trauma greater than second degree. Episiotomy alone conferred a trend toward an increased risk (increased odds ratio [OR]) of anal sphincter rupture (P = .060; OR 2.15, 95% confidence interval [CI], 0.97-4.76), although this was not statistically significant. Forceps delivery (plus routinely performed episiotomy), however, was significantly associated with perineal tear (P < .001, OR 5.62, 95% CI, 2.16-14.62). In addition, and high birth weight (P = .001, OR 1.68, 95% CI, 1.18-2.41) conferred an increased risk of severe perineal laceration in adjusted analysis. In addition, a strong correlation between head circumference and birth weight was observed (Pearson’s correlation coefficient 0.69, P < .001). Furthermore, a statistical significant interaction of these two variables (birth weight and head circumference, P = .012) was found that resulted in a decrease of the OR of birth weight (risk for third-/fourth-degree perineal laceration) with each additional centimeter of head circumference (OR 0.99, 95% CI, 0.98-0.99). Explained in other words, each centimeter of head circumference decreased the odds ratio of birth weight by 1% in the total sample. However, in a multiple model, the influence of the parameter head circumference did not reach statistical significance when the effect of interaction with birth weight was taken into account.

Comment

The present study depicts risk factors that are associated with severe perineal trauma during spontaneous and forceps-assisted vaginal deliveries at midsize central European referral hospital. The goal of the present
work was to identify risk factors for third and fourth degree perineal lacerations. Forty-seven (0.9%) out of 5377 patients experienced third-degree perineal tears during vaginal delivery. Forceps were used in 333 (6.2%) cases and led to severe perineal damage in 14 (4.2%) patients. Using cases and control subjects, univariate analysis revealed nulliparity, prolonged first and second stage labor, high birth weight, and the type of delivery as risk factors for disruption of the external sphincter. After adjustment in multivariate regression analysis, high birth weight and episiotomy in conjunction with forceps proved to be independent risk factors. Additionally, there was a significant interaction between birth weight and head circumference. The isolated use of episiotomy conferred an increased risk for the occurrence of sphincter damage but this was of marginal statistical significance. The overall incidence of third-/fourth-degree perineal tears (0.9%) regardless of parity is slightly higher than the 0.6% reported by Sultan and colleagues\textsuperscript{4} and the 0.8% in primigravid women presented by Gupta et al.,\textsuperscript{21} but lies far below the rates reported in various other studies ranging from 2.2%,\textsuperscript{13} 2.9%,\textsuperscript{22} 3.3%,\textsuperscript{23} and 5.8%\textsuperscript{24} up to 7.7%,\textsuperscript{11} 8.4%,\textsuperscript{25} 10.2%,\textsuperscript{14} and 10.8%.\textsuperscript{15} Although the comparison of rates of severe perineal lacerations between different institutions must be done with caution because of differences in the diagnostic approach for the detection of external sphincter damage (visible disruption vs use of anal endosonography), varying frequencies and types of episiotomies used (liberal or restricted, median or mediolateral), and different policies regarding the use of vacuum and/or forceps deliveries, the presently found

| Table I | Maternal characteristics of the study population and univariate analysis of cases and controls by Mann-Whitney’s U test and Fisher’s exact test |
|-----------------|-----------------|-----------------|-----------------|
| Characteristic  | 3\textsuperscript{rd}/4\textsuperscript{th} degree laceration (n = 46) | Controls (n = 155) | Statistical significance (P ≤ .05) |
| Median (quartiles) age (y) | 29.0 (26.0 to 31.3) | 29.0 (25.0 to 33.0) | n.s. |
| Primiparity (yes/no) | 32 (69.6%) | 71 (45.8%) | .003 |
| Median (quartiles) gestational age (wk + d) | 40 + 3 (39 + 4 to 41 + 1) | 40 + 0 (39 + 1 to 40.5) | n.s. |
| ≥35 + 0 – <36 + 0 (wk) | 0 (0%) | 1 (0.6%) | n.s. |
| ≥36 + 0 – <38 + 0 (wk) | 3 (6.5%) | 10 (6.5%) | n.s. |
| ≥38 + 0 – ≤40 + 0 (wk) | 15 (32.6%) | 72 (46.5%) | n.s. |
| Postdates > 40 wk gestation (yes/no) | 28 (60.9%) | 72 (46.5%) | n.s. |
| Tobacco use (yes/no) | 7 (15.2%) | 21 (13.5%) | n.s. |
| Gestational diabetes (yes/no) | 1 (2.2%) | 2 (1.3%) | n.s. |
| PIH (yes/no) | 2 (4.4%) | 5 (3.2%) | n.s. |

| Table II | Delivery details of the study population and univariate analysis of cases and controls by Mann-Whitney’s U test and Fisher’s exact test |
|-----------------|-----------------|-----------------|-----------------|
| Characteristic  | 3\textsuperscript{rd}/4\textsuperscript{th} degree laceration (%) (n = 46) | Controls (%) (n = 155) | Statistical significance (P ≤ .05) |
| Labor induction (yes/no) | 2 (4.3%) | 12 (7.7%) | n.s. |
| Augmentation (yes/no) | 11 (23.9%) | 43 (27.7%) | n.s. |
| Peridural anaesthesia (yes/no) | 2 (4.4%) | 11 (7.1%) | n.s. |
| Median (quartiles) duration of 1st stage (min) | 226.0 (182.3 to 356.3) | 196.0 (123.0 to 286.0) | .011 |
| Median (quartiles) duration of second stage (min) | 40.0 (19.5 to 72.3) | 21.0 (11.0 to 40.0) | .001 |
| Delayed 2nd stage (>2h) (yes/no) | 6 (13%) | 7 (4.5%) | .022 |
| Forceps (yes/no) | 13 (28.3%) | 14 (9.0%) | .002 |
| Episiotomy (alone) (yes/no) | 29 (63.0%) | 59 (38.1%) | .004 |
| Median (quartiles) birth weight (g) | 3570 (3325 to 3847) | 3336 (3060 to 3744) | .031 |
| below 3000 g | 5 (10.9%) | 32 (20.6%) | n.s. |
| 3000g to 4000 g | 34 (73.9%) | 105 (67.7%) | n.s. |
| above 4000 g | 7 (15.2%) | 18 (11.7%) | n.s. |
| Median (quartiles) head circumference (cm) | 34.0 (33.0 to 35.1) | 34.0 (33.0 to 35.0) | n.s. |
| Median (quartiles) shoulder circumference (cm) | 37.5 (35.0 to 39.0) | 37.0 (35.0 to 39.0) | n.s. |
incidence of 0.9% at an institution exclusively using forceps for vaginal operative delivery is relatively low. In accordance with previous data, high birth weight was an independent risk factor for the occurrence of third- and fourth-degree perineal lacerations. One simple reason may be the greater susceptibility and vulnerability to disruption of a perineum that is exposed to a greater tension with higher birth weight. Interestingly, nulliparity and thus lower perineal elasticity was only associated with a higher rate of sphincter damage in univariate analysis but did not prove to be an independent risk factor in the multivariate regression model. The use of episiotomy to prevent damage of the external anal sphincter is still subject of constant debate. Several lines of evidence indicate a strong association between the performance of midline episiotomy, and anal sphincter tears. This is supported by recent study done by Nager and co-workers who showed that midline episiotomy significantly increased perineal laceration length and incidence of sphincter disruption by adding nearly 3 cm to perineal tears. The role of mediolateral episiotomy as

**Table III** Outcome of multivariate logistic regression analysis on variables that potentially influence the incidence of 3rd/4th degree lacerations

<table>
<thead>
<tr>
<th>Regression model</th>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample</td>
<td>Birth weight (100g)</td>
<td>1.68^ (1.18 to 2.41)</td>
<td>.004</td>
</tr>
<tr>
<td></td>
<td>Head Circumference</td>
<td>1</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Type of delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Episiotomy (alone)</td>
<td>2.15 (0.97 to 4.76)</td>
<td>.060*</td>
</tr>
<tr>
<td></td>
<td>(vs. spontaneous delivery)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forceps plus Episiotomy</td>
<td>5.62 (2.16 to 14.62)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td></td>
<td>(vs. spontaneous delivery)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^ Each centimeter of increasing head circumference leads to a decrease in the OR of birth weight by 1cm (significant interaction between birth weight and head circumference, P = .012).
a contributing factor for third- and fourth-degree lacerations is discussed controversially. By investigating a total of 50,210 vaginal deliveries, Angioli et al.\(^1\) concluded that the episiotomy procedure per se, regardless of the type of episiotomy used, represents an independent risk factor for sphincter disruption. Bek et al.,\(^2\) Bodner et al.,\(^2\) and Bodner-Adler and colleagues\(^2\) found an increased risk of anal sphincter tear when mediolateral episiotomy was used. By contrast, Poen et al.,\(^3\) Shiono et al.,\(^4\) and de Leeuw et al.\(^5\) showed that mediolateral episiotomy was protective against anal sphincter damage and fecal incontinence after vaginal delivery, and Hendriksen and coworkers\(^3\) and Buekens et al.\(^6\) found no association between mediolateral episiotomy and lesions of the anal sphincter. In the present study, the use of mediolateral episiotomy conferred an increased risk of severe perineal damage although the result was of marginal statistical significance, possibly because of the small number of samples investigated. Accordingly, our results, although showing a trend toward an increased risk, are in concordance with the data presented by Hendrikson et al.\(^3\) and Buekens et al.\(^6\) suggesting that neither a restrictive nor a very liberal use of mediolateral episiotomy in spontaneous vaginal births yields optimal results. Vaginal operative delivery, especially the use of forceps, is a well-known cause of third- and fourth-degree perineal tears. The majority of research conducted in this field showed that forceps delivery significantly predicted anal sphincter injury.\(^4\),\(^11\)-14,20,33,34 However, in a retrospective study of 16,172 primigravid vaginal deliveries conducted by Gupta et al.,\(^21\) instrumental delivery by the aid of forceps was not found to be an independent risk factor for sphincter damage with 36 (1.6%) of 2311 forceps deliveries resulting in third-degree lacerations. In contrast to several studies showing that anal sphincter injury is likely to complicate more than 60% of forceps deliveries, the incidence of 1.6% presented by Gupta and colleagues\(^23\) and another 13% in a prospective study investigating 93 females undergoing forceps delivery by de Parades et al.\(^15\) is surprisingly low. The present study clearly identified forceps delivery routinely combined with mediolateral episiotomy as an independent risk factor for injury of the anal sphincter using cases and controls. However, only 14 of 333 (4.2%) deliveries conducted by forceps resulted in a third- or fourth-degree perineal laceration. One possible reason for the low frequency of anal injury in the present study may be that forceps delivery is a delicate technique that demands exact knowledge of the station and presentation of the descending caput. Thus, forceps delivery conducted in teaching institutions with a high turnover in trainees may yield a higher risk of perineal damage compared with hospitals staffed exclusively with fully trained obstetricians and low turnover in training personnel like our institution. The most significant risk factors found for severe perineal damage were high birth weight and assisted vaginal delivery. To reduce the occurrence of third- and fourth-degree perineal lacerations and its sequelae, the use of forceps, particularly in the presence of a large baby, should therefore be minimized whenever possible.

References


The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth

Israel Hendler, MD,* Robert L. Goldenberg, MD, Brian M. Mercer, MD, Jay D. Iams, MD, Paul J. Meis, MD, Atef H. Moawad, MD, Cora A. MacPherson, PhD, Steve N. Caritis, MD, Menachem Miodovnik, MD, Kate M. Menard, MD, Gary R. Thurnau, MD, Yoram Sorokin, MD

National Institute of Child Health and Human Development, Maternal-Fetal Medicine Units Network, National Institutes of Health, Bethesda, Md

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Objective: The purpose of this study was to evaluate the relationship between prepregnancy maternal body mass index and spontaneous preterm birth and indicated preterm birth. Study design: This was a secondary analysis of the Maternal-Fetal Medicine Units Network, Preterm Prediction study. Patients were classified into categories that were based on their body mass index. Rates of indicated and spontaneous preterm birth were compared. Results: Five hundred ninety-seven (20.5%) of 2910 women were obese. Obese women had fewer spontaneous preterm births at <37 weeks of gestation (6.2% vs 11.2%; P < .001) and at <34 weeks of gestation (1.5% vs 3.5%; P = .012). Women with a body mass index of <19 kg/m² had 16.6% spontaneous preterm birth, with a body mass index of 19 to 24.9 kg/m² had 11.3% spontaneous preterm birth, with a body mass index of 25 to 29.9 kg/m² had 8.1% spontaneous preterm birth, with a body mass index of 30 to 34.9 kg/m² had 7.1% spontaneous preterm birth, and with a body mass index of ≥35 kg/m² had 5.2% spontaneous preterm birth (P < .0001). Indicated delivery was responsible for an increasing proportion of preterm births with increasing body mass index (P = .001). Obese women had lower rates of cervical length <25 mm (5% vs 8%; P = .012). Multivariable regression analysis confirmed a lower rate of spontaneous preterm birth in obese gravid women (odds ratio, 0.57; 95% CI, 0.39-0.83; P = .003). Conclusion: Obesity before pregnancy is associated with a lower rate of spontaneous preterm birth.

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* Reprint requests: Israel Hendler MD, Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, Hutzel Hospital-Wayne State University, 3990 John R Rd, Detroit, MI 48201.

E-mail: ihendler@med.wayne.edu

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Obesity is defined by the National Institutes of Health as a body mass index (BMI) of ≥30 kg/m². The prevalence of obesity among adults in the United States has increased from 12% in 1991 to 20.9% in 2001. Obesity in nonpregnant women is a known risk factor for many disorders, including diabetes mellitus, atherosclerosis, and certain malignancies, and it is the second leading cause of preventable death in the United States. Previous studies have reported the association between maternal obesity and many adverse pregnancy outcomes, which include fetal anomalies, miscarriages, preeclampsia, gestational diabetes mellitus, cesarean deliveries, shoulder dystocia, and intrauterine fetal demise. However, evidence regarding the association of maternal obesity and spontaneous preterm birth (SPB) is conflicting. Although some studies suggest that obesity does not influence the rate of preterm birth at <37 weeks of gestation, other studies have found reduced rates of preterm birth in obese and morbidly obese patients. Finally, other studies have reported increased preterm birth in obese gravidas. Of note, in these retrospective studies, the main objective was to assess the adverse effects of obesity on pregnancy; preterm birth was one of many variables that were studied; the type of preterm birth (ie, after spontaneous preterm labor, preterm premature rupture of membranes, or indicated labor) was not distinguished, and confounding variables that were associated with preterm birth were not addressed.

At the other end of the spectrum, low maternal weight has been associated repeatedly with an increased risk of SPB. In a previous analysis from the Preterm Prediction Study of the Maternal-Fetal Medicine Units Network, we evaluated clinical risk factors for preterm birth and found a significantly increased risk of SPB with maternal BMI at <19.8 kg/m². The purpose of this analysis was to further evaluate the relationship between maternal BMI and the rate of spontaneous and indicated preterm birth, after controlling for potentially confounding factors, in a prospectively evaluated cohort of women.

### Material and methods

This was a secondary analysis of the prospective observational Preterm Prediction Study performed by the Maternal-Fetal Medicine Units Network of the National Institute of Child Health and Human Development. The primary study was conducted in 10 medical centers from 1992 to 1994. The overall study population and methods for this study has been described previously and will be briefly reviewed. Institutional Review Board approval was obtained at each of the 10 centers that participated in the original investigation. Each study participant provided informed consent. Exclusion criteria included multifetal gestation, prenatally detected major fetal anomalies, a history of cerclage in the current pregnancy and placenta previa. Gestational age was based on the last menstrual period, if the last menstrual period and the earliest ultrasound evaluation agreed within 10 days. If not, the earliest ultrasound evaluation was used to define gestational age. The initial study visit occurred at 23 to 24 weeks of gestation, with 3 additional visits scheduled at 2-week intervals until 31 weeks of gestation. Extensive demographic and outcome data were collected. Samples of maternal serum and cervical fluid, which included Gram stain for diagnosis of bacterial vaginosis, were collected at the initial (23-24 weeks of gestation) and the third visit (27-28 weeks of gestation).

We performed a secondary analysis on all patients for whom maternal height and pre-pregnancy weight were available. Prepregnancy maternal BMI was calculated as follows:

\[
BMI = \frac{weight \ (kg)}{height \ (m)^2}
\]

where

- **BMI** is the body mass index in kg/m²
- **weight** is the weight in kilograms
- **height** is the height in meters

### Table I: Characteristics and pregnancy outcome in obese and nonobese women

<table>
<thead>
<tr>
<th>Patients characteristic</th>
<th>Obese (n = 597)</th>
<th>Nonobese (n = 2313)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)*</td>
<td>36.2 ± 5.3</td>
<td>22.7 ± 3.5</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Maternal age (y)*</td>
<td>25.7 ± 5.6</td>
<td>23.3 ± 5.4</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Gravidity (n)*</td>
<td>3.0 ± 1.8</td>
<td>2.6 ± 1.6</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Nulliparity (%)</td>
<td>33</td>
<td>44</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Education (y)*</td>
<td>12.1 ± 1.7</td>
<td>11.8 ± 2.0</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Married (%)</td>
<td>33</td>
<td>27</td>
<td>.005</td>
</tr>
<tr>
<td>Previous SPB (%)</td>
<td>10</td>
<td>13</td>
<td>.019</td>
</tr>
<tr>
<td>Black race (%)</td>
<td>65</td>
<td>62</td>
<td>.15</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>29</td>
<td>31</td>
<td>.31</td>
</tr>
<tr>
<td>Family income &lt; $800/mo (%)</td>
<td>64</td>
<td>61</td>
<td>.15</td>
</tr>
<tr>
<td>Gestation at delivery (wk)*</td>
<td>38.6 ± 2.5</td>
<td>38.3 ± 2.6</td>
<td>.005</td>
</tr>
<tr>
<td>Cesarean delivery (%)</td>
<td>29</td>
<td>15</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Birth weight (g)*</td>
<td>3287 ± 660</td>
<td>3114 ± 633</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Birth weight &gt; 4000 g (%)</td>
<td>10</td>
<td>5</td>
<td>&lt; .0001</td>
</tr>
</tbody>
</table>

* Data are given as mean ± SD.
for each patient (weight in kilograms/height in meters$^2$). For some of the analyses, patients were classified as non-obese (BMI, $< 30$ kg/m$^2$) or obese (BMI, $\geq 30$ kg/m$^2$), for other analyses, women were classified into groups according to the National Institutes of Health guidelines,$^1$ underweight (BMI, $< 19$ kg/m$^2$), normal weight (BMI, 19-24.9 kg/m$^2$), overweight (BMI, 25-29.9 kg/m$^2$), class I obesity (BMI, 30-34.9 kg/m$^2$), and class II or morbid obesity (BMI, $\geq 35$ kg/m$^2$). Our primary outcome was SPB because of premature rupture of membranes or spontaneous labor before 37 weeks of gestation. Other outcomes included SPB at $< 34$ and $< 32$ weeks and the rate of indicated preterm birth.

Statistical analyses were performed with the SAS software (version 8.2; SAS Institute Inc, Cary, NC). Categoric variables were compared with the use of the chi-squared and Fisher’s exact tests. The Mantel-Haenszel chi-squared test was used to test for trends across BMI categories. Multivariable logistic regression was used to assess the relationship between SPB and maternal BMI, while being controlled for potential confounders (age, race, previous SPB, bacterial vaginosis, fetal fibronectin, and cervical length [CL]).

**Results**

A total of 2910 of the originally enrolled 2929 patients met eligibility requirements and were included in this analysis. There were 597 obese women (BMI, $\geq 30$ kg/m$^2$; 20.5%). Obese women were older than nonobese women, were more likely to be multiparous, had more years of education, were married more often, and were less likely to have had a previous spontaneous preterm delivery (10% vs 13%; $P = .019$) than nonobese women (Table I). However, there were no differences in the rate of smoking, race, or family income between groups. Obese women had larger infants (3287 $\pm$ 660 g vs. 3114 $\pm$ 633 g; $P < .0001$), delivered at a more advanced gestational age (38.6 $\pm$ 2.5 weeks vs 38.3 $\pm$ 2.6 weeks; $P = .005$), and had more frequent cesarean deliveries (29% vs 15%; $P < .0001$). Obese patients had significantly lower rates of SPB at $< 37$ weeks of gestation (6.2% vs 11.2%; $P = .0001$), and at $< 34$ weeks of gestation (1.5% vs 3.5%; $P = .012$; Figure 1). The odds ratio (OR; 95% CI) of an obese patient to have a SPB was approximately one-half that for a nonobese patient ($< 37$ weeks of gestation: OR, 0.5; 95% CI, 0.4-0.7; $< 34$ weeks of gestation: OR, 0.4; 95% CI, 0.2-0.8; $< 32$ weeks of gestation: OR, 0.5, 95% CI, 0.2-1.3).

When the patients’ BMIs were classified into groups according to the National Institutes of Health guidelines,$^1$ the risk of SPB at $< 37$ weeks of gestation progressively decreased with increasing BMI: underweight, 16.6%; normal weight, 11.3%; overweight, 8.1%; class I obesity, 7.1%; class II or morbid obesity, 5.2%; $P < .0001$; Figure 2).

The total rate of preterm deliveries, which included both spontaneous and indicated deliveries, was also lower in obese women (11.1% vs 15.3%; $P = .009$). The frequency of indicated preterm birth was comparable in obese and nonobese patients (4.9% vs 4.0%), but indicated preterm birth accounted for a higher percentage of preterm birth in the obese than nonobese patient (44% vs 24%; $P = .003$). Figure 2 shows the relative proportions of indicated preterm birth and SPB for each BMI group. In obese patients, 18 of 29 indicated preterm deliveries (62%) were due to maternal preeclampsia, compared with 33 of 92 indicated preterm deliveries (36%) for the nonobese women ($P = .013$). Among women who were delivered at $< 37$ weeks of gestation, we found indicated preterm birth to be responsible for an increasing proportion of preterm births with increasing maternal BMI ($P = .001$).

Obese women had a longer CL compared with nonobese women, (36.5 $\pm$ 8.4 mm vs 34.9 $\pm$ 8.1 mm;
P < .0001; Table II). Obese women had lower rates of CL < 30 mm (16% vs 21%; P = .02) and CL < 25 mm (5% vs 8%; P = .012). However, fetal fibronectin positivity and bacterial vaginosis were comparable among obese and nonobese gravidas.

Multivariable logistic regression analysis was performed to adjust for confounding variables that are known to be associated with SPB: maternal age, parity, education, history of SPB, black race, bacterial vaginosis, fetal fibronectin positivity, and CL at 23 to 24 weeks of gestation. Obesity was associated significantly with decreased SPB at < 37 weeks of gestation (OR, 0.57; 95% CI, 0.39-0.83; P = .003). The results were similar, but not significant, for SPB at < 34 weeks of gestation (OR, 0.58; 95% CI, 0.28-1.21; P = .15). When BMI was included in the model as a continuous variable, the adjusted odds ratio for SPB at < 37 weeks of gestation declined by 21% for each 5-unit increase in maternal BMI (P < .0001).

Comment

BMI, which is derived from the weight and height measurements, is one of the best markers of nutritional status and is used to classify populations from thin to obese. In this study, we evaluated the entire range of prepregnancy BMIs and compared them to the rates of indicated preterm births and SPBs. We found that prepregnancy obesity, defined as BMI ≥ 30 kg/m², was associated with fewer total preterm births and fewer SPBs. Maternal thinness on the other hand was associated with increased preterm birth and especially SPBs. A significantly high percentage of the preterm births of obese women were indicated, often in association with preeclampsia, compared with the preterm births of thin women. An additional finding is that obese women tended to have longer CLs than nonobese women.

In agreement with our results, Gross et al. found that obese patients (body weight > 90 kg) had a 9.9% rate of preterm birth at < 38 weeks of gestation compared with 19.9% for nonobese patients. Kumari et al. analyzed 488 morbidly obese patients (BMI ≥ 40 kg/m²) and found an OR of 0.1 (95% CI, 0.01-0.7) for preterm birth at < 37 weeks of gestation. Sebire et al. found that obese gravidas (n = 287,213) had a reduced risk for preterm birth at < 32 weeks of gestation (OR, 0.81; 95% CI, 0.69-0.95) but not at < 37 weeks of gestation; Cnattingius et al. found an increased risk for SPB (n = 167,750) at ≤ 32 weeks of gestation in obese nulliparous patients (OR, 1.6; 95% CI, 1.1-1.3). Alternatively, Cedergren et al. found an increased risk for PTB (n = 805,275) at < 37 and < 32 weeks of gestation (4.5% and 0.6% for BMI < 29 kg/m², compared with 5.4% and 0.8% for BMI 29.0-35 kg/m²).

The disparity in results between different studies may be due to the use of population registries, to different definitions of spontaneous and indicated preterm birth, or to the different populations that are studied. Our analysis has the benefit of being based on prospectively collected data in a study that was aimed to determine risk factors for SPB, and the results are corrected for confounding variables that are associated with SPB.

Many studies have found an association between low maternal weight and an increased risk of SPB. Ehrenberg et al. recently described a population of 15,196 patients in which low BMI (< 19.8 kg/m²) at conception and low BMI at the time of birth were associated with an increased risk for SPB. Using a multivariable analysis in a population of 17,000 patients, Wen et al. showed that a previous preterm delivery and very low maternal weight had the greatest association with preterm birth. Thus, because low maternal weight is associated with an increased rate of SPB, there may be a continuous inverse association between BMI and the risk for SPB.

Obese women had lower rates of CL of < 30 mm (17% vs 21%; P = .02) and CL of < 25 mm (5% vs 8%; P = .012). Similar results were found in Thai women, in whom the cervix was significantly longer in women with a BMI of > 26 kg/m². The longer CL may explain part of the reduced rate of SPB that is seen in obese women.

Preterm births that occur < 30 weeks of gestation are more often associated with intra-amniotic inflammation and infection. Maternal obesity is known to be associated with an increased production of systemic proinflammatory cytokines. Thus, the reduced rate of spontaneous preterm labor in the obese population is not likely due to a reduced systemic inflammatory process, but whether an actual infectious process is involved is unknown. Some studies describe malnutrition as a factor in the cause of SPB. Decreased intake of calories, proteins, vitamins, and minerals, which often are associated with decreased BMI, may explain the higher rate of SPB in thin patients. In obese women, the increased intake of various nutrients may be related to a reduced rate of SPB. However, by an unknown mechanism, obesity is associated with an increased risk

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**Table II** Tests predictive of SPB at 24 weeks of gestation in obese and nonobese women

<table>
<thead>
<tr>
<th>Clinical characteristic (visit 1)</th>
<th>Obese (n = 595)</th>
<th>Nonobese (n = 2301)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (mm)*</td>
<td>36.5 ± 8.4</td>
<td>34.9 ± 8.1</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>CL &lt; 30 mm (%)</td>
<td>16.5</td>
<td>21.0</td>
<td>.015</td>
</tr>
<tr>
<td>CL &lt; 25 mm (%)</td>
<td>5.2</td>
<td>8.3</td>
<td>.012</td>
</tr>
<tr>
<td>CL &lt; 20 mm (%)</td>
<td>2.7</td>
<td>3.2</td>
<td>.51</td>
</tr>
<tr>
<td>Fetal fibronectin positive (%)</td>
<td>3.4</td>
<td>4.2</td>
<td>.37</td>
</tr>
<tr>
<td>Bacterial vaginosis positive (%)</td>
<td>23.5</td>
<td>23.1</td>
<td>.84</td>
</tr>
</tbody>
</table>

* Data are given as mean ± SD.
of preeclampsia; thus, the rate of indicated preterm births is increased in obese women.

In summary, we found a strong inverse association between prepregnancy BMI and SPB at <37 weeks of gestation. Further research is needed to investigate the different mechanisms that are responsible for spontaneous and indicated preterm birth in obese and nonobese women.

Acknowledgments


References


Assessment of cervical antibody concentrations fails to enhance the value of cervical length as a predictor of preterm delivery

Rodney K. Edwards, MD, MS, Ronald J. Ferguson, PhD, Jonathan J. Shuster, PhD, Douglas Theriaque, MS, Susan Gentry, MSN, ARNP, Patrick Duff, MD

Departments of Obstetrics and Gynecology and Statistics, University of Florida College of Medicine, Gainesville, Fla

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KEY WORDS
Antibody concentration
Bacterial vaginosis
Preterm delivery

Objective: The purpose of this study was to determine if cervical fluid antibody concentrations can enhance the value of cervical length in predicting risk of preterm delivery.

Study design: We obtained cervical fluid samples with preweighed cellulose wicks from a prospective cohort of women 23 to 32 weeks’ gestation with signs and symptoms of preterm labor and intact membranes. Total immunoglobulin A and G (IgA and IgG) concentrations were determined by enzyme-linked immunosorbent assay. Bacterial vaginosis was diagnosed by Gram stain, and cervical length was measured with endovaginal ultrasound.

Results: For subjects with term (n = 77) and preterm (n = 24) deliveries, median IgA and IgG concentrations were 736 vs 643 μg/mL (P = .33) and 1528 vs 1769 μg/mL (P = .85). For subjects with normal flora (n = 71), intermediate flora (n = 14), and bacterial vaginosis (n = 16), median IgA and IgG concentrations were 717, 624, and 774 μg/mL (P = .90) and 1383, 1553, and 2731 μg/mL (P = .02). In a forward stepwise logistic regression model, cervical length was the only factor associated with preterm delivery (P < .001).

Conclusion: Measuring the concentrations of IgA and IgG in cervical fluid does not enhance the value of cervical length in predicting risk of preterm delivery.

More than 12% of all births occur before 37 weeks’ gestation. The complications of preterm birth cause more than 70% of the deaths of nonanomalous neonates, and are responsible for the majority of morbidity suffered by such infants.

Bacterial vaginosis is a polymicrobial infection that is an established risk factor for preterm delivery. Bacterial vaginosis is characterized by an increase in vaginal pH, an amine odor, and an increased bacterial count. In this disorder, there is a replacement of the naturally predominant bacteria in the vaginal flora (Lactobacillus sp.) by a complex mixture of anaerobic and facultative anaerobic bacteria. The mechanism by which bacterial vaginosis causes preterm delivery is thought to be due to ascension of organisms through the cervix and into the uterus.
Bacteria colonize the vagina both in patients with normal vaginal flora and in those with bacterial vaginosis. Because the uterus normally is sterile, it seems logical that factors at the level of the cervix are involved in maintaining the sterility of the upper genital tract. The length of the cervix, measured with endovaginal ultrasound examination, is inversely related to the risk of preterm delivery. However, the reason for this relationship is not well defined.

Cervical mucus displays antimicrobial properties in both nonpregnant and pregnant women. Secretory antibodies, or immunoglobulins, function to perform immune exclusion at mucosal surfaces. Immunoglobulin A (IgA) and immunoglobulin G (IgG) are the predominant classes of antibody recoverable from the endocervix. These antibodies act to exclude vaginal microorganisms from the upper genital tract. We speculated that the reason for the inverse relationship between cervical length and preterm delivery was that the cervix functions as an “immune tunnel” between the vagina and the uterine cavity. A shorter cervical canal and/or a lower concentration of antibodies within this cervical canal might allow organisms easier access to the uterine cavity. The objective of this study was to determine whether measuring the concentrations of antibodies in cervical fluid could further refine the risk of preterm delivery associated with cervical length.

Material and methods

We performed a prospective observational cohort study at Shands Hospital at the University of Florida. Subjects were enrolled from September 2001 to March 2003. Women were eligible for inclusion in this cohort if they presented to the labor and delivery unit for evaluation of uterine contractions, had a singleton gestation, and were between 23 and 32 weeks’ gestation. Exclusion criteria included ruptured membranes, human immunodeficiency virus (HIV) infection, placenta previa or abruption, cervical dilation ≥3 cm, or administration of antibiotics within the preceding 2 weeks. In addition, women with clinical chorioamnionitis at the time of presentation were excluded. We defined clinical chorioamnionitis as a temperature ≥38.0°C and 1 or more of: maternal heart rate >100 beats per minute, fetal baseline heart rate >160 beats per minute, or uterine tenderness. The study was conducted in accordance with the guidelines established by the University of Florida Health Center Institutional Review Board.

After informed written and oral consent was obtained, and before any manipulation of the cervix, a sterile speculum examination was performed on each subject. Cervical fluid samples were obtained by placing a cellulose acetate wick (UniWick™, Whatman, Clifton, NJ) approximately 5 mm into the cervical os with a sterile forceps, taking care not to contact the vagina or vaginal fluid. The wick was left in place for approximately 60 seconds and removed. Wicks were stored in preweighed microcentrifuge tubes before sample collection. After sample collection, the wicks were returned to the same tube, taken immediately to the laboratory, postweighed, and stored at –80°C until fluid was eluted from the wicks.

At the time of the speculum examination, a swab from the vaginal fornix was obtained from each subject and used to prepare a slide for Gram stain for diagnosis of bacterial vaginosis using the method of Nugent et al. The speculum then was removed, and the cervical length was measured with a real-time endovaginal ultrasound examination, according to the method described by Iams et al.

For elution of fluid from the cervical wicks, 600 µL of elution buffer was added to each microcentrifuge tube containing a wick. This solution was composed of phosphate-buffered saline (PBS) with 2% Triton X-100, 0.2 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 µmol/L leupeptin, 1 µg/mL aprotinin, and 3.25 µmol/L bestatin. Fluid was extracted on ice for 30 minutes, vortexing 3 to 4 times. Contents of the microcentrifuge tubes then were transferred into centrifuge filter tubes (VectaSpin 3™; Whatman, Maidstone, England) and centrifuged at 4°C for 12 minutes at 3000g. Supernatants were removed and used for analysis. Using known concentrations of antibodies absorbed by wicks (Cappel purified human secretary IgA and purified human IgG; ICN Pharmaceuticals, Aurora, Ohio), we determined that this elution process recovered >95% of antibody from the wicks (data not shown). We assumed that cervical fluid has a density equivalent to water (1 g/mL). Because we knew the difference in weight before and after sample collection and used a constant volume of elution buffer, true concentrations of antibodies in the cervical fluid could be calculated.

IgA and IgG enzyme-linked immunosorbent assays (ELISAs) were performed using modifications of the techniques described by Quesnel et al. Briefly, for the IgA ELISA, Fisherbrand high binding 96-well plates (Fisher Scientific, Pittsburgh, Pa) were coated with 2.5 µg/mL of Cappel goat affinity-purified antibody to human IgA (α-chain; ICN Pharmaceuticals) in carbonate buffer pH 9.6 as the primary antibody and a 1:2000 dilution of Cappel peroxidase-conjugated sheep affinity-purified antibody to human IgA (α-chain; ICN Pharmaceuticals) in PBS, pH 7.4, with 0.05% Tween 20 (PBS-T) as the secondary antibody solution. For the IgG ELISA, Fisherbrand high binding 96-well plates were coated with 5 µg/mL of Cappel goat affinity-purified antibody to human IgG Fc (ICN Pharmaceuticals) in carbonate buffer pH 9.6 as the primary antibody, and a 1:8000 dilution of Cappel
peroxidase-conjugated goat IgG fraction to human IgG Fab (ICN Pharmaceuticals) in PBS-T, 1% gelatin (Sigma-Aldrich, St. Louis, Mo) as the secondary antibody solution.

Demographic data and relevant maternal medical information were abstracted from each subject’s medical record. The primary outcome was delivery before 37 weeks’ gestation. Forward stepwise logistic regression was used to evaluate the association of preterm delivery with cervical length, cervical fluid IgA concentration, cervical fluid IgG concentration, and Nugent score. Univariate comparisons of timing of delivery and Nugent score-based groups are also presented using the Kruskal-Wallis test. All tests of statistical significance were two-tailed, and used an alpha of .05 to define statistical significance.

Power calculations for regression models are complex. We took the simplified approach of looking only at total IgA in conjunction with the BV factor in order to obtain an approximate estimate of the adequacy of 200 patients. Assuming that 24% of the subjects would have a preterm delivery, 40% would have BV, 50% of patients without BV would have IgA concentrations above the median, and 20% without BV would have IgA concentrations above the median, with that number of subjects, we would have had a power of .66 for the BV factor, .97 for the total IgA factor, and .80 for the interaction factor. Note that the test for each will become more efficient with the addition of demographic covariates to the model.

### Results

During the study period, 137 patients were enrolled in the study, and complete delivery information was available for 134. For this analysis, another 33 subjects were excluded because of visible staining of the cervical wick by blood. Therefore, 101 subjects were analyzed. Demographic data are displayed in Table I. Gestational age at study entry did not differ between groups. As would be expected, a higher proportion of subjects in the group delivering preterm had a previous preterm birth.

Median cervical fluid antibody concentrations stratified by term or preterm delivery and by vaginal Gram stain result, respectively, are shown in Tables II and III. The rate of preterm delivery in this cohort was 24% (24 of 101 subjects). Four of the subjects delivering before 37 weeks had “indicated” preterm births caused by maternal disease. Results were not substantially different if these subjects were included in the term group, rather than the preterm group (data not shown).

In a forward stepwise logistic regression model with preterm birth as the dependent variable and cervical length, IgA, IgG, and Nugent score (2 variables to include 3 levels) as independent variables, cervical length was highly correlated with preterm delivery ($P < .001$). After controlling for cervical length, none of the other variables were significantly prognostic for preterm delivery (residual overall $P = .75$). From this model, it was estimated that a 1-mm decrease in cervical length was associated with a 10% (95% CI 5%-15%) increase in the risk of preterm delivery, and that a 10-mm decrease in cervical length was associated with a 157% (95% CI 57%-319%) increase in the risk of a preterm birth. In other words, a patient with a cervical length of 20 mm is estimated to have 2.57 times the risk of preterm delivery as a patient whose cervical length was 30 mm.

### Comment

Previous reports have established that cervical length is inversely related to the risk of preterm delivery. Results from our cohort are consistent with the findings from those studies, and confirm the value of cervical length in predicting the risk of preterm delivery.
However, the reason for the association between short cervix and preterm delivery remains undefined.

The relationship may be due to biomechanical factors such as uterine contractions and the inherent tensile strength of the cervical tissues. However, bacterial vaginosis, an established risk factor for preterm delivery, is thought to cause preterm delivery because of ascension of organisms through the cervix and into the uterus. Furthermore, clinical or subclinical infection of the uterus is thought to play a major role in the majority of cases of spontaneous preterm birth, particularly those that occur before 34 weeks. These associations are not explained by biomechanical factors.

Measuring the concentrations of total IgA and IgG in the cervical fluid, as we did in this study, does not appear to enhance the predictive value of cervical length. The cohort that we reported in this manuscript is rather small, and type II errors are possible. In the univariate analysis, IgG concentrations were increased in the setting of bacterial vaginosis. This finding may be caused by the fact that bacterial vaginosis causes not only an inflammatory response in the host but also an immune response. However, although IgG concentrations were increased in the setting of bacterial vaginosis in univariate analysis, there was no trend indicating that the concentration of either antibody in the cervical fluid correlates with preterm delivery. Therefore, we think that clinically significant differences would be unlikely, even if we had enrolled the planned number of subjects.

The concentrations of immunoglobulins that we measured from the cervical fluid of subjects in this study were similar to the concentrations reported by Quesnel et al, using the same collection method in nonpregnant women. Kutteh and Franklin, using a different method for collection, reported concentrations of IgA and IgG in cervical fluid samples from pregnant women that were 2 to 5 times lower than those that we found. However, they also reported concentrations of these antibodies in the cervical fluid of nonpregnant women that were lower than the values that they measured in pregnant subjects, and therefore, differed even more with the nonpregnant values reported by Quesnel et al. Although we did not control for microscopic amounts of blood in samples, such contamination (if present) would not have affected the results for IgA because serum levels of IgA in pregnant women are similar to those in cervical mucus. Serum levels of IgG are higher than cervical fluid levels of this antibody, and microscopic blood contamination could elevate a given subject’s concentration of this antibody.

Despite the negative findings in this study, the concept that cervical length is inversely related to preterm delivery because the cervix functions as an “immune tunnel” between the vagina and the uterine cavity may still, at least partially, explain the relationship. It may be that antigen-specific, rather than total, immunoglobulins are important in modulating the risk of preterm delivery related to cervical length. One group of investigators has evaluated, with mixed results, IgA directed against a certain toxin of *Gardnerella vaginalis*, a bacterial vaginosis-related organism. Alternatively, factors associated with the innate, rather than the adaptive, immune response may warrant investigation. Other authors have demonstrated the presence of such factors as secretory leukoprotease inhibitor, lysozyme, lactoferrin, and defensins in cervical mucus at concentrations sufficient for antimicrobial activity. Further work measuring these and other factors may help to explain the pathophysiology of preterm delivery and the mechanism of the relationship between short cervix and this outcome. Such antimicrobial constituents of the innate immune response might become candidate agents for treatment or prevention of preterm delivery.

### References


Prenatal cardiovascular manifestations in the twin-to-twin transfusion syndrome recipients and the impact of therapeutic amnioreduction

Catherine Barrea, MD,a Fawaz Alkazaleh, MD,b Greg Ryan, MB,b Brian W. McCrindle, MD,a Anita Roberts, BSc,a Jean-Luc Bigras, MD,a Jon Barrett, MD,c Gareth P. Seaward, MB,b Jeffrey F. Smallhorn, MB, BS,a Lisa K. Hornberger, MDa,*

Department of Pediatrics, Division of Cardiology, Fetal Cardiac Program, The Hospital for Sick Children,a and the Division of Maternal-Fetal Medicine, Mount Sinai Hospitalb and Women’s College Hospital,c University of Toronto, Ontario, Canada

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KEY WORDS
Monochorionic twins
Twin-to-twin transfusion syndrome
Amnioreduction
Fetal echocardiography
Fetal hydrops

Objective: We evaluated the cardiovascular pathologic condition in the recipient twin in twin-to-twin transfusion syndrome and the influence of amnioreduction.

Study design: Fetal echocardiograms and medical records of 54 pregnancies that were complicated by twin-to-twin transfusion syndrome were reviewed. Recipient twin right and left ventricular wall thickness, diameters, systolic and diastolic function, valve regurgitation, and structural cardiac defects were assessed at examination and after amnioreduction.

Results: At examination (n = 28 pregnancies), cardiomegaly because of right ventricular and/or left ventricular hypertrophy was observed in 58% of recipient twins, and biventricular hypertrophy was observed in 33% of recipient twins, without ventricular dilation. Biventricular diastolic dysfunction was present in two thirds of recipient twins, and right ventricular systolic dysfunction and significant atrioventricular valve regurgitation was observed in one third of recipient twins. Serial assessment (n = 21 pregnancies) revealed progressive biventricular hypertrophy and right ventricular systolic and biventricular diastolic dysfunction in most recipient twins. Steeper progression of hypertrophy, diastolic dysfunction, and structural or functional right ventricular outflow disease (20% incidence) were associated with an increased perinatal mortality rate.

Conclusion: In twin-to-twin transfusion syndrome, the recipient twin has progressive biventricular hypertrophy with predominant right ventricular systolic and biventricular diastolic dysfunction. Despite amnioreduction, the cardiovascular disease persists and even progresses in many recipient twins.

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Severe twin-to-twin transfusion syndrome (TTTS) complicates about 15% of monochorionic, diamniotic twin pregnancies.\textsuperscript{1-5} It is the most common severe complication of monochorionic twins.\textsuperscript{1,6} TTTS is thought, at least partly, to be due to a net transfer of blood from 1 fetus (donor) to the other fetus (recipient) through unbalanced unidirectional arteriovenous anastomoses in a monochorionic placenta.\textsuperscript{1,7-10} TTTS usually manifests as an imbalance of amniotic fluid (polyhydramnios/oligohydramnios sequence). As TTTS progresses, the recipient experiences cardiomegaly, decreased cardiac function, and ultimately hydrops. Unlike the recipient, cardiac disease is unusual in the donor.\textsuperscript{1,2,9}

Conservative treatment of severe TTTS is associated with survival rates of \(<10%\).\textsuperscript{11} Death is due to extreme prematurity with growth restriction in the donor and cardiac dysfunction and hydrops in the recipient. A variety of therapeutic approaches have been proposed. Serial therapeutic amnioreductions and selective laser ablation of placental vascular anastomoses have lead to improved perinatal outcomes.\textsuperscript{12-17} Nevertheless, morbidity and mortality rates remain high, and mechanisms of reported improvements in survival are understood incompletely.

Over the past decade there has been an increasing interest in the noninvasive assessment of fetal heart function. It has become clear that ventricular diastolic function may be as important, if not more so, than systolic function in the preservation of the fetal circulation and the survival of a fetus with ventricular dysfunction.\textsuperscript{18} However, reports on the recipient twin’s cardiovascular disease in TTTS have focused on ventricular size, wall thickness, and systolic function.\textsuperscript{19-21} There is a paucity of information concerning the influence of therapeutic interventions for TTTS on cardiovascular parameters, and existing data are conflicting.\textsuperscript{20,21} We retrospectively investigated ventricular systolic and diastolic function of the recipient twin in a large cohort of pregnancies with severe TTTS before any intervention and assessed the impact of therapeutic amnioreduction on cardiovascular parameters.

Methods

From 1993 to 2001, 78 patients with TTTS were seen in our institutions, 61 of whom were referred for fetal echocardiography as part of their routine assessment; 54 of these patients were included in the analysis (Table 1). The diagnosis of TTTS was based on a monochorionic-diamniotic twin pregnancy (single placenta, thin dividing membrane, same gender) that was complicated by a “polyhydramnios (>8 cm depth of amniotic fluid)-oligohydramnios (<2 cm depth of amniotic fluid)” sequence, with the exclusion of other causes for amniotic fluid and growth discrepancy.\textsuperscript{2} Placentation was confirmed after birth by clinical examination and/or pathologic examination. When data were available, staging was assigned as previously described by Quintero et al.\textsuperscript{22} Given the small number of patients in stages I and II and the similarity between those 2 stages from a cardiovascular standpoint, these patients were grouped together.

Maternal medical records and fetal and postnatal echocardiograms of liveborn infants were reviewed retrospectively. Autopsies were reviewed when available. This study was approved by the ethics boards from participating hospitals.

Fetal echocardiograms were performed with linear and curved array transducers with variable frequencies (7-3.5 MHz) on ATL 3000 and HDI 5000 (Philips Ultrasound, Bothell, Wash). All studies included a complete 2-dimensional evaluation of cardiac anatomy with the use of standard fetal echocardiography views and spectral and color Doppler analysis.

Echocardiograms were reviewed retrospectively by 3 of the authors (C.B., A.R., L.K.H.), and offline measurements were performed. Fetal hydrops was defined as the presence of \(\geq 2\) fluid collections. Cardiothoracic index was calculated by dividing the cardiac diameter in diastole in a 4-chamber view by the external thoracic dimension. Cardiomegaly was defined as cardiothoracic index of \(\geq 0.40\). Ventricular chamber dimensions and wall thickness were measured on 2-dimensional images or M-mode tracings as previously described and compared with normal values for gestational age (GA).\textsuperscript{23,24}

We evaluated systolic and diastolic function parameters of the fetal left and right ventricles. The systolic function refers to the “pumping” function of the heart that occurs during ventricular contraction and ejection. The diastolic function describes the ability of the ventricle to fill. Diastolic function is made up of 2 phases, an early diastolic phase of relaxation (which is an active period of diastole) and a late diastolic phase (which reflects ventricular compliance). Changes in diastolic function and to a lesser extent in systolic function (with reduced ejection) result in increasing pressures to fill the heart during ventricular diastole. Increased ventricular filling pressures result in increasing atrial pressures during atrial contraction, which subsequently leads to disturbances in blood flow (with increasing a wave reversal) in the systemic veins, including the inferior vena cava (IVC), ductus venosus, and umbilical vein. Changes in ventricular filling, as such, ultimately impede venous return from the placenta, which results in placental edema, and from the lymphatics, which results in the evolution of fetal hydrops.

Systolic function was assessed by right ventricular (RV) and left ventricular (LV) shortening fractions (SFs; SF = [end diastolic dimension – end systolic dimension]/end diastolic dimension \(\times 100\)). Systolic dysfunction was diagnosed when the SF was \(<28%\) (<2 SD
below the normal mean\textsuperscript{25}). Atrioventricular valve (AV) valve regurgitation was assessed qualitatively by color mapping as absent, mild, moderate, or severe. When possible, and in the absence of outflow tract obstruction, systolic RV and LV pressures were measured by continuous wave Doppler interrogation of the AV valve regurgitant jet with the modified Bernoulli equation ($4 \times [\text{peak velocity}]^2$) with an estimated right or left
atrial pressure of 5 mm Hg. This permitted the assessment of systemic blood pressures, which were compared with data that were obtained invasively in fetuses with normal hearts. Diastolic function was evaluated by pulsed Doppler interrogation of the IVC or hepatic veins, ductus venosus, umbilical vein (umbilical vein), and RV and LV blood inflows (Figure 1). From simultaneous Doppler recordings of the LV blood inflow and outflow, LV isovolumic relaxation time was also measured as the interval from aortic valve closure to the onset of mitral valve blood inflow. Diastolic dysfunction was considered to be present when ≥ 2 of the following parameters were identified: abnormal early (E wave) to late (A wave) diastolic filling ratio through LV or RV inflows (± 2 SD from the mean for GA), abnormal LV isovolumic relaxation time (>2 SD of mean for GA), increased “a” wave reversal during atrial contraction (>20 cm/sec) in the IVC or hepatic veins, absence of diastolic blood flow or the presence of reverse blood flow during atrial contraction in the ductus venosus or umbilical vein pulsations. Whenever data were available, the Tei index or myocardial performance index of both ventricles was calculated \((a – b)/b\): \(a\) is the time interval from offset of AV valve blood inflow to onset of next AV valve inflow, \(b\) is the ejection time on Doppler tracings. The Tei index has been shown to reflect the global, systolic and diastolic ventricular function and is believed to be relatively independent of ventricular loading conditions. In patients in whom we had simultaneous blood inflow and outflow tracings through the LV, we measured the LV isovolumic contraction time (time interval between mitral valve closure and aortic valve opening). Results were compared with normal values. Spectral Doppler assessments were performed as parallel as possible to blood flow, and all variables that involved offline Doppler measurements were performed at least in triplicate and averaged. Only Doppler spectra that were obtained in a normal atrial rhythm and in the absence of fetal breathing were included. The presence of RV or LV outflow tract obstruction was assessed on 2-dimensional images and by spectral and color Doppler imaging. Pulmonary valve regurgitation and direction of blood flow in the ductus arteriosus were also evaluated.

All amnioreductions were performed under local anesthesia and ultrasound guidance with 15- to 18-gauge needles. As much fluid as possible was removed as quickly as possible with negative pressure suction from 1-L chest drain bottles until the amniotic fluid volume appeared to be normal.

Statistical analysis

Data were described as frequencies, mean ± SD, Z score (number of standard deviations a patient’s measurement is from the mean value), or median values with ranges, as appropriate. Paired and unpaired Student t test, analysis of variance, Kruskal Wallis test, and Fisher’s exact test were used as appropriate. Abnormalities and trends in serial prenatal normalized echocardiogram values in recipients were sought in mixed linear regression analysis. Statistical analyses were performed with SAS statistical software (version 8.02; SAS Institute Inc, Cary, NC); a probability value of <.05 was considered significant.

Results

Fifty-four patients with TTTS were included: 50 pairs of twins and 4 sets of triplets. GA at diagnosis of TTTS was 21 ± 3 weeks. More than one half of the patients were in Quintero stage III. Figure 1 summarizes the case, including the interventions where performed. Amnioreduction was the treatment of choice until 1999; laser was the treatment of choice thereafter, especially for advanced (stage III or IV) or when the diagnosis was made at < 22 weeks of gestation.

Cardiovascular findings before intervention

Twenty-eight recipient twins had echocardiography before any intervention, at a mean GA of 21 ± 3 weeks; in 83% of the twins, the first echocardiogram was performed within 1 week of the diagnosis of TTTS. Cardiomegaly was present in all recipients (cardiothoracic index, 0.49 ± 0.06); however, it was not associated with cardiac chamber dilatation because the LV and RV end-diastolic dimensions were small rather than dilated for GA (Figure 2). LV and/or RV hypertrophy was present in 58% of the patients (15/26 assessed), and biventricular hypertrophy was present in 33% of the patients (8/24 assessed). LV systolic dysfunction was present in 15% of the patients (4/27 assessed; mean LV SF, 37% ± 10%) and RV systolic dysfunction in 35% (9/26 assessed; mean RV SF, 30% ± 12%). Moderate to severe mitral regurgitation was present in 12% of patients (3/26 assessed), and tricuspid regurgitation was present in 27% (7/26 assessed). In 3 cases, LV or RV systolic pressure had been measured by Doppler interrogation of the AV valve; all suggested high systolic systemic blood pressure for GA (range, 49-63 mm Hg, with normal values for GA of approximately 25 mm Hg).

Diastolic ventricular dysfunction was present in 2 of 3 recipient twins who were assessed, with most frequently abnormal parameters being IVC blood flow and prolonged LV isovolumic relaxation time (mean, 61 ± 13 msec in 28 cases compared with a normal time of 42 ± 8 msec; Table II). With respect to global ventricular function assessment, LV Tei index was within normal limits for GA (mean, 0.63 ± 0.18; n = 14), although
Figure 1  The different Doppler parameters that were used to evaluate diastolic function in the recipient twin with normal (left panel) and abnormal (right panel) waveforms. A, Doppler tracings that were obtained in the IVC. In the presence of elevated ventricular and atrial filling pressures, the velocity pattern in the IVC and proximal hepatic veins goes from largely biphasic forward blood flow to increasing a wave reversal (arrow) and less forward blood flow. B, Ductus venosus Doppler spectra are normally characterized by continuous forward blood flow with reduced velocities during atrial systole. When abnormal, there is absent or reversed blood flow during atrial systole (arrow). C, Umbilical vein Doppler spectra that were obtained in the normal fetus and in a recipient twin with elevated central venous pressures in which notching occurs during atrial systole (arrow). D, Ventricular blood inflow patterns, which are usually biphasic, ultimately become uniphasic with the loss of early passive ventricular filling and only a very short period of filling during atrial contraction. E, The isovolumic relaxation time is the time interval from the offset of aortic blood flow to the onset of mitral valve blood inflow.
RV Tei index was significantly increased (mean, 0.87 ± 0.28; n = 10; P = .03), which suggests global RV dysfunction. Mean LV isovolumic contraction time in 12 recipient fetuses was 45 ± 17 msec, which was not significantly different from normal data. 

At initial assessment, 4 of 28 fetuses were hydropic. Two of the 4 hydropic fetuses had functional or “pseudo” pulmonary atresia because of severely reduced RV systolic function, and 2 of the fetuses had anatomic RV outflow tract obstruction.

Table III describes the cardiovascular findings according to the Quintero stage at assessment. The cardiac disease was more severe in higher stage patients. There was a statistically significant difference in the frequency of AV regurgitation and a trend towards more frequent ventricular hypertrophy.

### Influence of amnioreduction on the recipient’s cardiovascular pathologic condition

A total of 66 therapeutic amnioreductions were performed on 36 patients at a mean GA of 23 ± 4 weeks (range, 18–31 weeks) at the first procedure. A mean of 1.8 ± 1.3 amnioreductions/patient (range, 1–7 amnioreductions/patient) were performed with a mean volume of 2.4 ± 1.3 L/amnioreduction (range, 0.7–7 L/amnioreduction) removed and a total volume of 4.1 ± 3.0 L/patient (range, 1–14 L/patient). Median interval between the first amnioreduction and delivery was 6 weeks (range, 0–17 weeks). Seven patients (19%) were delivered within 1 week of the first amnioreduction. Mean GA at delivery was 30 ± 5 weeks (range, 21–38 weeks) in those patients who were treated with amnioreduction.

Serial assessment was performed in 21 patients over a 4–3-week period (Figure 1). There was progressive biventricular hypertrophy with significant increasing Z score for right ventricular anterior wall (RVAW; Figure 3), left ventricular posterior wall (LVPW), and interventricular septum (IVS) thickness (RVAW, Z-score = .017; LVPW, Z-score = .028; IVS, Z-score = .029). By general estimation equation analysis for repeated measures, there was a steeper progression of biventricular hypertrophy in those who died prenatally from cardiovascular compromise or hydrops (RVAW, Z-score < .05; LVPW, Z-score = .028; IVS, Z-score = .029). Despite the significant changes in ventricular wall thickness, there was no significant change in LV or RV end diastolic dimension Z scores.

Between the first and the last echocardiogram, RV systolic function remained or became abnormal in the majority (Figure 4, A). In contrast, LV systolic function remained normal in the majority of patients (Figure 4, B). Comparison of the systolic function between the first and the last echocardiogram among 11 cases that required >1 amnioreduction (total amount of fluid removed, 6.4 ± 3.8 L) for recurrent severe polyhydramnios showed that LV systolic function worsened significantly (LVSF, 37% ± 10% to 28% ± 12%; P = .025), although there was no significant change in RV systolic function (which was abnormal in most at initial assessment). In contrast, LV and RV systolic function of the recipient twin did not change in the 10 patients who required only 1 amnioreduction (fluid...
removed, 2.6 ± 1.0 L: LVSF, 39% ± 11% to 38% ± 11%; RVSF, 23% ± 9% to 25% ± 11%.

Diastolic dysfunction persisted or developed in 57% of recipients (8/14), regressed in 21% (3/14), and remained normal in 21% (3/14). In 1 recipient, diastolic function normalized after the spontaneous death of the donor. None of the 3 fetuses with regression of diastolic dysfunction died prenatally. Only 1 fetus without diastolic dysfunction died of cardiovascular compromise or hydrops prenatally (results not shown).

Four recipient fetuses had structural RV outflow obstruction, and 2 of them died. Two of these 4 fetuses have been reported previously.31 Three other fetuses had functional or “pseudo” pulmonary atresia because of severely reduced RV systolic function. They had significant systolic and diastolic pulmonary insufficiency that was associated with retrograde ductal blood flow. Two died prenatally of cardiovascular compromise, despite amnioreduction. Of the 5 fetuses in ongoing pregnancies who had anatomic or functional RV outflow obstruction, 4 fetuses (80%) died during the perinatal period. Excluding terminations of pregnancy, death was more frequent in the presence of structural or functional prenatal RV outflow obstruction (P = .05). No additional major structural heart defects were diagnosed in any recipient.

No major cardiovascular anomalies were seen in any donors at assessment or serial assessment, with the exception of absence of end-diastolic velocities in the umbilical artery. Two donors had small isolated pericardial effusions after amnioreduction that were unassociated with any functional cardiac abnormalities, and both donors survived.
Clinical outcome

In the amnioreduction group (n = 36 pregnancies), 58% of the fetuses survived beyond the neonatal period (28 days); 16 recipients and 14 donors died. Both twins survived the neonatal period in 16 pregnancies (44%); 1 twin survived in 10 pregnancies (28%), and both twins died in 10 pregnancies (28%). Cardiovascular compromise with severe hydrops was responsible for intrauterine death in 8 recipients. In 3 other recipients, the parents requested pregnancy termination, given the severity of cardiovascular compromise in the recipient. Cardiovascular compromise was thus the most common cause of prenatal death among recipient fetuses. Two recipients died postnatally, 1 of extreme prematurity (22 weeks) and 1 of cerebral damage and cardiac disease that prompted the withdrawal of care. Of 6 pregnancies without intervention, the mean GA at birth was significantly lower (23 ± 2 weeks), with a mortality rate of 88% (14/16 fetuses; 2 triplets). Finally, there were 5 prenatal deaths (45%) among the 11 patients who required >1 amnioreduction and only 2 prenatal deaths (20%) among the 10 patients who required only 1 amnioreduction, which further suggests a difference in the severity of disease between the 2 groups.

Comment

In TTTS, cardiovascular disease occurs in most recipient twins19-21,32 and is a major cause of death.33,34 The recipient’s cardiovascular disease also contributes significantly to the donor’s morbidity and death. Knowledge of the influence of TTTS on ventricular systolic and diastolic function in the recipient contributes to our understanding of pathophysiologic conditions and may ultimately assist in the identification of the most effective invasive or noninvasive therapies.

Most recipient fetuses present initially with cardiomegaly,19-21,32 which we have demonstrated to be the result of myocardial hypertrophy rather than ventricular dilation. The absence of cardiac chamber dilation in this study provides evidence against an actual “volume load.” Furthermore, others have shown no significant difference in cardiac output indexed to fetal weight between recipient and donor twins with TTTS and monochorionic twins without TTTS.3 In contrast, conditions in which there are increased “volume load” or ventricular preload (such as high cardiac output states in the fetus that include anemia,35 agenesis of the ductus venosus36 and arteriovenous malformations37) are characterized by cardiac chamber dilation and increased cardiac output early in the course of the disease. In addition to ventricular hypertrophy, we found that only one third of patients have evidence of ventricular systolic dysfunction and significant systolic AV valve regurgitation at initial assessment, which primarily involves the right heart. Although earlier reports suggested similar disease, the data that were provided were largely qualitative and, where quantitative, focused on the LV only.4,20,21 In our experience, when such significant RV dysfunction occurred in the absence of RV outflow obstruction, we observed “pseudo” pulmonary

<table>
<thead>
<tr>
<th>Table III</th>
<th>The cardiovascular findings at examination, according to the staging of Quintero et al22</th>
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<tbody>
<tr>
<td>Finding</td>
<td>Stage</td>
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<tr>
<td>Total recipients (n)</td>
<td></td>
</tr>
<tr>
<td>RVSF (%)*</td>
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</tr>
<tr>
<td>RVSF &lt;28% (n/N)</td>
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</tr>
<tr>
<td>LVSF (%)*</td>
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<tr>
<td>LVSF &lt;28% (n/N)</td>
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<tr>
<td>Moderate to severe</td>
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</tr>
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<td>tricuspid valve regurgitation (n/N)</td>
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<tr>
<td>Moderate to severe</td>
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<tr>
<td>mitral valve regurgitation (n/N)</td>
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<tr>
<td>Diastolic dysfunction (n/N)</td>
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<td>RV anterior wall thickness (Z score)*</td>
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</tr>
<tr>
<td>Interventricular wall thickness (Z score)*</td>
<td>+1.70 ± 0.87</td>
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<tr>
<td>LV posterior wall thickness (Z score)*</td>
<td>+1.67 ± 1.53</td>
</tr>
<tr>
<td>Right and/or left hypertrophy (n/N)</td>
<td>2/7 (29%)</td>
</tr>
</tbody>
</table>

NS, Not statistically significant (P ≥ 1.0).

* Data are given as mean ± SD.
atresia, in which the RV had such severe systolic dysfunction that it was unable to generate sufficient pressure to open the pulmonary valve. The presence of pulmonary insufficiency was a clue to valve patency, a feature that was also described in fetal Ebstein’s anomaly of the tricuspid valve with severe insufficiency.38

Most importantly, our study demonstrates for the first time that the predominance of ventricular diastolic dysfunction is present in approximately two thirds of recipients at initial assessment. Ventricular hypertrophy and diastolic dysfunction preceded systolic dysfunction in most cases. We suspect that diastolic dysfunction may occur, at least in part, because of ventricular hypertrophy, which we have observed also in primary fetal hypertrophic cardiomyopathies.18 The diastolic dysfunction may be sufficient to cause cardiovascular compromise, given that the fetoplacental circulation requires low downstream ventricular filling pressures to function effectively. In support of this, we found diastolic, but not systolic, dysfunction to be more common among recipient fetuses who died prenatally as a result of cardiovascular compromise. Assessment of global RV and LV function with the Tei index suggested greater compromise of RV than LV function, although in some cases there was also progressive LV dysfunction. That global RV function was most affected in TTTS may be the result of changes in peripheral vascular resistance having a greater impact on RV function. Finally, we observed more severe cardiovascular findings with higher Quintero stage at assessment.22 Limited patient numbers in each of the groups, however, resulted in an inability to demonstrate statistically significant differences for many of the parameters that were assessed. Worsening or persistent biventricular systolic dysfunction and worse outcome were also observed in pregnancies that underwent >1 amnioreduction, compared with those pregnancies that underwent only 1 amnioreduction, which suggests a correlation between the severity of polyhydramnios and the severity of the cardiovascular disease, particularly systolic dysfunction, in the recipient twin.

We found structural RV outflow obstruction prenatally or postnatally in 6 of our entire cohort of 54 fetuses (11.1%), which is similar to our previous findings in >70 pregnancies with TTTS.31 The development of structural RV outflow obstruction may be multifactorial. We suspect that it may, in part, be due to RV hypertrophy with development of muscular blood outflow obstruction. This pathologic condition alone potentially could regress after birth, as observed in 2 previous cases.31 Reduced RV filling because of diastolic dysfunction and reduced RV ejection because of systolic dysfunction may further contribute to the development of structural disease. That reduced RV ejection contributes to anatomic RV outflow obstruction is supported by the progressive anatomic RV outflow obstruction that has also been observed in tricuspid valve dysplasia with severe tricuspid insufficiency.39

Despite amnioreduction, cardiovascular disease persisted or worsened in most recipients who were assessed serially. In some, we observed progressive hydrops and even the development of structural and functional RV outflow disease, despite serial amnioreduction. Similarly, others have described progressive cardiovascular disease with worsening of systolic function in smaller cohorts of recipient twins.42,20 In contrast, Fesslova et al21 showed an improvement in cardiac function after amnioreduction in 11 of 17 recipients after normalization of the amniotic fluid index, but details of the impact in the 6 other patients were not provided. These earlier studies focused on changes in systolic function and did not address changes in ventricular wall thickness or diastolic function.

Given the lack of evidence for significant volume loading, the pathogenesis of the recipient’s cardiovascular disease is likely more complex. An alternative hypothesis is that cardiac disease evolves as a consequence of increased afterload. We have demonstrated evidence for elevated ventricular and thus systemic blood pressure in utero through Doppler interrogation of the AV valve regurgitation, using a standard postnatal echocardiographic technique. It has also been recognized that recipients often have elevated systemic blood pressure in the initial neonatal period.20,21 Histologically, there is a thickened vascular media of the systemic and pulmonary arteries that, in addition to vasoconstriction, likely contributes to changes in peripheral vascular resistance and thus ventricular afterload.40 Circulating factors that may be responsible for cardiovascular changes in the recipient have been elucidated recently. Bajoria et al41 demonstrated significantly higher endothelin-1 and atrial and brain natriuretic peptide levels42 in venous and arterial blood from recipients’ umbilical cords, compared with both their donor counterparts and normal twins. They further found that the levels of at least endothelin-1 and brain natriuretic peptide were highest in recipients with cardiac dysfunction.42 Endothelin-1 is a potent vasoconstrictor and a well-recognized mitogenic factor for human fetal vascular smooth muscle cells; moreover, it can also stimulate human fetal cardiac myocyte proliferation in vitro.43 As such, endothelin-1, and perhaps other vasoactive peptides (eg, angiotensin II44) and growth factors that are possibly released by the placenta in response to the abnormal vascular connections, may play a role in the development of cardiac disease. Cardiac myocyte proliferation that leads to ventricular hypertrophy has been demonstrated in the myocardium of recipient twins40 and may occur as an indirect consequence of increased afterload or through direct mitogenic effects of such factors. Endothelin-1 itself, and
changes in atrial filling pressures and hypoxia, can stimulate increased cardiac expression of atrial natriuretic peptide. Natriuretic peptides could account for changes in renal blood flow, polyuria, and polyhydramnios and even the polycythemia and hyperviscosity that are found frequently in recipients. The placenta may release endothelin-1 and other vasoactive peptides and growth factors in response to hypoxia. We hypothesize that the deep vascular anastomoses may expose the placental cotyledon through which they course to hypoxia and stimulate the release of such factors into the recipient, which sets off a cascade of events. It is also possible that blood flow through the recipient’s larger placental mass may be insufficient and results in relative placental hypoxia. Discordant amniotic fluid volume with elevated intra-amniotic pressure may further impair uteroplacental blood flow in the recipient, which ultimately results in a worsening of placental hypoxia.

Our study had certain limitations. Although we assessed most of the patients, we did not perform fetal echocardiograms on all pregnancies that were referred with TTTS, which could have resulted in recruitment bias, likely towards more severe disease. Our study was retrospective; as such, the full assessment of systolic and diastolic function was not performed in every case and not all cases underwent a fetal echocardiogram before intervention. Similarly, we did not have enough data to evaluate the evolution of LV and RV Tei indices and LV isovolumic contraction time after amnioreduction.

Cardiovascular findings in the recipient twin in TTTS are characterized by an early onset hypertrophic cardiomyopathy with predominance of diastolic dysfunction in two thirds of the cases and systolic dysfunction in one third of the cases. Congestive heart failure with hydrops and RV outflow obstruction, anatomic or functional, are common findings and are frequently progressive. The cardiovascular disease becomes more severe with advancing Quintero stage and may correlate with the severity of polyhydramnios. Our observations are potentially consistent with a primary role for increased ventricular afterload. Therapeutic amnioreduction does not appear to significantly impact on the recipient twin’s cardiovascular disease, although its impact on disease progression could not be evaluated. Further prospective assessment of the influence of amnioreduction and other therapies, in addition to more fundamental research that elucidates the primary factors that are responsible, are warranted in the identification of the optimal treatment of severe TTTS.

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We thank our obstetric and pediatric colleagues who referred patients and provided prenatal and postnatal follow-up both locally and in other Canadian provinces.

References
Tocolytic effect of a Rho-kinase inhibitor in a mouse model of lipopolysaccharide-induced preterm delivery

Masahiro Tahara, MD,* Rikako Kawagishi, MD, Kenjiro Sawada, MD, Kenichiro Morishige, MD, Masahiro Sakata, MD, Keiichi Tasaka, MD, Yuji Murata, MD

Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Yamadaoka, Suita, Osaka, Japan

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Objective: The small guanosine triphosphatase RhoA/Rho-kinase cascade has been implicated in uterine contraction. Our purpose was to evaluate the tocolytic effect of a Rho-kinase inhibitor, Y-27632, in lipopolysaccharide-induced preterm delivery in mice.

Study design: We used an animal model of lipopolysaccharide-induced preterm delivery in C3H/HeN × B6D2F1 pregnant mice. Y-27632 was delivered continuously through an osmotic pump that was implanted into the peritoneal cavity 6 hours before lipopolysaccharide treatment. The primary outcome was the preterm delivery rate. To further study the possible involvement of this cascade in lipopolysaccharide-induced preterm delivery, we determined the effect of lipopolysaccharide and prostaglandin F$_{2\alpha}$ on RhoA activation in mouse myometrial cells and uterine smooth muscle tissues.

Results: The rate of preterm delivery for lipopolysaccharide-treated animals was 94.4%. The administration of Y-27632 (1 or 10 mg/kg/d) significantly reduced the preterm delivery rate to 61.1% or 15.8%, respectively. The level of guanosine triphosphate–bound RhoA was increased after the addition of lipopolysaccharide or prostaglandin F$_{2\alpha}$.

Conclusion: The RhoA/Rho-kinase cascade is involved in lipopolysaccharide-induced preterm delivery, which suggests that Rho-kinase could be used as a new therapeutic target for the prevention of preterm labor.

Preterm delivery is the most important problem in perinatology because preterm birth is the major cause of neonatal morbidity and death. Chorioamnionitis is known to be related closely to preterm delivery, and intrauterine infection has been thought to play a critical role in the pathogenesis of preterm delivery. Lipopolysaccharide is a cell-surface component of Gram-negative organisms and is elevated in the amniotic fluid of chorioamnionitis. These results suggest that lipopolysaccharide is one of the major causes of preterm delivery. Therefore, it is crucial to elucidate the effects of lipopolysaccharide on uterine contraction for optimal therapies of preterm labor.
In recent years, there have been remarkable advances in our knowledge of the biochemical mechanisms underlying smooth muscle contractility. The small guanosine triphosphatase (GTPase) Rho, a member of the Ras superfamily of monomeric GTPases, is involved in various cellular functions, which include cell motility, smooth muscle contraction, and the determination of cell morphologic condition. It is widely accepted that Rho is responsible for the Ca$^{2+}$-sensitization in smooth muscle contraction that is induced by agonist stimulation. The expressions of RhoA and its effector Rho-kinase have been reported in the pregnant myometrium. We and others reported the potential role of the RhoA/Rho-kinase pathway in agonist-induced uterine contraction on the basis of studies that used a specific Rho-kinase inhibitor, Y-27632. However, whether Y-27632 is effective in in vivo models of lipopolysaccharide-induced preterm labor remains unknown. In addition, it has not been determined whether the RhoA/Rho-kinase pathway in myometrial cells is activated by lipopolysaccharide or prostaglandins, which are known to be stimulated by lipopolysaccharide.

In the present study, we evaluated the Rho-kinase inhibitor Y-27632 for its tocolytic effect using a preterm delivery mouse model that was induced by lipopolysaccharide. Next, to examine the possible involvement of RhoA in lipopolysaccharide-induced uterine contraction, we evaluated whether lipopolysaccharide or prostaglandin F$_2$g activates RhoA in myometrial cells and uterine smooth muscle tissues.

**Material and methods**

**Material**

C3H/HeN female mice and B6D2F1 male mice were purchased from Charles River Japan (Yokohama, Japan). Y-27632, a specific inhibitor of Rho-kinase, was provided by Mitsubishi WelFide Corporation (Osaka, Japan). Y-27632 was dissolved in distilled water as a stock solution (10 mmol/L) and stored at −20°C until used. Osmotic pumps (model 1007D; ALZET Osmotic Pumps) were loaded with a mini-osmotic pump (model 1007D; ALZET Osmotic Pumps) filled with 100 μL of saline solution. Continuous infusion of saline solution with a mini-osmotic pump. Group II (lipopolysaccharide group) received continuous infusion of saline solution intraperitoneally, with a 3-hour interval between injections (at 14:00 and 17:00), as reported previously.

The stock solution of Y-27632 was diluted with 0.9% (weight/volume) physiologic saline solution. Continuous administration was performed by loading a mini-osmotic pump (model 1007D; ALZET Osmotic Pumps) filled with 100 μL of saline solution or Y-27632 at different concentrations (6 or 60 mg/mL). On day 15 of gestation, mice were anesthetized by an injection of 0.2 mL of 10% sodium pentobarbital; an osmotic pump was then implanted into the peritoneal cavity under sterile conditions through a small dorsal incision, and the skin incision was closed with sutures. These pumps delivered a volume of 0.5 μL/h, which was equivalent to 1 or 10 mg/kg/d of Y-27632 for a 70-g pregnant mouse. There were no complications of insertion of the pumps (including bleeding, infection, or death) in the 10 mg/kg/d Y-27632- or saline solution–treated animals. Lipopolysaccharide was administered 6 hours after the pump implantation.

The mice were divided randomly into 4 groups. Group I (control group) received continuous infusion of saline solution intraperitoneally with a mini-osmotic pump. Group II (lipopolysaccharide group) received continuous infusion of saline solution with a mini-osmotic pump, and was treated with lipopolysaccharide, as described earlier. Group III (lipopolysaccharide + Y-27632 group 1) received continuous infusion of Y-27632 (1 mg/kg/d) with a mini-osmotic pump and was treated with lipopolysaccharide. Group IV (lipopolysaccharide + Y-27632 group 2) received continuous
Cultured mouse myometrial cells were previously. The Rho pull-down assay was performed as described by Boulet and Fortier. After the uteri were removed at day 15 of gestation, according to the modified method of Tahara et al. Myometrial smooth muscle cells were prepared for primary culture from uterine horns from pregnant mice at day 15 or 20 gestational days as full-term. Defined a delivery at every day throughout the experimental period. We performed in the early morning and the late afternoon determination of the presence or absence of delivery was pumped and was treated with lipopolysaccharide. The infusion of Y-27632 (10 mg/kg/d) with a mini-osmotic pump and was treated with lipopolysaccharide. The determination of the presence or absence of delivery was performed in the early morning and the late afternoon every day throughout the experimental period. We defined a delivery at <19 gestational days as preterm and at 19 or 20 gestational days as full-term.

**Preparation of myometrial muscle cell culture**

Myometrial smooth muscle cells were prepared for primary culture from uterine horns from pregnant mice at day 15 of gestation, according to the modified method of Boulet and Fortier. After the uteri were removed and dissected free of fat, they were placed in ice-cold Hanks solution that contained HEPES (0.5% wt/vol) and penicillin/streptomycin, and were cut into 1- to 2-mm³ fragments. The tissue was incubated at 37°C for 60 minutes with Hanks solution that contained trypsin (0.02% wt/vol), collagenase I (0.05% wt/vol), DNase (0.01% wt/vol), and ethylenediaminetetraacetic acid (0.02% wt/vol). Digestion was stopped with 2 mL of 10% fetal bovine serum, and the cells were filtered through a 500-μm nylon mesh. The suspension of the myometrial fraction was centrifuged at 300 g for 10 minutes and washed 3 times with Hanks’ solution. Dispersed cells were plated at a final concentration of 2 to 3 × 10⁶ cells/mL in RPMI 1640 that contained fetal bovine serum (5% vol/vol). The cell suspension was seeded into 60-mm–diameter wells and incubated for 16 hours at 37°C, which allowed fibroblasts to adhere to the culture flasks. Unattached myometrial cells were transferred to 60-mm–diameter wells (3 × 10⁶/well). The cells were maintained at 37°C in an atmosphere of 95% air and 5% carbon dioxide in RPMI 1640 medium that contained fetal bovine serum (10% vol/vol) that was supplemented with penicillin (200 U/mL)/streptomycin (200 μg/mL). They were used for experiments after 2 days.

**Pull-down assay for GTP-bound RhoA**

The Rho pull-down assay was performed as described previously. Cultured mouse myometrial cells were washed twice and incubated in fresh RPMI 1640 without serum for 3 hours. After incubation, cells were stimulated with 25 μmol/L lipopolysaccharide and 5 μmol/L prostaglandin F₂α for 10 minutes, washed twice with phosphate-buffered saline solution, and lysed in lysis buffer. Cell lysates were clarified by centrifugation at 13,000 rpm for 10 minutes, and equal volumes of lysates were incubated with rhokinin RBD–agarose beads (30 μg) at 4°C for 45 minutes. The beads were washed 4 times with washing buffer. Activated RhoA that was bound to beads or total RhoA in cell extracts was detected by Western blotting with a polyclonal antibody against RhoA. A protein assay was performed before beginning the pull-down assay to equalize the total protein concentration of each treatment group. GTP-bound activated RhoA in mouse uterine smooth muscle tissues was determined as described previously. Briefly, uterine horns were obtained from pregnant mice at day 15 of gestation and were cut into longitudinal strips approximately 5-mm long and 2-mm wide. The uterine tissues were incubated in RPMI 1640 without serum for 3 hours at 37°C. After incubation, the tissues were stimulated with lipopolysaccharide (25 μmol/L for 15 minutes) or prostaglandin F₂α (5 μmol/L for 15 minutes) and then quickly frozen by immersion in liquid nitrogen. Frozen tissues were homogenized in lysis buffer, and the supernatants were recovered by centrifugation at 14,000 g at 4°C for 10 minutes and were used for pull-down assay, as described earlier.

Quantitative densitometric analysis of immunoblots was performed with Fluor Chem IS-8000 (Alpha Innotech Corp, San Leandro, Calif), and the amount of GTP-RhoA was normalized for the total amount of RhoA in each sample. The quantitative data of normalized amounts of GTP-RhoA are expressed as multiples over a value in unstimulated cells or tissues, which is expressed as 1.0.

**Statistical analysis**

The results of each experiment were expressed as the mean ± SD. Statistical analysis was performed with Fisher’s exact probability test in the preterm delivery mouse model experiments and with 1-way analysis of

### Table Effect of Rho-kinase inhibitor on a lipopolysaccharide-induced preterm delivery mouse model

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice (n)</th>
<th>Incidence</th>
<th>Preterm delivery (day &lt;19)</th>
<th>Term delivery (day 19-20)</th>
<th>Rate of preterm delivery (%)</th>
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<tbody>
<tr>
<td>I (Control)</td>
<td>15</td>
<td></td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>II (Lipopolysaccharide)</td>
<td>18</td>
<td></td>
<td>17</td>
<td>1</td>
<td>94.4</td>
</tr>
<tr>
<td>III (Lipopolysaccharide + Y-27632 [1 mg/kg/d])</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td>61.1*</td>
<td></td>
</tr>
<tr>
<td>IV (Lipopolysaccharide + Y-27632 [10 mg/kg/d])</td>
<td>19</td>
<td>3</td>
<td>16</td>
<td>15.8†</td>
<td></td>
</tr>
</tbody>
</table>

* P < .05.
† P < .01 versus group II.
variance and subsequent Scheffe’s post-hoc test in the other experiments. A statistically significant difference was defined as a probability value of <.05.

Results

Effect of Y-27632 on lipopolysaccharide-induced preterm delivery

We evaluated the Rho-kinase inhibitor Y-27632 for its tocolytic effects on mice and used a lipopolysaccharide-induced preterm delivery mouse model. In group I, all pregnant C3H/HeN × B6D2F1 mice were delivered of pups at term at day 19 or 20 (Table). In group II, C3H/HeN × B6D2F1 pregnant mice had a high incidence (94.4%) of preterm delivery (Table). Delivery occurred without any maternal death in all cases. All preterm deliveries past 17 days of pregnancy, the offspring survived, and 84.2% live offspring were obtained in the Y-27632 administration group at a dose of 10 mg/kg/d.

RhoA activation by lipopolysaccharide and prostaglandin F2α

To further elucidate whether the effect of lipopolysaccharide on uterine contractility is due to the activation of rho GTPase, we measured the intracellular levels of the GTP-bound, active form of RhoA using the pull-down assay system.16 As shown in Figure 1, the level of GTP-bound RhoA at 10 minutes after lipopolysaccharide (25 μmol/L) addition, and the normalized level of GTP-RhoA was approximately 5-fold higher than that in unstimulated cells. We also determined whether prostaglandin F2α activates RhoA in...
myometrial cells, because lipopolysaccharide is known to stimulate prostaglandin production by macrophages or myometrial cells.\textsuperscript{18,19} The level of GTP-bound RhoA was also elevated at 10 minutes after the addition of prostaglandin F\textsubscript{2\alpha} (5 \textmu mol/L), and the normalized level of GTP-RhoA was significantly higher than that in unstimulated cells (Figure 1).

To confirm that RhoA/Rho-kinase cascade is activated in uterine smooth muscle tissues and in cultured myometrial cells, we determined the level of GTP-bound RhoA in uterine muscle tissues. We freeze-clamped uterine muscle tissues that were pretreated with or without lipopolysaccharide (25 \textmu mol/L; 15 minutes) or prostaglandin F\textsubscript{2\alpha} (5 \textmu mol/L; 15 minutes) and determined the amounts of GTP-RhoA with a pull-down assay. The level of GTP-Rho was elevated in uterine tissues that were treated with lipopolysaccharide compared with tissues that were not treated with lipopolysaccharide (Figure 2); the normalized level of GTP-RhoA was approximately 5-fold higher than that in unstimulated muscle tissues. Prostaglandin F\textsubscript{2\alpha} also significantly increased the level of GTP-bound RhoA (Figure 2). These results clearly show that both lipopolysaccharide and prostaglandin F\textsubscript{2\alpha} induce the activation of rhoA not only in cultured myometrial cells but also in uterine smooth muscle tissues.

Comment

In myometrium, as in other smooth muscles, an increase in free Ca\textsuperscript{2+} concentration is of great importance in the regulation of contractions.\textsuperscript{20} Calcium ions within smooth muscle cells bind with the Ca\textsuperscript{2+}-binding protein calmodulin and activate myosin light-chain kinase.\textsuperscript{19} Prostaglandin and oxytocin are known to stimulate uterine contractions by facilitating cytoplasmatic influx of calcium through Ca\textsuperscript{2+} channels and intracellular Ca\textsuperscript{2+} mobilization.\textsuperscript{20} Therefore, agents such as Ca\textsuperscript{2+} channel blockers have been used for tocolysis in threatened preterm delivery.\textsuperscript{21}

On the other hand, several lines of evidence indicate that the small GTPase RhoA is responsible for smooth muscle contraction by changing the Ca\textsuperscript{2+}-sensitivity of agonist stimulation.\textsuperscript{8} Recently, RhoA and its effector Rho-kinase have been implicated in smooth muscle contraction of the pregnant myometrium.\textsuperscript{11-13,22} As we\textsuperscript{11} and Woodcock et al\textsuperscript{23} reported previously, Y-27632 inhibits oxytocin-induced myometrial contraction without affecting the oxytocin-induced intracellular Ca\textsuperscript{2+} mobilization in myometrial cells. These results suggest that the attenuation of the RhoA/Rho-kinase pathway may lead to the inhibition of myometrial contraction, mainly by attenuating the Ca\textsuperscript{2+} sensitivity of the contractile apparatus. There are several reports that Y-27632 leads to the inhibition of agonist-induced contraction not only through a reduction in Ca\textsuperscript{2+} sensitivity but also by affecting intracellular Ca\textsuperscript{2+} mobilization in endothelial cells\textsuperscript{24} and in tracheal smooth muscle.\textsuperscript{25} Although we cannot exclude a possibility that Y-27632 leads to inhibition of uterine contraction by affecting intracellular Ca\textsuperscript{2+} mobilization, Rho-kinase could be a therapeutic target for the prevention of preterm labor.

In this study, we tested the preventive effect of a Rho-kinase inhibitor (Y-27632) in an animal model for experimental preterm delivery.\textsuperscript{14} As shown in the Table, the administration of Y-27632 significantly reduced the preterm delivery rate. This result supports the idea that RhoA/Rho-kinase plays a crucial role in inflammation-related premature labor. It has been reported that the expression of RhoA and Rho-kinase was up-regulated in the rat myometrium during the course of pregnancy.\textsuperscript{9,11} We also examined the effects of Rho-kinase inhibitor treatment on the length of full-term gestation in normal pregnancy. When Y-27632 was administered with an osmotic pump on day 15 of gestation, the gestation length was prolonged by treatment with Y-27632 at 10 mg/kg/d (average gestation length, 21.7 ± 0.8 days; Tahara M, unpublished data). These results suggest that the up-regulation of the expression of RhoA/Rho-kinase cascade may also play a physiologic role in normal pregnancy and in inflammation-related premature labor.

As for the mechanisms of lipopolysaccharide-induced uterine contraction, it has been shown that lipopolysaccharide stimulates Ca\textsuperscript{2+} influx into human\textsuperscript{20} and rat\textsuperscript{27} myometrial cells. It has been demonstrated that lipopolysaccharide activates RhoA in endothelial cells,\textsuperscript{28} which led us to speculate that lipopolysaccharide could induce RhoA activation in myometrial cells. As shown in Figures 1 and 2, lipopolysaccharide induced the activation of RhoA not only in cultured myometrial cells but also in uterine smooth muscle. Moreover, we determined whether prostaglandin F\textsubscript{2\alpha} activates RhoA in myometrial cells, because lipopolysaccharide is known to stimulate prostaglandin F\textsubscript{2\alpha} production by decidual macrophages or myometrial cells.\textsuperscript{18,19} Prostaglandin F\textsubscript{2\alpha} also increased GTP-bound RhoA in myometrial cells and uterine muscle tissues (Figures 1 and 2). These results suggest that the activation of the RhoA cascade may be an alternative mechanism of augmentation of uterine excitability by lipopolysaccharide or prostaglandin F\textsubscript{2\alpha}. Therefore, lipopolysaccharide can increase Ca\textsuperscript{2+} concentration directly and stimulate RhoA activation. In addition to these direct effects of lipopolysaccharide, we speculate that lipopolysaccharide could exert its indirect effect on uterine contractility by increasing prostaglandin F\textsubscript{2\alpha} production by uterine tissues, and these pathways may enhance uterine contractility cooperatively.

Our trials in an animal model system provide a requisite step before selective Rho-kinase inhibition.
is considered during human pregnancy. Rho-kinase inhibitors are very promising because the clinical application for the treatment of asthma, glaucoma, hypertension, and cancer metastasis is now under investigation. However, whether specific Rho-kinase inhibition results in decreased fetal morbidity remains a concern. Future studies that will be designed to assess the long-term viability of pups that are treated in utero with Rho-kinase–specific blockade are indicated.

In conclusion, the intraperitoneal administration of Rho-kinase inhibitor Y-27632 inhibited preterm delivery in mice. The present study may provide insight into possible treatment strategies that will involve the RhoA/Rho-kinase pathway.

Acknowledgments

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References

The cost of twin pregnancy: Maternal and neonatal factors

Barbara Luke, ScD, MPH, RD,a Morton B. Brown, PhD,b Pierre K. Alexandre, PhD,a Toyin Kinoshi, MS,c Mary Jo O’Sullivan, MD,d Dibe Martin, MD, d Ruta B. Misiunas, BA,e Clark Nugent, MD,e Cosmas Van De Ven, MD,e Roger B. Newman, MD,f Jill G. Mauldin, MD,f Frank R. Witter, MDg

Department of Epidemiology and Public Health, University of Miami School of Medicine, Miami, Fla,a Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, Mich, b Department of Knowledge Management, Jackson Memorial Hospital, Miami, Fla, c Department of Obstetrics and Gynecology, University of Miami School of Medicine, Miami, Fla, d Department of Obstetrics and Gynecology and Gynecology, University of Michigan School of Medicine, Ann Arbor, Mich, e Department of Obstetrics and Gynecology, Medical University of South Carolina, Charleston, SC, f Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, Md,g

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Objective: The purpose of this study was to evaluate factors affecting birth charges in twin pregnancies.

Study design: Clinical and financial data were obtained on 1486 twin pregnancies delivered between 1995 to 2002 at medical centers in Maryland, Florida, Michigan, and South Carolina. Maternal and neonatal length of stay (LOS) and charges were modeled by gestational age and other risk factors using a general linear model.

Results: Maternal and infant birth admission LOS and charges increased significantly with a decline in gestational age. Maternal LOS and charges were also significantly increased by cesarean delivery and preeclampsia. Newborn LOS and charges increased significantly by monochorionicity and slowed growth between 20 to 28 weeks. For mother and infants, the shortest LOS and lowest birth charges were at 37 to 38 weeks.

Conclusion: These findings reflect the substantial maternal and neonatal morbidity associated with twin pregnancies, and demonstrate that 37 to 38 weeks is their optimal gestation.

In 2002, there were more than 132,000 infants born from multiple pregnancies in the US, the highest number ever recorded.1 The rate of twin births, which accounts for more than 94% of all multiple births, has risen 38% since 1990 and 65% since 1980. This increase is due primarily to the related trends of childbearing at older ages and the widespread use of fertility therapies.2-4 Born an average of 3 weeks earlier and nearly 1 kg lighter than singletons, twins are disproportionately represented among the preterm and early preterm, low-birth-weight, and very-low-birth weight infant populations.5-7 At the lower range of viability, there is little debate regarding morbidity, although term for twins remains controversial. Population-based studies of twins...
have identified delivery at 36 to 37 weeks to be associated with the lowest risk of fetal death.\textsuperscript{8} Less is known about factors influencing neonatal morbidity in twin gestations. The purpose of this study was to use maternal and neonatal hospital charges, length of stay (LOS), and frequency of neonatal diagnoses as a means of estimating the optimal timing of delivery for twins.

**Material and methods**

**Study population**

The study population included all twin pregnancies delivered from 1995 to 2002 from Johns Hopkins University, Baltimore, Maryland; University of Michigan, Ann Arbor, Michigan; University of Miami/Jackson Memorial Hospital, Miami, Florida; and Medical University of South Carolina, Charleston, South Carolina. The study population was limited to pregnancies that met the following inclusion criteria: (1) both twins born alive and discharged alive; (2) \( \geq 20 \) weeks gestation by last menstrual period, first-trimester ultrasound scan, or best obstetric estimate (a combination of clinical and ultrasonographic estimates); (3) documented maternal height; (4) pregravid weight; (5) documented genders and birth weights of both infants in the twin pair; and (6) absence of major congenital anomalies as documented by normal findings in the newborn medical record. All data were abstracted from hospital charts. Hospital charges were obtained from the departments of finance, and the study was approved by the institutional review boards at the respective institutions.

**Study variables**

The abstracted data included maternal age, race and Hispanic ethnicity, smoking during pregnancy, parity, conception with assisted reproductive technology (ART), multifetal pregnancy reduction (MFPR), chorioicity, gender of each infant, maternal size (height, pregravid weight, weight at each prenatal visit), all fetal weights (estimated by ultrasonography), birth weights, gestational age, infant genders, and cesarean delivery. Maternal complications included preterm premature rupture of membranes, pregnancy-induced hypertension, and preterm labor. Length of stay and charges were calculated for antenatal admissions and the birth admission by gestation group. Neonatal procedures and complications included NICU admission, supplemental oxygen after delivery, ventilator support, phototherapy, blood transfusion, necrotizing enterocolitis, patent ductus arteriosus, respiratory distress syndrome or hyaline membrane disease, and retinopathy of prematurity. Major morbidity was defined as retinopathy of prematurity, necrotizing enterocolitis, ventilator support, or intraventricular hemorrhage (grades III and IV).

**Statistical analyses**

Maternal pregravid body mass index (BMI) was calculated as \( \frac{\text{weight}}{\text{height}^2} \). Women were characterized by their pregravid BMI as underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9), or obese (\( \geq 30 \)). Rates of maternal weight gain were estimated from regression curves fit to measure prenatal weights over time. From the regression equations, we predicted the rate of maternal weight gain to 20 weeks’ gestation, between 20 and 28 weeks’ gestation, and between 28 weeks’ gestation and birth. Fetal growth was estimated from regression curves fit to ultrasonographic fetal weight measures. From the regression equations, we predicted the rates of fetal growth to 20 weeks’

<table>
<thead>
<tr>
<th>Table I: Characteristics of the study population</th>
<th>% or mean (SEM)</th>
<th>Range (1486 twin pregnancies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study sites (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johns Hopkins University</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>University of Miami</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>University of Michigan</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>Medical University of South Carolina</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>Race and Hispanic ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>46.5</td>
<td></td>
</tr>
<tr>
<td>Black, non-Hispanic</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Maternal age (y)</td>
<td>28.5 (0.2)</td>
<td>13-50</td>
</tr>
<tr>
<td>13-50</td>
<td>1.02 (0.03)</td>
<td>0-10</td>
</tr>
<tr>
<td>&gt;35 (%)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>0-10</td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Length of gestation (weeks)</td>
<td>35.4 (0.09)</td>
<td>22-43</td>
</tr>
<tr>
<td>&lt;30 wk (%)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>&lt;32 wk (%)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>&lt;36 wk (%)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Height (in)</td>
<td>64.6 (0.07)</td>
<td>46-76</td>
</tr>
<tr>
<td>&lt;62 (%)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Pregravid weight (lb)</td>
<td>152.3 (1.10)</td>
<td>87-400</td>
</tr>
<tr>
<td>Pregravid body mass index (BMI, wt/ht$^2$)</td>
<td>25.5 (0.18)</td>
<td>15.5-63.7</td>
</tr>
<tr>
<td>Underweight (%)</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Normal weight (%)</td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>Overweight (%)</td>
<td>21.1</td>
<td></td>
</tr>
<tr>
<td>Obese (%)</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>Low birth weight (%)</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>(&lt;2500 g) (%)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Very low birth weight (&lt;1500 g) (%)</td>
<td></td>
<td></td>
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</tbody>
</table>
gestation, between 20 and 28 weeks’ gestation, and between 28 weeks’ gestation and birth. These gestational periods have been shown in our previous studies on weight gain in twin pregnancies to be more important for fetal growth, rather than traditional trimesters.9,10 Regression equations including quadratic terms with no intercept were found to fit the data well. In addition, we corrected for the proportional upward bias in ultrasound estimated fetal weights near birth, forcing the regression curve through the actual birth weight. Rates of fetal growth were calculated as the predicted gain in each gestational interval, with birth weight used as the last measurement. Fetal growth for the larger and smaller of the twin pair were characterized as slowed at rates $<90$ g per week between 20 and 28 weeks’ gestation and $<168$ g after 28 weeks’ gestation, which is below the 10th percentile using singleton growth standards.11 Length of gestation was based on the last menstrual period if it was within 10 days of the earliest ultrasonographic estimate; if not, the latter was used to calculate length of gestation. Infants were divided into gestation groups: $<28$, 28-29, 30-31, 32-33, 34-35, 36, 37, 38, 39 and $\geq 40$ weeks.

To account for the effects of inflation, all charges were converted into constant 2002 dollars.12 For better accuracy, when available, we used city-specific price indexes, with the exception of Charleston, for which we used price changes for the Atlanta area. More importantly, rather than using the general consumer price index, our analysis used the medical care price index, which includes prices for medical care commodities (prescription drugs, nonprescription over-the-counter drugs, and other medical equipment and supplies) and medical care services (professional medical services, Table II

Maternal characteristics, complications, and antenatal and birth admission length of stay (LOS) and charges by weeks of gestation*

<table>
<thead>
<tr>
<th>Week</th>
<th>Percentages within gestation groups</th>
<th>Antenatal admissions</th>
<th>Birth admission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N)</td>
<td>Primip</td>
<td>ART</td>
</tr>
<tr>
<td>&lt;28</td>
<td>57</td>
<td>53.6</td>
<td>30.2</td>
</tr>
<tr>
<td>28-29</td>
<td>52</td>
<td>53.8</td>
<td>22.4</td>
</tr>
<tr>
<td>30-31</td>
<td>107</td>
<td>56.6</td>
<td>26.7</td>
</tr>
<tr>
<td>32-33</td>
<td>172</td>
<td>49.7</td>
<td>22.9</td>
</tr>
<tr>
<td>34-35</td>
<td>312</td>
<td>42.9</td>
<td>23.1</td>
</tr>
<tr>
<td>36</td>
<td>226</td>
<td>43.3</td>
<td>23.6</td>
</tr>
<tr>
<td>37</td>
<td>253</td>
<td>39.1</td>
<td>27.5</td>
</tr>
<tr>
<td>38</td>
<td>177</td>
<td>43.5</td>
<td>19.7</td>
</tr>
<tr>
<td>39</td>
<td>38</td>
<td>43.4</td>
<td>7.2</td>
</tr>
<tr>
<td>$\geq$ 40</td>
<td>47</td>
<td>40.9</td>
<td>9.3</td>
</tr>
<tr>
<td>All</td>
<td>1486</td>
<td>44.9</td>
<td>22.7</td>
</tr>
<tr>
<td>Significance</td>
<td>0.078</td>
<td>0.007</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Primip, Primiparity; PTL, preterm labor; mono, monochorionic placentation; PIH, pregnancy-induced hypertension; ART, conception with assisted reproductive technology; PROM, premature rupture of membranes; MFPR, multifetal pregnancy reduction; LOS, length of hospital stay; C/S, cesarean delivery. Significance is chi-square or ANOVA $P$ value across gestation groups.

*Data presented as either percentages or means (SEM).
hospital services, nursing home services, and health insurance premiums). Gestational age groups included 28, 28-29, 30-31, 32-33, 34-35, 36, 37-38, 39 and 40 weeks. Birth weight, length of hospital stay, and birth charges were calculated by smaller and larger of the twin pair by gestation group.

Dummy variables were created for study site, year of delivery, and gestation group. Separate multivariate regression models were fitted to the LOS and charges for the mother, the larger twin, and the smaller twin of each pair (a total of 6 models); both LOS and charges were log-transformed due to skewness. Each model was fitted to gestational age and adjusted for year of birth, study site, and for the newborns, the genders of the twin pair. In models of maternal charges and LOS, dummy variables for cesarean delivery and pregnancy-induced hypertension were included. In models of infant charges and LOS, dummy variables for monochorionicity and slowed growth were included.

**Results**

Characteristics of the study population are given in Table I. Maternal characteristics, complications, and antenatal and birth admission length of stay and charges by gestation group are shown in Table II. Forty-five percent of women were primiparas; 23%
had infertility treatments; 16% were monochorionic; average twin pair birth weight was 2293 g at the average gestational age of 35.4 weeks. Twenty-nine percent of women had an antepartum admission for an average of 4.7 days, and a mean charge of $7,547. Birth admission LOS for the mother averaged 5.7 days at $10,251.

Factors that differed significantly across gestational groups included ART conceptions, MFPR, monochorionicity, preterm rupture of membranes, preterm labor,

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Log and antilog of factors affecting maternal and newborn birth admission length of stay and charges in twin pregnancies compared with delivery at 37 to 38 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birth length of stay</strong></td>
<td><strong>Birth charges</strong></td>
</tr>
<tr>
<td><em>β-coefficient</em></td>
<td><strong>SE</strong></td>
</tr>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.541</td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td>0.096</td>
</tr>
<tr>
<td>Pregnancy-induced hypertension</td>
<td>0.124</td>
</tr>
<tr>
<td>Delivery &lt;28 vs 37-38 wk</td>
<td>0.287</td>
</tr>
<tr>
<td>Delivery 28-29 vs 37-38 wk</td>
<td>0.236</td>
</tr>
<tr>
<td>Delivery 30-31 vs 37-38 wk</td>
<td>0.193</td>
</tr>
<tr>
<td>Delivery 32-33 vs 37-38 wk</td>
<td>0.142</td>
</tr>
<tr>
<td>Delivery 34-35 vs 37-38 wk</td>
<td>0.034</td>
</tr>
<tr>
<td>Delivery 36 vs 37-38 wk</td>
<td>0.022</td>
</tr>
<tr>
<td>Delivery 39 vs 37-38 wk</td>
<td>0.002</td>
</tr>
<tr>
<td>Delivery ≥40 vs 37-38 wk</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Newborn</strong></td>
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</tr>
<tr>
<td>Intercept</td>
<td>0.561</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.570</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.036</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.030</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.100</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.199</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.097</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.091</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>1.046</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.974</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.788</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.861</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.571</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.623</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.257</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.264</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.086</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.088</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.036</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.063</td>
</tr>
<tr>
<td>Smaller of twin pair</td>
<td>0.037</td>
</tr>
<tr>
<td>Larger of twin pair</td>
<td>0.056</td>
</tr>
</tbody>
</table>

* Models adjusted for year and study site.
* Models adjusted for year, study site, and genders of twin pair.

References


Association between vaginal 70-kd heat shock protein, interleukin-1 receptor antagonist, and microbial flora in mid trimester pregnant women

Mehmet R. Genc, MD, PhD, a Emre Karaşahin, MD, b Andrew B. Onderdonk, PhD, c Ann Marie Bongiovanni, AB, b Mary L. Delaney, MS, c Steven S. Witkin, PhD, b,* the Microbiology and Prematurity Study Group

Department of Obstetrics, Gynecology and Reproductive Biology, Brigham & Women’s Hospital, Harvard Medical School, Boston, Mass, a Department of Obstetrics and Gynecology, Weill Medical College of Cornell University, New York, NY, b Channing Laboratory, Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass c

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KEY WORDS
70-kd Heat shock protein
Cytokine
Vaginal microflora
Pregnancy
Bacterial vaginosis

Objective: This study investigated the association among the inducible 70-kd heat shock protein, cytokines, and microbial flora in the vagina in mid trimester pregnant women and subsequent preterm delivery.

Study design: Vaginal samples from 205 pregnant women, which were collected at 18 to 22 weeks of gestation, were analyzed for qualitative and quantitative vaginal microflora and for 70-kd heat shock protein, interleukin-1β, interleukin-1 receptor antagonist, and tumor necrosis factor-α by enzyme-linked immunosorbent assay. Pregnancy outcome data were obtained subsequently.

Results: The 70-kd heat shock protein was detected in 38 vaginal samples (18.5%). Its presence was associated with elevated vaginal pH, a diagnosis of bacterial vaginosis, and elevated interleukin-1 receptor antagonist levels (P < .001). Among women with bacterial vaginosis, 70-kd heat shock protein–positive subjects had a >80% increase in median vaginal concentration of interleukin-1 receptor antagonist (P < .05).

Conclusion: Vaginal 70-kd heat shock protein expression is associated with the down-regulation of the proinflammatory immune response to abnormal vaginal flora in mid trimester pregnant women.

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Heat shock proteins are highly conserved proteins that are present in every organism from bacteria to man. Synthesis of an inducible 70-kd heat shock protein (hsp70) is up-regulated in response to physiologic insults, such as elevated temperature, inflammation, infection, or toxic metabolites. Hsp70 enhances cell survival under adverse conditions by preventing protein denaturation and incorrect peptide folding and aggregation, by tagging...
denatured proteins for elimination, and by inhibiting the induction of apoptosis.1

Bacterial vaginosis (BV) describes the clinical condition that is characterized by a malodorous vaginal discharge, a vaginal pH > 4.5, and a shift away from a Lactobacilli-dominant vaginal flora towards a predominance of Gardnerella vaginalis, anaerobic bacteria, and Mycoplasma hominis.2,3 It remains unclear whether the decrease in lactobacilli is the primary event and precedes the overgrowth of the other micro-organisms or whether the proliferation of G vaginalis and the anaerobes occurs first and causes the disappearance of the lactobacilli. Furthermore, it is completely unknown what triggers this massive alteration in the vaginal ecosystem.

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are cytokine mediators of the host immune response to invading micro-organisms, increase in concentration in the vagina in response to alterations in the microbial flora.4,5 The concentration of IL-1 receptor antagonist (IL-1ra), a competitive inhibitor of IL-1β, also increases under these conditions.6 IL-1ra regulates the proinflammatory action of IL-1 so that pathogenic micro-organisms are destroyed while normal tissue architecture and function are maintained. Hsp70 also is elevated in the sera and vaginal secretion of women with BV.5,7 Detection of Hsp70 in the vagina was associated with a high concentration of the anti-inflammatory cytokine IL-10, but not with elevated levels of proinflammatory cytokines such as IL-1 and IL-8.7 These results suggest that the detection of Hsp70 in vaginal fluid is associated with an enhanced anti-inflammatory cytokine response to abnormal vaginal flora.

There is now a substantial body of evidence that BV increases the risk of preterm delivery by approximately 2-fold.8 However, antibiotic treatment of BV in pregnant women to prevent preterm birth has been largely, but not exclusively,9 unsuccessful.10,11 The detection of BV is related to adverse pregnancy outcome, particularly in some subsets of patients, and the treatment of BV early in the course of their pregnancy may lead to a significant decrease in the rate of BV-related adverse outcomes. A 4-fold increased risk for preterm delivery was calculated for women who were screened for BV at <20 weeks of gestation.8 Furthermore, there appears to be an inter-individual variability in the capacity to counteract the negative effects of BV-related micro-organisms or their products on gestation. An imbalance between pro- and anti-inflammatory cytokines that favors the prolongation and enhancement of proinflammatory immunity is likely to be one factor that is associated with adverse clinical events. We recently demonstrated that a disproportionate increase in vaginal levels of IL-1β over IL-1ra in women with altered bacterial flora in mid trimester was associated with subsequent spontaneous preterm delivery.9 Given that Hsp70 in vaginal fluid is associated with an enhanced anti-inflammatory cytokine response, women with Hsp70 that is detected in their vaginal fluid in mid trimester might be expected to have a lower rate of preterm deliveries. This study was performed to test this hypothesis.

Material and methods

Subjects
A detailed description of subject enrolment and data collection has been published previously.4 Eligible women who had their first prenatal visit to the Brigham and Women’s Hospital, Boston, from January 1, 2000, to March 1, 2002, were approached by the study nurse. Exclusion criteria at enrolment, which were designed to eliminate conditions that were associated with a high rate of preterm births and that were unrelated to infection were age <15 years, antimicrobial therapy within 4 weeks of initial sampling, multiple gestation, cervical cerclage, previous pregnancy loss at <24 weeks of gestation, placenta previa, isoimmunization, and/or other obstetric or chronic medical conditions that predisposed to preterm delivery. Although infection may have played a role in preterm delivery among women who underwent cerclage placement, inclusion of women with cerclage certainly may change microflora, cytokines, and hsp70 concentrations and thus is an appropriate exclusion criterion. Of 400 eligible patients, 300 women (75%) were approached at 18 to 22 weeks of gestation; 231 women agreed to participate. The most common reasons for failure to approach patients included the unavailability of study nurses or laboratory personnel or a failure to keep appointments. Written informed consent was obtained from all patients. Women with incomplete test results and women with fetal death, who were lost to follow up, or who delivered at <37 weeks of gestation for obstetric indications in the absence of preceding spontaneous labor or spontaneous rupture of membranes were excluded from the final analysis.

Microbiologic analysis

Four vaginal swab samples were collected for quantitative and qualitative microbiologic evaluation, pH determination, and Gram staining and culture, as previously described.2,3 Microbiologic counts were recorded as log10 colony forming units (CFU) per gram of sample. The Nugent Gram stain scoring system was used to assess vaginal smears for the presence of BV.12

Determination of hsp70 and cytokine concentrations

After the collection of swabs, cervicovaginal lavage was performed with 10 mL non-bacteriostatic saline solution in a 10-mL syringe to which a soft plastic cannula had
been attached. The pooled lavage was aspirated from the posterior fornix into a 15-mL conical polypropylene tube and transported on ice to the laboratory within 4 hours of collection. The specimen was centrifuged at 600g for 14 minutes. The vaginal lavage supernatants were analyzed for IL-1ra, IL-1β, TNF-α (BioSource International Inc, Camarillo, Calif) and Hsp70 (Stress-Gen, Victoria, British Columbia, Canada) concentrations by commercial enzyme-linked immunosorbent assays. The values were converted to picograms per milliliter (TNF-α, IL-1β) or nanograms per milliliter (IL-1ra, hsp70) by reference to a standard curve that was generated in parallel to the test samples.

**Statistical analysis**

Means of normally distributed data were compared using 2-tailed unpaired *t*-test. Chi-squared test or Fisher’s exact test were used to compare proportions. Cytokine values were not distributed normally; thus, a nonparametric test (2-tailed Mann-Whitney *U* test) was used to analyze such data. A comparison of cytokine concentrations was performed for the entire study population, and subpopulations were defined as hsp70-positive and -negative subjects with and without BV. A probability value <.05 was considered statistically significant. The software package StatsDirect (Cheshire, UK) was used for data analyses.

A power analysis was performed to estimate the sample size that was required to detect a 20% increase above a baseline preterm delivery rate of 15% that was observed in this population. Using these estimates and assuming 80% power and an α of .05, we calculated that we would need a total of 186 subjects.

**Results**

**Demographic characteristics**

Of the 231 subjects who were enrolled, 26 women were excluded subsequently from analysis for in utero fetal death at 19 weeks of gestation because of fetal cytomegalovirus infection (n = 1 woman), lost to follow up (n = 1 woman), vaginal lavage samples unavailable for analysis (n = 16 women), induction of labor <37 weeks of gestation because of pregnancy-induced hypertension (n = 5 women), and elective cesarean delivery at <37 weeks of gestation because of idiopathic fetal hydrops (n = 1 woman) or previous full-thickness myomectomy (n = 2 women). The final analysis therefore was performed on a total of 205 women, which included 83 white women (40%), 59 Hispanic women (29%), 44 black women (21%), and 19 women (9%) of other ethnicities.

Hsp70 was detected in vaginal secretions from 38 subjects (18.5%). The median hsp70 concentration in the positive samples was 0.64 ng/mL, with upper and lower quartiles of 0.35 and 1.31 ng/mL, respectively. Demographic and pregnancy outcome data in relation to hsp70 status are shown in Table I. There were no differences in maternal age at delivery, parity, outcomes of the current and previous pregnancies, smoking history, alcohol history, and insurance status between those who were tested positive or negative for hsp70.

**Vaginal pH and microflora**

The median vaginal pH was significantly higher for hsp70-positive than for hsp70-negative women (5.0 vs 4.4; *P* < .001; Table II). A Nugent’s score of ≥7 was used to establish a diagnosis of BV. On the basis of this criteria, BV was much more frequent in hsp70-positive than in hsp70-negative women (42% vs 9%; *P* < .001; Table II).

From the 205 subjects, 1934 bacterial isolates that represented 115 phenotypes were characterized. A comparison of frequency of isolation and mean counts of CFUs per gram of vaginal fluid for the major phenotypes (isolated from at least 20% of subjects) that comprised the vaginal microflora are shown in Table II. Differences in the isolation rates and mean CFU counts for certain bacteria in relation to hsp70 status were apparent. Hsp70-positive women had significantly higher mean CFU counts for *G. vaginalis* (9.40 ± 0.20 vs 8.54 ± 0.28 log10 CFU per gram of vaginal fluid; *P* = .008), *Mycoplasma* species (6.57 ± 0.20 vs 5.94 ± 0.20 log10 CFU per gram of vaginal fluid; *P* = .042), and anaerobic Gram-negative rods (*Prevotella, Bacteroides*,...
**Table II** Relation among hsp70 status, vaginal microflora, and cytokine levels

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Hsp70-negative (n = 167)</th>
<th>Hsp70-positive (n = 38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nugent score ≥7, %</td>
<td>9</td>
<td>42</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Vaginal pH*</td>
<td>4.4 (4.4, 4.7)</td>
<td>5.0 (4.4, 6.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td><em>Actinomyces</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>28</td>
<td>26</td>
<td>.895</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.55 ± .24</td>
<td>5.55 ± .67</td>
<td>.987</td>
</tr>
<tr>
<td><em>Candida</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>25</td>
<td>16</td>
<td>.253</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.31 ± 0.16</td>
<td>4.86 ± 0.38</td>
<td>.303</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>59</td>
<td>63</td>
<td>.670</td>
</tr>
<tr>
<td>Colony count†</td>
<td>4.71 ± 0.10</td>
<td>5.05 ± 0.27</td>
<td>.230</td>
</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>28</td>
<td>53</td>
<td>.004</td>
</tr>
<tr>
<td>Colony count†</td>
<td>8.54 ± 0.28</td>
<td>9.40 ± 0.20</td>
<td>.008</td>
</tr>
<tr>
<td><em>Lactobacilli</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>94</td>
<td>87</td>
<td>.158</td>
</tr>
<tr>
<td>Colony count†</td>
<td>8.91 ± 0.06</td>
<td>8.38 ± 0.06</td>
<td>.021</td>
</tr>
<tr>
<td><em>Mycoplasma</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>17</td>
<td>45</td>
<td>.001</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.94 ± 0.20</td>
<td>6.57 ± 0.20</td>
<td>.042</td>
</tr>
<tr>
<td><em>Peptostreptococcus</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>45</td>
<td>66</td>
<td>.026</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.33 ± 0.16</td>
<td>5.72 ± 0.41</td>
<td>.379</td>
</tr>
<tr>
<td><em>Prevotella, Bacteroides, Porphyromonas</em> spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>44</td>
<td>55</td>
<td>.205</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.61 ± 0.21</td>
<td>6.85 ± 0.44</td>
<td>.016</td>
</tr>
<tr>
<td><em>Staphylococcus</em> epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>31</td>
<td>34</td>
<td>.657</td>
</tr>
<tr>
<td>Colony count†</td>
<td>4.23 ± 0.13</td>
<td>4.14 ± 0.13</td>
<td>.612</td>
</tr>
<tr>
<td><em>Ureaplasma urealyticum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolation rate (%)</td>
<td>21</td>
<td>29</td>
<td>.289</td>
</tr>
<tr>
<td>Colony count†</td>
<td>5.79 ± 0.13</td>
<td>6.24 ± 0.16</td>
<td>.072</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>3.47</td>
<td>6.16</td>
<td>.195</td>
</tr>
<tr>
<td></td>
<td>(1.45, 9.16)</td>
<td>(1.81, 12.69)</td>
<td></td>
</tr>
<tr>
<td>IL-1ra (ng/mL)</td>
<td>30.9</td>
<td>98.8</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>(11.1, 93.1)</td>
<td>(44.5, 185.1)</td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>2.63</td>
<td>3.04</td>
<td>.389</td>
</tr>
<tr>
<td></td>
<td>(1.44, 5.03)</td>
<td>(1.33, 6.76)</td>
<td></td>
</tr>
</tbody>
</table>

* Values are expressed as median (lower-upper quartiles).
† Values are expressed as mean log_{10} colony-forming units/g of sample ± SEM.

Significantly less among hsp70-positive as compared with hsp70-negative women (8.91 ± 0.06 vs 8.38 ± 0.06 log_{10} CFU per gram of vaginal fluid; P < .042).

**Cytokine profiles**

Median vaginal IL-1ra concentrations were significantly higher in hsp70-positive as compared with hsp70-negative women (98.8 vs 30.9 ng/mL; P = .001). There were no significant differences in the median vaginal IL-1α and TNF-α levels between the 2 groups (Table II).

The relation between BV, hsp70 status, and median vaginal concentrations of IL-1α and IL-1ra is shown in Table III. The diagnosis of BV was associated with higher median vaginal levels of IL-1α and IL-1ra in both hsp70-positive and -negative women. In women diagnosed with BV, hsp70-positive subjects had a significantly higher median vaginal concentration of IL-1ra than did hsp70-negative subjects (185.2 vs 100.7 ng/mL, P = .047). In contrast, the median IL-1β concentration was similar in hsp70-positive and hsp70-negative women with BV (10.47 vs 15.47 pg/mL; P = .24). In the absence of BV, neither the mean IL-1β or IL-1ra concentration was significantly different between the hsp70-positive and -negative subjects.

**Comment**

Detection of hsp70 in vaginal fluids from mid trimester pregnant women was associated with BV and elevated vaginal pH. Qualitative and quantitative bacteriologic analysis revealed that the isolation of anaerobic Gram-negative rods, *G vaginalis*, *Peptostreptococcus*, and *Mycoplasma* spp was more frequent and with higher CFU counts; *Lactobacilli* were isolated with lower CFU counts from hsp70-positive women.

There is conflicting evidence regarding the intracellular and extracellular functions of hsp70 in the regulation of innate immunity. Induction of hsp70 gene transcription inhibits expression of the genes coding for...
the proinflammatory cytokines, IL-1β and TNF-α.\textsuperscript{14-16} Thus, activation of the hsp70 gene as a consequence of infection or other stressors results in the down-regulation of proinflammatory immune responses. Conversely, it has been proposed that the extracellular release of hsp70 may serve as an early warning “danger” signal that a nonphysiologic condition is present and that immune system activation is needed.\textsuperscript{17} Cell-free hsp70 has been shown to bind to specific receptors on antigen-presenting cells and to stimulate production of proinflammatory cytokines.\textsuperscript{18,19} We speculate that, in the vagina in the setting of pregnancy, hsp70 induces the anti-inflammatory cytokine, IL-1ra, to down-regulate the induction of cell-mediated immunity. This hsp70-mediated IL-1ra expression may be necessary to minimize the likelihood of premature myometrial contractions and localized tissue damage during gestation. The demonstration that the vaginal concentration of IL-1ra is elevated in the presence of hsp70, in women with BV, suggests that hsp70 may be a unique enhancer of the anti-inflammatory cytokine response to infection in the lower female genital tract during pregnancy. A correlation between progressive disappearance of lactobacillary morphotypes and an elevation in the vaginal IL-1ra concentration has been observed previously.\textsuperscript{20}

There are precedents for the association of hsp70 with anti-inflammatory immunity. Incubation of mycobacterial hsp70 with human peripheral blood mononuclear cells in vitro has been shown to result in induction of the anti-inflammatory cytokine, IL-10.\textsuperscript{21} The presence of vaginal hsp70 in healthy, asymptomatic nonpregnant women has also been associated with elevated IL-10 concentrations.\textsuperscript{7}

The reason that only some women with BV have detectable levels of hsp70 in their vagina remains unexplained. The inducible hsp70 gene is polymorphic,\textsuperscript{22} and women may be predisposed genetically to express greater or lesser amounts of hsp70 in response to environmental stress. This, in turn, may influence the capacity for bacterial proliferation in the vagina because of differential expression of pro- and anti-inflammatory immunity.

In the present study, there was no difference in the rate of spontaneous preterm delivery between the hsp70-negative and hsp70-positive women. However, the number of women with a preterm birth was too small for a definitive analysis. In addition, our previous study suggested that the risk of preterm delivery increases if the vaginal IL-1β concentration is >10 pg/mL and the IL-1ra:IL-1β ratio is >8632:1.\textsuperscript{4} In the present investigation, the median IL-1β concentration was similar in both the hsp70-positive and hsp70-negative women, and neither exceeded 10 pg/mL.

Murine intestinal epithelial cells have been shown to increase expression of hsp70 in response to the anaerobic Gram-negative micro-organism, \textit{Bacteroides fragilis}. Treatment with metronidazole led to a marked decrease in epithelial hsp70 expression. It was hypothesized that enteric bacteria-induced hsp70 expression enhanced cell viability in the presence of potential pathogenic microorganisms.\textsuperscript{23} It is interesting to speculate that a similar mechanism might be present in the vagina during pregnancy. Hsp70-negative women with BV might be less efficient at counteracting the negative effects of an altered vaginal microflora and might be at a greater risk for preterm delivery than would hsp70-positive women with BV. Women in the former group had almost a 50% higher vaginal IL-1β level and an almost 4-fold lower IL-1ra:IL-1β ratio than did women in the latter group. Further investigations are needed to evaluate this possibility. The present study was not designed to test this hypothesis and lacked power for this analyses.

In conclusion, the presence of hsp70 in vaginal secretions of asymptomatic pregnant women in mid trimester was associated with BV and elevated IL-1ra level. Although vaginal expression of hsp70 is an indicator of an altered vaginal flora in asymptomatic women, its value in the assessment of the risk in a general pregnant population for subsequent preterm birth remains unspecified. We speculate that the vaginal expression of hsp70 may provide a mechanism to down-regulate proinflammatory immunity that may be detrimental to the continuation of the pregnancy. Whether hsp70 is released in response to interactions between a microorganism and its host may aid in the determination of the specificity of cytokine response to that micro-organism. This, in turn, may influence whether a given micro-organism will be a benign commensal or will initiate clinical disease. Prospective studies are needed to assess whether the rate of a subsequent spontaneous preterm delivery will be decreased among women with BV in their mid trimester who are positive for vaginal hsp70 as opposed to BV-negative, hsp70-negative women.

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Members of the MAP study group include Andrea M. DuBois, Linda Steele, Dorothy Bender, Robin A. Ross, Mei-Ling Lee, Ellice Lieberman, and Amy Cohen.

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Aspirin use during early pregnancy and the risk of congenital abnormalities: A population-based case-control study

Bente Nørgård, MD, PhD, Erzsébet Puhó, MSc, Andrew E. Czeizel, MD, PhD, Mette V. Skriver, MSc, Henrik T. Sørensen, MD, PhD

Department of Clinical Epidemiology, Aarhus University Hospital, Aarhus, Denmark, Department of Human Genetics and Teratology, National Center for Epidemiology, Budapest, Hungary, and Foundation for the Community Control of Hereditary Diseases, Budapest, Hungary

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KEY WORDS
Aspirin
Congenital abnormalities
Pregnancy

For decades, it has been controversial whether aspirin use during pregnancy increases the risk of congenital abnormalities (CAs). Recently, a meta-analysis, based on 22 studies published between 1971 and 2002, has suggested an increased risk of neural tube defects (odds ratio [OR] = 2.2; 95% CI: 0.93-5.17), gastroschisis (OR = 2.37; 95% CI: 1.44-3.88), and cleft lip and palate (OR = 2.87; 95% CI 2.04-4.02) after aspirin use in early pregnancy. Such a meta-analysis, however, may be hampered by heterogeneity of study designs with a risk of, in particular, recall bias, inability to adjust for confounding, and different data collection methods. Because aspirin is widely used, any teratogenic effect has major public health implications.

We examined the association between maternal aspirin use in the 5 to 12 weeks of gestation (the critical period for most major CAs) and the above-mentioned specific CAs in a population-based dataset of the Hungarian Case Control Surveillance of Congenital Abnormalities (HCCSCA) from 1980 to 1996. This association has been examined in this database previously, but the paper cannot be found in PubMed MEDLINE (National Library of Medicine) and was not included in the meta-analysis. Here, we examined the association based on a new strategy of analysis, using a different statistical approach to reduce the risk of recall bias.

Material and methods

The HCCSCA is based on the Hungarian Congenital Abnormality Registry, which is a national-based registry of cases with CAs. Reporting of malformed fetuses...
after prenatal diagnosis, stillborns, and live born children is compulsory in Hungary for physicians.2

The 3415 children included in the study had 4 selected and isolated CAs diagnosed: neural-tube defects (N = 1202), exomphalos/gastroschisis (N = 238), cleft lip ± palate (N = 1374), and posterior cleft palate (N = 601).

Mothers of children with CAs tend to recall antenatal drug exposure differently from mothers of infants without CAs. To offset this potential bias, the control group comprised all children with CAs other than the 4 above-mentioned CA groups (N = 19,428). None of the included children had syndromes of known origin (eg, chromosome disorders).

Information on aspirin use was obtained from: (1) a detailed questionnaire with an explanatory letter as well as lists of diseases and drugs, including information on medication taken during pregnancy according to gestational week; and (2) the antenatal care logbook written by the obstetricians. Information on aspirin use was available for all 4 case groups and patient controls.

We used logistic regression models to estimate the relative risk (OR), with 95% CI, of 4 groups of CAs associated with aspirin use, adjusted for potential confounding factors (maternal age, birth order, use of folic acid during the first trimester of pregnancy, nausea/vomiting or common cold/influenza (during the first trimester of pregnancy), and mothers’ underlying diseases of diabetes mellitus or epilepsy.

Results

The adjusted relative risks for neural-tube defects, exomphalos/gastroschisis, cleft lip ± palate, and posterior cleft palate associated with aspirin use were 1.1 (95% CI: 0.7-1.6), 0.7 (95% CI: 0.2-2.2), 0.9 (95% CI: 0.6-1.3), and 1.0 (95% CI: 0.6-1.8), respectively (Table).

Comment

These data suggest that maternal use of aspirin in early pregnancy is not associated with increased risk of neural-tube defects, exomphalos/gastroschisis, cleft lip ± palate, or posterior cleft palate in the offspring compared with nonusers of aspirin.

The results, based on this large population-based case-control dataset, were not included in the meta-analysis. The use of a control group of mothers of children with CAs reduces the risk of recall bias, which is a major concern in case-control studies based on self-reported data. In our data, each case of CA was validated, and we had the ability to control for confounders. Analyses of neural-tube defects, cleft lip ± palate, and posterior cleft palate were controlled for maternal age, birth order, use of folic acid, maternal diseases of nausea/vomiting, common cold/influenza, and diabetes mellitus and epilepsy. The analysis of exomphalos/gastroschisis was adjusted for the same, except for diabetes mellitus and epilepsy. Unfortunately, we had no information on dose of aspirin or maternal smoking in all pregnancies.

Even though our results are reassuring regarding the safety of aspirin use in early pregnancy, it is important to report all available data on this subject to determine whether the associations are causal or influenced by change or bias.

References

Dose response of RU486 in a novel rabbit model of noninfectious preterm birth: Comparative efficacy of 3 routes of administration

David Gorenberg, MD,a,c,* Kay Beharry, BS,a Kenji C. Nishihara, BA,b Eileen Chang, BS,b Joshua Waltzman,b Aamir Akmal, MD,b Tamerou Asrat, MDa

Division of Maternal-Fetal Medicine, Women's Hospital,a and Department of Research Administration,b Long Beach Memorial Medical Center, Long Beach, Calif, and Department of Obstetrics and Gynecology, University of California, Irvine Medical Center, Orange, Calif

Objective: The purpose of this study was to examine whether the pregnant rabbit model can be used as a viable model for the study of non–infection-mediated preterm birth.

Study design: Timed pregnant New Zealand rabbits were injected with a single dose of RU486 on day 22 of gestation. Three doses (50 mg, 75 mg, and 100 mg) were administered intramuscularly, intraperitoneally, or subcutaneously. The rabbits were monitored for preterm delivery. Progesterone, cortisol, and cytokine levels were examined before the induction and after delivery. Uterine and cervical progesterone, cortisol, and cytokine levels were determined after delivery.

Results: RU486 resulted in 100% preterm delivery in all doses and modes of administration, compared with 0% of controls. Intramuscular administration appeared to generate the most favorable preterm delivery time. Rabbits that received 100 mg RU486 intramuscularly showed significantly decreased serum progesterone levels and uterine progesterone levels, compared with 100 mg subcutaneously and intraperitoneally.

Conclusion: RU486 that was administered intramuscularly appears to be a potent and effective method for inducing preterm birth. This model of hormonally mediated preterm birth might serve as a useful model for the investigation of the possible mechanisms of preterm labor.

Preterm birth continues to be a major cause of perinatal morbidity and death in the United States. Approximately 10% to 11% of infants in the United States are born prematurely. The exact mechanisms of preterm labor are not understood completely; therefore, the development of animal models is important not only to study the underlying mechanisms of preterm delivery, but also to evaluate potential intervention strategies.
The clinical conditions that are associated with preterm birth are (1) medically indicated or iatrogenic (hypertension, abruptio placentae, intrauterine growth restriction, fetal distress), (2) preterm premature rupture of membranes, and (3) spontaneous or idiopathic. Iatrogenic preterm birth accounts for 9% to 35% of all preterm births; preterm premature rupture of membranes accounts for 7% to 51% of all preterm births, and idiopathic preterm birth accounts for 23% to 64% of all preterm births. Inflammation is a major cause of preterm delivery, which complicates as much as 50%, especially those that occur at <30 weeks of gestation. Previous animal models of preterm pregnancy loss have focused on infectious-mediated strategies. Both rabbit and mouse models of bacterial induction preterm labor have been reported. However, animal models for the induction of preterm birth by noninfectious means are necessary to study the other mechanisms of preterm birth. These animal models are lacking in the current literature. Therefore, we examined the hypothesis that the pregnant rabbit model can be used as a viable model for the study of noninfection-mediated preterm birth.

The progesterone antagonist, RU486 (mifepristone), has been shown to induce preterm birth in both mice and rats. Murine pregnancy is characterized by a peak in serum progesterone levels at mid trimester, followed by a decline approximately 2 days before parturition. Although there is no corollary for this in humans, it is thought that human parturition is preceded by an effective decline in progesterone activity through decreased binding at the receptor level. Hence, the administration of RU486, in fact, may mimic the end result of decreased progesterone activity in humans. The applicability of this model across other species has not been studied. We chose to develop a model of noninfectious preterm birth in rabbits. Their larger size, as compared with mice and rat models, makes the examination of the tissue effects and fetal effects less difficult. The purpose of this study was to determine whether RU486 could induce preterm delivery reliably and predictably in the pregnant rabbit and, if so, the optimal dose and route of administration. In addition, because progesterone, cortisol, and cytokine production is highly involved in parturition, we attempted to characterize any changes as measured by serum levels and at the tissue level.

Material and methods

This study was approved by the Animal Care and Use Committee of Long Beach Memorial Medical Center, Long Beach, Calif.

Experimental design

Timed, pregnant female New Zealand white rabbits (>3 kg) were obtained from a local breeder (The Rabbit Source, Ramona, Calif). The day of vaginal plugging was designated as day 1 of pregnancy. The animals were housed in an animal facility with a 12-hour day-night cycle and provided food and water ad libitum. On day 22 of gestation (70% of rabbit pregnancy), each pregnant rabbit was induced with a single injection of RU486 (Sigma Chemical Co. St. Louis, Mo) that was dissolved in absolute ethanol to a concentration of 33 mg/mL. RU486 was administered in 1 of 3 doses: 50, 75, or 100 mg by 1 of 3 routes of administration (intramuscularly, intraperitoneally, or subcutaneously). The intramuscular doses were injected in the interior left or right hind leg. The intraperitoneal doses were injected as a bolus into the peritoneal cavity of each animal. The subcutaneous doses were injected into the subcutaneous region of the interior left or right hind leg of each animal. There was a total of 4 animals in each group. Control groups received equal volumes of ethanol. Animals were monitored daily for signs of preterm delivery, as indicated by the delivery of at least 1 pup. Immediately after delivery, animals were killed by carbon dioxide inhalation, and the number of nonviable pups that remained in the uterus was recorded.

Blood and tissue sampling

Blood samples were obtained before induction (2 mL) and after delivery (2 mL) for progesterone, cortisol, and cytokine (interleukin [IL]-1β, -6, -8, and tumor necrosis factor–α [TNF-α]) determination. Samples were obtained from the doe through the marginal vein of the ear with a 25-gauge phlebotomy needle and syringe (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ). For plasma samples, 0.5 mL blood was placed in equal volumes of an ice-cold solution of 28 mg/mL EDTA and 40 μg/mL indomethacin in sterile polypropylene tubes. The samples were centrifuged at 4°C for 20 minutes, and the resulting plasma was transferred to sterile Eppendorf tubes and frozen at –80°C until assay. For serum samples, 1 mL blood samples were collected in sterile polypropylene tubes and placed on ice for 30 minutes. The samples were centrifuged at 4°C for 20 minutes, and the resulting serum was transferred to sterile Eppendorf tubes and frozen at –80°C until assay. Uterine and cervical tissues were obtained by open laparotomy with sterile techniques for cortisol, progesterone, and cytokine levels. Approximately 200-mg tissue samples were biopsied, rinsed in ice-cold phosphate buffered saline solution (PBS), and placed in sterile polypropylene tubes that contained fresh sterile phosphate buffer on ice. Tissue samples were kept frozen at –80°C until analysis.

Measurement of cortisol and progesterone

Plasma cortisol and serum progesterone levels were performed with enzyme immunoassay kits (Assay Designs, Long Beach, Calif).
Table I  Average time of vaginal delivery after induction

<table>
<thead>
<tr>
<th>Dose of RU486</th>
<th>Mode of administration (hr)</th>
<th>Subcutaneously</th>
<th>Intramuscularly</th>
<th>Intraperitoneally</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>232 ± 28 (99%)</td>
<td>234 ± 12 (99%)</td>
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<tr>
<td>50 mg</td>
<td>72 ± 80 (78%)</td>
<td>48 ± 0 (75%)</td>
<td>60 ± 24 (77%)</td>
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<tr>
<td>75 mg</td>
<td>114 ± 86 (84%)</td>
<td>72 ± 0 (78%)</td>
<td>114 ± 102 (84%)</td>
<td></td>
</tr>
<tr>
<td>100 mg</td>
<td>48 ± 0 (75%)</td>
<td>48 ± 0 (75%)</td>
<td>60 ± 42 (77%)</td>
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</tr>
</tbody>
</table>

Measurement of cytokines

Serum and tissue IL-1β, -6, -8, and TNF-α levels were determined with commercially available ultrasensitive enzyme immunoassay kits (BioSource International, Camarillo, Calif). Blood samples (1.0 mL) were placed in pyrogen/endotoxin–free tubes on ice and allowed to clot for 30 minutes. Serum was obtained by centrifugation at 3000 rpm for 20 minutes. Tissue samples (200 mg) were homogenized in phosphate buffered saline solution (pH 7.4) on ice. The homogenates were centrifuged at 10,000 rpm for 20 minutes at 4°C, and the supernatant was filtered through a 0.45 μm/L filter (Millipore, Bedford, Mass), prewashed with high-performance liquid chromatography–grade water. For cortisol and progesterone determinations, the filtrate was diluted in assay buffer (1:10) and pretreated with steroid displacement reagent. Tissue levels were standardized with total cellular protein levels in the samples. Quantitative measurements of cortisol and progesterone were carried out with 96-well plates that were coated with a polyclonal antibody to cortisol or progesterone, which binds in a competitive manner to cortisol or progesterone in the samples. After being incubated and washed, a substrate was added, and a color developed, the intensity of which was inversely proportional to the concentration of cortisol or progesterone in the standards or samples. The plate was read on a microplate reader at 405 nm. A standard curve that ranged from 0 to 10,000 pg/mL (cortisol) or 0 to 2000 pg/mL (progesterone) was generated with a 4-parameter logistic curve. The inter- and intra-assay coefficient of variation was <10% for both steroids. The optical density for the samples was used to calculate the concentration of cortisol or progesterone in the samples.

Total cellular protein assay

Ten-microliter samples of the tissue homogenates were assayed for total cellular protein by the dye-binding Bio-Rad protein assay (Bio-Rad, Hercules, Calif) with bovine serum albumin as a standard. The standard curve was linear from 0.05 to 1.45 mg/mL of protein.

Statistical analysis

Categoric data were analyzed by the Fisher’s exact test or the Mann Whitney U test, as appropriate. A paired t-test was used to determine differences between the before and after levels of measured variables. One-way analysis of variance was used to determine differences between the vehicle and RU486 doses and among the different routes of administration. Post-hoc analysis was performed with the Student-Newman-Keuls multiple comparisons test or Dunnett’s test after Levene’s test for equality of variances. To better visualize the distribution of the values for the groups, the data are presented in a box plot as the median and 25th to 75th percentiles. Statistical analyses were performed with SPSS software (version 8.1; SPSS Inc, Cary, NC) and GraphPad Instat software (GraphPad Software Inc, San Diego, Calif). Data are presented as mean ± SEM, where applicable. Significance was set at a probability value of <.05.

Results

A total of 36 study animals were injected with RU486, and 12 controls were injected with vehicle. The litter sizes ranged from 3 to 13 pups. RU486 resulted in 100% preterm delivery of live pups in all doses and routes of administration. The time course of delivery after the administration of the various doses and routes of RU486 administration are shown in Table I. The mean time to delivery was less in all RU486-treated than vehicle-treated animals, which delivered at term. Table II shows that the number of animals that were delivered within 24 hours of induction was highest in the intramuscular groups. Table III lists the number of pups that were delivered vaginally. Of the 4 animals that...
received vehicle through intraperitoneal administration, 2 animals were delivered of 100% of her pups; 1 animal was delivered of 1 of 4 pups, with the remainder dead in the uterus; and 1 animal was delivered of 1 of 3 pups, with the remainder dead in the uterus. Of the smallest dose (50 mg), intramuscular administration resulted in the greatest percentage of pups delivered, with 100% animals being delivered within 48 hours.

**Effect on progesterone**

There were no differences in serum progesterone levels among the groups before induction (data not shown). Serum progesterone levels (picograms per milliliter) did not change appreciably in the groups that received RU486 by intraperitoneal and subcutaneous administration. However, in the group that received 75 mg intramuscularly, a substantial decrease was noted after delivery (805.6 ± 18.6 pg/mL; P < .05) compared with preinduction levels (1294.8 ± 84.3 pg/mL). Similarly, serum progesterone levels at delivery were decreased in the group that received 100 mg intramuscularly (1153.6 ± 44.4 pg/mL) compared with the 100 mg intraperitoneally (1576.6 ± 43.1 pg/mL; P < .001) and 100 mg subcutaneously (1375.1 ± 40.6 pg/mL; P < .05). Uterine progesterone levels (picograms per milligram of protein) were reduced only in the group that was treated with 100 mg RU486 intramuscularly (167.5 ± 29.5 pg/mg; P < .01) versus 100 mg intraperitoneally (563.5 ± 81.1 pg/mg) and 100 mg subcutaneously (610.7 ± 46.2 pg/mg). No differences were detected in cervical progesterone levels among the groups.

**Effect on cortisol**

The effects of RU486 on plasma and uterine cortisol levels are shown in Figures 1 through 4. In contrast to progesterone, plasma cortisol levels were elevated significantly at delivery, in all groups that received RU486 compared with preinduction levels. The groups that were administered RU486 by subcutaneous administration demonstrated a linear correlation between plasma cortisol levels at delivery and RU486 dose (Figure 1). In the intramuscular groups, the strongest response was noted with the 50 mg dose at delivery compared with preinduction (Figure 2). Similar increases in plasma cortisol levels were noted for the intraperitoneal doses, although a significant increase in mean plasma cortisol levels at delivery was also noted in the group that received vehicle by intraperitoneal administration compared with preinduction levels (Figure 3). A negative relationship was observed between the RU486 dose and mean uterine cortisol levels (Figure 4). All routes of
administration of 50 mg RU486 resulted in increased uterine cortisol levels, compared with vehicle. Of the 75-mg RU486 groups, only the intramuscular group achieved statistical significance. Mean cervical cortisol levels remained relatively unchanged in response to all doses and all routes of administration of RU486.

**Effect on cytokines**

Compared with their prestudy serum levels, all control animals had decreased serum IL-1β levels at delivery (subcutaneously: 0 ± 0 pg/mL vs 0.9 ± 0.1 pg/mL; P < .001; intramuscularly: 0.4 ± 0.1 pg/mL vs 0.9 ± 0.3 pg/mL; P < .05; intraperitoneally: 0.1 ± 0.1 pg/mL vs 0.8 ± 0.04 pg/mL; P < .02). Compared with preinduction serum levels, RU486 resulted in significantly decreased mean serum levels of IL-1β at preterm delivery in the intramuscular groups that received 50-mg (0.7 ± 0.02 mg vs 1.2 ± 0.2 mg; P < .05) and 75-mg (0.7 ± 0.003 mg vs 1.5 ± 0.3 mg; P < .05) doses. No changes in mean serum IL-1β levels were detected for any of the RU486 groups were administered subcutaneously or intraperitoneally between their before and after induction levels. Uterine IL-1β levels (picograms per milligram of protein) were comparable with controls for all groups. Cervical IL-1β levels (picograms per milligram of protein) increased in the group that was treated with 50 mg intramuscularly (0.9 ± 0.26 mg; P < .05) compared with the 50-mg intraperitoneal group (0.05 ± 0.1 mg). No other changes in tissue IL-1β levels were noted (data not shown).

In contrast to serum IL-1β responses, no significant effects were noted with serum IL-6 levels. Conversely, uterine IL-6 levels were decreased in all groups that were treated with RU486 compared with control, except for the group that received 50 mg intraperitoneally (Figure 5). Cervical IL-6 levels remained relatively unchanged, regardless of dose or route of administration. Serum IL-8 was undetectable. Tissue IL-8 levels were also undetectable in the vehicle-treated groups. Although all doses of RU486 resulted in increased levels of uterine IL-8 levels, significance was achieved only in the subcutaneous and intraperitoneal groups that were treated with 75 mg RU486 (0.19 ± 0.04 and 0.15 ± 0.002 mg; P < .05, respectively) compared with the 75-mg intramuscular group (0.07 ± 0.03 mg) and in the 100-mg intraperitoneal group (0.17 ± 0.002 mg; P < .05) compared with the 100-mg subcutaneous and intramuscular groups (0.05 ± 0.03 mg and 0.06 ± 0.03 mg, respectively). Cervical IL-8 levels were also elevated with all doses of RU486. However, only the dose of 75 mg intraperitoneally
resulted in significantly higher mean levels (0.25 ± 0.002 mg; P < .05) compared with the 75-mg subcutaneous and intramuscular groups (0.03 ± 0.04 mg and 0.13 ± 0.03 mg, respectively; data not shown).

Mean serum TNF-α levels (pg/mL) increased in all the control animals at delivery compared with their prestudy levels (subcutaneously: 8.8 ± 1.6 pg/mL vs 3.3 ± 0.4 pg/mL; P < .05; intramuscularly: 10.5 ± 1.9 pg/mL vs 3.8 ± 0.8 pg/mL; P < .05; intraperitoneally: 8.8 ± 1.2 pg/mL vs 4.1 ± 0.2 pg/mL; P < .01). Serum TNF-α levels were also elevated in the 100-mg subcutaneous group (5.4 ± 1.2 pg/mL; P < .05) and the 50-mg intramuscular group (4.9 ± 0.74 pg/mL; P < .05) compared with their preinduction levels (1.9 ± 0.3 pg/mL and 2.7 ± 0.08 pg/mL, respectively; data not shown). A different response to RU486 was noted in the tissue. All doses of RU486, regardless of route of administration, resulted in decreased TNF-α levels in the uterus, although significance was not achieved for the intramuscular doses and the 50-mg intraperitoneal dose (Figure 6). A similar response pattern was noted in the cervix (Figure 7).

**Comment**

The present study is the first to examine whether the treatment of pregnant rabbits with RU486 reliably produces preterm delivery within 48 hours of administration. Using 3 commonly used routes of administration, we examined the dose-response effects of RU486 on progesterone, cortisol, and cytokines in the blood before and after induction and in the uterine and cervical tissue at the time of delivery. The most significant finding was that, in contrast to progesterone, cortisol levels increased in response to progesterone receptor inhibition. Furthermore, we found no appreciable effects on serum progesterone levels. Of the 3 routes of administration, intramuscular doses appeared to generate the most favorable response in terms of delivery time and progesterone response. Given the possible adverse effects of RU486, the main objective was to identify the lowest dose that would give a reasonable degree of preterm delivery. Taken together, these data suggest that the 50-mg intramuscular dose was the most efficacious, which resulted in 100% preterm delivery within 48 hours.

Progesterone plays a role in preterm parturition in the setting of infection in mice and rabbits; however, there are no studies to demonstrate the role of progesterone in a noninfectious animal model of preterm birth. Progesterone maintains pregnancy, and in most animal species, a drop in maternal progesterone levels precedes parturition. Therefore, the response of progesterone levels to RU486 was surprising because we
expected to see a reduction in serum progesterone levels after the delivery. In humans, progesterone levels remain high during labor and delivery. Studies by Mesiano et al.\textsuperscript{12} have demonstrated that decreased progesterone responsiveness and estrogen activation that are mediated by changes in progesterone and estrogen receptors are key events that control parturition. Similar findings were reported by Haluska et al.\textsuperscript{13} Our data suggest that RU486-induced preterm birth in rabbits is not associated with progesterone withdrawal, but with decreased progesterone responsiveness and possibly with changes in progesterone and estrogen receptor binding.\textsuperscript{10,14,15} Therefore, our model of RU486-induced preterm birth may provide a reasonable model for examining the mechanisms of idiopathic preterm delivery.

The effect of RU486 on cortisol production in our animal model was interesting. RU486 is known to have antiglucocorticoid activity especially at higher doses by blocking the feedback mechanism of cortisol on corticotropin secretion.\textsuperscript{16,17} The adrenal axis compensates for this blockade by increasing the secretion of both corticotropin and cortisol. Elevation of fetal cortisol precedes labor in many species.\textsuperscript{9,18} In sheep, increased corticosterone production by the fetal adrenals causes increased estrogen production by the placenta and uterine synthesis of prostaglandins, thereby inducing labor.\textsuperscript{19,20} Progesterone and cortisol have significant effects on fetal or maternal corticotropin-releasing hormone. In culture, corticotropin-releasing hormone output is inhibited by progesterone and stimulated by glucocorticoids.\textsuperscript{21,22} Because RU486 has been shown to cross the placenta by simple diffusion in monkeys,\textsuperscript{23} it is possible that it has similar effects on the fetal hypothalamic-pituitary axis. Such an increase in fetal hypothalamic-pituitary axis function has been confirmed by RU486-induced labor in pregnant sheep.\textsuperscript{24} Cortisol increases prostaglandin dehydrogenase by reversing the effect of progesterone.\textsuperscript{26} In our animal model, the observation that progesterone receptor inhibition results in increased plasma cortisol production provides support for the association between cortisol and progesterone withdrawal in the setting of noninfection-mediated preterm birth. It was interesting to note a linear relationship between plasma cortisol levels and subcutaneous doses of RU486. This response paralleled the number of rabbits that were delivered within 48 hours of induction (Table II). A similar response pattern was not demonstrated for the intramuscular and intraperitoneal doses and may be due to differences in drug absorption.

The effects of RU486 on the concentrations of various cytokines were inconsistent and unexpected. It is well documented that cytokines play a key role in preterm and term parturition, even in the absence of infection. Cytokine production, particularly in the
cervix, is involved in remodeling of the cervix. Systemic administration of IL-1β has been shown to induce preterm labor in mice. In our model, the administration of RU486 resulted in significantly decreased IL-1 levels in maternal serum after delivery, but only in the intramuscular groups of 50 and 75 mg. However, uterine IL-6 and TNF-α levels were decreased more consistently in all treated groups. Although these responses were surprising, we believe that the drop in cytokine levels may be due to the loss of the fetuses and demonstrates that, in this model of noninfectious preterm delivery, cytokine levels do not remain elevated after delivery. In addition, other factors (such as the wide variations in delivery times and the differences in the number of pups delivered within each group) may have contributed to these inconsistent findings.

Our pilot study provides new information regarding the usefulness of the rabbit model for the study of noninfectious preterm birth. However, it suffers from a number of limitations, including small sample size. Furthermore, we did not measure estrogen levels or expression of progesterone and estrogen receptors to further elucidate the mechanisms that are associated with RU486-induced preterm birth. However, studies by Mesiano et al. have defined the response of these receptors during parturition. Also, we did not investigate the responses of prostanoids, which are key agents that regulate preterm and term parturition. That information would have expanded our knowledge regarding the interplay between progesterone and cyclo-oxygenase.

In conclusion, we have demonstrated that the administration of RU486 to the pregnant rabbit provides an excellent model for the study of the mechanisms of hormonally mediated preterm birth. Because it has become increasingly recognized that a cascade of hormones mediates parturition in humans, we believe that this novel model will provide an opportunity to investigate potential mechanisms that are associated with noninfectious preterm delivery.

References

Pregnancy outcome of women exposed to bupropion during pregnancy: A prospective comparative study

Brian Chun-Fai-Chan, MSc, a Gideon Koren, MD, a Ibrahim Fayez, MD, a Sanjog Kalra, BSc, a Sharon Voyer-Lavigne, MSc, b Andrew Boshier, MD, c Saad Shakir, MD, c Adrienne Einarson, RN a,*

The Motherisk Program, The Hospital for Sick Children, Toronto, Ontario, Canada, a Pregnancy Riskline, Farmington, Conn, b and The Drug Safety Research Unit, Southampton, UK c

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KEY WORDS
Pregnancy
Depression
Smoking
Birth defects
Spontaneous abortions

Objective: Bupropion was developed for the treatment of depression, but subsequently was found to be effective for smoking cessation. To date, there are no prospective comparative studies examining its safety in pregnancy. The primary objective was to determine whether bupropion increases the risks for major malformations above baseline. The secondary objective was to examine the rates of live births, stillbirths, spontaneous and therapeutic abortions, mean birth weight, and gestational age at birth.

Study design: Women who were pregnant or planning a pregnancy and taking bupropion were enrolled in the study. Follow-up of pregnancy outcome was carried out between 4 months and 1 year after delivery. Three comparisons were carried out: 1) women exposed to bupropion vs a nonteratogen group; 2) those taking for depression vs other antidepressants, vs a nonteratogen group; 3) spontaneous abortions were compared between those taking for depression, vs another antidepressant group vs a nonteratogen group.

Results: We completed follow-up on 136 women exposed to bupropion during the first trimester of pregnancy. There were (105) live births, no major malformations, the mean birth weight was (3450g), the mean gestational age at delivery was (40 weeks), the number of spontaneous abortions was 20, there were 10 therapeutic abortions, there was 1 stillbirth, and 1 neonatal death. There were no statistically significant differences between any of the end points we examined between the exposed and comparison groups, with the exception of significantly more spontaneous abortions in the bupropion group (P = .009).

Conclusion: These results suggest that bupropion does not increase the rates of major malformation above baseline. The higher rates of spontaneous abortions are similar to other studies examining the safety of antidepressants during pregnancy.

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Initially developed and marketed as an antidepressant, bupropion (Wellbutrin; Wellbutrin SR, bupropion hydrochloride sustained release tablets) is an aminoketone antidepressant related to phenylethylamines and chemically unrelated to tricyclics, tetracyclics, SSRIs, or other known antidepressant agents.\(^1\) It was later developed as a non-nicotine aid for smoking cessation in conjunction with behavior modification, and is marketed under the brand name of Zyban (bupropion hydrochloride).\(^2\) It is a relatively weak inhibitor of the neuronal uptake of norepinephrine, serotonin, and dopamine. The mechanism by which bupropion enhances the ability of patients to abstain from smoking is unknown, but appears to be mediated by its unique pharmacologic profile, particularly its effects on noradrenergic and dopaminergic systems.\(^3\)

Studies in rats at doses up to 45 mg/kg (14 times the human dose), and in rabbits at doses up to 150 mg/kg (10 times the human dose), have shown no evidence of teratogenicity.\(^4,5\)

Presently, there are no prospective controlled studies on its safety during pregnancy. The manufacturer, GlaxoSmithKline, has maintained a pregnancy registry of prospectively reported cases,\(^6\) and as of June 2003, they had data on 397 pregnancy outcomes with 10 reports of major malformations (2.5%).\(^7\) However, there is no comparison group in this industry registry, making it difficult to make any conclusions regarding the safety of this drug in pregnancy.

The recommended dose of Zyban is 300 mg/day, 150 mg twice daily for smoking cessation. When given for depression, doses range from 100 to 150 mg twice a day.\(^1,2\)

Despite the fact that it is well known that cigarette smoking causes adverse effects on the fetus, Canadian national health statistics documented that 12% of women continue to smoke during pregnancy.\(^9\) In addition, of the estimated 25% to 40% of smokers who are able to quit during pregnancy, many relapse within a few months of giving birth.\(^8\)

Depression is a common occurrence in women, with prevalences ranging from 10% to 25% throughout life, peaking during the childbearing years of 25 to 44 years of age. Recently, a prevalence study was published that involved screening 3472 pregnant women for depressive symptoms with the Centre for Epidemiologic Studies-Depression scale (CES-D). The authors found that 20% of the women surveyed scored above the cutoff score for depressive symptoms.\(^10\) Studies have also shown that untreated depression in pregnancy can cause preterm delivery,\(^11\) smaller head circumference,\(^12\) and lower Apgar scores.\(^13\)

Depression may also lead to poor nutrition and compliance to prenatal care, thus increasing the risk of poor pregnancy outcomes. As well, depressed individuals are more likely to be involved with other high-risk behaviors, such as alcohol, and/or illicit drugs.\(^14\)

The primary objective of this study was to determine whether bupropion increases the risks for major malformations above the baseline. The secondary objective was to examine the rates of live births, stillbirths, spontaneous and therapeutic abortions, mean birth weight, and gestational age at birth.

### Material and methods

Women who contacted The Motherisk Program in Toronto, Canada, and The Pregnancy Riskline in Farmington, Conn, in the first trimester of pregnancy were asked to participate in the study. In both services, information is given regarding the safety/risk of drugs, chemicals, radiation, and infectious diseases. Women who called either service, were taking bupropion, and were pregnant or planning a pregnancy at the time of call were enrolled. Women were also recruited from The Drug Safety Research Unit in Southampton, UK, a prescription event monitoring database of new drugs on the market. When a questionnaire is returned and it is indicated that a woman became pregnant while taking a new drug, the physician is contacted and asked to prospectively monitor these women, and subsequently complete a follow-up to ascertain pregnancy outcome.

Exposure history included: medical indication, dose used, frequency of administration, gestational age at exposure, and gravidity/parity. The primary outcome was the presence or absence of a major malformation. Secondary outcomes of interest included: outcome of the pregnancy defined as spontaneous or therapeutic abortion, stillbirth or live birth, presence or absence of minor anomaly, birth weight, gestational age at delivery, method of delivery, and presence or absence of neonatal distress. After the telephone administration of a follow-up questionnaire to each study participant, the researcher subsequently sent a letter to the patient’s physician asking for verification of the information.
obtained from the questionnaire regarding her and her baby’s health.

Two comparison groups were used in this study to compare results with the bupropion-exposed group. Women who were taking other antidepressants were used to match for depression. A nonteratogenic group, representing women who had contacted the Motherisk program, but were not exposed to any teratogens during pregnancy, was also matched with the group exposed to bupropion. The matching criteria included age of the participant (± 2 years), alcohol consumption (± 1 drink), and smoking. Gestational age at the time of call to the Motherisk program (± 2 weeks) was also matched to ensure that spontaneous abortion rates were compared correctly. We were unable to do this at the other 2 sites.

Individuals using bupropion for smoking cessation were also matched for the amount of cigarettes consumed per day before pregnancy with smokers in the nonteratogen group who were not using smoking cessation therapy. An additional comparison was made between women who were taking bupropion for depression, bupropion for smoking cessation, and a nonteratogen group, to compare rates of spontaneous abortions.

Data regarding patient demographics, rates of major malformations, spontaneous abortion, therapeutic abortion (defined as “elective abortions”), stillbirth, and neonatal complications were compared using chi-square test for dichotomous data. Mean gestational age at the time of delivery and birth weight was compared between groups using the Student $t$ test or Mann Whitney rank-sum test if the data were not normally distributed.

This study was approved by the Hospital for Sick Children Research Ethics Board in Toronto, Canada.

Results

We were able to ascertain the outcomes of (136) women who had taken bupropion during pregnancy, all of whom used the drug in the first trimester. Forty-five of these women also continued to take bupropion throughout their pregnancy. There were 118 cases from The Motherisk Program, 10 cases from The Pregnancy Riskline, and 8 cases from DSRU.

The maternal characteristics of the women did not differ because they had been matched for age ± 2 years, smoking, and alcohol. We first compared all of the women exposed for any reason to bupropion with a nonteratogen group. The only statistically significant result was the rate of spontaneous abortions, which was significantly higher in the bupropion-exposed group (Table I). We then compared women who were taking the drug for depression with those taking other antidepressants and a nonteratogen group, and there were no statistically significant differences among the 3 groups (Table II).

We also performed a subanalysis of spontaneous abortions between women using the drug for depression vs smoking cessation, vs nonteratogen comparison group. The rates were similar in both the depression (14/91 [15.4%]) and smoking group (6/37 [16.2%]), which were both higher than the NTC group (6/133 [4.5%], $P = .01$).

There was 1 neonatal death, the infant of a woman who presented at 22 weeks’ gestation with abruptio placenta, in the bupropion group. The baby was born alive, but died shortly after delivery, with no evidence of congenital malformations. In addition, there were no congenital abnormalities in the baby who was stillborn. There were also 3 sets of twins in the bupropion group, all of whom were born normal and healthy.

Comment

To our knowledge, this is the first prospective study of pregnancy outcome of women exposed to bupropion, with comparison groups for both smoking cessation and depression use. The results suggest that there does not appear to be an association between bupropion exposure during pregnancy and an increased risk for major malformations. One third of the women used the drug

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<tr>
<td>Birth weight (kg ± SD)</td>
<td>3398 ± 725</td>
<td>33536 ± 725</td>
<td>3433 ± 525</td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>39 ± 2.9</td>
<td>40 ± 1.7</td>
<td>40 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Neonatal death</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>.32</td>
</tr>
</tbody>
</table>

* Included 2 sets of twins.
throughout their pregnancy; however, we did not attempt to examine possible long-term neurodevelopmental adverse effects in these children. This would have to be carried out in a longitudinal fashion as the children grow older because these babies were less than a year old at time of follow-up, with a substantial number less than 6 months old.

The only significant difference in all the end points examined was in the rates of spontaneous abortions. We detected a significantly higher rate of spontaneous abortions in the group exposed to bupropion compared with the nonteratogen group ($P = .009$). To further explore this observation, the total number of spontaneous abortions experienced by the participants who had a spontaneous abortion was analyzed. This was done to exclude the possibility that the higher rate of spontaneous abortion could be a result of a higher number of participants with recurrent spontaneous abortion in the exposed group. The results in the exposed group revealed that for the majority of participants, the current outcome was their first spontaneous abortion. Only 2 of the 20 women had 3 or more spontaneous abortions before this pregnancy. We also matched for time of call to the service to allow for an accurate comparison of spontaneous abortions. If a woman suffered an abortion early on, she would not call the service, and therefore, would not be included in the study, thus lowering the rates.

The higher rate of spontaneous abortions in the exposed group has been observed in 4 other studies conducted by Motherisk when studying antidepressant drug use during pregnancy. A study of trazodone and nefazodone found a 13.5% spontaneous abortion rate vs 8% in the comparison group.15 Fluoxetine, a 13.5% rate in the exposed group, 12% rate in the tricyclic group compared with 7% rate in the comparison group.16 In the newer SSRIIs, 12% rate in the exposed to vs 7% rate in the comparison group.17 Finally, venlafaxine also had a 12% vs a 7% rate in the comparison group.18 It must be noted that none of these results were statistically significant because of the relatively small sample sizes. However, in a recent meta-analysis conducted by our group that pooled all of these studies along with others, we found a significantly increased risk (RR 1.22–1.89, n = 3567) for spontaneous abortions.19

These observations suggest that antidepressants as a group may be associated with an increased risk of spontaneous abortions. However, there are studies on antidepressant use in pregnancy that have suggested that most depressed pregnant women are not adequately treated for their condition.20 Thus, it is difficult to separate whether the higher rate of spontaneous abortions in the women taking antidepressants is associated with the medication or the disease.

Bupropion has 2 distinct indications for use. As such, studying this drug in pregnancy gave us a unique opportunity to attempt to determine the mechanism behind the increased rate of spontaneous abortion in antidepressant studies. This drug is an antidepressant that is frequently prescribed to patients for assistance in smoking cessation, not for depression. In fact, in our cohort, only 1 woman who was taking the drug for smoking cessation was also diagnosed with depression. However, after comparing the rates of spontaneous abortions in women using bupropion as an antidepressant vs smoking cessation, we found similar rates between the 2 groups. This may reflect an insufficient sample size in the smoking cessation group (37) vs (91) in the depression group. It would be useful to recruit a larger sample size to explore this possibility further. The main limitation of this study to examine the rates of major malformations is the sample size, which is small for statistical purposes. It only has an 80% power to detect a 5-fold increased risk for malformations, with an alpha of 0.05. Approximately 800 cases in each group would be required to detect a 2-fold increase in risk of relatively common malformations, and thousands would be required to detect rare defects.

In summary, the results of this study do not suggest that bupropion increases the risk for major malformations above the baseline of 1% to 3%. The increase in the rates of spontaneous abortions requires further examination before any conclusion can be drawn regarding an association between the antidepressant or the underlying depression. With the paucity of data in the literature, this new information will assist both pregnant women and their health care providers to weigh the benefits and risks of taking this drug during pregnancy.

References

Reference ranges for serial measurements of umbilical artery Doppler indices in the second half of pregnancy

Ganesh Acharya, MD,a,* Tom Wilsgaard, PhD,b Gro K. Rosvold Berntsen, MD, PhD,b Jan Martin Maltau, MD, PhD,a Torvid Kiserud, MD, PhDc

Department of Obstetrics and Gynecology, University Hospital of Northern Norway, Tromsø, Norway,a Institute of Community Medicine, University of Tromsø, Tromsø, Norway,b Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Bergen, Bergen, Norwayc

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Doppler index
Umbilical artery
Fetus
Placenta

Objective: The purpose of this study was to construct new reference ranges for serial measurements of commonly used umbilical artery Doppler indices (pulsatility index, resistance index, and systolic:diastolic ratio).

Study design: This was a prospective longitudinal study of the umbilical artery Doppler indices that were obtained serially at the free-loop of umbilical cord at 4-week intervals at 19 to 42 weeks of gestation in 130 low-risk singleton pregnancies. A total of 513 observations were used to construct the reference ranges with the use of multilevel modeling.

Results: Longitudinally established percentiles of Doppler indices from the present study show a continuous reduction throughout the second half of pregnancy without any plateau or increase near term, as reported previously. There was a significant negative association between Doppler indices and placental weight and neonatal birth weight, but not with gender. The intraobserver coefficients of variation for the umbilical artery pulsatility index, resistance index, and systolic:diastolic ratio were 10.5%, 6.8%, and 13.0%, respectively.

Conclusion: New reference ranges for umbilical artery Doppler indices that are based on longitudinal observations appear to be slightly different from cross-sectional studies and are more appropriate for serial evaluation of fetal hemodynamics.

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ranges. Several reference ranges of these waveform indices have been published. However, the studies with an adequate number of observations are cross-sectional\textsuperscript{6-8} and mostly use routinely collected clinical data.\textsuperscript{9,10}

For serial measurements, appropriate reference ranges must be derived from longitudinal studies rather than cross-sectional studies. However, the few longitudinal studies that have been published in the English language are small\textsuperscript{11-14} or use continuous wave Doppler imaging without any knowledge of site or angle of insonation\textsuperscript{15,16} and the data are mostly analyzed and presented as if they were derived from a cross-sectional study.

The aim of this study was to establish reference ranges for serial measurements of the umbilical artery Doppler indices in the second half of pregnancy based on longitudinal data. In addition, we wanted to examine the effect of neonatal weight, gender, and placental weight on the Doppler indices.

### Material and methods

This was a longitudinal study of 130 low-risk pregnancies that were recruited for a detailed study of the umbilical circulation according to a research protocol approved by the Regional Committee for Medical Research Ethics; written informed consent was obtained from all participants.

Inclusion criteria were gestational age confirmed by ultrasound measurement of <20 weeks and no complications in the current pregnancy before recruitment. Maternal smoking, multiple pregnancy, a diagnosed fetal abnormality before recruitment, previous history of preeclampsia, intrauterine growth retardation, abruptio placenta or preterm delivery, and history of any pre-existing medical condition (such as hypertension, diabetes mellitus, renal disease) were reasons for not being included. Each woman was examined 3 to 5 times at approximately 4-week intervals between 19 and 42 gestational weeks.

Doppler ultrasonography was performed with an ultrasound system with a 2.5- to 6-MHz curvilinear transducer (Sequoia 512; Acuson; Mountain View, Calif). A single operator (G.A.) performed all examinations. Color Doppler imaging was used to optimize the insonation by the pulsed Doppler examination. The angle of insonation was kept at <15 degrees in all cases, and angle correction was used if the angle was not zero. The high-pass filter was set at minimum, and a large sample volume (10-12 mm) was used for the pulsed Doppler recording. The Doppler velocity waveforms were obtained from the free-floating loop of the umbilical cord during fetal quiescence. Five to 6 uniform waveforms were obtained ≥3 times in succession, and online measurements were performed. The values that were recorded were an average of 3 consecutive cardiac cycles. The waveform envelope that had the highest measured peak systolic velocity was considered for analysis, assuming that the highest measured velocity represents the lowest angle of insonation. The guidelines of the International Perinatal Doppler Society\textsuperscript{17} were followed during Doppler sonographic examinations. The mechanical index was kept at <1.9, and the thermal index was kept at <1.5. Doppler waveform indices were calculated from the maximum velocity waveform with the following computerized planimetry:

\[
\text{PI} = \frac{\text{Peak systolic velocity} - \text{end-diastolic velocity}}{\text{time-averaged maximum velocity}}
\]

\[
\text{RI} = \frac{\text{Peak systolic velocity} - \text{end-diastolic velocity}}{\text{peak systolic velocity}}
\]

\[
\text{S:D ratio} = \frac{\text{Peak systolic velocity}}{\text{end-diastolic velocity}}
\]

The outcome of pregnancy was noted and included any complications, gestation at delivery, mode of delivery, neonatal birth weight, sex, Apgar score, umbilical cord blood gases, perinatal complications, and placental weight. All the placentas were collected immediately after delivery and inspected for completeness and any gross abnormalities. The umbilical cord was cut flush with the placental surface, but the membranes were not trimmed. Blood was allowed to drain from the placenta, and the clots were removed. The placenta was weighed on a precision balance by the midwife shortly after delivery. A pediatrician routinely examined the newborn infants on the third postnatal day and noted any abnormalities, if present.

Data analysis was performed with SAS software (version 8.2; SAS Institute Inc, Cary, NC). Normality was checked for each outcome variable, and logarithmic

---

**Table I** Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>Age (median, range) 30 Y (18-43 Y)</td>
</tr>
<tr>
<td></td>
<td>Nulliparous (n) 60 (46%)</td>
</tr>
<tr>
<td></td>
<td>Body mass index at booking (mean ± SD) 25.81 ± 3.98 kg/m(^2)</td>
</tr>
<tr>
<td>Fetal</td>
<td>Gestational age at delivery (mean ± SD) 39.8 ± 1.36 wk</td>
</tr>
<tr>
<td></td>
<td>Birth weight (median, range) 3665 g (1645-4590 g)</td>
</tr>
<tr>
<td></td>
<td>Placental weight (mean ± SD) 673 ± 145 g</td>
</tr>
<tr>
<td></td>
<td>Umbilical arterial pH 7.23 ± 0.148 (mean ± SD)</td>
</tr>
<tr>
<td></td>
<td>Umbilical arterial base excess −4.18 ± 3.507 mmol/L (mean ± SD)</td>
</tr>
<tr>
<td></td>
<td>Umbilical venous pH 7.33 ± 0.084 (mean ± SD)</td>
</tr>
<tr>
<td></td>
<td>Umbilical venous base excess −4.63 ± 3.572 mmol/L (mean ± SD)</td>
</tr>
</tbody>
</table>

---
or power transformations were performed as appropriate (ln transformation for PI and S:D ratio, square root for RI) to reduce the skewness of residuals. Intraobserver coefficients of variation were calculated from 3 sets of measurements obtained from 513 observations as:

Coefficient of variation \( = 100 \times \sqrt{\frac{\sum_i (S_i^2 / X_i^2)}{n}} \)

where \( S_i^2 = \) within-subject variance, \( X_i = \) mean of all measurements, \( n = \) number of observations. Multilevel modeling was used to estimate the reference percentiles. Fractional polynomials were fitted to find the best relationship between Doppler indices and gestational age.

**Results**

Of a total of 133 recruited participants, 3 participants withdrew because they moved their residence, which left 130 participants with complete data sets for the statistics. All the participants were white. Characteristics of the study population are presented in Table I. Three women (2.3%) had preeclampsia, and 1 woman had gestational diabetes mellitus. Onset of labor was spontaneous in 110 women (84.6%) and was induced in 13 women (10%); 7 women (5.4%) had an elective cesarean delivery before the onset of labor. Seventeen women (13.1%) had an emergency cesarean delivery; 5 women (3.8%) had vacuum delivery, and 1 woman (0.8%) had forceps delivery. Three women (2.3%) were delivered preterm (34-36 weeks of gestation). Four babies (3%) were below the 5th percentile for the gestational age. One fetus was diagnosed with a transposition of the great arteries at 37 weeks of gestation, and 1 fetus was diagnosed with tetralogy of Fallot after birth. There were 66 male (50.8%) and 64 female (49.2%) babies. There was 1 intrauterine fetal death at 42 weeks of gestation. Of 129 liveborn infants, 5 infants (3.9%) had an Apgar score of <7 at 5 minutes. Three babies (2.3%) required resuscitation at birth, and 9 babies (6.9%) were admitted to the neonatal care unit.

Gestational age-specific reference values for the 2.5th, 5th, 10th, 25th, 50th, 75th, 90th, 95th, and 97.5th percentiles of the umbilical artery PI, RI, and S:D ratio are presented in Tables II, III, and IV. Curve-fitted percentile charts for each of these variables are shown in Figures 1, 2, and 3. The statistical formulas and the regression equations are presented in the Appendix.

The coefficients of variation for PI, RI, and S:D ratio were 10.5% (95% CI, 9.9%-11.1%), 6.8% (95% CI, 6.4%-7.2%), and 13.0% (95% CI, 12.1%-13.9%), respectively.

The Doppler indices decreased continuously with advancing gestational age (\( P < .0001 \)). When the individual values of the Doppler indices that were
940
Table III

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Reference values for serial measurements of the umbilical artery resistance index:
Percentile

Gestation (wk)

2.5th

5th

10th

25th

50th

75th

90th

95th

97.5th

19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

0.64
0.63
0.62
0.60
0.59
0.58
0.56
0.55
0.54
0.53
0.51
0.50
0.49
0.47
0.46
0.45
0.44
0.42
0.41
0.40
0.39
0.38
0.36

0.66
0.65
0.64
0.62
0.61
0.60
0.58
0.57
0.56
0.55
0.53
0.52
0.51
0.50
0.48
0.47
0.46
0.45
0.43
0.42
0.41
0.40
0.39

0.68
0.67
0.66
0.65
0.63
0.62
0.61
0.59
0.58
0.57
0.56
0.54
0.53
0.52
0.51
0.50
0.48
0.47
0.46
0.45
0.44
0.43
0.41

0.72
0.71
0.70
0.68
0.67
0.66
0.65
0.64
0.62
0.61
0.60
0.59
0.58
0.56
0.55
0.54
0.53
0.52
0.51
0.50
0.48
0.47
0.46

0.77
0.75
0.74
0.73
0.72
0.71
0.69
0.68
0.67
0.66
0.65
0.64
0.63
0.61
0.60
0.59
0.58
0.57
0.56
0.55
0.54
0.53
0.52

0.81
0.80
0.79
0.78
0.76
0.75
0.74
0.73
0.72
0.71
0.70
0.69
0.68
0.67
0.66
0.65
0.64
0.63
0.62
0.61
0.60
0.59
0.58

0.85
0.84
0.83
0.82
0.81
0.80
0.79
0.78
0.77
0.76
0.75
0.74
0.73
0.72
0.71
0.70
0.69
0.68
0.67
0.66
0.65
0.65
0.64

0.88
0.87
0.85
0.84
0.83
0.82
0.81
0.80
0.79
0.78
0.77
0.76
0.76
0.75
0.74
0.73
0.72
0.71
0.70
0.70
0.69
0.68
0.67

0.90
0.89
0.88
0.87
0.86
0.85
0.84
0.83
0.82
0.81
0.80
0.79
0.78
0.77
0.77
0.76
0.75
0.74
0.73
0.73
0.72
0.71
0.70

Table IV

Reference values for serial measurements of the umbilical artery systolic:diastolic ratio
Percentile

Gestation (wk)

2.5th

5th

10th

25th

50th

75th

90th

95th

97.5th

19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

2.73
2.63
2.51
2.43
2.34
2.25
2.17
2.09
2.02
1.95
1.89
1.83
1.77
1.71
1.66
1.61
1.57
1.52
1.48
1.44
1.40
1.36
1.33

2.93
2.83
2.70
2.60
2.51
2.41
2.33
2.24
2.17
2.09
2.03
1.96
1.90
1.84
1.79
1.73
1.68
1.64
1.59
1.55
1.51
1.47
1.43

3.19
3.07
2.93
2.83
2.72
2.62
2.52
2.43
2.35
2.27
2.20
2.13
2.06
2.00
1.94
1.88
1.83
1.78
1.73
1.69
1.64
1.60
1.56

3.67
3.53
3.36
3.24
3.11
2.99
2.88
2.78
2.69
2.60
2.52
2.44
2.36
2.29
2.23
2.16
2.11
2.05
2.00
1.95
1.90
1.85
1.81

4.28
4.11
3.91
3.77
3.62
3.48
3.35
3.23
3.12
3.02
2.92
2.83
2.75
2.67
2.60
2.53
2.46
2.40
2.34
2.28
2.23
2.18
2.13

5.00
4.80
4.55
4.38
4.21
4.04
3.89
3.75
3.63
3.51
3.40
3.30
3.20
3.11
3.03
2.95
2.87
2.80
2.74
2.67
2.61
2.56
2.50

5.75
5.51
5.22
5.03
4.82
4.63
4.45
4.30
4.15
4.02
3.89
3.78
3.67
3.57
3.48
3.39
3.30
3.23
3.15
3.08
3.02
2.96
2.90

6.26
5.99
5.67
5.45
5.22
5.02
4.83
4.66
4.50
4.36
4.22
4.10
3.98
3.87
3.77
3.68
3.59
3.51
3.43
3.36
3.29
3.22
3.16

6.73
6.43
6.09
5.85
5.61
5.38
5.18
5.00
4.83
4.67
4.53
4.40
4.27
4.16
4.06
3.96
3.86
3.78
3.69
3.62
3.54
3.48
3.41


obtained at the first and last visits were divided into quartiles, 76% of the fetuses remained within ±1 quartile during the second half of pregnancy. We found no significant association between neonatal gender and the slopes of PI, RI, and S:D ratio ($P = .63, .93, \text{and} .32$, respectively) or their gestational age-specific values ($P = .21, .06, \text{and} .16$, respectively). The slope of the PI was associated negatively with placental weight. The PI was estimated to decrease by 0.01 per gestational week, but this negative slope of PI was associated with placental weight so that, for an increase of 100 g, the slope decreased by $-0.002$ per week ($P = .009$). The RI was estimated to decrease by 0.005 per week, but this association was modified by placental weight so that, for an increase of 100 g, the slope of RI decreased by $-0.001$ per week ($P = .005$). The association between placental weight and the S:D ratio was not statistically significant ($P = .14$).

We found a significant negative association between birth weight and gestational age-specific values of PI, RI, and S:D ratio ($P = .003, .011, .024$, respectively) so that the PI, RI, and S:D ratio decreased by 0.035, 0.01, and 0.12, respectively for every 500-g increase in the birth weight. There was no association between the linear slopes of these Doppler indices and birth weight ($P = .32-.96$).

**Comment**

We have established reference ranges that were based on longitudinal observations that are suitable for serial measurements of 3 commonly used umbilical artery Doppler indices (ie, PI, RI, and S:D ratio). Currently used references are either based on cross-sectional studies or have common methodologic problems (such as inappropriate design, insufficient information about the study population, inadequate sample size, and handling of the longitudinal data as cross-sectional during statistical analysis without accounting for within-subject changes in Doppler measurements). Our study was designed to overcome many of the methodologic weaknesses that have been noted in fetal measurement studies.\textsuperscript{23}

Our longitudinal study showed a continuous reduction of Doppler indices with advancing gestational age, which confirms previous observations. However, our data demonstrate that this reduction continues beyond term, which is in contrast with some of the previously reported large cross-sectional\textsuperscript{7} and longitudinal\textsuperscript{16} studies that report plateau or a small increase in Doppler

---

**Figure 1** Umbilical artery pulsatility index at the free loop with p2.5, p5, p50, p95, and p97.5. The dotted lines represent 95% confidence limits for the mean.

**Figure 2** Umbilical artery resistance index at the free loop with p2.5, p5, p50, p95, and p97.5. The dotted lines represent 95% confidence limits for the mean.

**Figure 3** Umbilical artery systolic:diastolic ratio at the free loop with p2.5, p5, p50, p95, and p97.5. The dotted lines represent 95% confidence limits for the mean.
Doppler indices are well known. Some authors have advised recording the waveforms from the fetal end of the umbilical cord, and other authors have advised to record from the placental end. We chose the free-floating loop of the umbilical cord because it seems to be the preferred technique in many centers. Semiquantitative Doppler indices are not angle dependent, and an insonation angle of <60 degrees does not have any significant effect in their calculation. However, we kept the angle at <15 degrees in all cases.

We had a rare possibility of determining intraobserver variability in 3 sets of examinations in 513 observations at different gestational ages between 19 and 42 weeks. Intraobserver variability was acceptable (coefficients of variance, 6.8%-13% for different indices) and similar to previously reported variability.

Choosing a representative population is important in studies that are intended to construct reference ranges. Our sample was a low-risk population with a uniform ethnic background. Additionally, we did not exclude any of them because of complications that developed during the project to reduce the shift towards a supernormal population. Other characteristics that included an even distribution of the babies’ gender and the mean birth weight of 3662 g (median, 3665 g; range, 1645-4590 g), which is at approximately 50th percentile according to Norwegian standards, suggest that this can be considered as a reference population. Because our study population had a relatively uniform socioeconomic and ethnic background, it could be argued that our nomograms may not be entirely applicable to other populations. Umbilical artery PI is known to vary with birth weight and placental size, which are parameters that may vary with ethnicity. Taking into account such factors, the impact of other ethnic and socioeconomic characteristics of the population on the Doppler indices is likely to be small. Furthermore, because our data are based on longitudinal observations, conditional reference ranges can be calculated for any individual fetus on the basis of a previous measurement. Therefore, we believe that our nomograms are applicable also outside the Nordic population.

Some investigators would argue that pregnancies that had complications after inclusion should be excluded. However, such a study design has been criticized previously for producing supernormal ranges that were less applicable in the general population. Accordingly, we chose not to exclude such complications in the present study.

A close linear relationship between birth weight and umbilical artery Doppler velocity waveforms has been described previously. The present longitudinal data confirm that relationship.

In clinical practice, the umbilical artery Doppler indices are usually obtained serially when a fetus is deemed to be at increased perinatal risk. The advantage of longitudinal data is that they may be used to calculate conditional reference percentiles (Appendix); ie, it is possible to predict a value including 95% CI for a given gestational age on the basis of a previous measurement, which may be more appropriate in the assessment of individual fetuses.

In short, we have constructed new reference ranges for umbilical artery Doppler indices that are based on longitudinal data. They differ slightly from previously published studies and are more appropriate for serial evaluation of fetal hemodynamics.

Acknowledgments

We thank Bjørn Odvar Eriksen, MD, PhD, Inger Sperstad, and Ingrid Dorthea Stanstad at the Department of Clinical Research, University Hospital of Northern Norway, and Mrs Regina Fernando at the University of Hertfordshire, UK, for their help with this study.

References

Appendix

Reference interval

If $Y_i =$ velocity at gestational age $T_i$, then the mean and variance of a transformed velocity $Z_i$ at a transformed time $X_i$ are

$$
\mu_i = E(Z_i) = \beta_0 + \beta_1 X_i
$$

$$
\sigma_i^2 = Var(Z_i) = \sigma_{int}^2 + \sigma_{time}^2 X_i^2 + 2\sigma_{int, time} X_i + \sigma_e^2
$$

where $\beta_0$, $\beta_1$ are the fixed parameter estimates and $\sigma_{int}^2$, $\sigma_{time}^2$, $\sigma_{int, time}$, $\sigma_e^2$ are the estimated variance components from the multilevel analysis.

The time specific reference value for $Y_i$ with 95% coverage is

$$
(\mu_i \pm 1.96\sigma_i)^{1/2}
$$

if transformation of velocity is $Z_i = Y_i^{1/2}$

$$
\exp(\mu_i \pm 1.96\sigma_i)
$$

if transformation of velocity is $Z_i = \ln(Y_i)$

Conditional reference interval

The conditional mean and variance of $Z_2$ given $Z_1$ is

$$
E(Z_2|Z_1) = \mu_{2|1} = \mu_2 + (Z_1 - \mu_1)\sigma_{12}/\sigma_2^2
$$

$$
Var(Z_2|Z_1) = \sigma_{2|1}^2 = \sigma_2^2 - \sigma_{12}^2/\sigma_1^2
$$

where

$$
\sigma_{12} = cov(Z_1, Z_2) = \sigma_{int}^2 + (X_1 + X_2)\sigma_{int, time} + X_1 X_2\sigma_{time}^2
$$

The conditional reference interval of $Y_2$ given $Y_1$ with 95% coverage is

$$
(\mu_{2|1} \pm 1.96\sigma_{2|1})^{1/2}
$$

if transformation of velocity is $Z = Y^{1/2}$

$$
\exp(\mu_{2|1} \pm 1.96\sigma_{2|1})
$$

if transformation of velocity is $Z = \ln(Y)$

Pulsatility Index (PI)

PI is log transformed (ie, $Z = \ln[PI]$).

$$
\mu_i = E(Z_i) = 1.5075 - 0.2843T_i^{0.5}
$$

$$
\sigma_i^2 = Var(Z_i) = 0.0667 + 0.00398T_i - 0.0276T_i^{0.5}
$$

The conditional mean and variance of $Z_2$ given $Z_1$ is
The transformation of RI is $Z = \text{RI}^{0.5}$.

$\mu_{21} = 1.5075 - 0.2843T_2^{0.5} + \ln(P) - 1.5075 + 0.2843T_1^{0.5} \left( \frac{0.04616 - 0.0138(T_1^{0.5} + T_2^{0.5}) + 0.00398T_1^{0.5}T_2^{0.5}}{0.0667 + 0.00398T_1 - 0.0276T_1^{0.5}} \right)$

$\sigma_{21}^2 = 0.0667 + 0.00398T_2 - 0.0276T_2^{0.5}$

$\sigma_{21}^2 = \left( \frac{0.04616 - 0.0138(T_1^{0.5} + T_2^{0.5}) + 0.00398T_1^{0.5}T_2^{0.5}}{0.0667 + 0.00398T_1 - 0.0276T_1^{0.5}} \right)$

**Resistance Index (RI)**

The transformation of RI is $Z = \text{RI}^{0.5}$.

$\mu_i = E(Z_i) = 1.0079 - 0.007T_i$

$\sigma_i^2 = \text{Var}(Z_i) = 0.0016 - 0.0000623T_i + 0.00000272T_i^2$

The conditional mean and variance of $Z_2$ given $Z_1$ is

$\mu_{2|1} = 1.0079 - 0.007T_2 + (\text{RI}^{0.5} - 1.0079 + 0.007T_1) \left( \frac{0.000249 - 0.0000312(T_1 + T_2) + 0.00000272T_1T_2}{0.0016 - 0.0000623T_1 + 0.00000272T_1^2} \right)$

$\sigma_{2|1}^2 = 0.0016 - 0.0000623T_2 + 0.00000272T_2^2 - \left( \frac{0.000249 - 0.0000312(T_1 + T_2) + 0.00000272T_1T_2}{0.0016 - 0.0000623T_1 + 0.00000272T_1^2} \right)$

**Systolic:Diastolic Ratio (SDR)**

SDR is log transformed (ie, $Z = \ln[\text{SDR}]$).

$\mu_i = E(Z_i) = 4.16676 - 0.9188\ln(T_i)$

$\sigma_i^2 = \text{Var}(Z_i) = 0.4851 - 0.2678\ln(T_i) + 0.04115\ln(T_i)^2$

The conditional mean and variance of $Z_2$ given $Z_1$ is

$\mu_{2|1} = 4.16676 - 0.9188\ln(T_2) + (\ln[\text{SDR}_i] - 4.16676 + 0.9188\ln(T_1)) \left( \frac{0.45537 - 0.1339(\ln(T_1) + \ln(T_2)) + 0.04115\ln(T_1)\ln(T_2)}{0.4851 - 0.2678\ln(T_1) + 0.04115\ln(T_1)^2} \right)$

$\sigma_{2|1}^2 = 0.4851 - 0.2678\ln(T_2) + 0.04115\ln(T_2)^2 - \left( \frac{0.45537 - 0.1339(\ln(T_1) + \ln(T_2)) + 0.04115\ln(T_1)\ln(T_2)}{0.4851 - 0.2678\ln(T_1) + 0.04115\ln(T_1)^2} \right)$
Maternal gestational protein-calorie restriction decreases the number of glomeruli and causes glomerular hypertrophy in adult hypertensive rats

Jorge R. Almeida, MD, Carlos A. Mandarim-de-Lacerda, MD, PhD*

Laboratory of Morphometry and Cardiovascular Morphology, Biomedical Center, Institute of Biology, State University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

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Objective: This work analyzed the renal function and structure in offspring rats that were submitted to maternal protein-calorie restriction during prenatal or lactation periods.

Study design: Kidneys from adult offspring were studied. Animals from mothers that were submitted to food restriction were separated in 3 groups: control, prenatal restriction, and lactation restriction. Blood pressure, microalbuminuria, and glomerular filtration rate were determined. Kidney cortical remodeling was analyzed with stereology; volume-weighted glomerular volume and the number of glomeruli were estimated.

Results: Adult prenatal restriction offspring showed enhanced microalbuminuria, decreased glomerular filtration rate, and hypertension; their kidneys showed a smaller number of hypertrophied glomeruli than control and lactation restriction animals.

Conclusion: Maternal prenatal protein-calorie restriction in rats causes kidney disease in adult offspring, which is characterized by hypertension and renal dysfunction and suggests secondary kidney remodeling because of an impairment of glomerulogenesis.

Intrauterine malnutrition plays a pivotal role in the impairment of renal function and glomerulosclerosis in adult rats,1 which supports the association between protein and/or calorie restriction during pregnancy with higher systolic blood pressure in adult offspring.2,3 Among the proposed mechanisms to explain the effects of maternal malnutrition on the arterial blood pressure (BP) in adult offspring, one could underline a decrease in hypothalamus-pituitary-adrenal sensitivity, a decrease of placental activity of 11-β steroids, and changes in renal development.4,5 Offspring from rats that were submitted to calorie restriction (50%) in the first half, in the second half, or during all the pregnancy period showed a decrease in the glomerular filtration rate, glomerular number, and increased nephrosclerosis at 3 months of age.1,6

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* Reprint requests: Carlos A. Mandarim-de-Lacerda, MD, PhD, Universidade do Estado do Rio de Janeiro, Centro Biomédico, Instituto de Biologia, Laboratório de Morfometria e Morfologia Cardiovascular, Av 28 de Setembro 87 (fds) – 20551-030 Rio de Janeiro, RJ, Brazil.

E-mail: mandarim@uerj.br, URL: www2.uerj.br/~lmmc

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The kidney needs a long time to be developed fully compared with other organ organogenesis. This fact frequently creates a site for potential pathologic alterations during nephrogenesis. Murine nephrogenesis has been divided into a prenatal period of glomerular formation and an early postnatal period of multiplication development that occurs during the lactation period. In rats, whose fetal period initiates on gestational day 17.5 and birth corresponds to gestational day 21, this process extends up to 7 to 10 days after birth.

Modest maternal protein restriction leads to hypertension in both sexes and a reduced number of glomeruli in adult male, but not the female offspring. Severe protein restriction reduces glomerular number and causes hypertension in both female and male offspring; the window of sensitivity of adult BP to prenatal protein restriction seems to fall within the period of nephrogenesis.

The purpose of the present work is to study the renal cortex remodeling and the renal function in adult male rats under protein-calorie restriction during 2 development periods: intrauterine and lactation.

**Material and methods**

**Experimental groups**

All procedures were carried out in accordance with the conventional guidelines for experimentation with animals (National Institutes of Health Publication No. 85-23, revised 1996). The experimental protocols that were used in this study were approved by the Ethics Committee for Animal Experimentation at the State University of Rio de Janeiro.

Multiparous (second and third pregnancies) female Wistar rats that weighed 274.8 ± 27.3 g (from 6 to 9 months old) were mated overnight (1 female to 1 male; copula was confirmed based on the analysis of the vaginal plug and vaginal smear). All animals were housed individually in controlled temperature (21°C ± 1°C), with artificially reversed dark-light cycle (lights on from 7:00 PM to 7:00 AM), humidity-controlled (60% ± 10%) room with air exhaustion cycle (15 min/h). Once pregnancy was confirmed, the day after the mating was considered the first gestational day. Standard diet for rats (Nuvilab-NUVITAL, Curitiba, Brazil) was used in accordance with the American Institute of Nutrition AIN-93G recommendation for rodent diet. The experimental groups received 50% of daily needs, which were calculated in grams per kilogram of their body mass (BM); the daily reference was the intake of 1 of the paired and matched animals of the control group. This approach allowed us to progressively, day-by-day, adjust on half of both the calories and the components of diet. Three groups of matrices were assigned randomly to 1 of the following groups: the control group (with free access to a standard rat diet [23% protein] during pregnancy and lactation); the prenatal restriction (PR) group (protein-calorie restriction occurred from gestational day 1 to day 21 [matrices received standard rat diet in restricted quantities that equaled 50% of the needs, adjusted in accordance with the control group]; after the birth, mothers received standard rat diet ad libitum and offspring had normal lactation period); lactation restriction (LR) group (a restricted quantity of rat diet was offered to mothers during the lactation [21-day] period [50% of the needs that were adjusted in accordance with the control group]; the protein-calorie restriction was started at birth and was ended at weaning; after being weaned, offspring had free access to food and water until euthanasia day (day 121); in this group, excess pups were removed at birth; only 6 male pups were kept per dam).

Three groups of 120-day-old adult male Wistar rats were studied; 1 rat was from each mother, according to the previous detailing (n = 6 in each group): control, PR, and LR. Conscious and trained animals had their BP verified weekly by the noninvasive method of tail-cuff plethysmography (Leticia LE 5100; Panlab, Barcelona, Spain) and were weighed (BM, in grams) before they were placed in metabolic cages.

**Tissue processing and renal function analysis**

Adult offspring were kept in metabolic cages for 24 hours for urinary protein excretion measurements 1 day before death, and urine was collected. The 24-hour urinary volume allowed us to calculate the urinary flow. In the morning of day 121, animals were anaesthetized deeply (intraperitoneal sodium pentobarbital) and killed by exsanguination. The left kidneys were dissected carefully, and the kidney volume was measured according to Scherle’s method (immersed in physiologic saline solution). The kidney mass index was determined with the following formula: kidney (grams)/BM (grams).

Microalbuminuria concentration was measured by nephelometric method (Labtest Diagnostica, Lagoa Santa, Minas Gerais/Brazil). Blood samples were taken during death. Serum and urinary creatinine levels were determined by alkaline picrate method (Labset kit; Labtest Diagnostica) with the automatic analyzer (Mega Bayer; Merck, Darmstadt, Germany). The creatinine clearance was calculated as urinary creatinine (milligrams per deciliter) × urinary flow (milliliters per minute)/serum creatinine (milligrams per deciliter) and corrected to the BM (kilograms).

**Light microscopy and stereologic analysis**

The left renal cortex from adult rats was analyzed stereologically. Kidneys were longitudinally divided in
2 halves and placed on the cut surface. After that, one half of the kidney was cut in several vertical consecutive fragments and placed in fixative (freshly prepared 4% weight/volume formaldehyde in 0.1 mol/L phosphate buffer pH 7.2) for 48 hours at room temperature, embedded in Paraplast plus (Sigma Chemical Company, St Louis, Mo), sectioned 3 μm thick, and stained with Masson’s trichrome. The analysis used video-microscopy (Leica model DMRBE microscope; Leica, Wetzlar, Germany; Kappa CF 15/5 video camera; Gleichen, Germany; Sony Trinitron monitor; Sony, Pencoed, UK). Ten fields were analyzed by section, 6 sections per animal, and 6 animals per group (360 fields per group). A transparent test-system with points and a frame of 324,900 μm² was put on the monitor. The reference volume was estimated by point counting that used the test points that hit the renal cortex (PT). Points hitting glomeruli (Pp) were counted to estimate the glomerular volume density (V_g = Pp/PT [an estimation]). The adult cortical-to-medullary ratio was estimated for the right kidneys of each animal through the Cavalieri principle. Then, the absolute cortical volume was calculated as cortical-to-medullar ratio × kidney volume.

The total glomerular volume was calculated as: V_g × the absolute cortical volume. The volume-weighted mean glomerular volume (VWGV) estimate was made through the point-sampled intercepts method that analyzed a minimum of 50 glomeruli per animal. The random sampling of glomeruli is important in stereologic analysis. Therefore, the procedure that was used in this study did not separate cortical or juxta-medullary glomeruli; it was a blind study in which animal groups were not identified to the observer (J.R.A.).

A test-system that consisted of parallel lines that were associated with testing points was superposed on each microscopic field. The direction of the lines on the sample was determined by lottery. Then, the intercepts lengths over the glomeruli were measured with a 32-mm long logarithmic rule that was composed of a series of 15 classes. Each individual intercept was cubed, and the mean of all values was multiplied by π/3 in every case to obtain the VWGV. 16

The total glomerular number per kidney was estimated as the ratio between the product of the cortical volume and V_g by the VWGV.

Statistical analysis

BP, biometry, and kidney function were tested using the 1-way analysis of variance and the Student-Newman-Keuls post-hoc test. Stereologic evidence was analyzed with non-parametric tests (Kruskal-Wallis analysis of variance and Mann-Whitney test). In all cases, the level of significance was set at a probability value of <.05. All analyses were performed using GraphPad Prism software (version 4.01 for Windows; GraphPad Software Inc, San Diego, Calif).

Results

Biometric and functional data

At birth the PR animals showed the smallest BM (P < .001). However, as adults, the PR animals became significantly heavier than control and LR animals (P < .01; Figure 1). The kidney volume was smaller in the PR group than in the control and LR groups (P < .05; Table I). BP levels (Table I) were not different between control and LR groups. BP was higher in the PR group from day 90 than in the control and LR groups (P < .05; Figure 2). Serum creatinine and urinary excretion of microalbumin were higher, and creatinine clearance that was corrected by BM was lower in the PR animals than in the control and LR groups (Table I). The kidney mass index was smaller in the PR animals than in the control animals (P < .05; Table I). The cortical-to-medullar ratio was not different among the groups. However, the absolute cortical
volume was smaller in the PR and LR groups ($P < .001$ and $P < .01$, respectively; Table I) when compared with the control group.

**Glomerular stereology of adult kidney**

The $V_{V[\text{glomerus}]}$ was not different among the groups. The $V_{W[\text{glomerus}]}$ was greater in the PR group than in the control and LR groups ($P < .01$, respectively; Figure 3). The total number of glomeruli per kidney was significantly smaller in PR animals than in control and LR animals (35% and 29% smaller, respectively; $P < .01$; Figure 4).

**Comment**

This work studied the effects of maternal protein-calorie restriction in rats during prenatal and lactation period and the influence on BP and renal morphofunctional aspects in the later life of the offspring. Previous experimental evidence shows that fetal malnutrition during different stages of gestation has diverse long-term effects.
effects on the offspring’s physiologic condition and metabolism. Fetal malnutrition may occur if the mother is malnourished or may result from the failure of the fetal supply line (from the placenta). Therefore, birth weight could be just a result of fetal growth retardation.

The rat glomerulogenesis extends some days after birth; consequently, immature glomerular structures can be observed at the end of the gestational period like comma-shaped and S-shaped bodies and vascularized glomeruli (when a fully developed mesangial region with capillaries is observed). Experimental studies have applied maneuvers to impose fetal malnutrition in animal models; those data have supported the concept that the effect of the intrauterine environment on fetal growth is a mechanism for the development of hypertension. Kidney is a target organ of hypertension, and animals that were submitted to prenatal protein-calorie restriction had hypertension with consequent renal cortex alteration. The renal disease caused by hypertension develops progressively up to chronic renal failure, with loss of glomeruli and several morphologic and quantitative alterations.

However, the evidence that developed from epidemiologic investigations on humans seems to be less conclusive. Several authors have used food intake restriction in a large range of intensities. In the present study, intrauterine protein-calorie restriction protocol could be considered severe. Furthermore, the restriction was not a selective food restriction and probably approached the human malnutrition conditions. We found that the adult kidney can be programmed by intrauterine protein-calorie restriction as a result of impaired glomerulogenesis and a decrease of glomeruli, but no effects were observed when the restriction was imposed during the lactation period.

The greater BM in the PR group might mean different roles and endocrine adjustments. As an adaptation to malnutrition in fetal life, permanent metabolic and endocrine changes occur, if the food that is available becomes plentiful, those changes predispose to a gain of weight in adulthood.

The exact mechanisms to explain hypertension that are induced by fetal exposure to a maternal low-protein diet in rats are unknown. However, experimental evidence suggests the role of the maternal glucocorticoid synthesis and a link between impaired nephrogenesis and decreased activity of the gestational renin-angiotensin system. Besides, adult hypertension was prevented by early administration of an angiotensin-converting enzyme inhibitor. In rats, whose fetal period initiates on the gestational day 17.5 and birth corresponds to the gestational day 21, this process extends up to 7 to 10 days after birth. However, in humans, the glomerulogenesis extends for several months, which covers a large part of the fetal period and ends before birth.

Modifications in absolute glomerular volume, as determined by stereologic analysis, may be due to an increase in the number of glomeruli or to an increase of the average glomerular volume. The increase in the volume of the glomerular compartment in rats, and in humans, corresponds to a progressive increase in the number of nephrons during the fetal period. After a precocious postnatal period, progressive age-related modifications in the glomerular compartment occur,
characterized by increased glomerular volume and a progressive reduction in the number of glomeruli. Glomerulogenesis works in a centrifugal way: The most mature glomerular structures are found out deeper in the cortical region; in-developing structures are more and more superficial. This could explain the smaller absolute cortical volume seen in the PR animals. Therefore, with the secondary glomerular hypertrophy, characterized by a greater VWGV, the PR offspring had a smaller number of glomeruli per kidney. Alterations in glomerular volume density could be mainly the result of an increase in average glomerular volume, an event that has been proposed as a key stage in the pathogenesis of secondary renal lesions, including arterial hypertension, which represents a common final pathway for the eventual development of glomerulosclerosis.

In the present study, prenatal protein-calorie restricted rats, when adults, showed (1) a small number of big glomeruli, (2) a small index of glomerular filtration rate, and (3) microalbuminuria. Testing for microalbuminuria is recommended to detect early kidney damage. Proteinuria as a marker of kidney disease also has prognostic value; individuals with more proteinuria have more morbidity, which is related both to renal and to cardiovascular causes in general.

The relationship between the number of nephrons (which could represent renal mass) and hypertension (which results from intrauterine retardation of kidney development) has been proposed. Most studies on the effects of prenatal malnutrition-inducing hypertension have placed the decrease of the number of nephrons as a central event that starts the pathogenetic mechanisms. Furthermore, experimental evidences have shown a reduction in the nephron number of approximately 30%, regardless whether the protein-deficient diet was given throughout the pregnancy or only in the second half of the pregnancy. However, it might be possible that compensatory maladaptive changes of ultrastructural and metabolic ways in the kidney by itself occur intrarenally when nephrogenesis is compromised. This is consistent with the fact that uninephrectomy in the adult does not necessarily cause hypertension, and in the adult, no compensatory nephrogenesis occur.

The reasons that male offspring from modestly protein-restricted mothers are more sensitive to the reduction of the number of glomeruli when adults than do female offspring are understood poorly. Future studies to elucidate the different intrauterine programmed effects that are not related to the glomerular number and gender interaction are of interest.

In conclusion, prenatal protein-calorie restricted rats showed a different development after birth from normally nourished rats; they became hypertensive and proteinuric animals with a consequent decrease in the glomerular filtration rate. Prenatal protein-calorie restriction might induce structural damage on filtration barrier and a progressive hypertensive and proteinuric state associated with or the result of an impairment of glomerulogenesis. The effects on the renal function and BP are not evident when the protein-calorie restriction takes place after birth.

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References


Antihypertensive effects of flutamide in rats that are exposed to a low-protein diet in utero

Pandu R. R. Gangula, PhD, Luckey Reed, BS, Chandrasekhar Yallampalli, DVM, PhD*

Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Tex

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Objective: We investigated whether gestational age of in utero low-protein diet played a role in the subsequent development of adult hypertension and whether it is gender dependent and examined whether flutamide (a specific, nonsteroidal competitive antagonist of the androgen receptor) reduces blood pressure in rat offspring that are exposed to in utero low-protein diet (6%).

Study design: Pregnant rats were fed either with 20% protein (control) or 6% protein (low-protein diet) from day 1 or day 12 of gestation. Fetoplacental weights and mortality rates of pups were assessed. Systolic blood pressure, mean arterial blood pressure, and circulatory hormone levels in offspring were determined. In addition, male and female hypertensive offspring were treated with flutamide, and their blood pressure was monitored.

Results: After delivery, pup weights were reduced, and pup mortality rates increased in the low-protein diet–day 1 group. Systolic blood pressure and mean arterial blood pressure were elevated in low-protein diet–day 1 males and females and low-protein diet–day 2 males. Significant ($P < .05$) reduction in blood pressure was achieved with flutamide in low-protein diet–day 1 females. Serum estradiol levels were decreased ($P < .05$) in low-protein diet–day 1 females; flutamide attenuated this effect.

Conclusion: The day of in utero insult by low-protein diet is critical in the induction of adult hypertension; the severity is gender dependent. Flutamide was found to protect against hypertension only in females.

The concept that adult diseases may result from in utero “programming” has been subject of much recent investigation. Studies in humans have documented that low birth weight is associated with an increased risk of the development of cardiovascular disease,$^{1-3}$ noninsulin-dependent diabetes mellitus,$^{4}$ and hypertension$^{5,6}$ in later life. Several factors (including unbalanced maternal nutrition, inappropriately low or high body composition, or other maternal “stress” before or during early pregnancy) may alter fetal cardiovascular development. Studies that used maternal protein restriction during pregnancy in rats resulted in offspring that had high blood pressure (BP).$^{7}$ Many potential mechanisms through which hypertension may be initiated by fetal undernutrition have been proposed. Ashton$^{8}$ demonstrated that alterations in maternal glucocorticoids and

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* Reprint requests: Chandrasekhar Yallampalli, DVM, PhD, Departments of Obstetrics & Gynecology and Neuroscience & Cell Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1062.
E-mail: cyyallam@utmb.edu

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deficiency in placental 11β-hydroxysteriod dehydrogenase activity increase BP in adult offspring. Further, in utero low-protein diet (LPD) alters the renin-angiotensin system either in newborn infants or adults and causes hypertension. Hypertensive male and female rats that are exposed to maternal LPD display a deficit in total nephron numbers, up-regulation of renin aldosterone axis, and inappropriate renal Na⁺ retention that leads to increased extracellular volume. Previous studies of isolated aortic rings demonstrated that vasoconstrictor effects of norepinephrine are elevated, whereas vasodilator sensitivity to acetylcholine is decreased in adult male offspring with nutrition-restricted mothers. These findings further suggest that altered vasodilator responses to acetylcholine in female offspring might be a consequence of the reduction in estrogen levels that lead to reduced endothelial nitric oxide synthase activity. Together, these studies suggest that impaired vascular function or alterations in kidney development result in the development of adult hypertension.

Considerable evidence shows that androgens play a pivotal role in gender-associated differences in BP regulation. In several experimental models, androgens can promote hypertension. Elucidation of the mechanisms by which these hormones regulate cardiovascular homeostasis continues to be the subject of contemporary interest. Flutamide, an androgen receptor antagonist, has been used widely to investigate the effects of androgens in several cardiovascular models, which includes lowering BP. Hyperandrogenism in men or women is considered a risk factor for cardiovascular disease. Studies in the patients with polycystic ovarian syndrome revealed that flutamide can regulate blood flow to the uterine artery. These studies suggest that flutamide not only reduces BP but also regulates blood flow to the reproductive organs, at least in patients with polycystic ovarian syndrome. To date, there are no studies available that examine whether flutamide exhibits hypotensive effects in hypertensive adult offspring who were exposed in utero to LPD.

Previous studies that used an LPD model demonstrated that the fetoplacental weights were either decreased, increased, or unchanged. Moreover, various groups of investigators suggested that LPD exposure develops hypertension in the adult offspring if the diet is implemented during the preimplantation, the second half of pregnancy, or throughout gestation. However, it is unclear whether the timing of the insult with the LPD in utero influences the severity of the BP and whether it is gender specific. Further, data are incomplete whether sex steroid hormone levels are altered in the rat offspring that are exposed to the in utero LPD.

In this study, we have investigated (1) whether fetoplacental weights are altered by maternal LPD, (2) whether the maternal LPD intake causes hypertension in both male and female offspring, (3) whether gender or gestational age at in utero LPD plays a role in the subsequent development of adult hypertension, (4) whether flutamide treatment decreases BP in both LPD male and female offspring, and (5) whether circulatory steroid hormones are altered in rats with maternal LPD beginning at day 1 and day 12 of gestation.

Material and methods

Adult, nonpregnant (180-220 g body weight [BW]) rats were purchased from Harlan Sprague Dawley (Houston, Tex). All animals were housed in a climate-controlled room with a 12:12 hour light-dark schedule and were fed standard rat diet with water to drink ad libitum. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas. Virgin female rats were mated, and the day of observation of a vaginal plug with the presence of sperm was designated day 1 of gestation. After acclimatization, pregnant rats were fed a normal protein diet (20% casein, control) or a LPD (6% casein) from day 1 (LPD-1) or day 12 (LPD-2) gestation. The dams were delivered spontaneously at term and were then immediately switched back to the standard rat diet. The offspring were nursed by their mothers until they were weaned (21 days old) to regular diet. The isocaloric synthetic low-protein and normal-protein diets were obtained from Harlan Teklad (Madison, Wis). The composition of the diets for the 2 groups, except the protein content, was identical as described previously. Both diets also contained equal amounts of standard vitamins and minerals.

To determine the effects of a LPD on fetal growth and placental development, a group of 3 pregnant rats each from the low-protein and normal-protein groups were killed on day 18 of gestation. Placentae and fetuses were isolated, blotted to remove fluids and blood, and weighed immediately. After spontaneous delivery of pups on day 22 of pregnancy (n = 5-6), pup and placental weights and pup mortality rate were recorded (within 1 hour after delivery). On all adult offspring animals (n = 10-12), systolic BP was measured monthly by the pneumatic tail cuff method (Narco-Biosystems, Houston, Tex) from 2 to 9 months old. After being restrained, the rats were allowed to stabilize. Three consecutive measurements, at 10-second intervals, were obtained and were averaged for each animal. All measurements were made at the same time of the day beginning at 9 A.M. When the pups were 9 months old, mean arterial BP was measured in groups (n = 3 each) of anesthetized rats (ketamine, 45 mg/kg BW; Fort Dodge Laboratory, Fort Dodge, Ind; xylazine, 5 mg/kg BW, Burns Veterinary Supply, New York, NJ) at 30 minutes after surgery with indwelling catheters (PE-50; Clay Adams, Parsippany, NJ) that were inserted into the left carotid artery.
Flutamide treatment

Both male and female offspring that were exposed in utero to LPD and control offspring (n = 4) at the age of 9 months were selected for this study. Hypertensive LPD rats were treated subcutaneously with flutamide (specific nonsteroidal competitive antagonist of the androgen receptor) either for 20 days (LPD males; 10 or 30 mg/kg BW/d/rat) or 4 days (LPD-1 females; 10 mg/kg BW/d/rat). The doses of flutamide (Sigma Chemical Company, St. Louis, Mo) were chosen on the basis of previous studies.18,19,22 A group (n = 4) of hypertensive LPD male and female rats also received vehicle (sesame oil, 0.3 mL, subcutaneously). Systolic BP was taken before and during the treatment period.

Hormone analysis

Blood was collected from 9-month-old rats (n = 4) into heparinized tubes from the vena cava immediately after carbon dioxide inhalation. Blood was centrifuged at 2000g for 20 minutes at 4°C, and serum was collected and stored at −70°C until used. In a similar manner, serum was also collected from flutamide-treated rats (n = 3) from females of the LPD-1 group. In all of these animals, serum estradiol, progesterone, and testosterone levels were measured with the use of specific I125 label for serum estradiol, progesterone, or testosterone, respectively, by radioimmunoassay method as per instructions provided by supplier (Diagnostic Systems Laboratories, Webster, Tex). In another experiment, to compare age-related changes in testosterone levels, serum was also collected from 6-month-old male rats. To avoid variations in endogenous steroid hormone levels because of the estrous cycle, only females in estrous were used.

Statistical analysis

The weight (in grams) of fetuses, placentae, and pups are expressed as means ± SEM and compared by analysis of variance or the Student t test. Differences in BP between groups were determined by either analysis of variance followed by the Tukey-Kramer multiple comparisons test or the Student t test. The differences were considered significant at a probability value of <.05.

Results

Effect of in utero LPD on fetoplacental weight and pup mortality rate

To determine whether maternal LPD alters fetoplacental weights during pregnancy, groups of rats on day 18 of gestation were killed, and fetoplacental weights were measured. No differences in fetoplacental weights were observed either in LPD-1 or LPD-2 group compared with control animals (data not shown). Next, we examined whether the weights of these tissues and pup mortality rate were altered after spontaneous delivery on day 22 of gestation. Results show that a decrease in pup weight and placental weights (data not shown) and an increase in pup mortality rate were observed in the LPD-1 group but not in LPD-2 animals, compared with controls (Table).

Effect of in utero LPD on systolic BP and mean arterial BP in adulthood

In both male and female offspring, systolic BP was measured from 2 to 9 months of age. Systolic BP was higher (P < .05) in both genders in the LPD-1 offspring compared with their respective controls. This phenomenon was noticeable from 2 months of age, with a maximal effect observed by 4 months (Figure 1). In contrast, 70% to 80% of LPD-2 male offspring were shown to be hypertensive (P < .05), beginning from 6 months of age (Figure 1, A). The magnitude of increases in systolic BP was greater in LPD-1 males compared with LPD-2 males or LPD-1 females. On the other hand, systolic BP remained unchanged in LPD-2 females compared with the control group (Figure 1, B).

To confirm whether the increases in systolic BP were correlated with arterial pressure, groups (n = 3) of either hypertensive LPD rats or normotensive control rats were anesthetized, and PE-50 catheters were inserted into the left carotid artery for the measurement of mean arterial BP. The mean arterial BP was measured after 30 minutes of surgery while the animals were under anesthesia. As shown in Figure 2, the increases in mean arterial BP was significantly (P < .05) greater in LPD-1 (24 ± 2.0 mm Hg) compared with LPD-2 (16 ± 1.5 mm Hg) males (Figure 2). Similarly, in females, significant (P < .05) elevation in mean arterial BP (Figure 2) occurred with in utero-exposed LPD from day 1 (15 ± 1.3 mm Hg), compared with day 12 of gestation (5 ± 0.5 mm Hg). The magnitude of increases in MAP in utero because of LPD was higher in males compared with female offspring (Figure 2).

Effect of flutamide on systolic BP in in utero LPD-exposed hypertensive offspring

We next investigated whether nonsteroidal antiandrogen flutamide reverses hypertension that is induced by
maternal LPD in offspring. Groups (n = 4 each) of LPD-1 and LPD-2 male or LPD-1 female offspring were treated with either 10 mg or 30 mg of flutamide (subcutaneously) for 20 days or 4 days, respectively. Systolic BP was taken for all of these animals before or during the treatment period. As shown in Figure 3, flutamide was ineffective in decreasing systolic BP in males that were exposed to in utero LDP (data not shown for 10 mg flutamide dose). In contrast, flutamide (10 mg/kg BW) administration reduced the systolic BP of LPD-1 females to control levels by day 1 of treatment, which was maintained during the entire treatment period (Figure 3, B).

### Measurement of circulatory sex steroid hormones in adult rats

As shown in Figure 4, females have higher circulatory serum estradiol levels compared with male controls at 9 months of age. Significant (P < .05) decreases in the serum estradiol levels in serum were observed in the female LPD-1 group, compared with the controls (Figure 4). However, no differences in serum estradiol concentrations were observed in LPD-1 males, compared with control group (data not shown). Flutamide treatment in the female LPD-1 group reversed the decreased serum estradiol levels in the circulation (Figure 4). Next, we determined whether serum testosterone levels were altered either with age or with maternal LPD in male offspring. As shown in Figure 5, A, males have high (P < .05) circulatory testosterone levels compared with female control groups at 9 months of age. Significant (P < .05) decreases in testosterone levels in circulation were also observed in males at 9 months compared with at 6 months (Figure 5, B); however, LPD did not alter the testosterone levels in serum either in males (Figure 5, C) or in female offspring (Figure 5, D). The progesterone levels in circulation were greater (P < .05) in the 9-month-old female group, compared with male controls (Figure 6). However, in utero insult by LPD did not alter
these hormone levels either in male or female offspring (Figure 6).

Comment

The major findings of the present study are that (1) exposure to in utero LPD throughout pregnancy (LPD-1) reduced fetoplacental weights and increased pup mortality rates, when assessed on day 22 of pregnancy immediately after spontaneous delivery; (2) the offspring raised from LPD-1 exhibited severe hypertension from 2 to 9 months; (3) LPD-2 males, but not LPD-2 females, developed hypertension; (4) the magnitude of increases in systolic BP is higher in LPD-1 males compared with LPD-2 males or LPD-1 females; (5) flutamide treatment significantly decreased BP in LPD-1 females, but not in either of the LPD male groups; (6) serum estradiol levels in circulation are lower in LPD-1 females, and flutamide treatment increased; and (7) neither progesterone nor testosterone levels were altered in both male and female LPD groups. However, testosterone levels were decreased significantly with age in male controls. These results, together with previous findings, 10,26,35,36 suggest that “programming” in utero by LPD leads to fetal growth restriction and increases BP in later life.

Poor growth in utero has been linked to the development of type-2 (noninsulin dependent) diabetes mellitus and hypertension.4,5 Fetal growth is a complex, dynamic process, that is dependent on a continuous supply of nutrients from the mother. In the present study, maternal LPD-1 significantly (P < .05) decreased fetoplacental growth and increased the mortality rate of the pups (Table) on day 22 of gestation. When contrasting with other studies,10,36 we found that the weight and mortality rate of the pups were no different from controls (Table) in the animals that were exposed to low-protein during the second half of pregnancy (LPD-2). Moreover, we observed that fetoplacental weights were similar in

Figure 3  The effect of androgen receptor antagonist (flutamide) on systolic BP in maternal LPD-exposed rat offspring. The LPD was administered in utero either from LPD-1 or LPD-2 of pregnancy until delivery. The control group received 20% protein. Groups of male (n = 4; A) or female (B) hypertensive LPD-1 rats at the age of 9 months were given 30 mg or 10 mg, respectively, flutamide subcutaneously (per kilogram body weight per rat per day). Males were treated with flutamide for 20 days; females were treated for 4 days. Systolic BP was measured before (0 days) or during treatment period by the tail cuff method. Values are expressed as mean ± SEM. The asterisk denotes P < .05 compared with vehicle-treated (sesame oil) LPD-1 group.

Figure 4  The effect of in utero LPD exposure on circulatory estradiol-17ß (E2) concentrations (picograms per milliliter) in rat offspring. The LPD (6% protein) was given from day 1 of pregnancy until delivery (LPD-1). The control group received 20% protein. Groups of male and female rats (controls) and LPD-1 female rats that were treated with flutamide (10 mg/d/ kg BW) subcutaneously at 9 months old were used in this study (n = 3). Blood was collected for the measurement of circulatory estradiol levels. Values are expressed as mean ± SEM. The asterisk denotes P < .05 compared with control male group; the dagger denotes P < .05 versus control; the double dagger denotes P < .05 versus LPD-1 female rats.
both LPD and control groups on day 18 of pregnancy (data not shown). These observations suggest that the adverse effects of LPD-1 are exhibited during the later pregnancy, when the fetal growth is exponential. The detailed mechanisms through which LPD caused feto-placental growth restriction are not addressed in the present study. Rees et al.\textsuperscript{31} postulated that a diet-induced decrease of threonine in both maternal serum and the fetal free amino acid pool may be responsible for fetal growth restriction. Furthermore, these studies suggest that alterations in the serum threonine-methionine-homocysteine group of amino acids early in pregnancy may affect fetal development, which leads to insulin resistance and hypertension in later life.\textsuperscript{31} Recently, it has been reported\textsuperscript{32} that maternal protein restriction from day 1 of pregnancy imposes changes in maternal levels of glucose, insulin, prolactin, progesterone, serum estradiol, and leptin. These studies, together with our current findings, suggest that alterations in metabolic and/or endocrine environment by in utero LPD could influence the feto-placental development and elevate fetal mortality rates.

To reiterate, our findings clearly demonstrate that fetal exposure to LPD elevates BP in adult offspring. The results presented in Figure 1 indicate that the LPD-1 group was hypertensive beginning from 2 months of age and that the severity of this disease increased progressively until 9 months. Further, this group showed higher BP compared with LPD-2 males or LPD-1 females, which suggests that the day of in utero programming is critical and that the severity is gender dependent (Figure 1). The increases in systolic BP were confirmed by mean

\begin{figure}[h]
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\caption{The effect of in utero LPD exposure on circulatory testosterone (T) concentrations (nanograms per milliliter) in rat offspring. The LPD (6\% protein) was given from LPD-1 or LPD-2 until delivery. The control group received 20\% protein. Groups of male and female rats at 6 or 9 months old were killed, and blood was collected for the measurement of circulatory testosterone levels. A, Circulatory testosterone levels in male and female offspring. B, The effect of age on serum testosterone levels in male offspring. The effect of maternal LPD on circulatory testosterone levels in male (C) and female (D) rat offspring (n = 3). Values are expressed as mean \pm SEM. The asterisk denotes \( P < .05 \) compared with control male group (A); the dagger denotes \( P < .05 \) versus 6-month-old male offspring (B).}
\end{figure}
arterial BP measurements in both male and female LPD offspring (Figure 2). In contrast, 2 separate studies that used Sprague Dawley rat model demonstrated that the exposure of fetuses to LPD during the second half of pregnancy causes hypertension to a similar magnitude in both male and female adult offspring. Reports in humans indicate that males are more inclined to be hypertensive than women of similar age. Similarly, in hypertensive rat models, investigators have found that males have higher BP than females, and androgens may play a role in gender-associated differences in BP regulation. In contrast, some studies show that estrogen replacement therapy lowers BP in postmenopausal women and in several animal models of hypertension. Substantial evidence supports the theory that renal dysfunction that was observed as the result of maternal undernutrition or protein restriction may be responsible for the developing hypertension. At the present time, it is unclear whether the changes in BP are steroid hormone-dependent or whether males are more susceptible for renal dysfunction compared with females. Further studies are needed to address the mechanisms that are involved in this model.

Next, we examined whether a flutamide blockade of androgen receptors would attenuate the elevated BP in both male and female LPD offspring. As shown in Figure 3, subcutaneous treatment with flutamide had no effect on BP in male LPD offspring. However, the low dose of this drug (10 mg in female vs 30 mg in male LPD) significantly decreased the BP in LPD-1 females (Figure 3, B). Previous studies that used spontaneous hypertensive rat models demonstrated that BP was lower in flutamide-treated male animals and that the mechanisms that were involved in this process appeared to be decreased levels of catecholamines in the adrenal medulla. Moreover, flutamide has been shown to decrease BP in both male and female hypertensive transgenic rats that harbor the mouse Ren-2 renin gene. Furthermore, this effect appears to be mediated through a decreased renin-angiotensin system. In addition, the BP-lowering effects of flutamide are found to be less in male rats that lack a functional androgen receptor (testicular feminization mutation) than those of malignant hypertensive rats. Recent observations that used male or female Sprague Dawley rats or testicular feminization mutation rats reported that flutamide dose-dependently relaxes aorta and small resistance blood vessels through the nitric oxide–cyclic guanosine monophosphate pathway. Caplea et al suggest that flutamide can reverse sodium-induced rise in BP in male spontaneously hypertensive Y chromosome rat strains. These studies, together with the present findings, suggest that the antihypertensive effects of flutamide in female LPD-1 offspring are androgen independent. Further investigations are warranted to better understand the mechanism of action of flutamide on vascular tone, especially in male studies that use another antiandrogen (such as cyproterone acetate) and will delineate whether the effects of flutamide on BP in females is related to its antiandrogenic properties.

Some epidemiologic studies suggest that estrogen replacement therapy has a protective effect on the cardiovascular system in postmenopausal women. The protective effects of estrogen on vasculature appeared to be mediated by intracellular receptors and thus by alterations of gene expression. Reports indicate that, in addition to the genomic effect, estrogens can have a direct vasorelaxing action without altering gene expression. Further, estrogen therapy down-regulated the renin-angiotensin system in both postmenopausal women and animal models. In the present study, the circulatory estrogens are decreased significantly in LPD-1 female (Figure 4) but not in the LPD-1 male (data not shown) offspring. These studies further demonstrated that flutamide treatment attenuated decreased estrogen levels in the serum of LPD-1 female offspring (Figure 4). Taken together, these data suggest that the vasodilator effects of flutamide appear to be modulated by increased estrogens and perhaps that the renin-angiotensin system may be involved in this process in LPD-1 female offspring. Studies are underway currently to examine the mechanisms that are involved in the BP-lowering effects of flutamide in the LPD-1 female hypertensive model.

Figure 6 Effect of in utero LPD exposure on circulatory concentrations (nanograms per milliliter) of progesterone (P₄) in rat offspring. The LPD (6% protein) was given from LPD-1 until delivery. The control group received 20% protein. Groups of male and female rats at 9 months old were killed, and blood was collected for the measurement of circulatory progesterone levels (n = 3). Values are expressed as mean ± SEM. The asterisk denotes P < .05, compared with male control group.
Present studies have further shown that testosterone levels in male circulation are decreased with age (Figure 5, B). However, no differences in circulatory testosterone concentrations were observed in LPD male (Figure 5, C) and female offspring (Figure 5, D) compared with control values. Previous reports postulated that both circulatory androgen levels and androgen receptors in renal vasculature are decreased with age in the male rat.49-52 We speculate that androgen receptors may also be altered with age; therefore, treatment with flutamide in LPD male offspring does not affect BP in the present study. Moreover, studies of Ganten et al53 that used a spontaneous hypertensive rat model demonstrated that the removal of circulating testosterone receptor-mediated mechanisms is effective only during the early phase of the development of high BP and not in established hypertension in male rats. Also, circulatory levels of progesterone did not change in LPD male and female offspring (Figure 6), which suggests that progesterone may not be involved in BP regulation in this animal model.

In summary, these results confirm that adult hypertension in the rat can be programmed by prenatal intrauterine events. The severity of increases in BP is dependent on the timing of in utero insult by maternal LPD. The male offspring appeared to have a greater risk of the development of hypertension. Another interesting finding in this study is that males, unlike females, do not respond to flutamide in attenuating the elevated BP. We speculate that estrogen may be involved in the flutamide effects of reducing BP in female LPD-1 offspring. This is the first study to show that flutamide decreases BP in LPD females, but not in LPD males. LPD is relevant to certain populations in which carbohydrate-protein imbalances often result in programmed diseases, particularly metabolic syndrome.54 We speculate that androgen receptor blockers, such as flutamide, may be useful in countering hypertension in programmed female adults.

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References

Placental vascular disease and toll-like receptor 4 gene expression

Xin Wang, PhD, Neil Athayde, MBBS, Brian Trudinger, MD

Department of Obstetrics and Gynaecology, University of Sydney at Westmead Hospital, Sydney, New South Wales, Australia

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Objective: Vascular disease in the placenta, which is identified by the study of umbilical artery Doppler flow velocity waveforms, is associated with endothelial cell activation and a proinflammatory cytokine response in the villous placental circulation. We studied toll-like receptor 4 expression (the ligand is lipopolysaccharide) to examine whether infection may cause these inflammatory components of placental vascular disease through an innate immune response.

Study design: Microvessel endothelial cells were isolated from human placentae with collagenase digestion and then extracted with Dynabeads that were coated with monoclonal antibody against CD31. We studied 13 placentae from normal pregnancies that were delivered at term and 15 pregnancies with umbilical placental vascular disease that was defined by an abnormal umbilical artery Doppler study. We extracted RNA from the isolated endothelial cells. The messenger RNA expression of toll-like receptor 4 production was assessed by reverse transcriptase–polymerase chain reaction and factored relative to the glyceraldehyde-3-phosphate dehydrogenase and 18S ribosomal RNA genes.

Results: Microvessel endothelial cells from placental villi with placental vascular disease showed up-regulation of toll-like receptor 4 expression (toll-like receptor 4/18S, 1.92 ± 0.37 vs 0.99 ± 0.19; P < .05; toll-like receptor 4/glyceraldehyde-3-phosphate dehydrogenase, 2.20 ± 0.36 vs 1.25 ± 0.22; P < .05) in comparison with normal pregnancy.

Conclusion: Up-regulation of toll-like receptor 4 gene in the endothelium of the placental villi is present in placental vascular disease, which may result from exposure of this endothelium to the toll-like receptor 4 ligand lipopolysaccharide in vivo. Directly extracted endothelial cells were used to avoid the possibility for change in behavior in tissue culture. We conclude that Gram-negative infection and lipopolysaccharide stimulation may cause placental vascular disease.

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crucial to the activation of adaptive immunity that then develops over 4 to 7 days and involves antigen-presenting dendritic cells and lymphocytes. The most widely known signaling receptors of mammalian innate immunity are the Toll-like receptor (TLR) family, which are homologous to the Toll receptors that were first described in the fruit fly. Specifically, TLR4 recognizes molecular patterns of the cell wall of gram-negative bacteria that constitute lipopolysaccharide.

Thrombotic vascular disease in the placenta can be identified by a high resistance pattern on the umbilical artery blood flow velocity waveform that is studied with Doppler ultrasound. We have shown this to be associated with the activation of endothelial cells in the placental villous circulation. Up-regulation for gene expression for proinflammatory cytokines and cell adhesion molecules is associated with an increase in circulating protein product. We have surmised that these findings are an inflammatory reaction, a response of the endothelium to injury. We have noted the similarities between these changes and the features of atherosclerosis, which has been defined as an inflammatory process and a response of the vascular endothelium to injury. This leads to the question of cause. We now postulate that infection may be the underlying cause. We are aware that immune responses and infectious agents have been proposed as instigators or potential initiators of atherogenesis through an innate immune response to infection. It is also relevant that we have shown recently that fetal plasma from pregnancy with premature rupture of membranes will cause endothelial cell activation and a proinflammatory cytokine response similar to our findings with placental vascular disease. This condition has been associated with infection and a fetal inflammatory response. There are recent descriptions of vascular lesions in the placenta in premature rupture of membranes that are similar to those lesions that are seen in placental vascular disease.

We recently have developed a technique to isolate microvascular endothelial cells from the umbilical placental circulation and extract messenger RNA (mRNA). This allows the direct study of endothelial cell behavior and avoids the possibility of alteration on passage in the tissue culture. The endothelium is one of the barriers that microbial agents must cross to gain access to the tissues of the body. Endothelium can act as phagocytic cells that express TLRs and initiate an innate immune response. If an innate immune response underlies the vascular lesion that we have studied, then it is likely to be a response to gram-negative infection and lipopolysaccharide. Because exposure to lipopolysaccharide up-regulates TLR4 expression, we examined endothelium from placentas with antenatal evidence of thrombotic vascular disease for TLR4 expression.

Material and methods

Study population

Two groups of pregnant women were studied. In the first group, we collected placentae from 13 normal control pregnancies. In the second group, we studied placentae from 15 pregnancies in which an abnormal umbilical artery Doppler study indicated umbilical placental vascular disease. This group was subdivided according to the absence (n = 7 pregnancies) or presence (n = 8 pregnancies) of maternal preeclampsia with abnormal umbilical artery Doppler evidence. All infants in this group were delivered by elective caesarean delivery. In the normal group, all pregnancies were uncomplicated with no identifiable medical or obstetric diseases and were delivered by spontaneous vaginal delivery or elective cesarean delivery at term (for reasons not associated with fetal compromise). The umbilical placental vascular disease group was identified by an abnormal umbilical artery Doppler study carried out within 1 to 4 days before delivery. The systolic diastolic ratio was >95th percentile with our previously reported normal range. Maternal preeclampsia was defined as a blood pressure of ≥140/90 mm Hg on at least 2 occasions that occurred after week 20 of gestation that was accompanied by proteinuria (>300 mg/24 hours).

This study was conducted with the approval of the Western Sydney Area Health Service Ethics Committee.

Preparation of placental villi, isolation, and purification endothelial cells

The placentas from normal term pregnancies after vaginal delivery or caesarean deliveries were collected and immediately processed. Four placental lobes (30 g) were immediately excised and cleared of their main vascular trunks and stored in cold phosphate buffer (PBS; Gibco BRL, Gaithersburg, Md). We previously have described our method for the isolation and purification of endothelial cells. This study was conducted with the approval of the Western Sydney Area Health Service Ethics Committee.

Reverse transcription and polymerase chain reaction (PCR)

Total RNA was isolated from these purified endothelial cells that were extracted from the placental microvessels with the use of RNAzol B (Tel-Test, Inc, Friendswood,
The optical density at 260 nm/280 nm of RNA was always >1.6. The integrity of RNA was confirmed by the presence of intact 18s and 28s bands on 1% agarose gel. The first-strand complementary DNA synthesis reaction was performed with Perkin-Elmer GeneAmp PCR Systems’ Reverse Transcription kit (Roche, Branchburg, NJ). Semiquantitative PCR was used to examine the expression of the mRNA of TLR4 in microvascular endothelial cells. Primers for TLR4, 18S recombinant RNA and glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) were chosen from the published sequences. TLR4: 5′-TTC TAC AAA ATC CCC GAC AA-3′ and 5′-GGC TCC CAG GGC TAA ACT-3′; 18S recombinant RNA: 5′-AGC TTC CGG GAA ACC AAA GT-3′ and 5′-CAA TCT CGG GTG GCT GAA C-3′; GAPDH: 5′-GAC CCC TTC ATT GAC CTC AA-3′and 5′-CAT GGA CTG TGG TCA TGA GC-3′. Amplification reactions were performed with a thermostable DNA polymerase kit (Advanced Bio-technologies, Surrey, UK). Housekeeping gene 18S recombinant RNA and GAPDH were used as internal controls. Preliminary experiments were done to optimize the amount of complementary DNA and the reaction conditions. The conditions for each PCR were optimized with respect to the linear range of the amplification reaction. The TLR4 and 18S recombinant RNA or GAPDH products were then loaded respectively into 2% agarose gel. Separation of DNA fragments was achieved by electrophoresis with ethidium bromide staining. The gel were scanned and analyzed by Fluor-S MultiImager using Bio-Rad Multi-Analyst software (version 1.0.2; Bio-Rad Laboratories, Hercules, Calif).

### Statistical analysis

The quantitative data about clinical characteristics and mRNA expression of TLR4 between the patient groups were analyzed by independent Student t test. The expression of mRNA in the normal control group and those patients with placental vascular disease with and without maternal preeclampsia were assessed with a 1-way analysis of variance with Dunnett post-hoc comparison. The categoric data about parity, low percentile birth weight, and delivery mode among the groups was analyzed by a Fisher’s exact test. A probability value <.05 was considered statistically significant. An analysis of covariance on our results with gestational age and delivery method as covariates was carried out because these parameters differed between our study group and the control group.

### Results

#### Clinical outcome of the pregnancies studied

In all pregnancies, the last Doppler study and delivery occurred in the third trimester. The group with umbilical placental vascular disease were delivered earlier, and the infant birth weight was reduced in comparison with normal. The percentile birth weight for gestational age and the placental weight were also lower in this group. The clinical characteristics of our investigation groups are summarized in the Table.

#### MRNA expression of TLR4 in microvascular endothelial cells

In our study group with umbilical placental vascular disease, the expression of TLR4 mRNA was up-regulated (TLR4/18S, 1.92 ± 0.37 vs 0.99 ± 0.19; P < .05; and TLR4/GAPDH, 2.20 ± 0.36 vs 1.25 ± 0.22; P < .05) in comparison with normal pregnancy. The expression of TLR4 mRNA by endothelial cells from placental villi in the study group with umbilical placental vascular disease and our normal pregnancy group are shown in Figure 1.
In the group with umbilical vascular disease, the difference in TLR4 mRNA expression in the subgroups with maternal preeclampsia in comparison with pre-eclampsia absent was not significant, although the pre-eclampsia group was greater (TLR4/18S, 2.16 ± 0.48 vs 1.63 ± 0.59; TLR4/GAPDH, 2.50 ± 0.59 vs 1.85 ± 0.36; P < 0.05; Figure 2).

We carried out an analysis of covariance for both the normal group and the study group with respect to gestational age and method of delivery for mRNA expression of TLR4. In both groups, there was no correlation with gestational age. The study group did not labor (elective caesarean delivery); however, in the normal group, the occurrence of labor did not correlate with TLR4 expression.

Comment

TLR4 is the transmembrane lipopolysaccharide receptor that initiates the innate immune response to common gram-negative bacteria. In this study, we have demonstrated that endothelium from the microcirculation of the placenta is up-regulated for the expression of the TLR4 gene in placental vascular disease. Up-regulation of the TLR4 gene follows ligand stimulation.12 Our results therefore are consistent with the presence of microbial infection and specifically gram-negative infection and lipopolysaccharide in the fetal compartment and TLR4 receptor activation. However, we have not demonstrated the presence of infection, lipopolysaccharide, or receptor activation. This evidence is required before we can conclude that infection is the cause of the vascular pathologic condition in placental vascular disease. We carried out this study to seek an explanation for endothelial cell activation and proinflammatory cytokine production, which we had shown in these placentae in our previous work. We used strict Doppler criteria to identify pregnancy with placental vascular disease for study inclusion. The high resistance pattern in the umbilical artery blood flow velocity waveform recorded using Doppler ultrasound has been correlated with placental vascular disease on subsequent histologic examination of the placenta by ourselves14 and others.15

Sixty to seventy per cent of babies who are born small for gestational age show a high resistance pattern on umbilical artery Doppler study, and this is the group of growth-restricted fetuses with the most morbid outcome. These are the pregnancies in our study group.

Innate immunity is the immediate defense of the body to infection. The TLR family is pre-eminent among the pattern recognition receptors that identify the repeating conserved sequences of the outer cell wall of the invading micro-organisms. These are located widely on phagocytic cells through the body and on epithelial surface cells, endothelium, and blood cells. These transmembrane receptors have 2 main functions that are responsible for the initiation of phagocytosis of the invading micro-organism and the stimulation of a program of gene expression that constitutes the innate immune response. This latter includes triggering signals for intercellular messaging through lipid (prostaglandin) and protein (cytokine) release to produce an inflammatory response and marshalling forces for an adaptive immune response.1 The transmembrane receptor TLR4 binds with CD14 and its ligand (the complex of lipopolysaccharide and its specific binding protein). TLR4 receptor activation up-regulates receptor expression, which is our explanation for the findings of the present study. TLR ligation indicates the presence of infection and leads to a signaling cascade,16 which includes the gene expressions that we have demonstrated in placental vascular endothelium. There is uncertainty about TLR stimulation in the absence of infection. Endogenous ligands, which include heat shock protein, β defensin, and oligosaccharides from the breakdown of extracellular matrix, are recognized; however, their presence is associated with infection and the possibility of lipopolysaccharide contamination.17

Innate immunity and infection have been associated with vascular damage. Intravascular inflammation has putative proatherogenic effects, and atherosclerosis has been defined as the response of vascular endothelium to injury.8 Endothelium plays a pivotal role in recruiting inflammatory cells to the site of tissue injury or infection. It has been suggested that atherosclerosis may be the result of an inflammatory reaction to bacterial infection.18 It has been demonstrated that endothelial cell activation follows TLR4 receptor ligation with gram-
negative bacteria and polymorphisms that attenuate receptor signaling and diminish the inflammatory response to gram-negative pathogens that are associated with a decreased risk of atherosclerosis. Proinflammatory polymorphisms of components of the inflammatory cascade (such as interleukin-6, E-selectin and tumor necrosis factor–α) are associated with increased risk. Seropositivity for Chlamydia has been associated with risk of cardiovascular disease; these bacteria can be detected within atherosclerotic lesions. There are many reports that associate the acute-phase inflammatory response with the acute events of cardiovascular disease. We previously have noted the presence of inflammation of vascular endothelium in the placenta and commented on similarities with atherosclerosis.

Clinical problems in the fetal compartment in the second half of pregnancy have been separated into 2 groups that have been encapsulated eloquently in the phrase “born too soon or born too small.” Current opinion associates thrombotic placental vascular disease with a baby who is born too small and associates chorioamnionitis with a baby who is born too soon. In the present report, we have studied a group of infants who were born too small and investigated the vasculature of the placenta. The novel feature of our results is the potential association of infection with the vascular disease that leads to fetal growth restriction and a baby who is born too small. More usually, infection, inflammation, and adverse fetal outcome have been linked in the preterm labor syndrome that leads to a baby who is born too soon. Ascending maternal infection from the vagina through the cervix and along the uterine decidua is identified as a major cause of preterm labor. Preterm labor and preterm rupture of the membranes before labor have been associated with an exuberant inflammatory response with elaboration of cytokines and matrix metalloproteinases. Adverse outcome, particularly for the fetus, is related to the presence of a fetal inflammatory response. The presence of proinflammatory cytokines in fetal blood predicts morbidity, which includes the risk of neurologic injury. Interestingly, it appears that it is the fetal response, not the presence of infection, that is most important, because clinical and/or histologic indicators of intrauterine infection have not been shown to be independent risk factors for morbidity. There are points of similarity between these 2 fetal syndromes that lead to a “too soon” or “too small” birth that have been overlooked in the past. The preterm labor syndrome usually has not been associated with umbilical placental vascular disease, which is defined by a high resistance umbilical artery Doppler blood flow velocity waveform study. However, a vasculopathy in the placental villous umbilical circulation that is similar to that seen with abnormal umbilical artery Doppler study and the growth-restricted fetus may also be seen with preterm rupture of membranes. The birth weight of fetuses from spontaneous preterm delivery is less than normal. Placental vascular disease is a cause of preterm labor. Similar long-term neurologic handicaps may be present in fetuses who are born both too soon and too small. A recent American College of Obstetricians and Gynecologists and American Academy of Pediatrics task force on Neonatal Encephalopathy and Cerebral Palsy noted the association among intrauterine infection and inflammatory cytokines and the risk of cerebral palsy and also noted the association between proinflammatory cytokine activation and thrombophilia.

There is other recent work from a quite different direction that adds credence to the concept that infection may occur in the fetal compartment. This is critical to our hypothesis that vascular damage in the placenta and in target fetal organs may be the result of infection. Both preterm labor and fetal growth restriction have been associated with maternal periodontal disease and with a fetal (not maternal) antibody response to oral organisms. Amniotic fluid PCR for bacterial particles is positive in pregnancy with adverse fetal events. This work shows that micro-organisms of maternal origin do enter the fetal compartment and may stimulate an immune response. Second, this work suggests that this process occurs in mothers who do not mount a protective antibody response. Because there are similarities in the micro-organism populations of the oral cavity and vagina, it is also possible that the infection is from the vagina and not the oral cavity.

In this study, we have demonstrated that thrombotic placental vasculopathy is associated with the up-regulation of TLR4 gene expression in the endothelium of the placental villi. Inflammation and activation of endothelium are present when the umbilical artery Doppler study indicates placental vascular disease. The results of the present study support the hypothesis that this may be a response to infection through an innate immune reaction. To confirm our hypothesis, we need to demonstrate TLR4 ligation and activation in the endothelium.

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Aberrant patterns of cellular communication in diabetes-induced embryopathy in rats: II, Apoptotic pathways

E. Albert Reece, MD, PhD, MBA,a,* Xiang-Dong Ma, MD, PhD,a Zhiyong Zhao, PhD,a Ying-King Wu, MD, PhD,a Danny Dhanasekaran, PhDb

Departments of Obstetrics and Gynecology and Biochemistry and Molecular Biology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Ark,a and The Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pa b

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KEY WORDS
Diabetic embryopathy
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Apoptosis

Objective: The objective was to test the hypothesis that hyperglycemia-induced injury of yolk sac cell membranes is associated with disruption of cellular apoptotic signaling pathways.

Study design: Pregnant rats were induced to become diabetic by injection of streptozotocin. Fourteen normal control and 24 diabetic rats were killed on day 12 of gestation. Yolk sac membranes in 3 conceptus groups (nondiabetic, diabetic with normal, or diabetic with malformed conceptus) were collected for study. DNA was extracted from yolk sac cells and assayed for fragmentation by using gel electrophoresis, which indicates apoptosis. Protein expression was evaluated by Western blot assays. Statistical analyses were performed with the Student t-test.

Results: The level of phosphorylated Akt was significantly decreased, whereas that of the proapoptotic protein Bax was increased. These changes were correlated with the presence of DNA fragmentation in yolk sac cells of the diabetic malformed conceptuses.

Conclusion: Hyperglycemia-induced embryopathy involves apoptosis, during which the expression of proapoptotic protein Bax is upregulated and the activity of the cell-survival factor, Akt kinase, is decreased in yolk sac cells. These observations suggest that hyperglycemia of maternal diabetes triggers apoptotic signaling pathways and inhibits cell survival pathways, leading to embryonic malformations.

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Diabetes mellitus is associated with a 6% to 10% incidence of major congenital anomalies and an increased risk for neonatal deaths in infants of diabetic mothers (IDM).1 The incidence of these malformations is 3 to 5 times higher than that observed in the general population.2 Malformations of the central nervous system, cardiovascular system, digestive system, urogenital, and skeletal systems are often encountered,3 although their precise molecular mechanisms still remain unknown. Previous studies revealed that the risk of
congenital malformations in IDM is related to multiple factors, including the imbalance of maternal fuels early in gestation,\textsuperscript{4,5} the deficiency in essential fatty acid, such as arachidonic acid (AA), and proteins, such as myo-inositol (MI).\textsuperscript{5,6} as well as the excess of embryonic oxygen-free radicals.\textsuperscript{9} Morphologic analyses indicate that the primary damage to yolk sac cell membranes induced by hyperglycemia occurs during a critical period of organ formation. This is believed to be the vulnerable stages of organogenesis for hyperglycemic insult. The most frequently observed embryonic malformation is the incomplete closure of the neural tube. It is referred to as neural tube defects (NTD).

Recently, it has been hypothesized that hyperglycemia-induced injury of the membranes of yolk sac cells is associated with the disruption of cellular signal transduction pathways,\textsuperscript{10-12} especially the signal-mediating apoptotic pathways.\textsuperscript{13}

Apoptosis or programmed cell death is found in many pathologic conditions, including diabetic complications and embryopathy. However, the molecular pathways for apoptosis in diabetic embryopathy have not been delineated. In this study, we focused on a proapoptotic factor, Bax, and an antiapoptotic factor, Akt, because these factors have been found to be affected by hyperglycemia in diabetic condition.\textsuperscript{14,15}

The aim of this study was to determine whether activation of apoptotic signaling pathways and inhibition of cellular survival signaling pathways play important roles in diabetes-induced embryopathy.

Material and methods

Animal experimentation

The procedures of animal use were approved by the University of Arkansas Medical Sciences (UAMS) Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass), age 70 to 90 days and weighing 250 to 300 g, were mated overnight with nondiabetic males of the same strain. Vaginal smears were examined the next morning, and the presence of spermatozoa in the examination indicated the day zero of gestation. Diabetes was induced in the pregnant rats on the day 6 by intravenous injection of streptozotocin (STZ) in 65 mg/kg of body weight. Saline-injected rats served as normal control. The animals were killed on day 12, and the embryos and yolk sac were dissected out of the uteri and examined morphologically under a dissecting microscope. The embryos were categorized as being either morphologically normal or showing evidence of neural tube malformation. Embryos were defined as normal if examination revealed correct body flexure and both anterior and posterior neural pole closure. Embryos were categorized as having a NTD if closure of the mesencephalon was not observed after other portions of the neural tube had fused and/or malrotation was present. Yolk sac membranes and embryos were categorized into 3 tissue study groups: normal control offspring, diabetic with normal offspring, and diabetic with malformed offspring.

DNA fragmentation assay

Yolk sac membranes were homogenized in 10 volumes of lysis buffer (pH 8.0) consisting of 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 0.5% sodium dodecylsulfate (SDS), and 100 μg/mL protein kinase, and centrifuged at 15,000g for 20 minutes. DNA in the supernatant was extracted with a phenol-choroform-isomyl alcohol mixture (25:24:1) and precipitated with 70% ethanol at –20°C. Contamination of RNA in the DNA samples was removed by using DNase-free RNase one (Promega, San Luis Obispo, Calif) (100 u/mL) at 37°C for 30 minutes. Resolving electrophoresis was performed in 1.0 μg/mL of ethidium bromide; 20 μg of DNA per well was loaded on 1.8% agarose gel along with λDNA-Bst E II digest marker (Cell Signaling Technologies Inc, Beverly, Mass) and a low DNA mass ladder (Gibco/BRL, Gaithersburg, Md) was run for 1 hour with at 100V and visualized under ultraviolet light. A distinct DNA ladder was identified as an index of apoptosis.\textsuperscript{16}

Western blot analysis

Yolk sac specimens were randomly collected from each of the 3 study groups and protein was extracted from these samples. Equal amount of protein (20 μg) was separated on 10% SDS-PAGE in Tris-glycine electrophoresis buffer at 15 to 20 mA for 2 hours. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, Mass) at 500 mA for 30 minutes using a mini-PROTEAN blotting apparatus (Bio-Rad, Hercules, Calif) in 10 mmol/L CAPS buffer (pH 11) containing 20% methanol. The protein-transfer was verified by Ponceau S staining (0.1% Ponceau S in 5% acetic acid).

The PVDF membrane was blocked in TBST (20 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, and 0.1% Tween-20) containing 5% fat-free milk and 1% bovine serum albumin (BSA) at room temperature for 1 hour. Membranes were quickly rinsed 3 times for 5 minutes each time with TBST, then incubated in TBST containing 5% BSA at 4°C overnight. Blocked membranes were incubated with primary rabbit antibodies specific to phospho-Akt kinase (Cell Signaling Technologies Inc) and to Bax (Santa Cruz Biotechnology, Santa Cruz, Calif) (1:5000). The immunoblots were washed with TBST and incubated at room temperature for 1
hour with the horseradish peroxidase conjugated anti-rabbit secondary antibody (1:10,000, Promega) in TBST containing 5% milk. Membranes were finally washed completely with TBST, and the immunoblots were developed with the use of the Renaissance Western Blot Chemiluminescence Reagent plus (Perkin Elmer Life and Analytical Sciences, Boston, Mass) (NEN) according to manufacturer’s instructions.

Chemiluminescence was detected by exposing the blots to Kodak scientific imaging film (X-Omat, Kodak, Rochester, NY). The signals in the immunoblots were scanned and analyzed by NIH Image software (download available at: http://rbs.info.NIH.gov/NIH-image/index.html). All experiments were repeated 3 times with the use of independently prepared cell lysates.

Statistical analyses were performed with the Student t test, and the data were expressed as mean ± SD.

Results

Morphologic changes

A total of 210 embryos were harvested from 14 rats in the nondiabetic control groups, and 333 embryos were harvested from 24 rats in the diabetic groups. The mean
glucose level in the diabetic dams during the experiment (gestational days 9-12) was $332.8 \pm 59.6 \text{ mg/dL}$, 2 to 3 times higher than that in the nondiabetic control group ($89.6 \pm 13.5 \text{ mg/dL}$). Examination of the embryos from the diabetic rats showed higher rates of NTD (21.5% $\pm$ 9.6% per rat) (Figure 1, B and C) compared with the rats in nondiabetic control (6.6% $\pm$ 5.0% per rat) (Figure 1, A) ($P < .001$). The growth of the embryos was delayed, as indicated by the crown-rump length and number of somite, in comparison with control. Embryonic resorption rate was significantly different between diabetic and nondiabetic groups ($P < .01$) (Table).

### Table: Maternal glucose levels and embryonic development

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>No. of embryos</th>
<th>Glucose level (mg/dL)</th>
<th>Grown-rump length (mm)</th>
<th>Somite number</th>
<th>Resorption rate (%)</th>
<th>NTD rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetes</td>
<td>14</td>
<td>210</td>
<td>84.2 $\pm$ 0.9</td>
<td>57.1 $\pm$ 6.3</td>
<td>36.8 $\pm$ 2.8</td>
<td>6.54 $\pm$ 4.23</td>
<td>6.63 $\pm$ 5.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>24</td>
<td>333</td>
<td>82.4 $\pm$ 9.2</td>
<td>45.9 $\pm$ 0.2*</td>
<td>33.5 $\pm$ 3.3*</td>
<td>21.37 $\pm$ 20.39*</td>
<td>21.48 $\pm$ 9.6*</td>
</tr>
</tbody>
</table>

Value is mean $\pm$ SD.
* Indicates significant difference ($P < .01$).

**Apoptosis**

To examine apoptosis in the yolk sac under hyperglycemic condition, fragmentation of DNA was assessed using electrophoresis. DNA samples from the control yolk sacs showed no distinct bands, but high molecular weight mass retained on the top portion of the gel (Figure 2, lane 1). In contrast, a number of lower molecular weight bands were observed in the DNA samples from the diabetic groups, indicating DNA fragmentation (Figure 2, lane 2).

**Changes in Bax and Akt**

The above experiments show that maternal diabetes generates abnormal embryos and the embryonic malformations are associated with excessive apoptosis. To further delineate the molecular mechanisms, we examined Bax, a proapoptotic protein, and Akt, an anti-apoptotic protein. Expression of Bax was assessed using Western blotting and showed a dramatic increase in the yolk sacs of abnormal embryos from the diabetic groups (Figure 3, panel 1, lane AB; and panel 2), when compared with nondiabetic control (Figure 3, panel 1, lane NC; and panel 2). A moderate increase was also seen in the yolk sacs of the normal embryos in the diabetic groups (Figure 3, panel 1, lane AB; and panel 2), in comparison with nondiabetic control (Figure 3, panel 1, lane NC; and panel 2). These results suggest that Bax and Akt are involved in hyperglycemia-induced apoptosis in the embryonic cells.

**Comment**

The teratogenic process of diabetic embryopathy is multifactorial. These adverse factors induce primary injury to the yolk sac cell membrane as evidenced by histologic, ultrastructural, and biochemical studies, and secondly affect the embryo in early embryogenesis. There is now convincing evidence from clinical and experimental studies indicating those disturbances in fuel metabolism during diabetes-associated pregnancy leads to sustained generation of reactive oxygen species (ROS) and depletion of antioxidant defense mechanisms. When ROS generation exceeds the antioxidant defense capacity of the cell (redox imbalance), it causes intracellular oxidative stress and results in cell injury and the activation of a variety of stress-sensitive signaling pathways that allow the cell to adapt and survive, or alternatively, induce apoptotic cell death. Apoptotic signal pathways may play a critical role in the pathogenic mechanism of diabetic embryopathy. It has been hypothesized that apoptosis in the mammalian preimplantation blastocyst is a normal process, one designed to protect the early embryo by eliminating abnormal cells. To determine whether maternal diabetes affects rat embryo and yolk sac apoptosis during the postimplantation period, Forsberg et al found that severely malformed and growth-retarded embryos of gestational day 12 from diabetic rats exhibited pronounced DNA laddering on agarose gels, and no DNA laddering was observed in nonmalformed embryos from control and diabetic rats, or in their corresponding yolk sacs. In the experimentally induced hyperglycemic situation, we noted that diabetic malformed embryonic cells underwent changes consistent with apoptosis. This indicates that hyperglycemia is linked to the induction of cell death in yolk sac cells in the diabetic environment, one occurring, most probably, through the activation of the apoptotic signaling pathways and the inhibition of cellular survival signal pathways. This process occurs by upregulating the expression of proapoptotic genes such as Bax and caspase-3, along with downregulating the
expression of antiapoptotic genes, such as Akt and Pax-3. These altered gene expressions trigger the onset of apoptotic signaling pathways while inhibiting the cell survival pathways. Phelan et al. studied the role of the Pax-3 gene and apoptosis in neural tube defects embryos of diabetic mice. They used a semiquantitative reverse transcription-polymerase chain reaction to detect Pax-3 messenger RNA (mRNA) and fibronectin mRNA, and found that Pax-3 were underexpressed in the embryos of diabetic mice, not fibronectin. They also examined the expression and location of Pax-3 mRNA along the length of the neural tube by using in situ hybridization technique and found that the expression of Pax-3 gene is significantly reduced and that in the same neural tube regions subsequently contain high concentrations of cells undergoing apoptosis. Consistent with this idea, our most recently unpublished studies reveal an increase in the activity of caspase-3 in embryopathic yolk sac cells. The roles of the proapoptosis protein Bax and antiapoptotic kinase Akt have been well defined. The activity of Akt is significantly inhibited in yolk sac cells harvested from malformed conceptuses. The overexpression of Bax correlates with the inhibition of activated Akt, which may contribute to chromatin degradation in yolk sac cells from experimentally induced embryopathy as shown in Figure 3. These aberrant patterns of cellular communication are believed to mediate an abnormal developmental signal associated with embryopathy. It is postulated that hyperglycemia is the major cause of not only congenital malformations, such as NTD, but also of early and late pregnancy losses as well. The increased risk of spontaneous abortion and of major congenital malformations may result from an advanced sequence of apoptotic processes in conceptuses of women with diabetes mellitus. Finally, our studies serve to illustrate the precise molecular mechanisms underlying the apoptotic signal pathways in diabetic embryopathy as hypothesized in Figure 4. Accumulating data suggest that, in diabetic conceptuses, hyperglycemia triggers apoptotic signal pathways by upregulating the expression of the proapoptotic genes, Bax, and caspase-3, and by downregulating the expression of the antiapoptotic genes, Akt, and Pax-3, which may lead to a sequence of maldevelopmental events, resulting in clinically recognizable defects. In contrast, euglycemia activates cellular survival signal pathways in reverse, resulting in normal developmental processes (Figure 4).

References


Impaired $K_{\text{ATP}}$ channel function in the fetoplacental circulation of patients with type 1 diabetes mellitus

Tanya M. Bisseling, MD, Marieke G. Versteegen, MD, Selina van der Wal, MD, Jenny J. H. Copius Peereboom-Stegeman, PhD, Joop M. P. M. Borggreven, Eric A. P. Steegers, PhD, Jeroen A. W. M. van der Laak, PhD, Frans G. M. Russel, PhD, Paul Smits, PhD

Departments of Pharmacology-Toxicology, Pathology, and Internal Medicine University Medical Center, Nijmegen, and Department of Obstetrics and Gynecology, Erasmus University Medical Center, Rotterdam, The Netherlands

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KEY WORDS
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Glibenclamide
Fetoplacental vasculature
Endothelium
Pregnancy

Objective: The increased perinatal morbidity in diabetes may be partly related to vascular dysfunction. Because potassium channels play an important role in the regulation of vascular tone, this study explores the impact of diabetes on potassium channel function in the fetoplacental vascular bed.

Study design: Vascular potassium channel function was investigated by ex vivo dual perfusion of isolated placental cotyledons (n = 47). Appropriate control experiments were carried out to exclude nonspecific effects.

Results: Glibenclamide ($K_{\text{ATP}}$ channel blocker) increased perfusion pressure to a maximum fetoplacental arterial pressure of 37 ± 6 mm Hg in controls versus 15 ± 6 mm Hg in diabetes ($P < .05$). 4-Aminopyridine ($K_V$ channel blocker) equally increased fetoplacental arterial pressure in controls, and in diabetes (21 ± 4 mm Hg vs 22 ± 2 mm Hg). Apamin and Charybdotoxin ($K_{\text{Ca}}$ channel blockers) caused a negligible rise in fetoplacental arterial pressure.

Conclusion: In the fetoplacental circulation, $K_{\text{ATP}}$ channels and $K_V$ channels significantly contribute to baseline vascular tone. In diabetes, vascular $K_{\text{ATP}}$ channel function is impaired.

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dysfunction, in particular with respect to the nitric oxide negative (NO) and the cyclooxygenase pathways, have been observed in different vascular beds including fetoplacental arteries. Apart from this, recent research points toward an impaired release of endothelium-derived hyperpolarizing factor (EDHF) in diabetes. Opening of the vascular smooth muscle K\(_{\text{Ca}}\) channel seems to be a final common pathway in the mechanism of action of EDHF. Independent from the EDHF pathway, diabetes has been associated with dysfunction of the cardiovascular K\(_{\text{ATP}}\) channel, an ion channel that has an important role in the regulation of vascular tone during ischemia. With a constant fetal arterial inflow, the fetoplacental arterial pressure was considered to be a reflection of net downstream fetoplacental vascular resistance. The contribution of 4 different vascular potassium channels to baseline fetoplacental vascular tone was investigated by adding increasing concentrations of the selective blockers for these channels both in control and in diabetic placentas. These blockers were as follows: glibenclamide for the ATP-dependent K channel (K\(_{\text{ATP}}\) channel); apamin for the small conductance K\(_{\text{Ca2+}}\) channel (SK channel); charybdothxin for the intermediate conductance K\(_{\text{Ca2+}}\) channel (IK channel), large conductance K\(_{\text{Ca2+}}\) channel (BK channel), and some voltage-dependent K channels (K\(_{\text{V}}\) channel); and 4-aminopyridine for the K\(_{\text{V}}\) channel. These blockers were added in 6 to 9 cumulative log-dose steps in concentration ranges from (log concentration [mol/L]): −8.0 to −3.5 (glibenclamide), −9.0 to −6.0 (apamin), −10.0 to −7.0 (charybdothxin), and −7.5 to −3.5 (4-aminopyridine).

Control experiments on vascular compliance of isolated resistance arteries
To assess the elasticity of the resistance arteries (compliance) of control and diabetic placentas, fetoplacental resistance arteries were isolated from 4 healthy and 4 diabetic patients. On isolation, all arteries were transferred to the 10-mL pressure myograph organ bath, where they were immersed in Ca-free medium as described previously by Smits et al. The artery was gradually pressurized to 50 mm Hg in a pressure myograph (Danish MyoTech P100) over a period of 5 minutes, and the arterial diameter was studied by stepwise increasing the intraluminal pressure from 1 to 60 mm Hg for a period of 2 minutes at each pressure step. This was done twice in succession for each artery. The vessel diameter values for these 2 series were then averaged for each pressure. In this way, 2 or 3 arteries were studied per placenta. These vessel diameter values were averaged to one representative value for each placenta. All preparations were gassed with 95% \(\text{O}_2/5\% \text{CO}_2\) to maintain pH at 7.4 throughout the experiment.

Control experiments on morphometry of the fetoplacental arterial circulation
To quantify the vascular diameters and wall thickness we used the computerized image analysis system (eg, Vidas PLUS system, Carl Zeiss GmbH, Jena, Germany). To perform this analysis, the vascular endothelial CD34 antigen was detected by the monoclonal

**Material and methods**

**Study population**
Pregnant women with type 1 diabetes were eligible to participate. Controls were healthy pregnant women with uncomplicated pregnancies. Exclusion criteria for both groups were multiple pregnancy, premature birth (<37 weeks’ gestation), retained placenta, pregnancy-induced hypertension (diastolic pressure >90 mm Hg on 2 following occasions), and preeclampsia or HELLP syndrome (hemolysis, elevated liver enzymes and low platelets).

From all participants maternal and umbilical venous blood samples for insulin and C-peptide assay were taken within 15 minutes after delivery. All women gave written informed consent. The local medical ethics committee approved this study.

**Classification of diabetes mellitus in pregnancy**
In pregnancy, diabetes mellitus is classified according to the White Classification. This classification consists of 7 groups: A, B, C, D, F, R, and H. Group A is the classification for gestational diabetes mellitus. Groups B, C, D, F, R, and H are classifications for pregestational diabetes mellitus. In groups B, C, and D, the age of onset of diabetes mellitus in years (>20, 10-19, and <10, respectively), and duration of the disease in years (<10, 10-19, >20) determine the classification. Groups B and C are not associated with vascular complications; group D is associated with a benign nephropathy. In groups F, R, and H, vascular complications determine the classification. Group F is associated with nephropathy, group R with retinopathy, and group H with heart disease.

**Cotyledon perfusion**
Over a period of 2 years, all placentas from women with type 1 diabetes were obtained immediately after delivery and transported to the laboratory within 10 minutes after delivery. Controls matched for mode of delivery, parity, and maternal age were acquired throughout this period. After arrival at the laboratory, a suitable cotyledon was selected from the placenta for ex vivo dual perfusion as described extensively previously. With a constant fetal arterial inflow, the fetoplacental arterial pressure was considered to be a reflection of net downstream fetoplacental vascular resistance. The contribution of 4 different vascular potassium channels to baseline fetoplacental vascular tone was investigated by adding increasing concentrations of the selective blockers for these channels both in control and in diabetic placentas. These blockers were as follows: glibenclamide for the ATP-dependent K channel (K\(_{\text{ATP}}\) channel); apamin for the small conductance K\(_{\text{Ca2+}}\) channel (SK channel); charybdothxin for the intermediate conductance K\(_{\text{Ca2+}}\) channel (IK channel), large conductance K\(_{\text{Ca2+}}\) channel (BK channel), and some voltage-dependent K channels (K\(_{\text{V}}\) channel); and 4-aminopyridine for the K\(_{\text{V}}\) channel. These blockers were added in 6 to 9 cumulative log-dose steps in concentration ranges from (log concentration [mol/L]): −8.0 to −3.5 (glibenclamide), −9.0 to −6.0 (apamin), −10.0 to −7.0 (charybdothxin), and −7.5 to −3.5 (4-aminopyridine).

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Control experiments on morphometry of the fetoplacental arterial circulation
To quantify the vascular diameters and wall thickness we used the computerized image analysis system (eg, Vidas PLUS system, Carl Zeiss GmbH, Jena, Germany). To perform this analysis, the vascular endothelial CD34 antigen was detected by the monoclonal
mouse-anti-CD34 antibody (QBEnd). The Vidas PLUS system is organized in such a way that it is able to discriminate the CD34-stained endothelium from the surrounding tissue. In 25 images of each placenta, the following vascularization variables were calculated by the computer system: (a) per image: vascular area as a percentage, vascular perimeter, and vascular number; and (b) per vascular element: the area, the perimeter, and the diameter.

Materials

Apamine, 4-aminopyridine, and H2O2 were obtained from Sigma (St. Louis, Mo), charybdotoxin was obtained from Alomone Laboratories (Jerusalem, Israel), glibenclamide was obtained from Hoechst Marion Roussel/Aventis (Bridgewater, NJ). Formalin was obtained from JT Baker (Deventer, The Netherlands), QBEnd was obtained from Biogenex (San Ramon, Calif), and horse antimouse and the ABC Elitekit were obtained from Vector Laboratories (Burlingame, Calif). Before each experiment the blockers were dissolved in Krebs-Henseleit buffer to a solution of 0.8 mmol/L, except for 4-aminopyridine, which was dissolved in water.

Statistical analysis

All data were tested for normality with the Shapiro-Wilk test. Comparison of the clinical characteristics of the diabetic women versus the healthy controls was performed by a Mann-Whitney U test. Data on cotyledon perfusion pressures were analyzed with the Prism 3.0 (Graphpad Software, San Diego, Calif) by fitting individual concentration-response curves for each experiment. Maximal percentage changes were calculated by use of the quotient:

\[
\frac{1}{2}\max - \text{baseline fetoplacental arterial pressure} / \text{baseline fetoplacental arterial pressure} \times 100
\]

The resulting parameters did not show Gaussian distribution and were therefore tested by a Mann-Whitney U test. Differences were considered to be significant at P-values less than .05. Data concerning vascular compliance were compared by paired t test. Vidas PLUS data were tested by an unpaired t test. All statistics were performed in SPSS (SPSS 10.0, SPSS Inc, Chigaco, Ill).

Results

In total, 37 placentas were included in this study (19 controls, 18 diabetes). When possible, we investigated 2 cotyledons of each placenta simultaneously. In those cases, the 2 cotyledons were used for different series. As such, 26 control cotyledons versus 21 diabetes cotyledons were measured. For the separate potassium channel blocker series, all cotyledons originated from different placentas.

Table I summarizes the clinical characteristics of the participants. Women with diabetes had a shorter gestational

| Table I Clinical characteristics of the participants (median and range) |
|------------------------|------------------------|
| Control | Diabetic women |
| Number of women/placentas | 19 | 18 |
| Number of cotyledons tested | 26 | 21 |
| Maternal age (y) | 33.1 (25.0-40.1) | 30.5 (21.0-38.2) |
| Parity (n) | 1 (0-3) | 1 (0-3) |
| Gestation (wks) | 40 (38-42) | 38 (37-39)* |
| Vaginal delivery (n) | 14 | 13 |
| CS + locoregional anesthetic | 4 | 5 |
| CS + general anesthetic (n) | 1 | 0 |
| Birth weight (g) | 3097 (2555-3970) | 3525 (2435-4875) |
| Placental weight (g) | 548 (370-700) | 670 (400-950)* |
| Body mass index (kg/m²) | 23.0 (17.7-26.8) | 23.9 (17.2-45.9) |
| Diastolic BP (mm Hg) | 79 (60-90) | 87 (55-90)* |
| Smokers (n) | 1 | 2 |
| White class | — | B, C, D, F, G |
| HbA1c 1st trimester (%) | — | 6.5 (5.8-8.8) |
| HbA1c 3rd trimester (%) | — | 6.5 (4.8-7.1) |
| Insulin (mE/mL) maternal umbilical | 15.5 (0.2-161.0) | 10.0 (0.0-124.0)* |
| C-peptide (nmol/L) maternal umbilical | 1.02 (0.16-2.35) | 0.12 (0.00-0.48)* |

CS, Cesarean section; BP, blood pressure.

* P < .05 vs healthy controls.

1 Diastolic BP was measured 0-6 days before delivery.

Normal value for HbA1c in our laboratory is 4.2%-6.3%; in patients with diabetes is aimed at a value <7.2%.
age at time of delivery. As expected, maternal venous C-peptide concentration was lower in diabetic patients versus controls. There was no difference in maternal insulin concentration between controls and patients with diabetes. In umbilical venous plasma, the C-peptide concentration was lower in the patients with diabetes compared with the controls, but as expected, higher than the maternal C-peptide concentrations. In umbilical venous plasma, insulin concentration was increased in patients with diabetes compared with the controls.

Overall, baseline fetoplacental arterial pressure was comparable in controls (n = 19) versus patients with diabetes (n = 18) (22 ± 1 mm Hg vs 24 ± 1 mm Hg, mean ± SEM).

\[ \Delta \text{Fetoplacental arterial pressure (mmHg)} \]

\[ \text{Log [glibenclamide (mol/L)]} \]

\[ \text{Log [4-aminopyridine (mol/L)]} \]

**Figure 1** The increase in fetoplacental arterial pressure in response to glibenclamide (left panel) and 4-aminopyridine (right panel) in the placenta in controls (solid line) and in women with diabetes mellitus type 1 (dotted line) (mean ± SEM).

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Group</th>
<th>Baseline</th>
<th>At maximum</th>
<th>Maximum increase</th>
<th>Log EC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glibenclamide</td>
<td>Control (n = 7)</td>
<td>20 ± 1</td>
<td>56 ± 6</td>
<td>37 ± 6</td>
<td>−6.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diabetes (n = 6)</td>
<td>23 ± 3</td>
<td>38 ± 4*</td>
<td>15 ± 6*</td>
<td>−6.4 ± 0.2</td>
</tr>
<tr>
<td>4-Aminopyridine</td>
<td>Control (n = 7)</td>
<td>22 ± 2</td>
<td>43 ± 4</td>
<td>21 ± 4</td>
<td>−5.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diabetes (n = 7)</td>
<td>26 ± 2</td>
<td>48 ± 2</td>
<td>22 ± 2</td>
<td>−5.0 ± 0.2</td>
</tr>
<tr>
<td>Apamin</td>
<td>Control (n = 6)</td>
<td>27 ± 2</td>
<td>37 ± 3</td>
<td>10 ± 2</td>
<td>−7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diabetes (n = 4)</td>
<td>30 ± 3</td>
<td>38 ± 5</td>
<td>9 ± 3</td>
<td>−7.6 ± 0.3</td>
</tr>
<tr>
<td>Charybdotoxin</td>
<td>Control (n = 6)</td>
<td>23 ± 3</td>
<td>26 ± 3</td>
<td>4 ± 1</td>
<td>−8.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diabetes (n = 4)</td>
<td>23 ± 3</td>
<td>24 ± 3</td>
<td>1 ± 1</td>
<td>−10.2 ± 2.1</td>
</tr>
</tbody>
</table>

* \( P < .05 \) compared with control.

**Table II** Mean ± SEM characteristics of the concentration-response curves for the 4 potassium channel blockers in the fetoplacental circulation

\[ \text{Fetoplacental arterial pressure (mm Hg)} \]

\[ \text{Log EC}_{50} \]

\[ \text{Mean ± SEM} \]

\[ \Delta \]

\[ \text{Control (n = 7)} \]

\[ \text{Diabetes (n = 6)} \]

\[ \text{Control (n = 7)} \]

\[ \text{Diabetes (n = 7)} \]

\[ \text{Control (n = 6)} \]

\[ \text{Diabetes (n = 4)} \]

\[ \text{Control (n = 6)} \]

\[ \text{Diabetes (n = 4)} \]

\[ \text{Control (n = 6)} \]

\[ \text{Diabetes (n = 4)} \]

**K\(_{ATP}\) channel blockade by glibenclamide**

Glibenclamide induced a concentration-dependent rise in fetoplacental arterial pressure to a maximum of 56 ± 6 mm Hg in the controls (n = 7) versus 38 ± 4 mm Hg in the patients with diabetes (n = 6) (Table II). As a consequence, the absolute glibenclamide-induced increase in fetoplacental arterial pressure was significantly lower in the patients with diabetes (Figure 1). Figure 2 shows the percentage increments in fetoplacental arterial pressure for all K channel blockers in both groups. This figure also shows the impaired vasoconstrictor response to glibenclamide in patients with diabetes. The Log EC\(_{50}\) for glibenclamide was similar in both groups.

**K\(_{V}\) channel blockade by 4-aminopyridine**

Addition of 4-aminopyridine caused a significant rise in fetoplacental arterial pressure, which was equal in the controls (n = 7) and in the patients with diabetes (n = 7) (\( P < .001 \)) in the controls as well as in the patients with diabetes (Figures 1 and 2). Log EC\(_{50}\) was similar in both groups (Table II).
SK channel blockade by apamine and BK/IK channel blockade by charybdotoxin

Both apamine and charybdotoxin only caused minor increments in fetoplacental arterial blood pressure (Table II, Figure 2), both in the controls (n = 6) and in the patients with diabetes (n = 4).

Comparison of fetoplacental vascular compliance between diabetes and control experiments

In theory, the observed differences between the controls and the patients with diabetes in the response to glibenclamide may relate to diabetes-induced structural changes in the vascular wall. Therefore, fetoplacental vascular compliance was studied.

The diameter at an intraluminal pressure of 0 mm Hg was 90 ± 20 μm in fetoplacental arteries from the controls and 90 ± 3 μm (average ± SEM) in those from the patients with diabetes. The maximum diameter, measured at an intraluminal pressure of 60 mm Hg, averaged 260 ± 35 μm (n = 4) in controls and 270 ± 10 μm (n = 4) in patients with diabetes. The pressure-diameter relationship was similar between the controls and the patients with diabetes. Figure 3 shows the absolute pressure-diameter relationship. The figure for the relative diameter as percentage of the diameter at maximal tested pressure of 60 mm Hg is comparable (not shown).

Figure 2 Maximal percentage increase in fetoplacental arterial pressure induced by the highest dose of the different potassium channel blockers in controls (open bars) and women with diabetes mellitus type 1 (dotted bars) (mean ± SEM). The maximum concentrations given were 0.3 mmol/L for glibenclamide, 1 μmol/L for apamin, 0.1 μmol/L for charybdotoxin, and 0.3 mmol/L for 4-aminopyridin. *P < .05 compared with controls.

Figure 3 Inner diameter of fetoplacental resistance vessels from healthy controls (open bars) and women with diabetes mellitus type 1 (mean ± SEM).

Comparison of placental vascular morphometry between diabetes and control experiments

The impaired vasoconstrictor response to glibenclamide in patients with diabetes may also relate to anatomic differences in the vascular beds between controls and patients with diabetes. Therefore, we performed control experiments on morphometry in placentas from controls (n = 5) and from women with diabetes (n = 8).

The diabetes placentas originated from patients with white class B (n = 2), class C (n = 4), and class D (n = 2). Consequently, none of the women with diabetes had a history of vascular disease. As shown in Table III, all values for the individual vessels were comparable between controls and patients with diabetes.

Comment

The key observation in this study is that the vascular $K_{ATP}$ and $K_V$ channel function significantly contributes to the baseline vascular tone of the ex vivo human fetoplacental circulation. In diabetes, this effect appears to be impaired for the vascular $K_{ATP}$ channel, but not for the vascular $K_V$ channel. Under resting conditions, the $K_{Ca}$ channels hardly contribute to baseline fetoplacental vascular tone, neither in women with diabetes nor in healthy women.

Opening of potassium channels in vascular smooth muscle cells induces vasodilation by hyperpolarizing the cell membrane. By this hyperpolarization, voltage-sensitive calcium channels will close resulting in a fall in calcium influx, a fall in intracellular calcium concentration, and subsequent vasorelaxation. In resting conditions, vascular potassium channels may be open or closed depending on the type of tissue. In our setup, the fetoplacental vascular bed shows a clear
vasoconstrictor response to pharmacologic blockade of the $K_{ATP}$ channel by glibenclamide. From these observations, we conclude that the $K_{ATP}$ channel is opened in the ex vivo perfused fetoplacental vascular bed, and contributes to baseline vascular tone. A similar line of reasoning concerns the $K_V$ channel because we observed a relevant vasoconstrictor response to 4-aminopyridine.

Our observation on an impaired function of the vascular $K_{ATP}$ channel in diabetes mellitus is unique as far as the fetoplacental vascular bed is concerned. However, other investigators have observed similar data in other vascular beds in diabetes. In theory, the attenuated response to glibenclamide in diabetes may be related to dysfunction of the vascular $K_{ATP}$ channel itself, for example, as a result of glycosylation.

The ATP/ADP ratio is a major determinant of the open state probability of $K_{ATP}$ channels. As such, hypoxia or metabolic stress is a well-known trigger for the opening of $K_{ATP}$ channels. From a theoretical point of view, the vasoconstrictor response to glibenclamide may be explained by the fact that the cotyledon is relatively hypoxic in our perfusion model. An argument against this mechanism is that the perfusion fluid is oxygenated intensively resulting in a $P_{O_2}$ in the inflow perfusion fluid between 400 and 550 mm Hg. However, the tissue oxygenation may be different from normal values, because the perfusion fluid does not contain an oxygen carrier, and we were not able to measure the tissue oxygen content during perfusion. Although previous experiments from our laboratory have shown a constant lactate production in the perfused cotyledon model, this does not imply a hypoxic state because the placenta has been shown to use the glycolysis pathway independent of the oxygen status. Recently, the $K_V$ channel has been shown to play a role in the vasoconstrictor response to hypoxia in the human fetoplacental vascular bed. These investigators observed a hypoxia-induced increase in the perfusion pressure from 27 to 33 mm Hg. In our test with the $K_V$ channel blocker 4-aminopyridine, the perfusion pressure rose from 22 to 43 mm Hg. Because the baseline perfusion pressure was low in our model, and the response to 4-aminopyridine was more pronounced than the reported response to hypoxia, we think that hypoxia could not have played an important role in our setup.

During pregnancy, the fetoplacental vascular bed may be exposed to ischemic insults, which can harm fetal development or even result in fetal death. In theory, the effects of these ischemic periods on the vascular bed might be comparable to those in cardiac and brain tissue. In the latter organs, powerful endogenous mechanisms have been described against ischemic injury, for example, hypoxic vasodilation and ischemic preconditioning, both mechanisms that contribute to the optimal match between oxygen use and metabolic demand. The $K_{ATP}$ channel has been shown to play a crucial role in these protective mechanisms. Interestingly, diabetes mellitus has been associated with impaired ischemic preconditioning. Our observation of an impaired vascular $K_{ATP}$ channel function in the fetoplacental vascular bed in patients with diabetes implies that the matching between oxygen supply and demand may be less optimal in these patients. Such a defect may contribute to a poor outcome of ischemic insults in the fetoplacental circulation in patients with diabetes. Along this line of reasoning, the vascular $K_{ATP}$ channel may be an interesting pharmacologic target to improve perinatal morbidity and mortality in women with type 1 diabetes mellitus.

Apart from effects on the vascular smooth muscle cell, potassium channel blockers may affect the endothelium. Studies on the contribution of potassium channels to endothelium-dependent vascular responses in human vessels show that this contribution is small or does not play a role at all. As such, the primary site of action of potassium channel blockers is more likely to be the vascular smooth muscle cell than the endothelial cell.

In theory, the impaired vasoconstrictor response to glibenclamide in patients with diabetes might reflect

<table>
<thead>
<tr>
<th>Table III</th>
<th>Mean (± SEM) of some fetoplacental vascular parameters as calculated by the Vidas PLUS image analysis system in controls and diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls (n = 8)</td>
<td>Diabetes mellitus type 1 (n = 7)</td>
</tr>
<tr>
<td>Per field</td>
<td></td>
</tr>
<tr>
<td>Vascular area (%)*</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Perimeter (μm/mm²)†</td>
<td>0.064 ± 0.003</td>
</tr>
<tr>
<td>Vascular count (N/mm²)‡</td>
<td>960 ± 70</td>
</tr>
<tr>
<td>Per vascular element</td>
<td></td>
</tr>
<tr>
<td>Area (%)</td>
<td>207 ± 23</td>
</tr>
<tr>
<td>Perimeter (μm)</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>8.5 ± 0.4</td>
</tr>
</tbody>
</table>

* CD34 positively stained area/total chorionic area × 100.
† Total perimeter of vascular elements/total chorionic area × 10⁶.
‡ Total number of vascular elements/mm² chorionic area.
a more nonspecific defect in vasoconstrictor capacity. However, this seems not to be the case because the vasoconstrictor response to other stimuli (for example, 4-aminopyridine) is similar in patients with diabetes versus in controls. For the NO-synthase inhibitor L-NAME, we previously observed an even more pronounced vasoconstrictor response in patients with diabetes as compared with controls. As such, our observation on an impaired vasoconstrictor response to glibenclamide in patients with diabetes seems to be specific. This conclusion is supported by the fact that our compliance and morphometric studies did not reveal structural differences of the fetoplacental vessels between patients with diabetes and controls.

In 2001 Langer et al presented a study, which concluded that oral treatment with glibenclamide is a clinically effective alternative to insulin for the treatment of gestational diabetes. Our study shows that vascular KATP channels do have a substantial role in the regulation of baseline fetoplacental vascular tone. Because glibenclamide blocks these KATP channels, this treatment could compromise placental vascular function. Nevertheless, the transfer of glibenclamide across the human placenta seems to be minimal. Oral treatment with glibenclamide may therefore be expected to only minimally affect fetoplacental vascular function, and as such, may be preferred over other sulfonylurea derivatives, which may cross the placenta.

In conclusion, our human ex vivo study in the fetoplacental vascular bed shows that both the vascular KATP channel and the KV channel significantly contribute to the regulation of baseline vascular tone. In type 1 diabetes, the function of the fetoplacental vascular KATP channel appears to be impaired.

References

LETTERS TO THE EDITORS

Ethical response to liability crisis

To the Editors: A major medical liability crisis is unfolding in the United States today. It is definitely happening in many Western countries as well. Indeed, in France since 2002 and the institution of the law on patients' rights, insurance premiums have skyrocketed, and many medical insurers have abandoned the medical-malpractice market, especially in obstetrics and gynecology. We totally agree with Chervenak and McCullough1 when they state that developing a culture of fiduciary professionalism is a response to this crisis. Nevertheless it seems to us that, even if it may influence both macro and micro levels, it is but a part of the answer to this issue.

The authors explain that the concept of fiduciary professionalism has been developed to correct the dominance of self-interest in a highly competitive and market-driven world. According to them, fiduciary professionalism was the only viable response, because the market could not be changed easily. However, in the present crisis, we believe that patient behavior ( needless to say, less a competitive market) has a great influence on physician attitudes.

Since the second half of the 20th century, medical practice has been moving towards more and more autonomy for the patients.2 Consequently, an ethical response to our current malpractice issue could be to respect patient autonomy and to inform the public about this problem, as we should do for any medical problem.

First, we could explain merely the problems, such as skyrocketing insurance premiums, threats concerning access to care in some specialties (eg, obstetrics/gynecology),3 and inefficiency of the present system to compensate negligence.3 Second, we should propose and develop solutions, such as a no-fault compensation system, capping damages, and increasing the price of care. Last but not least, for each problem, we would have to explain to the public the benefit/risk ratio. The actual difficulty would be to be really neutral and not to try and influence the patients in the solutions we think better fit the situations. Then at the end, the public would make an informed choice that would probably be specific to each country, depending on cultural differences.

Once again, the actual key point is to be strictly neutral and not to try to influence the patient. We have to be very careful, because lawyers challenge the claim that there is a real malpractice crisis,4 and we must not forget that many legislators are lawyers.

Sébastien Tassy, MD*
Guillaume Gorincour, MD
Espace Ethique Méditerranéen Hôpital de la Timone
13005 Marseille, France
*E-mail: drsebastientassy@yahoo.fr

References

To the Editors: We thank Drs Tassy and Gorincour for their comments on our ethical analysis of and arguments concerning how physicians should respond to the professional liability crisis and for their strong endorsement of our views. It is important to recognize, as they point out, that ethical challenges of the professional liability crisis transcend national boundaries.

Drs Tassy and Gorincour’s proposal to invoke respect for patient autonomy and then inform patients about the nature and scope of the professional liability crisis would help to lead to a more well-informed and reasoned public policy debate in democratic societies.

We would add 2 important caveats about such an effort. First, this public education program should not take place in doctors’ offices or in any health care organizations. We take this view because undertaking the education of patients in the clinical setting may be perceived by some patients as creating political conditions for treatment, namely, their agreement with the information presented and the proposed public policy solutions, such as no-fault compensation. Reasonable people, including physicians, can and do disagree about the public policy solutions; even the appearance of a requirement of agreement to controversial public policy proposals should be prevented in the clinical setting. Fiduciary responsibility requires strong and steady focus on the well-being of patients and the excellence in their medical care. The main point of our article was that, even if strong feelings about the professional liability crisis are warranted, these are matters of individual and professional self-interest and therefore have no place in the clinical setting. This information should be presented through the public media and other mechanisms that are completely separated from the clinical setting.

Second, reasonable people, including physicians, can and do disagree about what information is pertinent to provide to patients. Consider 3 examples that Drs Tassy and Gorincour do not mention. (1) Insurance company investment experience can have an adverse impact on premiums, which is a factor that has nothing to do with the quality of patient care. (2) An interesting feature of professional liability insurance in the United States is that the individual premiums of physicians who have had judgments against them, especially those physicians who have had multiple well-founded judgments against them, do not increase; the increase is spread among all physicians. Unlike automobile insurance, this practice provides economic protection to those few physicians whose repeated, well-documented malpractice results in multiple large awards that, in turn, result in large premium increases. (3) In some states in the United States, there has been a failure of the medical profession to regulate itself adequately. It could be argued reasonably that licensing boards in these states are unduly controlled by the guild interests of the medical profession, thereby allowing physicians to retain licenses when the evidence strongly supports the conclusion that these physicians are incompetent and habitually dangerous to their patients. In other words, the fiduciary responsibility of the medical profession as such, to protect the health and well-being of all patients, requires that the package of information to be presented to the public should be comprehensive and not biased by the self-interests of the medical profession.

Frank A Chervenak, MD*
Laurence B. McCullough, PhD
Department of Obstetrics and Gynecology, The New York Presbyterian Hospital, Weill Medical College of Cornell University, New York, NY 10025
*E-mail: fac2001@med.cornell.edu

Risk factors for recurrent vulvovaginal candidiasis

To the Editors: These comments on the study by Patel et al1 on risk factors for recurrent vulvovaginal candidiasis (VVC) in 65 women who received maintenance antifungal therapy. Among other factors, the use of pantiliners was associated positively in this study with a symptomatic VVC episode, both in the week of and the week before an episode.

This association does not imply causation. Pantiliner use in the same week as an episode may reflect a general wearing practice of such product to absorb the accompanying discharge. Because protection from discharge is a principal reason that women wear pantiliners, usage may increase during any urogenital infection that leads to abnormal discharge, regardless of its cause.
Pantiliner wear may account for an association in the week before a VVC episode. Pantiliners are often used for hygienic protection in anticipation of the onset of menses, and patients frequently report an exacerbation of VVC symptoms just before menstruation. This temporal coincidence could give rise to a spurious association that is unrelated to the risk of recurrence. The use of pantiliners to absorb postcoital discharge also could have contributed to the temporal link, because intercourse frequency in the 4 weeks preceding infection is a risk factor for VVC.

Systematic, examiner-blind clinical investigations in the general population have failed to show a connection between pantiliner use and an increased risk of vulvovaginal infection. A 6-month, prospective trial in 204 women that compared daily pantiliner use to no use found no increase in the prevalence or semiquantitative cell densities of vaginal or vulvar Candida species and no evidence for symptomatic infection, as assessed by visual signs, symptoms, and culture results.

An important limitation of the study by Patel et al was the need to rely largely on health care provider diagnoses (one fifth of which was based on solely on telephone consultation) without laboratory confirmation. Such diagnoses are unreliable because symptoms are not pathognomonic. In family practice, it is estimated that one-half of routine diagnoses without the benefit of microscopy or culture are inaccurate.

The investigators rightly stress that their study does not examine the natural history of candidiasis and that the characteristics of this patient subpopulation may not be broadly relevant. The potential misdiagnosis of VVC episodes and the relatively small sample size further complicate study interpretation.

Miranda A. Farage, PhD
Angela Stadler, PhD
The Procter and Gamble Co
Cincinnati, OH 45224

References

Reply

To the Editors: As noted by Drs Farage and Stadler, pantiliner use by women with vulvovaginal candidiasis (VVC) may be a result, rather than a cause, of vaginal symptoms. In our study of women with recurrent VVC who received maintenance antifungal therapy, we did observe an association between VVC and pantiliner use in the week of a VVC episode (relative risk, 3.3; 95% CI, 1.7-6.2); however, we also observed a significant association in the week before a symptomatic episode (relative risk, 2.3; 95% CI, 1.2-4.3), which suggests a potentially causal association in this highly susceptible group. By contrast with studies among women with sporadic VVC, sexual activity was not a confounder in our study population; we observed no significant associations between sexual practices (vaginal, active oral, or receptive oral intercourse) and symptomatic VVC episodes.

Fundamental differences in study populations and outcome assessment are likely reasons that our findings differed from those of Farage et al. First, although our study focused on women with recurrent VVC, Farage et al studied 224 healthy female patients and excluded women with a history of chronic vulvovaginal disorder. Second, VVC incidence in our study was determined by participant self-report of a diagnosis that was made either in person or by telephone by clinicians at vaginitis specialty clinics. Farage et al assessed frequency and density of Candida albicans or other yeasts at the labia, vagina, and pantiliner itself at baseline and 6 months and found no significant changes. Although Farage and Stadler state in their letter that they found no evidence of symptomatic infection as assessed by visual signs and symptoms, these results were not included in their published manuscript.
Twenty percent to 25% of healthy, asymptomatic women have positive vaginal cultures for *C. albicans.* The transformation from asymptomatic colonization to symptomatic VVC may be enhanced by factors that promote or facilitate germination. Local hypersensitivity or allergic reactions that are triggered by feminine hygiene practices, including pantiliner use, may predispose some women to colonization with *Candida* organisms or symptomatic infections. We agree that our study was not definitive and had limitations, which we freely acknowledge. For example, future studies should examine whether menstrual cycle stage confounds the association between pantiliner use and VVC.

Recurrent VVC causes considerable morbidity, and risk factors for repeated episodes are poorly understood. Suggesting that women with recurrent VVC consider a trial of not using pantiliners will do no harm and might potentially be beneficial.

Divya A. Patel, PhD
Betsy Foxman, PhD*
Paul Nyirjesy, MD
Department of Epidemiology
University of Michigan School of Public Health
Ann Arbor, MI 48109-20291
*E-mail: bfoxman@umich.edu

References

A comparative review of the risks and benefits of hormone replacement therapy regimens

To the Editors: The review of Michelle P. Warren, MD, which is based on a detailed list of literature, offers a wealth of information on the risks and benefits of hormone replacement therapy. However, the substance of the paper is somewhat limited, for the crucial question is not which therapy regimen may be associated with a greater or smaller number of risk, but whether hormone replacement therapy is administered only in those cases in which a replacement is necessary.

The Women’s Health Initiative study started from the premise that all women with amenorrhea in menopause also have an estrogen deficiency, while overlooking the large potential of extragenital steroid synthesis, which must be regarded as sufficient in patients without menopausal problems, as in the Women’s Health Initiative study. Only by listing papers according to this criterion to examine the risks and benefits would it be possible to assess the true value of hormone replacement therapy. The interactions between the different ovarian steroids are so complex that the hitherto existing studies that are based on high simplicity do not deal with this complexity. Encouraging another review with such a focus of investigation may not be a bad idea.

J. C. Huber, MD, PhD*
C. Schimitzek, MD
Department of Obstetrics and Gynecology
Division of Gynecologic Endocrinology and Reproductive Medicine
General Hospital of Vienna
Währinger Gürtel 18-20
A-1090 Vienna, Austria
*E-mail: johannes.huber@meduniwien.ac.at

References
Reply

To the Editors: Professor Huber and Dr Schimitzek are right when they observe that hormone replacement therapy should be offered only when necessary. However, we treat women on the basis of their symptoms not on the basis of their hormone levels. Hormone therapy is approved for vasomotor symptoms and genitourinary atrophy, and women appear to have different hormonal baselines for the development of these problems. There are, of course, great individual differences that may account for the differences that we see in this population in terms of risk/benefit. Extra glandular synthesis is an important source of hormones, in particular estrogens, and may protect patients from such chronic disease as osteoporosis, which is the only chronic disease for which hormone therapy is approved for prevention. In these cases, it is important to evaluate the patient condition carefully. At the present time, we have no guidelines for measuring extra glandular synthesis as a guideline for therapy. It would be indeed helpful if we did, particularly if it related to risk benefit.

Michelle P. Warren, MD
Department of Obstetrics and Gynecology
Columbia University
New York, NY 10032
E-mail: mpw1@columbia.edu

Research of surgical outcome of incontinence surgery in women

To the Editors: The article by Ward and Hilton1 has to be acknowledged as a milestone in the research of surgical outcome of incontinence surgery in women. It is by far the largest randomized controlled trial that, 2 years after the surgery, compares subjective and objective outcome after abdominal colposuspension and tension-free vaginal tape, which is a newly developed procedure.2 Although meticulously designed, its ability to enlarge our understanding about objective and subjective success rates after both of these operations is flawed for 2 reasons: a lack of study power (which was uncovered by an editorial about the authors’ 6 months results3) and more importantly an outcome variation that was based on the multicentricity of this study. Unfortunately, the tremendous variations in outcome between the participating centers were unmasked by Hilton4 only in a commentary to the British Journal of Obstetrics and Gynecology in 2002. Objective cure rates varied between 0 and 92% for colposuspensions and 0 and 90% for tension-free vaginal tape. Taking these inaccuracies into consideration, it is obvious that this study will not give further insight into the best possible cure rates of tension-free vaginal tape and colposuspension, respectively, no matter which statistical analysis is applied to deal with the missing data.

Apart from that, however, there are important lessons to be learned. To my knowledge this is the first study that shows that a variation of success rates for incontinence surgery exists, even among centers that are incorporated into a randomized controlled trial. Because better results seem to be correlated with higher recruiting rates per center,4 it is obvious that these findings may have repercussions as far as preoperative information and informed consent for patients is related.

What this study also adds is a further example that randomized controlled trials in surgery although highly necessary are very difficult to performed successfully. Based on this, prospective nonrandomized designs that minimize biases should be considered sympathetically by journals and founding bodies.5 This should also be kept in mind by the growing number of clinicians who tend to believe only in outcome data that are derived from randomized controlled trials.

B. Schuessler, MD
Department of Obstetrics and Gynecology
3rd Floor Leazeswing
Kantonsspital Luzern CH-6000
Luzern 16, Switzerland
E-mail: schuessler@tic.ch

References
Reply

To the Editors: We are grateful to Professor Schuessler for his kind comments about our recent publication. We recognize that, although this is one of the largest trials of surgery for stress incontinence, the statistical power is limited because of a failure to recruit up to our calculated sample size. Although this was raised in an editorial in the British Medical Journal, it was certainly not “uncovered,” as Prof Schuessler suggests; it had already been emphasized in our discussion, in both the 6-month publication and subsequent correspondence and the current publication. It does remain, of course, one of the few surgical trials in this area with any statistical power to support its conclusions.

The variation in cure rates for the different centers that participated in the trial was discussed in a commentary that illustrated the potential pitfalls of trials of surgery for stress incontinence. Although this was an interesting observation in this context, the trial was not designed to assess differences in individual centers’ performances, and as such, this analysis was not performed when the trial was reported; conclusions from such subgroup analyses have no statistical power whatever. The design of the trial was intentionally pragmatic to reflect the outcome of surgery across a range of surgeons, whether they be urologists, urogynecologists, or gynecologists. It was never intended to gain insight into the “best possible” cure rates for these procedures but to optimize the extent to which the results could be generalized outside of the trial setting.

Karen Ward, MRCOG
Paul Hilton, MD, FRCOG*
Urogynaecology Unit, 3rd Floor, Leazes Wing
Directorate of Women’s Services, Royal Victoria Infirmary
Newcastle-upon-Tyne, NE1 4LP UK
*E-mail: paul.hilton@ncl.ac.uk

References


Effects of gastroschisis on gastric dilation

To the Editors: We read with interest the article of Aina-Mumuney et al, in which the authors describe increased morbidity and mortality among infants with gastroschisis who have a prenatally dilated stomach. These infants had a significantly prolonged time to full oral feedings, and a longer mean overall length of hospital stay. Despite the clinical importance of gastric dilation, the authors did not discuss the potential pathophysiologic mechanism for the dilation or potential obstetric preventative approaches. Of the 13 infants with gastric dilation, 8 demonstrated no evidence of gastrointestinal (GI) obstruction. Furthermore, among the 21 infants without a dilated stomach, there were 5 cases of GI obstruction. Thus, it appears that GI obstruction does not
explain the development of fetal gastric dilatation in gastroschisis.

Our laboratory has recently used a rabbit model of gastroschisis created by a fetal abdominal incision at day 24 of gestation (term 31 days). At delivery, none of the pups demonstrated evidence of GI obstruction. Although the fetal stomach remained inside the abdominal cavity throughout gestation, gastroschisis pups were noted to have reduced in vitro gastric contractility responses to cholinergic stimulation. Thus, gastric dilatation in humans, and reduced gastric contractility in rabbit fetuses with gastroschisis occur in the absence of gastric evisceration from the abdominal cavity. Gastric effects may result from direct inflammatory damage to the GI muscle caused by exposure to intraperitoneal amniotic fluid, or resulting from intestinal injury and altered production of ileal gastric motility factors (eg, ghrelin, gastric inhibitory polypeptide). Alternatively, injury to GI neural pathways may temporarily or permanently alter GI motility.

These findings are consistent with an inflammatory response to amniotic fluid, either localized to the ileum or throughout the GI tract. Further studies on the mechanism of fetal gastric dilatation and GI dysmotility, the association with maturational changes in amniotic fluid composition, and the potential therapeutic use of amniotic fluid exchange are essential to develop strategies for obstetric management so as to prevent GI dysmotility and reduce neonatal morbidity.

References

Reply

To the Editors: We applaud these authors’ experiments. The role that GI contractility plays in dilated fetal bowel in gastroschisis is, as yet, uncertain.

Although it is possible that decreased GI motility contributes to GI dilatation in fetal gastroschisis, it is also possible that volvulus or partial obstruction could be responsible. We would favor the latter theory as more likely.

We saw an increase in volvulus (resulting in proximal GI dilation) in fetuses with both gastric dilatation and obstruction (5/5). We believe, moreover, that obstruction occurs more frequently in human gastroschisis than diagnosed postnatally; partial obstruction may occur either at the site of the fascial defect or from malrotation, and may be more difficult to diagnose prenatally. Prenatal volvulus has been a purported etiology of intestinal atresia, and in human gastroschisis there is a know incidence (23%) of intestinal atresias.

We also speculate that there was an absence of gastric dilatation despite diagnosing a form of obstruction in 5 of 21 fetuses (Table II) because of the timing of the event that led to obstruction. In 4 of the 5 cases, atresias were seen that appeared to have resulted from events early in gestation. We postulate that the single case of volvulus may have occurred at birth or postnatally; thus, the event occurred after prenatal evaluation.

The authors’ model differs from human gastroschisis in that the fascial defect was created late in gestation and, as most animal models, after normal bowel rotation has already occurred. In contrast, in human gastroschisis, the abdominal wall defect occurs during the embryonic period, and precedes the normal rotation of the GI tract. Thus, malrotation is virtually always seen in fetuses with gastroschisis, and malrotation is associated with a higher incidence of volvulus.
One opinion: An IRA for pregnant women

Photographs are stronger than words. When what is being conveyed is new and complex, one picture can replace 1000 words to educate the reader instantly. However, if the desired target is missed, a powerful image can create confusion that requires years to undo. AJOG has chosen an ultrasound image of a nuchal translucency measurement for the cover of this volume. Our choice reflects recent studies that show that nuchal translucency measurement can be combined with ultrasound evaluation of the embryo-fetus and maternal serum assays between 11 and 14 weeks to produce a personal estimate of the risk of Down syndrome, similar to current practice is the second trimester.1

The Journal is not alone in its enthusiasm for providing information about nuchal translucency: in the United States, the Society for Maternal-Fetal Medicine, the American College of Obstetricians and Gynecologists, the March of Dimes, and the National Institute of Child Health and Human Development (NICHD) have been working together to create a common vision of how to take the best “picture” of nuchal translucency in order to introduce it to American women and their health care providers. Representatives of these organizations recently met with international experts to discuss first and second trimester testing in two days of meetings under NICHD’s leadership; a summary of the proceedings will be published later this year. As one who was privileged to attend, I want to offer my own view of the picture I hope will emerge from these efforts, something I’ll call “an IRA for pregnant women”. IRA in this instance does not refer to individual retirement account but instead to Individualized Risk Assessment. Instead of relying on maternal age, family history, and/or a second trimester serum screen that without proper counseling can become a nightmare, pregnant women can soon be offered their own personal individual risk assessment, particularly for trisomy 21, in either the first or second trimester. Although there are many scientifically valid ways to combine maternal age, personal history, blood testing and ultrasound to produce a risk estimate, I believe the best “picture” to present to the public and practitioners will be something like this:

“If you wish, your age plus blood tests plus ultrasound in either the first or second trimester can be combined to give you your own “IRA”. This process is similar to forecasting the weather – it’s generally accurate but not perfect, and the more data we have, the more accurate we can be. You may not want to make a decision about whether to have an invasive procedure (chorionic villus sampling or amniocentesis) until you’ve obtained your own IRA based on your age and history, blood tests, and ultrasound.”

This is a simple and accurate message for every pregnant woman. It avoids the confusion that will occur if we present detailed trees instead of this overview of the forest, and even better, it avoids the scientifically accurate but permanently tarnished word “screening,” a word that should never be used in communicating prenatal risk assessment results to pregnant women. If we keep it simple (age + blood tests + ultrasound = best individual risk assessment), I think our message will be easily understood.

Jay D. Iams, MD
Associate Editor

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