**EDITORS’ CHOICE**

**Pediatric presence at cesarean section: Justified or not?**

Adrienne Gordon, MBChB, MRCP, FRACP,
Elizabeth Jane Mckechnie, MBBS, MRCP,
Heather Jeffery, MBBS, MRCP, FRACP, MPH, PhD
Sydney, Australia

The need for resuscitation is not significantly greater in infants delivered electively by cesarean section under regional anesthesia compared with those delivered vaginally provided there are no additional risk factors.

**Commentary**

Many hospitals require the presence of a pediatrician for all cesarean sections. This practice impacts the cost of health care, but the practice has largely evolved as dogma with little care taken to break down the need for the pediatrician’s presence based on the reason for the operative delivery. This thoughtful report by Gordon et al finds no increased need for resuscitation in Cesarean sections done under regional anesthesia except when the indication for the operation is for “fetal distress” or malpresentation. These findings would logically lead to the conclusion that there is no more need for a pediatrician in the operating room than in an otherwise uncomplicated spontaneous vaginal delivery.

**High-dose methadone maintenance in pregnancy: Maternal and neonatal outcomes**

John J. McCarthy, MD, Martin H. Leamon, MD, Michael S. Parr, MD,
Barbara Anania, PsyD
Sacramento and Davis, CA

Pregnant women who received a mean of 132 mg/day of methadone had less drug use than a group of pregnant women who received 62 mg/day; there were no differences in treatment for neonatal abstinence.

**Commentary**

The papers by McCarthy et al and Jansson et al in this issue provide contrasting information about the benefits and risks of methadone maintenance in pregnancy. McCarthy et al found that higher dosages of methadone were associated with less illicit drug use in mothers without increasing significant neonatal withdrawal symptoms. Jansson et al caution however that methadone may have neurobehavioral and cardiorespiratory effects on the fetus beyond withdrawal.
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Fetal response to maternal methadone administration 611
Lauren M. Jansson, MD, Janet DiPietro, PhD, Andrea Elko, PA-C
Baltimore, MD

Maternal methadone administration is associated with significant changes in fetal heart rate and movement that are independent of maternal effects.

Commentary
The papers by McCarthy et al and Jansson et al in this issue provide contrasting information about the benefits and risks of methadone maintenance in pregnancy. McCarthy et al found that higher dosages of methadone were associated with less illicit drug use in mothers without increasing significant neonatal withdrawal symptoms. Jansson et al caution however that methadone may have neurobehavioral and cardiorespiratory effects on the fetus beyond withdrawal.

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George A. Macones, MD, MSCE, Series Editor
Philadelphia, PA

Issues in clinical trial design: Stopping a trial early and the large and simple trial 619
Elizabeth A. Thom, PhD, Mark A. Klebanoff, MD, MPH
Rockville and Bethesda, MD

We review the decision process of a Data and Safety Monitoring Committee in recommending early termination of a clinical trial, and discuss the “large and simple clinical trial” design.

CLINICAL OPINION

Research agenda for preterm birth: Recommendations from the March of Dimes 626
Nancy S. Green, MD, Karla Damus, RN, PhD, Joe Leigh Simpson, MD, Jay Iams, MD, E. Albert Reece, MD, PhD, MBA, Calvin J. Hobel, MD, Irwin R. Merkatz, MD, Michael F. Greene, MD, Richard H. Schwartz, MD, and the March of Dimes Scientific Advisory Committee on Prematurity
White Plains, Bronx, and Brooklyn, NY, Houston, TX, Columbus, OH, Little Rock, AR, Los Angeles, CA, and Boston, MA

A national research agenda to address the etiology and prevention of preterm birth is proposed by The Scientific Advisory Committee on Prematurity of the March of Dimes.

REVIEW ARTICLE

Headache as a side effect of combination estrogen-progestin oral contraceptives: A systematic review 636
Elizabeth W. Loder, MD, Dawn C. Buse, PhD, Joan R. Golub, MD
Boston, MA

There is limited evidence to support the common belief that combination oral contraceptives have an important influence on headache in most women.
GENERAL OBSTETRICS AND GYNECOLOGY: GYNECOLOGY

Colposcopic and histopathologic evaluation of women participating in population-based screening for human papillomavirus deoxyribonucleic acid persistence

Kristina Elfgren, MD, PhD, Eva Rylander, MD, PhD, Thomas Rådberg, MD, PhD, Björn Strand, MD, Anders Strand, MD, PhD, Kirsti Paajanen, MD, Inga Sjöberg, MD, PhD, Walter Ryd, MD, PhD, Ilvars Silins, MD, PhD, Joakim Dillner, MD, PhD, for the Swedescreen Study Group

Stockholm, Gothenburg, Uppsala, Malmö, and Umeå, Sweden

Among women with a normal Papanicolaou smear participating in population-based cervical screening, human papillomavirus deoxyribonucleic acid persistence had a positive predictive value for detection of cervical intraepithelial neoplasia 2 or 3 of 29%.

The effect of bright light therapy on depression associated with premenstrual dysphoric disorder

Catherine Krasnik, PhD, Victor M. Montori, MD, Gordon H. Guyatt, MD, Diane Heels-Ansdell, MSc, Jason W. Busse, DC, for the Medically Unexplained Syndromes Study Group

Hamilton, Ontario, Canada, and Rochester, MN

This meta-analysis suggests that the role of bright light therapy in the management of premenstrual dysphoric disorder remains uncertain.

Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer

Elizabeth M. Swisher, MD, Melissa Wollan, BS, Sarita M. Mahtani, BS, Julia B. Willner, MD, Rochelle Garcia, MD, Barbara A. Goff, MD, Mary-Claire King, PhD

Seattle, WA

Tumor-specific p53 DNA sequences may be identified in free DNA from blood and peritoneal fluid of women with ovarian cancer.

Melanoma, thyroid, cervical, and colon cancer risk after use of fertility drugs

Michelle D. Althuis, PhD, Bert Scoccia, MD, Emmet J. Lamb, MD, Kamran S. Moghissi, MD, Carolyn L. Westhoff, MD, Jerome E. Mabie, BS, Louise A. Brinton, PhD

Bethesda and Rockville, MD, Chicago, IL, Stanford, CA, Detroit, MI, and New York, NY

Fertility drugs do not increase risk of melanoma or thyroid, cervical, or colon cancer.

First glimpse of the functional benefits of clitoral hood piercings

Vaughn S. Millner, PhD, Bernard H. Eichold II, MD, DR PH, Thomasina H. Sharpe, MD, Sherwood C. Lynn Jr, MD

Mobile, AL

This exploratory, descriptive study provides the first empirical glimpse of the relationship between female genital piercing and sexual functioning.

Arcus tendineus fascia pelvis: A further understanding

Todd S. Albright, DO, Alan P. Gehrich, MD, Gary D. Davis, MD, Farzaneh L. Sabi, MD, Jerome L. Buller, MD

Bethesda, MD

An average length for the arcus tendineus fascia pelvis is obtained, and associations to fascial attachments, cadaver height, and pelvis type are explored.
Characterization of vaginal microflora of healthy, nonpregnant women by chaperonin-60 sequence-based methods

Janet E. Hill, PhD, Swee Han Goh, PhD, Deborah M. Money, MD, FRCSC, Melissa Doyle, Andra Li, BSc, William L. Crosby, PhD, Matthew Links, BSc, Amy Leung, Debbie Chan, Sean M. Hemmingsen, PhD
Saskatoon, Saskatchewan, and Vancouver, British Columbia, Canada

The application of cpn60 sequence-based methods to profiling vaginal microflora led to the identification of significant diversity within traditionally identified taxa and novel vaginal organisms.

A national probability survey of American Medical Association gynecologists and primary care physicians concerning menopause

Betsy Singh, PhD, Xiao-Dong Liu, PhD, Claudia Der-Martirosian, PhD, Mary Hardy, MD, Vijay Singh, BS, Neil Shepard, MA, Sonal Gandhi, MD, Raheleh Khorsan, MA
Whittier and Los Angeles, CA, and Bowling Green, OH

Physicians' views on various treatment options available to menopausal women and their interaction with menopausal women during their office visits are presented.

GENERAL OBSTETRICS AND GYNECOLOGY: OBSTETRICS

A randomized trial of amnioreduction versus septostomy in the treatment of twin-twin transfusion syndrome

Kenneth J. Moise Jr, MD, Karen Dorman, MS, Georgine Lamvu, MD, MPH, George R. Saade, MD, Nicholas M. Fisk, MD, PhD, Jan E. Dickinson, MD, R.D. Wilson, MD, Alain Gagnon, MD, Michael A. Belfort, MD, Richard O. O'Shaughnessy, MD, Usha Chitkara, MD, Sonia S. Hassan, MD, Anthony Johnson, DO, Anthony Sciscione, DO, Daniel Skupski, MD
Chapel Hill, NC, Galveston, TX, London, United Kingdom, Mumbai, India, Vancouver, British Columbia, Canada, Salt Lake City, UT, Columbus, OH, Stanford, CA, Ann Arbor, MI, Newark, DE, and New York, NY

Amnioreduction and septostomy are associated with similar rates of survival of at least 1 twin fetus in cases of severe twin-to-twin transfusion syndrome.

Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only

Jacqueline Lau, BSc, Fergall Magee, MD, FCRCP(C), Zhenguo Qiu, PhD, Jill Houbé, MD, FRCPC, MPhil, Peter Von Dadelszen, MBChB, MRCOG, FRCSC, DPhil, Shoo K. Lee, MBBS, FRCPC, PhD
Vancouver, British Columbia, Canada

Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than when only a maternal inflammatory response is present.

Early discharge from obstetrics-pediatrics at the Hospital de Valme, with domiciliary follow-up

José Antonio Sainz Bueno, María Ruiz Romano, Rogelio Garrido Teruel, Antonio Gutiérrez Benjumea, Ana Fernández Palacín, Carmen Almeida González, Manuel Caballero Manzano
Sevilla, Spain

Early discharge is not implicated in increased maternal or neonatal diseases.
Impact of maternal-fetal surgery for myelomeningocele on the progression of ventriculomegaly in utero
Amy Adelberg, MD, Angela Blotzer, BS, Gary Koch, PhD, Rachael Moise, Nancy Chescheir, MD, Kenneth J. Moise Jr, MD, Honor Wolfe, MD
Chapel Hill, NC
In utero repair of myelomeningocele does not affect the rate of progression of fetal ventriculomegaly when compared with control fetuses with myelomeningocele undergoing postnatal repair.

Isolated fetal pyelectasis and chromosomal abnormalities
Claudio Coco, MD, Philippe Jeanty, MD, PhD
Nashville, TN, and Rome, Italy
In a review of 12,672 unselected patients, karyotyping of fetuses with isolated pyelectasis of greater than 4 mm is not justified.

Endometrial microbial colonization and plasma cell endometritis after spontaneous or indicated preterm versus term delivery
William W. Andrews, PhD, MD, Robert L. Goldenberg, MD, John C. Hauth, MD, Suzanne P. Cliver, BA, Michael Conner, MD, Alice R. Goepfert, MD
Birmingham, AL
Microbial colonization of the endometrium and plasma cell endometritis are similar 3 months after spontaneous or indicated preterm or term births.

Variation in microbiologic profiles among pregnant women with bacterial vaginosis
Leonardo Pereira, MD, Jennifer Culhane, PhD, MPH, Kelly McCollum, MPH, Kathy Agnew, BS, Paul Nyirjesy, MD
Portland, OR, Philadelphia, PA, and Seattle, WA
The presence of Mobiluncus is associated with black race, clue cells on wet mount, and a positive amine odor after KOH preparation in pregnant women with bacterial vaginosis.

Use of DNA hybridization to detect vaginal pathogens associated with bacterial vaginosis among asymptomatic pregnant women
Kim A. Boggess, MD, Thomas N. Trevett, MD, Phoebus N. Madianos, DDS, PhD, Lorna Rabe, BS, Sharon L. Hillier, PhD, James Beck, PhD, Steven Offenbacher, DDS, PhD
Chapel Hill, NC, and Pittsburgh, PA
Microbial DNA hybridization may be a useful method to study bacterial vaginosis during pregnancy.

Is zygosity or chorionicity the main determinant of fetal outcome in twin pregnancies?
Stephen G. M. Carroll, MD, Linda Tyfield, PhD, Louise Reeve, PhD, Helen Porter, MD, Peter Soothill, MD, Phillipa Mm Kyle, MD
Bristol, United Kingdom
Fetal outcome in twin pregnancies is related to chorionicity rather than zygosity.

Sonographic myometrial thickness predicts the latency interval of women with preterm premature rupture of the membranes and oligohydramnios
Catalin S. Buhimschi, MD, Irina A. Buhimschi, MD, Errol R. Norwitz, MD, PhD, Anna K. Sfakianaki, MD, Benjamin Hamar, MD, Joshua A. Copel, MD, George R. Saade, MD, Carl P. Weiner, MD
New Haven, CT, Galveston, TX, and Baltimore, MD
Myometrial thickness is directly correlated with latency interval in non-laboring women with PPROM.
Use of over-the-counter medications during pregnancy
Martha M. Werler, ScD, Allen A. Mitchell, MD, Sonia Hernandez-Diaz, MD, DrPH, Margaret A. Honein, PhD, and the National Birth Defects Prevention Study
Boston, MA, and Atlanta, GA

Findings show that over-the-counter medications are used by most pregnant women.

Comparison of the TDx-FLM II and lecithin to sphingomyelin ratio assays in predicting fetal lung maturity
Tamina Winn-McMillan, MD, Brad S. Karon, MD, PhD
Las Vegas, NV

We retrospectively analyzed results of 218 consecutive paired TDx-FLM II and L/S ratio tests, and compared the utility of the two tests in predicting fetal lung maturity.

Increasing maternal parity predicts neonatal adiposity:
Pune Maternal Nutrition Study
Niranjan P. Joshi, MD, DNB, Smita R. Kulkarni, MSc, Chittaranjan S. Yajnik, MD, FRCP, Charudatta V. Joglekar, MS, Shobha Rao, PhD, Kurus J. Coyaji, MD, Himangi G. Lubrec, MSc, Sonali S. Rege, MSc, Caroline H. D. Fall, DM, FRCP, FRCPCH
Pune, Maharashtra, India, and Southampton, United Kingdom

Increasing parity predicts higher adiposity in the offspring of thinner rural Indian mothers.

Misoprostol induces cervical nitric oxide release in pregnant, but not in nonpregnant, women
Mervi Väisänen-Tommiska, MD, Tomi S. Mikkola, MD, PhD, Olavi Ylikorkala, MD, PhD
Helsinki, Finland

Vaginal misoprostol stimulates cervical NO release in early and late pregnancy, but not in nonpregnant women.

N-acetyl-transferase phenotype and risk for preeclampsia
Petra L. M. Zusterzeel, René H. M. te Morsche, Maarten T. M. Raijmakers, Eva Maria Roes, Wilbert H. M. Peters, Régine P. M. Steegers-Theunissen, Eric A. P. Steegers
Nijmegen and Rotterdam, The Netherlands

Women with the fast acetylating N-acetyltransferase phenotype, which may result in altered N-acetyltransferase detoxification capacity, may have enhanced susceptibility to preeclampsia.

Profound hypotension and associated electrocardiographic changes during prolonged cord occlusion in the near term fetal sheep
Bert Wilbrens, MD, Jenny A. Westgate, MD, PhD, Laura Bennet, PhD, Vincent Roelfsema, Harmen H. De Haan, MD, PhD, Christian J. Hunter, MD, PhD, Alistair J. Gunn, MBChB, PhD
Auckland, New Zealand, Groningen and Zwolle, The Netherlands, and Loma Linda, CA

During profound asphyxia fetal T/QRS ratio and ST segment height reach their greatest elevation shortly after the start of asphyxia and reduce in amplitude as hypotension develops.

Hyperemesis gravidarium: Epidemiologic findings from a large cohort
Jennifer L. Bailit, MD, MPH
Cleveland, OH

Hyperemesis of pregnancy complicates 473 of 100,000 live births. Fetal and neonatal death rates are similar to the general population.
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Intrauterine therapy of goitrous hypothyroidism in a boy with a new compound heterozygous mutation (Y453D and C800R) in the thyroid peroxidase gene. A long-term follow-up

Kirsten Börgel, MD, Joachim Pohlenz, MD, Wolfgang Holzgreve, MD, Jurgen H. Bramswig, MD
Münster and Mainz, Germany, and Basel, Switzerland

Spontaneous delivery was possible after intrauterine L-thyroxine therapy improved thyroid size and thyroid function in a fetus with goitrous hypothyroidism caused by TPO gene defect.

CLASSIC PAGES

Syndrome of hemolysis, elevated liver enzymes, and low platelet count: A severe consequence of hypertension in pregnancy

Louis Weinstein, MD
Philadelphia, PA

An excerpt from the American Journal of Obstetrics and Gynecology 1982;142:159-67, with a commentary by Lawrence D. Longo, MD, followed by It has been a great ride: The history of HELLP syndrome, by Louis Weinstein, MD.

CASE REPORTS

Recombinant human activated protein C treatment of septic shock syndrome in a patient at 18th week of gestation: A case report

Lásló Medve, MD, István Kis Csáráti, MD, Zsolt Molnár, MD, PhD, Ádám László, MD, PhD
Salgótarján, Pécs, and Budapest, Hungary

Septic shock syndrome in a pregnant patient at the 18th week of gestation was successfully treated with recombinant human-activated protein C.

Clinicopathologic features of six cases of primary cervical lymphoma

John K. Chan, MD, Vera Loizzi, MD, Alessandra Magistris, MD, Mark I. Hunter, MD, Joanne Rutgers, MD, Philip J. DiSaia, MD, Michael L. Berman, MD
Stanford, Orange, and Long Beach, CA

Cervical lymphoma is a rare disease. Most patients present with localized stage IE disease and will usually respond to various combinations of surgery, chemotherapy, and radiotherapy.

Port site ischemic necrosis: An unforeseen complication of laparoscopic surgery

R. Oliver, MBBS, MRCOG, A. Coker, MBBS, MRCOG, Cert Lap Sur (RCOG), Cert BSCCP
Romford, United Kingdom

This case report of necrosis of tissue at the site of a laparoscopy port insertion highlights an unforeseen and previously unreported complication of laparoscopic surgery.

Spontaneous epidural hematoma of the spine in pregnancy

Ashley S. Case, MD, Patrick S. Ramsey, MD
Birmingham, AL

We report a case of spontaneous epidural hematoma of the spine that complicated a term pregnancy.
Incisional hernia on the 5-mm trocar port site and subsequent wall endometriosis on the same site: A case report

Rodolfo Sirito, MD, Andrea Puppo, MD, Maria Grazia Centurioni, MD, Claudio Gustavino, MD
Genova, Italy

One year after the removal of an endometrial cyst and, a hernia developed at the trocar site; 2 years later, a wall endometriosis and another endometrial ovarian cyst developed.

Prenatal diagnosis of amniotic sheets by magnetic resonance imaging

Kiyoshi Kato, MD, Tanri Shiozawa, MD, Takashi Ashida, MD, Nobuya Unno, MD, Ikuo Konishi, MD
Nagano, Japan

Magnetic resonance imaging is useful for the diagnosis of pregnancies complicated by amniotic sheets.

Posterior reversible leukoencephalopathy in a case of postpartum eclampsia

Maryam Pariaei, MRCOG, Iris Derwig, MBBS, Jeannie Yoon, MRCOG, Katrina J. Erskine, MRCOG, Paul R. Jarman, PhD
London, United Kingdom

An atypical presentation of eclampsia.

Twin-to-twin transfusion syndrome at 11 weeks of gestation

Marieke Sueters, MD, Johanna M. Middeldorp, MD, Dick Oepkes, PhD, Enrico Lopriore, MD, Frank P. H. A. Vandenbussche, PhD
Leiden, The Netherlands

Presumed vascular connections on the surface of a monochorionic twin placenta are a possible cause of fetal co-twin death in the first trimester of pregnancy.

Spontaneous closure of the hymen during pregnancy

M. A. Onan, MD, A. B. Turp, MD, C. Taskiran, MD, C. Ozogul, MD, O. Himmetoglu, MD
Besevler, Ankara, Turkey

Case study of spontaneous formation of an imperforate hymen during pregnancy in the absence of previous regional surgeries.

Small bowel obstruction due to adhesive disease observed after uterine fibroid embolization

Jay Goldberg, MD, MS, Kristyne Boyle, MD, Monica Choi, CRNP, PhD, Narhari Panchal, MD
Philadelphia, PA, and Buffalo, NY

Small bowel obstruction caused by adhesive disease should be included in the differential of patients with abdominal pain having a history of uterine fibroid embolization.

LETTERS TO THE EDITORS

Selective fetocide reverses preeclampsia in discordant twins

Francois Audibert, MD, Laurent J. Saloman, MD, René Frydman, MD
Montreal, Quebec, Canada, and Clamart, France

Reply

Kent D. Heyborne, MD, Richard P. Porreco, MD
Englewood, CO
Is circulating extracellular VEGF increased in preeclamptic women?
Simone Ferrero, MD
Genoa, Italy

Cytokines, preeclampsia, and uterine denervation?
M. J. Quinn, MD
Salford, UK

Reply
Gareth C. McKeeman, Joy E. S. Ardill, Carolyn M. Caldwell, Neil McClure
Belfast, Northern Ireland

Accidental fetal lacerations during cesarean delivery: Experience in an Italian level III university hospital
Koji Nishijima, MD, Ken-ichi Shukunami, MD, Fumikazu Kotsuji, MD, PhD
Fukui, Japan

Reply
Salvatore Dessole, MD, Giampiero Capobianco, MD, PhD, Erich Cosmi, MD
Sassari, Italy

CORRECTION
Metabolites of progesterone and the pregnane X receptor: A novel pathway regulating uterine contractibility in pregnancy?
Mitchell et al

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Editors’ Choice

Pediatric presence at cesarean section: Justified or not?

Adrienne Gordon, MBChB, MRCP, FRACP, Elizabeth Jane Mckechnie, MBBS, MRCP, Heather Jeffery, MBBS, MRCP, FRACP, MPH, PhD

Department of Neonatal Medicine, Royal Prince Alfred Hospital, Sydney, Australia

Received for publication November 28, 2004; revised March 23, 2005; accepted June 24, 2005

Objectives: This study was undertaken to determine the incidence and type of resuscitation required for infants delivered by both elective and emergency cesarean section relative to spontaneous vaginal delivery.

Study design: A hospital-based cohort study from 1990 to 2002. Information was extracted from a prospectively collected database on term (≥37 weeks) singleton infants delivered by cesarean section and spontaneous vaginal delivery. Analysis was performed on type of cesarean section, type of anesthetic, fetal presentation, and evidence of fetal distress. Outcomes assessed were resuscitation and Apgar scores.

Results: There were 44,938 eligible deliveries. There was no significant difference in need for resuscitation between infants born by elective cesarean section under regional anesthetic compared with spontaneous vaginal delivery ($\chi^2 = 0.998; df = 1; P = .318$). General anesthesia, fetal distress, and noncephalic presentation increase the need for resuscitation.

Conclusion: An advanced skills practitioner does not need to be present at elective cesarean sections under regional anesthesia provided there are no additional risk factors.

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The rate of cesarean sections is increasing in most developed country hospitals placing extra demands on currently strained workforces. International guidelines on neonatal resuscitation and Pediatric and Obstetric Colleges state that an appropriately trained practitioner should be present at all births by cesarean section and an advanced skills practitioner present for high risk deliveries. Evidence suggests that term infants born after elective cesarean section (CS) under regional anesthetic have no greater need for resuscitation than those born vaginally. A summary of the evidence from the published randomized, cohort and case-control studies is attached as Appendix A. All studies were reviewed independently by 2 reviewers and scored for 4 subscales of a Quality Index for randomized and nonrandomized studies. Despite the evidence, many hospitals still require an advanced skills practitioner (most commonly the pediatrician) to be present at all cesarean deliveries. We undertook this study to assess the need for resuscitation in a tertiary hospital that has this policy to facilitate implementation of evidence-based practice and appropriately allocate staff resources.

Methods

Royal Prince Alfred Hospital (RPAH), Sydney, Australia, is the major obstetric tertiary referral center for Central Sydney Area Health Service (CSAHS). It covers...
an inner city, multicultural population of approximately 500,000 people. Within the CSAHS population, 39.6% of residents are born overseas and 41.3% speak a language other than English at home, compared with 18.7% in NSW as a whole. Of mothers giving birth, 41.4% were born in a non-English speaking country, with Chinese, Arabic, Greek, and Vietnamese the most commonly spoken languages. During the study period, the average number of live births per year ranged from 3,591 to 4,969. The Obstetric Information Service (OIS) computer database was initiated at RPAH in 1990. Information on all deliveries is entered prospectively by the attending practitioner. Information collected includes demographic data, fetal and maternal condition before delivery, type of delivery, and fetal outcomes. Ethics approval was not sought because all data were obtained from this de-identified established database used for audit purposes.

The OIS was analyzed for a 13-year period, from January 1, 1990, to December 31, 2002. Data were extracted on all singleton term deliveries (≥37 weeks) born by vaginal delivery and CS. Multiple, preterm, and instrumental deliveries were excluded because of the mandatory requirement for an advanced skills practitioner. CS was defined as emergency or elective by the presence or absence of labor. Information was collected on type of anesthetic (regional or general), fetal presentation, and the presence of fetal distress. Fetal distress was diagnosed if 1 or more of the following was present: fetal bradycardia, fetal tachycardia, meconium stained liquor, late decelerations, or fetal scalp pH less than 7.2. The main outcome measures assessed were resuscitation and Apgar scores. Resuscitation was classified into 4 categories: bag and mask ventilation, endotracheal tube and positive pressure ventilation, endotracheal tube, and cardiopulmonary resuscitation. The Apgar score, although known to be subjective, was recorded consistently for all deliveries throughout the study period. An Apgar score of less than 6 was used as an indicator of the need for resuscitation and not as a marker for subsequent neonatal outcome.

Statistical analysis was performed with SPSS (Statistical Package for Social Sciences) version 11.1 (SPSS, Inc, Chicago, Ill). Independent proportions were compared with the use of the χ² test.

### Results

There were a total of 44,938 eligible singleton term deliveries during the study period. This comprised 79% of all deliveries at RPAH. There was a trend to increasing CS rates throughout the 13 years with an average rate of 21.2% (range 18.3%-27.8%). There were 35,753 spontaneous vaginal deliveries, 4,968 emergency CS, and 4,487 elective CS. The demographic, presentation, and resuscitation details are shown in Table I. Women delivered by elective CS were older than the emergency CS or vaginal delivery groups. There were no significant differences in mean birth weight or gestational age between the groups.

### Emergency versus elective CS

As expected significantly more infants required resuscitation when delivered by emergency CS when compared with an elective procedure (966 [20.6%] vs 683 [15.2%]). χ² = 44.4, df = 1, P < .0001). The number of infants with Apgar scores less than 6 at 1 minute are consistent

### Table I Total cohort—demographic, presentation, and resuscitation details

<table>
<thead>
<tr>
<th></th>
<th>SVD</th>
<th>Emergency CS</th>
<th>Elective CS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regional</td>
<td>General</td>
<td>Regional</td>
</tr>
<tr>
<td>Total no. of deliveries</td>
<td>35,753</td>
<td>3,694</td>
<td>1,004</td>
</tr>
<tr>
<td>Demographic details*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean maternal age (y)</td>
<td>30.2 (5.4)</td>
<td>31.7 (5.2)</td>
<td>30.9 (5.8)</td>
</tr>
<tr>
<td>Mean birth weight (g)</td>
<td>3,430 (464)</td>
<td>3,420 (522)</td>
<td>3,429 (605)</td>
</tr>
<tr>
<td>Mean gestation (wk)</td>
<td>39.4 (1.2)</td>
<td>39.4 (1.3)</td>
<td>39.3 (1.4)</td>
</tr>
<tr>
<td>Presentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalic</td>
<td>35,661</td>
<td>3,230</td>
<td>839</td>
</tr>
<tr>
<td>Breech</td>
<td>13</td>
<td>392</td>
<td>106</td>
</tr>
<tr>
<td>Other</td>
<td>79</td>
<td>72</td>
<td>59</td>
</tr>
<tr>
<td>Apgar &lt;6 at 1 min</td>
<td>2,636 (7.4%)</td>
<td>528 (14.3%)</td>
<td>380 (37.8%)</td>
</tr>
<tr>
<td>Total requiring resuscitation</td>
<td>2,830 (7.9%)</td>
<td>530 (14.3%)</td>
<td>436 (43.3%)</td>
</tr>
<tr>
<td>Bag and mask ventilation</td>
<td>2,286</td>
<td>375</td>
<td>268</td>
</tr>
<tr>
<td>ETT and IPPR</td>
<td>284</td>
<td>92</td>
<td>123</td>
</tr>
<tr>
<td>ETT</td>
<td>233</td>
<td>55</td>
<td>32</td>
</tr>
<tr>
<td>CPR</td>
<td>27</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

* Mean (SD).

ETT and IPPR, Endotracheal tube insertion plus positive pressure ventilation; ETT, endotracheal tube insertion; CPR, cardiopulmonary resuscitation.
with this finding (908 [19.3%] vs 386 [8.6%] $\chi^2 = 218.1$, $df = 1$, $P < .0001$).

**General versus regional anesthesia**

For both emergency and elective CS significantly more infants require resuscitation when delivered under general anesthetic. For emergency CS, 436 infants (43.3%) required resuscitation after general anesthesia compared with 530 (14.3%) under regional anesthesia ($\chi^2 = 408.7$, $df = 1$, $P < .0001$). For elective CS, 409 infants (33.3%) required resuscitation after general anesthetic compared with 274 (8.4%) under regional anesthesia. The Apgar scores were generally consistent with the need for resuscitation with the exception of elective CS under regional anesthesia. An unexpectedly small number of infants had a low Apgar score compared with the number of infants requiring resuscitation (122 [3.7%] vs 274 [8.4%]). The above results clearly demonstrate an increased need for resuscitation with emergency CS and general anesthetic. After these findings, the remaining results compare elective CS under regional anesthetic with vaginal deliveries.

**Fetal distress versus no fetal distress**

Vaginal deliveries and elective CS were compared with regard to fetal distress. The results are shown in Table II. Fetal distress significantly increased the need for resuscitation in both groups. In infants without fetal distress, those born by elective CS under regional anesthetic were more likely to require resuscitation (242 [7.8%] vs 908 [4.2%] $\chi^2 = 77.0$, $df = 1$, $P < .0001$). However, there were significantly more infants with a low Apgar score in the vaginal delivery group compared with the elective CS group (1012 [4.7%] vs 104 [3.3%] $\chi^2 = 11.7$, $df = 1$, $P = .001$). The need for active resuscitation (intubation and/or cardiac massage) was not significantly different between the elective CS regional anesthesia and vaginal delivery groups in the absence of fetal distress (9 [0.3%] vs 50 [0.2%] $\chi^2 = 0.365$, $df = 1$, $P = .546$).

**Cephalic versus noncephalic presentation**

Vaginal and elective CS deliveries without fetal distress were next examined with regard to fetal presentation. The results are shown in Table III. There was no significant difference in need for resuscitation between vaginal deliveries and elective CS deliveries under regional anesthetic with cephalic presentation in the absence of fetal distress (906 [4.2%] vs 100 [4.5%] $\chi^2 = 0.362$, $df = 1$, $P = .547$). Noncephalic presentation, significantly increased the need for resuscitation in elective CS deliveries (100 [4.5%] vs 142 [16.0%] $\chi^2 = 117.2$, $df = 1$, $P < .0001$).

**Comment**

This study was performed to assess the need for resuscitation at CS with particular regard to type of anesthetic, elective or emergency procedures, presence of fetal distress, and fetal presentation. This was primarily to determine whether the need for an advanced skills practitioner was justified for all CS. Previous studies and international guidelines have indicated that the need for resuscitation in term infants delivered by elective CS under regional anesthesia is not significantly different to those delivered vaginally.1-15 Many of these studies, however, have not assessed all relevant factors contributing to the need for resuscitation or have not had a control group of vaginal deliveries. This represents the largest hospital-based study on this subject and confirms the findings of the only other large population-based cohort study by Parsons et al4 in Tasmania. In addition, we were able to assess the type of resuscitation required, the presence or absence of fetal distress, and whether the CS was an emergency or elective procedure. The results clearly demonstrate the increased need for resuscitation for infants delivered after emergency CS, general anesthesia, and fetal distress. The crude rates for resuscitation between those infants delivered vaginally and those delivered by elective CS under regional anesthetic were not significantly

<table>
<thead>
<tr>
<th>Table II</th>
<th>SVD and elective CS—effect of fetal distress on resuscitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVD</td>
</tr>
<tr>
<td>No fetal distress</td>
<td>Total no.</td>
</tr>
<tr>
<td></td>
<td>No. (%) requiring resuscitation</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td></td>
<td>No. (%) requiring active resuscitation</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>Fetal distress</td>
<td>Total no.</td>
</tr>
<tr>
<td></td>
<td>No. (%) requiring resuscitation</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td></td>
<td>No. (%) requiring active resuscitation</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td></td>
<td>Apgar &lt; 6 at 1 min</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td></td>
<td>Apgar &lt; 6 at 1 min</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
</tbody>
</table>

NS, Not significant ($P > .05$).

* Compared with vaginal deliveries.
different consistent with published data. It is imperative, however, particularly when planning hospital guidelines and offering practice recommendations to compare CS relative to an appropriate control group. This control group consisted of vaginal deliveries with no fetal distress, as these deliveries would not routinely call an advanced skills practitioner. When we compared these groups, we found a significant difference between the need for any type of resuscitation. However, when the Apgar score at 1 minute was assessed as an indication of the need for resuscitation, significantly more infants in the control group had low Apgar scores. Also, when fetal presentation was taken into account, there was no significant difference in need for resuscitation between the 2 groups with respect to cephalic presentation. Furthermore, the need for active resuscitation, i.e., intubation and/or cardiac massage, was not significantly different between the groups. We would therefore suggest that the increased number of infants requiring bag and mask resuscitation at elective CS under regional anesthetic may be primary to the fact that an advanced skills practitioner was present. Another possibility is that the larger number of infants with nonvertex presentation in the elective CS group may have required more bag and mask resuscitation, although the initial Apgar scores were not significantly different between the 2 groups making the former suggestion more likely. Competency at bag and mask resuscitation is a recommended standard requirement for all those practitioners involved in CS. In the absence of fetal distress, the extra need for an advanced skills practitioner competent at intubation and cardiac massage would not have been required in the elective CS group any more often than the low-risk vaginal deliveries.

Importantly, we assessed fetal presentation within the regional elective CS group to assess whether noncephalic presentation affected the need for resuscitation in the absence of fetal distress. Surprisingly, we found a 4-fold increased need for resuscitation in those infants with a noncephalic presentation. This may relate to a technically more difficult delivery if presentation is noncephalic, despite no fetal distress before delivery. This finding has also been shown by Ng et al who demonstrated a similar increased risk to this study (5% vs 19%) if presentation was noncephalic within an elective regional anesthetic CS group but did not analyze the elective CS findings with respect to fetal distress.

There are several factors that may influence the findings of this study. The hospital-based population within a tertiary referral center will be of higher risk than a wider population-based cohort. This population will, however, be similar to that of many tertiary centers with rising CS rates that need to allocate staff and resources appropriately. We did not analyze the significance of ethnicity on need for resuscitation between the 2 groups; however, the previous literature has not indicated that this would make a significant difference within the context of delivery and anesthetic type. An important factor that may influence the interpretation of our findings is the classification of emergency or elective CS as being the presence or absence of labor. Although the absence of labor is a common classification for elective CS, it does not accurately define true planned cesarean deliveries in low-risk patients. It is feasible that some infants delivered “electively” as defined by the absence of labor may be at increased risk of resuscitation secondary to other factors such as maternal illness, antepartum hemorrhage, or abnormal placental Doppler flow studies. We were unable to assess all the above factors via the OIS database; however, the exclusion of such cases from our elective CS group would favor our interpretation of the results.

### Conclusions

The results of this study suggest that an advanced skills practitioner need not be present at uncomplicated elective CS under regional anesthesia provided there are no other risk factors, namely, fetal distress and nonephalic presentation. Conversely, an advanced skills practitioner is required at emergency CS, CS under general anesthesia, and in the presence of fetal distress and nonephalic presentation.

### Acknowledgments

We acknowledge the help of Dr Phillip Beeby, Neonatologist, for data retrieval from the Obstetric Information Systems Database.

<table>
<thead>
<tr>
<th>Presentation</th>
<th>SVD—no fetal distress</th>
<th>Elective CS—no fetal distress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. deliveries</td>
<td>Regional</td>
</tr>
<tr>
<td></td>
<td>No. requiring resuscitation</td>
<td>21,497</td>
</tr>
<tr>
<td>Cephalic</td>
<td>906 (4.2%)</td>
<td>100 (4.5%)</td>
</tr>
<tr>
<td>Breech</td>
<td>9</td>
<td>127 (16%)</td>
</tr>
<tr>
<td>Other</td>
<td>41</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>1 (2.4%)</td>
<td>15 (15%)</td>
</tr>
</tbody>
</table>

Table III: SVD and elective CS—effect of presentation on resuscitation
References


### Appendix A

Review of published literature in descending order of level of evidence by study type* and quality index† rating

<table>
<thead>
<tr>
<th>Author and year</th>
<th>Study type</th>
<th>Country</th>
<th>Study subjects</th>
<th>No. of patients</th>
<th>Quality rating* (x/27)</th>
<th>Outcome assessed</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kolatat 1999</td>
<td>Randomized trial</td>
<td>Thailand</td>
<td>Uncomplicated pregnant women undergoing CS at term randomized to general, epidural or spinal anesthesia.</td>
<td>341</td>
<td>17</td>
<td>Apgar scores</td>
<td>Lower Apgar scores in GA group No difference in NACS between groups.</td>
<td>Anesthetic changed in 39 patients and not documented whether intention-to-treat analysis used.</td>
</tr>
<tr>
<td>Parsons et al 1998</td>
<td>Statewide population-based cohort study</td>
<td>Australia</td>
<td>All singleton deliveries ≥37 wks’ gestation and/or ≥2,500 g in state over 10-y period 1980-1989.</td>
<td>64,739</td>
<td>20</td>
<td>Need for intubation Apgar scores.</td>
<td>Low intubation rate and lower incidence of Apgar &lt;4 in repeat CS under epidural group compared with vaginal delivery.</td>
<td>Large population-based study of prospectively collected data.</td>
</tr>
<tr>
<td>Gordon 2004 (current study)</td>
<td>Hospital-based cohort study</td>
<td>Australia</td>
<td>All singleton deliveries ≥37 wks’ gestation born by CS or vaginal delivery over 13-year period 1990-2002.</td>
<td>44,938</td>
<td>20</td>
<td>Need and type of resuscitation Apgar scores.</td>
<td>Lower incidence of Apgar &lt;6 and no significant difference in need for resuscitation in elective CS under regional group compared with vaginal delivery.</td>
<td>Large hospital-based study of prospectively collected data. Assessment of elective or emergency CS, fetal distress and anesthetic on need for resuscitation compared with vaginal deliveries.</td>
</tr>
<tr>
<td>Annibale et al 1995</td>
<td>Cohort study in 2 hospitals</td>
<td>United States</td>
<td>Low-risk population at term with CS and vaginal deliveries.</td>
<td>11,702</td>
<td>19</td>
<td>Need and type of resuscitation Apgar scores Respiratory support, NICU admission.</td>
<td>Higher incidence of low Apgar scores, bag and mask ventilation and intubation in all CS.</td>
<td>CS not classified by indication, presence of fetal distress or type of anesthesia.</td>
</tr>
<tr>
<td>Posen et al 2000</td>
<td>Retrospective hospital-based cohort study</td>
<td>United States</td>
<td>≥37 wks’ gestation and delivered by CS over 3-year period. Exclusion of maternal and fetal conditions felt to increase fetal risk.</td>
<td>499</td>
<td>19</td>
<td>Need for ventilation, circulatory support, fluid resuscitation or medications Apgar scores NICU admission.</td>
<td>Increased risk of resuscitation if fetal distress present.</td>
<td>No control group of vaginal deliveries.</td>
</tr>
<tr>
<td>Levine et al 1999</td>
<td>Hospital-based cohort study</td>
<td>United States</td>
<td>≥2,500 g infants delivered by CS or spontaneous vaginal delivery over a 5-year period.</td>
<td>12,923</td>
<td>18</td>
<td>Need and type of resuscitation Apgar scores NICU admission.</td>
<td>No higher incidence of low Apgar score in infants delivered by CS under epidural for nonfetal indication compared with SVD.</td>
<td>Did not assess need for or type of resuscitation.</td>
</tr>
<tr>
<td>Ng et al 1995</td>
<td>Hospital-based cohort study</td>
<td>UK</td>
<td>Term singleton deliveries born by CS over a 1-year period.</td>
<td>520</td>
<td>18</td>
<td>Need and type of resuscitation Apgar scores NICU admission.</td>
<td>Increased need for active resuscitation with GA. Increased need for active resuscitation with fetal distress and noncephalic presentation in both regional and general anesthetic groups.</td>
<td>No control group of vaginal deliveries.</td>
</tr>
</tbody>
</table>
### Appendix A

(Continued)

<table>
<thead>
<tr>
<th>Author and year</th>
<th>Study type</th>
<th>Country</th>
<th>Study subjects</th>
<th>No. of patients</th>
<th>Quality rating (x/27)</th>
<th>Outcome assessed</th>
<th>Results</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>Zogorzycki and Brinkmann 1982</td>
<td>Retrospective hospital-based cohort study</td>
<td>United States</td>
<td>Elective repeat CS deliveries with infants &gt;2,000 g over a 1-year period.</td>
<td>195</td>
<td>15</td>
<td>Apgar scores</td>
<td>No difference between Apgar scores in general and epidural anesthetic groups.</td>
<td>Nonrandomized study of intervention-based on patient preference. Need for resuscitation not documented. No control group of vaginal deliveries.</td>
</tr>
<tr>
<td>Primhak et al 1984</td>
<td>Retrospective hospital-based cohort study</td>
<td>UK</td>
<td>&gt;37 wk’s gestation vaginal, instrumental and operative deliveries over 1-year period.</td>
<td>1,781</td>
<td>13</td>
<td>Apgar scores</td>
<td>Increased incidence of low Apgar score if fetal distress and instrumental or operative delivery. However, if no fetal distress incidence of low 1-min Apgar score similar in general and epidural groups.</td>
<td>For the 1 year period only 1781 records from the total of 2,086 term deliveries were available. Need and type of resuscitation not analyzed.</td>
</tr>
<tr>
<td>Press et al 1984</td>
<td>Prospective hospital-based cohort study</td>
<td>United States</td>
<td>Term infants delivered by CS to private patients over a 2-year period.</td>
<td>377</td>
<td>12</td>
<td>Type of resuscitation</td>
<td>Increased need for intubation if fetal distress and/or cephalopelvic disproportion.</td>
<td>No control group of vaginal deliveries.</td>
</tr>
<tr>
<td>Hogston 1987</td>
<td>Retrospective hospital-based cohort study</td>
<td>UK</td>
<td>Consecutive deliveries ≥37 wks’ gestation</td>
<td>460</td>
<td>11</td>
<td>Need for resuscitation</td>
<td>In the 55 CS with no fetal distress and cephalic presentation similar rates of resuscitation as vaginal deliveries.</td>
<td>Numbers too small to compare type of anesthetic. No period or population documented for the study subjects.</td>
</tr>
<tr>
<td>Burt et al 1988</td>
<td>Statewide case-control study</td>
<td>United States</td>
<td>Singleton uncomplicated births in state over 3-year period delivered by repeat CS or vaginal delivery. Cases- 5 min Apgar scores of 0-6. Controls- 5-min Apgar scores of 7-10.</td>
<td>Cases 1,030 Controls 998</td>
<td>21</td>
<td>Delivery type in cases and controls</td>
<td>Infants of repeat CS more likely to have low Apgar score.</td>
<td>Type of anesthetic for the repeat CS not analyzed. Fetal distress not analyzed.</td>
</tr>
<tr>
<td>Jacob and Pfenniger 1997</td>
<td>Retrospective case-control study in 2 hospitals</td>
<td>United States</td>
<td>Singleton term newborns (37-42 wks’ gestation) over 2-year period. Cases-CS deliveries with exclusion of maternal or fetal risk factors Controls-low-risk vaginal deliveries.</td>
<td>Cases 834 Controls 834</td>
<td>15</td>
<td>Need and type of resuscitation Apgar scores</td>
<td>Need for active resuscitation similar for repeat CS and low-risk vaginal deliveries. Increased need for resuscitation if general anesthesia.</td>
<td>Types of populations from each hospital not described.</td>
</tr>
</tbody>
</table>

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* Descending order of level of evidence as per NHMRC How to review the evidence 2000.17

1 Quality Index published by Downs and Black J Epidemiol Community Health 1998;52:377-384.16
High-dose methadone maintenance in pregnancy: Maternal and neonatal outcomes

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KEY WORDS
Methadone
Pregnancy
Neonatal abstinence syndrome
Drug abuse

Objective: This study assesses the effect of higher doses of methadone during pregnancy on maternal and fetal outcomes.

Study design: We retrospectively reviewed clinical data for 81 mothers who received methadone and their 81 offspring. The cohort was divided into high-dose (≥ 100 mg) and low-dose (<100 mg) groups.

Results: There were no differences in the rate of medication treatment for neonatal abstinence symptoms or days of infant hospitalization between the high-dose (mean, 132 mg) and low-dose (mean, 62 mg) groups. Despite longer histories of opiate abuse, the high-dose group had less illicit drug use at delivery. The whole cohort, which received an average of 101 mg/d, had an 81% rate of negative toxicology screens at delivery.

Conclusion: High doses of methadone were not associated with increased risks of neonatal abstinence symptoms but had a positive effect on maternal drug abuse. Arbitrarily limiting methadone dose as a way of minimizing the risks of neonatal abstinence symptoms may be unwarranted.

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Methadone maintenance treatment in opiate-addicted pregnant women reduces maternal morbidity and mortality rates and promotes fetal stability and growth, compared with mothers who use heroin.1,2 Methadone is associated with better compliance with obstetric care and better preparation for parenting responsibilities.3 The baby, however, is at risk for symptoms of neonatal abstinence syndrome (NAS) that is associated potentially with withdrawal from methadone at birth. Abstinence symptoms can occur in gastrointestinal, metabolic, and neurologic domains. Mild symptoms may not require medication treatment, although moderate or severe symptoms usually require medication-assisted withdrawal and 3-5 weeks of hospital monitoring.

There are conflicting studies on whether the higher methadone doses that are often needed to eliminate maternal withdrawal symptoms and drug abuse may increase the level of fetal pharmacologic dependence, potentially leading to more severe NAS.4-9 Berghella et al.10 in a retrospective review of 100 women maintained on methadone during pregnancy, found no difference in severity, duration, or treatment of NAS between...
infants of mothers who received <80 mg/d of methadone and those who received ≥80 mg/d. In contrast, Dashe et al., in their retrospective review of 70 women (mean dose, 20 mg/d) who were withdrawn or tapered just before delivery, found significant correlations between methadone dose and NAS.

Therapeutic response to methadone is dose related. Higher doses are associated with the better treatment outcomes in nonpregnant patients, and federal guidelines recommend increasing methadone doses in pregnant patients with withdrawal symptoms. Pregnant patients have required 50 to 150 mg/d to suppress withdrawal symptoms.

We retrospectively reviewed mothers and infants in a specialized methadone maintenance pregnancy program with individualized dosing to assess whether higher doses of methadone were associated with adverse neonatal outcomes.

Material and methods

The study’s narcotic treatment program maintains an active census of approximately 1100 methadone maintenance patients in a California metropolitan area with a population of 1.5 million. It is the only specialized provider of pregnancy services for opiate-addicted women in the area. Women in the program are assigned to a specially trained counselor, are all linked with obstetric care, and give written consent for providers to share information. All of the women participate in and receive a psychiatric assessment, supportive psychotherapy, 1 hour of individual drug treatment counseling per week, and participate in a weekly support group for both pregnant and early postpartum patients. All patients provide random weekly urine drug screens. As part of the clinic’s ongoing quality assurance program, maternal and infant data are collected from program entry until 1 month after delivery.

Patients were maintained on divided doses of methadone, given twice or occasionally 3 times a day, because the sustained plasma levels that are achieved with split dosing are associated with fewer withdrawal symptoms and less illicit drug use during pregnancy. As a quality control measure, methadone trough serum levels are measured after women reach stable methadone dosing and are repeated in patients who require unusually high doses. Although there is a therapeutic range for methadone trough levels in nonpregnant patients of 150 to 600 ng/mL, there is no attempt to achieve “target” serum levels. Methadone doses are clinically adjusted, without arbitrary limits, in response to illicit opiate use, withdrawal symptoms, or side effects.

NAS was evaluated with an objective scoring system, and treatment of the infant was initiated clinically when repeated scores were in the 6 to 8 range.

The study was approved by the University of California, Davis, Institutional Review Board. SPSS software (version 11.5; SPSS Inc, Chicago, Ill) was used for all analyses, and probability values >.05 were selected for statistical significance. Data were analyzed with independent samples (between-subjects) 2-tailed *t*-tests, chi-squared analyses, and Mann-Whitney tests.

Results

There were 94 admissions to the pregnancy program from February 1999 to May 2003. Thirteen subjects were excluded: 4 women miscarried; 3 women decided to terminate pregnancy; 2 women left treatment; 2 patients requested to taper off methadone, and 2 patients had unavailable outcome information. Eight women had 2 pregnancies during the study; each pregnancy was considered a separate admission. Data were analyzed for 81 admissions and 81 offspring.

The study group was 64% white, 25% Mexican/Hispanic, 6% African-American, 4% Asian, and 1% other. The average maternal age on admission was 23 ± 5.6 years, and the average years of use was 10 ± 6.5 years. Twenty-five admissions had conceived while on methadone maintenance. All others (n = 56 women) were acutely addicted to heroin (n = 49 women), prescription opiates (n = 5 women), or opium (n = 2 women). Seventy-seven percent of the women were cigarette smokers, with 28% of the smokers using >1 pack/day. Polydrug abuse (alcohol, cocaine, methamphetamine, or marijuana) was reported by 38% of the women on admission. Seventy-eight percent (n = 1188/1528 specimens) of all maternal urine toxicology screens before delivery were negative for illicit drugs.

The average maternal methadone dose at delivery was 101 mg/d (range, 14-190 mg/d). Trough serum methadone levels were obtained at different gestational ages on only 59 of 81 women during pregnancy because of the difficulty of peripheral venous access in heroin injectors. The mean trough serum level was 146 ng/mL (median, 115 ± 101.5 ng/mL; range, 20-478 ng/mL). Forty-six percent of mothers nursed their babies. The Figure shows the number of babies who were treated for NAS at each maternal dose range.

The infants had a mean gestational age at delivery of 37.3 weeks and a mean birth weight of 2792 g. No major developmental abnormalities were noted. Eighty-one percent of infant toxicology screens (n = 66/81 screens) at the time of delivery were negative for illicit drugs. The 15 positive screens detected opiates (n = 4 women), amphetamines (n = 9 women), cocaine (n = 4 women), diazepam (n = 2 women), marijuana (n = 1 woman), and alcohol (n = 1 woman). Six infants tested positive for 2 drugs. Thirty-seven babies (46%) required
medication for treatment of NAS symptoms. Infants were treated with paregoric (n = 20 infants), phenobarbital (n = 10 infants), both paregoric and phenobarbital (n = 4 infants), methadone (n = 1 infant), ativan (n = 1 infant), and both paregoric and ativan (n = 1 infant).

Because of custody issues, length of stay information was not available on 10 infants. The median length of stay for the 71 infants on whom data were available was 10.0 days (range, 1-105 days). There was no significant correlation between maternal dose and length of stay (Pearson correlation co-efficient, .066; \( P = .586 \)). When divided into NAS-treated (n = 37 infants) and untreated (n = 44 infants) groups, the untreated babies spent a median of 3 days (range, 1-44 days) in the hospital, while babies who were treated for NAS spent a median of 25 days (range, 8-105 days). We observed no cases of post-hospitalization NAS in untreated babies during the 1-month postpartum period.

To assess whether higher doses resulted in increased risks of NAS, the cohort was divided into 2 dose groups: mothers who were treated with <100 mg of methadone (n = 36 mothers) and mothers who were treated with \( \geq 100 \) mg (n = 45 mothers). The cut-off of 100 mg for the groups was chosen to achieve approximately equal cohort size. Comparison of maternal dose groups revealed a mean dose in the \( \geq 100 \) mg group of 132 mg and 62 mg in the <100-mg group. Independent samples \( t \)-tests showed no significant differences between groups in maternal age, age of onset of drug use, or time in treatment, although the high-dose group had significantly longer histories of opiate abuse (mean, 11.6 years vs 7.8 years in the low-dose group; \( t = -2.6 \pm 66.6 \); \( P < .05 \)). Chi-squared analyses showed no significant differences between groups in ethnicity, polydrug use history, and smoking history.

The Table shows infant outcome data by maternal dose group. Chi-squared analyses revealed that the higher dose group had significantly less drug use at delivery: 11% of infant toxicology screens were positive for illicit drugs in the high-dose group versus 27% positive screens in the low-dose group (\( P = .05 \)). There were no significant differences in the incidence of treated NAS between infants of high- and low-dose methadone mothers; 51% of the high-dose babies and 49% of the low-dose babies required treatment. Mann-Whitney tests for non-normal distributions revealed no significant differences in gestational age (\( U = 735; N_1 = 36; N_2 = 45; P = .47 \)), birth weight (\( U = 775; N_1 = 36; N_2 = 45; P = .74 \)), or days of infant hospitalization (\( U = 600; N_1 = 31; N_2 = 40; P = .81 \)) between high- and low-dose groups.

**Comment**

This retrospective records review of methadone-maintained pregnant women and their offspring found no evidence of an increased incidence of adverse outcomes in babies who were exposed to higher, clinically determined...
methadone doses. The rate of treatment for NAS and length of infant hospitalization was similar for both high-dose (mean, 132 mg/d) and low-dose (mean, 62 mg/d) groups that were studied. Our results extend the findings of Berghella et al to higher average dose ranges.

Importantly, our high-dose group had significantly less detected illicit drug use at delivery, even though this group had significantly longer histories of addiction. Berghella et al found a trend toward less drug use at doses of 80 mg. Our study suggests that, as in nonpregnant populations, higher doses of methadone do lead to less drug use. Any theoretic goal of reducing NAS by using low doses or tapering schedules may well be off-set by the adverse effects of more illicit drug use. For example, Brown et al reported on a low-dose methadone-treated pregnant population (41% were maintained on <50 mg) in which 84% of newborn infants tested positive for illicit drugs at the time of delivery, which led them to question the efficacy of methadone treatment.

The dose range (14-190 mg/d) in our cohort was quite wide, possibly reflecting individual differences in methadone metabolism. Accelerated methadone metabolism and decreases in methadone bioavailability occur during pregnancy. Consistent with these metabolic effects, the mean maternal methadone serum level during pregnancy in this study was in the low range for methadone, despite the high average dose.

The overall 46% rate of treated NAS for the infants is comparable to, or better than, studies in which lower doses were used. Doberczak et al reported a 78% rate of treated NAS, where the average maternal dose was 50 mg/d. The overall rate of treatment in the Dashe et al study (median, 20 mg/d) was 46%.

We used treated NAS as an outcome measure. We did not assess other variables that might affect the severity of NAS because our study relied on readily available measures that were used in routine clinical practice. Doberczak et al found that the severity of NAS was related to the decline of the neonatal plasma methadone level from day 1 to day 4 of life. Kushel et al confirmed this finding and further found that both low maternal and low cord methadone concentrations at delivery were associated with more severe NAS. These studies underscore the importance of infant variables in the determination of the risks of NAS. Furthermore, almost one half of our mothers nursed their babies. Methadone levels in milk are small and normally not sufficient to prevent NAS. However, Ballard found that frequent small feedings in the neonatal period were associated with reduced symptoms of NAS. Finally, it is speculative, but the more stable serum levels that are achieved by split doses may have some protective effect against NAS.

The role of non-opiate fetal drug exposure (alcohol, cocaine, amphetamine, and benzodiazepine) in effecting the expression of NAS has not been studied systematically and remains a potential confounder in our study, as in others.

Maternal recovery from illicit drug abuse is critical for the long-term health and safety of both the mother and the child. The use of adequate doses of methadone during pregnancy in a specialized program such as the one described in this study can increase the likelihood of the mother achieving recovery early in treatment. Continued methadone maintenance after delivery may further reduce the risks of maternal relapse during the critical and often stressful period of parenting a newborn child.

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References

Fetal response to maternal methadone administration

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KEY WORDS
Methadone
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Fetal heart rate
Fetal movement

Objective: The purpose of this study was to investigate the effect of methadone on fetal neurobehavioral functions and maternal physiologic indicators.

Study design: Forty women attending a substance abuse treatment facility with otherwise uncomplicated pregnancies were evaluated at peak and trough methadone levels. Fetal measures included heart rate, variability, periodic accelerations/decelerations, motor activity, and fetal movement-heart rate coupling. Maternal measures included maternal heart period, variability, electrodermal skin conductance, respiration, and respiratory sinus arrhythmia (RSA). Repeated measure analysis of variance was used to evaluate within-subject changes.

Results: At peak methadone, fetal heart rate was slower, less variable, and displayed fewer accelerations. Fetuses displayed less motor activity, and the integration between heart rate and motor activity was attenuated. Maternal heart rate and skin conductance were unchanged, but methadone administration was associated with lower respiratory rate and RSA, an indicator of parasympathetic tone.

Conclusion: Maternal methadone administration has significant effects on fetal behavioral functions that are independent of maternal effects.

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fetal mortality, and increased chances of discharging the infant into the care of his/her parents upon hospital discharge. Conversely, methadone use during pregnancy is associated with a significant risk of neonatal abstinence syndrome (NAS). Approximately 75% of opiate-exposed infants develop NAS after birth, and methadone-exposed infants display more profound NAS symptoms than heroin-exposed infants. When comparing methadone with heroin-exposed infant outcomes, methadone-exposed infants are heavier at birth, but weigh less than non–drug-exposed infants.

Methadone crosses the placenta readily, reaching concentrations in the umbilical cord of one fourth the maternal serum. A number of studies have found less reactivity in nonstress testing in methadone-exposed fetuses after maternal methadone administration when compared with controls. Although consistent in their findings, these reports are variously limited by reliance on visual inspection of fetal tracings, subtherapeutic maternal methadone dosing during pregnancy, relatively small sample sizes, and potential confounding because of recent, or failure to evaluate for, illicit drug or alcohol use.

The present study was designed to ascertain the maternal and fetal effects of therapeutic methadone administration by simultaneously monitoring fetal neurobehavioral indicators and maternal physiologic responses at peak and trough methadone levels. Assessment of subtle indicators of fetal functioning provides a window into the developing nervous system and quantification of fetal response to episodic methadone exposure.

Material and methods

Study population

Forty-two methadone-maintained women were selected from a population of women engaged in substance abuse treatment at an urban, comprehensive substance abuse treatment program for pregnant and postpartum substance abusing women and their children. The program has been described elsewhere. Subjects were selected based on recommendation from their counselors for compliance to program standards and staff confidence in their abstinence from illicit substances and alcohol during treatment, but were not different demographically (race, parity age), or by substance use history from program participant means for women meeting criteria for methadone maintenance. At the start of treatment, all women met DSM IV-R criteria for opiate dependence, and federal guidelines for methadone maintenance. To minimize confounds based on pregnancy risk factors, inclusion was limited to women with uncomplicated singleton pregnancies who were HIV negative and free of significant pregnancy complications, such as hypertension and gestational diabetes. The research was approved by the governing institutional review board, and informed consent was obtained from all participants.

Procedures

Data collection involved 2 60-minute fetal monitoring sessions on one day during the 36th week of gestation. Estimation of gestational age was based on a sonogram performed between 16 and 20 weeks’ gestation in all cases, and was confirmed by postnatal assessment. The initial test session took place at trough maternal methadone level, typically occurring at 1 hour before oral dose, near 09:30; the second, at peak methadone, 2½ hours after oral methadone, near 13:30. Peak plasma methadone levels in individuals chronically treated with oral methadone occur between 2 to 4 hours after an oral daily dose. Methadone was administered once daily, at the conclusion of the first monitoring session (M dose = 78.4 mg, sd 17.8, range 40 to 115 mg). Methadone administration was confirmed by pupillometer photograph, which revealed pupillary constriction at peak (vs trough) methadone in all cases.

Women were asked to eat a meal and refrain from caffeine and/or cigarettes 2 hours before each session in an effort to control for maternal glycemic status and the effects of caffeine and nicotine on the fetus. Recent cigarette smoking was evaluated at both sessions by breathalyzer testing for carbon monoxide (CO) using the Micro Smokerlyser (Bedfont Scientific Limited, Kent, UK). Carbon monoxide levels in parts per million were recorded; values were not adjusted for background levels. Recent alcohol use was similarly evaluated using a Digitox-D.O.T. (Sound Off, Inc, Hudsonville, MI) screening breath tester, which measures blood alcohol content to 0.02%, or the equivalent of 1 drink. Urine was obtained for toxicology at both sessions. Participants’ perceived symptomatology indicative of opiate withdrawal was surveyed at each test session using the Subjective Opiate Withdrawal Scale (SOWS). 18

Primary outcome measures

Fetal neurobehavioral assessment

Fetal data were collected using a Toitu (MT325, Tokyo, Japan) fetal actocardiograph. This monitor detects fetal movement (FM) and fetal heart rate (FHR) using a single-wide array transabdominal Doppler transducer, and processes this signal through a series of filters. The actograph detects fetal movements by preserving the remaining signal after band-passing frequency components of the Doppler signal that are associated with FHR and maternal somatic activity. Reliability studies comparing actograph-based vs ultrasound-visualized fetal movements have found the performance of this monitor to be highly accurate in detecting both fetal
motor activity and quiescence. Fetal data were collected from the output port of the monitor and digitized at 1000 Hz through an internal A/D board using streaming software. Data were analyzed offline using customized software (James Long Company, Caroga Lake, NY). Digitized heart rate data underwent error rejection procedures based on moving averages of acceptable values as needed. Fetal variables included 4 cardiac measures: fetal heart rate and variability (root mean squared) computed for each 1-minute epoch averaged over time, and accelerations and decelerations. Accelerations were identified when FHR values attained 10 bpm above baseline for ≥15 seconds; decelerations defined as ≤15 bpm below baseline for ≥15 seconds.

Fetal movement measures were based on the acto-graph signal, which ranges from 0 to 100 in arbitrary units. A movement bout was considered to begin when the first spike of the actograph attained an amplitude of 15 units, and ended when there was a cessation of 15 unit signals for at least 10 seconds. The number of movement bouts was counted, and the duration of each movement was measured. Total motor activity was computed as the number of movement bouts multiplied by the mean movement duration (seconds) divided by 3600 (number of seconds per 60-minute recording), yielding the total time the fetus spent moving. FM-FHR coupling was calculated as the percentage of fetal movements associated with excursions in FHR ≥5 bpm over baseline within 5 seconds before the start of a movement or within 15 seconds after the start of a movement.

Maternal physiologic assessment
Maternal physiologic signals were amplified using a multichannel, electrically isolated bioamplifier. Electrocardiogram (ECG) was recorded from 3 carbon fiber disposable electrodes in triangulated placement (right mid subclavicle, left mid axillary thorax, and upper left thigh for ground lead). Electrodermal activity (ie, skin conductance) was monitored from 2 silver-silver chloride electrodes with a gelled skin contact area placed on the distal phalanxes of the first and index fingers of the nondominant hand affixed with adhesive collars to limit gel contact to a 1-cm diameter circle and Velcro.

Maternal data were time synchronized and analyzed in conjunction with fetal data. ECG data underwent R-wave detection, manual editing for artifact, and interbeat interval computation. Skin conductance was measured by administering a constant 0.5-V root mean square 30 Hz AC excitation signal, and detecting the current flow. Skin conductance was scaled from 0 to 25 microsiemens and detrended to remove the mean, thereby amplifying the signal to noise ratio (−2.5 to +2.5 microsiemens). Maternal ECG values were quantified as heart period (ie, interval between R-waves in milliseconds), and heart period variability (standard deviation of successive heart periods). Both cardiac measures are influenced by both neural and non-neural factors, so spectral analysis was used to extract the variation in heart period within the high frequency oscillations coincident with spontaneous breathing (James Long Company), yielding a measure of respiratory sinus arrhythmia (RSA; msec²). RSA is mediated through the sinoatrial node, and thus, is frequently used as an indicator of neural activation of parasympathetic processes and vagal tone. Maternal variables were averaged during the recording.

Data analysis
Univariate repeated measure analysis of variance was used to evaluate changes in fetal and maternal values from trough to peak maternal methadone levels. Change scores from peak to trough in each measure were computed and Pearson correlations were used to determine whether methadone dosage was related to the magnitude of fetal and maternal baseline values and responses.

Results
Participants tended to be multigravid (75%), near 30 years of age (mean = 29.4 years, sd = 5.7), varied in race and ethnicity (40% African American, 57.5% Caucasian, 2.5% Hispanic), and unmarried (92.5%). Prescribed substances included psychotropic medications, primarily fluoxetine and sertraline (40%), asthma medications (5%), and antibiotics (12.5%). Most (85%) smoked cigarettes regularly. Two women were retrospectively removed from study inclusion as a result of positive urine toxicology at the time of testing, leaving a sample of 40 women.

Half (52.5%) of the fetuses were male, and 92.5% (37) were born at term. Of the remainder, 2 were born in the 36th week of gestation, and there was 1 fetal death at term from unspecified causes. This fetus was not outstanding in terms of location within the distribution of any fetal or maternal parameter measured in the study, so was retained in the final sample. Birth weights of the surviving neonates were appropriate for gestational age (M birth weight = 3078.50 g, sd 415.50 g); there was no evidence of growth retardation for any infant. All 5-minute Apgar scores were >7. Infants were without significant neonatal morbidity aside from NAS, which occurred with treatable significance in 51.4%.

Fetal parameters
There were highly significant changes in all aspects of fetal neurobehavioral functioning from trough to peak methadone; data are presented in Table I. At peak methadone, fetuses displayed significantly slower fetal heart rate, reduced variability, and fewer heart rate...
accelerations. Decelerations were too infrequent to be analyzed: 3 were observed at trough, and 1 at peak. Although the number of movement bouts was unchanged, the duration of each movement and the resultant total amount of fetal motor activity were both significantly reduced, approximately by half, at peak maternal methadone. In addition, the degree of coupling between fetal movements and fetal heart rate (FM-FHR coupling) was significantly lower at peak.

Although these quantitative differences are statistically robust, mean values do not adequately capture the degree to which methadone exerted an effect on fetal functioning. The Figure presents representative data at peak and trough from a single fetus. To better define the totality of the effect of methadone, a clinician expert in fetal actocardiograph tracings who was blinded to condition was asked to judge under which condition (trough or peak) the fetus looked “less well.” The rater identified the peak methadone tracing as the one in which the fetus looked less well in all 40 cases.

### Maternal measures

Despite the significant differences in fetal measures, methadone administration generated few effects on maternal physiologic parameters (Table II). Maternal heart period and variability were not significantly different at trough vs peak methadone, nor was skin conductance. Maternal RSA was significantly lower at peak methadone, indicating lower parasympathetic tone. Respiratory period was slightly higher at peak methadone. Maternal reports of withdrawal symptomatology as determined by the Subjective Opiate Withdrawal Scale declined from trough (M = 5.0, sd 7.4) to peak (M = 2.5, sd 4.1), F (1, 38) = 8.84, P < .01. However, SOWS values below middle-teen scores reflect minimal withdrawal discomfort; thus, despite this decline, women at trough levels were experiencing very little to no withdrawal symptomatology.

### Methadone dosage effects

Maternal methadone doses ranged from 40 to 115 mg daily. Correlation coefficients were computed between dosage and change scores from trough to peak for each fetal variable. To control for the Law of Initial Values, trough levels were partialled from each correlation. Methadone dosage was significantly associated with greater changes from trough to peak only for fetal heart rate variability (r (38) = −.34, P < .05), indicating that those receiving more methadone showed greater changes in variability. Correlations for methadone dosage and mean fetal measures at peak (not change scores) indicated the following: higher dosages were associated with lower heart rate (r (38) = −.32, P < .05), greater variability (r (38) = −.37, P < .05), and a trend towards more fetal heart rate accelerations (r (38) = .29, P < .10). No significant associations were detected with fetal motor variables or FM-FHR coupling. Maternal measures were unrelated to dosage.

### Effects of other substances

No appreciable levels of alcohol use were detected via alcohol screening by breathalyzer testing for any subject at either visit. Women were asked to refrain from smoking, and carbon monoxide testing indicated no substantial recent cigarette use. Trough values (M = 6.80 ppm, sd 4.8) did not differ from peak values (M = 7.5, sd 5.3), F (1,39 = 1.27, P > .10).

### Comment

Administration of methadone to pregnant women is associated with profound effects on fetal neurobehavioral functioning. These changes were evidenced despite minor effects on indicators of physiologic functioning in pregnant women, suggesting that methadone may be mediating these fetal effects directly. The effects were pervasive and evidenced in fetal heart rate, motor activity, and their interrelation. Fetal neurobehavior serves as a window to the developing nervous system, and its disruption implies threat to the neural development of the fetus.

In opiate-exposed newborns, evidence of neonatal behavioral disorganization does not stipulate that neurologic harm has been done during pregnancy, but rather may reflect more transient effects of methadone administration or withdrawal. Support for this view is provided by the lack of association between the severity of neonatal abstinence and later developmental outcome. The goal of this study was to evaluate the transient effects of maternal methadone administration.

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**Table I** Fetal neurobehavioral measures at trough and peak methadone (n = 40)

<table>
<thead>
<tr>
<th></th>
<th>Trough Mean (SD)</th>
<th>Peak Mean (SD)</th>
<th>F</th>
</tr>
</thead>
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<tr>
<td>Heart rate (bpm)</td>
<td>136.47 (7.56)</td>
<td>128.29 (5.22)</td>
<td>52.09*</td>
</tr>
<tr>
<td>Heart rate variability</td>
<td>5.93 (1.26)</td>
<td>3.72 (1.05)</td>
<td>100.33*</td>
</tr>
<tr>
<td>Number of accelerations</td>
<td>3.63 (3.03)</td>
<td>0.47 (0.75)</td>
<td>48.21*</td>
</tr>
<tr>
<td>Movement bouts</td>
<td>66.80 (16.45)</td>
<td>63.55 (18.94)</td>
<td>0.79</td>
</tr>
<tr>
<td>Duration of movements</td>
<td>26.88 (15.38)</td>
<td>13.69 (9.05)</td>
<td>26.17*</td>
</tr>
<tr>
<td>Total motor activity</td>
<td>1627.81 (670.33)</td>
<td>880.05 (588.85)</td>
<td>36.66*</td>
</tr>
<tr>
<td>FM-FHR coupling (%)</td>
<td>22% (8%)</td>
<td>16% (9%)</td>
<td>18.01*</td>
</tr>
</tbody>
</table>

* P < .0001.
Figure  Representative fetal heart rate and fetal movement tracing at trough (A) and peak (B) maternal methadone level.
on fetal behavior. The long-term sequelae of daily, repeated depression of motor activity and heart rate are unknown, but potentially present a distinct mechanism through which methadone may exert an effect on development beyond neurotoxic effects of the substance itself. Without a control group, we are unable to evaluate the neurobehavioral toxicity of methadone exposure. However, 36-week FM-FHR coupling trough values from fetuses in this sample, when compared to previously published values from a comparable population of socioeconomically disadvantaged, but nondrug-using women from the same city, differ by more than a standard deviation (22% vs 38%). This suggests persistent effects of methadone exposure even when methadone levels are at their nadir.

A limitation of this study was that we were unable to counterbalance dosage (ie, have half of the participants undergo peak methadone at 10:30, and the other half at 13:30) to control for potential circadian influences and the effect of meals. This was due to the strict dosing and programmatic guidelines for methadone outpatient treatment. To achieve appropriate peak and trough levels at comparable times would require providing women with take home doses for a prolonged period in order to achieve pharmacologic stabilization, which is prohibited by federal regulations. Moreover, we would be unable to ascertain compliance in timing of dose administration. However, based on existing literature and our experience in fetal assessment, there is little reason to expect that either the 4-hour span between trough and peak or the transition from breakfast to lunch would generate the striking effects observed here. Although circadian rhythms in fetal behaviors from morning to night have been documented, the preponderance of literature finds minimal, if any, alterations in fetal behavior and heart rate during daylight hours. Second, despite common clinical perception to the contrary, there is no empirical support for the notion that maternal food intake or blood glucose levels affect fetal behavior or heart rate. Thus, while we are confident that interpretation of these results is not obscured by the nature of the design, subsequent investigations would benefit from inclusion of a non-opiate-dependent control group evaluated at the same times of day.

Although our results indicate that methadone maintenance is clearly not innocuous to the fetus, the broader issue, the use of methadone as the treatment of choice for pregnant opiate addicts, is a complex one. This strategy has persisted despite evidence that methadone generates effects on the neonate that are as deleterious, if not more so, than heroin, on neonatal abstinence symptoms. The benefits of methadone use during pregnancy have been well documented for the mother, but not conclusively so for the infant. This study evaluated single-dose therapy, the most common approach to methadone administration. Findings suggest that while this may be the most convenient management strategy for the mother, other approaches may be less disruptive to the fetus. Split-dosing administration has been shown to decrease fetal effects in a small study of 7 methadone maintained women at 26 to 37 weeks’ gestation. Fetuses of women on a split dose displayed less decrease in ultrasound-observed fetal body movements and breathing rate in contrast to those receiving a single dose. More intensive investigation is needed to establish the dosing strategy that provides least disruption to the fetus, while offering the opiate-dependent woman the multiple benefits of methadone therapy. In addition, evaluation of the degree to which new treatments on the horizon for use during pregnancy, including buprenorphine, are more or less disruptive to fetal neurobehavioral development than methadone should be conducted concurrently with efficacy studies for their use in pregnant women.

### Acknowledgments

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### References


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### Table II: Maternal physiologic measures at trough and peak methadone (n = 40)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Trough Mean (SD)</th>
<th>Peak Mean (SD)</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart period</td>
<td>884.41 (152.10)</td>
<td>881.07 (155.48)</td>
<td>0.16</td>
</tr>
<tr>
<td>Heart period variability</td>
<td>32.68 (30.57)</td>
<td>32.17 (30.50)</td>
<td>0.08</td>
</tr>
<tr>
<td>Respiratory sinus arrhythmia</td>
<td>61.32 (29.09)</td>
<td>51.30 (30.48)</td>
<td>5.28*</td>
</tr>
<tr>
<td>Skin conductance</td>
<td>6.19 (4.63)</td>
<td>5.76 (3.72)</td>
<td>1.33</td>
</tr>
<tr>
<td>Respiratory period</td>
<td>4.43 (1.24)</td>
<td>4.69 (1.43)</td>
<td>4.82*</td>
</tr>
</tbody>
</table>

* P < .05.
It is a pleasure to introduce a new series that will be appearing in the American Journal of Obstetrics and Gynecology - Research Methods: State of the Science. With the recent explosion of research methods - clinical, basic, and translational - physicians and investigators are faced with an increasingly complex body of research literature in obstetrics and gynecology. The goal of this series is to provide in depth reviews and discussions of emerging themes and controversies in research and will include manuscripts focused on both basic and clinical research topics. Manuscripts from this series will be appearing intermittently in the journal over the next several years and will be solicited and written by experts in these specific content areas of research. It is our hope and belief that this series will aid both clinicians and researchers in their understanding of the literature related to obstetrics and gynecology.

In this edition of the Journal, Thom and Klebanoff eloquently discuss 2 critical issues related to the design and execution of randomized clinical trials. The first is the role of Data Safety and Monitoring Committees (DSMC) in the context of clinical trials and how decisions are made to stop studies. This topic has gained in importance in recent years, as concerns regarding the protection of human subjects in research have increased. In fact, any National Institutes of Health (NIH)-funded clinical must have provisions made for monitoring data on an interim basis. One question unanswered by the manuscript by Thom and Klebanoff is whether there is need for data safety and monitoring of smaller clinical trials, such as those commonly performed at a single site. For the Maternal-Fetal Medicine Units (MFMU) Network, which is supported by NIH, funds are available for this aspect of clinical research. In addition, there are certainly “economies of scale,” such that the same DSMC can assess all of the ongoing clinical trials within the MFMU Network. But the situation is very different for a single-site, investigator initiated study. In this case, the development of a plan for interim analysis and composing a DSMC can be daunting. Still, to ensure adequate protection of human subjects in our research studies, we do have an obligation to assess efficacy and safety of our clinical trials. On a smaller scale, this can be accomplished by consultation with a statistician before commencing with the study and construction of a DSMC using clinicians and researchers at local institutions.
Issues in clinical trial design: Stopping a trial early and the large and simple trial

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KEY WORDS
Research methods
Clinical trial
Biostatistics
Study monitoring

During the conduct of a clinical trial, a primary function of the Data Safety and Monitoring Committee is to select the trial conduct and the accumulating data to determine whether the trial should continue or be discontinued earlier than planned. Reasons for early discontinuation of a trial include: evidence of benefit, evidence of harm, and evidence of futility. More than 1 of these elements will often be present. These principles will be illustrated with examples from National Institute of Child Health and Human Development–Maternal-Fetal Medicine Units clinical trials. The “large and simple clinical trial” is a study design rarely undertaken in the United States but commonly used elsewhere. The principles of this type of trial will be introduced and contrasted with those of the “conventional clinical trial.”

This essay, one in a series on research methods, will discuss 2 issues in the design and analysis of randomized clinical trials. The issues are (1) when a Data and Safety Monitoring Committee (DSMC) should recommend early termination of a randomized trial and (2) the complexity of the study protocol itself. Although these topics are not directly related, both can lead to controversy when clinicians decide whether to incorporate trial results into practice.

Stopping a trial early

It is generally acknowledged that most randomized trials should have an independent DSMC. Indeed, such oversight is required for all National Institutes of Health (NIH)-sponsored trials that entail potential risks to participants. The role of the DSMC is often thought by investigators to be restricted to monitoring the safety of the subjects enrolled in the trial. In fact, these committees play a broader role during the development and conduct of randomized trials. As an example, the National Institute of Child Health and Human Development (NICHD) Division of Intramural Research delineates the following as functions of DSMCs for their clinical trials: (1) to review new intervention protocols to suggest modifications in design, reporting and analysis relating to safety and efficacy endpoints; (2) to review and provide guidance regarding major study design changes; (3) to monitor endpoint and toxicity data, based on a specific and predetermined schedule; (4) to make recommendations concerning continuation, termination, or modification of ongoing studies; and (5) to assist in resolving problems that may arise during conduct of the study.1

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Here we focus on the role of the DSMC in a clinical trial after patient recruitment has started, and in particular on how the committee approaches the decision to stop a trial at an interim point. For the purposes of this discussion, we will consider a randomized trial designed to test a new treatment against a control treatment such as placebo to determine whether the new treatment reduces the risk of an adverse medical outcome, designated as the primary outcome. The DSMC meets during the course of the trial to review the emerging data—not only safety data such as adverse events, but also the frequencies of primary and secondary outcomes.

At each interim review, the DSMC may recommend continuing the trial as originally designed, or continuing with a modification of the protocol. Alternatively, for several reasons the committee may recommend stopping the trial before the estimated sample size has been reached, or before the expected follow-up period is complete. First, it is possible that the original sample size was overestimated. Second, the new treatment may have a beneficial or a detrimental effect that is already apparent. Third, the trial may be deemed to be futile; in other words, it is unlikely that an answer to the primary question will be attained in a reasonable time, if ever. This might be due to a previously unrecognized flaw in the trial design, or to inadequate patient recruitment. Fourth, there may be external influences such as changing practice or newly learned results from other studies. Fifth, the question under study may no longer be relevant; the new treatment may even be “obsolete.”

We discuss these reasons for early discontinuation of recruitment in more detail below, with examples derived mainly from the NICHD’s Maternal-Fetal Medicine Units (MFMU) Network, a multicenter study group that conducts trials to answer timely clinical questions in obstetrics and perinatal medicine. Of the 13 randomized clinical trials completed by the MFMU Network to date, 8 have been stopped before the estimated sample size was reached. Of the 8 trials, 2 were discontinued for futility, and 1 for evident benefit. The rest were more complicated. Some of these have been previously discussed\(^2\) and some are presented here.

**Reduction in sample size**

The DSMC may determine that sufficient power to show equivalence between the treatments, or to exclude benefit or harm of the new treatment, will be achieved before the full sample size has accrued. This can occur when the primary outcome occurs more frequently than originally anticipated. In our experience this is unusual; it is more common to underestimate the required sample size when planning a trial. However, in one instance the MFMU Network DSMC did recommend a reduction in sample size. In a trial of low-dose aspirin to prevent preeclampsia among four separately powered strata of women at high risk,\(^3\) the stratum of women with insulin-dependent diabetes was predicted to have a preeclampsia rate of 10% in the placebo group. After 3 interim analyses, it was observed that the rate of preeclampsia in the placebo group was consistently greater than 20%, and the sample size for this cohort was reduced from 980 to 450.

**Evidence of benefit**

If the new treatment shows a significant beneficial effect at an interim analysis it would be unethical not to stop the trial so that the new treatment may be made available. However, it is important to define in advance what is meant by a “significant benefit” and to consider the effect of conducting multiple interim data analyses on the type I error. Using a rough analogy, imagine a 1-mile race between 2 horses. We agree before starting that if 1 of the horses is ahead by at least 1 length at the end of the race, then that horse is truly faster. If the margin of victory is less than that, then we ascribe the victory to luck. Now suppose that to spare the horses, we also decide to stop the race early if at some point one of them has such a large lead that the other would be very unlikely to close to within a length by the end of the race, let alone pull ahead. There may be times during the race when 1 horse has a 1-length lead, but how confident are we that this lead will be maintained? To stop the race after a quarter mile, 1 of the horses would have to be far behind and almost have to break a leg leaving the starting gate for us to be confident that the ultimate victory margin for the other would be by at least 1 length. At the half mile mark, the lead could be less overwhelming, but an interim lead of only 1 length would still not be enough to convince most of us that the final margin will be at least 1 length, or that the lagging horse could not catch up. We require even less of a lead at 3/4 mile, but we still acknowledge a possibility that the trailing horse might catch up.

Interim analyses are very similar to this analogy. At the end of a trial, a treatment is declared to be of significant benefit if the calculated test statistic exceeds a prespecified statistical boundary or critical value, generally analogous to the usual Z-value of 1.96 (\(P = .05\)) for a single test. For interim analyses, it is not acceptable to stop the trial merely because the test statistic exceeds 1.96. This is because looking at the data more than once increases the chance of finding a significant difference when there is none (type I error). Therefore, for interim analyses, a more conservative boundary is used—one example is the Peto boundary of a Z-value of 3.5 (\(P = .0005\)).\(^4\) In the MFMU Network, we use a boundary that is unlikely to be crossed early in the trial unless there is a huge treatment difference, but becomes closer to 1.96 near the end of the trial; the exact value depending on the previous number of looks at the data, and the percentage of the final sample...
size that is included in the analysis. In the recently completed trial of progesterone versus placebo to prevent recurrent preterm delivery, an interim analysis of 351 delivered patients out of a projected 500 patient sample size revealed a preterm delivery rate of 54% in the placebo group and 37% in the progesterone treatment group, a significant Z-value of 3.0 \((P = .0027)\). The boundary was at \(Z = 2.43\), corresponding to a \(P\)-value of .0149. At the time the DSMC met, another 112 patients had already been randomized (463 total patients) but were not yet delivered. Although trials should not be stopped for benefit until the predefined boundary has been crossed, DSMCs may opt to continue the trial once significance has been reached to collect data on important but less frequent secondary outcomes. In the case of this progesterone trial, the primary outcome was delivery less than 37 weeks of gestation; but neonatal morbidity and mortality were very important secondary outcomes. In fact, the committee did consider completing recruitment of the total 500 patient cohort to examine neonatal outcome, but decided that it was unlikely that data derived from 37 patients remaining to be randomized warranted a delay in publishing the results, and recommended discontinuation of study recruitment.

Evidence of harm

Interim data analysis may reveal a negative impact of the new treatment on the primary outcome, rather than a positive effect. Again, a statistical boundary is usually determined before recruitment starts such that if it is crossed, the new treatment is deemed to be harmful. The boundary may be symmetric with the boundary for benefit (ie, analogous to \(-1.96\)) but generally a lesser degree of evidence is required to declare a negative trend than to declare a beneficial effect. In other words, DSMCs will usually require less evidence to stop study recruitment when a new treatment might be harmful than when it might be beneficial. How asymmetric, ie, how small a negative effect is needed to cross the boundary, would depend on whether the new treatment is already in general use for this or another indication, and whether there is other evidence of benefit. However, care should be taken not make the boundary for determination of harm too easy to cross.

Discontinuation of a study too early for harm may result in a new treatment that has promise with regard to secondary outcomes being ignored, or the trial results being dismissed if the new treatment is already being used. In an MFMU Network trial of metronidazole to prevent preterm delivery in women with asymptomatic *Trichomonas vaginalis*, the DSMC reviewed the data after 516 women (of the final sample size of 1900) had been delivered. The preterm delivery rate in the active treatment group was higher than in the placebo group (18.8% vs 11.5%, \(P = .02\)) as it had been consistently at three previous interim analyses. In this case, we did use a boundary for harm that was symmetric with the one used for benefit and so we would have required a \(P\)-value of .0002 to declare harm. In retrospect this was much too conservative. Nevertheless, the committee recommended discontinuation of the trial because of the trend toward harm. Also factored into their decision was the declining recruitment, which meant the trial would take another 7 years to complete. In fact this decision to stop this trial was based on a combination of reasons, evidence of both harm and futility.

Futility and other reasons

Interim analysis may reveal a neutral effect or even a trend toward a negative effect of the new treatment, and it may appear increasingly unlikely that a positive effect will be demonstrated by the end of the trial. It is often useful to determine the conditional power of the observed difference, calculated under the assumption that the current trend in the data will continue until the end of the trial. Not surprisingly, if a negative or neutral trend is observed at interim analysis, the power to show a beneficial effect assuming the same trend in the remainder will be slim to nonexistent. Caution should be exercised in discontinuing a trial on this basis alone, however. There are many instances in the published literature, and even in the MFMU Network, where trends in the data have changed over time—going from positive to negative or vice versa. In the trial of repeated weekly versus single course steroids conducted by Guinn et al., a sample size of 1000 women was originally planned to give 90% power to detect a one-third reduction in a composite outcome of neonatal morbidity and mortality, from an estimated 25% in the single-course group to 16.5% in the weekly course group. An interim analysis after 308 women had been recruited, revealed very little difference between the 2 treatment groups. The rate of composite neonatal morbidity/mortality was 27% in the single-course group and 24% in the weekly course group, only an 11% reduction. The group calculated the conditional power assuming this trend in the remainder of the patients and of course, found it to be very low, less than 2%. They used this as a justification to stop after 500 rather than the originally calculated 1000 patients had been enrolled. In fact, had they calculated the conditional power under the assumption of the positive trend that became evident in the next cohort of recruited patients (which was actually quite close to the one third reduction assumed in the sample size calculation) there would have been more than 75% power to show a difference by the time that 1000 patients had accrued.

We usually recommend calculating conditional power for more than one scenario, that is, not just assuming the observed trend to date, as Guinn et al. did. We also
recommend calculating the conditional power assuming that there will be a positive effect for the remainder of the trial. The positive effect that we frequently use is the same as that used for the original sample size calculation. As a hypothetical example, if a trial’s sample size were chosen to detect a 20% reduction in the primary outcome for a new treatment compared with a control treatment, then we would calculate the conditional power assuming that for the remainder of the trial there is a 20% reduction in the primary outcome in the new treatment group. This can be described as “calculating the conditional power under the alternative hypothesis.” If the conditional power under the alternative hypothesis is low, there can be considerably more confidence about stopping the trial.

In the case of Guinn et al.,10 external evidence, albeit retrospective studies, had shown some evidence of harm and this, in addition to the results of the conditional power analysis, was a factor in the decision. It is also likely, as has been the case in several of the MFMU Network trials, that it was not just these considerations but the uphill battle to keep recruitment going for another number of years that led to the decision to terminate the trial earlier than first planned.

The MFMU Network also initiated a randomized trial of repeated versus single dose prenatal corticosteroids,12 using a similar design to that of Guinn et al. An interim analysis was conducted on the data from 282 randomized patients, the planned sample size being 2400. The DSMC recommended discontinuation of the trial at this interim review. The committee was concerned regarding safety as infants in the repeat course group tended to be smaller, which was concordant with external evidence of harm in recent publications. However, there was no difference between the groups in head circumference, the parameter of most concern. The committee noted the slight suggestion of benefit in some of the pulmonary parameters, but absolutely no trend for benefit in the primary outcome of neonatal morbidity and mortality. The committee also had concerns regarding the ongoing relevance of the question being studied, as the regimen of repeated steroids had been abandoned by many obstetricians since the start of recruitment. In addition, recruitment was slow, although it had more than doubled since the year before. It was estimated that it could take another six years to finish recruitment.

Finally, the outcome rate in the placebo group was somewhat less than expected, implying that a larger sample size would be required, and this was also taken into account in their decision. Nevertheless, because only 12% of the final sample size was included in the interim analysis cohort, there was still reasonable power to show a difference under the alternative hypothesis. This last case had elements of benefit, harm and futility and is an excellent illustration of the difficulty in conducting and monitoring clinical trials.

**Summary**

In summary, data and safety monitoring committees are charged with monitoring not only adverse events during the conduct of clinical trials, but also with monitoring the trial’s progress, likelihood of coming to a successful completion, and the need for modification or early termination based on data derived from interim analyses. Although stopping rules for benefit and harm should be specified before starting the trial, in practice, the decision to stop a trial is usually not clear-cut, and cannot be reached solely on statistical grounds but rather on a number of complex statistical and clinical considerations,13 including changes in prevailing practice or new data available in the literature, and invariably incorporates some subjective considerations. When a trial is stopped early, we believe that it is important for the readers of the report of the trial to have the reasons given, and especially to have the importance of each reason clarified, if there is more than 1.

**The large and simple clinical trial**

As generally conducted in the United States, clinical trials are detailed, complex and expensive undertakings, limiting the number of that can be attempted. Due to the vigorous advocacy of statistician Richard Peto, much of the rest of the world employs a different approach—the Large and Simple Clinical Trial.14-18 These different approaches to clinical trial development have inherent benefits and problems. In this discussion, we compare 2 studies evaluating interventions to improve perinatal outcomes—the NIHCD MFMU Network Trial of 17alpha hydroxyprogesterone caproate to prevent preterm birth,6 and the UK-based ORACLE studies19,20 of maternal antibiotic therapy to improve neonatal outcome to illustrate these different philosophies.

The progesterone study was conducted in 19 US academic medical centers that had competed successfully for membership in the MFMU Network. As described previously, 463 women were enrolled in this trial. The study was restricted to women who had a previous spontaneous preterm birth and were within a relatively narrow gestational age window in the current pregnancy. The study design required that the prior spontaneous preterm birth used to determine eligibility be confirmed by reviewing the original medical records, and the gestational age at randomization in the current pregnancy had to be confirmed by sonography before inclusion. There was an extensive list of conditions in the current pregnancy that rendered a woman ineligible. Treatment was intensive—weekly injections of 17-alpha hydroxyprogesterone caproate or placebo; if a woman missed her weekly clinic appointment a research nurse visited her at home to administer the injection. Weekly saliva specimens for assay of steroid hormones were
obtained from all women. The study required up to 13
different forms (some of which were used on multiple
occasions) for each woman enrolled; the primary study
outcome was the prevention of birth before 37 weeks of
gestation. Infant outcomes were evaluated secondarily.

In contrast, the ORACLE studies were conducted in
161 volunteer sites and enrolled 8710 women in multiple
countries. The eligibility criteria for the ORACLE I
trial included a pregnancy less than 37 weeks of
gestation complicated by preterm premature rupture of
membranes (preterm PROM), and managing clinician
uncertainty regarding the need to prescribe antibiotics.
The eligibility criteria for the companion ORACLE II
trial included a pregnancy at less than 37 weeks of
gestation in suspected or definite labor with intact mem-
branes, and the managing clinician was uncertain as to
whether to prescribe antibiotics. No definitions were
provided for how membrane rupture or labor was to be
diagnosed, how gestational age was to be ascertained, or
why the clinician did not believe antibiotics to be either
indicated or not indicated. The lists of exclusions for both
trials were short: antibiotics already being prescribed,
immediate delivery desirable or unstoppable, fetus not
sufficiently preterm to cause concern (all of these as
determined by the enrolling clinician), and specific con-
traindications (such as allergy) to the antibiotics being
used. The treatment was not intensive—women were
given packs of pills to be taken for 10 days or until
delivery. The study used only 3 single-sided data
forms—1 collected at randomization, 1 at delivery, and
1 at death or hospital discharge of the neonate. The
primary study outcome was not merely prevention of
preterm birth, but rather fetal/neonatal death, major
adverse outcome (such as chronic lung disease) or major
cerebral abnormality on ultrasound, all determined by
the time of hospital discharge of the newborn infant.

Philosophy of the large and simple trial

As demonstrated over the years about how large
and simple trials should be conducted and why they are
valid. The 2 types of trials are compared in the Table. In
contrast with the conventional trial, the large and simple
trial uses few specific inclusion or exclusion criteria,
relying instead on each individual clinician’s level of
uncertainty. It collects minimal data, puts minimal
burden on the enrolling clinicians and participating
women, and uses a study protocol that is very simple
and easy to follow. Large and simple trials enroll many
more participants than conventional trials; and their
primary outcomes, rather than being surrogate measures
such as preterm birth or prolongation of pregnancy, are
often uncommon but important outcomes such as fetal/
neonatal death, chronic lung disease, or brain damage.
Surrogates such as preterm birth are relegated to
secondary outcome status. The large and simple trial is
based on the belief that as long as proper randomization
is used, the outcome is important and assessed in an
unbiased manner, and an intent-to-treat analysis is
followed, large numbers are more important than highly
standardized data quality.

Yusuf et al advance 3 major underlying principles to
guide development of the large and simple trial. First,
identification of effective treatments is likely to be relevant
if the disease being treated is common and the condition
being prevented is important. In perinatology, important
outcomes include maternal, fetal and/or neonatal death;
serious, long-term neonatal outcomes such as chronic
lung disease or cerebral palsy. Except in populations at
very high-risk, these outcomes are uncommon.

Prolongation of a pregnancy and reduction of the
frequency of preterm birth are merely surrogates for
these more important outcomes. Second, “miracle treat-
ments” like penicillin come along perhaps once in a
lifetime. The real difference between 2 treatments, or
between treatment and no treatment, will probably not
be large. However, even moderate reductions in impor-
tant outcomes (say, from 5% to 4%) may be worth
achieving. Third, although some readily identifiable
subgroups of patients might respond more favorably
than others to a given treatment, the effect of treatment
is unlikely to be reversed in different subgroups. In other
words, treatments might be more helpful in some preg-
nancies than in others, but it is unlikely that a treatment
will help some while harming others. Peto et al suggest
that when the overall study result indicates no benefit,
any benefit observed in a particular subgroup of partic-
ipants is probably a false-positive result. When the over-
all result indicates a benefit, failure to find a benefit in a
particular subgroup is probably a false-negative result.

If one accepts these principles (and not everyone
does), then the need for and design of large and simple
trials is clear. The rarity of important outcomes and the
likely modest benefits that one can expect of treatment
mean that clinical trials must be large to achieve
adequate power. The need to accrue large numbers of
patients means that common conditions must be stud-
ied, and that the study protocol must be simple,
collecting only minimal prognostic and outcome data
from each individual patient. These principles also imply
that inclusion and exclusion criteria can be minimal,
usually limited to clinician uncertainty regarding the
need for treatment and the absence of a few absolute
contraindications to use of the treatment. The underly-
ing assumption that a beneficial treatment will benefit
everyone also supports broad inclusion and minimal
exclusion criteria. The broad, nonspecific inclusion crite-
ria and simple treatments are also suggested to improve
generalizability of the trial’s results, as they might mimic actual practice more closely. Peto et al have noted that “There is no need to collect large amounts of data on prognostic features, nor for extensive ‘quality’ review, since detailed analyses of such data add little to the statistical power of the trial, and are generally counterproductive by discouraging participation.” Because the outcomes are “hard” endpoints such as death, complex diagnostic algorithms are not necessary.

### Arguments against large and simple trials

Some of the most cogent arguments against the use of the large and simple trial were made by the late internist and clinical epidemiologist Alvan Feinstein who drew the distinction between evaluating the average performance of 2 treatments and evaluating treatments for the care of patients. Feinstein complained that lumping together heterogeneous groups of patients sharing nothing more than a broad, unverified definition of some disease and clinician uncertainty of the need for treatment may produce results with excellent statistical stability but which will be relatively worthless when clinicians try to apply the results in practice. In other words, the large and simple trial may gloss over important clinical distinctions, and as such would not be helpful to clinicians in deciding how to treat any particular patient.

In their textbook of clinical trials, Friedman et al provide a balanced discussion of the advantages and disadvantages of large and simple trials. They note that this design works best when the intervention is simple, the condition being treated is easily diagnosed, both the outcome and important prognostic variables can be determined without the need for specialized tests, and the follow-up time is relatively short. When these conditions cannot be met, the large and simple trial may not be feasible. Although large and simple clinical trials in perinatal medicine are rarely done in the United States, they are the most common type of clinical trial in the rest of the world.

In practice, the complexity and size of a trial depend on the specific question being answered and the setting where the trial will be conducted. Although there have been exceptions, new treatments that are complicated, expensive, or acutely risky should probably be tested in a relatively small number of centers where skilled, specifically trained personnel can be assembled, and protocol adherence and participant safety can be closely monitored. The cost and recruitment difficulties of such a study will probably mandate a small-to-moderate sample size, and therefore a surrogate outcome. Conversely, trials relying on volunteer physicians (as might be the case in countries where funds to establish the elaborate structure of a conventional trial are not available) are probably best served when they evaluate

### Table: Comparison of Conventional and Large and Simple Clinical Trials

<table>
<thead>
<tr>
<th></th>
<th>Conventional Trial</th>
<th>Large and Simple Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>Small to moderate</td>
<td>Large</td>
</tr>
<tr>
<td>Number of clinics</td>
<td>Few (usually &lt;20)</td>
<td>Many (up to hundreds)</td>
</tr>
<tr>
<td>Support for clinics</td>
<td>Clinics compete for award</td>
<td>Mainly volunteer, minimal compensation</td>
</tr>
<tr>
<td>Who benefits from treatment</td>
<td>Specific subgroups benefit. Therefore detailed inclusion and exclusion criteria needed.</td>
<td>Everyone benefits or no one benefits. Therefore few inclusion and exclusion criteria needed. Main criterion: ‘clinician uncertainty regarding need for treatment’</td>
</tr>
<tr>
<td>Treatment protocol</td>
<td>Complex, defined in detail</td>
<td>Simple, minimally defined</td>
</tr>
<tr>
<td>Reliance on routine clinical care</td>
<td>Low. Often requires special research clinics</td>
<td>High</td>
</tr>
<tr>
<td>Quality control</td>
<td>Extensive</td>
<td>Minimal</td>
</tr>
<tr>
<td>Number of forms</td>
<td>Many</td>
<td>Few</td>
</tr>
<tr>
<td>Biospecimens saved</td>
<td>Often</td>
<td>Rarely</td>
</tr>
<tr>
<td>Study outcome</td>
<td>Surrogate conditions (preterm birth, preeclampsia, etc)</td>
<td>Severe conditions (fetal, neonatal, maternal death; convulsions, stroke, cerebral palsy, etc.)</td>
</tr>
<tr>
<td>Advantages</td>
<td>Entry criteria well defined - applicability clear</td>
<td>Severe outcomes studied</td>
</tr>
<tr>
<td></td>
<td>Extensive quality control</td>
<td>Patients more like those in routine practice</td>
</tr>
<tr>
<td></td>
<td>Extensive data and specimen collection can help explain study results</td>
<td>Simple, practical protocol</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Complex protocol might not be practical in routine setting</td>
<td>Entry criteria vague - applicability to any given patient uncertain</td>
</tr>
<tr>
<td></td>
<td>Surrogate outcomes studied</td>
<td>Little quality control</td>
</tr>
<tr>
<td></td>
<td>Patients often highly selected</td>
<td>Minimal data and specimen collection - difficult to explain study results</td>
</tr>
</tbody>
</table>
relatively simple, inexpensive, low-risk interventions and use a protocol that does not interfere with routine clinical care. A more severe primary outcome is well-advised to maintain clinical credibility in the face of a relatively nonspecific study protocol; fortunately, the low cost and burden of such a study makes the required large sample size feasible.

Most MFMU Network trials follow the middle road of a “larger and less complex” protocol. A relatively few critical entry criteria, such as history of preterm birth in the progesterone trial,\(^6\) history of preeclampsia or chronic hypertension in the high-risk aspirin trial,\(^7\) the diagnoses of bacterial vaginosis and trichomoniasis in the BTVT trials,\(^8,23\) and gestational age in virtually all trials, are defined according to a specific and detailed protocol. Specific, detailed protocols are developed to diagnose the primary and often a few key secondary study outcomes; centrally done chart reviews are used as needed to verify these outcomes. Other measures are standardized only when deemed absolutely necessary. Wherever feasible, the remainder of patient care is left to routine practice, and relevant data are abstracted from the regular clinic chart. Albeit more complex than trials such as ORACLE,\(^19,20\) this approach is considerably less complex than that used by trials such as the multiple risk factor intervention trial.\(^24\) We believe this strikes a favorable balance between cost, complexity, and clinical credibility.

Which is the “correct” way to conduct a trial? In the final analysis, for a clinical trial to be of value, its results must influence practice. Clinicians might question the validity of a large and simple trial because of the vagueness of the entry criteria or the lack of quality control. Conversely, clinicians might question the relevance of a conventional trial because the detailed entry criteria excluded the very patients whom that clinician believes might have benefited from treatment, because the complex trial procedures could never be accomplished in the “real world,” or because the outcome of the study was not sufficiently severe to be worth preventing in its own right. Ultimately, whether large numbers of patients and severe study outcomes can compensate for reduced data collection and quality control is a question of judgment, not of statistics.

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Research agenda for preterm birth: Recommendations from the March of Dimes

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Preterm birth (PTB) is a common, serious, and costly health problem affecting nearly 1 in 8 births in the United States. Burdens from PTB are especially severe for the very preterm infant (<32 weeks’ gestation), comprising 2% of all US births. Successful prevention needs to include newly focused and adequately funded research, incorporating new technologies and recognition that genetic, environmental, social, and behavioral factors interact in complex pathogeneses and multiple pathways leading to PTB. The March of Dimes Scientific Advisory Committee created this prioritized research agenda, which is aimed at garnering serious attention and expanding resources to make major inroads into the prevention of PTB, targeting six major, overlapping categories: epidemiology, genetics, disparities, inflammation, biologic stress, and clinical trials. Analogous to other common, complex disorders, progress in prevention will require incorporating multipronged risk reduction strategies that are based on sound scientific discovery, as well as on effective translation into clinical care.

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Preterm birth (PTB) is the major determinant of early childhood mortality and morbidity and is the leading cause of neonatal mortality (<28 days of life) and of black infant mortality (<365 days of life) in the United States.1 As much as half of all pediatric neurodevelopmental problems can be ascribed to preterm birth.2 Moreover, the severity and incidence of adverse outcomes inversely correlates with gestational age, especially birth before 32 completed weeks of gestation. Beyond the enormous impact on affected families, the
economic costs are staggering. Although only 9% of all infant hospitalizations in 2002 were related to prematurity, the charges were $15.5 billion, representing nearly half of all charges for infant hospitalization (unpublished data from March of Dimes Perinatal Data Center. Agency for Healthcare Research and Quality, National Inpatient Sample, 2002.)

Despite decades of research and clinical efforts, the US preterm birth rate (<37 completed weeks’ gestation) rose to an all time high of 12.1% in 2002, a 29% increase over the previous 2 decades.3 Most of this increase has been reported in moderately preterm births (32-36 weeks of gestation), as the rate for very preterm births (<32 completed weeks’ gestation) has remained virtually stable at about 2%.3 Contributing factors include the increasing frequency of births to women older than 35 years, and the use of infertility treatments,4 with their significant enhancement of multiple birth rates.5,6 Preterm births in singleton pregnancies result most frequently from spontaneous preterm labor and preterm premature rupture of membranes (PPROM).7-10 In addition to spontaneous PTB, 20% to 30% of preterm births are considered medically indicated to avoid or minimize maternal and/or fetal complications, such as intrauterine growth abnormalities. Advances in maternal, fetal, and neonatal management have led to an increased willingness to deliver high-risk pregnancies preterm.11,12 Patient and provider preferences may also be promoting clinical interventions that are further increasing PTB rates at later gestations, such as scheduled deliveries.13,14

Rates of PTB in the United States differ profoundly among racial/ethnic groups, with the largest and most persistent disparities occurring between non-Hispanic white and non-Hispanic black births. In 2002, the rates of preterm and very preterm births for Hispanics were only modestly higher than for non-Hispanic white infants, but respective rates for non-Hispanic black infants were 60% and 250% higher (Table I, NCHS data). Native Americans have intermediate rates, whereas Asian PTB rates are the lowest of all these groups. These disparities remain even after stratification by plurality and adjusting for possible confounders such as education and occupation, thus likely reflecting a combination of genetic, environmental, and social factors.15-20 Racial/ethnic disparities in PTB are associated with persistent gaps in chronic health outcomes throughout life.15-20

### Table I

<table>
<thead>
<tr>
<th>Race/ethnicity</th>
<th>Total US (%)</th>
<th>Non-Hispanic black (%)</th>
<th>Non-Hispanic white (%)</th>
<th>Hispanic (%)</th>
<th>Non-Hispanic Asian (%)</th>
<th>Non-Hispanic Native American (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTB rate (&lt;37 wks)</td>
<td>12.1</td>
<td>17.7</td>
<td>11.0</td>
<td>11.6</td>
<td>10.4</td>
<td>13.0</td>
</tr>
<tr>
<td>Very PTB rate (&lt;32 wks)</td>
<td>2.0</td>
<td>4.0</td>
<td>1.6</td>
<td>1.7</td>
<td>1.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

All race categories exclude Hispanic births. Source: National Center for Health Statistics.

### Table II

<table>
<thead>
<tr>
<th>Data collection variables for PTB</th>
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</thead>
<tbody>
<tr>
<td>Plurality</td>
</tr>
<tr>
<td>Maternal race/ethnicity</td>
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<tr>
<td>Maternal age</td>
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<tr>
<td>Maternal education</td>
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<tr>
<td>Paternal race/ethnicity</td>
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<tr>
<td>Medical interventions</td>
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<td>Paternal age</td>
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<td>Mode of conception</td>
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<tr>
<td>Paternity information</td>
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<tr>
<td>Socioeconomic status</td>
</tr>
<tr>
<td>Neoplastic outcomes</td>
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<tr>
<td>History of preterm labor/PTB</td>
</tr>
<tr>
<td>(spontaneous and iatrogenic)</td>
</tr>
<tr>
<td>History of preterm labor/PTB</td>
</tr>
<tr>
<td>Micronutrient and supplement use</td>
</tr>
</tbody>
</table>

Research has identified numerous risk factors for spontaneous PTB, although accurate prediction and prevention remain elusive.2,8-10,21-25 A few of the major risk factors have biologic plausibility, such as multifetal gestation, a history of prior PTB or preterm labor and certain cervical, uterine, and placental structural or physiologic abnormalities. Much research has focused on the roles of infection and the resultant cascade of immunologic effects. However, environmental interactions—broadly defined to include behavioral factors and social underpinning—are also likely to play important but less well understood roles. Other factors include race/ethnicity, maternal age, socioeconomic status, interconceptional interval, maternal medical conditions, maternal weight, nutritional status, substance abuse, paternal effects, stress, depression and other factors (Table II). The precise mechanisms by which these risk factors influence the preterm birth rate and the potential impact of their successful modification on PTB are
largely unknown. Unfortunately, most biomarkers assessing risk of PTB have poor positive predictive value for guiding clinical interventions.

PTB is now best understood as the clinical endpoint for a number of potential causes and pathogeneses (Figure). In this context, at least 4 major pathophysiologic pathways have been described toward the shared outcome of PTB: inflammation/infection with its associated maternal and fetal cytokine response; maternal/fetal stress with generation of placental and fetal membrane-derived corticotrophin-releasing hormone, which in turn, enhances placental estrogen and fetal adrenal cortisol production; abruption or decidual hemorrhage with thrombin-induced protease expression and disturbances in uterine tone; and mechanical stretch due to multifetal pregnancy or polyhydramnios-induced abnormal uterine and cervical distention. These pathophysiologic pathways may occur independently but more commonly are present in various degrees of combination. Research is needed to define both the biologic pathways of PTB and successful clinical interventions.

In contemporary terms therefore, PTB is increasingly conceptualized as a “common, complex disorder,” defined as a condition stemming from heterogeneous composites of multiple gene-environment interactions. The prototype of such disorders is cardiovascular disease, which arises from multiple inherited factors, including metabolism of cholesterol, fat, homocysteine, and insulin, acting often synergistically with modifying environmental and behavioral influences such as diet, weight, exercise, and smoking. The evidence supporting this conceptualization of PTB includes findings of familial aggregation, nonmendelian heritability, high rates of recurrence, and the existence of ethnic/racial disparities. For PTB, 2 corollaries thus follow: (1) that etiologic investigation should include consideration of the broad range of factors and mechanisms that need to be prioritized by frequency and by level of impact; and (2) that successful interventions will likely need to simultaneously and/or sequentially target multiple risk factors and will require tailoring for individuals and/or specific communities. This work will be aided by use of the tools of genomics, proteomics, and genetic epidemiology and by an improved understanding of pathophysiology in preterm birth. As a common, complex disorder, PTB also meets the criteria recently set forth for problems of high research priority and of major public health significance.

Consistent with this conceptual framework, March of Dimes initiated its Perinatal Research Initiative (PERI) in 1998 to fund multidisciplinary research targeting the interactions between epidemiologic and biologic bases of prematurity. Most recently, the Foundation launched its National Prematurity Campaign in 2003 to stimulate public concern for prematurity and to reduce the nation’s rate of PTB, with campaign partners of the American College of Obstetricians and Gynecologists (ACOG), American Academy of Pediatrics (AAP), and Association of Women’s Health, Obstetrics and Neonatal Nursing (AWHONN). A principal aim of the campaign is to foster research on PTB through increased support from federal and other funding sources, including the March of Dimes.

**Development of a research agenda**

A Scientific Advisory Committee (SAC) on Prematurity was created to advise the National March of Dimes Campaign (membership listed in the Appendix). The SAC has interdisciplinary representation of national experts in PTB research and treatment, from fields of obstetrics-gynecology, pediatrics, nursing and public health, with representation from the 3 campaign partner organizations. It was charged to develop a national research agenda that included prioritized recommendations. After deliberation, the SAC concluded that research efforts should focus primarily on cause and prevention of very preterm birth (<32 weeks). The rationale was that the study of the most severely affected would likely be more biologically distinct and thus most amenable to discovery, and may translate into the greatest impact on child health. Four subcommittees were formed to address the interrelated research categories of: basic science, clinical management, social and behavioral interactions, and racial/ethnic disparities. Committee processes included formal reviews of extant publications from relevant disciplines, based on the members’ experience as investigators and clinicians. External review of the resultant document was then obtained from additional experts representing the Prematurity Campaign partner organizations ACOG, AAP, and AWHONN, as well as the National Institute of Child Health and Human Development (NICHD),
Centers for Disease Control (CDC), and Society of Maternal Fetal Medicine (SMFM).

The current report summarizes recommendations for addressing specific research strategies, methodologies and approaches needed to stimulate and implement the prioritized prematurity research agenda. These recommendations emphasize the most promising experimental paradigms to elucidate the complex contributions of genetic and environmental influences. Research on PTB needs to: (1) better define the etiologic mechanisms responsible for PTB; (2) identify biomarkers for PTB to improve clinical risk assessment; (3) develop clinical interventions that lead to reduction in rates of PTB; and (4) eliminate disparities in PTB among racial/ethnic groups in the United States. Genetics is a relatively new aspect of prematurity research that brings an exciting array of possibilities for new avenues of investigation through new technologies. Ultimately, translating the findings from genetic research into modification of risk for PTB for individuals and for communities will need to take into account the effects of complex environmental influences.

By establishing a national emphasis on strategies that have proven successful for other complex common disorders, the Foundation hopes to attract investigators to this important arena, including some not previously vested in prematurity research. The March of Dimes thus seeks to inspire novel innovative approaches to address the challenges inherent in translating science into clinical practice. A second March of Dimes report on prematurity will be forthcoming to address broader research topics within public, provider and health care delivery issues, such as the impact of education, clinical management, racial/ethnic disparities, stress, and behavioral modifications, particularly as they impact the large group of moderately premature infants (32-36 weeks’ gestation).

Major recommendations

Preface

Decreasing PTB will require enhanced efforts that explore mechanisms of specific contributing genetic and environmental factors and their interactions, as well as a better understanding of uterine and placental pathophysiology. These findings should lead to hypothesis-driven, controlled clinical trials for prediction and prevention of PTB, focussed on those births occurring before 32 weeks’ gestation.

The SAC recommends 6 main targets for research initiatives (Table III):

1. Contemporary epidemiologic studies of very preterm births (≤32 weeks’ gestation). The traditional epidemiology of PTB is rooted in decades-old clinical data, much of which was acquired outside of the United States\(^9,41\) and does not focus as heavily on early preterm births. Some of the latter were historically considered stillbirths, but now constitute the source of the majority of perinatal and infant morbidity and mortality. Robust epidemiologic studies should be conducted on heterogeneous populations for which data are collected on all relevant biologic and environmental risk factors and clinical management variables. These studies must be adequately powered to establish sample sizes large enough to accommodate appropriate stratification, adjustment, and multivariate analytic techniques. Although cross-sectional and case-control studies are important, prospective cohort studies and cohort studies of sequential pregnancies should also be performed to generate positive and negative predictive profiles of very preterm births.

   a. Data collection variables. To provide uniformity, comparability, and to conduct appropriate adjustments and stratification that was based on probable factors that afford risk or protection,
epidemiologic studies of preterm births should collect at least the clinical variables listed in Table II.

b. Adequate sample size. Study designs should take into account genetic and other heterogeneity, with adequate case ascertainment and patient selection that meet specific inclusion and exclusion criteria. Sample sizes should be specified to ensure that studies have adequate power to address the hypotheses in question.

c. Biomarkers of PTB. Prediction of PTB should include the identification of specific markers of risk and their incorporation into epidemiologic analyses. Such markers include genetic polymorphisms (see below), alterations in level of expression of specific genes and susceptibility to adverse effects from smoking, genital tract infection, fetal fibronectin, cervical length, and contour and other influential variables.

d. Epidemiologic overlap between PTB and other adverse birth outcomes. Other adverse perinatal outcomes commonly occur with PTB, such as birth defects and intra-uterine growth restriction. The association between PTB with other adverse birth outcomes need definition to delineate overlapping mechanisms and to facilitate predictions of treatment efficacy and outcomes.

2. Genes and gene-environment interactions. A search for biologic regulatory substrates and circuits integral to preterm delivery is required. The qualitative and quantitative roles of host susceptibility in infectious and other environmental processes, the effects of specific genes, genetic pathways, and epigenetic regulation on host responses and on the biology and molecular biology of parturition are all promising areas for research. Likely pathways of focus include inflammation, which is mediated through proinflammatory and anti-inflammatory pathways involving cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β. Other pathways include those regulating pro-apoptotic and anti-apoptotic pathways, tissue maintenance, remodeling and stretch, hemostasis and hemorrhage, cellular turnover and degradation. Promising strategies include:

a. High-risk phenotypes. Studies of individuals and families with recurrent, idiopathic very PTB should be given priority. Such cohorts provide opportunities for identifying relevant genetic and environmental factors and predictive biomarkers, including some that may be amenable to intervention or individualized risk assessment.

b. New analytic techniques and bio-information systems. New techniques for genetic, functional genomic, and proteomic analysis should be developed and applied. Although candidate gene approaches may be useful, microarray technolo-

gies permit comprehensive and pathway-based genomic/proteomic analyses without presupposed identification of target genes and proteins. Applications should be paired with collection and storage of biologic specimens and bioinformatics support. These tools should be applied in conjunction with epidemiologic analyses, family studies, cohorts of severely affected subjects, community-based intervention trials, and relevant animal models.

c. Biobanking. Research studies on PTB should include collection and storage of biologic specimens with the minimum of collection of maternal blood/DNA samples. Sampling should include other maternal samples (urine, vaginal secretions, placenta, uterine tissue), paternal samples, and fetal samples (amniotic fluid, cord blood). Samples should be appropriately banked and made available to future investigators.

d. Animal models. Better use of animal models is needed to elucidate the underlying mechanisms of preterm labor and PPROM and to identify promising interventions. Rodent models are useful for some areas of investigation, despite not being ideal models of PTB. “Knock out” and “knock down” models of known and novel genes are needed. Primates and other mammals are useful and require political as well as financial support.

3. Racial/ethnic disparities. PTB is among the most disparate health outcomes in the United States, especially for black Americans (Table I). Research on PTB should work toward attaining the Healthy Peoples 2010 goal of eliminating health disparities. Innovative research must address both the underlying biology of disparate gestational length and inequities in systems of health care delivery so that responsive, culturally sensitive interventions can be developed to reduce and eventually eliminate those inequities. Multidisciplinary approaches should combine biologic, sociologic, and epidemiologic paradigms, including prospective family and community cohorts. These studies should encompass quantifiable issues involving prior reproductive outcomes, medical conditions, infection, immune regulation, stress, and other biomarkers (described previously), poverty, depression, racism, substance and physical abuse, weight, and nutrition. Socio-medical interventions, including those applied at the community level should be studied. Similar approaches have had demonstrable success in prevention and therapeutic intervention in other common complex disorders such as cardiovascular disease. Specific research priorities include:

a. Risk factor analysis. Develop improved measures of risk factors to investigate the potential for higher
prevalence of specific risk factors among black women.

b. Genetic factors. Assess differential ethnic effects on biologic responses of specific genetic factors, such as polymorphisms in genes regulating immune responses and steroid receptor polymorphisms and their interactions with various environmental (including social and behavioral) factors.

c. Behavioral factors. Determine the differential and cumulative effects of behavioral risk factors such as multiple partners, interval between pregnancies, sexual activity during pregnancy, use of barrier contraceptive methods that may prevent ascending infections, douching, substance abuse (tobacco, alcohol, and other drugs), nutrition, preconception weight, and pregnancy weight gain on PTB.

d. Health care delivery. Study how components of health care delivery systems including access, quality of care, content, nontreatment, unequal treatment, cost, and reimbursement impact on rates of PTB.

4. Role of inflammatory responses in PTB. The microbial environment and host response play pivotal roles in both preterm labor and PPROM. Infections are implicated in at least 40% of PTB and as much as 70% for PTB less than 32 weeks, as documented by clinical signs, histologic chorioamnionitis, and microbial cultures of fetal membranes and amniotic fluid, consistent with an ascending pathway. Inflammation may play a primary or secondary role in PTB. For example, treatment of infections often does not ameliorate, and may even paradoxically increase the risk of PTB. This unexpected effect may reflect further stimulation by endotoxin, and/or by-products of microbial demise of inflammatory pathways already triggered by infection, or may reflect a shared pathway between susceptibility to infection and an underlying exaggerated inflammatory response. These responses to infection reflect an integration of genetic and environmental influences that need elucidation, with development of predictive biomarkers. Important research areas are as follows:

a. Micro-organisms. Document the presence and chronology of micro-organisms, including mycoplasma species, chlamydia, and viruses in the endometrium and decidua that may contribute to PTB.

b. Infection. Elucidate the role of chronic endometritis, ascent of vaginal flora and of inflammation at distant sites, eg, periodontal disease on preterm labor and birth.

c. Effects of antibiotics. Investigate the results of prenatal and preconception antibiotic treatment on women with genital infection and/or prior PTB.

d. Role for the cervix. Study the mechanical and immunologic role played by the uterine cervix.

e. Immune pathways. Identify pathways that regulate maternal and fetal humoral and cell-mediated immune responses to infection and noninfectious inflammatory processes.

f. Immune modulation. Study the effects of suppression or augmentation of inflammation on PTB. Modulation may need to be general or targeted to particular inflammatory mechanisms, possibly affecting associated neurodevelopmental problems such as cerebral palsy.

5. Stress responses and PTB. Pregnancy itself is a stress on normal physiology and endocrinology of women, with simultaneous yet competing demands to sustain the fetus and to adapt to foreign antigens. Some pregnancy states may be more susceptible to effects from stress, such as in multifetal pregnancies, given the extra physiologic demands placed on the maternal, fetal, and placental systems. Maternal emotional, psychosocial, nutritional, or other environmental stress may increase risk of PTB via effects on several interacting mechanisms: (1) neuroendocrine pathways via maternal and/or fetal hypothalamic-pituitary-adrenal (HPA) neuroendocrine axes (eg, ACTH, cortisol) that result in premature triggering of labor and/or a greater degree of activation of the placental-fetal endocrine systems, including corticotropin releasing hormone (CRH) and estrogens; (2) immune mechanisms regulated by inflammatory responses, likely affecting susceptibility to maternal infection such as bacterial vaginosis; (3) intrauterine/fetal inflammatory processes, thereby promoting PTB through proinflammatory mechanisms; and (4) maternal-placental-fetal vascular function, including both uterine and umbilical blood flow.

Studies should be performed to delineate the role that biologic and other types of acute and chronic stressors play in PTB and in racial/ethnic disparities, functioning within the context of the common complex disorder of PTB. These studies should include:

a. Biologic assessment of stress, its timing and its effects on maternal and fetal health. Focus on improved biologic definition and quantification, and identification of functional links between the biologic, social, and environmental risk factors. This should include validated quantitative biomarkers, such as those described previously, as well as CRH, plasma and salivary cortisol, and other relevant markers in the neuroendocrine stress and inflammatory pathways; mechanisms underpinning the associations between stress, the HPA axis and the autonomic nervous system, including the effects of glucocorticoids and catecholamines; immunologic changes such as type 1/type 2 cytokine/cellular
responses; vascular effects in the placental circulation, and related physiologic mechanisms.

b. Effects of stress on racial/ethnic disparities. Biologically relevant and quantified markers of stress should be examined regarding their role in disparate outcomes among sub-populations, as described in target area 4 (above).

6. Clinical Trials. In light of the overall paucity of prior success with clinical trials and interventions in reducing the incidence of PTB, a change in expectation is needed. The investigative approach should be broadened so that the research is no longer organized to reduce PTB by eliminating the effect of single risk factors. Instead it should be guided by the overriding concept of PTB as a common complex disorder, in which interacting environmental and heritable factors must be addressed, concomitantly with the clinical milieu. Moreover, progress may prove to be incremental rather than monumental. Promising interventions need to be tested in controlled trials of sufficient sample size for stratification, enroll well-defined populations, and include a clinical database and anticipatory maternal and fetal/neonatal biologic sampling. Studies should be primarily randomized trials with clearly established mechanistic underpinnings and biologic analyses. Opportunities to clarify areas of known disparities should receive priority. Appropriate outcome measures for these studies should include perinatal morbidity and mortality, in addition to duration of prolongation of pregnancy and gestational age at delivery.

A comprehensive agenda for clinical investigation requires: (a) better timing and protocols for treatments based on improved techniques for diagnosis of preterm labor; (b) identification of high-risk women both before (preconception) and during early pregnancy for risk reduction and early intervention; and ultimately, (c) reduction of risk among all women of reproductive age through education, risk reduction programs, and innovative community-based interventions with contemporary standards. These efforts should be population-based, culturally sensitive, and appropriate for race, ethnicity, parity, and educational level. Successful interventions will need to address multiple risk factors simultaneously or sequentially, and will require tailoring for individuals and/or specific communities. They should also be optimally timed during the preconception, prenatal, and interconception periods to attain the highest impact on rates of PTB and neonatal morbidity and mortality.

Clinical investigations of women at increased risk of PTB should integrate the areas described in the major target areas 1 through 5 above, as well as:

a. Biomarkers. Determine effective biologic markers with positive and negative predictive value for risk of preterm labor and/or PPROM, as described previously.

b. Progesterone supplementation. Administered empirically in the second trimester, progesterone appears to reduce the risk of recurrent PTB for a select group of women at very high risk for PTB. This recent clinical success should be used to stimulate studies on the mechanisms of action, optimal form, dose and route of administration, identification of women who will, and just as importantly, who will not benefit from progesterone prophylaxis use in stratified risk groups, and clinical management and follow-up of offspring.

c. Infections. Screening and management of possible bacterial and nonbacterial pathogens. These studies should examine effects from local, systemic and distant infection such as bacterial vaginosis, sexually transmitted diseases, urinary tract infections, or periodontal disease. In addition to novel strategies for antibiotic therapies, treatment options may include manipulation of the maternal and fetal cytokine milieu.

d. Abruptio and impaired uterine-placental blood flow. Screening and intervention for causes and consequences of compromised placental blood flow, and/or decidual hemorrhage abruption, including acquired and inherited thrombophilias and other coagulation abnormalities, aberrant response to protease-activated receptor (PAR)-ligand interactions.

e. Multifetal pregnancies. Determine optimal management for spontaneous and artificially conceived multifetal pregnancies, including the traditional approaches, such as bed rest, tocolysis, cerclage, and potential strategies to prevent PTB, such as supplemental progesterone, anticoagulation, enhanced nutrition, and/or antioxidants.

f. Assisted reproductive technologies (ART). Elucidate the relationship between ART and PTB for both multiple and singleton gestations, including the attributable contribution of underlying maternal and paternal pathology. Technical aspects should be studied to optimize the yield of healthy births.

g. Risk reduction strategies. Studies should evaluate the impact on rates of PTB of comprehensive individual risk assessment and, where possible, intervention with both genetic and environmental variables (Table II). Interventions include eliminating or minimizing environmental factors such as smoking and exposure to other toxic agents and stressors, treating underlying medical disorders, and optimizing preconceptional and prenatal maternal medical and mental health.
Conclusion

Productive research on PTB requires several essential elements included in this report: promising paradigms to frame investigative approaches; innovative methodologies to address the problem; well-delineated genotypes and phenotypes; sufficient access to research participants, patient data, and clinical samples, facilitated by biobanks; clinical animal models and in vitro materials; adequate funding; and trainee support. As for other common, complex disorders, research on PTB must consider the contributions of genetic, psychosocial, cultural, racial/ethnic, nutritional, behavioral, and environmental factors. Research programs already aimed at prevention of prematurity and that are consistent with this report’s recommendations include the March of Dimes PERI research portfolio and the newly created Prematurity Research Initiative (PRI), which specifically focuses on the causes of PTB,79 existing federally funded research programs on approaches such as the use of progesterone, studies on racial/ethnic disparities, community-based programs, and a new NICHD program, “Genomic and Proteomic Network for Premature Birth Research,”80 as well as being incorporated in the National Children’s Study championed by NICHD. Sufficient funding must be made available to support and sustain these efforts. To expand the ranks of scientists focused on PTB, new investigators in PTB need to be recruited through sustained efforts to attract and retain culturally competent, junior and multidisciplinary investigators.

Design and testing of promising methods to prevent PTB are best served by enhanced understanding of basic biologic mechanisms that inform well-focused, robust, controlled, clinical trials and innovative community-based, participatory research. Ideally, basic, clinical, and community research programs are designed to reciprocally inform all responsible research on PTB. However, identification of effective interventions to prevent PTB based on sound epidemiologic approaches may be possible, despite limits in the understanding of specific biologic mechanisms. Two recent, notable examples of successful perinatal interventions are the periconceptional intake of folic acid to prevent serious neural tube defects,81 and the supine positioning and safe sleep environment to prevent Sudden Infant Death Syndrome.82,83 Promising treatments such as supplemental progesterone to prevent recurrent PTB also exemplify the need to expeditiously test the most promising interventions based on epidemiologic, laboratory, and clinical research. The urgent problem of PTB, with its profound impact on health throughout life and on health disparities, mandates intense, priority research attention through a multipronged “full court press.” The March of Dimes and its SAC are hopeful that approaching PTB as a common complex disorder will catalyze research, improvements in clinical care, and the education of communities to increasingly respond to this serious birth outcome.

Acknowledgments

We acknowledge the thoughtful input from the leadership of ACOG, AAP, and AWHONN, SMFM, as well as Dr Catherine Spong at NICHD. We also thank several March of Dimes staff members for their valuable contributions: Drs Jennifer Howse, Michael Katz, Joann Petrini, Siobhan Dolan; and Motoko Oinuma, Ann Umemoto, Lorraine Gore.

References


Appendix

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REVIEW ARTICLE

Headache as a side effect of combination estrogen-progestin oral contraceptives: A systematic review

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Objective: We conducted a 2-part systematic review of published studies to examine the evidence that combination oral contraceptives can aggravate or cause headache.

Study design: We used trials with a control group to assess headache risk that was attributable to oral contraceptive use and prospective cohort trials to answer important clinical questions about the natural history and treatment response of headache that occurs with oral contraceptive use.

Results: Because of differences in study populations, oral contraceptive formulations, trial endpoints and trial duration, it was not possible to pool data; but the evidence supports several conclusions. There is little indication that oral contraceptives have a clinically important effect on headache activity in most women.

Conclusion: Headache that occurs during early cycles of oral contraceptive use tends to improve or disappear with continued use. No evidence supports the common clinical practice of switching oral contraceptives to treat headache; however, manipulating the extent or duration of estrogen withdrawal may provide benefit.

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 KEY WORDS
 Oral contraceptives
 Migraine
 Headache
 Systematic review

It is believed widely that the use of combination estrogen-progestin oral contraceptives (OCs) can aggravate pre-existing headache or trigger the onset of troublesome headaches. Headache is consistently among the most common side effects that are reported with OC use and is a frequently cited reason for discontinuation.1-3 Women who consult physicians because of headaches frequently are advised to discontinue or avoid OCs.

An OC effect on headache is biologically plausible, but because the background incidence and prevalence of headache is high in the population of women who are most likely to use OCs, it is difficult to evaluate the true risk of headache as a potential or actual consequence of OC use. Migraine affects up to 28% of women during their childbearing years; in 1 study in which placebo was administered to 147 women who believed they were taking OCs, headache, which was the second most frequent complaint, was reported in 16% of cycles.4,5

OC use and migraine are independent risk factors for ischemic stroke. Good quality evidence and guidelines support the belief that, for some women with headache (principally those who have migraine with aura or additional risk factors for stroke), the risk of OC use is unacceptably high. The risk of stroke in women who have migraine without aura is increased by a factor of approximately 3; in women who have migraine with aura, the risk of stroke is increased by a factor of...
approximately 6 to 8, and the stroke risk increases even further with the addition of other risk factors. For example, the odds ratio for stroke in women who have migraine, who smoke, and who use OCs is approximately 34.6 Guideline from both the American College of Obstetrics and Gynecology and the World Health Organization discourage the use of OCs in women who have migraine with aura.7,8 For many women, though, especially those who have migraine without aura or other primary headache disorders, the benefits of OC use may outweigh the drawbacks. Better information about the effect of OCs on the clinical course of headache would be useful for physicians and patients who are attempting to balance the benefits and disadvantages of OC use. This is important because OCs are the most effective form of contraception and provide important noncontraceptive benefits.9

Previous reviews of this subject have not been systematic or have focused on the safety, rather than the tolerability, of OC use in women with headache.10-12 Because expert opinion on this matter conflicts, we carried out a 2-part systematic review to assess the strength of the evidence for a causal association between combination estrogen-progestin OC use and headache and to answer important clinical questions about the treatment and natural history of headache that occurs during OC use. In the first part of the review, we evaluated controlled studies to assess the risk of headache that was attributable to OC use and to distinguish it from the high background level of headache in women who use OCs. In the second part of the review, we evaluated studies that provided information on the natural history of OC-associated headache or its response to specific treatment strategies.

Sources

We collected peer-reviewed articles that assess the association between migraine or headache and the use of combination estrogen-progestin OCs. Studies were identified by several methods. We searched MEDLINE 1966 through June 2004, MEDLINE (R) In-Process and other non-indexed citations, the Cochrane Database of Systematic Reviews, and the Cochrane Register of Controlled Trials through the second quarter of 2004 using the following search terms: oral contraceptives, headache, migraine, adverse effects, and side effects. In addition, the reference lists of articles that were identified were hand-searched. We did not attempt to contact study authors.

Study selection

To be included in the first part of this review, articles were required to meet the following criteria: (1) be written in English; (2) be published fully in a peer-reviewed medical journal between 1966 and June 2004; (3) be described as a prospective study with a contemporaneous placebo, untreated, or active treatment control group; (4) be involved in the use of combined estrogen-progestogen OCs that provide active treatment for 21 days followed by 7 days of placebo or no treatment; (5) have elicited information about changes in headache or migraine; (6) reported complete and interpretable data for headache or migraine during at least an initial cycle of OC use and reported data on headache or migraine in the control group. To be included in the second part of this review, articles were required to meet the following criteria: (1) be written in English; (2) be published fully in a peer-reviewed medical journal between 1966 and June 2004; (3) have as a predetermined study goal the prospective assessment of the treatment or natural history of headache occurring with OC use; (4) provide complete and interpretable data.

In the first part of our review, we did not include studies of progestin-only contraceptives or extended duration use of OCs because most OCs in use today are estrogen-progestin combinations given on a cyclic basis.13 We did not include studies without a contemporaneous placebo, untreated, or active control group because they do not allow separation of the headache risk that was attributable to OC use from that that was attributable to a high background incidence and prevalence of headache. In the second part of this review, we did not include studies in which headache or side effects were not a specific focus of a prospective study because information about adverse events in such studies is collected in nonstandardized ways. In evaluating the natural history of headache that occurs with OC therapy, we did not include studies that lacked information about reasons for study dropout, because such dropouts could account for an apparent headache improvement with continued OC use.14

Data were abstracted independently from each study by 2 authors and compared. Differences in interpretation were resolved by consensus and through consultation with a third author. The results were summarized as narrative. For each study in which it was reported, we calculated the number of women with existing headache or migraine who reported improvement, no change, or worsening in headache or migraine during at least the first cycle of OC use. We also calculated the number of women without existing headache or migraine who experienced new onset of headache or migraine. From studies that reported only percentages, we obtained the number of women by multiplying the percentage of patients who experienced the outcome of interest by the total number of patients in the group and rounded to the nearest whole number. From studies that provided only graphic information, we estimated the numbers or percentages that were involved by measuring the graph. In crossover studies, we considered data from the first...
period of OC use only, to minimize carryover and dropout effects.

Methods quality was assessed with the Newcastle-Ottawa Quality Assessment Scale. The studies were also assessed against the following quality criteria: whether an explicit definition of migraine or headache was provided and used for diagnosis; whether information on headache change was obtained from patient report or was based on recorded diary information; whether information was collected on the magnitude or severity of headaches; and whether the study adjusted for confounding variables or predictors of headache activity. Trials were also stratified according to whether they studied a headache or general contraceptive-seeking population and whether they used high- or low-dose OCs. Because the dose of estrogen that is used in OCs has declined over the years, we categorized studies from the 1960s and 1970s as using “high dose” OCs and studies reported in 1980 or later as using “low dose” OCs.

Results

The literature search identified 121 articles. Most of the studies reported the results of trials that were performed to evaluate the contraceptive efficacy of OCs, in which information about study dropouts or adverse events such as headache inconsistently was obtained and reported. Few studies provided information about the baseline prevalence of headache in the population that was studied, and even fewer included a placebo, untreated, or active control group that allowed assessment of headache risk that was attributable to OC use. Details of studies excluded for these and other reasons are contained in the Appendix.

Part one: headache attributable to OC use

Seven studies that investigated the association between combination OC use and headache or migraine met our criteria for the first part of this review. One article reported the pooled results of 2 placebo-controlled trials that were conducted with identical methods and is treated here as a single study. Three studies were prospective, placebo-controlled trials. One study used a crossover design in which patients served as their own controls and received placebo. Two studies were prospective trials with other control groups. One study was prospective with an untreated control group. No study met all methods criteria. All but 1 study were conducted and published in the 1960s or 1970s and studied OCs with higher estrogen contents than OCs that are now in common use. Six studies examined the effect of OC use on headache or migraine in women who attended contraceptive clinics, and 1 study examined the effect of OC use in women who attended a migraine clinic. The sample size ranged from 40 to 3179 women. The Table summarizes the characteristics and findings of these 6 studies.

Placebo-controlled studies

Cullberg reported a double-blind, placebo-controlled study of 301 subjects, who used 3 different OCs that all contained 50 μg of ethinyl estradiol but had different types of progesterone. One hundred forty of 301 women (47%) reported baseline headache, which is a high figure that may reflect the systematic and careful questioning in this study. Information about new onset headache was not obtained, but during the 2-month duration of the study, 16 of 105 women (15%) in the OC group who had pre-existing headache reported improvement in headaches, compared with 7 of 35 women (20%) in the placebo group. Sixty of 105 women (57%) in the OC group who had pre-existing headache reported no important change in headache, compared with 21 of 35 women (60%) in the placebo group. Twenty-nine of 105 women (28%) in the OC group who had pre-existing headache reported worsening of headache, compared with 7 of 35 women (20%) in the placebo group. There were no statistically significant differences between the OC and the placebo groups.

A double-dummy, crossover trial of 4 different OC preparations in a general, contraceptive-seeking population used pills that were formulated to appear identical to avoid “profound” changes in reports of headache and other side effects that were demonstrated with changes in pill appearance. All subjects experienced a placebo cycle. The duration of observation was 4 cycles for the initial preparation and 2 cycles after crossover. As they entered the study, subjects were assigned to coded treatment regimens. Headache and other symptoms specifically were inquired about with “yes-no” questions at a baseline and subsequent monthly visits. The presence or absence of a symptom was scored and then averaged over 4 cycles. The first report of any complaint was also tracked, which provided a method of estimating new onset headache. No specific definition of headache or migraine was provided. In this study, 32 of 398 women (8%) women reported headache at baseline. Four of 76 women (5%) in the placebo group reported headache during the first cycle of use, compared with 15 of 79 women (19%) in the OC group. This difference was statistically significant. However, after the first cycle of use, headache complaints in the OC group declined and were not significantly different from those in the placebo group. Because no dropouts for headache were recorded, it is unlikely that this is the reason for the decline in headache complaints over time. Of note, complaints of headache did spike slightly in a group of women who were switched from a progesterone-only to an estrogen-containing OC. The authors concluded from
their averaged scores over all cycles that “headaches occur spontaneously in about 6-12% of cycles in this group of subjects, down from a pretreatment level of 20-30%. A significant increase in nausea and vomiting, headache, and nervousness could be demonstrated statistically only in the first treatment cycle with high-estrogen agents; the frequency of all other symptoms in all other cycles fell within the placebo range...It is concluded that the customary uncontrolled clinical trials of OCs attribute to these agents a substantial incidence of side effects which, in fact, would be present without the medication. Side effects such as nausea, headache...can be produced by high-estrogen agents, but the true incidence is far less than generally supposed.” The number of patients whose headaches improved or who had no change in headaches was not reported.

Ryan19 conducted a prospective, open-label, crossover study of 40 women who attended a headache clinic. Patients with contraindications to OCs were excluded. All patients had moderate or severe migraine at baseline, according to commonly accepted criteria for that condition. Twenty women were assigned randomly to receive a combination OC therapy that contained 0.5 mg norgestrel and 0.05 mg ethinyl estradiol for 2 months; the other 20 women received placebo. After 2 months, patients crossed over to the other study treatment. There were no dropouts. Using a proprietary headache index, the author calculated that headaches worsened in 28 of 40 women (70%) and improved in 12 of 40 women (30%).

Another study pooled data from 2 placebo-controlled randomized trials in a general contraceptive-seeking population; double blind, placebo-controlled (n = 301); 3 OCs, all with 50 μg ethinyl estradiol but different progestins. NOS = 5

Complaints of headache significantly higher in OC group only for first cycle of use; no significant difference when results averaged over duration of the study; 4/76 women (5%) in 1st cycle of placebo had new-onset headache compared with 31/238 women in 1st cycle of OC (13%).

Migraine worse in 70%, improved in 30% over 2-month duration of study.

### Table: Summary of trials included in Part I

<table>
<thead>
<tr>
<th>Study</th>
<th>Characteristics and Newcastles-Ottawa Quality Score (NOS)*</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cullberg17</td>
<td>General contraceptive-seeking population; double blind, placebo-controlled (n = 301); 3 OCs, all with 50 μg ethinyl estradiol but different progestins. NOS = 5</td>
<td>No significant difference between OC and placebo group on measures of worsening, improvement, or no change in headache over 2 months; no significant difference among preparations with different progestins.</td>
</tr>
<tr>
<td>Goldzieher et al18</td>
<td>General contraceptive-seeking population; double dummy (vaginal foam), placebo-controlled, cross over (n = 398); 4 OCs, 3 with ≥50 μg ethinyl estradiol, 1 progestin only. NOS = 8</td>
<td>Complaints of headache significantly higher in OC group only for first cycle of use; no significant difference when results averaged over duration of the study; 4/76 women (5%) in 1st cycle of placebo had new-onset headache compared with 31/238 women in 1st cycle of OC (13%).</td>
</tr>
<tr>
<td>Ryan19</td>
<td>Migraine population; open label, placebo-controlled, crossover (n = 40); single OC with 50 μg ethinyl estradiol, 500 μg norgestrel. NOS = 4</td>
<td>Migraine worse in 70%, improved in 30% over 2-month duration of study.</td>
</tr>
<tr>
<td>Coney et al16</td>
<td>General contraceptive-seeking population; single-blind, placebo-controlled (n = 684); single OC with 20 μg ethinyl estradiol, 100 μg levonorgestrel. NOS = 8</td>
<td>No significant difference between OC and placebo group on measure of headache reported as adverse event over 6-month duration of study.</td>
</tr>
<tr>
<td>Herzberg and Coppen20</td>
<td>General contraceptive-seeking population; open label (n = 152); 6 OCs, all with ≥50 μg ethinyl estradiol, and different progestins; barrier method control group. NOS = 7</td>
<td>No significant difference in headache complaints between OC users and control group over 11-month study; 4% of OC group reported new onset or worsened headache, compared with none of control group.</td>
</tr>
<tr>
<td>Herzberg et al21</td>
<td>General contraceptive-seeking population; open label (n = 272); 3 OCs, all with ≥50 μg ethinyl estradiol or equivalent and different progestins; IUD control group. NOS = 6</td>
<td>Slightly more women in OC group reported moderate to severe headaches than in the IUD group at 3 of 4 follow-up visits; statistical comparisons not performed; 25/86 women (30%) who discontinued OCs did so because of headache, compared with 0 in the IUD group; depression did not predict discontinuation because of headache.</td>
</tr>
<tr>
<td>Diddle et al22</td>
<td>General contraceptive-seeking population; open label (n = 3179, with 7710 women in untreated control group); multiple OCs, all ≥50 μg ethinyl estradiol. NOS = 5</td>
<td>No significant difference in headache complaints between OC users and untreated control subjects.</td>
</tr>
</tbody>
</table>

* Score on Newcastle-Ottawa scale for assessing the quality of cohort studies: Studies are awarded up to 9 stars for meeting measures of quality in study selection procedures, comparability of groups, and outcome measures; the higher the number of stars, the higher the quality of a study.15
population. The trials were aimed at assessing weight change and other adverse events that were attributable to an OC that contained 20 μg of ethinyl estradiol and 100 μg of levonorgestrel. Six hundred eighty-four women were assigned randomly in blocks of 4 to placebo or OC. The 2 groups were similar with respect to baseline characteristics, including headache frequency. More than one half of the women were former OC users. Subjects kept daily diary cards and recorded any symptoms that occurred; in addition, at each monthly visit subjects were asked, “How have you been feeling since your last visit?” Over the course of this 6-month study, 31% of the women in the OC group and 32% of the women in the placebo group reported headache as an adverse event, a difference that was not statistically significant ($P > .05$). The authors concluded that possible estrogen-related side effects such as headache occurred with a similar incidence between groups, and low comparable numbers of participants discontinued because of these adverse events.

**Active control studies**

A prospective cohort study followed 152 women who started OCs for the first time or restarted after at least 12 months off. Six different types of OCs were used, all of which contained $\geq 50 \mu g$ of estrogen, predominantly ethinyl estradiol. A control group of 40 women who were beginning a barrier method of contraception was also studied. Questionnaires that obtained specific information on headache (including information on whether the headache was moderate or severe, which is a good proxy for migraine) were administered at baseline. On follow-up side effects were noted when symptoms appeared for the first time or worsened during the course of the survey. One hundred thirty-six women (89%) in the OC group responded to the baseline and first-month questionnaire, compared with 27 women (68%) in the control group. Thirty-five women (23%) in the OC group reported moderate or severe headache at baseline, compared with 14 women (35%) of the control group. At the first follow-up questionnaire, which was administered at 5 weeks, 4% of the OC group and none of the control group reported new onset or worsened headache. Thirteen of 31 women (42%) who discontinued OC use during the 11-month period identified headache as the reason. Of those who continued, moderate or severe headache was reported by 7% of the OC group at 5 months and by 2% at 11 months.

Another study evaluated 272 women who were using an intrauterine device (IUD) or 1 of 3 OCs, 2 of which contained ethinyl estradiol 50 μg and 1 that contained 50 μg of mestranol. Women completed a baseline questionnaire regarding depression, headaches, and libido before starting contraception and at intervals during the first year of use. Side effects caused 25% of OC users and 13% of IUD users to discontinue. Depression, headaches, and loss of libido were the most common reasons for stopping OCs. None of the IUD users gave headache as a reason for stopping contraception; 25 of 86 women (30%) who discontinued OCs gave headache as the reason. At the baseline visit, 17% of women in the OC group reported headache, compared with 30% at the second visit, 25% at the third visit, 27% at the fourth visit, and 20% at the final visit. At the baseline visit, 17% of women in the IUD group reported headache, compared with 13% at the second visit, 26% at the third visit, 14% at the fourth visit, and 13% at the final visit. The authors did not report whether these differences were statistically significant. This study also examined the percentage of women with moderate or severe headaches within the group that continued OCs and the group that stopped or changed OCs. No differences were detected, which led the authors to conclude that some women were prepared to tolerate the side effects. They also noted that those women who changed because of headaches did not have a baseline depression score that was significantly different from those who changed for other reasons. This study did not distinguish between new onset headache and headache that improved or worsened.

**Untreated control studies**

Diddle et al studied 3179 women who used several different OCs, all of which contained $\geq 50 \mu g$ estrogens. The duration of observation is unclear from the study description, and it is not clear how questions were asked about headache or what the interval of visits was. Patients who attended a family planning clinic were enrolled consecutively in the study, and the definition of migraine that was used was a good approximation of current International Headache Society criteria (headaches defined as migraine if they were described as throbbing and had 2 of 5 of the following symptoms: unilateral location, associated with nausea, preceded by aura, family history migraine, history of cyclic vomiting. The incidence and prevalence of headaches in these women was compared with 7710 untreated control subjects. Demographic characteristics of the 2 groups were not reported. At baseline, 33 of 3179 of OC subjects (1%) had migraine, and 44 of 3179 women (1%) had “tension” headache, a remarkably low prevalence that suggests that subjects with headache were excluded systematically from or chose to avoid study participation. One hundred three women in the OC group (3%) complained of headache at some point during OC use, which included all patients who had pre-existing headache. Twenty-six of 103 women had new onset headache, for an incidence of $< 1\%$. Eight percent
of the women in the untreated control group reported headache, although no breakdown is given of those who had migraine compared with tension headache. No significant differences in headache were demonstrated among the many different OC preparations that were used in the study or between OC users and those in the control group. Twenty-three of the 103 women who had headache on the OC therapy discontinued because of that, but the other 82 women voiced less degree of headache when reassured of the relative safety of taking the medication. The authors commented that “...if headache occurred in relation to the pill the discomfort generally appeared shortly after the medication was withheld. A third of the complaining treated patients had migraine...There was a small number of women where the contraceptive medication either accentuated the discomfort or produced it for the first time.”

Part two: clinically relevant evidence about the clinical course and treatment response of headache that occurs with OC

Nineteen studies were identified that met criteria for inclusion in this part of the review and provided clinically relevant evidence about the natural history or treatment of headache that occurred with OCs. Results are grouped according to clinical topic.

Influence of expectations and belief

Wimberly et al. assessed 218 women to determine whether expectations about side effects were associated with experiencing those side effects. Twenty-five subjects (15%) anticipated having more headaches before taking OCs; 32 women (19%) reported more headaches at 3 months, a correlation no greater than that expected by chance alone. Most participants previously had used OCs.

The natural history of OC-associated headache

Larsson-Cohn and Lundburg followed 1676 women from a general contraceptive-seeking population for an average of 17 cycles. This study involved 16 different OCs, all of which were taken cyclically. Baseline and periodic information on headache was obtained, and the study used explicit criteria for migraine. Subjects were divided into a group of women who had headache continuously, periodically, or recurrently and a group who had headaches only occasionally or not at all. The authors commented that they used the results from the first group “to see what happened to women with pre-existing headaches” and the results from the second group “to see if OCs induced headache.” The first group was further subdivided into “1m” (defined as women with migraine on the basis of a detailed questionnaire) and “1h” (defined as women with “headache.” Thus, the 1m group contained rather well-defined cases of migraine only.

Before treatment there were 226 women (14%) in the 1m group and 88 women (5%) in the 1h group, a total of 314 of 1676 women (19%) with headache. Of the 1676 women who were studied, 1362 women (81%) had no pre-existing headache. Sixty-six of 362 women (18%) with pre-existing migraine (group 1m) experienced a worsened condition while undergoing OC therapy; the authors commented that there was a trend for women to take sequential rather than combined pills to improve the condition but that the numbers were too low for definite conclusions to be drawn. Fifteen of 199 patients (8%) in the 1h group reported worsening of headache; 42 of 362 women (12%) with pre-existing migraine (1m group) had no headaches during treatment, and 87 of 362 women (24%) had fewer headaches. In the 1h group, 24 of 119 women (20%) had fewer headaches. The differences between the 2 groups were not statistically significant.

This study also provided information on new onset headache with OC use. Of the 1352 women with no headache before treatment, 140 women (10%) had headache during treatment. The authors had calculated an age-weighted “expected frequency” of 1.14%, and determined that the difference was “highly significant” with a probability value of <.001. They also examined whether there was a family history of migraine in the women who had headache on OCs and found a family history in 22.7% versus 12.5% in the 2 groups, a statistically significant difference.

Ernst et al. studied 3679 subjects, all of whom were new users of a 20-μg ethinyl estradiol/150-μg desogestrel pill for a mean of 3.4 cycles. Of these women, 1327 (36%) had headache at baseline; 191 women (14%) reported worsening headache during OC use. New onset of headache was reported in 0.5%. Seven hundred fifty-nine women (57%) who received OCs reported improvement in headache, although 377 women (28%) reported no change in headache. No dropouts because of headache were reported.

Brill et al. studied 3267 women for up to 18 cycles who received an OC that contained 30 μg ethinyl estradiol and 0.075 μg gestodene; 513 of 3226 women (16%) reported pre-existing headaches. Improvement after 3 cycles of treatment was reported by 63% of subjects, although no definition of “improvement” is provided. No information is provided on the percentages of women with pre-existing headache who had no change or worsening in headache. Headache emerged as a new complaint in 8.8% of the women between cycles 1 and 3, in 3.9% of women in cycles 4 through 6, in 3.9% of women in cycles 10 through 12, and in 2.0% of women between cycles 16 and 18. Headache was not among the reasons for study dropout. The authors commented that “headaches, including
migraine-like symptoms...lessened in severity and had a very low incidence after the initial cycles.”

Berger et al. performed a study that examined the persistence of side effects that were reported during OC use. One hundred sixty women were assigned randomly to receive 1 of 3 OCs, all of which contained \( \geq 50 \mu g \) of ethinyl estradiol. Subjects were specifically questioned about the presence or absence of each symptom at 2-week intervals. The percentage of women who reported any side effect during the first 3 cycles of OC use was approximately 50% in all groups and did not differ significantly depending on OC type. This study examined the probability of experiencing a side effect in the second cycle of use if it occurred in the first. The probability of experiencing headache in the second cycle if it was experienced in the first was .33 (1/3 women), and the probability of experiencing headache in the third cycle if it was experienced in the first was .10 (1/10 women).

Rekers conducted a study of 1613 women and divided them into “starters” who had never used OCs before and “switchers” who had previously used OCs. He noted that “...especially for nausea and headache there were distinct differences between the two groups. Whereas the OC starters had higher incidences in the first treatment cycle compared with pretreatment, the incidences decreased already in the first cycle of use in the ‘switcher’ group.” He concluded that those women who had not used OCs previously needed some time to adjust to the exogenous hormones, as indicated by the increased incidence of nausea and headache in the first treatment cycle.

Skouby reported the results of a multicenter clinical trial with 1921 subjects that compared 2 low-dose OCs. This is one of the few trials for which dropouts because of headache and other side effects were carefully accounted. The 24-month dropout rate because of headache was 2.2%, and the prevalence of headache dropped from 6.7% of subjects after the first cycle of OC use to 3.1% after 24 months of use. The author concluded that this decline could not be explained by the dropouts alone, which indicated that the occurrence of such side effects during hormonal treatment is time-dependent and decreases during OC treatment.

Schramm and Steffens performed a multicenter trial of an OC that contained 20 \( \mu g \) of ethinyl estradiol in 2620 women. This study specifically assessed not only worsening headache but also the possibility of improvement in women who noted “migraine/headache” at baseline. In this study, 61.6% of women with previous headache said it no longer existed as a problem after 12 cycles; 20% reported worsening; 3% reported improvement, and 4% reported that the condition was unchanged. The remainder did not answer the question.

Nilsson et al. performed a randomized, double-blind, crossover study of 4 high-dose OCs. Headaches were classified as “slight,” “marked,” or “disabling.” The authors noted that the prevalence of headaches decreased significantly over the time of the entire study and that their statistical calculations did not show any significant difference between baseline headache and headache during OC treatment. They noted no significant differences in headache complaints among the various preparations that were evaluated. No dropouts because of headache were reported.

In another trial that was undertaken to assess the side effects (including headache) of 5 OCs, the authors noted that “There was a significant reduction (\( P < .10 \)) in the proportions of...users reporting most side effects between cycles 1 and 3 and cycles 1 and 6.” Generally, the reduction was larger between cycles 1 and 3 than between cycles 3 and 6.

Privrel and Daubenfeld evaluated an OC with 30 \( \mu g \) ethinyl estradiol and 75 \( \mu g \) gestodene in 246 women for up to 6 months. Only 4 subjects discontinued because of headache. The baseline prevalence of headache was 7.5%; this decreased to 4.4% at cycle 3 and to 4.8% at cycle 6.

**Effects of age**

Headache prevalence in women peaks during later childbearing years. Susceptibility to OC-associated headache also appears to increase with age. In the previously reported Larsson-Cohn and Lundberg study, the highest incidence of new onset headache was in women aged 36 to 40 years. Guillebaud reported the incidence of headache from a large pharmaceutical database of subjects who used a 30-\( \mu g \) ethinyl estradiol OC. When stratified by age, the percentage of cycles in which headache was reported was lowest in subjects aged 16 to 24 years, intermediate in women aged 25 to 34 years, and highest in women who were \( \geq 35 \) years.

**Effects of different progesterone types and doses**

The dose and type of progesterone in OCs does not appear to influence headache. A trial by Koetsawang et al. compared 2 OCs that contained 30 \( \mu g \) ethinyl estradiol but different progesterone (desogestrel or gestodene). Over the 6-cycle duration of the study, headache complaints in the 2 groups did not differ significantly. Dunson et al. assigned 892 subjects randomly to 1 of 2 OC preparations. Both preparations contained 30 \( \mu g \) of ethinyl estradiol, but one preparation contained norgestrel, and the other contained norethindrone. Subjects were specifically questioned about headache at each follow-up visit. No significant differences in headache activity were reported between the 2 groups. The previously discussed placebo-controlled study by Cullberg also examined whether women whose OC contained a higher dose of progesterone were more likely to report headache, which was not the case.
Effects of treatment strategies that manipulate the magnitude or duration of estrogen exposure or withdrawal

Headache related to OC use is widely believed to be an estrogen withdrawal symptom. A number of studies examined the effect of treatment strategies that alter the magnitude or duration of estrogen exposure or withdrawal. One such study, whose principal intent was to compare symptoms that were experienced during the 21 days on active pills compared with the 7 days off, separated headache complaints into “any headache” and “headaches rated ≥5” (on a 0-10 point pain scale). Two hundred sixty-two subjects returned prospectively recorded daily diary information about symptoms, including headache, that were attributed commonly to estrogen withdrawal. The authors separated subjects into “new starts,” which included women who had never used OCs or who had used OCs previously but had been off at least 3 months before study entry, and “current users,” who were already using other OCs but agreed to be switched to a study preparation that contained ≤35 μg ethinyl estradiol and a progestin. In most evaluated cycles, severe headache was statistically significantly more likely to occur during the placebo pill week and the few days leading up to it, compared with the 3 weeks on active OC. The authors concluded that headache activity in the last few days of active pills could be explained by the fact that combination low-dose OCs with <35 μg of ethinyl estradiol do not reliably produce complete ovarian suppression and that estrogen levels decline during the last week of active pills before the hormone-free interval.

The observation of Sulak et al about incomplete ovarian suppression with OCs that contained very low estrogen doses may explain the reason that switching to such OCs does not appear to reduce headache complaints. Gerais and Rushwan studied 165 women who used an OC that contained 50 μg of mestranol for 3 months, after which 81 subjects continued that OC for 3 months and 84 subjects were switched to an OC that contained 30 μg of ethinyl estradiol. The switch did not reduce complaints of headache or other symptoms.

In another study, there was a statistically significant increase in headache in patients who were switched from an OC that contained 50 μg ethinyl estradiol to an OC that contained 35 μg. (P < .1). It has been suggested that ovarian suppression is more complete with a 5-day hormone-free interval and that “shortening the hormone-free interval might increase the contraceptive safety margin and decrease prevalence of symptoms such as breast tenderness and headaches.” Continuous or extended duration OC use to prevent OC-related headache was first suggested in the 1960s. This method of OC use is becoming more popular, although no study to date has carefully evaluated its effect on headache. Other strategies that affect the duration or magnitude of estrogen withdrawal appear to minimize subsequent headache or migraine during OC use. One small trial evaluated estrogen supplementation during the pill-free week of OCs and concluded that this had a beneficial effect on migraine.

Ziaei et al studied 143 women who took an OC that contained 30 μg ethinyl estradiol and 150 μg levonorgestrel for 3 months. After a 1-month washout period, subjects were given the same preparation vaginally for 3 months. Subjects were questioned about symptoms at baseline and then during the last month of use of each method. The side effects of vertigo and headache were reported together. Eighty-three subjects (58%) reported headache when they used OCs, compared with only 11 women (8%) with vaginal use, a difference that was highly statistically significant. The authors ascribed these differences to the fact that vaginal steroids are “absorbed gradually into the systemic circulation and can reach the target organs in the hypothalamic-pituitary–ovarian axis without first undergoing passage through the liver.”

Nonhormonal treatment strategies for OC-related headache

Two studies that met our criteria evaluated nonhormonal treatment strategies to prevent OC-related headache. In the first, a multivitamin supplement that was given to 500 women in conjunction with OCs had no effect on a number of adverse events, including headache. This study excluded subjects with “frequent or severe migraine headaches.” Diddle et al found that the use of diuretics for those women with headache on OCs did not produce relief.

Clinical significance of headache that occurred with OC use

There is little information on the clinical significance or consequences of headache that occurred in association with OC use. One large study of >16,000 women in a large health maintenance organization was designed to evaluate major diseases that would require hospitalization or that might lead to death. The authors concluded that results that were based on the few cases of migraine or tension headache that were reported on the hospital discharge records showed no evidence of a higher risk among users that could not be accounted for by chance.

Comment

Good evidence for this issue is scarce. Because of differences in study populations, OC formulations, trial end points, and trial durations, it is not possible to pool data for quantitative analysis. There is considerable uncertainty about optimal methods of assessment of
harmful treatment effects in systematic reviews, which we considered in developing review questions and in judging study aims and quality. Despite these difficulties, we believe our results support a number of clinically relevant conclusions:

1. Controlled trials provide the strongest evidence of a cause and effect relationship. Those trials that exist do not suggest a strong, durable relationship between OC use and headache for most women. In controlled trials, increases in headache activity occur in the early cycles of OC use, but few or no persistent statistical differences can be demonstrated in headache activity among groups of women who receive OCs and those in control groups. Furthermore, this improvement is not due to subject dropout. This evidence from controlled trials is consistent with evidence and investigator impressions in numerous less rigorous trials.

2. Some women do appear to have a higher risk of headache exacerbation or new-onset headache attributable to OC use. This higher risk is most apparent in women with a strong personal or family history of troublesome headaches, particularly migraine. The incidence also increases with age. Even within these higher risk groups, some women note improvement in headache with OC use; most women report no change in overall headache activity, and headache complaints decrease with continued use.

3. The dose or type of progestin does not appear to influence headache. The relationship between headache and the estrogen dose of OCs is more complex. Headache that is related to OC use generally is precipitated by estrogen withdrawal during the pill-free or placebo pill week of treatment. It is not surprising then that estrogen supplementation during the pill-free week or vaginal administration of contraceptives may decrease headache. However, switching to OCs that contain a very low dose of estrogen (eg, 20 or 25 μg of ethinyl estradiol), which minimizes the magnitude of estrogen withdrawal, does not improve headache. This paradox may be due to the fact that OCs with very low estrogen doses do not suppress ovarian function completely. This lack of benefit on headache activity is in addition to lower rates of patient satisfaction with and adherence to very low-dose OCs because of breakthrough bleeding. Thus, an OC that provides 30 or 35 μg of ethinyl estradiol appears to be the most reasonable recommendation for women with headache who choose to use OCs. No nonhormonal treatment strategies have been demonstrated to benefit OC-associated headache.

4. Regardless of cause, when headache begins or worsens in conjunction with OC use, it tends to improve or disappear despite continued use. Women who experience headache in the first cycle of OC use can be counseled that they have only a 1 in 3 chance of experiencing headache the following month and only a 1 in 10 chance of experiencing headache in the third month.

Our review has several limitations. The search strategy may have missed some European studies that were not captured through a MEDLINE search. The decision to exclude non-English articles may also have affected our results. We also did not contact study authors to inquire about non-published studies. Among the studies that we identified, most studies reported simply on “headache,” and few used accepted diagnostic criteria to classify headache. None adequately adjusted for important predictors of headache activity (such as age or the subject population enrolled in study), and this is a possible explanation for some of the findings. In most studies, no information was available regarding the severity or functional consequences of the headaches that were reported. Statistics on the number of women who discontinue trial participation because of headache or migraine would be a useful way of assessing the clinical relevance of such symptoms, but these statistics were reported infrequently.

The studies included in this review used different OCs with varying types and amounts of estrogen. In most, the dose was higher than that in currently used combination OCs, which generally contain estrogen doses of <50 μg of ethinyl estradiol or occasionally mestranol. Because a higher dose of estrogen may be more likely to lead to subsequent headache on withdrawal, the results of this review probably overestimate the possibility of headache from OC use. If anything, this limitation would strengthen, rather than weaken, the main conclusions of this review.

Some studies that were included in our review may also have overestimated headache risk by the method in which the information about headache was elicited. A prospective, randomized investigation of the effect of study design on reported rates of symptoms with OCs showed that asking about specific symptoms increased the reporting of those symptoms compared with symptoms elicited by general inquiry. At the same time, it is also possible that some of the observed improvements in headache over time are due to placebo or other non-specific effects that were related to participation in a study.

Despite these limitations, it seems reasonable to conclude that concerns about OC effects on headache should not weigh heavily in the decision about whether to use them. Rather, the contraceptive and noncontraceptive benefits of OCs must be weighed against other tolerability and safety concerns. For women with migraine, that discussion should focus on the risk of stroke that is associated with the interaction of migraine, OC use, and their individual risk factors for stroke. Further study of this issue is important because there have been no large methodologically sound studies that used the OC formulations that are currently most
prescribed. Future studies should use precise definitions of headache and migraine and capture prospective, systematic information about the magnitude and functional effects of any headache changes in carefully matched OC and control groups.

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Appendix: Excluded studies

<table>
<thead>
<tr>
<th>Citation</th>
<th>Reason for exclusion*</th>
</tr>
</thead>
<tbody>
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**Appendix (Continued)**

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*Reasons for exclusion.
1. No control group.
2. No baseline headache data.
3. Cannot extract data.
4. Duplicate publication.
5. Did not inquire systematically about or capture information on headache.
6. Nonstandard OC regimen.
7. Not prospective.
8. Not a clinical trial.
9. Inadequate information on patient dropout or flow throughout trial.
10. Headache change not a prespecified study outcome.
11. Not an inception cohort.
12. Not in English or not within specified time period.
Colposcopic and histopathologic evaluation of women participating in population-based screening for human papillomavirus deoxyribonucleic acid persistence

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KEY WORDS
Human papillomavirus
Deoxyribonucleic acid
Colposcopy
Cervical cancer screening

Objective: Evaluation of colposcopic and histopathological findings in women screened for cervical human papillomavirus deoxyribonucleic acid persistence.

Study design: A total of 12 527 women, aged 32 to 38 years old, attending the population-based cervical cancer screening program in Sweden were randomized 1:1 to mock testing or human papillomavirus deoxyribonucleic acid testing by general primer 5+/6+ polymerase chain reaction and subsequent typing. Human papillomavirus deoxyribonucleic acid–positive women with a normal Papanicolaou smear (n = 341) and an equal number from the control group were human papillomavirus tested on average 19 months later. One hundred nineteen women with type-specific human papillomavirus persistence and 111 controls were referred to colposcopy, and 84.8% attended.

Results: Histopathology from colposcopically directed biopsies confirmed cervical intraepithelial neoplasia grade 2 or 3 in 28 of 100 of the women with human papillomavirus deoxyribonucleic acid persistence and in 2 of 95 among controls.

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doi:10.1016/j.ajog.2005.01.056
Carcinoma of the uterine cervix is a major health issue for women, being the leading cause of death in cancer among women in the developing world. The most successful preventive strategy has been population-based Papanicolaou smear screening. Management of women with abnormal Papanicolaou smears includes colposcopy and colposcopy-directed biopsies and surgical removal of histologically verified precancerous lesions (cervical intraepithelial neoplasia [CIN]).

However, there are limitations of the Papanicolaou smear. The poor specificity for high-grade CIN results in overtreatment and diagnostic labeling of healthy women, whereas the limited sensitivity necessitates repeated screening.

Infection with human papillomavirus (HPV) is considered necessary for the development of the disease. The HPV genome from a genital high-risk type is detected in up to 96.6% of the cervical carcinomas. HPV infections are common, with the highest prevalence at 20 to 24 years of age, with change of male sexual partners as the main risk factor for acquiring an HPV infection. Most HPV infections will clear spontaneously within 5 years. At the age of 35 years, the prevalence is less than 10% in most populations. Persistence of the viral infection is found mainly among women infected with high-risk types like HPV 16. Development of CIN and eventually cervical cancer will occur among women with persistent oncogenic HPV infection.

Several studies have shown that HPV deoxyribonucleic acid (DNA) testing has a higher sensitivity than the Papanicolaou smear for the identification women with cervical intraepithelial neoplasia 2 or 3 was 29%. The specificity of HPV DNA testing may be improved by restricting the screening to women >30 years of age and requiring repeated HPV DNA positivity (ie “persistence”) before referral to colposcopy.

A major question when considering HPV screening is whether the clinical management of women referred from HPV screening programs should differ from the conventional management being applied to women referred from Papanicolaou smear screening programs. However, few studies have specifically studied the colposcopic assessment of women referred from population-based HPV screening programs. To find out whether colposcopic assessment is sensitive in this group of women, we took “blind” biopsies in case of normal colposcopic findings. To address the issue of verification bias as well as the issue of specificity, a control group of women referred at random from the same cohort was subjected to the same colposcopic assessment in a double-blinded fashion. To ensure generalizability, the study was performed within a randomized intervention trial that is entirely nested within the real-life population-based, invitational screening program in Sweden. We report the results of the histopathological and colposcopic outcome of screening for HPV persistence, compared with random referral of women from the general population.

Material and methods

Study group

In the organized screening program, women aged 23 to 50 years are invited by letter for screening at 3-year intervals. The files of the population registry are first checked against cytology registries and women who have had a Papanicolaou smear taken within the previous 18 months are not invited. In the present study, the study base was defined as the entire population aged 32 to 38 years resident in 5 different regions in Sweden (Stockholm, Gothenburg, Malmö, Umeå, and Uppsala), with the following additional inclusion criteria: not having been sorted out from the organized screening program because of recent opportunistic Papanicolaou smear; having responded to the invitation letter, and having provided informed consent to participate in the HPV screening trial. The study was approved by the Institutional Review Board of the Karolinska Institute (decision number 96/305).

Altogether 12,527 women were enrolled. A complete account of the quality assessment of the enrollment will be the subject of a separate publication. Examples of incorrect enrollments that were detected and excluded from the study include an illegible or incorrect personal identification number of the woman, women showing up for HPV screening without having been invited, and women moving between cities who were invited twice.

At enrollment, both a Papanicolaou smear and a brush sample for HPV DNA testing were collected. The Stockholm, Gothenburg, Uppsala, and Malmö organized screening programs use an endocervical brush sampling for cytology. After obtaining the Papanicolaou smear, the endocervical brush with cells from both the endo- and ectocervix was placed in 1 ml NaCl, frozen at
–20°C at the enrollment center, shipped frozen, and stored frozen at –80°C at the laboratory. The screening program in Umeå uses a broom type of brush (Cervex Brush, Rovers Medical Devices B.V. Oss, The Netherlands). A pilot study determined that the most appropriate HPV DNA sampling method in that setting was to take a second sample with the endocervical brush after the Cervex sample. Testing for inhomogeneity in HPV DNA results (prevalence and sensitivity) have not revealed any differences that could be attributable to this difference in sampling strategy.

Women were randomized 1:1 to the intervention arm (HPV testing) (N = 6257) or the control arm (sample stored frozen without analysis) (N = 6270). The randomization file was made at the Stockholm Regional Cancer Registry using computer-generated random numbers. Randomization results were not released until the HPV DNA samples had arrived at the virus laboratory.

The samples from the intervention arm were screened by a general HPV general primer 5′–3′ ENH–C–ACATGAGTCTCTGACTTCCTGAT 3′–5′[22,23] Samples from Stockholm, Malmo¨, and Umea˚ were screened at the regional virus laboratories, whereas the samples from Uppsala and Gothenburg were sent to the Malmo laboratory for screening. All positive samples were sent to Malmo for repeat analysis and HPV typing by reverse-dot blot hybridization.[15] Samples were scored as positive only if confirmed by successful typing by reverse-dot blot hybridization.[15]

Among the 6089 HPV DNA tests that fulfilled the sample adequacy test (β-globin PCR), 433 were HPV DNA positive (Figure). A control group of 409 women was selected at random from the control arm. Ninety-two of the 433 HPV DNA-positive women and 11 of 409 control women were referred to colposcopy because of concomitant abnormal cytological findings. The remaining 739 women were invited for a second Papanicolaou smear and a new HPV test at least 12 months later (on average 19 months). Repeated contact attempts were made to nonresponding women for the entire time the study was open. The longest time interval between enrollment test and follow-up test was 54 months. The randomization code was not revealed to the women or the personnel managing the visits and the sampling, neither at the second smear nor at the invitation to colposcopy. Two hundred seventy of 341 (79.2%) of HPV DNA-positive women and 337 of 398 (84.7%) of the control women attended the second Papanicolaou smear and HPV test.

Reasons for nonparticipation were active refusal (68 women), relocation to city not covered by trial (18 women), no response at all to letters and also not possible to reach by phone (40 women), pregnancy (4 women), other (1 woman).

Type-specific HPV persistence was detected in 119 of 268 (44.4%) of the women in the intervention arm that had an adequate (β-globin PCR positive) second HPV test done. Fourteen women were HPV positive only for other HPV type(s) and were classified as having cleared the original infection. The 119 women who were positive for the same type of HPV DNA in both tests as well as 111 women, selected at random from the 337 control women, were referred to colposcopy (Figure 1). Finally, 195 of the 230 invited women (84.8%) attended the colposcopy at 1 of the 5 colposcopy centres. Thirty-three women declined participation (18 women with HPV persistence and 15 control women). One woman (case subject) had been referred to routine colposcopy because of a CIN diagnosed between the 2 HPV tests. One woman (control group) had never had sexual intercourse. The study flow is summarized in the Figure.

The cervical samples from the 95 control women, taken both at enrollment and at the second sampling visit, were retrieved and HPV tested and typed by the same methods as described above. The HPV tests that were performed in the control group had no influence on the selection of patients for the study.

The 195 colposcopies were performed according to a standardized protocol by 8 different gynecologists with specialist training in colposcopy. Both women and colposcopists were blinded for the HPV test results. The protocol included judgment of the transformation zone (TZ) for maturation, visibility, and border to the columnar epithelium, acetowhitem and iodine staining. First, 5% acetic acid was applied to assist in identifying undifferentiated epithelia or inflammation as well as true CIN. Thereafter, 5% potassium iodine was applied to distinguish the dimension and borderline of an abnormal area. Biopsies were taken from all acetowhite and iodine-negative lesions. If colposcopy was considered normal (ie, no acetowhite or iodine-negative lesions were identified), 2 biopsies were taken from an area at 12 o’clock and 6 o’clock on the ectocervix close to the squamocolumnar junction. All acetowhite areas, including metaplasia (undifferentiated epithelium), inflammation, and neoplasia were considered as abnormal colposcopy.

An endocervical cell sample for cytology was taken using a cytobrush. Endocervical cytobrush cytology in combination with ectocervical biopsies has been shown to have an equal or higher sensitivity than endocervical curettage in combination with ectocervical biopsies.[24]

The Papanicolaou smears taken at the time of the second HPV test of the 195 women who attended colposcopy were evaluated only after the colposcopies had been performed.

All histopathological specimens underwent routine histopathological evaluation, which formed the basis of clinical management of the patients. All histopathological specimens were sent for review by a single expert reviewer (W. R.) who was blinded to the HPV status of the women. When the routine diagnoses and expert
Women participating in the population-based cervical screening program and consenting to be enrolled
12,527 women

**Intervention group:**
HPV test performed
N = 6257

- HPV negative
  N = 5656
  - Pap smear negative:
    Invited for a second HPV test
    N = 341
    - Attended the second HPV test
      N = 270
      - HPV negative or HPV type change
        N = 149
      - Type-specific HPV persistence:
        Invited for colposcopy
        N = 119
        - Attended colposcopy
          N = 100
      - HPV test not adequate
        N = 2
      - Attended colposcopy
        N = 2

- HPV positive
  N = 433
  - Pap smear positive
    N = 92

**Control group:**
Sample for HPV test frozen
N = 6270 women

- HPV test not adequate
  N = 168
  - Pap smear negative:
    Invited for a second HPV test
    N = 398
    - Attended the second HPV test
      N = 337
      - Invited for colposcopy
        N = 111
        - Attended colposcopy
          N = 95
  - Pap smear positive
    N = 11

Figure Selection of patients for the study.
Results
Altogether 95 of 195 (48.7%) of the women who underwent colposcopy had an abnormal colposcopy (including all acetowhite lesions), 59 of 100 (59%) in the intervention group with persistent HPV infection and 36 of 95 (37.9%) in the population-based control group (Table I). These lesions predicted CIN 2 or 3 in colposcopy-directed biopsy verified by expert histopathological re-review in 23 of 58 (39.7%) of the women in the intervention group but in only 2 of 36 women in the control group (Table I). Among the women with normal colposcopy, blind biopsies revealed 1 case of CIN 3, 4 cases of CIN 2, 4 cases of CIN 1, and 2 cases of unspecified atypia (Table I).

Of 100 women in the intervention group, biopsies were taken in 98 women. Specimens from 28 of 98 (29%) contained CIN 2 or 3, whereas 57 of 98 (58%) had histopathological diagnoses within normal limits (normal, inflammatory, or koilocytosis without atypia) (Table I). In the control group, the biopsies showed CIN 3 in 1 woman, CIN 2 in 1, and CIN 1 in 6 women (6.3%). Analysis of the stored consecutive HPV tests in the control group revealed that the woman with CIN 3, but none of the other control women with CIN, had had a persistent HPV infection (Table I).

The blinded expert review resulted in some changes in histopathological diagnoses. According to routine histopathology, only 22 women in the intervention group and 1 woman in the control group had CIN 2 or 3. Furthermore, specimens reported to have koilocytosis was reduced from 42 to 12 after review. Koilocytosis had no significant association with HPV status, neither before nor after expert review (Table I).

Twenty-eight of 90 endocervical cytologies in the intervention group and 1 of 81 in the control group were abnormal (Table IIIa). One case of CIN 3 and 2 cases of CIN 2 were detected only by the endocervical cytology and not by the histopathological examination of the ectocervical biopsies (Tables II, IIIa, and IIIb).

The population-based positive predictive value of referral because of HPV persistence for detection of histopathologically confirmed CIN 2 or 3 among women with a prior negative smear was 29% (28/98 referred women), an 18-fold increased risk, compared with random referral from the population (relative risk 18.2; 95% CI 4.0-114.5). The risk of detection of CIN 2 or 3 in HPV-positive women, compared with referral of HPV-negative women was 34.4 (95% confidence interval 4.8-696.3).

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<th>Intervention group: HPV persistence, N (%</th>
<th>Control group: HPV positive, N (%)</th>
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<tr>
<td>Benign</td>
<td>50 (50)</td>
<td>79 (83.2 %)</td>
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<tr>
<td>Koilocytosis</td>
<td>7 (7.0)</td>
<td>5 (5.3 %)</td>
</tr>
<tr>
<td>Atypia</td>
<td>1 (1.0)</td>
<td>1 (1.1 %)</td>
</tr>
<tr>
<td>CIN 1</td>
<td>12 (12.0)</td>
<td>6 (6.3 %)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>12 (12.0)</td>
<td>1 (1.1 %)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>16 (16)</td>
<td>1 (1.1 %)</td>
</tr>
<tr>
<td>Missing diagnosis</td>
<td>2 (2.0)</td>
<td>2 (2.1 %)</td>
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<tr>
<td>Total</td>
<td>100</td>
<td>95</td>
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* The woman with CIN 3 in the control group and 1 of 5 HPV-positive women with benign histopathology in the control group were found to have type-specific HPV persistence in both tests; the other 4 HPV-positive women in the control arm were HPV negative in their baseline cervical sample.
The positive predictive value of abnormal colposcopy for presence of CIN 2 or 3 was 39.7% (23/58 biopsied women) in women referred because of HPV persistence but only 5% (2/36) among women with abnormal colposcopy in the control group.

“Blind” biopsies among women with normal colposcopy revealed 1 CIN 3 and 4 CIN 2 in the 40 women referred because of HPV persistence but in none of 57 women referred at random (Table I).

In 156 of 195 women who attended colposcopy, the second Papanicolaou smear taken concomitantly with the second HPV test was normal. Among these, 16 (10%) had CIN 2 or 3 in the biopsies (Table IV). All of these women belonged to the HPV screening group. Therefore, the positive predictive value of HPV DNA persistence for detection of histopathologically confirmed high-grade CIN 2+ among women who both had a normal smear at baseline and also are cytologically normal before the colposcopy is 16 of 73 (22.5%). Nine of the 17 women (53%) with cytological signs of atypia or CIN 1 in cytology had histopathological CIN 2 or 3 (Table IV). Almost all the abnormal smears at the second sampling occasion were found among the women in the HPV screening group (Table IV).

**Comment**

We found that population-based screening for HPV persistence and subsequent referral to colposcopy has a high predictive value (29%) for the presence of CIN 2 or 3. Even among women with 2 consecutive normal cytologies, the positive predictive value was 10%, supporting the concept that additional or alternative screening tools may be required. By design, all women with atypical Papanicolaou smears at enrollment into the study (whether HPV positive or negative) were taken care of according to routine clinical practice. Our study is therefore performed in the setting of screening for HPV persistence as an additional screening test on top of cytology.

Our studies of the clinical management of women with screening-detected HPV persistence found that “blind” biopsies in women with HPV persistence, but
with a normal colposcopy, resulted in detection of CIN 3 in 1 of 40, CIN 2 in 4 of 40, and CIN 1 in 3 of 40 women. It is debatable whether this suggests that blind biopsies may have a place in the clinical management of women with screen-detected HPV persistence. However, the fact that there were some CIN lesions detected, in conjunction with the known risk for future development of high-grade CIN in women with HPV persistence, 25 emphasizes that women with HPV persistence, but no detectable lesion, need to be closely followed up.

The TZ was only partially visible in 31% of the colposcopies. With age the TZ extends into the endocervix and hence more often will be out of reach for colposcopic judgment and biopsy. The fact that 28 of 90 women with HPV persistence (31%) had abnormal endocervical cytology, only partially overlapping with the results of the ectocervical biopsies, confirms that endocervical cytology should be a part of the clinical management of women referred because of screen-detected HPV persistence.

The proportion of women with koilocytosis according to histopathology was similar among those with HPV persistence and among those randomly referred. Histopathologic diagnosis of koilocytosis is currently classified as a finding within normal limits in Sweden, and the nonexistent specificity of the diagnosis found in the present study indicates that this is appropriate.

According to the results in our population-based study of more than 12 000 women, repeat HPV testing in primary screening for cervical cancer seems promising because a substantial number of high-grade CIN lesions that had been missed by cytology were detected. Although screening based on etiology-oriented principles is considered preferable, the fact that at most a few percent of initially HPV-infected individuals actually develop cervical cancer has resulted in doubts about whether HPV-based screening has sufficient specificity. The current study has found a high positive predictive value (29%) of HPV-based screening, even among women with normal Papanicolaou smears believed to be a low-risk group for CIN 2 or 3 development.

Several features of the study design that tend to improve the positive predictive value should be noted. The study was restricted to an age group (32 to 38 years) with rather low population-based HPV prevalence. 13 An HPV test with documented high sensitivity and specificity 24 was used and subjected to extensive interlaboratory comparisons of reliability before use. 25 Repeatedly positive HPV tests taken at rather long intervals (average 19 months) were required before referral,
and, finally, the testing included HPV typing and required that repeatedly HPV-positive tests should contain the same HPV type before referral.

In conclusion, our results imply that the proposed HPV screening algorithm has an acceptable specificity and positive predictive value and results in detection and treatment of a significant number of women with the established precursor for cervical cancer (CIN 2 or 3), even among women with a normal Papanicolaou smear.

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The participants of the Swedescreen study group were: Ann Kristin Andersson, Ola Forslund, Bengt-Göran Hansson, Anna Palmstierna-Bengtsson, Björn Hagmar, Anders Hjerpe, Bo Johansson, Hilde Larsson, Sven Törnberg, Charlotte Wistrand, Karin Edlund, Göran Wadell, and Margareta Larsson.

References

The effect of bright light therapy on depression associated with premenstrual dysphoric disorder

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KEY WORDS
Premenstrual syndrome
Premenstrual dysphoric disorder
Bright light therapy
Meta-analysis

Objective: This systematic review summarizes the evidence from randomized clinical trials of bright light therapy for treatment of premenstrual dysphoric disorder.

Study design: The authors performed a systematic review and meta-analysis of randomized clinical trials. They searched MEDLINE, AMED, CINAHL, Digital Dissertations, EMBASE, and the Cochrane Central Register of Controlled Trials. The main outcome measure was the change in depressive symptom scores as measured by the Hamilton Depression Rating Scale and the Beck Depression Inventory.

Results: Four crossover trials studying a total of 55 participants met inclusion criteria. Three trials showed similar results; one fully unblinded trial showed a much larger effect. The pooled effect size from the random-effects model of the 3 higher quality trials was $0.20 (95\% CI 0.07$ to $0.48$).

Conclusion: The small size of trials and correspondingly wide confidence limits, and methodologic limitations of the trials, leaves the impact of bright light therapy for relief of premenstrual depressive symptoms uncertain. The current evidence justifies neither enthusiastic dissemination nor confident rejection of this therapeutic modality.

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Premenstrual syndrome comprises periodic behavioral and somatic symptoms that include tension, irritability, hyperphagia, hypersomnia, carbohydrate craving, and dysphoria. Seventy-five percent of menstruating women experience mental or somatic symptoms during the luteal phase of the menstrual cycle. Six to 8% of North American women (particularly young women) have symptoms severe enough that they
impair social or work-related function, resulting in premenstrual dysorphic disorder (PMDD), according to DSM-IV. PMDD can lower quality of life to an extent similar to that of major depression, and may be associated with an increase in suicidal ideation and suicide attempts.3,6,7

The current first-line treatment for PMDD is selective serotonin reuptake inhibitors.1 These antidepressants may be intolerable or result in serious adverse effects, particularly in adolescents.8-10 Treatments such as bright light therapy, which may also act to increase brain serotonin levels and production, may represent a safe and attractive alternative.11 Although widely promoted as an effective intervention in the lay literature, randomized controlled trials of bright light therapy performed to date have failed to resolve the controversy regarding effectiveness.12 To further inform this issue, we conducted a systematic review and meta-analysis to determine the extent to which bright light therapy reduces depressive symptoms in patients with PMDD.

Material and methods

Search strategy and eligibility criteria

We searched the following electronic databases, from their inception to September 11, 2004: MEDLINE, AMED, CINAHL, Digital Dissertations, EMBASE, and the Cochrane Central Register of Controlled Trials. We used the terms phototherapy, light, light therapy, light exposure, premenstrual syndrome, late luteal-phase dysphoric disorder, and premenstrual dysphoric disorder. We also searched the reference sections of included trials, and contacted experts in the field requesting information about unpublished or ongoing trials. We posted a query in the electronic list for members of the Society for Light Treatment and Biological Rhythms (www.sltbr.org). Eligible studies were placebo-controlled randomized trials investigating the effect of bright light therapy on depressive symptoms, measured using validated instruments, in patients with PMDD. Two reviewers (CK and JWB) independently assessed study eligibility and achieved perfect agreement.

Data extraction and quality assessment

Two reviewers (CK and JWB) extracted the relevant data using a standardized protocol and data collection form. Data extracted included the method of random treatment allocation, concealment of allocation, blinding of study participants, intensity, duration and timing of light therapy used, outcome measures, and adverse effects. Reviewers resolved disagreements through discussion. Two trials lacked sufficient data for analysis, and the authors were contacted directly to acquire the missing information. Two reviewers (CK and JWB) independently assessed study quality by appraising the method of randomization, concealment of allocation, blinding, and handling of withdrawals.13

Statistical analysis

For each trial, we computed an effect size for the change in depressive symptoms using Hedges’ adjusted g method, a computation of Cohen’s d (effect size) that adjusts for small-sample-size bias.13 This method expresses the difference between treatment arms in terms of the standard deviations for the outcome measure. Following Cohen, we considered an effect size of ≤0.2 as small, of 0.3 to 0.5 as moderate, and of >0.5 as large.13 For 2 studies for which we could not obtain the relevant standard deviations,14,15 we imputed these using the correlation coefficient estimated from a study for which we had complete individual patient data.16 The estimation of these studies’ effect sizes did not change the interpretation of the pooled estimates across the entire range of values for the correlation coefficient (0.1–0.9). In one instance, authors reported the treatment effect using the odds ratio for a 50% reduction in Hamilton scores.15 We converted this odds ratio to an effect size.17 We did not have access to period-specific data, and treated crossover studies as if they were parallel design trials, which is a conservative assumption.

We used the Review Manager software13 to conduct random-effects meta-analyses. Heterogeneity between studies was assessed by using the Q statistic (χ²),18 and we used the I² statistic to quantify inconsistency, the proportion of between-study variability that was not due to chance.19 The a priori potential explanations for heterogeneity in this meta-analysis that provided the basis for subgroup analyses were: lack of blinding, severity of depression at baseline, and the bright light therapy dosing regimen, ie, intensity, duration, and whether participants received bright light therapy during the morning or the afternoon. We evaluated the effect of the intervention in these subgroups of trials using a test for interaction.20

Because both the Hamilton Depression Rating Scale (HDRS) and the Beck Depression Inventory (BDI) measure the same underlying domain (depression), we pooled these data within trials that reported both outcomes1,6,21,22 to improve the precision of each trial’s weighted average. We calculated a measure of variance of these weighted averages by assigning a correlation of 0.61 between the HDRS and BDI. Correlations between the BDI and the HDRS have been reported in the literature to range from 0.61 to 0.86,23,24 and we elected to use the most conservative estimate. Further, we elected to perform sensitivity analyses by assuming correlations of 1 and 0 in order to investigate the impact of this approach on our findings.
Our search identified 102 articles; 8 published studies\textsuperscript{14,15,21,22,25-28} and 2 unpublished studies\textsuperscript{16,29} were retrieved for determination of eligibility. A participant in The Society for Light Treatment and Biological Rhythms listserve\textsuperscript{30} provided one unpublished study\textsuperscript{16}; the other was located through a dissertation database.\textsuperscript{26} Of the 8 studies, 2 were observational studies and did not examine depressive symptoms,\textsuperscript{27,28} and 1 did not make use of a validated instrument to measure depression.\textsuperscript{29} This left 7 eligible trials, each of which measured depressive symptoms as an outcome, by use of either the HDRS or the BDI. Four of these studies shared the same participants.\textsuperscript{14,15,25,26} The most recent trial that reported on all previous participants\textsuperscript{15} provided data for analysis.

All eligible trials lacked an adequate description of randomization procedures. Allocation concealment was either not reported\textsuperscript{15,21,22} or reported as not done.\textsuperscript{16} Only after contacting the authors were we able to clarify blinding and allocation concealment status: only 1 trial\textsuperscript{22} blinded participants as well as clinical and research personnel. Two trials\textsuperscript{15,21} blinded research personnel but not participants, and in 1 trial\textsuperscript{16} neither the participants nor the research or clinical personnel were blinded. Only 1 study concealed allocation.\textsuperscript{22}

Three trials\textsuperscript{16,21,22} specified DSM diagnostic criteria for establishing PMDD among participants. Only 1 study mentioned adverse effects, which were increased agitation and eye strain.\textsuperscript{15} One participant reported agitation severe enough to cause her to withdraw from treatment, and another reduced her light treatment time in half. All 4 trials reported that bright light therapy was effective in reducing depressive symptoms\textsuperscript{15,16,21,22}; however, these conclusions were based on the differences in baseline and post-treatment scores within the treatment phase rather than the appropriate comparison, end of treatment vs end of control phase results.

Assuming a correlation of 0.61 between the HDRS and the BDI, the pooled standardized mean difference in depression scores from 4 trials\textsuperscript{15,16,21,22} was $-0.39$ (95% CI $-0.85$ to 0.06); however, heterogeneity was substantial ($I^2 = 45.3\%$; \chi$^2 = 5.49$, $P = .14$). After excluding the fully unblinded trial,\textsuperscript{16} the $I^2$ was reduced to 0% ($\chi^2 = 0.34$, $P = .84$), and the estimate of effect size was $-0.20$ (95% CI $-0.48$ to 0.07) (Figure). Our sensitivity analyses, assuming extremes in correlations between the HDRS and the BDI, did not substantially alter these findings.

Evidence from small trials with limited safeguards against bias suggests a small effect of bright light therapy in the treatment of depressive symptoms in women with PMDD, but the imprecise results remain

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{The impact of bright light therapy versus control on depression associated with PMDD, assuming a correlation of 0.61 between the HDRS and the BDI.}
\end{figure}
consistent with no effect and with a moderate effect. Clinical trials of PMDD have shown a substantial placebo effect, and including only randomized trials strengthens our findings. Our analysis revealed that claims of superiority in the included trials were based on changes from baseline during the treatment phase and not on comparisons of treatment against the control phase. This misinterpretation of the data may have contributed to the numerous claims in the lay literature for the efficacy of bright light therapy in management of PMDD. A recent Internet search using the terms “bright light therapy” and “premenstrual syndrome” (Google, Dec 27, 2004) yielded 5310 hits, with the vast majority promoting this therapy, and a number citing the trials we evaluated as proof of efficacy (eg, http://www.outsidein.co.uk/res_pms.htm). The available evidence, while suggesting the possibility of small effects of bright light therapy, is limited by methodologic quality and sample size. Larger trials are needed to define what role, if any, bright light therapy has in treatment of depression associated with PMDD.

Acknowledgments

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References

Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer

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KEY WORDS
Ovarian cancer
Tumor DNA
p53 mutations
Prognosis
Peritoneal fluid

Objective: Free tumor DNA in body fluids may be an important biomarker. We tested whether tumor-specific mutated p53 DNA can be detected in blood and peritoneal fluid from women with epithelial ovarian cancer.

Study design: Sequencing of tumor DNA identified somatic p53 mutations. Free DNA from matched blood or peritoneal fluid was evaluated for the tumor-specific p53 mutation using a ligase detection reaction.

Results: Sixty-nine of 137 tumors (50%) had p53 mutations. Plasma or serum from 21 (30%) of the 69 informative cases contained the tumor-specific p53 mutation. Circulating tumor was an independent predictor of decreased survival in multivariate analysis (P = .02). We detected tumor DNA in peritoneal fluid in 28 of 30 (93%) cases, including all 6 cases with negative cytology.

Conclusion: One third of women with ovarian cancer have circulating tumor DNA and an associated reduced survival. Free tumor DNA can be detected in the majority of peritoneal fluid samples.

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widely between studies, likely secondary to differences in tumor types, stage distribution of patients studied, target alterations evaluated, and assays used. The relative proportion of ovarian cancer patients with tumor-specific circulating DNA is not certain. The identification of tumor DNA in blood or other body fluids is a promising biomarker for a variety of cancers. Identifying tumor-specific molecular alterations in urine, saliva, sputum, and stool can be a noninvasive diagnostic test for cancer. 6-9 Most ovarian cancers spread through local dissemination in the peritoneal cavity or through local lymphatic channels, and ovarian cancers differ from other solid tumors in the rarity of hematogenous metastasis. Conceivably, the differences in spread patterns of ovarian cancer might lessen the likelihood of finding associated circulating tumor DNA compared with other malignancies.

p53 mutations are the most common single somatic alteration in ovarian cancer, and occur in early as well as advanced staged disease. 10,11 Mutations in p53 may be a sensitive indicator of the presence of circulating tumor DNA. 12,13 We tested whether tumor-specific p53 mutations could identify circulating tumor DNA in women with epithelial ovarian cancer. We evaluated the association of tumor DNA in plasma or serum with patient prognosis. We likewise tested paired peritoneal fluid samples to identify tumor DNA in peritoneal fluids.

**Material and methods**

**Tissues: DNA extraction and quantitation**

Blood and tumor tissue was collected by the University of Washington Gynecologic Oncology Tissue Bank as approved by the Human Subjects Committee of the Institutional Review Board. Platinum resistance was defined as less than a complete response to chemotherapy or relapse within 6 months of completing chemotherapy. Tumors were surgically staged according to the International Federation of Obstetrics and Gynecology (FIGO) criteria. 14

DNA was extracted from lymphocytes and 137 tumors that were microdissected, if necessary, to attain a neoplastic cellularity \( \geq 70\% \). Plasma, serum, and cell-free peritoneal fluid DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, Calif) as previously described. 2 Some samples were concentrated in a speed vac \( 2-4 \times \). Ten to 15 \( \mu L \) of plasma DNA was used in amplification reactions. Plasma DNA was quantified after extraction using the PicoGreen dsDNA Quantification Kit (Molecular Probes, Inc, Eugene, Ore) according to the manufacturer’s instructions. Total DNA in plasma was calculated based on 100% extraction efficiency.

**DNA sequencing**

DNA was polymerase chain reaction (PCR) amplified for all p53 coding exons (2-11) and flanking regulatory regions. Primer sequences and PCR conditions are available from the authors. PCR products were purified and sequenced using Big Dye Terminator chemistry (Perkin-Elmer, Boston, Mass), and run on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif). Sequencing data were analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, Mich). All chromatograms from tumor and plasma sequences were reviewed and compared with those from corresponding normal DNA.
Ligase detection reaction

For each p53 exon harboring a mutation, nested PCR products were used in the ligase detection reaction plasma or serum, tumor, peritoneal fluid (if available), and normal DNA. For each p53 mutation, 2 10-15 base oligomers were designed containing the mutant sequence at the 3 prime end of the 5 prime oligomer. The second oligomer was specific to the sequence just adjacent (3 prime) to the mutant sequence. The mutation-specific oligomer was end labeled with $\gamma^{32}$P using phosphonucleotide kinase (Roche, Indianapolis, Ind). Ligase reactions included 4 μg Salmon sperm DNA (Invitrogen, Carlsbad, Calif), 5 nmol $^{32}$P- labeled mutation-specific oligomer, 5 nmol unlabeled adjacent oligomer, 0.25 μmol dithiothreitol, 12.5 U thermo-stable ligase (Ampligase, Epicentre, Madison, Wis), Ampligase buffer (Epicentre), 5 μL purified PCR product and water in a 25 μL reaction. Ligase reactions were cycled 35 times at 94°C for 30 seconds and 37°C for 10 minutes. Ligase was inactivated at 99°C for 10 minutes. Specific oligomer sequences are available from the authors. Radiolabeled ligated products were separated on a 6% polyacrylamide gel, and detected with autoradiography.

Every plasma sample was evaluated by ligase detection reaction in tandem with a positive control (tumor DNA known to contain the mutation) and a negative control (normal DNA from case known not to contain the mutation). To test the sensitivity of the assay we diluted tumor DNA into normal DNA. We reliably detected the mutation-specific oligomer, 5 nmol unlabeled adjacent oligomer, 0.25 μmol dithiothreitol, 12.5 U thermo-stable ligase (Ampligase, Epicentre, Madison, Wis), Ampligase buffer (Epicentre), 5 μL purified PCR product and water in a 25 μL reaction. Ligase reactions were cycled 35 times at 94°C for 30 seconds and 37°C for 10 minutes. Ligase was inactivated at 99°C for 10 minutes. Specific oligomer sequences are available from the authors. Radiolabeled ligated products were separated on a 6% polyacrylamide gel, and detected with autoradiography.

Statistics

Contingency tables were evaluated with the Fisher exact tests or with chi-square using InStat (Graphpad Software, San Diego, Calif). Survival curves were generated according to the Kaplan-Meier method using Prism 4 (Graphpad Software). Differences between survival curves were tested with the log-rank method. Multivariate analysis was performed using Cox proportional hazards modeling with Statview (SAS, Cary, NC).

Results

Tumor-specific variants in p53 sequences

Somatic p53 mutations were detected in 69 of 137 tumors (50%) (Table I). Forty-eight (70%) mutations were missense, occurring exclusively in exons 5-8. Twenty-one (30%) mutations were null mutations, consisting of 10 nonsense (14%), 9 deletion (13%), and 2 splice site (3%) mutations. Twelve (17%) mutations occurred in exons 4 (n = 7), 9 (n = 2), or 10 (n = 3).

Of the 69 cases with somatic p53 mutations, the tumor-specific p53 sequences were detected in 21 plasma or serum samples (30%, Figure 1). Detection of free tumor DNA was similar in plasma (18/61, 30%) and serum samples (3/8, 38%). Free DNA concentration in plasma of women with ovarian cancer ranged from 1 to 99 ng/mL (median 35 ng/mL), and was similar between cases with and without tumor DNA in plasma.

All women received primary therapy containing a taxane and platinum agent. Table II indicates the clinical characteristics of the tumors relative to the absence or presence of tumor DNA in plasma. There was no difference between the distribution of stage, grade, or adequacy of surgical cytoreduction. One of 3 informative FIGO stage I cases demonstrated circulating tumor DNA. Preoperative serum CA-125 levels were similar between cases with and without tumor DNA in plasma.

Tumor DNA in plasma and serum predicts poor survival

Overall survival was significantly reduced in cases with tumor DNA in plasma (log rank test: $P = .01$, Figure 2, median survival 28 vs 56 months). Among the 64 FIGO stage III and IV cases, surgical cytoreduction ($P = .0004$) and stage ($P = .06$) were additional predictors of survival. Cox multiple logistic regression of stage III and IV cases revealed circulating tumor DNA ($P = .02$) and surgical cytoreduction ($P = .003$), but not stage ($P = .2$), as significant in predicting overall survival. Circulating tumor DNA was not associated with likelihood of complete response to initial chemotherapy (77% vs 73%) or platinum resistance (29% vs 47%, $P = .23$). Two patients with, and no patients without tumor DNA in plasma developed brain metastases ($P = .09$, Fisher exact test, 95%CI 1.2-30.4), exceeding the predicted <1% incidence of brain metastases in ovarian cancer.15

<table>
<thead>
<tr>
<th>Table I</th>
<th>Ovarian tumor characteristics and the presence of circulating tumor DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No tumor DNA</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>3</td>
<td>45 (94%)</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>II</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>III</td>
<td>39 (81%)</td>
</tr>
<tr>
<td>IV</td>
<td>5 (11%)</td>
</tr>
<tr>
<td>Surgical cytoreduction*</td>
<td></td>
</tr>
<tr>
<td>Optimal (≤1 cm)</td>
<td>27 (61%)</td>
</tr>
<tr>
<td>Suboptimal (≥1 cm)</td>
<td>17 (39%)</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
</tr>
</tbody>
</table>

* Data for volume of residual disease after initial surgery were not available for 7 cases. Adequacy of surgical cytoreduction is assessed by the largest residual tumor diameter at completion of surgery.
Tumor DNA in peritoneal fluid

In 30 cases with somatic p53 mutations, we evaluated free DNA in peritoneal washings or ascites for the tumor-specific sequence using the ligase detection reaction. We identified the tumor-specific p53 sequence in the peritoneal fluid of 28 of 30 (93%) women with intraperitoneal ovarian cancer, including all 6 cases with no malignant cells identified on cytopathology (Table II), and 22 of 24 cases with malignant cytology. We identified tumor DNA in the peritoneal fluid of 2 early stage cases that were limited to a single ovary without surface involvement and had negative cytology. Overall, cytology was positive in 24/30 cases (80%), while tumor DNA was identified in 28/30 (93%). Cytology identified malignant cells in 1 of 3 FIGO stage I cases, while tumor DNA was identified in all 3 stage I cases. All 30 cases were correctly identified by either cytology or the presence of tumor DNA.

**Comment**

Tumor-derived DNA has been identified in the body fluids of patients with a variety of cancers, including colorectal, head and neck, lung, bladder, kidney, and prostate.6-8,16 Because the ovary is in direct contact with the peritoneal cavity, we hypothesized that the detection of tumor-specific alterations in peritoneal fluid would be a sensitive indicator of disease. Tumor DNA has been previously identified in cytologically malignant peritoneal fluid samples from women with ovarian cancer, but rarely in cytologically negative or early stage samples.17,18 Using the p53 ligase detection reaction, we identified tumor DNA in 28 of 30 peritoneal fluids, including 6 cases that were negative for malignant cells as determined by the cytopathologist (Table II).

Tumor DNA was identified in peritoneal fluid from all 3 stage I cases, while cytology identified malignant cells in only 1 case. Combining cytology and tumor DNA analyses correctly identified all 30 cases. Our increased detection rate in peritoneal fluid compared to previous studies may be secondary to our use of cell-free instead of cellular peritoneal DNA.18 The detection of free tumor DNA in peritoneal fluid could augment cytologic detection of malignant cells. However, the use of tumor DNA in peritoneal fluid as a diagnostic tool is limited at present by the lack of a p53 mutation in half of tumors and a dearth of other specific somatic DNA sequence alterations in ovarian cancer. More than 80% of ovarian cancers associated with germline mutations in BRCA1 or BRCA2 contain p53 mutations.19,20 Thus, p53 mutations are likely to be more sensitive for identification of tumor DNA in women at elevated genetic risk of ovarian cancer.

Free tumor DNA in plasma or serum was present in one third of women with advanced ovarian cancer, and was a strong independent predictor of decreased survival. Presence of circulating tumor DNA has also been associated with worse prognosis for patients with melanoma, colorectal, breast, and esophageal cancer.21-24 The quantity of total DNA among women with ovarian cancer did not predict the presence of tumor-derived DNA sequences in plasma. Thus, simply quantifying DNA in plasma does not predict survival, nor does it substitute for specific assays that identify tumor-derived sequences.

**Table II** Detection of tumor DNA in peritoneal fluid in those cases with negative cytology

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Fluid source</th>
<th>Surgery</th>
<th>Grade</th>
<th>FIGO stage</th>
<th>Histology</th>
<th>p53 alteration nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>Ascites</td>
<td>1st surgery</td>
<td>3</td>
<td>IIIC</td>
<td>Papillary serous</td>
<td>535 C&gt;T</td>
</tr>
<tr>
<td>131</td>
<td>Wash</td>
<td>1st surgery</td>
<td>2</td>
<td>IA</td>
<td>Endometrioid</td>
<td>818 G&gt;A</td>
</tr>
<tr>
<td>178</td>
<td>Wash</td>
<td>1st surgery</td>
<td>3</td>
<td>IA</td>
<td>Carcinoma (NOS)</td>
<td>976 del31</td>
</tr>
<tr>
<td>187</td>
<td>Wash</td>
<td>1st surgery</td>
<td>1</td>
<td>IIIA</td>
<td>Borderline serous</td>
<td>742 C&gt;T</td>
</tr>
<tr>
<td>124</td>
<td>Wash</td>
<td>Recurrence</td>
<td>3</td>
<td>IIIIC</td>
<td>Carsinosarcoma</td>
<td>817 C&gt;T</td>
</tr>
<tr>
<td>97</td>
<td>Wash</td>
<td>Second look</td>
<td>3</td>
<td>IIIIC</td>
<td>Endometrioid</td>
<td>833 C&gt;T</td>
</tr>
</tbody>
</table>

NOS, Not otherwise specified.

**Figure 2** Kaplan-Meier analysis of overall survival relative to the presence of circulating tumor DNA. Tumor DNA in plasma or serum was a significant predictor of decreased survival among all 69 informative cases (P = .01, log rank test), with a median survival of 28 months vs 54 months in women without tumor DNA in plasma.

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We anticipated that the sensitive ligase detection reaction would detect tumor-specific p53 mutations in a greater proportion of cases than DNA sequencing. However, an identical proportion of plasma samples were positive for tumor alterations using each method. The consistency of the proportion of patients with circulating tumor DNA, regardless of the assay, and the strong association with clinical outcome suggests an underlying biological difference in ovarian cancers associated with circulating tumor DNA.

A recent study identified tumor-specific methylation changes in the serum of most women with ovarian cancer. Methylation-sensitive PCR (MSP) has been shown to be highly sensitive, and the differences in assays and target sequences may explain the lower detection rate in this study. However, MSP results may be difficult to replicate between laboratories, and may lead to false-positive results secondary to incomplete conversion of DNA during bisulfite treatment. Further studies are needed to compare the detection of tumor-specific methylated promoter sequences and somatically mutated sequences with various techniques to optimize identification of circulating tumor DNA in women with ovarian cancer.

A higher proportion of patients with pancreatic, colon, and lung cancers have circulating tumor DNA than we found in women with ovarian cancer using comparably sensitive assays. Pancreatic, lung, and colon cancers have much higher rates of hematogenous spread than is seen with ovarian cancer. The lower proportion of women with ovarian cancer with circulating tumor DNA in this study may relate to the low rate of hematogenous dissemination seen in ovarian cancer patients. If this is true, understanding the differences between those cases with and without circulating ovarian tumor DNA may provide insight into the unusual spread patterns of epithelial ovarian cancer. The relatively high incidence of brain metastases in our small set of patients with circulating tumor DNA supports the hypothesis that circulating tumor DNA is associated with a more aggressive tumor biology and an increased risk of hematogenous spread.

Tumor DNA in plasma is a significant, independent predictor of poor prognosis, and is associated with more aggressive tumor behavior. The identification of free tumor DNA in peritoneal fluid compares favorably to cytopathologic evaluation, and warrants further investigation as a diagnostic marker. Free tumor DNA in blood or peritoneal fluid may represent an important new biomarker in ovarian cancer.

References


Melanoma, thyroid, cervical, and colon cancer risk after use of fertility drugs

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Objective: This study was undertaken to evaluate melanoma, thyroid, colon, and cervical cancer risks after clomiphene or gonadotropins.

Study design: Retrospective cohort of 8422 women (155,527 women-years) evaluated for infertility (1965-1988). Through 1999, cancers were ascertained by questionnaire, cancer and death registries. Poisson regression estimated adjusted rate ratios (RRs).

Results: Clomiphene use did not significantly increase risk of melanoma (RR = 1.66; 95% CI, 0.9-3.1), thyroid (RR = 1.42; 95% CI, 0.5-3.7), cervical (RR = 1.61; 95% CI, 0.5-4.7), or colon cancer (RR = 0.83; 95% CI, 0.4-1.9). We found no relationship between clomiphene dose or cycles of use and cancer risk at any site. Clomiphene use may impart stronger effects on risks of melanoma (RR = 2.00; 95% CI, 0.9-4.6) and thyroid cancer among women who remained nulliparous (RR = 4.23; 95% CI, 1.0-17.1). Gonadotropins did not increase cancer risk for these sites.

Conclusion: Fertility drugs do not appear to have strong effects on these cancers. Nonetheless, follow-up should be pursued to assess long-term risks and to monitor effects among women who remain nulliparous.

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the risk of these cancers associated with fertility treatments among infertile women, with small numbers of cancers and inconsistent results.\textsuperscript{10-13} This report summarizes melanoma, thyroid, cervical, and colon cancer risk among a large cohort of women from different clinical sites in the United States. Strengths of the study included nearly 20 years of average follow-up and information on other predictors of cancer risk, including specific causes of infertility and reproductive status through follow-up.

Materials and methods

Brinton et al\textsuperscript{5} previously described this retrospective cohort study, which was conducted at 5 large reproductive endocrinology practices in the following metropolitan areas: Boston, Mass; New York City, NY; Chicago, Ill; Detroit, Mich; and the San Francisco Bay Area, Calif. The institutional review boards at the collaborating centers as well as at the National Cancer Institute (NCI) approved the study protocol. Briefly, eligible patients were evaluated for infertility between 1965 and 1988 at participating centers where they were seen more than once, or if seen only once, were referred by another physician who provided substantive medical information. Patients with either primary or secondary infertility (nulligravid and gravid, respectively) were eligible for the study, whereas those who were evaluated for reversal of a tubal ligation were not. Medical records for 12,193 eligible women were abstracted for information pertaining to all procedures and tests (allowing a determination of different causes of infertility), medications prescribed (including clomiphene citrate and a variety of human gonadotropins, namely, Pergonal, Humegon, or Metrodin), menstrual and reproductive histories, and other factors that might affect health status.

A total of 9751 (80.0\%) of the patients were successfully traced with the use of several sources, including clinic records, telephone directories, credit bureaus, postmasters, motor vehicle administration records, and the National Death Index. A total of 1319 of the eligible women (10.8\%) indicated on contact that they did not want to participate in the study. For these women, we retained only information on calendar year and age at study entry, and race.

For the patients traced as alive (we identified 272 patients as deceased), clinic records, completed questionnaires, and cancer registries provided information on the development of cancers. We mailed questionnaires to patients beginning in 1998, with telephone follow-up attempted for nonrespondents. A total of 5597 of the patients completed the questionnaire that ascertainment information on sociodemographic factors, updated health status, and lifestyle factors, including menopausal, pregnancy, and breastfeeding history; use of exogenous hormones; and anthropometric factors. For patients for whom we were unable to obtain questionnaire data, we had accurate location information on cancer status through clinic records (216), or cancer registries (2347) if the women last resided in California, Florida, Illinois, Massachusetts, Minnesota, New Jersey, New York, and Texas (eg, the states in which the majority of patients were last known to reside). We attempted to medically verify cancers reported in the questionnaires by obtaining discharge summaries, operative reports, and pathology reports from the institutions where the diseases had been diagnosed and/or treated. Eleven of the self-reported melanomas and 1 colon cancer were found on medical record review to be benign and were excluded from analytic consideration.

Statistical methods

Person-years accrual began 1 year after clinic registration and continued through the earliest date of cancer diagnosis, death, or date last known alive and free of cancer (as indicated by the last clinic visit, questionnaire completion, or linkage against cancer registry data). Patients having cancer registry searches had variable study ending dates, depending on the completeness of registration, which ranged from the end of 1997 to 1999. Otherwise, December 31, 1999, defined the end of the study period. Patients lost to follow-up after their initial clinic visit, those who denied access to their records, and 10 woman who had cancer diagnosed within 1 year of their registration clinic visit were excluded, leaving 8,422 analytic study subjects and 155,527 person-years (mean = 18.8 years) of follow-up.

We used 2 analytic approaches to assess cancer risk among the cohort members. We first calculated standardized incidence ratios (SIRs) and 95\% CI comparing cancer rates of infertile women with those of US women. SIRs were computed as the number of observed cancer events divided by the expected number of events that were based on age, race, and calendar year-specific incidence disease rates for women from cancer registry rates available through the Surveillance Epidemiology and End Results (SEER) Program of the NCI. The SEER program has population-based catchment areas and is widely used to estimate cancer burden in the United States.

The second analytic approach involved analyses within the cohort of infertile women, which allowed multivariate adjustment for potential confounding factors. Rate ratios (RRs) and their 95\% CIs for developing cancer associated with administration of ovulation-stimulating drugs (ever use, total dosage, cycles prescribed, interval since first use) as compared with nonusers were estimated by Poisson regression with the use of standard methods.\textsuperscript{14} For all analyses, the RRs were adjusted for study site, age at follow-up (<40, 40-49, 50+), and
calendar year of follow-up (before 1980, 1980-1989, 1990, or later), and gravidity at entry. Factors that were available from the medical records, such as cause of infertility, smoking history, and body mass at entry, were included in the regression models, as necessary, to evaluate their roles as potential confounding or modifying factors. For the subjects who completed the questionnaire, we evaluated additional predictors of risk, such as parity and gravidity at follow-up and hormone replacement use.

**Results**

**Description of subjects included in analysis**

The median year of first evaluation was 1978 and the median age of the study subjects at first evaluation was 30 years. Nearly 80% of the subjects were known to be white and 43% were evaluated for primary infertility. A total of 3276 (39%) of the study subjects were prescribed clomiphene to treat their infertility, whereas 865 (10%) received gonadotropins. Subjects included in the analyses and those excluded were not significantly different according to calendar year and age at first evaluation; however, a larger proportion of the subjects excluded from analysis had missing information on race (30% vs 11%).

**SIRs analysis of cancer**

Infertile study subjects developed cancers at higher rates than women in the general SEER population (SIR = 1.23; 95% CI, 1.1-1.3) (Table I). Elevation in risk was evident for tumors that are most well-recognized as having hormonal causes, namely, cancers of the uterine corpus (SIR = 1.57), breast (SIR = 1.29), and ovary (SIR = 1.98), as well as colon cancer (SIR = 1.76) and melanoma (SIR = 1.57). Thyroid (SIR = 0.99) and cervical cancer risk (SIR = 0.61) were the only malignancies postulated to have a hormonal cause that were not more frequently diagnosed among infertile women in the study cohort compared with the general population. Infertile women did not have a higher risk of cancers at other sites, when assessed individually or in aggregate (data not shown).\(^{15}\)

Clomiphene-exposed women were at higher risk of tumors at only 2 sites: uterus (SIR = 2.14; 95% CI, 1.3-3.3 discussed in detail in Althuis et al\(^3\)) and melanoma (SIR = 2.00; 95% CI, 1.3-3.1). Risk among women not exposed to clomiphene was similar to the general population for both of these cancer sites, with SIRs of 1.25 (0.8-1.9) and 1.28 (0.8-2.0), respectively. We found no difference in risk among “ever used” compared with “never used” clomiphene users for the remaining cancer sites. In addition, cancer risk did not vary by gonadotropin use when comparing cohort members were compared with the US population (data not shown).

**Internal analyses of cancer risk**

To assess the influence of ovulation-stimulating drugs on cancer risk while adjusting for other predictors of cancer risk (including gravidity), subsequent analyses were within the population of infertile women, comparing drug users with nonusers. We found little evidence to suggest that melanoma, thyroid, cervical, or colon cancer risk was increased by ovulation-stimulating drugs within the cohort of infertile women (Table II). Although point estimates for melanoma (RR = 1.66; 95% CI, 0.9-3.1), thyroid cancer (RR = 1.42; 95% CI, 0.5-3.7), and cervical cancer risk (RR = 1.61; 95% CI, 0.5-4.7) were modestly elevated among clomiphene users, these findings were not statistically significant and there was no dose-response with more detailed parameters of drug usage (dosage and cycles) for any site. Clomiphene use appeared to impart stronger effects on risks of melanoma, thyroid, and cervical cancer among women who were followed for 15 or more years, with RRs relative to never users of 2.08 (0.9-4.9), 1.54 (0.3-8.0), and 2.67 (0.6-11.9), respectively. However, these findings were based on small numbers and did not reach statistical significance. Risk associated with ever compared with never clomiphene use was 0.83 for colon cancer, with no increase with dose, cycles of use, or latency. The models for these data were stable, with little difference in risk estimates after adjustment for potential confounding factors or when we restricted our outcomes to medically validated cases (that is, 11 melanoma [high proportion were based on self-report], 13 thyroid, 6 cervical, and 24 colon cancers).

Although limited by small numbers of events, we assessed if the relationship between clomiphene use and cancers varied according to other predictors of cancer

| SIRs were computed as the number of observed cancer events divided by the expected number of events based on age, race, and calendar year-specific incidence disease rates for women from cancer registry rates available through the SEER Program. |  |
|---|---|---|---|---|
| **Observed** | **Expected** | **SIR** | **95% CI** |
| All sites, excluding Hormone-sensitive skin cancers | 581 | 473.8 | 1.23 | 1.1-1.3 |
| Uterine corpus | 39 | 24.9 | 1.57 | 1.1-2.1 |
| Breast | 292 | 226.5 | 1.29 | 1.2-1.4 |
| Ovary | 45 | 22.7 | 1.98 | 1.4-2.7 |
| Postulated hormone-sensitive cancers |  |
| Colon | 28 | 15.9 | 1.76 | 1.2-2.6 |
| Melanoma | 42 | 26.7 | 1.57 | 1.1-2.1 |
| Thyroid | 18 | 18.1 | 0.99 | 0.6-1.6 |
| Cervical | 14 | 23.0 | 0.61 | 0.3-1.0 |
risk (Table III). Melanoma (RR = 1.72), thyroid cancer (RR = 1.83), and cervical cancer risk (RR = 2.89) associated with clomiphene usage was highest among women who were nulligravid at entry when compared with unexposed gravid women. For melanoma (RR = 2.00) and thyroid cancers (RR = 4.23), risk was also elevated among women who remained nulliparous at follow-up, with no elevation among parous women. Cervical cancer risk associated with clomiphene use was not modified by parity at follow-up. Interpretation of these findings is complicated by small numbers of cancer and missing information on parity at follow-up that was available primarily from women who completed the questionnaire. Colon cancer risk associated with clomiphene usage was not modified by either gravidity at entry or parity at follow-up. All models adjusted for attained age, calendar time, study sites, and gravidity at entry. Estimates of risk associated with clomiphene were also adjusted for anovulatory disorders, a primary indication for use. Additional adjustment for other causes of infertility did not appreciably change risk estimates.

Analysis of the relationship between gonadotropins and cancer risk was limited by the small number of exposed women (Table II). Fewer than 5 cases at each site were exposed to gonadotropins, with no apparent elevation in cancer risk.

**Comment**

Infertile women, as shown in this study and other cohort investigations, are at higher risk of cancer than women from the general population. Whether excess cancers diagnosed among infertile women are attributable to underlying causes of infertility, to the women's low parity, or to fertility drugs has been unclear. We previously reported the possible association of fertility treatment with hormone sensitive cancers of the breast, ovary, and uterus in this cohort. This report examines risks related to melanoma, thyroid, cervical, and colon cancers, which have been suggested as having possible hormonal causes. Consistent with previous investigations, infertile women in our study were at elevated risk of melanoma (SIR = 1.57) and colon cancer (SIR = 1.76), but not thyroid (SIR = 0.99) or cervical cancer (SIR = 0.61).

In addition to comparisons with the general population, we were able to make comparisons within the population of infertile patients, allowing us to attempt to disentangle the effects of fertility drugs with other predictors of cancer risk, such as parity and specific causes of infertility (such as anovulatory disorders or endometriosis) or attained age (data not shown).

Analysis of the relationship between gonadotropins and cancer risk was limited by the small number of exposed women (Table II). Fewer than 5 cases at each site were exposed to gonadotropins, with no apparent elevation in cancer risk.

**Table II** Ovulation stimulating drug use and cancer risk among infertile women

<table>
<thead>
<tr>
<th>Infertility treatment</th>
<th>Melanoma (n = 42)</th>
<th>Thyroid (n = 18)</th>
<th>Cervical (n = 14)</th>
<th>Colon* (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clomiphene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>21</td>
<td>10</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Ever</td>
<td>21</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Dosage (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-900</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>901-2250</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>&gt;2250</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cycles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6</td>
<td>16</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6+</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Year since first use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>11</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>15+</td>
<td>9</td>
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<td>3</td>
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</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonadotropins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>38</td>
<td>16</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>Ever</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

All models adjusted for attained age, calendar time, study sites, and gravidity at entry. Estimates of risk associated with clomiphene were also adjusted for anovulatory disorders, a primary indication for use. Additional adjustment for other causes of infertility did not appreciably change risk estimates.

* Additionally adjusted for body mass index at study entry (quartiles, kg/m²).
with time because clomiphene usage with RRs of 2.08 (0.9-4.9), 1.54 (0.3-8.0), and 2.67 (0.6-11.9), respectively, among women followed for 15 years or more. These hints of a possible latency effect may suggest that clomiphene is an initiator of carcinogenesis and is consistent with the fact that carcinogenesis is a long process, which takes many years. Accrual of more cancer events via continued follow-up of this and other infertile cohorts is necessary for clarification.

We also found that clomiphene use appeared to impart stronger effects on the risks of melanoma (RR = 2.00) and thyroid cancer (RR = 4.23) among women whom remained nulliparous through follow-up, a finding that is based on few cancers and that also requires confirmation. Clomiphene-associated cancer risk among nulliparous women persisted after adjustment for underlying causes of infertility such as anovulatory disorders, which have been associated with melanoma,12,15 and endometriosis, which has been associated with melanoma, thyroid, and colon cancers.15 We cannot entirely exclude the possibility that women who remained nulliparous and who used clomiphene had more severe underlying disease for which we were unable to account.

Early studies linking parity, oral contraceptives, and postmenopausal hormone therapy to melanoma postulated a hormonal cause for this cancer.24 A relationship between ovulation-stimulating drugs and melanoma was first suggested by case reports25,26 and by elevations in melanoma risk seen among cohorts of infertile women.11,12,16,17,23 For the latter, too few cancers have been diagnosed to disentangle the effects of infertility medications from their indications for usage. Of the larger investigations, a cohort study in Seattle found no elevation in melanoma risk overall, but risk was elevated among women who had used clomiphene for 12 or more menstrual cycles (RR = 2.2; 95% CI, 0.5-10.2, based on 4 exposed cases, 3 of whom had ovulatory abnormalities).23 Consistent with our results, 2 other prior studies that evaluated the relationship between ovulation-stimulating agents and melanoma found no increase in cancer risk,23,27 although only 1 (a case-cohort design) was able to examine the effect of specific fertility drugs.27

Thyroid cancer has been postulated to have a hormonal cause because it is 3 times more frequent in women than men and because risk has been linked to oral contraceptive use10 and reproductive factors, particularly among those diagnosed at young ages.8 To date, little evidence of an association between ovulation-stimulating agents and thyroid cancer risk has emerged. A pooled-analysis of case-control studies reported a nonsignificant increased risk of thyroid cancer after infertility drug use (odd ratio [OR] = 1.6; 95% CI, 0.9-2.9),10 although 1 of the included studies reported a significant 4-fold excess risk.28 Unlike the case-control design that relies on patient reports of complex infertility treatment, the cohort design enables obtaining information directly from medical records. This allows for more detailed assessment of specific agents and doses. Cohort studies before ours have not evaluated thyroid cancer risk after use of ovulation-stimulating agents because of the few cancers diagnosed.

Although cervical cancer is not generally viewed as a hormonally-related tumor, relationships of the disease with increasing parity and long duration of oral contraceptive use8 have raised concerns regarding effects of other hormonal agents. The most informative data derive from a retrospective cohort study by Rossing et al conducted in Seattle in which 36 in situ and invasive cervical cancers were detected.13 In the current study,

<table>
<thead>
<tr>
<th>Table III</th>
<th>Modification of cancer risk associated with ever compared with never clomiphene use by reproductive status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melanoma (n = 42)</td>
</tr>
<tr>
<td></td>
<td>Cancers</td>
</tr>
<tr>
<td>Gravidity at entry</td>
<td></td>
</tr>
<tr>
<td>No clomiphene</td>
<td>13</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>12</td>
</tr>
<tr>
<td>Nulligravid</td>
<td></td>
</tr>
<tr>
<td>No clomiphene</td>
<td>8</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>9</td>
</tr>
<tr>
<td>Parity at follow-up</td>
<td></td>
</tr>
<tr>
<td>Parous</td>
<td></td>
</tr>
<tr>
<td>No clomiphene</td>
<td>18</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>11</td>
</tr>
<tr>
<td>Nulliparous</td>
<td></td>
</tr>
<tr>
<td>No clomiphene</td>
<td>3</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>8</td>
</tr>
<tr>
<td>Parity missing</td>
<td>2</td>
</tr>
</tbody>
</table>

Models estimated cancer risk associated with ever compared with never clomiphene use adjusted for attained age, calendar time, and study site.
which is in line with other studies that have shown that parity is a risk factor for this cancer, infertile women were at a decreased risk of having cervical cancer develop compared with the general population. Contrary to the study by Rossing et al, which reported a reduction in cervical cancer risk associated with clomiphene (RR = 0.4), we found the risk among women who had taken clomiphene was elevated relative to nonusers (RR = 1.61; 95% CI, 0.5-4.7). Neither our study nor the study by Rossing et al reported any apparent relation according to dose or duration of use. It remains unclear as to whether clomiphene may act predominately as an estrogen agonist or antagonist on the cervix.

Hormonal factors have been inconsistently associated with colon cancer risk in women, with some studies showing an inverse relationship with parity and hormone replacement therapy. Colon cancer risk associated with infertility and its treatment has received little attention with only 1 other published study, which reported results similar to ours. Specifically, in an Australian cohort of infertile women, 1 colon cancer was diagnosed among women exposed to in vitro fertilization compared with 3 cases among unexposed women.

Whether fertility drugs other than clomiphene increase cancer risk requires further investigation. We previously reported a non-significant elevation in breast cancer risk among women who used gonadotropins for 6 or more menstrual cycles (RR = 1.50) and who were first exposed 20 or more years ago (RR = 1.54). Melanoma, thyroid, cervical, and colon cancer sites had fewer than 5 exposed cancers and did not show an increased cancer risk with gonadotropin use (RRs from 0.9-1.39).

Although our study had a number of strengths, there were some notable limitations. Although larger than previously published studies, the total number of cancers for each site was small. Given the retrospective nature of the study, we were unable to locate 20% of the study population and 11% did not agree to release their information about later drug use was obtained via questionnaire, we could not account for drugs subsequently prescribed by other providers among women who did not complete the questionnaire. Finally, the pattern and dose of drug exposures for many women that we evaluated were quite different from those in current use. In summary, after accounting for independent contributions of causes of infertility and reproductive status, findings from this study do not suggest an association between ovulation-stimulating drug use and melanoma, thyroid, cervical, or colon cancers. Nonetheless, because clomiphene is one of the most widely used drugs in the management of infertility, extended follow-up of infertile patients is necessary to accrue more cancer events and to clarify the relationship between clomiphene citrate and cancer risk, particularly among infertile women who remain nulliparous.

References

First glimpse of the functional benefits of clitoral hood piercings

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In this exploratory study, we identify a positive relationship between vertical clitoral hood piercing and desire, frequency of intercourse and arousal. There were no dramatic differences in orgasmic functioning. Clinicians can play key roles in educating patients about potential outcomes and risks of genital piercing.

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Female genital piercing is an emerging form of body art in western culture. Vertical clitoral hood piercing, one of several genital piercing techniques, is apparently popular and functional. A review of the literature reveals little more than anecdotal information, including the belief that it is useful in the treatment of types of female sexual dysfunction such as anorgasmia.

Objective and study design

The investigators conducted an exploratory study to describe the population and determine whether vertical clitoral hood piercing had associations with female sexual functioning. In a University of South Alabama Institution Review Board–approved study, 33 female participants were recruited from a piercing studio in New Orleans, La, between September 2001 and July 2003. The participants were offered an opportunity to complete the Female Sexual Functioning Inventory both before piercing and 7 weeks after piercing. The instrument requested information about the domains of arousal, desire, lubrication, pain, orgasm, and overall sexual satisfaction.

Of the 77 participants initially enrolled in the study, 33 (42%) completed and returned both questionnaires. The mean age was 31 (SD = 9.4) and ages ranged from 19 to 55. Over half (55%) were between the ages of 19 and 29. Nine (27%) were between the ages of 31 and 40 years with an additional 6 (18%) between the ages of 41 and 55 years. Fourteen (42%) of the participants were married and 14 (42%) were single. Three participants (9%) were divorced and the remainder were separated or widowed. The majority (85%) were white and heterosexual (76%). The sample was generally well educated, with 11 (33%) participants possessing a college degree and 4 (12%) with some college education. Four (12%) participants had masters degrees and 4 (12%) had doctorates. Eight (24%) earned a high school degree; 1 (3%) less than high school.
The piercing materials were implant-grade surgical stainless steel, implant-grade titanium or 18-karat gold. The majority of piercings were steel. All participants kept the piercing.

Results
We calculated 2-tailed bivariate analyses to test the degree of relationship between the before and after scores. For the individual items comprising the domains, we observed increased frequency of sexual desire (0.451, \(P = .008\)), level of desire (0.394, \(P = .023\)), and level of arousal (0.429, \(P = .013\)). We observed significance in only 1 of the domains, desire (0.414, \(P = .017\)). Other sexual functioning indexes were negligible. We expected to see a change in the orgasm frequency and/or satisfaction. Yet, contrary to popular belief, we saw no dramatically significant difference in orgasm.

The lack of significant differences between some of the sexual functioning domains before and after piercing may reflect a true lack of difference or may be the result of insufficient statistical power to detect significant relationships. We considered \(P < .05\) to be statistically different. An interim power analysis using a power of 80% indicated the need for a sample size greater than 200 to detect significant differences in the other domains of sexual functioning. Desire’s statistical significance is noteworthy, considering the small sample size.

Comment
Vertical clitoral hood piercing’s influence on desire can be understood by considering the physiology involved in the sexual response cycle established by Masters and Johnson as well as bearing in mind the more subjective element of desire that so often eludes sexuality researchers. Genital piercing may address both objective and subjective elements of desire.

The clitoral hood or prepuce serves a protective function and plays a critical role in sexual activity. During sexual arousal, the labia minora become engorged with blood pulling down on the clitoral hood and stimulating the clitoris. At the same time, the clitoris enlarges and becomes erect elevating the clitoral hood, which in turn acts as a direct stimulant to the clitoris via friction. Penetration causes movement and stretching of the labia minora that pull down on the prepuce and further stimulate the clitoris.

Piercing of the clitoral hood, either vertically or horizontally, acts to lift the clitoral hood. The intention is to enhance sexual sensitivity and stimulation of the clitoris, the only organ in the human body that serves the single purpose of providing pleasure. In addition to lifting and retracting the clitoral hood, piercings can be fitted with jewelry meant to directly stimulate or ornament the clitoris. Lesbians and heterosexual women experience both coital and noncoital sexual activity, including self masturbation and other types of genital stimulation, wherein clitoral hood piercing can perform a role.

This limited survey encourages further study on the desire enhancing benefits of vertical clitoral hood piercing and the role of medical practitioners in treating and counseling female patients seeking intimate piercing. We found no indications to counsel against clitoral hood piercing, but urge practitioners to inform patients of the obvious health risks associated with body piercing such as infection and viral hepatitis as well as the possibility of streptococcal toxic shock syndrome associated with piercing mucosal surfaces. We caution against generalizing outcomes until other studies produce deeper understanding.

We thank Elayne Angel with Rings of Desire for her assistance with data collection as well as Beth Mitchell, CRNP, and Peggy Hamlin, CRNP.

References
Objective: The study was undertaken to further define the anatomy of the arcus tendineus fascia pelvis (ATFP).

Study design: Thirty cadavers were dissected to find the average length, SD, and range of the ATFP. Comparisons were made to height and pelvis type. The average distance between the ischial spine and the attachment of the fascia of the rectovaginal septum (RVF) to the ATFP was measured.

Results: The average length, SD, and range in centimeters for the ATFP are 9.0, 0.70, and 7 to 10.5, respectively. The length of the ATFP increased with height. No associations could be made regarding pelvis type. The average distance between the ischial spine and the attachment of the RVF to the ATFP is 2.15 cm with a SD and range of 0.21 and 1.75 to 2.5, respectively.

Conclusion: In this study, an average length for the ATFP is established and the distance between the ischial spine and the attachment of the RVF to the ATFP is redefined.

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The arcus tendineus fascia pelvis (ATFP), originally termed the “white line,” is a fibrous thickening that is made up of parietal fascia from surrounding muscles. These muscles are the pubococcygeous and iliococcygeous portions of the levator ani and the obturator internus. The ATFP runs along the pelvic sidewall from its origin near the pubic symphysis to its insertion on the ischial spine bilaterally. Discussion regarding the ATFP is sparse in anatomic texts and only 2 articles have been dedicated to defining its anatomy. However, neither study has accurately determined its length or attempted to correlate its length to the pelvic morphology. Even some of the most widely used anatomic texts do not give an average length.

The ATFP has 3 functional parts—the anterior, middle, and posterior segments. It is customary to refer to the anterior segment as the most proximal and the posterior segment as the most distal. The anterior segment is attached to the lower posterior side of the body of the pubic bone approximately 1 cm from the pubic symphysis and extends posterior for approximately 3 cm. It has attachments to the proximal urethra and anterior vaginal wall. It functions as a lateral support for these structures. This portion of the ATFP,
including the histology, has been extensively studied by DeLancey.8,9

The middle segment is approximately 3 cm long. It is attached anteriorly to the anterolateral vagina. This attachment becomes less prominent toward its midpoint. At the midpoint of the middle segment, vessels from the internal iliac artery running to the obturator internus muscle lie laterally. When placing sutures in this area, care must be taken to avoid going too deep into the obturator internus muscle. At the distal end of the middle segment are the fascial attachments of the arcus tendineous levator ani (ATLA) and the fascia of the rectovaginal septum (RVF).10 These attachments distinguish the middle from the posterior segment of the ATFP.

The posterior segment is approximately 2 to 2.5 cm in length and terminates on the ischial spine. It functions as the anchor for the fascia and all of its attachments. DeLancey noted that the distal attachment of the posterior segment to the ischiopubic spine is avulsed in 96% of parous women.11 This avulsion occurs primarily in childbirth and it is remarkable that the effects of prolapse are not seen for decades. This may in part be due to the “anchors” provided by the ATLA and the RVF. Aging, estrogen deficiency, muscle atrophy, and/or lifestyle factors involving chronic increases in intraabdominal pressure may ultimately be responsible.

The ATFP is an important factor in the support of pelvic structures. In 1912, White first noted its role in the support of the bladder and proposed a technique for cystocele repair.12 Many different modifications have occurred since White’s proposal. Richardson enhanced our surgical correction with his concept of the site-specific repair. He noted that the wide variety in presentations of prolapse correlated with defects in the different segments of the ATFP. He proposed the repair of both cystocele and rectocele by correcting site-specific defects in the ATFP.13-15 Given the increasing importance of the ATFP in the repair of pelvic organ prolapse, a better understanding of this fascia is needed. The goal of this study is to further define the anatomy of the ATFP and from these observations draw conclusions concerning its function and repair.

Material and methods

Seven fresh and 23 embalmed cadavers in the anatomic teaching laboratory at the Uniformed Services University of Health Sciences were dissected to visualize the ATFP. Visualization of the ATFP allowed for the following measurements to be taken: length of the ATFP bilaterally, point of attachment of the RVF, obstetric conjugate, biischial spine diameter, and patient height. Information on patient age and race was also noted. Because this information was obtained while teaching pelvic anatomy to medical students and is consistent with the intent of the donors, the Anatomic Materials Use Committee and the Department of Clinical Investigation exempted this study from Institutional Review Board approval.

An abdominal incision allowed access to the pelvis. The dome of the bladder was removed, and the lateral attachments of the midline organs were dissected, allowing visualization of the tendon’s entire path along the pelvic sidewall. The path originates 1 cm lateral to the pubic symphysis on the posterior, inferior border of the pubic bone, proceeds lateral to the urethra in a posterior direction, extends along the pelvic sidewall, and ends at its insertion on the ischial spine. In many cases, the tendon was not clearly defined along its entire length. However, by using remnants of the tendon and by using the ischial spine as an anatomic landmark allowed for accurate measurement. No cadaver was found to have detachment of the tendon from its origin on the pubis.

First, the length of the ATFP was measured bilaterally in centimeters while maintaining the curve of the pelvis. Keeping the measuring tape in place, the distance between the midpoint of attachment of the RVF to the ATFP and the ischial spine was measured. The RFV attaches to the ATFP throughout its length. However, this fascia condenses posteriorly. We like to refer to this condensation as the arcus tendineous rectovaginalis. By our observations, this posterior condensation of the RFV is approximately 1 to 1.5 cm in width. It is the midpoint of this condensation that attaches to the ATFP and is where we began our measurement. Furthermore, the obstetric conjugate and biischial spine diameter were measured. The obstetric conjugate was measured from the posterior inferior margin of the pubic symphysis to the sacral promontory. The biischial spine diameter was taken as the distance between the tips of the bilateral ischial spines. Finally, the patient’s height was obtained measuring heel to crown. Measurements of the obstetric conjugate and the biischial spine diameter along with vaginal evaluation at dissection were used to diagnose pelvis type. Information regarding pelvis type and height were consistently obtained following the measurements of the ATFP, and demographic data regarding the cadavers were collected after all measurements were taken. Demographic data were limited to the information available to the anatomy laboratory.

Given the reported length of the ATFP to be 10 cm, we assumed a significant difference in length of 0.5 cm and a SD of 1 cm. Setting power at 80% and alpha at .05, the necessary sample size was calculated to be 32 using the above parameters. However, because no prior SD for the ATFP existed and noting a SD of 0.7 after obtaining our measurements, it was found that only 16 cadavers were needed for statistical significance. Therefore, our results from 30 cadavers exceed the number needed to meet statistical significance. The same is true for the distance at which the RVF attaches to the ATFP.
The average length of the ATFP and the average distance of the attachment of the RVF to the ATFP were computed from the measurements. SDs and ranges were then computed. Comparisons between the lengths of the ATFP and patient height and pelvis type were made. Also, to account for possible differences between the fresh and embalmed cadavers, their average length of the ATFP was compared. Statistical computations were performed with SPSS 12.0 (SPSS Inc, Chicago, Ill).

### Results

The 30 cadavers used for dissection were made up of 27 white and 3 black women. Their average height was 64.35 inches with a range of 59 to 71 inches. A total of 24 gynecoid, 5 anthropoid, 1 android, and no platypelloid pelvis types were diagnosed. Cadaver demographic information can be seen in the Table.

The average length of the ATFP was 8.99 cm with a SD of 0.703 cm and a range of 7 to 10.5 cm. Measurements of the right and left ATFP were obtained for each cadaver and can be seen in the Table. Of note, the length of the ATFP on the right and left sides showed variability. The difference in length between the 2 sides ranged from 0 to 0.75 cm. The average length of the ATFP was then compared with the height of the cadavers. Although an exact association between height and length could not be determined, a trend toward increasing length of the ATFP can be seen in the Table as cadaver height increases. As for comparing length and pelvis type, there were not enough anthropoid, android, or platypelloid pelvises to draw any conclusions. The

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Average of right and left measurements:

- SD of right and left measurements: 0.703
- SD of right and left measurements: 0.206

| W, White; B, black; G, gynecoid; A, anthropoid; AND, android. |
average length of the ATFP of the fresh and embalmed cadavers was 8.88 and 9.02, respectively, \( P = .678 \). By using \( P < .05 \) as statistically significant, there was no statistically significant difference between the fresh and embalmed cadavers.

The average distance of the attachment of the RVF to the ATFP as measured from the ischial spine was 2.154 cm with a SD of 0.206 cm and a range of 1.75 to 2.5 cm. Measurements of the right and left sides of attachment were obtained for each cadaver and can be seen in the Table. Again, small discrepancies were observed between the left and right distances.

Regarding the general pelvic measurements, the average length of the bischial spine diameter was 11.317 cm with a SD of 1.044 cm and a range of 9.25 to 13 cm. The average length of the obstetric conjugate is 11.617 cm with a SD of 1.006 cm and a range of 10 to 14 cm. The individual measurements can be seen in the Table. No comparison was made between the average length of the ATFP and these measurements. Rather, these measurements helped in the diagnosis of the pelvis type of the cadaver and are noted as points of interest.

### Comment

The ATFP is a point of attachment for many gynecologic procedures. As this tendon is increasingly used in prolapse and incontinence procedures, it becomes more important to have a thorough understanding of its anatomic course and attachments. Ocelli et al\(^3\) dissected 2 cadavers and determined the ATFP length to be 10 cm. This is the length that is quoted by most gynecologists in discussions regarding the ATFP. Leffler et al\(^10\) dissected a total of 24 cadavers and determined the lengths for anterior and posterior segments of the ATFP. A total length was not stated, but adding the anterior and posterior segments would give a length of 8.55 cm. This more closely resembles our finding of 8.99 cm, which is significantly less than the often-quoted 10 cm.

The rectovaginal septum separates the vagina from the rectum. Its origin has been vigorously debated in earlier literature.\(^16-20\) Here, we are concerned with its attachment to the ATFP. We now know that defects in this attachment result in rectocele formation. Leffler et al\(^10\) obtained an average distance of 4.8 cm for the attachment of the RVF to the ATFP as measured from the ischial spine. However, our observations in this study place this point at 2.15 cm from the ischial spine. It might be that Leffler et al chose a different point of reference than what was indicated in the article. Our illustration, Figure 1, is a modification of the picture in the article by Leffler et al.\(^10\) It should be noted that the rectovaginal septum has minor attachments to the ATFP all along its lateral border. We measured only the condensation of these attachments at its distal end. We were able to consistently identify this condensation in all cadavers. Also, we noted that condensations of the ATLA and the RVF attach at the same point on the ATFP (Figure 2). This observation makes inherent sense from an engineering standpoint (Figure 3). The attachment of the RVF at a distance of 4.8 cm from the ischial spine is consistent with our findings.
spine would change the forces applied to the ATFP, making the tendon less efficient. It also places the attachment of the RVF to the ATFP very near, if not directly over, vessels leading to the obturator internus muscle. This is also stated by Ocelli et al. Therefore, placing sutures in an attempt to reattach RVF to the ATFP during a posterior repair would entail a high risk of potential catastrophic bleeding. With our findings, such risky placement would not be warranted. Our results indicate no need to place sutures beyond this attachment during a paravaginal repair. This issue has previously been questioned by Cornella. We surmise that there may be no structural benefit of placing the sutures as far back as the ischial spine.

Making associations to cadaver height further adds information when building a model of the pelvis. Even though a direct correlation to height could not be made, an obvious trend toward increasing length with increasing height was seen. Because this seems to follow reasonable logic, we believe the exceptions are most likely caused by differences in cadaver morphology, such as fat, atrophy, and edema.

There are aspects of this study that could be improved. First, our measurements were taken to the nearest quarter of a centimeter. Taking the measurements to the nearest tenth of a centimeter would have increased accuracy. However, the change in the average value would have been small. Second, slicing the relevant area of the pelvis into sections would provide for a more detailed measurement, but this would have added significant time and cost to the study. Our dissections did allow complete visualization for these measurements. Third, when comparing the length of the ATFP to cadaver height, one must consider that osteoporosis would have affected the results in this age group. It would have been more accurate to compare the length of the ATFP with a hip to heel length measurement of the cadavers. Fourth, even though our study reaches statistical significance, studies involving a larger number of dissections that are more diverse in ethnicity and pelvic types would truly define a standard length of the ATFP. Finally, the study would have been more complete by measuring the distance of all the attachments to the ATFP as these measurements are needed for a complete reconstruction of a pelvic model.

Our dissection of 30 cadavers is the largest study to date to specifically examine the length of the ATFP. We therefore propose our finding of approximately 9 cm to be used as the current standard length. This study also redefines the attachment of the RVF. By establishing an average length for the ATFP and points of attachment, computer modeling of the pelvis will become more accurate and in turn will help improve on medical and surgical therapies.

References

Characterization of vaginal microflora of healthy, nonpregnant women by chaperonin-60 sequence-based methods

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KEY WORDS
Vaginal flora
Chaperonin-60
Molecular method

Objective: The purpose of this study was to use a novel method that was based on the application of chaperonin-60 sequencing to describe the vaginal microflora of 16 healthy women.

Study design: Asymptomatic women consented for vaginal swabs to be collected at the time of a clinical pelvic examination. Total genomic DNA was isolated from the vaginal swabs. Degenerate, universal polymerase chain reaction primers were used to amplify an approximately 555 base pair region of the universal chaperonin-60 gene, which is found in all eubacteria and eukaryotes, from the total genomic DNA and libraries of cloned polymerase chain reaction products were constructed. Library clones were sequenced, and the resulting sequences were assigned to taxonomic groups on the basis of similarity to reference sequence data. Presence of Chlamydomphila psittaci sequences in the samples was confirmed by species-specific polymerase chain reaction.

Results: Sixteen of the 23 women who were enrolled had normal flora by Nugent’s score of <4 and had adequate polymerase chain reaction product for assessment. Vaginal flora libraries were dominated by a variety of sequences with similarity to Lactobacillus spp L. crispatus, L. iners, L. gasseri, L. jensenii, and L. buchneri. Other sequences that were identified included representatives of Gardnerella spp, sequences with similarity to Porphyromonas spp and Megasphaera spp and sequences identical to C. psittaci.
Human vaginal flora plays a profound role in reproductive health and disease. However, our primitive understanding of the complex microbial ecosystem of the genital tract greatly hampers our ability to develop appropriate, focused therapies for genital infections. Given the current limitations in our diagnostic abilities, it is naive to assume that we know all of the organisms that are involved in genital tract health. It is likely that the microorganisms that are responsible for reproductive health and disease remain to be discovered. To date, no exhaustive, culture-independent survey has been done of this important microbial community.

The use of conventional culture repeatedly has been found to be unhelpful, because approximately 5% of normal flora is comprised of multiple organisms that are implicated as genital pathogens; thus, their presence alone is insufficient information. In addition, many are extremely difficult or impossible to culture routinely, and there may be organisms that have yet to be detected. The organisms that are associated most often with bacterial vaginosis include anaerobic bacteria, in particular Bacteroides spp, Peptostreptococcus spp, Gardnerella vaginalis, and Mycoplasma hominis. The fastidious nature of these organisms makes culture-based methods impractical. This major limitation of culture-based methods has been described as “the great plate count anomaly” because only a small fraction of microorganisms that are present in a population can be cultured. Studies that are based on culture have characterized vaginal lactobacilli or other specific organisms of interest, such as G vaginalis. A few new organisms have been recognized but these generally are associated with disease, not with healthy flora. There is a reasonable expectation that organisms that are important to reproductive health may have evaded detection with standard methods.

The development of culture-independent, gene-based methods has facilitated small-scale studies of a wide variety of complex microbial communities. Molecular methods have been applied in previous studies to identify and enumerate vaginal organisms. However, the relatively small scale and often generally descriptive nature of these studies leaves us with a somewhat superficial understanding of vaginal flora. Recently, results of a larger study demonstrated the potential usefulness of the application of high-throughput molecular methods to the characterization of vaginal microflora.

Chaperonin-60 is a molecular chaperone essential for the folding and assembly of proteins and protein complexes in all eubacteria and in the plastids and mitochondria of eukaryotes. The gene encoding chaperonin-60 (cpn60) offers several advantages over the widely used 16S recombinant RNA (rRNA) gene as a target for microbial species identification and phylogenetics. A robust molecular method for the identification of microorganisms, which is based on the amplification of a 549– to 567–base pair (bp) portion of the cpn60 gene (the “universal target”) with universal degenerate polymerase chain reaction (PCR) primers, and the comparison of amplified sequences to a reference database of cpn60 sequences has been applied previously to phylogenetic studies, the identification of clinical isolates, and studies of the microbial ecologic condition of the animal gastrointestinal tract. The cpn60 universal target region generally provides more discriminating and phylogenetically informative data than the 16S rRNA target, particularly between closely related species. Sequence variation extends quite uniformly throughout the cpn60 coding region, whereas variable regions of 16S rRNA genes are dispersed between regions of highly conserved sequence that result in stable secondary structure and facilitates PCR artifacts. Cpnl60 genes generally are present in a single copy in prokaryotic genomes, which makes an attractive target for quantitative methods. The relatively small size of the universal target facilitates high throughput sequencing approaches. Finally, a reference database of cpn60 sequences is available.

**Conclusion:** Culture-independent, chaperonin-60 sequence-based molecular methods can lead to the identification of greater diversity within defined taxa compared with those that are identified by standard culture-based methods and to the identification of novel organisms that were not previously associated with vaginal flora.

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**Material and methods**

**Subjects**

This project received University of British Columbia Research Ethics Board approval. Women who attended
an outpatient Sexually Transmitted Diseases clinic, at the British Columbia Centre for Disease Control, Vancouver, British Columbia, who self-identified as not having symptoms were offered enrollment. Written informed consent was obtained; at the time of the standard speculum examination, an additional swab for vaginal secretions was taken from the posterior fornix of the vagina. All women had clinical specimens that were evaluated for bacterial vaginosis by standardized Nugent scoring at the British Columbia Centre for Disease Control clinical laboratory. As part of routine clinical assessments, samples were taken for routine light microscopy assessment for yeast and Trichomonas vaginalis, Chlamydia trachomatis PCR, and Neisseria gonorrhoeae culture.

**Total genomic DNA isolation**

Each clinical Dacron swab was processed within 24 hours of receipt from the clinic. The contents of each swab were extracted in 800 µL of DNazol (MRC Inc, Cincinnati, Ohio). The extract was vortexed vigorously, with pulsing for 2 minutes. Ethanol (600 µL) was added, mixed, and incubated at room temperature for 5 minutes. Precipitated nucleic acid was pelleted by centrifugation and washed twice with 1 mL of 75% ethanol. One hundred microliters of 8 mmol/L NaOH was used to solubilize the nucleic acid, which was followed by the addition of 3 µL of 1 mol/L HEPES to neutralize the purified DNA solution.

**PCR and creation of PCR product libraries**

Each DNA sample (2 µL) was used as template in PCR reactions with 0.5 µg of each universal cpn60 PCR primer (H279 5’-GAI III GCI GGI GAY GGI ACI ACI AC-3’ and H280 5’-YKI YKI TCI TCI CCR AAI CCI GGI GGY TT-3’), 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 1.5 mmol/L MgCl2, 200 mmol/L of each deoxynucleoside triphosphate, 2 U Taq DNA polymerase in a final volume of 50 µL. After the addition of paraffin oil, PCR amplification with a robocycler (Stratagene, La Jolla, Calif) was carried out for 3 minutes at 95°C for 1 cycle, followed by 40 cycles of 1 minute at 94°C, 2 minutes at 40°C, 5 minutes at 72°C, and completed with 1 cycle of 10 minute at 72°C. PCR products from each template were agarose gel purified and ligated into T-A cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, Calif). Ligation mixtures were used to transform Escherichia coli strain JM109. The 16 resulting libraries were plated on Luria Broth (LB)/ampicillin/X-gal, and 480 white colonies were picked for each library. Colonies were picked into 96-well plates that contained 100 µL of LB with 100 µg/mL ampicillin. After overnight incubation at 37°C, 100 µL of 30% (vol/vol) glycerol was added to each well, and the cultures were stored at −80°C.

**Template preparation and sequencing**

The sequencing template was prepared by the TempliPhi system (Amersham Pharmacia, Piscataway, NJ) at a 1 to 8 recommended reaction scale in 384-well plates (total reaction volume 2.5 µL). Sequencing reactions were performed by direct addition of ET terminator reaction mix (Amersham Pharmacia) to 384-well plates that contained the TempliPhi products. Sequencing reactions were thermocycled according to the manufacturer’s recommended protocol. Sequencing reactions were resolved on an ABI PRISM 3730XL DNA Analyzer system (Applied Biosystems, Foster City, Calif) at the McGill University and Genome Québec Innovation Centre.

**Sequence data processing and bioinformatics**

Raw sequence data was processed using the Phred program, which assigns quality values to the bases and trims poor quality regions. The resulting sequences were clustered on the basis of sequence identity with the d2_cluster.23 Clusters of identical sequences were assembled, with the use of Phrap,24 which incorporates the base quality information from Phred. Manual confirmation of contig assembly was done with Gap4 (version 4.6) in the Staden software package (release 2000.0; J. Bonfield, K. Beal, M. Betts, M. Jordan, R. Staden, 2000). Sequence data, template information and similarity results were placed in a mySQL database (mySQL AB, Uppsala, Sweden) for storage and further analysis. Sequence manipulations, such as format changes and amino acid translations, were done with the European Molecular Biology Open Software Suite software.25 Sequence

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**Table I** Numbers of clones and unique sequences from 16 vaginal flora cpn60 libraries

<table>
<thead>
<tr>
<th>Library</th>
<th>Number of clones sequenced</th>
<th>Number of unique nucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>hvf3233</td>
<td>430</td>
<td>5</td>
</tr>
<tr>
<td>hvf3238</td>
<td>410</td>
<td>1</td>
</tr>
<tr>
<td>hvf3244</td>
<td>436</td>
<td>1</td>
</tr>
<tr>
<td>hvf3245</td>
<td>440</td>
<td>3</td>
</tr>
<tr>
<td>hvf3246</td>
<td>441</td>
<td>2</td>
</tr>
<tr>
<td>hvf3247</td>
<td>439</td>
<td>6</td>
</tr>
<tr>
<td>hvf3257</td>
<td>431</td>
<td>9</td>
</tr>
<tr>
<td>hvf3258</td>
<td>446</td>
<td>5</td>
</tr>
<tr>
<td>hvf3265</td>
<td>434</td>
<td>12</td>
</tr>
<tr>
<td>hvf3266</td>
<td>449</td>
<td>1</td>
</tr>
<tr>
<td>hvf3267</td>
<td>338</td>
<td>3</td>
</tr>
<tr>
<td>hvf3268</td>
<td>386</td>
<td>1</td>
</tr>
<tr>
<td>hvf3269</td>
<td>419</td>
<td>1</td>
</tr>
<tr>
<td>hvf3271</td>
<td>451</td>
<td>3</td>
</tr>
<tr>
<td>hvf3272</td>
<td>466</td>
<td>17</td>
</tr>
<tr>
<td>hvf3273</td>
<td>367</td>
<td>4</td>
</tr>
</tbody>
</table>
alignments were done using Clustal W. To determine the putative taxonomy of each contig and singleton that arises from the assembly step, each sequence was compared with a reference set of cpn60 sequences with the sequence alignment program FASTA.

Phylogenetic analysis was done with programs in the Phylogeny Inference Package software package (version 3.5c; Distributed by the author [Felsenstein J], 1993, Department of Genetics, University of Washington, Seattle, Wash). Specifically, alignments were sampled for bootstrap analysis with seqboot; distances were calculated with the maximum likelihood option of dnadist. Dendrograms were constructed from distance data by neighbor-joining with neighbor. Consensus trees were calculated with consense, and branch lengths were superimposed on consensus trees using fitch.

**Species-specific PCR primer design and amplification of C. psittaci cpn60**

Primers H1520 (5'- GCT CAG GTA GCC ACC ATT TC -3') and H1521 (5'- GCT AGA AAG GTA TCC AGC -3') were designed based on the phylogenetic analysis.
TCG GTT G –3′) were designed with Oligo6 (Molecular Biology Insights Inc, Cascade, Colo) and Signature Oligo (LifeIntel, Port Moody, BC, Canada). Amplifications were performed in a 50 μL reaction that contained 20 mmol/L Tris-HCl pH 8.0, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxyribonucleoside triphosphate, 20 pmol of H1520, 20 pmol of H1521, and 2 units Taq DNA polymerase. Reactions were incubated at 95°C for 5 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 62°C, and 30 seconds at 72°C. A final extension of 10 minutes at 72°C followed the last cycle. Amplifications were performed with a thermocycler instrument (BioRad iCycler; Bio-Rad Laboratories, Hercules, Calif).

**Results**

**Clinical samples**

Twenty-three nonpregnant, sexually active women who were being seed for a sexually transmitted infection screening examination, without genital symptoms, were enrolled after written informed consent. Their ages ranged from 19 to 35 years. All of the women underwent the clinic’s standardized questionnaire and examination. Two women had abnormal findings on pelvic examination and were excluded immediately. All remaining women tested negative for N gonorrhoeae, and C trachomatis. Yeast and T vaginalis were not detected by light microscopy in any subjects. One sample was “intermediate” for bacterial vaginosis, with a Nugent’s score of 4, and was excluded. Two samples were eliminated because they had been delayed in transit to the research laboratory by >24 hours, and 3 samples did not yield sufficient PCR product in the initial reactions to generate an adequate library. The remaining 16 women for whom samples were used had completely normal flora by all standard clinical testing.

**Cpn60 gene sequences amplified from vaginal swabs**

High-quality sequence data were obtained for 6869 of the 7680 clones that were picked randomly from 16 libraries. Data from the remaining 811 clones were excluded from the analysis because of incomplete or partially ambiguous sequences. As summarized in Table I, pairwise comparisons of the sequences that were determined for each library indicated that from 1 to 17 different nucleotide sequences were identified in each of the 16 libraries. Pooling of the sequence data from all 16 libraries resulted in the identification of a total of 57 different nucleotide sequences (GenBank accessions AY581720-AY581776). Phylogenetic analysis of the 57 different nucleotide sequences that were recovered from the pooled library data resulted in the identification of 13 distinct clusters of sequences (Figure 1). Clusters were classified on the basis of similarity of the sequences to reference sequence data (Table II). Most clones that were analyzed (5361 clones, representing 32 distinct sequences) fell into clusters L1 to L6. Sequences in L1 to L5 were at least 92% identical to Lactobacillus spp. Sequences in L6 were consistent with the Lactobacillales family but had weaker sequence similarity (83%-84%) to any reference Lactobacillus sp. Four distinct clusters (A1-A4) of Actinobacteria-like sequences were identified. These sequences (22 sequences, represented by 742 clones) were all at least 88% identical to G vaginalis ATCC 14018 (American Type Culture Collection, Manassas, Va). Single sequences were identified with similarity to the Clostridiales family (cluster CL1), the Bacteroidetes family (cluster B1), and the Chlamydiales

### Table II: Taxonomic identification of cloned cpn60 sequences, based on comparison to reference sequence data

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>Nearest reference sequence neighbor</th>
<th>Range of sequence identity (%)</th>
<th>Number of sequences recovered</th>
<th>Number of clones recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>G vaginalis ATCC14018</td>
<td>89</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A2</td>
<td>G vaginalis ATCC14018</td>
<td>88-95</td>
<td>12</td>
<td>629</td>
</tr>
<tr>
<td>A3</td>
<td>G vaginalis ATCC14018</td>
<td>90-100</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>A4</td>
<td>G vaginalis ATCC14018</td>
<td>94-97</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>B1</td>
<td>Porphyromonas levii ATCC29147</td>
<td>72</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CH1</td>
<td>C psittaci 6BC ATCCVR-125</td>
<td>100</td>
<td>1</td>
<td>678</td>
</tr>
<tr>
<td>CL1</td>
<td>Megasphaera elsdenii ATCC25940</td>
<td>70</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L1</td>
<td>L crispatus A3M75</td>
<td>93-100</td>
<td>8</td>
<td>2393</td>
</tr>
<tr>
<td>L2</td>
<td>L gasseri ATCC9857</td>
<td>93-97</td>
<td>6</td>
<td>393</td>
</tr>
<tr>
<td>L3</td>
<td>L jensenii ATCC25258</td>
<td>93-96</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>L4</td>
<td>L gasseri ATCC9857</td>
<td>92</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L5</td>
<td>L iners A3M7</td>
<td>98-99</td>
<td>12</td>
<td>2503</td>
</tr>
<tr>
<td>L6</td>
<td>L buchneri ATCC4005</td>
<td>83-84</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>
Comparison of sequence profiles of individual libraries

Figure 2 shows the taxonomic composition of the 16 vaginal flora libraries. Nine of the libraries were composed exclusively of *Lactobacillus*-like sequences. Library hvf3273 contained one sequence with similarity to the Bacteroidetes family and one sequence with similarity to the Clostridiales family and was otherwise composed completely of *Lactobacillus*-like sequences. The major *Lactobacillus* constituent in 6 libraries (hvf3233, hvf3238, hvf3245, hvf3267, hvf3268 and hvf3273) was the L1 cluster (93% identical to *L. crispatus*). The L5 group (98%-99% identical to *L. iners*) was most abundant in hvf3244, hvf3246, hvf3266 and hvf3271. Library hvf3265 was the only library to be dominated by L2 type sequences (93%-97% identical to *L. gasseri* ATCC 9857). Libraries hvf3247, hvf3257, hvf3258, and hvf3272 contained sequences in the A1 to A4 clusters (88%-100% identical to *G. vaginalis* ATCC 14018). Sequences identical to the type strain of *C. psittaci* were identified in libraries hvf3257, hvf3267 and hvf3269.

Variation within *G. vaginalis* sequence cluster

Twenty-two sequences (represented by 742 clones) with strong similarity to *G. vaginalis* were identified. A phylogenetic analysis of these sequences and other related Actinobacteria showed that these sequences reliably cluster with *G. vaginalis* (Figure 3). Pairwise nucleotide sequence identities within this group of sequences ranged from 86% to 99%. A multiple sequence alignment of the 22 *G. vaginalis*-like sequences resulted in the identification of 107 positions of difference in the 552-bp alignment (data not shown). Seven of these differences were found in codon position 1; 1 difference was found in position 2, and 99 differences were found in the third codon position.
Specific amplification of C. psittaci-like sequences

Phylogenetic analysis of the C. psittaci-like sequence that were derived from the vaginal flora libraries and reference sequence data from additional Chlamydiales family members was performed to confirm that C. psittaci formed a distinct taxon based on the 555-bp region of cpn60 (Figure 4) and could be discriminated from closely related species such as C. abortus (95% identical to C. psittaci in the 555-bp amplified region of cpn60). Primers for the specific amplification of a 174-bp region of the C. psittaci cpn60 gene (from positions 160-333 of the 555-bp cpn60 universal target) were designed on the basis of a multiple sequence alignment of partial cpn60 sequences from C. pneumoniae J138 (Genbank accession NC_002491), C. muridarum (NC_002620), C. trachomatis D/UW-3/CX (NC_000117), C. pneumoniae CWL029 (NC_002491), C. abortus (NC_002491), and C. trachomatis (NC_000117). The tree was calculated from 500 bootstrap iterations with maximum likelihood distance calculation and neighbor-joining. Bootstrap values (of 500) for major branch points in the tree are indicated. Scale bar indicates 0.1 substitutions per site.
**C. abortus** ATCC VR-656\(^T\) (AF109790)

**C. abortus** AB7 (AY052785)

**C. abortus** s26/3 (Sanger Institute cab-6c07.p1c)

<table>
<thead>
<tr>
<th>C. suis R27 (AY581778)</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. suis H7 (AY581779)</td>
<td>277</td>
</tr>
<tr>
<td>C. trachomatis D/UW-3/CX (NC_000117)</td>
<td></td>
</tr>
<tr>
<td>C. muridarum MoPn ATCC VR-123(^T) (NC_002620)</td>
<td></td>
</tr>
<tr>
<td>Simkania negevensis Z(^T) (AY219919)</td>
<td></td>
</tr>
</tbody>
</table>

| C. pneumoniae AR39 (NC_002179) |
| C. pneumoniae CWL029 (NC_000922) |
| C. pneumoniae J138 (NC_002491) |
| C. pneumoniae TW-183 ATCC VR-2282\(^T\) (NC_005043) |

<table>
<thead>
<tr>
<th>C. felis (AF448139)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. caviae GPIC ATCC VR-813(^T) (NC_003361)</td>
</tr>
</tbody>
</table>

HVF cloned sequence

<table>
<thead>
<tr>
<th>C. psittaci (avian isolate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. psittaci ATCC VR-125(^T) (AY581777)</td>
</tr>
</tbody>
</table>

*Figure 4*  Phylogenetic tree shows the relationships of *Chlamydia psittaci*-like library sequences to closely related *Chlamydiaeae*. The tree was calculated from 500 bootstrap iterations with maximum likelihood distance calculation and neighbor-joining. Bootstrap values (of 500) for major branch points in the tree are indicated. *Scale bar* indicates 0.1 substitutions per site.

(NC_000922), *C pneumoniae* AR39 (NC_002179), *C felis* FEIS (AF448139), *C pecorum* (AF109789), *C abortus* B577 (AF109790), *C caviae* GPIC (NC_003361), *C abortus* AB7 (AY052785), *C suis* R27 (AY581778), *C suis* H7 (AY581779) and *C psittaci* ATCC VR-125 (AY581777). *Figure 5* shows the results of PCR reactions that were performed with *C psittaci*-specific primers on total genomic DNA samples that were used to generate the vaginal flora libraries. The expected product size was obtained from samples 3247, 3267, and 3269. Product was also detected in template 3246. The identity of amplified products from these 4 templates was confirmed by sequence analysis (data not shown). All sequences were 100% identical to library clone sequences.

**Comment**

Previous studies of the vaginal flora of healthy individuals that were based on culture or sequence-based methods have led to the understanding of this microbial community as relatively homogenous and dominated by a small subset of the *Lactobacillus acidophilus* complex, particularly *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners*. The shortcomings of exclusively culture-based studies
are illustrated by the case of *L iners*. This organism, unlike other *Lactobacillus* spp, can be cultured only on blood agar and was thus overlooked in most studies of vaginal lactobacilli that rely on Man, Rogosa, and Sharpe agar. It is now apparent that *L iners* is a major component of the vaginal flora. 4

Consistently with previous findings, we found that most of the *cpn60* sequence libraries that were constructed in the current study were dominated by sequences with strong similarity to the *L acidophilus* complex, specifically *L crispatus*, *L gasseri*, *L jensenii* and *L iners*. Most of the libraries were found to be composed of representatives of 1 or 2 of the *Lactobacillus* clusters, frequently L1 (*L crispatus*) and L5 (*L iners*; Figure 2). A similar result was obtained in a study of the vaginal *Lactobacillus* flora of 23 healthy Swedish women with the use of randomly amplified polymorphic DNA analysis, where most individual samples were found to contain only 1 or 2 randomly amplified polymorphic DNA patterns, which indicated the dominance of 1 or 2 species. 4 The results of a recent 16S rRNA sequence-based study of the vaginal flora of 3 healthy subjects support the observation that healthy vaginal flora is dominated by 1 or 2 *Lactobacillus* spp. 13

Five of the libraries that we examined contained only 1 sequence each (Table I). We do not suggest that these subjects are colonized only by 1 organism but rather consider that this apparent lack of diversity is the result of a combination of factors that are related to the application of a PCR-based method to a microbial community that is dominated largely by a small number of species. The relative abundances of organisms in any complex microbial community can vary over many orders of magnitude so that, in a total DNA preparation from the community, genomes of the most abundant organisms far outnumber those of rare organisms and will be over represented correspondingly in the PCR product pool. Given that we sequenced only a few hundred clones from each library, it is not surprising that only 1 sequence was recovered in some libraries. In a much more diverse community (pig feces), most sequences were detected at a frequency of approximately 0.1% (1 occurrence in 1125 sequences), 20 a level that would make the sequences likely undetectable in a smaller study. It is also likely that there is bias in the PCR reaction, in which some templates are favored on the basis of composition (especially the guanine-cytosine content) or priming efficiency. To address these issues and identify rarer, potentially unculturable organisms in vaginal flora, technical advances that include subtraction methods and modified PCR protocols are being pursued.

In addition to identifying gross taxonomic clusters, we also identified a large amount of variation within these defined taxa. The 6 identified *Lactobacillus*-like clusters, L1 to L6, each contained from 1 to 12 distinct sequences (Table II; Figure 1). This potentially biologically significant “intraspecies” diversity would not be apparent with the use of culture-based methods or molecular methods (such as denaturing gradient gel electrophoresis) in which banding patterns are often identical for closely related species of *Lactobacillus*. 11 Similar “intraspecies” variation was observed in the *G vaginalis*-like taxa, clusters A1 to A4. *G vaginalis* can be isolated from the vaginal flora of individuals with healthy vaginal ecosystems and individuals who receive a diagnosis with bacterial vaginosis, although the reported proportions of healthy individuals harboring *G vaginalis* varies widely. 5,28,29 Results of previous studies of *G vaginalis* isolates from the vagina suggest that the “biotypes” of *G vaginalis* that are associated bacterial vaginosis are distinct from the *G vaginalis* that are found to varying degrees in healthy individuals. 5 The data presented in Figure 3 certainly support the idea that there is tremendous variability within the *G vaginalis* taxon and that a quantitative assessment of the occurrence of these organisms in healthy and non-healthy vaginal ecosystems certainly would provide clues to the significance of the variability.
The most surprising finding in the current study was the detection of sequences that are identical to C. psittaci in 3 of the libraries. Of the 8 characterized serovars of C. psittaci, serovars A, C, D, and E have been identified as human pathogens. However, C. psittaci has not been found previously in the vaginal mucosal flora. Two other species of the family Chlamydiaceae have been isolated from human vaginal flora. Chlamydia trachomatis causes trachoma, sexually transmitted disease, some types of arthritis, neonatal conjunctivitis, and pneumonia; Chlamyphila abortus has been found in sporadic zoonotic infections that cause abortion in women who work with sheep. However, all of these species can be distinguished readily by cpn60 sequence (Figure 4). The detection of C. psittaci-like cpn60 sequences raises the question of the role of C. psittaci in the vagina and also suggests that the application of molecular methods will likely lead to the identification of other organisms that have not been associated previously with vaginal flora in culture-based studies.

This study presents an evaluation of a small number of healthy women and demonstrates the usefulness of cpn60 sequence-based methods to enhance detailed evaluation of human vaginal flora. The application of this method resulted in the identification of "intraspecies" sequence variation that likely would be undetectable with the use of culture-based or sequence-based methods that use the 16S rRNA target. Clearly, further studies of larger populations of women with and without normal flora in concert with technical developments to improve the detection of rarer sequences will be needed to expand our understanding of this complex microbial community. The specific sequence data that were generated in these studies can be used to quantify and monitor individual population members so that their contributions to the function of vaginal microflora can be assessed and understood.

Acknowledgments

We thank Jennifer Town for excellent technical assistance and Tony Rees, RN, for clinical support and subject recruitment.

References

A national probability survey of American Medical Association gynecologists and primary care physicians concerning menopause

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KEY WORDS
Survey
Complementary and alternative medicine
Traditional therapy
Menopause
Health care professionals

Objective: This survey intended to clarify physicians’ understanding of the issues surrounding women, menopause, alternative medicine, and drug therapy for the treatment of menopause.

Study design: This study was designed as a national probability sample survey of primary care physicians and gynecologists nationwide. Its focus was to identify major concerns and issues identified by patients about menopause and perceived communication with effectiveness how to communicate with their patients. Physicians were also asked to rate their comfort level in recommending the use of herbal remedies and which herbal remedy they felt comfortable recommending to interested patients.

Results: Data indicated that a patient’s complaint about menopausal symptoms was the most common factor leading to discussion of menopausal issues with physicians (91%) and that the primary concern to the patient was management of menopausal symptoms. Other factors were controversies about hormone replacement therapy, long-term health implications of menopause, and hormone replacement therapy. Eighty percent of the physician found confusing messages with regard to menopause to be the most challenging aspect in patient communication. The second most challenging issue is “inconclusive data about hormone replacement therapy” (56%). Seventy-six percent of the physicians found “showing sympathy” to be the most important factor for the physicians to communicate effectively with patients, whereas “being honest and open” was the most important patient attitude cited for the same purpose. When it comes to herbal therapy for menopausal symptom control, only 4% of the physicians indicated that none of their patients take any remedies. Only 18% were not very comfortable in discussing or recommending herbal therapies, whereas the rest ranged from fairly comfortable to completely comfortable.

Conclusion: This study has provided data with regard to physician understanding of menopause treatment options and their primary interaction with patients on this issue. More in-depth studies concerning efficacy and/or side effects of each available treatment will be the relevant next step, given the controversies about both hormone replacement therapy and alternative therapies. The
Menopause is a universal phenomenon that typically occurs between the ages of 40 and 58 years in the Western world, with an average age of 51 years. Factors that influence the course, timing, duration, level, and severity of associated symptoms or health risks, from symptoms as commonly experienced as hot flashes and night sweats to osteoporosis and cardiovascular diseases are: age of onset, the cause of menopause (natural or induced), health and nutritional status, physical fitness, sexual activity, emotional state, stress level, use of medication, or alternative supplements. Perimenopausal symptoms, a transition phase that immediately precedes true menopause and is marked by irregular menstrual periods, include vasomotor responses such as hot flashes, night sweats, headaches, palpitations, and insomnia; and neurological or mental responses such as irritability, depression, anxiety, mood swings, and memory loss. Other manifestations include joint and muscle pain, dryness of skin and vagina, dyspareunia, and increased facial hair. Most of the symptoms wane over time but can persist in a small percentage of females.

Women’s attitudes toward menopause range from feeling relieved, happy, and having more control over health to anxious, depressed, or plagued by physical and mental aggravation brought on by prolonged symptomatic periods. Regardless, the long-term consequence of reduced estrogen, progesterone, and other female hormones following menopause, complicated by the natural course of aging, are inevitably linked to more severe health conditions such as osteoporosis and cardiovascular diseases. Some factors that play important roles in long-term health in postmenopausal years are: balanced nutrition, especially adequate intake of calcium, vitamin D, and vitamin E; physical activity, particularly weight-bearing exercises; use of hormone replacement therapy; and use of herbal supplements.

Estrogen or hormone replacement therapy (HRT) has long been used to help relieve menopausal-related symptoms such as hot flashes and night sweats. Long-term usage has also been shown to decrease serum low-density lipoprotein and cholesterol and reduce risk of heart disease, osteoporosis, colorectal cancer, Alzheimer’s disease, and macular degeneration and delay aging of skin. Common side effects include bloating, breast tenderness, irritability, depression, and sometimes prolonged menstrual bleeding. Furthermore, long-term use of estrogen replacement has also been known to increase risk of breast cancer and endometrial cancer because of prolonged estrogen exposure to the corresponding tissues. The addition of progestin (or other progesterone analogs) to estrogen in the so-called HRT protects against estrogen-induced endometrial hyperplasia, which is linked to malignancy. Yet prolonged use of HRT with combined progesterone and estrogen (more than 5 to 10 years) is still associated with increased risk of breast and endometrial cancers.

As a remedy in response to postmenopausal drop of testosterone, testosterone has also been included in HRT to increase muscle strength, appetite, physical well-being, and sexual desire.

In recent years a new treatment regimen has been developed: selective estrogen receptor modulators (SERMs). SERMs have the advantage of combining the positive skeletal protective effects of estrogen with estrogen-antagonistic effects on sex tissues as to reduce the risk of breast cancer.

There has been rising use of alternative therapies, especially herbals, in recent years. Commonly used herbals include black cohosh for hot flashes, night sweats, insomnia, and vaginal dryness; valerian for sleep disturbance; chaste berry for heavy menses and hot flashes; dong quai for easing common menopausal symptoms; Ginkgo biloba for memory loss; licorice for support of adrenal gland in producing low level of sex hormones; St. John’s wort for depression; red clover, soybeans, and flaxseeds as sources of isoflavones/phytoestrogens, which has estrogen-like effects; and wild yam for regulating progesterone/estrogen ratio in body. Use of phytoestrogens are an important option according to research. Facing the overwhelming available treatment options, physicians have traditionally been playing the most significant role in channeling the appropriate information to female patients concerning menopause, including the discussed modifiable lifestyle-related risk or benefits, supplement use, and HRT. Low compliance has been known for HRT, especially in cases in which strong side effects are encountered. Additional reasons include risks of cancer associated with long-term use and lack of long-term study of newer HRT alternatives such as SERMs. Under such circumstances, there has been a rapidly increasing use of alternative medicine among postmenopausal women for various symptom relief. However, it has been shown that physicians are poor at providing information on complementary and alternative therapies to ease postmenopausal symptoms and
diseases, there have been limited regional studies on these subjects, whereas national probability sample surveys are still scarce. Relevant information will greatly help to guide researchers and policy makers to steer relevant research directions.

This study was designed to facilitate more understanding of menopausal topics and communication between patients and physicians by carrying out a national probability survey with a hierarchical sample drawn proportionally from 2 American Medical Association membership groups (primary care physicians and gynecologists) and by geographical region of the United States.

Methods

The survey sample was from the American Medical Association membership list (2000) of family practice, general practice, and internal medicine physicians. Using a power calculation set at a 95% confidence level and a confidence interval of 4, a sample of 1137 was required. A stratified hierarchical sampling technique was adopted. Multiple waves of mailed questionnaires were sent out. The questionnaires were administered from May 2001 to September 2001.

Two hundred seventy-two physicians responded to the mail questionnaire. The physician response rate was 24%. The response was lower than desired. However, physicians constitute a relatively homogenous group, compared with the general public, and therefore the required sample size to ensure external validity is smaller.

The questionnaire consisted of 23 questions covering various issues about menopause. Respondents were asked to discuss major concerns and issues that they receive from patients about menopause. Several questionnaire items addressed the ways in which physicians can communicate effectively with their patients. Physicians were also asked to rate the qualities for effective patient communication and discuss how patients can communicate more effectively. The study also addressed recommendations for treatment of menopause symptoms. Respondents were asked to rate their comfort level in using herbal remedies and to indicate which herbal remedy they would feel comfortable recommending to interested patients. The last set of questions addressed the quality of information available to patients regarding menopause and alternative treatment.

Results

Physician profiles

Among the 128 primary care physicians and 144 gynecologists who responded to the questionnaire, the mean years in practice were 23.5, and more than two thirds of the sample (67%) were male physicians. Almost half (49%) worked in group practices, 38% had a solo practice, and 11% worked in a hospital setting. Size of practice was measured by number of patients seen daily. The average number was 20 patients. The mean daily working hours were 7.6.

Patient profiles and general attitudes and concerns about menopause

The average age of menopausal women in the surveyed practice was 56 years, according to the responses. Physicians were also asked to report the percentage of menopausal women who were on HRT. Only 1% of the physicians surveyed indicated that none of their patients were on HRT. Thirty-one percent indicated that 1% to 48% of their patients were on HRT. Twelve percent of physicians reported that 50% of their patients were on HRT. Almost one quarter reported between 51% and 75% being on HRT. The remaining 40% of the sample indicated that 76% to 100% of their patients were on HRT. Overall, physicians reported a high compliance rate. The majority of physicians (92%) indicated that their patients take products as prescribed.

Table I lists the most common factors that lead to discussion of menopause. Menopausal symptom relief is of great concern to most women (91%), and 95% of the physicians indicated that they get unsolicited questions concerning menopause from asymptomatic patients. Eighty-three percent of physicians bring up menopause to all patients of a certain age. Seventy-four percent of patients have questions about HRT; 63% have questions about information they received from friends, family, and the media; and 55% of patients have questions about herbal supplements or over the counter (OTC) preparations.

Table II ranks the top 3 concerns and/or questions patients have about menopause. At the top of the list, managing menopause symptoms ranks first at 82%. Questions about HRT were ranked second at 50%, with long-term health implications at third place at 33%. The following factors rated 16% or below at second or third rank: weight management, quality-of-life concerns, herbal supplements and OTC supplements, other alternative therapy, and reactions to information from the media.

Challenges to communication

In the survey, physicians were asked to indicate which of the following (listed in Table III) poses challenges to communicating effectively with patients. “Confusing messages from media” (80%) was the most commonly reported challenge. The second most common response was “inconclusive data about HRT” (56%). At third and fourth were “complicated issues surrounding treatment
“options” (45%) and “physician time constraints” (43%). A “lack of available information about treatment options” and “patients are not open,” rated at 27% and 23%, respectively. Seventeen percent or less of physicians ranked the next 3 statements as a challenge: “patients appear to manage menopause on their own,” “physicians’ lack of knowledge about alternative treatments,” and “patients are embarrassed to ask questions.”

Table IV ranks the elements of physician attitudes that are important for effective communication with patients. Seventy-six percent of the physicians agreed that showing sympathy is very important. Providing useful information to help the patient make decisions was reported to be very important by 66% of the sample. Half of the physicians rated “allowing plenty of time to discuss the patients’ concerns” and “not interrupting when the patient is speaking” as being very important. “Probing physical and emotional symptoms carefully” (49%) and “making the patient feel her experiences are normal” (45%) were also considered very important.

The last column of Table IV represents physicians’ self-rating with respect to the same set of qualities. Half of the physicians rated themselves “excellent” for showing sympathy toward their patients, and 42% of the physicians rated themselves as “excellent” on providing useful information to help the patient make decisions. The range of “excellent” rankings for self-rating of physicians was 50% for believing that they showed sympathy and 24% for believing that they do not interrupt when a patient was speaking.

Table V shows how physicians responded to questions addressing patient attitudes in facilitating discussion. In terms of the elements of patient attitudes that are important for effective communication, 76% of physicians reported “being honest and open” as a very important communication quality from patients. To “understand why a certain therapy is recommended” was ranked second by physicians (58%). Sufficient time to discuss questions and concerns (55%) was ranked third. Sharing information read, heard, or seen on the Internet was not considered to be important by 27% of the physicians. Similarly, having a list of goals and concerns was considered not important for 16% of the sample.

Physicians were also asked to rate their patients for each of these communication qualities, as listed in the last column of Table V. Only 17% of the physician sample gave the patients an “excellent” rating for being “honest and open.” The range for physicians’ rating their patients as excellent was 19% for “willing to trust my advice” and 9% at “having a list of goals and

Table I  Regular checkup: genesis of discussion about menopause

| Patient complains about symptoms | 91% |
| I bring it up with all my patients who are a certain age | 83% |
| Patient questions about hormonal replacement therapy | 74% |
| Patient questions about information they received from friends, media, family | 63% |
| Patient seeking information on herbal supplements and over-the-counter treatments | 55% |
| I don’t bring it up unless the patient raises a concern | 3% |
| Other | 10% |

Table II  Top three concerns/questions from patients about menopause

| Ranked first | Specific menopause symptom management/relief 82% |
| Hormone replacement therapy 8% |
| Weight management 5% |
| Long-term health implications 3% |
| Ranked second | Hormone replacement therapy 50% |
| Long-term health implications 18% |
| Weight management 16% |
| Herbal supplements and over-the-counter treatments 7% |
| Reactions to news stories, information on the Internet 6% |
| Ranked third | Long-term health implications 33% |
| Reactions to new stories, information on the Internet 21% |
| Quality-of-life issues 13% |
| Herbal supplements and over-the-counter treatments 11% |
| Hormone replacement therapy 9% |
| Alternative therapy other than herbal options 6% |

Table III  Challenges to effective communication between physicians and patients

| Confusing messages from media, health care professionals, and friends | 80% |
| Inconclusive data about hormone replacement therapy | 56% |
| Complicated issues surrounding treatment options | 45% |
| My time constraints | 43% |
| Lack of available information about alternative treatment options | 27% |
| Patients are not open about what they know/don’t know about menopause | 23% |
| Patients appear to be managing their menopause on their own | 17% |
| My lack of knowledge about alternative treatment options | 17% |
| Patients are embarrassed to ask questions | 14% |
"concerns" and "sharing information from media." This demonstrates a discrepancy in physicians’ attitude toward their own communication skills and their patients’ communications skills.

**Recommendations for herbal remedies**

When asked what percentage of menopausal women takes some type of herbal remedy or OTC treatment to relieve symptoms, only 4% of the physicians indicated that none of their patients take herbal remedies. Forty-four percent of the physicians indicated that less than 10% of their patients take herbal remedies. Twenty-three percent of the physicians indicated that 11% to 20% take herbal remedies. The remaining physicians indicated that more than 21% of their patients take herbal remedies. Physicians were also asked how comfortable they were discussing or recommending herbal remedies. Only 16% reported being “completely comfortable.” Thirty percent of the physicians reported being “very comfortable,” 32% reported being “fairly comfortable,” and the rest reported being “not very comfortable.”

Among the herbal remedies that physicians are comfortable recommending, soy products (62%) and vitamin E (52%) were on the top of the list. Black cohosh (31%), St. John’s wort (29%), and evening primrose berry (22%) ranked next. Red clover, combination products (Estroven), valerian, dong quai, and chaste tree berry all ranked at or below 16% (Table VI).

Physicians were also asked what course of action they would recommend to patients who are interested in purchasing herbas. The 2 most common responses were that patients “learn about ingredients and their safety/effectiveness” and “carefully read labels.”

**Sources and quality of menopause treatment**

The majority of physicians (81%) would feel more comfortable in discussing or recommending herbal remedies to patients if there were more information from clinical trials and other studies about herbal remedies. For these respondents, information about the following items would be most helpful: safety (82%), efficacy (73%), dosing (64%), effectiveness (61%), quality (49%), and reproducibility (42%).

When asked to rank the top 3 qualities that are most important when recommending any treatment for menopause, safety was ranked first by 65% of the

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**Table IV** Important physician qualities for good physician/patient communication and physician self-rating of behavior

<table>
<thead>
<tr>
<th>Important physician qualities</th>
<th>Very important (%)</th>
<th>Important (%)</th>
<th>Not important (%)</th>
<th>Physician self-rating “excellent” (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Showing sympathy</td>
<td>76</td>
<td>21</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Providing useful information to help the patient make decisions</td>
<td>66</td>
<td>32</td>
<td>2</td>
<td>42</td>
</tr>
<tr>
<td>Allowing plenty of time to discuss the patient’s concerns</td>
<td>50</td>
<td>47</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Not interrupting when a patient is speaking</td>
<td>50</td>
<td>38</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>Probing physical and emotional symptoms carefully</td>
<td>49</td>
<td>47</td>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td>Making the patient feel her experience is normal</td>
<td>45</td>
<td>48</td>
<td>6</td>
<td>35</td>
</tr>
</tbody>
</table>

**Table V** Important patient qualities for good physician/patient communication and physician rating of patients’ behavior

<table>
<thead>
<tr>
<th>Important patient qualities</th>
<th>Very important (%)</th>
<th>Important (%)</th>
<th>Not important (%)</th>
<th>Physician rating of patient “Excellent” (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be honest and open</td>
<td>76</td>
<td>23</td>
<td>0.8</td>
<td>17</td>
</tr>
<tr>
<td>Have a list of goals and concerns</td>
<td>35</td>
<td>47</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Share information read, heard, or seen on the Internet</td>
<td>21</td>
<td>39</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>Be willing to trust me and follow my advice</td>
<td>33</td>
<td>45</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Understand why a certain therapy is recommended</td>
<td>58</td>
<td>40</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Sufficient time to discuss questions and concerns</td>
<td>55</td>
<td>42</td>
<td>2</td>
<td>—</td>
</tr>
</tbody>
</table>

**Table VI** Types of herbal remedies and/or supplements recommended by physicians

<table>
<thead>
<tr>
<th>Herbal remedies and/or supplements</th>
<th>Recommended (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy products</td>
<td>62%</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>52%</td>
</tr>
<tr>
<td>Black cohosh (RemiFemin)</td>
<td>31%</td>
</tr>
<tr>
<td>St. John’s wort</td>
<td>29%</td>
</tr>
<tr>
<td>Evening primrose berry</td>
<td>22%</td>
</tr>
<tr>
<td>Red clover (Promensil)</td>
<td>16%</td>
</tr>
<tr>
<td>Combination products (Estroven)</td>
<td>13%</td>
</tr>
<tr>
<td>Valerian</td>
<td>11%</td>
</tr>
<tr>
<td>Dong quai</td>
<td>7%</td>
</tr>
<tr>
<td>Chaste tree berry</td>
<td>5%</td>
</tr>
</tbody>
</table>
respondents. Being supported by science or research was ranked second by 38%, and secondarily providing protection from other diseases was ranked third by 39% of the physicians.

In terms of the satisfaction rating of physicians of currently available treatments or remedies for menopause symptoms, approximately 34% of the physicians reported being “somewhat satisfied,” and only 13% reported being “very satisfied.” The rest of the sample reported being “somewhat dissatisfied” (31%) and 16% “very dissatisfied.”

Sources of information about menopause and treatments were the last topic of the questionnaire. Physicians were asked to indicate where they thought patients get their information on menopause. The most common response was friends or family (85%). The second and third most common response was newspaper articles or news programs (74%) and magazines (70%). Doctors were ranked fourth as a source of information at 61%. Media, such as television or print advertising (57%) or the Internet (44%), were also ranked by physicians. Pamphlets in doctors’ offices and other health care professionals ranked less than 30%.

Rating of the quality of these sources of information was the last item on the questionnaire. The “doctor” category received an “excellent” rating (13%). Only 1 source of information, pamphlets in a pharmacy or doctor’s office, received a “very good” rating from 40% of the sample. The majority of the categories received ratings of “satisfactory” or “not good.” Magazine articles (54%), advertisements in journals (52%), other health care professionals (49%), journal articles (43%), the Internet (41%), and doctors (39%) received satisfactory ratings from physicians. Friends and family (57%), television (53%), advertisements in magazines (52%), and newspapers (45%) received ratings of “not good” (see Table VII). It is interesting to note that physicians consider those sources of information that they commonly perceive patients relying on most as being of poor quality, suggesting that the physicians may view many patients as misinformed about menopause.

Future concerns for menopause symptom management

When physicians were asked what improvements they would like to see in menopause symptom management remedies, responses were divergent. The majority focused on the following few issues: (1) better understanding of the benefits and side effects of HRT and SERMs; HRT alternatives with fewer side effects (especially the risk of breast cancer) and the same potency; (2) better studies in alternative treatment effectiveness that would limit unsubstantiated claims; (3) more research in alternative medicines with more holistic approaches that take into account the condition of the whole person, instead of just symptoms; (4) research-based guidelines to individualize or customize treatment options and dosage for different patients; and (5) better patient education for efficacy and safety.

When asked when and how herbal treatments or OTC remedies are recommended or discussed, physician responses were more unanimous: herbal therapies or OTC treatments are discussed when HRT is contraindicated (eg, breast cancer, adverse reaction); a patient is reluctant in taking HRT; or a patient brings up the topic.

Differences in responses between gynecologists and primary care physicians

When comparing the responses to the SENSEI questionnaire, some notable differences were found between the 2 groups (primary care versus gynecology physicians). First, there were significant differences in average age. Gynecologists were older (mean 68 years), and the mean age for primary physicians was 48 years ($P < .0001$).

<table>
<thead>
<tr>
<th>Source of information</th>
<th>Physician thinks patients rely on info, %</th>
<th>Excellent, %</th>
<th>Very good, %</th>
<th>Satisfactory, %</th>
<th>Not good, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internet</td>
<td>44</td>
<td>0</td>
<td>15</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td>Magazine articles</td>
<td>70</td>
<td>0</td>
<td>8</td>
<td>54</td>
<td>32</td>
</tr>
<tr>
<td>Pamphlets in pharmacy/doctor office</td>
<td>30</td>
<td>4</td>
<td>40</td>
<td>37</td>
<td>14</td>
</tr>
<tr>
<td>Newspaper</td>
<td>74</td>
<td>0</td>
<td>6</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Friends/family</td>
<td>85</td>
<td>0</td>
<td>3</td>
<td>23</td>
<td>57</td>
</tr>
<tr>
<td>Television</td>
<td>57*</td>
<td>0</td>
<td>3</td>
<td>31</td>
<td>53</td>
</tr>
<tr>
<td>Advertisements in magazines</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>31</td>
<td>52</td>
</tr>
<tr>
<td>Advertisements in journals</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>52</td>
<td>32</td>
</tr>
<tr>
<td>Journal articles</td>
<td>8</td>
<td>8</td>
<td>33</td>
<td>43</td>
<td>10</td>
</tr>
<tr>
<td>Doctor</td>
<td>61</td>
<td>13</td>
<td>37</td>
<td>39</td>
<td>9</td>
</tr>
<tr>
<td>Other health care professionals</td>
<td>36†</td>
<td>2</td>
<td>30</td>
<td>49</td>
<td>15</td>
</tr>
</tbody>
</table>

* Question for patients’ source of information combined television or print advertisements.
† Of the 36%, 14% was from the subfield of “nurse.”
Given the older age of the gynecologists, they had been in practice for a longer time (27 years versus 18 years for primary). This difference was significant at $P < .0001$. There were significant differences in type of practice as well, with 56% of primary care physicians in group practice and 18% in hospital based. For gynecologists 90% were either in solo or group practice ($P = .0016$). With regard to number of hours in practice, average age of menopausal women in their practice or whether the majority of patients take products as prescribed, no significant differences were found. For gynecologists, more patients reported being on a hormone replacement product than primary care physicians ($P = .0003$).

In terms of self-rating, gynecologists consistently scored higher on showing empathy, allowing plenty of time to discuss patients' concerns, and making the patient feel her experience is normal. When asked about “when recommending treatment for menopause symptoms, what are the top 3 qualities that are most important to you?” no significant differences were found. Gynecologist’s reported being more satisfied with available remedies/treatments for menopause symptom management ($P = .0024$). In terms of how comfortable they feel discussing or recommending herbal remedies, no significant differences were found between primary care physicians and gynecologists. In terms of what course of action, if any, they would recommend to patients in purchasing herbals, no significant differences were found in their responses.

**Comment**

In this national probability sample study, we investigated the opinions of gynecologists and primary care physicians with regard to menopause. Findings in this study presented primary evidence of physicians’ first-hand experience with menopause issues in daily practice as well as their opinions on a variety of menopause-related topics. It also allowed physicians a chance to reflect on patient needs and attitudes toward menopause and to have their findings collectively analyzed statistically. It will serve as an important feedback tool to guide physicians’ communication with patients with regard to menopausal issues.

Data indicated that patients’ complaint about menopausal symptoms is the most common lead to discussion of menopausal issues with physicians (91%), and that of top concern to patients are management of menopausal symptoms, controversies around HRT, and long-term health implications of menopause and HRT. Confusing messages with regard to menopause was found by 80% of the physicians to be the most challenging aspect in patient communication. The second most challenging issue is “inconclusive data about HRT” (56%). Seventy-six percent of physicians found “showing sympathy” to be the most important factor for physicians to communicate effectively with patients, whereas patients being “being honest and open” was the most important patient attitude for the same purpose.

When it comes to herbal therapy for menopause symptom control, only 4% of the physicians indicated that none of their patients take any remedies. Only 18% of physicians are not very comfortable in discussing or recommending herbal therapies, whereas the rest range from fairly comfortable to completely comfortable. Soy products turned out to be the most commonly recommended herbal remedies by physicians for menopause (62%) and vitamin E the second (52%). Eighty-one percent of physicians would feel more comfortable in recommending herbal products to patients if there were more information from clinical trials or other studies. Safety was ranked as the most important quality (65%) when recommending herbal to patients, and support by research was ranked the second (38%). Only 13% of the physicians are “very satisfied” by the currently available treatment options for menopause, and 34% are “somewhat satisfied.” In the physician’s opinion, patients most likely get their information on menopause from friends and family, newspapers, and magazines.

There were 2 open-ended questions soliciting opinions on what improvement they would like to see in menopause management and when they would recommend herbal remedies. The answers to the first question were boiled down to a few key issues: better understanding of the pros and cons of HRT and SERMs, better HRT alternatives, and more research on alternative medicines. The physicians were more in agreement about the second question: They most likely recommend herbal products when HRT is contraindicated. This study has provided data concerning physician understanding of menopause treatment options and their primary interaction with patients on this issue.

Some limitations to this study need to be addressed. First, a larger response rate would have been preferable, although cited literature indicates that high compliance is unusual among physicians. Second, this survey was limited in scope by addressing only gynecologists and primary care physicians for reasons of cost. Other members of the medical field are solicited for advice by women facing menopause. Their attitudes about their patients, menopause, and menopausal treatment would have added to the generalizability of physicians’ attitudes and behaviors toward menopausal issues reported here.

Regardless, more in-depth studies regarding efficacy and/or side effects of each available treatment will be the relevant next step, given the controversies around both HRT and alternative therapies. The relative efficacy, safety, and cost-effectiveness of different treatments should also be put into the context of both clinical diagnosis and physicians’ clinical judgment.
References


A randomized trial of amnioreduction versus septostomy in the treatment of twin-twin transfusion syndrome

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Objective: Left untreated, severe twin-to-twin transfusion syndrome (TTTS) presenting in the early second trimester of pregnancy is often associated with significant maternal morbidity and almost universal perinatal loss. Removal of excessive amounts of amniotic fluid through serial amniocenteses (amnioreduction) has been the mainstay of therapy. We sought to compare amnioreduction to intentional perforation of the intervening twin membrane (septostomy).

Study design: Pregnant women with TTTS before 24 weeks’ gestation were randomly assigned to serial amnioreduction or septostomy. A single puncture technique under ultrasound guidance was used for the septostomy. The primary outcome measure was survival to neonatal discharge, and was assessed based on the number of pregnancies or the number of fetuses as appropriate.

Results: The study was terminated at the planned interim analysis stage after 73 women were enrolled. This was because the rate of survival of at least 1 infant was similar in the amnioreduction group compared to the septostomy group (78% vs 80% of pregnancies, respectively; RR = 0.94, 95% CI 0.55–1.61; P = .82). Patient undergoing septostomy were more likely to require a single procedure for treatment (64% vs 46%; P = .04).

Conclusion: Although overall perinatal survival is not enhanced, septostomy offers the advantage of often requiring a single procedure compared to serial amnioreduction in the treatment of severe twin-to-twin transfusion syndrome.

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Although monochorionic twin placenta occurs in only one fifth of all twin gestations, they account for the majority of the resultant perinatal morbidity and mortality. Approximately 15% are complicated by discordance of amniotic fluid volume—the “donor” fetus becomes “stuck” with minimal to absent amniotic fluid, while its “recipient” sibling is surrounded by excessive amount of fluid.1 This condition has been described as twin-to-twin transfusion syndrome (TTTS), reflecting the proposed causation of unequal blood volumes mediated by unbalanced intertwin transfusion through placental anastomoses. Monochorionic placentas that result in TTTS are less likely to have compensatory bidirectional vascular channels, and recent reports document resolution of the syndrome after laser ablation of deep unilateral intraplacental anastomoses.2,3

Left untreated, severe TTTS presenting in the second trimester has been associated with a perinatal survival of 0% to 30%.4,5 Until recently, serial amnioreduction has been the mainstay of treatment, with survival rates of approximately 60%.6 Amnioreduction aims to normalize amniotic fluid volume and reduce the rate of preterm labor and amniorrhexis, and there is some evidence that it may improve uteroplacental perfusion and, on occasion, lead to regression of the TTTS.7 In 1998, Saade et al8 proposed the concept of intentionally perforating the intertwin membrane (septostomy) to allow equilibration of the amniotic fluid volume in severe cases of TTTS. Indeed, allowing the donor twin to swallow excess amniotic fluid produced by the recipient is one explanation for the extreme rarity of TTTS in monoamniotic twins. In a feasibility study of 12 women treated with septostomy at 5 centers, the overall perinatal survival was 86%, with a mean gain of 8.3 weeks in utero. All procedures were successful on the first attempt. In only 3 of the 12 cases was an amnioreduction necessary concurrently with the septostomy; a single amnioreduction performed at a later date was necessary in only 2 of these cases. Johnson et al9 later reported a retrospective series of 14 women treated with septostomy or amnioreduction. Pregnancy was prolonged an average of 12 weeks in the septostomy group compared to 6.5 weeks in the amnioreduction group (P < .01).

These data led us to consider a multicenter randomized trial to compare the standard therapeutic option of amniodrainage with the newer technique of septostomy for the treatment of TTTS.

Material and methods

The study was approved as a multicenter trial by the human investigational review board of the University of North Carolina School of Medicine, as well as each of the local institutional review boards of the 11 participating centers.

Women with monochorionic twin gestations at less than 24 weeks’ gestational age with ultrasound evidence of TTTS were offered enrollment in the trial. Monochorionicity was defined as the presence of concordant fetal sex, a single placenta, and the lack of a projection of the chorionic tissue at the interface between the intertwin membrane and the chorionic surface. Chorionicity was confirmed after delivery by examination of the placenta. Standard inclusion criteria for TTTS included polyhydramnios in 1 amniotic cavity (deepest vertical pocket of >8 cm at less than 20 weeks’ gestation, >10 cm at 20 to 22 weeks’ gestation, and >12 cm after 22 weeks’ gestation), and oligohydramnios in the second amniotic cavity (deepest vertical pocket of <2 cm).

Exclusion criteria were fetal structural anomalies, premature contractions associated with cervical change, premature rupture of the membranes, suspected chorioamnionitis, or the presence of other indications for delivery.

Computer randomization was undertaken in advance utilizing a block size of 10. No stratification was used. Each participating center was given access to a secure web site for randomization as previously described.10

Amnioreduction procedure

Amnioreductions were performed using real-time ultrasound guidance within the recipient’s amniotic sac. An effort was made to avoid the intertwin membrane and, thus, the gestational sac of the donor twin so as not to perform an unintentional septostomy. An 18-gauge needle was introduced into the amniotic cavity with polyhydramnios, and then connected to either wall suction or a syringe attached to extension tubing in an effort to actively drain the amniotic fluid, thereby reducing the total procedure time. Fluid was removed until the deepest vertical pocket was less than or equal to 6 cm, or a total of 5 L was removed. Ultrasound assessment of amniotic fluid was then repeated weekly. A repeat amnioreduction was undertaken when the woman developed symptoms of excessive uterine activity or maternal respiratory compromise, or when the deepest vertical amniotic fluid pocket met the original inclusion criteria.

Septostomy procedure

The intertwin membrane was purposefully perforated under ultrasound guidance with a single puncture using a 22-gauge needle. This was usually introduced though the donor twin’s gestational sac into the recipient twin’s amniotic cavity, as preliminary experience before the trial indicated that visualization of the needle tip for confirmation of perforation was easier with this approach.
All women undergoing septostomy underwent a repeat ultrasound in 48 hours. If reaccumulation of amniotic fluid in the donor twin’s amniotic cavity did not occur, a repeat septostomy was undertaken. Occasionally, the treating clinician decided that a second amnioreduction was also necessary at the time of the repeat septostomy. If a repeat septostomy was accompanied by an amnioreduction at the same setting, this was considered a single additional procedure. Ultrasound was repeated 48 hours after successful septostomy. If the oligohydramnios had not resolved in the donor twin’s sac, or if the deepest vertical pocket in the recipient’s twin’s sac had increased by 30% over baseline value, crossover to the amnioreduction treatment arm was allowed.

If the amniotic fluid volume in the polyhydramnios sac was significant enough to be associated with maternal symptoms, a salvage amnioreduction was
undertaken concurrently with the septostomy. In these cases, the 22-gauge needle used to create the septostomy was inserted through the 18-gauge needle used for the amnioreduction in an effort to create only 1 puncture of the amniotic cavity.

New therapeutic interventions such as laser ablation of anastomotic vessels and umbilical cord occlusion were introduced into clinical care during the time course of this study.3,11 These modalities were employed in a minority of women who had progression of TTTS despite amnioreduction or septostomy. Outcomes of all women enrolled in the trial were analyzed on an intent-to-treat basis.

Statistical analysis

The primary outcome variable for the study was at least 1 infant surviving until hospital discharge. Based on the pilot trial of septostomy, we utilized a perinatal survival rate of 85% for the septostomy group and 65% for the amnioreduction group.6,8 Using an alpha value of 0.05 and a 1-beta of 0.80, a sample size of 140 subjects (70 in each arm) was required. An interim analysis was planned at the midway point of the trial utilizing the O'Brien-Fleming stopping rule12 for discontinuing enrollment.

The Shapiro-Wilk test was employed to test for normality of distribution for continuous variables. Data were analyzed using contingency tables, Student t test, Mann-Whitney test, and multiple logistic regression. A P value of less than .05 was considered statistically significant. Relative risks and 95% CIs were calculated.

Results

Between September 1997 and July 2002, a total of 73 women were enrolled in the trial. At the time of the interim analysis, 36 women were enrolled in the amnioreduction arm and 37 women in the septostomy arm. A decision was made to terminate the trial by the Data Safety Monitoring Officer after the interim analysis because of slower than projected enrollment that had been anticipated, and almost identical perinatal survival in the 2 treatment arms of the trial, rendering it unlikely that the primary end point would be achieved.

The mean gestational age at enrollment in the amnioreduction group was 20.9 ± 2.6 weeks compared to 20.8 ± 2.7 weeks in the septostomy group (P = .85). The trial was initiated before the description of the Quintero staging system, and therefore cases were not specifically staged at the time of enrollment in the trial.13 However, a review of the ultrasound parameters at entry revealed that only 2 pregnancies (one in each arm of the trial) were complicated by the finding of a hydropic fetus at the time of entry (stage 4 disease). Therefore, 97% of the gestations in the trial represented stage 1 to 3 disease.

Figure 1 depicts the total number of women in the trial. Two women were lost to follow-up in the septostomy arm. One patient was randomized at 23 weeks’ gestation, was treated with 2 septostomies, and then moved to Korea at 24 2/7 weeks’ gestation. The second patient was randomized to septostomy but all data, including follow-up information, were lost. In the amnioreduction arm, 11 women required only 1 procedure. In the remaining 25 women, there were a total of 80 procedures: 77 amnioreductions (range: 1-12), 1 laser therapy and 2 umbilical cord occlusions. In the septostomy arm of the trial, 18 (51%) of the women required only a single septostomy for treatment. One woman randomized to the septostomy arm received a single amnioreduction as her only therapeutic procedure. In 16 of the 35 women (46%), a salvage amnioreduction was undertaken in conjunction with the initial septostomy. Sixteen women in this treatment group required a total of 54 additional procedures: 8 repeat septostomies (3 of these accompanied by an amnioreduction), 44 additional amnioreductions (range: 1-8), 1 umbilical cord occlusion, and 1 laser therapy.

Perinatal survival in the trial is shown in Figure 2. The survival of at least 1 infant occurred in 78% of the pregnancies in the amnioreduction group vs 80% in the septostomy group (RR = 0.94, 95% CI 0.55–1.61; P = .82). Survival of a single infant only occurred in 28% vs 20% of pregnancies (RR = 1.22, 95% CI 0.75–2.00; P = .44; amnioreduction vs septostomy), while survival of both infants was noted in 50% vs 60% of pregnancies (RR = 0.82, 95% CI 0.52–1.30; P = .40; amnioreduction vs septostomy). Fetal and neonatal deaths occurred with equal frequency in the amnioreduction and septostomy groups (P = .40). There was no difference in the frequency of fetal or neonatal deaths in either the donor or recipient twin (Table).
Secondary outcome analysis revealed that women undergoing septostomy were less likely to require more than the initial additional procedure for treatment \( (P = .04) \).

Minimal complications were reported for both amnioreduction and septostomy. There were no cases of placental abruption or chorioamnionitis. Total disruption of the intervening membrane creating a monoamniotic twin gestation occurred in 1 case in each treatment arm of the study. However, entanglement of the umbilical cords was not observed at delivery in either case.

In multivariate analysis, perinatal survival was not related to treatment group after adjusting for gestational age at randomization, gestational age at delivery, birth weight, and donor vs recipient twin status (adjusted \( RR = 1.1; 95\% CI 0.60–1.94 \)).

**Comment**

Severe polyhydramnios due to second-trimester TTTS is often associated with preterm labor and preterm prematurity rupture of the membranes, leading to perinatal loss. This has led many investigators to propose serial amnioreduction as the treatment of choice. Multiple cases have been reported in which a single amnioreduction was the only therapeutic maneuver required for TTTS. In one series, 17% of cases presenting in the second trimester responded to a single amnioreduction.\(^\text{14}\) This was seen in the present study in almost one third of the 38 cases enrolled in the amnioreduction group. Saade et al.\(^\text{8}\) proposed that the likely explanation for this effect is that the dividing membrane, which is compressed against the uterine wall and therefore not visualized, is unintentionally perforated during amnioreduction. This led these authors to propose the use of an intentional fenestration of the intervening membrane as a treatment option for severe TTTS.

In the present investigation, the survival of at least 1 infant did not differ between amnioreduction and septostomy (78% vs 80%, respectively). However, other findings in the study would point to a slowing of the disease process of twin-to-twin transfusion with septostomy. More than half of the women in the septostomy arm required only the initial invasive intervention.

The mechanism by which septostomy potentially retards the progression of TTTS is speculative. One must first consider what causes the rapid reaccumulation of amniotic fluid around the donor twin. The rapid time course of this finding points to movement of amniotic fluid from the polyhydramnios sac to the oligohydramnios sac. The law of fluid dynamics of Pascal indicates that 2 compartments in the same vessel separated by an elastic membrane should exhibit equal pressures. Thus, fluid should not move between the amniotic sacs in TTTS even after the membrane has been breached. In practical terms, however, once the intervening membrane becomes transposed against the stuck twin, it serves as an inelastic wall within the uterus, thereby creating 2 separate compartments. A minor difference in hydrostatic pressure is therefore the most likely explanation for the movement of fluid through the septostomy from the higher-pressure polyhydramnios sac to the lower-pressure oligohydramnios sac. This proposed mechanism is consistent with a case reported by Bruner and Crean.\(^\text{15}\) Indigo carmine injected at the time of amnioreduction of the recipient’s sac was rapidly detected in the amniotic fluid surrounding the donor twin. Examination of the intertwin membrane at delivery revealed a 3-cm rent.

Equilibration of the amniotic fluid volumes may then have 1 of 2 subsequent effects. Polyhydramnios in TTTS has been associated with alterations in the pulsatility index of the uterine artery due to increased amniotic fluid pressure, reflecting reduced uterine perfusion, and there is an association between raised amniotic fluid

<table>
<thead>
<tr>
<th>Outcome data</th>
<th>Amnioreduction</th>
<th>Septostomy</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>36</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>GA at delivery (wk)</td>
<td>29.5 (3.5)</td>
<td>30.7 (5.0)</td>
<td>.24</td>
</tr>
<tr>
<td>Days gained</td>
<td>59.9 (26.4)</td>
<td>69.2 (37.5)</td>
<td>.12</td>
</tr>
<tr>
<td>Birth weight of donor twin (g)</td>
<td>996 (408)</td>
<td>1291 (731)</td>
<td>.12</td>
</tr>
<tr>
<td>Birth weight of recipient twin (g)</td>
<td>1075 (146–3189)</td>
<td>1455 (214–3335)</td>
<td>.27</td>
</tr>
<tr>
<td>Fetal deaths</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor</td>
<td>5 (14%)</td>
<td>4 (11%)</td>
<td>.76</td>
</tr>
<tr>
<td>Recipient</td>
<td>4 (11%)</td>
<td>5 (14%)</td>
<td>.69</td>
</tr>
<tr>
<td>Neonatal deaths</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor</td>
<td>9 (25%)</td>
<td>6 (17%)</td>
<td>.42</td>
</tr>
<tr>
<td>Recipient</td>
<td>8 (22%)</td>
<td>6 (17%)</td>
<td>.59</td>
</tr>
<tr>
<td>No. of additional procedures</td>
<td>2 (1–12)</td>
<td>2 (1–9)</td>
<td>.07</td>
</tr>
<tr>
<td>More than 1 procedure necessary</td>
<td>25 (69%)</td>
<td>16 (46%)</td>
<td>.04</td>
</tr>
</tbody>
</table>

Data provided as mean (SD), median (range), or numbers (%).
pressure and abnormal fetal blood gases. Amnioreduction or equilibration of amniotic volumes may improve blood flow to the uterus. Alternatively, high amniotic fluid pressure may be transmitted to placental plate vessels, causing increased uteroplacental resistance in the recipient twin. Doppler evidence of fetal hypertension in the recipient twin has been recently documented. A reduction in amniotic fluid pressure due to resolution of the polyhydramnios could alleviate this increased afterload.

Septostomy was associated with minimal complications in this trial. Critics of septostomy have cautioned that purposeful fenestration of the intertwin membrane could lead to creation of a monoamniotic gestation with a significant risk for entangled umbilical cords, and subsequent perinatal loss. Sporadic case reports of disruption of the dividing membrane have been reported after genetic amniocentesis using a single puncture technique. However, large series of amniocenteses in twins have failed to identify this as a complication. In the present trial, only 2 cases of disruption of the intervening membrane occurred, one in each treatment arm. In both cases, entanglement of the umbilical cords was not seen at birth. Nevertheless, both amnioreduction and septostomy present the potential for intertwining of the umbilical cords, which could lead to perinatal loss.

One of the limitations of the current trial was that long-term neurologic outcome was not assessed. Since it was first introduced in 1990 by De Lia, proponents of laser ablation of the communicating vessels for the treatment of TTTS have suggested that both amnioreduction and septostomy do not treat the underlying condition. Although perinatal outcomes with amnioreduction are reasonable in early stage (I/II) disease, the outcome is poorer with advanced and progressive disease, such that other treatment modalities are now warranted. In a recent randomized trial, laser coagulation of communicating vessels in TTTS was reported to improve both perinatal survival, as well as short-term neurologic outcome. This is supported by a large comparative series showing that laser was associated with better survival in stage III and IV disease than amnioreduction, albeit with worse survival in early stage disease.

The best therapeutic option for all women with severe second-trimester TTTS has yet to be determined. In a recent editorial that accompanied the report of the randomized trial of laser therapy, Fisk and Galea proposed that although laser treatment of TTTS appeared promising, further investigation particularly in early-stage disease is warranted. In addition, one study of the progression of early stage TTTS indicated that only 31% of stage I and 20% of stage II cases advanced to a higher stage disease, and there is evidence that adverse neurologic outcome is associated with advanced disease. Amnioreduction and septostomy, therefore, still remain viable treatment options for stage I and II disease. Septostomy offers the advantage over amnioreduction of more frequently requiring only a single procedure with minimal complications. If progression of disease is noted after a septostomy or amnioreduction, laser therapy would seem the treatment of choice. However, we acknowledge that septostomy may rarely impede subsequent laser therapy treatment if the resultant floppy membrane hinders visualization of the chorionic plate. We suggest that a randomized trial of combination amnioreduction/septostomy vs laser is indicated in early stage TTTS.

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Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only

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KEY WORDS
Chorioamnionitis
Neonatology
Outcome
Morbidity
Resources

Objective: This study was undertaken to evaluate whether the proximity of infection of the chorion/amnion and fetal vessels affects neonatal outcomes.

Study design: We examined all (n = 2012) infants admitted to the British Columbia’s Children’s Hospital Neonatal Intensive Care Unit, from January 1996 to October 1997. We included infants with a placental examination (n = 1296), and stratified those with histologic chorioamnionitis into cases displaying a maternal inflammatory response only and cases also displaying a fetal inflammatory response (funisitis and/or fetal surface vessel angiitis).

Results: Histologic evidence of chorioamnionitis was present in 31% of placentas. Of those, 38% exhibited maternal inflammation only, whereas 62% also exhibited fetal inflammation. Neonatal mortality (9.2% vs 7.2%), morbidity, and resource use were significantly (P < .05) higher when fetal inflammation was present compared with when only maternal inflammation was present.

Conclusion: Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than when only a maternal inflammatory response is present.

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The term chorioamnionitis refers to acute inflammation of the placental membranes (amnion and chorion) and is a leading cause of maternal and fetal complications, including preterm birth and neonatal infection. The inflammatory process is generally regarded as a continuum. During the early stage, the neutrophils involved in this inflammatory response are usually maternal (migrating from intervillous sac and/or decidual vessels) in origin. During later stages, fetal neutrophils (migrating from fetal surface vessels of the chorionic plate or umbilical cord) are involved. The incidence of histologically identified chorioamnionitis among infants born preterm has been reported to be about 33%. Although chorioamnionitis among infants admitted to the neonatal intensive care unit (NICU) has been previously reported, few studies have examined the correlation between the proximity of the inflammatory response (fetal vs maternal inflammatory response) in chorioamnionitis and neonatal outcomes. The objective of this study was to compare outcomes of infants whose placenta exhibited histologic chorioamnionitis with fetal response with those exhibiting maternal inflammatory response only.

**Material and methods**

**Study population**

The study cohort included all infants (n = 2012) admitted to the British Columbia’s Children’s Hospital NICU from January 1996 to October 1997. Infants were identified from standardized data collected by the Canadian Neonatal Network as part of a larger study of 17 tertiary NICUs across Canada, and which has been previously described. Trained research assistants abstracted patient information from the mothers’ and infants’ charts at each participating hospital on a daily basis. Data were entered directly into laptop computers at the bedside by using a customized data entry program with built-in error checking and a standard manual of operations and definitions. Identified infants were matched with placental histopathology reports from the Department of Pathology database. The pathology slides were read by 4 surgical pathologists in the hospital Department of Pathology and reviewed by a single perinatal surgical pathologist. Acute inflammation of the membranes was reported as present or absent. A further comment was then made as to whether there was evidence of a fetal inflammatory response (fetal surface vessel angiitis, funisitis). Pathology reports were reviewed by using a standardized data form and data definitions by a single reviewer who was blinded to the maternal and neonatal outcomes. Institutional ethics approval for the study was obtained.

**Definition of terminology**

Chorioamnionitis was defined histologically as an infiltration of the chorion and/or amnion (membranes) by polymorphonuclear leucocytes. These polymorphs are maternal and may migrate from the decidual vessels and/or intervillous space. Diagnosis was based on histologic features (not gross pathology) and required neutrophil infiltration involving at least 50% of membrane section submitted. The cases of chorioamnionitis were stratified into those that showed the inflammatory response confined to membranes only (maternal response) and those that showed both inflammation of the membranes with a fetal surface vessel angiitis and/or umbilical vessel inflammation (maternal and fetal response).

Indices of neonatal outcome were defined according to the Canadian Neonatal Network Data Abstractor Manual. Gestational age was defined as the best obstetric estimate that was based on early prenatal ultrasound, obstetric examination, and obstetric history, unless the postnatal pediatric estimate of gestation differed from the obstetric estimate by more than 2 weeks. In that case, the pediatric estimate was used instead. An infant was defined as small-for-gestational age (SGA) if the birth weight was less than the third percentile for gestational age according to the British Columbia provincial growth charts established by Whitefield et al in 1992. SNAP-II is a neonatal illness severity score calculated from 6 empirically weighted physiologic measurements made during the first 12 hours of admission to the NICU. NTISS is a score of neonatal therapeutic intensity calculated from a checklist of 63 NICU therapies used in a 24-hour period, weighted according to invasiveness and cost. Intraventricular hemorrhage was defined according to the criteria of Papile et al from head ultrasound performed before 14 days of life. Necrotizing enterocolitis was defined according to the criteria of Bell et al (stage II or higher) and was classified as medical (clinical symptoms and signs plus evidence of pneumatosis on abdominal radiographs) or surgical (histologic evidence on surgical specimen of intestine). Retinopathy of prematurity was defined according to the International Classification for Retinopathy of Prematurity and the Reese Classification of cicatrical disease. Nosocomial infection was defined by using blood and cerebrospinal fluid culture results according to criteria by Freeman et al. Primary infection was defined as positive single organism cultures from blood or cerebrospinal fluid.
obtained from an infant with signs or risk factors for sepsis during the first 48 hours of life. Patent ductus arteriosus was defined as clinical diagnosis plus treatment with indomethacin or surgical ligation or both. Respiratory distress syndrome was defined as the presence of respiratory symptoms, such as grunting and chest retraction, typical chest x-ray findings, or treatment with surfactant and the need for mechanical ventilation for greater than 24 hours. Chronic lung disease was defined as oxygen dependency at 36 weeks’ corrected gestational age for an infant who was born at 32 weeks’ or less gestation.19 Seizures were defined as clinically significant episodes witnessed by a nurse or physician and for which anticonvulsant treatment was given. Outborn infants were those born at a hospital different from the hospital in which the NICU was located.

Data analysis

Infants with histologic evidence of chorioamnionitis were stratified into those with maternal inflammation only and those with maternal and fetal inflammation. These groups were also compared with those who had a placental examination but did not have chorioamnionitis. Descriptive statistics were used to examine the data. Analysis of variance and likelihood ratio tests were used to compare differences in the characteristics and outcomes of infants with maternal and fetal inflammation, maternal inflammation, and those cases with no histologic evidence of chorioamnionitis. Multivariable logistic regression analysis was used to examine the relationship between the extent of inflammation and neonatal outcomes after adjusting for confounding factors, and multivariable linear regression modeling was used to analyze resource consumption and length of NICU stay.

Results

Of the 2012 cases, 1296 had a record of placental pathology, representing 64% of all NICU admissions. Histologic evidence of a maternal inflammatory response was identified in 403 cases, or 31% of those with a pathology report. Of those 403 cases, 153 (38%)
had maternal inflammation only, whereas 250 (62%) also displayed evidence of fetal inflammation. Infants who had histologic examination of their placentas were significantly ($P < .05$) more likely than infants who did not have a placental examination to be inborn (86% vs 76%), male (44.3% vs 38.5%), of lower mean gestational age (33.2 ± 4.3 weeks vs 37.3 ± 3.1 weeks) and lower mean birth weight (2.1 ± 0.9 kg vs 3.0 ± 0.8 kg), receive antenatal corticosteroid treatment (46.8% vs 6.7%), born of a mother with maternal hypertension (15.5% vs 8.6%), and born by cesarean section (46.2% vs 30.4%). However, the incidence of multiple births was similar between the groups (12.5% vs 11.7%, $P = .61$). Cases with histologic evidence of fetal inflammatory response were associated with a higher incidence of villous edema as identified by histology (20.0% vs 9.2%, odds ratio [OR] = 2.48; 95% CI 1.32-4.67), when compared with those displaying maternal inflammation only.

Infants with a fetal inflammatory response had significantly ($P < .01$) lower mean gestational age (31.7 ± 0.3 weeks, 33.0 ± 0.4 weeks, 33.7 ± 0.1 weeks, respectively) and birth weight (1894 ± 65 g, 2028 ± 74 g, 2124 ± 28 g, respectively) than those with only a maternal inflammatory response or no chorioamnionitis. They also had significantly ($P < .01$) lower incidence of maternal hypertension (2.4%, 13.7%, and 17.6%, respectively), cesarean section (33.2%, 40.5%, and 50.7%, respectively), and multiple births (10%, 14.4%, and 30.3%, respectively) and were more likely to have received antenatal corticosteroid treatment (63.5%, 47.8%, and 42.1%, respectively) than infants with a maternal inflammatory response and no chorioamnionitis.

Table I compares outcomes and resource use of infants without chorioamnionitis, with chorioamnionitis involving a maternal response only, and with chorioamnionitis also displaying a fetal response. Mortality, morbidity, and resource use were highest among infants with evidence of a fetal inflammatory response and lowest among those with no evidence of chorioamnionitis.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Risk factors for outcomes among NICU infants, using multivariable logistic regression analysis (n = 1296) (ORs and 95% CIs shown)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>Death in NICU</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>Fetal inflammation</td>
<td>2.9 (1.6-5.1)</td>
</tr>
<tr>
<td>Maternal inflammation</td>
<td>2.1 (1.1-4.2)</td>
</tr>
<tr>
<td>Antenatal steroids</td>
<td>NS</td>
</tr>
<tr>
<td>Apgar &lt;7 at 5 min</td>
<td>NS</td>
</tr>
<tr>
<td>Outborn status</td>
<td>2.6 (1.5-4.6)</td>
</tr>
<tr>
<td>SGA (3rd percentile)</td>
<td>2.5 (1.1-6.2)</td>
</tr>
<tr>
<td>Multiple birth</td>
<td>NS</td>
</tr>
<tr>
<td>Cesarean section</td>
<td>NS</td>
</tr>
<tr>
<td>Variables</td>
<td>Death in NICU</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>Fetal inflammation</td>
<td>1.8 (1.1-3.0)</td>
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<tr>
<td>Maternal inflammation</td>
<td>2.6 (1.7-3.8)</td>
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<tr>
<td>Antenatal steroids</td>
<td>NS</td>
</tr>
<tr>
<td>Apgar &lt;7 at 5 min</td>
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<tr>
<td>Outborn status</td>
<td>2.6 (1.5-4.6)</td>
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<tr>
<td>SGA (3rd percentile)</td>
<td>2.5 (1.1-6.2)</td>
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<td>Multiple birth</td>
<td>NS</td>
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<td>Cesarean section</td>
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<td>Variables</td>
<td>Death in NICU</td>
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<tr>
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<tr>
<td>Fetal inflammation</td>
<td>3.9 (1.1-14.1)</td>
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<td>Antenatal steroids</td>
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<td>Apgar &lt;7 at 5 min</td>
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<td>SGA (3rd percentile)</td>
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<td>Cesarean section</td>
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<td>Maternal hypertension</td>
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</table>
Table II shows independent predictors of neonatal outcomes, after adjustment for baseline population characteristics by using multivariable logistic regression analysis. Maternal only inflammatory response was independently predictive of mortality, nosocomial infection, necrotizing enterocolitis, and patent ductus arteriosus. However, fetal inflammatory response was even more highly predictive than maternal inflammation only for these same outcomes, as well as for primary infection, respiratory distress syndrome, need for assisted ventilation, need for oxygen treatment, chronic lung disease, severe intraventricular hemorrhage, and decreased survival without major morbidity. A proportional odds model showed that fetal (but not maternal only) inflammation was also significantly associated with higher NTISS scores, after adjustment for baseline population risk factors. A multivariate linear regression model demonstrated that fetal inflammation (but not maternal only) was associated with increased risk adjusted duration of NICU stay.

Comment

The importance of placental pathology for the diagnosis and management of neonatal conditions has long been recognized. As early as 1892, a Scottish obstetrician, Ballantyne stated that “During the intrauterine life, the fetus, the membranes, the cord, and the placenta form an organic whole, and disease of any part must react upon and affect the others.” Our findings that 31% of placentas examined in our cohort showed histologic evidence of chorioamnionitis, and that chorioamnionitis is associated with higher incidence of adverse neonatal outcomes are consistent with reports by previous authors.5,21

Our finding of increased mortality and morbidity among infants with increasing stages of chorioamnionitis is consistent with previous reports by Van Hoevan et al27 and others.23–25 This is in contrast to a recent report by Lahra and Jeffrey,26 who found that chorioamnionitis with a histologic fetal response was more prevalent among preterm survivors of 25 to 34 weeks’ gestation (but not 20-24 weeks’ gestation) compared with cases of perinatal death. Lahra and Jeffrey26 attributed their finding to increased secretion of cortisol production secondary to intrauterine exposure to infection, which facilitates fetal lung maturation in preterm infants and reduces respiratory distress syndrome during the neonatal period. However, because their cohort included infants born between 1984 and 1999, the majority of infants studied were from the presurfactant era. It is also unclear to what extent antenatal corticosteroid treatment was used in the cohort. Finally, their results were not risk-adjusted for either population risks or use of antenatal corticosteroids. These factors could account for the difference in findings reported by Lahra and Jeffrey.26 Our finding that increasing stages of chorioamnionitis are correlated with chronic lung disease is also consistent with previous reports by Watterberg et al27 and others, and suggests that the severity of pulmonary injury may be associated with severity of chorioamnionitis.

Although chorioamnionitis with fetal response has usually been regarded as a later stage of infection than chorioamnionitis with maternal response only,26 it is unclear whether this is really the case, or whether they might be manifestations of different infections (eg, type of organism, route of infection), modification by treatment (eg, antenatal corticosteroids), and host defenses of both the mother and fetus. It is also unclear whether infants whose placentas show fetal inflammation should be treated differently than those with maternal inflammation only because of the higher associated mortality, morbidity, and resource use. It is possible that more aggressive treatment of chorioamnionitis or earlier delivery of an infant before the development of a fetal inflammatory response may result in improved outcomes. Clarification of these questions through prospective clinical trials is important for determining optimum management of chorioamnionitis and reducing associated mortality and morbidity. Placental histologic findings may have a role to play in risk assessment and prognostication. Addition of placental findings to known demographic risk factors and clinical presentation may improve our ability to more accurately predict neonatal outcomes and counsel parents. Further research is also needed to determine whether clinical correlates of maternal and fetal inflammation among infants with chorioamnionitis can provide further insights into identification of antenatal risk factors that might guide interventions aimed at reducing neonatal mortality and morbidity.

This study was a single institution report and therefore the results reflect the unique diagnostic patterns and pathology referral practices of one hospital. Although neonatal data were abstracted by a single individual using standardized definitions and protocols, placental pathology was reported by 4 pathologists. However, interobserver variability was minimized because half the placental specimens were read by a single perinatal surgical pathologist, who also reviewed the specimens read by the other pathologists. Because only 64% of the cohort had histologic examination of the placenta, there might be bias in the results. However, the incidence of chorioamnionitis in our study was consistent with other studies.

Acknowledgments

We acknowledge the assistance of Ruth Little in preparing this article, and the Canadian Neonatal Network for use of their data.
References


Early discharge from obstetrics-pediatrics at the Hospital de Valme, with domiciliary follow-up

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KEY WORDS
Early discharge
Postpartum
Newborn infants
Mothers

Objectives: This study was undertaken to evaluate the advantages and disadvantages of a program of early obstetric-pediatric discharge (24 hours postpartum) with domiciliary follow-up, compared with the traditional postpartum hospital stay (more than 48 hours), according to the criteria described by reviewers of the subject.

Study design: A randomized controlled trial of early obstetric discharge for healthy mothers and term infants, with postpartum randomization, with no prenatal preparation and with observational and clinical follow-up was performed. The participants were mothers with healthy, term neonates (37-42 weeks) weighing more than 2500 g and produced via vaginal delivery and with a verified normal evolution before discharge. The sample consisted of 430 cases (213 cases with early discharge, and 217 control cases) in which the following variables were evaluated: existence of complications in the mother and/or child that required rehospitalization or a medical consultation, existence of maternal problems of fatigue or anxiety/depression after the birth, continuity of lactation and its problems, satisfaction of the mother and family, and relative costs.

Conclusion: After demonstrating the homogeneity of the groups, no significant differences were found in the rates of maternal rehospitalization (1.9% in the early discharge group vs 2.3% in the control group, relative risk 0.81, 95% CI 0.21-3.03) or in the rates of rehospitalization of the neonates (1.4% in the early discharge group vs 2.3% in the control group, relative risk 0.16, 95% CI 0.15-2.56). No increases were observed in maternal or neonatal disease, puerperal fatigue, or maternal anxiety/depression. A prolongation of maternal lactation to 3 months was observed in the early discharge group ($P = .016 < .05$ Fisher exact test). When the cost of early discharge is compared with that of traditional discharge with a minimum of 48 hours hospital stay, we find a saving of 18% to 20%. The level of maternal satisfaction with early discharge is better than 90%.

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The 2 reasons for postpartum hospitalization are to identify maternal or neonatal complications after the birth and to offer professional assistance to mother and child ensuring that the mother has recovered sufficiently from the birth to be able to care for both herself and her child when they return home. For these purposes, in cases of uncomplicated vaginal birth, the postpartum hospital stay recommended by the American College of Obstetricians and Gynecologists (ACOG) and the American Academy of Pediatrics (AAP) is a minimum of 48 hours.¹

Early discharge, in cases of vaginal delivery, may therefore be defined as the release of the mother and/or the neonate in less than 48 hours postpartum.²

Since the 1970s, there have been many programs of early obstetric-pediatric discharge, and various reviews of the medical literature on this topic exist with 1 conclusion in common: The existence of important errors in the methodology of the studies, which devalues their conclusions.³⁻⁷ No evidence exists that supports or denies the usefulness of the practice, and with the available data, it is not possible to define the ideal period of postpartum hospitalization for mother and child, nor the necessity of domiciliary follow-up in the event of early discharge.

Our objective was to perform a program of early obstetric-pediatric discharge with domiciliary follow-up and to evaluate its advantages and disadvantages compared with the traditional postpartum hospital stay, according to the established criteria for early.¹²,⁸

Material and methods

Study population

The study consisted of puerperae and their neonates, at the Hospital de Valme de Sevilla during the period of April 1999 to April 2001, who fulfilled the criteria in Table I.

Study description

The studies were nonexperimental with observational and clinical follow-up with no prenatal preparation and with postpartum randomization. The 2 study groups, intervention and nonintervention, received information, and consent was obtained in writing.

Intervention group

Puerperae discharged in the first 24 hours postpartum, monitored at home over the next 24 to 48 hours by a nurse qualified in puerperal and neonatal care (physical revision of the mother with arterial pressure, oral temperature (T°), uterine involution, metrorrhagia, and evaluation of the episiotomy; physical revision of the neonate with T°, and evaluation of icterus; informing the mother and family members about puerperal and neonatal care and maternal lactation), at 7 to 10 days in the practice and at 1, 3, and 6 months with a follow-up telephone consultation to the home.

Nonintervention group

Puerperae discharged after the usual minimum of 48 hours postpartum, monitored at 7 to 10 days in the practice and at 1, 3, and 6 months with a follow-up telephone consultation to the home.

Variables measured are as follows:

- Primary results of mother (in the first 6 weeks): Proportion of readmissions, proportion of cases with high scores for depression (Appendix A.)
- Primary results of newborn infant (in the first 28 days): Proportion of neonates readmitted, proportion of cases with breastfeeding, exclusive or mixed (evaluation at 1, 3, 6, and 9 months).
- Secondary results of mother (in the first 6 weeks): Total duration of rehospitalization, proportion of cases requiring emergency consultation, proportion of women with complaints of fatigue (Appendix B), proportion of women with physical problems, such as perineal.
- Secondary results of newborn infant (in the first 28 days): Length of stay of readmissions, total duration of rehospitalization, proportion of cases requiring emergency consultations.
- Evaluation of satisfaction (surveyed for satisfaction at 6 weeks).
- Economic evaluation: Cost of stay from birth to discharge, cost of the postnatal care required, such as health or lactation consultations, home visit programs, readmissions in hospitals (first 6 weeks), cost of the maintenance of the early discharge programs (first 6 weeks).

Statistical method

The sample size was fixed at 213 women in each group for a potential of 80%, an alpha error of 5%, and a percentage difference in maternal or neonatal readmission of 5.5% for a unilateral test. A randomization by blocks (opaque sealed envelops) was performed within the 2 strata defined by the parity variable (primiparous, multiparous); the sample size within each group in these 2 strata was fixed, taking into account the distribution of the parity variable within our area of study.

Statistical analysis

Means and SDs or interquartile ranges for the numerical variables were determined, whereas percentages were obtained for the qualitative variables. The Student t test, or the Mann-Whitney U test, was used to compare the numerical information in the 2 groups “early discharge” and “traditional discharge.” Significant results were
complemented by confidence intervals for means to 95%. For qualitative variables, the tests used were the \( \chi^2 \) test for \( r \times c \) tables, and the \( \chi^2 \) test with correction for continuity or Fisher exact test; in 2 \( \times \) 2 tables, the odds ratio and confidence intervals of the odds ratio were obtained for significant results.

### Results

#### Homogeneous study groups

A total of 430 cases were evaluated (Table II); 15 cases (3.5%) did not wish to participate in the assigned group, although they had previously given their consent; 14 cases withdrew their consent (7 did not want the follow-up at 1 week, and 7 were scared of this type of practice). We consider a total of 22 cases lost (5.1%), those who did not accept the study group, and those who did not want the follow-up at 1 week (Table III). All the cases who did not accept the assigned study group were included in the results (analysis by intention to treat).

#### Maternal puerperal pathology

In the study there was 2% maternal readmission (9 cases) in the first 6 weeks postpartum, with 1.9% in the early discharge group (ED) and 2.3% in the control group (CO). Between the sixth week and 6 months, readmissions amounted to 0.94% (1.4% ED, 0.5% CO) (Tables IV and V). We found puerperal pathology in 21.1% (16.6% ED, 22.9% CO) (Tables IV and V). If late puerperal pathology is included, then the rate rises to 24.8% (19.9% ED, 25% CO) (Table V).

#### Anxiety-depressive pathology

We found a score of more than 7 points on the hospital anxiety and depression (HAD) scale in 15.4% of the cases at 1 week, 2.8% of cases (1.4% ED, 4.2% CO) with a score of more than 10 points (requiring psychiatric monitoring), with 0.5% (0.5% ED, 0.5% CO) being serious cases (with psychiatric admission) (Table IV). At 1 month postpartum, only 2.3% (0.9% ED, 3.7% CO) were considered as pathologic and corresponded to the patients that were under psychiatric care (Tables IV).

#### Puerperal fatigue

Puerperal fatigue increased in the week of birth in 21.9% of the patients (20.1% ED, 23.5% CO), with 1.9% serious cases. At 1 month, only 1.2% of puerperae referred to an increased puerperal fatigue, none of which were serious (0.5% ED, 1.8% CO) (Table IV).
comparison of puerperal fatigue between the patients who presented a puerperal pathology (mammary, infectious, vascular, traumatic, psychiatric, or episiotomial effects) and those who did not is expressed in Table V.

Health service consultations

Of the cases, 9% (8% ED, 10.1% CO) consulted a health service system in the 6 months postpartum (Table V).
Maternal lactation

Breastfeeding was monitored up to 9 months postpartum, the results being expressed in Table IV. The reasons for the abandonment of breastfeeding are as follows: the infant was still hungry in 57.9% (162 cases), working mother 11.4% (32 cases), the infant did not want the breast 7.9% (22 cases), the infant did not put on weight 6.4% (18 cases), and other reasons 12.8% (46 cases) (n = 280; distinct variables such as type of discharge, parity, cultural level were not measured.)

Neonatal pathology

The rate of neonatal rehospitalization in the first 28 days was 1.9% (1.4% ED, 2.3% CO) (relative risk [RR] 0.61 with 95% CI 0.15-2.56) (Table IV). The reasons for readmission in the early discharge group were as follows: 1 case of fever, 1 case of dehydration, and 1 for icterus; in the control group: 3 cases of icterus, 1 case of fever, and 1 for important weight loss. The neonatal pathologies that did not need readmission in the first 28 days were as follows: 15 cases (7%) in the ED group (2 cases of suspected cardiac pathology, 4 cases of infectious pathology, 6 cases of orthopedic pathology, 1 case of congenital spherocytosis, 1 case of cephalohematoma, and 1 case of hydrocele) and 10 cases (4.5%) in the CO group (6 cases of infectious pathology, 2 cases of orthopedic pathology, 1 case of cephalohematoma, and 1 case of umbilical hernia).

Evaluation of satisfaction

In the satisfaction survey, a favorable valuation of this type of practice is observed: “early puerperal discharge with home visit” (Table VI). Of those patients who were in the CO group, more than 40% confirmed that they would have been more satisfied with early discharge and a home visit.

In the intervention group, more than 90% indicated they were satisfied or very satisfied with the practice; 92% indicated that they felt better at home and that they preferred the home visit to the usual hospital monitoring. Furthermore, they referred to having obtained more information about the care of the infant, and themselves, with the home visit. Up to 90% of this group indicated they would repeat the practice in a future birth.

Evaluation of costs

In the GDR (Grupos de Diagnóstico Relacionado) database of the COANH program (Contabilidad Analítica de Hospitales), the unit GDR cost for hospital obstetrics is $1725.20 US and $2269.50 US for pediatrics (average euro value $1.03 US with a range of 0.89-1.16. Average estimated between July 1999 and August 2000. http://www.europa.eu.int). Starting with this GDR value, the cost estimates for intervention types are given in Table VII.

The formula to calculate the daily cost per case is as follows: GDR weighting × GDR value/average stay. For the economic estimations in this study of early discharge, we measured all those parameters recommended by the reviewers.

The results of the cost evaluations are expressed in Tables VIII, IX, and X. A saving of 18.3% was obtained, representing $59,207.59 US over a total of 202 cases, and a saving of $293.11 US per patient by an early discharge program with home visit and telephone follow-up.

Comment

All the reviews of the practice of early discharge have 1 conclusion in common, the existence of important methodologic errors in the studies, which devalue their conclusions. Among the most common errors are that many were observational, the lack of control groups, the objectives were measured on a few occasions, the majority of studies have too few cases to be statistically significant, families were carefully selected for low socioeconomic risk, the inclusion of families with motives in favor of early discharge, and in many cases careful monitoring was applied after discharge. Various authors describe important negative effects for the neonate and mother in programs of early discharge. Other authors, however, consider this practice to be a family-based medical exercise that can potentially lead to lower risks of adverse effects.

For these reasons the reviewers indicate that well-designed studies should be performed to evaluate the impact of different periods of postpartum hospitalization, and that the studies should be randomized, prospective with control groups of mothers and neonates. Participants should be mothers with healthy neonates of more than 2500 g weight at term (37-42 weeks) and have demonstrated normal development before discharge. The variables that should be evaluated in all studies on early discharge are the existence of complications in

<table>
<thead>
<tr>
<th>Table III</th>
<th>Lost cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>n = 430</td>
</tr>
<tr>
<td>Reasons</td>
<td></td>
</tr>
<tr>
<td>Fear</td>
<td>7 (1.63%)</td>
</tr>
<tr>
<td>Too far</td>
<td>1 (0.23%)</td>
</tr>
<tr>
<td>No to follow-up</td>
<td>7 (1.63%)</td>
</tr>
<tr>
<td>Total</td>
<td>15 (3.5%)</td>
</tr>
<tr>
<td>Follow-up not attended</td>
<td>22 (5.1%)</td>
</tr>
</tbody>
</table>

ED, Early discharge; CO, control group.
the mother or child that require rehospitalization or a medical consultation; the existence of maternal factors such as fatigue, anxiety, level of self-confidence after the birth; problems of maternal lactation, satisfaction of the mother and family; and an evaluation of the costs.

### Homogeneous study groups

In our study, no differences were observed between the intervention and control groups in epidemiologic characteristics (marital status, age, in the educational level a small unimportant difference existed in favor of the intervention group), gestational histories, birth details (weeks of gestation, onset to completion of birth, dilation times, expulsion or broken sack, reason for induction), or details of the neonate (sex, Apgar score, weight) (Table II). It is demonstrated that both study groups were homogeneous and that there was no patient selection by important variables such as cultural level, age, onset to evolution to completion of birth, or by neonatal characteristics. In our study selections were made after birth, and no previous contact existed with the patients, only 1 visit was made to the home, which is considered indispensable in this type of discharge. Easy access to health services was available to both the study groups.

### Maternal puerperal pathology

The readmission rate (2%) in the first 6 weeks postpartum for maternal reasons (Table IV) is similar to that described in the literature on early discharge programs. In our study there was neither specific selection of patients from a low-risk population nor exhaustive monitoring of the patients, aspects that have been much criticized in other studies of this type (Table II). Studies of early discharge indicate the existence of 5% to 20% puerperal pathology. In the studies these pathologies are neither described nor differentiated by clinical importance. In our study we included all the puerperal pathologies that occurred and found an incidence of 21.1% (Tables IV and V) from which we found no statistically significant differences (in the evaluation of the diverse maternal pathologies, average or late, possible in the puerperium) between the 2 study groups. A diminution in health service consultations for maternal reasons in the 6 months postpartum is found in the early discharge group (Tables IV and V).

### Anxiety-depressive pathology

In general, studies of early discharge report no increase in anxiety-depressive pathology caused by early discharge. Only Carty and Bradley and James et al cite an increase in depressive problems in cases of early discharge. Brown et al conclude that depression problems do not vary and value scores do not increase with the length of hospital stay, but may do so with other variables, such as low economic level where there may be dissatisfaction with a longer hospital stay, thus increasing the degree of maternal anxiety and discomfort and where there may have been a complicated pregnancy. Grullon and Grimes in their meta-analysis

---

### Table IV  Maternal and neonatal results

<table>
<thead>
<tr>
<th>Results</th>
<th>ED (%)</th>
<th>Traditional discharge (%)</th>
<th>RR</th>
<th>95% CI</th>
<th>Statistical difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal readmission</td>
<td>4/213  (1.9%)</td>
<td>5/217 (2.3%)</td>
<td>0.81</td>
<td>0.21-3.03</td>
<td>None</td>
</tr>
<tr>
<td>Neonatal readmission</td>
<td>3/213  (1.4%)</td>
<td>5/217 (2.3%)</td>
<td>0.61</td>
<td>0.15-2.56</td>
<td>None</td>
</tr>
<tr>
<td>Maternal puerperal pathology</td>
<td>35/213 (16.6%)</td>
<td>50/217 (22.9%)</td>
<td>0.73</td>
<td>0.30-2.20</td>
<td>None</td>
</tr>
<tr>
<td>Maternal consultation to the health system</td>
<td>17/213 (8.0%)</td>
<td>22/217 (10.1%)</td>
<td>0.78</td>
<td>0.30-2.10</td>
<td>None</td>
</tr>
<tr>
<td>Increase in anxiety-depressive pathology at 1 wk</td>
<td>17/213 (8.0%)</td>
<td>27/217 (12.5%)</td>
<td>0.64</td>
<td>0.25-1.63</td>
<td>None</td>
</tr>
<tr>
<td>Increase in anxiety-depressive pathology at 1 mo</td>
<td>2/213 (0.9%)</td>
<td>8/217 (3.7%)</td>
<td>0.30</td>
<td>0.33-3.20</td>
<td>None</td>
</tr>
<tr>
<td>Increase in puerperal fatigue at 1 wk</td>
<td>43/213 (20.1%)</td>
<td>51/217 (23.5%)</td>
<td>0.85</td>
<td>0.43-1.64</td>
<td>None</td>
</tr>
<tr>
<td>Increase in puerperal fatigue at 1 mo</td>
<td>1/213 (0.5%)</td>
<td>4/217 (1.8%)</td>
<td>0.50</td>
<td>0.04-5.54</td>
<td>None</td>
</tr>
<tr>
<td>Breastfeeding at 1 wk</td>
<td>205/213 (96.2%)</td>
<td>200/217 (92.2%)</td>
<td>0.48</td>
<td>0.14-1.65</td>
<td>None</td>
</tr>
<tr>
<td>Breastfeeding at 1 mo</td>
<td>190/213 (89.2%)</td>
<td>182/217 (83.9%)</td>
<td>0.58</td>
<td>0.25-1.36</td>
<td>None</td>
</tr>
<tr>
<td>Breastfeeding at 3 mo</td>
<td>141/213 (66.2%)</td>
<td>119/217 (54.8%)</td>
<td>0.62</td>
<td>0.42-0.91</td>
<td>Yes P = .016 &lt; .05. Fisher exact test</td>
</tr>
<tr>
<td>Breastfeeding at 6 mo</td>
<td>94/213 (44.1%)</td>
<td>76/217 (35.0%)</td>
<td>0.68</td>
<td>0.46-1.006</td>
<td>None</td>
</tr>
<tr>
<td>Breastfeeding at &gt; 9 mo</td>
<td>42/213 (19.7%)</td>
<td>36/217 (16.6%)</td>
<td>0.81</td>
<td>0.49-1.32</td>
<td>None</td>
</tr>
</tbody>
</table>

ED, Early discharge; CO, control group.
refer to quality evidence (type II-2, recommendation C) with respect to lack of increase in anxiety-depression problems in cases of early discharge.

We used the HAD scale\textsuperscript{25} for measurement, as it is easy to apply and gives rapid results with a high degree of reliability and validity.\textsuperscript{26,27} The main criticism of this test is that it is not easy to distinguish anxiety from depression with the factors that are measured.\textsuperscript{27} This point was not considered to be a problem because we did not attempt to distinguish the 2 pathologies but only to identify any problem that could be assessed later by psychiatrists. In our study we found an incidence of

<table>
<thead>
<tr>
<th>Table V</th>
<th>Maternal results. Description of maternal pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal risk in the first 6 wks</td>
<td>Description</td>
</tr>
<tr>
<td>Early discharge (day of readmission)</td>
<td>1 case of postepisiotomial dehiscence (3)(P), 2 cases of puerperal fever (1 bartolin and 1 endometritis) (2,5)(P, M) and 1 case of puerperal depression (10)(M)</td>
</tr>
<tr>
<td>Control (day of readmission)</td>
<td>1 case of postepisiotomial dehiscence (3)(P), 3 cases of puerperal fever (1 mastitis and 1 endometritis) (2,4,5)(P, P, M) and 1 case of puerperal depression (25)(P)</td>
</tr>
</tbody>
</table>

Distribution, by study groups, of puerperal pathology in the first 6 mo

<table>
<thead>
<tr>
<th>Pathology in %</th>
<th>ED</th>
<th>CO</th>
<th>Statistical difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary</td>
<td>6.1</td>
<td>8.4</td>
<td>None</td>
</tr>
<tr>
<td>Infectious</td>
<td>1.6</td>
<td>1.5</td>
<td>None</td>
</tr>
<tr>
<td>Vascular</td>
<td>0.5</td>
<td>0.5</td>
<td>None</td>
</tr>
<tr>
<td>Orthopedic</td>
<td>3.3</td>
<td>2.3</td>
<td>None</td>
</tr>
<tr>
<td>Psychiatric</td>
<td>1.4</td>
<td>4.2</td>
<td>None</td>
</tr>
<tr>
<td>Episiotomial</td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>changes</td>
<td>16.6</td>
<td>22.9</td>
<td>None</td>
</tr>
<tr>
<td>Late puerperal</td>
<td>3.3</td>
<td>4.1</td>
<td>None</td>
</tr>
<tr>
<td>pathology</td>
<td>19.9</td>
<td>25.0</td>
<td>None</td>
</tr>
</tbody>
</table>

Relation between puerperal fatigue and the existence or not of puerperal pathology. In the evaluation of maternal fatigue, the cases that did not go to the follow-up (7 cases) were not included, because they did not complete the appropriate questionnaire.

FSS Puerperal pathology No puerperal pathology

| Normal (7-12) | 48 (51.6%) | 265 (84.4%) |
| Slight-moderate (13-29) | 41 (44.1%) | 45 (14.3%) |
| Serious (30) | 4 (4.3%) | 4 (1.3%) |
| Lost | 23.22 for not responding to the FSS and 1 lost to the system when making the statistical study |

Maternal pathology between 6 wks and 6 mo from birth

<table>
<thead>
<tr>
<th>Distribution</th>
<th>ED</th>
<th>CO</th>
<th>Statistical difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission</td>
<td>3</td>
<td>1</td>
<td>None</td>
</tr>
</tbody>
</table>

Consultations for maternal pathology between 6 wks and 6 mo

<table>
<thead>
<tr>
<th>Distribution</th>
<th>ED</th>
<th>CO</th>
<th>Statistical difference</th>
</tr>
</thead>
</table>

Health service consultations for neonate pathology in the first 28 days

<table>
<thead>
<tr>
<th>Distribution</th>
<th>ED</th>
<th>CO</th>
<th>Statistical difference</th>
</tr>
</thead>
</table>

\textit{ED}, Early discharge; \textit{CO}, control group; FSS, fatigue severity scale; \textit{P}, primiparous; \textit{M}, multiparous.
anxiety-depressive pathology in the study groups. (We did not find differences in the incidence of anxiety-depression between the study groups).

### Puerperal fatigue

All the programs of early discharge recommend that maternal puerperal fatigue be measured, but the required number of controls is not stated nor which scale should be used. Smith-Hanrahan and Deblois found that early discharge with adequate home follow-up did not affect maternal fatigue or functional ability to any significantly greater extent than traditional care. We used the “Fatigue Severity Scale” of Krupp et al., which is widely used for the measurement of fatigue in medical pathologies and is easy to apply. There were no differences in puerperal fatigue because of early discharge at 1 week or at 1 month postpartum (Table IV). If puerperal fatigue is measured in patients who present a puerperal pathology and in those who do not, then as would be expected, statistically significant differences are found ($P < .0005 \chi^2$, Pearson) (Table V).

### Maternal lactation

It is interesting to note that there are statistically significant differences between the groups with regard to the continuation of breastfeeding to 3 months in favor of the early discharge group ($P = .016 < .05$ Fisher exact test). At 6 months there is the same statistical trend that is almost significant ($P = .053 > .05$ Fisher exact) (Table IV).

Most of the studies on early discharge that give information on breastfeeding indicate that this is not affected by the short stay in hospital. Brown et al conclude that breastfeeding is not affected by the length of hospital stay, but is by other variables. Dershewitz and Marshall mention that in cases of early discharge there is a tendency for the discontinuation of breastfeeding, and they advise domiciliary aid for these mothers.

### Table VI Evaluation of satisfaction

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>ED</th>
<th>CO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Were you satisfied with your postnatal treatment?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14 (4.7%)</td>
<td>2 (1.2%)</td>
<td>12 (9.6%)</td>
</tr>
<tr>
<td>Adequate</td>
<td>65 (22%)</td>
<td>28 (16.3%)</td>
<td>37 (29.6%)</td>
</tr>
<tr>
<td>Satisfied</td>
<td>131 (44%)</td>
<td>69 (40.1%)</td>
<td>62 (49.6%)</td>
</tr>
<tr>
<td>Very satisfied</td>
<td>87 (29.3%)</td>
<td>73 (42.4%)</td>
<td>14 (11.2%)</td>
</tr>
<tr>
<td><strong>Were you satisfied with the answers to your question?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (7.8%)</td>
<td>5 (2.9%)</td>
<td>18 (14.4%)</td>
</tr>
<tr>
<td>Adequate</td>
<td>41 (13.8%)</td>
<td>29 (16.9%)</td>
<td>12 (9.6%)</td>
</tr>
<tr>
<td>Satisfied</td>
<td>170 (57.2%)</td>
<td>96 (55.8%)</td>
<td>74 (59.2%)</td>
</tr>
<tr>
<td>Very satisfied</td>
<td>63 (21.2%)</td>
<td>42 (24.4%)</td>
<td>21 (16.8%)</td>
</tr>
<tr>
<td><strong>For cases of 2 or more days stay. Do you think you would have found it better to have a shorter stay and a home visit?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>38 (30.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indifferent</td>
<td>33 (26.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Better</td>
<td>54 (43.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>For early discharge cases. How did you value the program?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsatisfied</td>
<td>5 (2.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indifferent</td>
<td>12 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Satisfied</td>
<td>94 (54.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very satisfied</td>
<td>61 (35.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>For early discharge cases. What did you find better?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrahospital monitoring</td>
<td>13 (7.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Home visit by health personnel</td>
<td>159 (92.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>For early discharge cases. When did you receive most information about care for yourself and your infant?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The hospital follow-up</td>
<td>13 (7.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Home visit by health personnel</td>
<td>147 (85.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equal</td>
<td>12 (6.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In the event of another pregnancy, and all went well, would you prefer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional monitoring</td>
<td>59 (19.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early discharge</td>
<td>193 (65%)</td>
<td>5 (2.9%)</td>
<td>38 (26.4%)</td>
</tr>
<tr>
<td>Indifferent</td>
<td>45 (15.2%)</td>
<td>155 (90.1%)</td>
<td>54 (43.2%)</td>
</tr>
</tbody>
</table>
Cooper et al.\(^{31}\) report severe cases of dehydration in breastfed children despite good maternal preparation or good post discharge follow-up. In the review made by the Cochrane Library, it was concluded that early discharge can be of benefit in the continuation of breastfeeding, with a valuation II-2 and recommendation type C, although more studies are needed to determine which factors influence the duration of breastfeeding, and which is the best type of domiciliary aid for those mothers that have had an early discharge.\(^{32}\)

In our study, we believe that the difference between the continuation of breastfeeding at 3 and 6 months is due to the combination of the recommendations made by the specialized staff who visited the mothers at home, giving information about the technique and the benefits of breastfeeding, particularly when doubts and problems about breastfeeding have started. Regarding the reasons for the abandonment of breastfeeding, we have only made a descriptive valuation without considering the diverse influencing factors. We found that the reasons for abandonment were very similar to those in the consulted literature,\(^{33-35}\) the most frequent reason (57.8%) being that “the infant is still hungry.”

### Neonatal pathology

Hospital readmission rates are between 0.6% and 5%.\(^{10,11,36}\) Liu et al.\(^{11}\) in a large, well-designed study, gives a rate of neonatal rehospitalization of 2%. Significant differences in the rates of rehospitalization between infants from early discharge groups and controls have not been found.\(^{6,10,18,37-41}\) Some studies observe that the rate of rehospitalization of neonates

---

### Table VII: Intervention values

<table>
<thead>
<tr>
<th>GDR</th>
<th>Description</th>
<th>Weighting</th>
<th>Average stay (d)</th>
<th>Daily case cost in US dollars ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>373</td>
<td>Vaginal birth with no complications.</td>
<td>0.484</td>
<td>2.78</td>
<td>303.60</td>
</tr>
<tr>
<td>376</td>
<td>Postpartum and postabortion diagnoses without surgery</td>
<td>0.559</td>
<td>4.09</td>
<td>235.80</td>
</tr>
<tr>
<td>628</td>
<td>Neonate; birth weight &gt;2500 g, with minor problems but without significant surgery.</td>
<td>0.689</td>
<td>7.17</td>
<td>218.08</td>
</tr>
<tr>
<td>627</td>
<td>Neonate; birth weight &gt;2,500 g, with major problems but without significant surgery.</td>
<td>1.026</td>
<td>8.79</td>
<td>264.90</td>
</tr>
<tr>
<td>427</td>
<td>Septicemia, &lt;18 y</td>
<td>1.004</td>
<td>8.17</td>
<td>278.89</td>
</tr>
<tr>
<td>423</td>
<td>Other diagnoses of infectious and parasitic illnesses.</td>
<td>1.074</td>
<td>8.33</td>
<td>292.61</td>
</tr>
<tr>
<td>422</td>
<td>Viruses and fever of unknown origin, age &lt;18 y</td>
<td>0.590</td>
<td>4.17</td>
<td>321.10</td>
</tr>
<tr>
<td></td>
<td>Cost of external consultation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cost of emergency consultation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cost of home visit</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from GDR 1999.\(^9\)

---

### Table VIII: Costs in US dollars for ED and CO groups

<table>
<thead>
<tr>
<th></th>
<th>ED No.</th>
<th>Average</th>
<th>Cost (US$)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of stay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>202</td>
<td>1.13</td>
<td>(1.13 × 303.60) + 39.15</td>
<td>77,208.00</td>
</tr>
<tr>
<td>CO</td>
<td>202</td>
<td>2.13</td>
<td>2.13 × 303.60</td>
<td>130,626.90</td>
</tr>
<tr>
<td>Maternal readmission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>20</td>
<td></td>
<td>235.80</td>
<td>4,716.00</td>
</tr>
<tr>
<td>CO</td>
<td>39</td>
<td></td>
<td>235.80</td>
<td>9,196.20</td>
</tr>
<tr>
<td>Neonatal readmission</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>16</td>
<td></td>
<td>218.08</td>
<td>3,489.20</td>
</tr>
<tr>
<td>CO</td>
<td>22</td>
<td></td>
<td>218.08</td>
<td>4,797.70</td>
</tr>
<tr>
<td>Maternal consultations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to the health systems</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>17</td>
<td></td>
<td>23.36</td>
<td>397.10</td>
</tr>
<tr>
<td>CO</td>
<td>22</td>
<td></td>
<td>23.36</td>
<td>513.90</td>
</tr>
<tr>
<td>Neonatal consultations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to the health systems</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>18</td>
<td></td>
<td>23.36</td>
<td>420.40</td>
</tr>
<tr>
<td>CO</td>
<td>13</td>
<td></td>
<td>23.36</td>
<td>303.60</td>
</tr>
<tr>
<td>Maternal-neonatal review</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at 1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>2 × 202</td>
<td></td>
<td>39.27</td>
<td>15,865.10</td>
</tr>
<tr>
<td>CO</td>
<td>2 × 202</td>
<td></td>
<td>39.27</td>
<td>15,865.10</td>
</tr>
<tr>
<td>10-min interprovincial telephone call (141)</td>
<td></td>
<td></td>
<td>US $/min = 0.68 US $</td>
<td>412.08</td>
</tr>
<tr>
<td>ED</td>
<td>3 × 202</td>
<td></td>
<td>0.08 US $</td>
<td>412.08</td>
</tr>
<tr>
<td>CO</td>
<td>3 × 202</td>
<td></td>
<td>US $/min = 0.68 US $</td>
<td>412.08</td>
</tr>
<tr>
<td>Total sum</td>
<td></td>
<td></td>
<td>264,223.73</td>
<td></td>
</tr>
</tbody>
</table>
from early discharge groups is lower than that of neonates from traditional discharge groups. However, these low rates were produced in low-risk groups in which all the neonates were receiving good monitoring care.18-21,23,41

Hyperbilirubinemia is the most frequent medical problem. According to Catz et al15 between 1% and 4% of suckling term neonates are rehospitalized during the first week of life. Of these readmissions, 45% to 85% were due to jaundice.4,6,36,42,43 Many studies do not specify what concentration of total bilirubin is sufficient to justify readmission. Seidman et al13 suggest the adoption of a uniform definition of serious hyperbilirubinemia, treatable in the home by phototherapy with a reduction in the need for readmission. After the change of therapeutic approach in these infants, by deferring phototherapy until the serum bilirubin levels increase beyond 18 mg/dL,13,44 jaundice ceased to be an important problem.

In our study we did not find statistically significant differences in the rate of readmission between the study groups. The rate of neonatal rehospitalization in the first 28 days’ postpartum in the early discharge group was 1.4%, and 2.3% in the control group (RR 0.61 with 95% CI 0.15-2.56). It must be emphasized that this difference is due to hyperbilirubinemia (33% ED vs 66% CO). The home visit by staff qualified in the control and assessment of neonatal care seems to be the cause of this benefit. Among the reasons for readmission, the most frequent is hyperbilirubinemia at 50% (with readmission levels of 16-18 mg/dL), the second most frequent being fever at 25%. Thus, the data found in our study agree with those in the global literature for early discharge.3,5,11,16,18,30,37-43

As far as other data are concerned, it should be emphasized that the time of readmission depends on the type and severity of the pathology and the response of the neonate to treatment. Regarding consultations with the health services, we did not find any diminution for early discharge.

### Evaluation of satisfaction

In the satisfaction survey, a favorable valuation of this type of practice is observed: “early puerperal discharge with home visit” (Table VI). The studies of Lieu et al45 and Escobar et al46 also find high maternal satisfaction in this practice.

Our aim is to provide the mother and child with the best possible health care and not only to achieve a cost saving, for this reason we believe that the home visit by qualified health personnel is fundamental and indispensable for early discharge programs. We base this affirmation on the fact that health personnel:

- Monitor puerperal pathology in the 48 to 72 hours postpartum;
- Monitor neonatal pathology in the 48 to 72 hours postpartum;
- Resolve doubts and problems about neonatal and puerperal care when they occur, and in the comfort of the maternal home environment;
- Check and correct the breastfeeding technique, resolve problems, and consolidate the recommendations about breastfeeding.

Brown et al,24 in their protocol “Early postnatal discharge from hospital for healthy mothers and term infants,” proposes a division between the study subgroups of those with and without home visits. Although it has been demonstrated that neonatal and puerperal pathology do not increase in early discharge groups without home visits, we believe that the home visit is necessary for improvement in the quality of assistance for the mother. Also, we believe that the increase in cases of neonatal jaundice and dehydration in some early discharge protocols3,11-15 are due to patients foolishly abandoning the Health Systems. With monitoring by, and contact with, health practitioners we do not think that this happens and that attention for these patients is secured.

### Evaluation of cost

Gazmararian and Koplan,47 in their review of the topic, conclude: The frequent supposition is that early

---

<table>
<thead>
<tr>
<th>Table IX</th>
<th>Costs in the ED group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED group</td>
<td>Cost (US$)</td>
</tr>
<tr>
<td>Hospital stay</td>
<td>77,208.03</td>
</tr>
<tr>
<td>Maternal readmission</td>
<td>4,716.00</td>
</tr>
<tr>
<td>Neonatal readmission</td>
<td>3,489.28</td>
</tr>
<tr>
<td>Maternal consultations to the health systems</td>
<td>397.10</td>
</tr>
<tr>
<td>Neonatal consultations to the health systems</td>
<td>420.48</td>
</tr>
<tr>
<td>Maternal-neonatal review at 1 wk</td>
<td>15,865.10</td>
</tr>
<tr>
<td>Telephone calls</td>
<td>412.08</td>
</tr>
<tr>
<td>Total sum</td>
<td>102,508.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table X</th>
<th>Costs in the CO group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO group</td>
<td>Cost (US$)</td>
</tr>
<tr>
<td>Hospital stay</td>
<td>130,626.94</td>
</tr>
<tr>
<td>Maternal readmission</td>
<td>9,196.20</td>
</tr>
<tr>
<td>Neonatal readmission</td>
<td>4,797.76</td>
</tr>
<tr>
<td>Maternal consultations to the health systems</td>
<td>513.90</td>
</tr>
<tr>
<td>Neonatal consultations to the health systems</td>
<td>303.68</td>
</tr>
<tr>
<td>Maternal-neonatal review at 1 wk</td>
<td>15,865.1</td>
</tr>
<tr>
<td>Telephone calls</td>
<td>412.08</td>
</tr>
<tr>
<td>Total sum</td>
<td>161,715.66</td>
</tr>
</tbody>
</table>
discharge is cost effective. However, the true cost of these programs, when compared with traditional hospital stay costs, have not been analyzed with great care. The few studies that have examined the topic of cost, regularly do so in terms of costs to the hospital, the patients, or to third-party payers, and do not consider the true costs of an early discharge program. The few attempts to make an analysis of cost-effectiveness do not adjust by the standards formulated for these studies.48,49

In theory, the cost savings for an early discharge program should be equal to the extra costs of a traditional stay for mother and child together with the program development costs.50 Lukacs51 notes 4 essential points for determining the true costs of these programs: (1) cost of program development, (2) cost of home visits (including the carer’s time in the home and in transportation), (3) number of visits per patient, and (4) cost of legal responsibility.

Most programs of early discharge, in which costs have been evaluated, report a saving,47,52-55 but other authors, such as Lieu et al.,45 Escabar et al.,46 and Amnas,56 agree; others, such as Gazmararian and Koplan,47 criticise on the grounds that the cost evaluations were not performed in an adequate manner. Grullon and Grimes6 qualify the scientific evidence on the cost evaluations in these programs as type C or inadequate. Brown et al24 and Lukacs51 make reference to those parameters that should be included in cost evaluations.

The economic evaluations in our study of early discharge included all those parameters recommended by the reviewers,24,47,48 and we obtained a saving of 18.3% (for a program of early discharge with home visit and telephone follow-up).

If the early discharge program includes a home visit and a hospital review of mother and child at 1 week, a saving over traditional discharge is obtained of $43,342.49 US for 202 cases, representing a 13.4% saving or $214.57 US per patient. Thus, for the 2,800 births, without complications, that occurred in the Hospital de Valme de Sevilla in 1999,9 a saving might have been obtained in:

- A system of early discharge with home visit and telephone follow-up: $820,680 US.
- A system of early discharge with home visit and hospital review of mother and child at 1 week: $600,768 US.

Our data indicate that a saving in the range of 18% to 20% exists over the cost of traditional discharge with a minimum stay of 48 hours.

In conclusion, we approached the study of early obstetric-pediatric discharge according to the recommendations in the meta-analysis of the topic and measured the indicated variables. It is demonstrated that both study groups were homogeneous and that there was no patient selection by important variables such as cultural level, age, initiation-evolution-completion of the birth, or by neonatal characteristics.

We made no previous preparation for the early discharge group. Neither a more rigorous follow-up if there was easy access to health services for any type of problem.

We did not find differences between the study groups in: the rate of maternal or neonatal rehospitalization, maternal puerperal pathology, maternal fatigue, anxiety-depressive pathology, or consultations to the health systems for maternal or neonatal pathology.

There were statistically significant differences between the study groups in the maintenance of breastfeeding at 3 months in favor of the early discharge group. At 6 months the trend was maintained but lacked statistical significance.

Early obstetric-pediatric discharge gives a verified improvement in the well-being of mother and family.

Early obstetric-pediatric discharge carries an important saving for Health Services.

References


Appendix A
We used the “Hospital Anxiety and Depression scale (HAD)” at weeks 1 and 4 to measure anxiety and...
depression. Designed by Zigmond and Snaith in 1983,\textsuperscript{25} it is an autoevaluation scale with 14 points of measurement, is easy to apply, and gives rapid results with a high degree of reliability and validity.\textsuperscript{26,27} A score of 0 to 7 is considered normal, 8 to 10 indicates a probable case of pathology, and more than 11 a pathologic case of anxiety or depression.\textsuperscript{27}

Appendix B

We used the “Fatigue Severity Scale” of Krupp et al\textsuperscript{29} in the first 6 weeks. It is commonly used for the measurement of fatigue in medical pathologies and is easy to use. A score of 12 to 19 is considered as slight fatigue, and 20 or more as serious. A score of 5 or more in any 1 item of measurement is also considered a pathologic factor.

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Impact of maternal-fetal surgery for myelomeningocele on the progression of ventriculomegaly in utero

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KEY WORDS
Myelomeningocele
In utero repair
Fetal surgery
Ventriculomegaly

Objective: Intrauterine myelomeningocele (MMC) repair decreases hindbrain herniation and the need for postnatal ventriculoperitoneal shunting. We examined the impact of intrauterine repair on the progression of ventriculomegaly in utero.

Study design: Fetuses with MMC were identified through computerized databases from June 1988 to April 2003. A retrospective cohort design was used to evaluate the impact of intrauterine repair on ventricular progression with a multivariate linear regression model that included baseline ventricle measurement, gestational age, level of lesion, and gender.

Results: Fourteen fetuses with intrauterine repair and 39 fetuses with postnatal repair were identified. The natural history of progression of ventricular diameter increased in a linear fashion throughout gestation (0.57 mm/week). After adjusting for confounding variables, no transient or sustained difference was observed in the rate progression of ventriculomegaly between intrauterine and postnatal repair (0.27 ± 0.35 mm/week; P = .45).

Conclusion: Intrauterine MMC repair does not impact the progression of ventriculomegaly.

Neural tube defects are common and serious birth defects that contribute to infant morbidity and mortality. Despite folic acid supplementation, the incidence of neural tube defects in the United States is estimated at 3000 per year.1 The estimated annual medical and surgical cost is 200 million dollars, underscoring the importance of research efforts to decrease the significant morbidity and mortality associated with this disease.

In utero repair of myelomeningocele (MMC), first reported in 1997, was proposed in an effort to limit damage to exposed fetal neural tissue from mechanical trauma and the toxic effect of amniotic fluid.3 Subsequent studies have demonstrated additional benefits, including an almost universal reversal of hindbrain herniation, and a decreased need for postnatal ventriculoperitoneal shunting.6, 7 Although the benefits to in utero repair have been shown in neonates, there is little information on its influence on fetal brain development.

It has been observed that some neonates who do not have overt hydrocephalus at birth will acutely develop...
hydrocephalus in the days or weeks following postnatal repair. Deleterious effects of hydrocephalus such as symptoms of stridor, facial palsy, and poor feeding will resolve in many cases after treatment. It is uncertain if this acute increase in ventricle size following postnatal repair occurs in fetuses after in utero repair. To date, no study has examined changes in ventricular size during in fetuses with MMC after in utero repair compared with control fetuses.

The purpose of this study is to evaluate our hypothesis that in the weeks following in utero repair, there would be an acute increase in the ventricular size when compared with fetuses with MMC undergoing postnatal repair.

Material and methods

Following approval by the University of North Carolina Institutional Review Board, computerized departmental ultrasound and perinatal hospital discharge databases from June 1988 to April 2003 were queried to identify fetuses and newborns with prenatally diagnosed MMC. Ultrasound reports were reviewed for gestational age and ventricular size at each ultrasound study, as well as fetal gender and level of the spinal lesion. Maternal medical records were reviewed for maternal age, race, and gestational age at delivery. Postnatal imaging studies were reviewed to confirm the diagnosis of MMC.

The maximum width of the atria of the lateral ventricle was measured from the medial to the lateral wall across the posterior aspect of the choroid plexus. This measurement was recorded at baseline and at each subsequent ultrasound. Mild, moderate, and severe ventriculomegaly were defined as 10 to 14 mm, 15 to 25 mm, respectively. Sonographers performed all prenatal ultrasounds at the University of North Carolina Prenatal Diagnostic Center, and were directly reviewed by Maternal-Fetal Medicine physicians. Examinations were performed with ATL HDI 5000 (Phillips Medical Systems, Bothell, Wash), Ultramark 4 (Advanced Technology Laboratories, Bothell, Wash), General Electric RT3200 Advantage (Milwaukee, Wis), or Corometrics 650 (Wallingford, Conn) machines with 5 MHz or 7 MHz transabdominal probes. All baseline ultrasounds and follow-up ultrasounds were performed at UNC, with the exception of the follow-up scans in 3 fetuses undergoing in utero repair. These ultrasound reports were obtained from their referring institutions and reviewed. Maternal-Fetal Medicine specialists supervised each of these referring institution’s ultrasounds.

The control group was composed of fetuses with MMC who underwent postnatal repair. Cases were fetuses that underwent in utero repair. The karyotype was normal for all cases but was not obtained prenatally for all control fetuses. Fetuses with anomalies were excluded from the control group unless a clubbed foot was present. Each patient in the cohort had a baseline ventricle measurement performed at our institution. For the case and control fetuses, this measurement was obtained at the initial ultrasound performed at our institution. For cases, this measurement was obtained before in utero repair. Both case and control fetuses had a minimum of 1 subsequent measurement at least 2 weeks apart.

Statistical analysis

The progression of ventricular size of the control fetuses was first determined using a multivariate linear regression model that controlled for the following variables: level of spinal cord lesion, gender, and ventricular measurement at baseline and at each follow-up visit. Because there was not a standardized interval for repeat ultrasound assessments of ventricular size, the generalized estimating equation method was applied to the analysis to account for the differences in the timing of these measurements.

The cases of in utero repair were then added and analyzed with the control fetuses in the same linear regression model. An additional interaction term of type of repair was added to evaluate for differences in the rate of ventricular progression between case and control fetuses. We considered that an increase in ventricular size with advancing gestational age might not occur in a linear fashion. In order to detect an acute or transient change in ventricular progression after in utero repair, a quadratic equation (the square of gestational age) and its interactions with the other variables were included in the full model.

Comparisons of demographic data were made using chi-square and Fisher exact tests for categoric variables, and the Wilcoxon rank sum test for continuous variables. P values of less than .05 were considered to indicate statistical differences, and 95% CI were used to describe comparisons.

Results

A total of 78 fetuses with MMC were identified (Figure). Seventeen patients (15 control fetuses and 2 cases) were excluded from analysis because they did not have a minimum of 2 ventricular measurements. Thirty-nine control fetuses that underwent postnatal repair, and 16 cases undergoing in utero repair were included in the analysis. Ten cases of maternal-fetal surgery were performed at the University of North Carolina. One of these patients delivered preterm and did not have a follow-up ventricular measurement, and was excluded.
from the analysis. Five patients were diagnosed with MMC and followed postoperatively at our institution after intrauterine repair at Vanderbilt University. The gestational age of the in utero repair ranged from 21 to 25 weeks. The number of ventricular measurements taken throughout the gestation is summarized in Table I. On average, cases had more frequent prenatal assessment of ventricular size than control fetuses.

Characteristics of the maternal and fetal population are shown in Table II. The control group who underwent postnatal repair presented and delivered at a later gestational age than fetuses that underwent in utero repair ($P < .01$). Males comprised a greater percentage of the control group ($P < .05$). There was no difference in the distribution of thoracic, lumbar, and sacral lesions between the 2 groups ($P = 1.0$). However, the mean ventricle size at baseline was 4.6 mm larger in control fetuses than cases ($P = .02$). The overall prevalence of mild (100% vs 94%), moderate (36% vs 26%), and severe ventriculomegaly (33% vs 21%), $P = .98$, was similar for in utero and postnatal repair groups.

The overall rate of progression of ventriculomegaly was 0.57 mm/week for fetuses with postnatal repair. However, the rate of progression differed by level of lesion (thoracic and lumbar lesions 0.70 mm/week; sacral lesions 0.08 mm/week [$P = .07$]). Although this difference only approaches statistical significance, we felt that reporting one rate for the entire control group would overestimate the rate of progression for sacral lesions ($P = .94$).

After adjusting for confounders, the fetuses that underwent in utero repair did not exhibit a statistically significant increase in ventricular progression over control fetuses (0.27 mm/week; $P = .45$) (Table III). Females had an increased rate of progression of ventricular size over the males by 0.04 mm/week, but this difference was not statistically significant ($P = .85$). Similarly, the rate of progression was not affected by baseline ventricle size ($P = .94$).

Comment

This study is the first to describe the natural progression of ventriculomegaly in fetuses with MMC, and to better define the impact of the baseline degree of ventriculomegaly, fetal gender, and level of lesion on this progression. When compared with a large group of fetuses with MMC undergoing postnatal repair, we found that in utero repair did not impact the rate of progression of fetal ventriculomegaly.
In the present study, ventricular size progressed in fetuses affected by MMC in a linear fashion with advancing gestational age. This finding confirms that of Babcock et al., who noted worsening ventriculomegaly in 17 fetuses with ventricular measurements after 24 weeks' gestation. However, Babcock's study was limited by the small number of patients with serial ultrasounds throughout gestation. Only 4 fetuses had serial scans, with 3 of 4 showing worsening ventriculomegaly in utero.

We found that thoracic and lumbar lesions were associated with a more rapid increase in ventricular size than sacral lesions. Because of the small sample size in our study, we were unable to perform a more detailed analysis of specific vertebral levels of MMC. A previous study of 33 fetuses with MMC confirmed our findings of an association between level of lesion and degree of hydrocephalus at birth. Tulipan and Rintoul independently found that higher lesions were more likely to require placement of neonatal ventriculoperitoneal shunts, suggesting either a greater baseline level of ventriculomegaly and/or more rapid progression in utero. In contrast to these findings, previous investigators reviewing a series of prenatal sonograms in MMC fetuses found that when one controls for gestational age and degree of posterior fossa deformity, the level of lesion did not predict ventricular diameter. This disparity with our findings may be explained by the fact that we did not assess or control for the degree of hindbrain herniation in the present investigation.

Previous studies have shown that in utero repair of MMC decreases the incidence of shunt-dependent hydrocephalus. The initial publication from Bruner et al. demonstrated a decrease in shunt placement after in utero repair; however, their study did not evaluate the baseline degree of ventriculomegaly and/or the impact of level of lesion. A subsequent publication from the same group showed that higher lesions, later gestational age at the time of repair, and larger baseline ventricle size are predictive of the need for neonatal ventriculoperitoneal shunt placement. Tulipan also showed that fetuses with larger ventricles at baseline and higher level lesions were more likely to require shunting. However, neither study included prenatal ultrasounds of control fetuses with MMC for comparison.

Our findings are in direct contrast to a recently reported study by Danzer et al., who found an improvement in the cortical index (head circumference/ lateral ventricular diameter) following in utero surgery for MMC. The authors reported an alteration in head growth and a slowing of the progression of ventriculomegaly with a decrease in frequency of severe ventriculomegaly. Comparisons were made to a cross-sectional control group of fetuses without MMC, making conclusions difficult to interpret.

Our study found that the ultimate degree of ventriculomegaly was not improved with in utero repair. To date, no other study on the effects of in utero repair for MMC has compared findings with prenatal information gathered from a control group of affected fetuses without prenatal intervention. An observational study of serial magnetic resonance imaging (MRI) of fetal brains in 10 cases of in utero repair has shown either an arrest or slowing of ventricular size as soon as 3 weeks after in utero surgery. We did not find an acute or transient effect on ventricular size following in utero repair for MMC, in contrast to this, and previously reported worsening in ventriculomegaly following postnatal MMC repair.

There are several limitations in this study. Although a standardized and accepted method of measuring the lateral ventricle was used, the results would be strengthened if a single observer had performed all of the measurements. Because of the retrospective study design, there was not a standardized interval for the sonographic assessments of ventricle size. Statistical methods were applied to account for this limitation, yet the irregular timing of these measurements may be responsible for the difference in findings from previous studies.

The number of cases of in utero repair was small, limiting our ability to analyze this group alone. Given the number of confounding factors that were controlled for in the logistic regression analysis, we could not report, with statistical confidence, results of this group alone. An alternative approach was chosen using cases and controls in the same model. First, the type of repair was included as a variable in the logistic regression, allowing a direct comparison between the groups. Second, the rate of ventricular progression was compared between the control group and that of the entire cohort. Neither analysis showed an impact of in utero repair on the progression of ventriculomegaly.

Our data suggest that the decrease in the need for ventriculoperitoneal shunting in the first year of life after in utero repair of MMC may not be caused by a slowing in the progression of ventriculomegaly in utero. An explanation for decreased rates of ventriculoperitoneal shunting requires further exploration. The long-term implications of in utero repair on the developing

<table>
<thead>
<tr>
<th>Table III</th>
<th>Results of multivariate regression for progression of ventriculomegaly for entire cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progression (mm/wk)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Level of lesion</td>
<td>0.55 ± 0.22</td>
</tr>
<tr>
<td>Gender (females &gt; males)</td>
<td>0.04 ± 0.24</td>
</tr>
<tr>
<td>Ventricular size baseline</td>
<td>0.002 ± 0.03</td>
</tr>
<tr>
<td>In utero repair</td>
<td>0.27 ± 0.35</td>
</tr>
<tr>
<td>Gestational age²</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>
brain are yet to be elucidated. The completion of the NICHD-sponsored randomized clinical trial will hopefully answer such questions.

References

Isolated fetal pyelectasis and chromosomal abnormalities

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KEY WORDS
Aneuploidy
Trisomy
Low-risk population
Pyelectasis
Dilated renal pelvis
Soft markers
Prenatal diagnosis
Fetal ultrasound

Objective: The primary objective of this study was to determine if isolated pyelectasis is a risk factor for trisomy 21.

Study design: Twelve thousand, six hundred and seventy-two unselected singleton fetuses were examined by prenatal ultrasound during the second trimester at a single institution. The sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio of pyelectasis (either isolated or in association with other soft markers/structural anomalies) to detect trisomy 21 were calculated.

Results: Pyelectasis (anteroposterior pelvic diameter ≥4 mm) was detected in 2.9% (366/12,672) of the fetuses. Among these, 83.3% (305/366) were isolated, and 16.7% (61/366) were associated with other markers/structural anomalies. The prevalence of trisomy 21 was 0.087% (11/12,672) and, among these fetuses, 2 (18.1%) had pyelectasis, 1 isolated, and 1 associated with other markers/structural anomalies. The presence of isolated pyelectasis had 9.09% sensitivity, 97.6% specificity, 0.33% positive predictive value, and 99.9% negative predictive value to detect fetuses with trisomy 21. The likelihood ratio of trisomy 21 in this group of fetuses was 3.79 (95% CI 0.582–24.616). Among fetuses with pyelectasis and other associated markers/structural anomalies, the sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio for trisomy 21 were 9.09%, 99.5%, 1.64%, 99.9%, and 19.2 (95% CI 2.91–126.44).

Conclusion: In the absence of other findings, isolated pyelectasis is not a justification for the performance of an amniocentesis.

Pyelectasis, which is a dilatation of the renal pelvis visible with ultrasound, is an anatomic variant that rarely has pathologic significance for fetal and postnatal renal function. Generally, the renal pelvis is collapsed and, thus, undetectable with ultrasound.

The renal pelvis diameter may be affected by maternal hydration,1–3 although this view is not shared by all investigators.4,5 Persutte et al6 reported that repeated measurements of the renal pelvis performed within a period of 2 hours present high variability, and that this observation was independent from the maternal hydration status. A possible genetic predisposition to pyelectasis in consecutive pregnancies has been proposed by Degani et al.7

In 1990, Benacerraf et al8 were first to suggest an association between pyelectasis and aneuploidy. They estimated a 3.3% risk for Down syndrome when pyelectasis was present. In this study, we investigated the prevalence and possible association of pyelectasis with
aneuploidy in an unselected population of patients. We determined whether the finding of fetal pyelectasis in the second trimester justified alteration in patient management, in particular, whether or not patients should be subjected to a karyotype.

Various criteria have been used to define fetal renal pyelectasis. For example, Benacerraf et al defined pyelectasis as a renal pelvis anteroposterior diameter greater or equal to 4 mm in fetuses between 15 and 20 weeks; an anteroposterior renal pelvis diameter greater than or equal to 5 mm in fetuses between 20 and 30 weeks; and a renal pelvis anteroposterior diameter greater or equal to 7 mm in fetuses between 30 and 40 weeks. Other authors used a cutoff of 4 mm to define pyelectasis. In our study, the criterion used to select fetuses with pyelectasis was an anteroposterior renal pelvis diameter of 4 mm or more.

Material and methods

In contrast to most published series, our patients came from a homogeneous base low-risk population from our practice, which serves the needs of a group of about 30 obstetricians. Patients referred by physicians outside our ultrasound practice were not included in the study because they were more likely to have been referred for a suspected anomaly and, therefore, may not have represented a low-risk population.

From the 16,272 midgestation patients referred to our center from January 1998 to December 2002, 12,672 patients between 16 and 23 gestational weeks were included in the study. The other patients were removed because their initial examinations do not fall within the 16 to 23 weeks frame. None of the patients had a first trimester aneuploidy screening.

All patients underwent a thorough ultrasound examination which, aside from the AIUM-ACR guidelines, sought as many soft markers as possible, including nuchal thickening, pyelectasis, echogenic intracardiac foci, brachymesophalangia of the fifth digit, as well as a simian crease, whenever possible, for these more subtle markers were only looked at for a few seconds and, if not seen, simply passed over. In only a small percentage of fetuses did we identify this sign in accord with previous work.

Hands, feet, and limb segments were included. Outflow tracts, return of pulmonary veins to the left atrium, and, when possible, both arches were also part of the routine.

Examinations were performed using either Acuson XP 128 (Siemens Medical Systems, Mountain View, Calif), Sequoia (Siemens Medical Systems), or Voluson 730 (General Electric Medical Systems, Kretztechnik, Zipf, Austria) scanners.

The results only include data from 2D examinations, not from 3D.

The aim of this study was to determine if isolated pyelectasis is a risk factor for trisomy 21. Because of the compounding risk in the presence of other markers, we analyzed the population with isolated pyelectasis and the population with other markers separately. There is a general consensus that pyelectasis is only a marker for trisomy 21 and not for other aneuploidy, and thus, our analysis only considered cases of trisomy 21. We also reported the total cases of aneuploidies recognized in the study period and the presence of pyelectasis in these cases.

The sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratios with 95% CIs of pyelectasis to detect trisomy 21 were calculated. Separate analyses were conducted for fetuses with isolated pyelectasis, and for those with pyelectasis and other associated markers/structural anomalies. The following markers/structural anomalies were included: choroid plexus cysts, echogenic heart focus, 2-vessel cord, nuchal thickening, heart defects, diaphragmatic hernia, esophageal atresia, omphalocele, facial cleft, micrognathia, myelomeningocele, growth restriction, shortening of the limbs, radial aplasia, (for long bones we routinely measured humerus and femur, and we looked at ulna-radius and tibia-fibula, but only measured them if they appeared abnormal), overlapping fingers, talipes, rocker bottom feet, clinodactyly, and brachymesophalangia of the fifth digit.

Likelihood ratios were also calculated by the formula: Likelihood ratio + = sensitivity/(1 – Specificity); likelihood ratio − = (1-Sensitivity)/Specificity.

The likelihood ratio expresses the odds that pyelectasis occurs in fetuses with Down syndrome versus the odds that pyelectasis occurs in fetuses without Down syndrome (Figure 1).

Follow-up information in all the cases was obtained by amniocentesis, infant postnatal reports, or by

![Figure 1](image)
Measurement of the renal pelvis in the anteroposterior dimension in a fetus with bilateral pyelectasis of 4.6 mm.
contacting the referring physician or pediatrician. If these options failed, the mother was interviewed. The follow-up was obtained in every case of pyelectasis.

Results

The mean maternal age was 27.2 ± (standard deviation 5.5), ranging from 15 to 42 years old. The prevalence of pyelectasis in our population was 2.9% (366/12,672). In 57% (208/366) of cases, the anteroposterior pelvis diameter measured 4 to 5 mm, in 23% (85/366) the renal pelvis measured 5 to 6 mm, in 13% (48/366) the dilatation was 6 to 7 mm, in 5% (19/366) it was 7 to 8 mm, and in 1% (4/366) the renal pelvis measured 8 mm, 1 fetus had 12 mm dilatation, and 1 had 16 mm dilatation.

The sex of the affected fetus was identified in 315 cases (in the other cases, the parents requested not to know the sex of the fetuses), with 206 (65%) fetuses being male and 109 (35%) being female. This indicated an increased prevalence of pyelectasis among males, with a male to female ratio of 1.9:1.

The prevalence of aneuploidy in the study population was 0.19% (24/12,672). Eleven fetuses had Down syndrome, including a 14-21 translocation, 6 had trisomy 18, 2 trisomy 13, and 5 had miscellaneous karyotypic anomalies including monosomy X, trisomy X, triploidy, and ring 14 chromosomes. Among cases of aneuploidy, pyelectasis was present in only 2 fetuses, both with trisomy 21: in 1 case pyelectasis was associated with other anomalies and, in the other, pyelectasis was an isolated finding.

Table I The prevalence, sensitivity, specificity, positive predictive value, negative predictive value, the likelihood ratio, and the diagnostic odds ratio of isolated pyelectasis to detect trisomy 21

<table>
<thead>
<tr>
<th></th>
<th>Total cases 12,672</th>
<th>Trisomy 21</th>
<th>Normal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated pyelectasis</td>
<td>1</td>
<td>304</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td>Absence of isolated</td>
<td>10</td>
<td>12,357</td>
<td>12,367</td>
<td></td>
</tr>
<tr>
<td>pyelectasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>12,661</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prevalence: 0.09%; sensitivity: 9.09% (95% CI 1.62–37.74); specificity: 97.6% (95% CI 97.32–97.85); PPV: 0.33%; NPV: 99.9%; +LR: 3.7862 (95% CI 0.582–24,616); −LR: 0.9315 (95% CI 0.773–1123); Diagnostic odds ratio: 4065 (95% CI 0.519–31,853).

Table II The prevalence, sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio, and diagnostic odds ratio of pyelectasis in association with other anomalies to detect trisomy 21

<table>
<thead>
<tr>
<th></th>
<th>Total cases 12,672</th>
<th>Trisomy 21</th>
<th>Normal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyelectasis</td>
<td>1</td>
<td>60</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Absence of pyelectasis</td>
<td>10</td>
<td>12,601</td>
<td>12,611</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>12,661</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prevalence: 0.09%; sensitivity: 9.09% (95% CI 1.62–37.74); specificity: 99.53% (95% CI 99.39–99.63); PPV: 1.64%; NPV: 99.92%; +LR: 19.1833 (95% CI 2.91–126.44); −LR: 0.9134 (95% CI 0.758–1.101); diagnostic odds ratio: 21,002 (CI 2647–166,637).
In the other aneuploidy encountered (the 6 cases of trisomy 18, the 2 trisomy 13, and 5 miscellaneous karyotypic anomalies), none had pyelectasis and were considered “normal” for statistical analysis.

Table I shows the prevalence, sensitivity, specificity, positive predictive value, negative predictive value, the likelihood ratio, and the diagnostic odds ratio of isolated pyelectasis to detect trisomy 21.

Table II shows the prevalence, sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio, and diagnostic odds ratio of pyelectasis in association with other anomalies to detect trisomy 21.

Of the total patients with pyelectasis, 83.3% (305/366) were isolated cases with no other associated anomalies, which was 2.41% (305/12,672) of the total population. In one case of isolated pyelectasis with a renal pelvis dilatation of 4 mm in both kidneys, the female fetus was affected with translocation 14-21. This patient was 23 years old at the examination, 24 years old at the time of delivery, and the ultrasound was performed at 18 weeks gestation. The prevalence of trisomy 21 in the group with isolated pyelectasis was 1:305, or 0.33%. In the other patients, no other chromosomal anomalies were found.

Of the total patients with pyelectasis, 16.7% (61/366) had other associated anomalies, which was 0.48% (61/12,672) of the total population. Most of these cases were minor anomalies, such as heart echogenic focus, choroid plexus cyst, 2-vessel cords, and short humerus. Trisomy 21 was identified in a case of bilateral pyelectasis with a right dilatation of 4 mm and left dilatation of 6 mm. The other minor signs present in this fetus included a cardiac echogenic focus in the left ventricle and a sandal gap on the right foot. The prevalence of trisomy 21 in fetuses with pyelectasis and associated anomalies was 1:61, or 1.64% (Figure 2 and Table III).

In the present study, trisomy 21 was found nearly 8 times more often in association with pyelectasis (2 of 366, or 0.546%) compared with fetuses without pyelectasis (9 of 12,306, or 0.0731%).

Comment

This is one of the first large studies examining the prevalence of pyelectasis in a low-risk unselected population. We found that 2.9% of the 12,672 fetuses examined had pyelectasis: 83.3% of these were isolated cases, whereas in 16.7% other anomalies were present. Only 2 cases of pyelectasis were associated with aneuploidies (0.55%), one of which had isolated pyelectasis and Down syndrome, while the other had pyelectasis with associated anomalies and trisomy 21.

In the present study, only 1 case out of 305 fetuses with isolated pyelectasis had trisomy 21; this prevalence was higher than the expected 1 in 1100 in the general population reported from the Centers for Disease Control and Prevention. However, although the likelihood ratio for fetuses trisomy 21 with isolated pyelectasis was 3.79, which appeared to be increased, if the 95% CI is calculated (0.582–24.616), the estimated risk was actually not significantly increased.

The male prevalence found in our work (male:female 1.9:1) confirmed previous reports. Comparing our work with previous studies was rather difficult because of the various standards and parameters used. Different studies have defined pyelectasis with a range of dilatation sizes, and used populations of different compositions (high-risk, low-risk, or a mix of both). Gestational age and patient age also varied from one study to another. In some cases, authors did not differentiate between isolated pyelectasis and pyelectasis with other associated anomalies.

The prevalence of pyelectasis in our patients was 2.9%, which was similar to values calculated in other studies (see Table IV), (range between 0.72% and 2.84%). Although the true prevalence of pyelectasis in

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Pyelectasis</th>
<th>Other findings</th>
<th>Maternal age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trisomy 21</td>
<td>Bilateral</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Trisomy 21</td>
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<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Trisomy 21</td>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
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<td>None</td>
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</tr>
<tr>
<td>5</td>
<td>Trisomy 21</td>
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</tr>
<tr>
<td>6</td>
<td>Trisomy 21</td>
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<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>Trisomy 21</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>9</td>
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<td>35</td>
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<tr>
<td>10</td>
<td>Trisomy 21</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>11</td>
<td>Translocation 14-21</td>
<td>Bilateral</td>
<td>23</td>
</tr>
</tbody>
</table>

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the general population was difficult to ascertain, this study provided evidence that is as close to the baseline as possible because none of the patients were preselected or referred from other centers. A summary of the previous study is provided in Table IV.

Chudleigh19 used the percentage of pyelectasis to the number of births, not among the total ultrasound exam, and different parameter inclusions (anteroposterior diameter of the renal pelvis of 5 mm up to and including 10 mm, while we used 4 mm).

Though there was variability in the prevalence of pyelectasis in past studies (Table IV), there was a relative uniformity of result for the prevalence of trisomy 21 among fetuses with pyelectasis. Because of the small prevalence of trisomy 21, 1 or 2 cases could have strongly affected the statistical analysis in most studies.

In our study the prevalence of trisomy 21 in isolated pyelectasis was 1:305: Chudleigh19 reported similar results (1:217, and 1:324 in women under 36 years) for isolated pyelectasis, but suggested a higher incidence for pyelectasis in association with other anomalies. In the study of Corteville,12 among 4 cases of trisomy 21, 3 were in association with ultrasound abnormalities and, in the remaining case of trisomy 21, the abnormality was suspected. Previous studies did not find any aneuploidy for isolated fetal pyelectasis.16 Others20,21 concluded that the presence of pyelectasis increased the risk of aneuploidy only if it was in association with other anomalies, such as nuchal fold thickening and a short humerus.

Although Persutte et al15 did not specify if his cases were associated with other structural anomalies or other soft signs, he found a high incidence of chromosomal anomalies (2 trisomy 21 and 1 recombinant 8 syndrome, 3 multiple congenital anomaly, 1 IUGR, 5 fetal or infant losses.

Table IV Comparison of several studies on pyelectasis

<table>
<thead>
<tr>
<th>Author</th>
<th>Prevalence of pyelectasis</th>
<th>Population</th>
<th>Gestational age</th>
<th>Cut Off</th>
<th>Pyelec.</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benacerraf (8)</td>
<td>2.84%</td>
<td>7400</td>
<td>&gt;16</td>
<td>≥4 mm between 15-20 weeks</td>
<td>210</td>
<td>2.84%</td>
<td></td>
</tr>
<tr>
<td>Corteville (12)</td>
<td>2.10%</td>
<td>5944</td>
<td>&gt;14</td>
<td>≥4 mm before 33 weeks</td>
<td>127</td>
<td>2.14%</td>
<td></td>
</tr>
<tr>
<td>Nicolaides (18)</td>
<td>NA</td>
<td>Selected</td>
<td>15-38</td>
<td>≥5 mm</td>
<td>258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wickstrom (10)</td>
<td>0.80%</td>
<td>11340</td>
<td>17-39</td>
<td>≥4 mm</td>
<td>82</td>
<td>0.72%</td>
<td></td>
</tr>
<tr>
<td>Wickstrom (25)</td>
<td>0.72%</td>
<td>7481</td>
<td>≥15</td>
<td>≥4 mm &lt;33 weeks</td>
<td>121</td>
<td>1.62%</td>
<td></td>
</tr>
<tr>
<td>Ouzounian et al. (28)</td>
<td>0.73%</td>
<td>—</td>
<td>—</td>
<td>≥7 mm &gt;33 weeks</td>
<td>84+</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Chudleigh et al. (19)</td>
<td>0.73%</td>
<td>101600</td>
<td>16-26</td>
<td>≥5 mm</td>
<td>737</td>
<td>0.73%</td>
<td></td>
</tr>
<tr>
<td>Havutcu et al. (16)</td>
<td>1.25%</td>
<td>25586</td>
<td>18-24</td>
<td>≥5 mm</td>
<td>320</td>
<td>1.25%</td>
<td></td>
</tr>
<tr>
<td>Persutte et al. (15)</td>
<td>5.50%</td>
<td>5529</td>
<td>16-38</td>
<td>≥4 mm</td>
<td>306</td>
<td>5.53%</td>
<td></td>
</tr>
<tr>
<td>Nyberg (23)</td>
<td>NA</td>
<td>Selected</td>
<td>14-20</td>
<td>≥3 mm</td>
<td>186</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotmensch (29)</td>
<td>NA</td>
<td>Selected</td>
<td>14-28</td>
<td>≥4 mm between 15-20 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sairam (30)</td>
<td>2.34%</td>
<td>12672</td>
<td>16-23</td>
<td>≥4 mm</td>
<td>366</td>
<td>2.89%</td>
<td></td>
</tr>
</tbody>
</table>

* No distinction there were among isolated and not isolated pyelectasis.
† The distinction was made only for major anomaly: 12 had concomitant anomaly: 2 T21, 1 recombinant 8 Syndrome, 3 multiple congenital anomaly, 1 IUGR, 5 fetal or infant losses.
‡ Analysys of 155 fetuses with Down syndrome.
isolated finding and 5.2 overall if in combination with other markers. The reported risk was higher in the study of Whitlow \(^2\) in the first trimester by a likelihood ratio of 8 (95% CI 2.0–31.7) for all aneuploidy and 9.6 (95% CI 1.4–64.7) for trisomy 21, although it was not clearly specified if this risk was only for isolated pyelectasis or in association with other soft markers or structural anomalies. The analysis made on 99 women by Wickstrom et al \(^2\) indicated an increased risk for isolated fetal pyelectasis over that related to age for both Down syndrome and all chromosomal abnormalities: 3.9-fold increase in Down syndrome risk, and a 3.3-fold increase in risk for all chromosomal abnormalities.

Two different studies showed results very similar to ours among the total cases of aneuploidy and association with pyelectasis.\(^{26,27}\)

Table IV summarizes the comparison with other previous study for number of population examine and different inclusion criteria.

Our data do not suggest an increased risk of aneuploidy for isolated pyelectasis compared with the general population.

Although the observed 3.79-fold appeared as an increase, the CIs were so large (to the point of including a decreased risk) that this study does not support an increased risk. We concluded that the recognition of an isolated pyelectasis is not an indication for amniocentesis, but an indication for a detailed exam to assess the presence of other ultrasound markers for aneuploidy. For fetuses with multiple markers (only 1 in our study, with an echogenic heart focus and a right-sided sandal gap) our likelihood ratio demonstrated an increased risk for aneuploidy.

### References


Endometrial microbial colonization and plasma cell endometritis after spontaneous or indicated preterm versus term delivery

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Objective: This study was undertaken to determine whether endometrial microbial colonization or plasma cell endometritis is increased after spontaneous versus indicated preterm delivery or a spontaneous term delivery.

Study design: Postpartum, endometrial specimens were obtained after a spontaneous (mean 83, ± 17.6 days) or indicated (mean 83, ± 16.7 days) preterm delivery before 34 weeks’ gestation and after a spontaneous term delivery (mean 82, ± 15.7 days; P = .980). Cultures for aerobic and anaerobic bacteria, Trichomonas vaginalis, and genital mycoplasmas were performed. Histologic endometritis was defined as the presence of plasma cells.

Results: The study population (n = 820) was 71% black, 29% white, 69% unmarried, and 31% had less than 12 years of education. Endometrial cultures were positive for at least 1 microorganism in 82% of the women. No significant difference in positive endometrial cultures were observed among women after a spontaneous versus an indicated preterm delivery (85% vs 79%, P = .102), or a spontaneous preterm versus a spontaneous term delivery (85% vs 81%, P = .123). Plasma cell endometritis was present in 39% of 506 specimens sufficient for histologic examination and was also similar in the three groups (P = .160).

Conclusion: Microbial colonization of the endometrium and plasma cell endometritis are similar 3 months after spontaneous or indicated preterm or term births. Therefore, chronic infection and inflammation of the endometrium (documented at 3 months postpartum) do not appear to be risk factors for subsequent delivery in women with a prior spontaneous delivery less than 34 weeks’ gestation.

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Preterm birth complicates up to 12% of all pregnancies and remains the primary cause of perinatal mortality and approximately one half of long-term neurologic morbidity. The majority of this mortality and morbidity is concentrated among very early preterm births.

Considerable data implicate a clinically silent upper genital tract infection and histologic chorioamnionitis as key components of the pathophysiology of a majority of very early spontaneous preterm births. The bacteria isolated from the upper genital tract among women who have preterm births are all microorganisms that are generally believed to be of low virulence and are typically representative of the normal microbial flora of the cervix and vagina.

The prevailing hypothesis is that microbial colonization of the chorioamnion and amniotic fluid results from ascending infection by bacteria initially localized in the cervix and vagina. These bacteria may reside for some time in the upper genital tract producing a clinically silent inflammatory response before ultimately resulting in a spontaneous preterm birth. The timing of the upper genital tract infection is not completely understood. However, available evidence indicates that bacterial ascension from the lower to the upper genital tract occurs early in gestation. This is supported by evidence that there is an inverse relationship between upper genital tract infection or inflammation with both birth weight and gestational age at delivery among women with a spontaneous, but not indicated preterm delivery. In addition, reports indicate that an activated host immune response, as evidenced by elevated concentrations of interleukin-6 in amniotic fluid, can be demonstrated as early as the midtrimester and, when present, is associated with an increased risk of early pregnancy loss and spontaneous preterm delivery.

Very early preterm delivery in an index pregnancy has been significantly associated not only with preterm delivery in the subsequent pregnancy, but also with delivery at earlier gestational ages. The mechanism(s) that results in the high rate of repeat preterm birth in these women remains uncertain. However, the studies linking spontaneous preterm birth to intrauterine inflammation that can be demonstrated as early as the midtrimester and data revealing the potential for asymptomatic plasma cell endometritis to be present in nonpregnant women led us to speculate that microbial colonization of the endometrial cavity may actually precede conception. If this were true, then chronic interpregnancy interval microbial colonization or inflammation of the endometrium in nonpregnant women may partially explain the high incidence of repeat spontaneous preterm birth in women with a previous spontaneous preterm delivery. Therefore, we hypothesized that nonpregnant women with a recent spontaneous preterm delivery would have higher frequencies of endometrial microbial colonization and plasma cell endometritis compared with nonpregnant women with a recent indicated preterm or spontaneous term delivery. The objective of the current study was to test this hypothesis.

**Material and methods**

The study was conducted at the University of Alabama at Birmingham between August 1995 and August 2001. Women with singleton pregnancies who presented with spontaneous labor or preterm premature rupture of membranes that ultimately resulted in a preterm birth or pregnancy loss between 16 weeks 0 days’ and 33 weeks 6 days’ gestation were recruited and offered enrollment by research personnel. These women represented the spontaneous preterm birth group. After discharge from the hospital, these women received follow-up care at a clinic specifically dedicated to this study. Also recruited were women delivered between 16 weeks 0 days’ and 33 weeks 6 days’ gestation because of maternal medical or obstetric indications. These women represented the indicated preterm delivery group. Research personnel also recruited a control group of women that included women with the spontaneous onset of labor who delivered a singleton infant at or beyond 37 weeks’ gestational age. To eliminate bias in the selection process for the term control group, this group was selected from among all women delivered at term at our institution. After recruitment of women with a preterm delivery, the next woman with a spontaneous term delivery who met inclusion criteria and who agreed to participate was recruited. The goal of this process was to allow the term control group to reflect the overall population delivered at our institution. All women were subsequently monitored in the Center for Research in Women’s Health at the University of Alabama at Birmingham where written informed consent was obtained before initiation of the study. The study was approved by the Institutional Review Board.

The women enrolled in all 3 groups received an initial evaluation at approximately 3 months postpartum during a scheduled visit to the Center for Research in Women’s Health. We chose to perform this initial evaluation at 3 months postpartum: (1) because we believed there would be resolution of the normal physiologic changes from the previous pregnancy and (2) there would likely be an opportunity to obtain this information before a subsequent pregnancy. Presence of pregnancy was excluded before the interval evaluation by menstrual history, physical examination, and pregnancy test when necessary. The initial evaluation consisted of a history and physical examination, including a speculum examination (described later). Specimens obtained included a vaginal smear to be Gram stained for determination of bacterial vaginoses and cervical and endometrial cultures. Specific cervical cultures included those for *Ureaplasma urealyticum, Mycoplasma* species,
**Microbiologic sampling of the cervix and endometrium**

After confirming the absence of pregnancy by menstrual history and a pregnancy test (if indicated), a sterile speculum was placed in the vagina to expose the cervix. Appropriate swabs for the individual cervical cultures were then obtained as were specimens from the posterior vaginal fornix to perform the assessment for bacterial vaginosis. After all vaginal and cervical specimens were obtained, the vagina and cervix were liberally prepped with a povidone-iodine 10% solution. A sterile plastic endometrial suction device (Pipelle, Cooper Surgical, Wilton, Conn) was then used to obtain an endometrial sample for culture by using a technique designed to minimize the possibility of microbial contamination of the endometrial specimen with microorganisms from the cervix and vagina. The technique used careful attention to the placement of the Pipelle into the cervix and endometrial cavity so that it did not come in contact with the vaginal walls. This technique protected against contamination with vaginal bacteria until the tip of the Pipelle was passed beyond the internal cervical os. Once within the endometrial cavity, the Pipelle plunger was then completely withdrawn to the hub end and the endometrial specimen was obtained. After obtaining the specimen, and before removing the Pipelle from the endometrial cavity, the hub end of the Pipelle was cut with a pair of sterile scissors. This released the vacuum within the Pipelle lumen and prevented aspiration of cervical microorganisms (and contamination of the endometrial specimen) as the Pipelle tip was withdrawn through the internal os. A sterile syringe and connector was then used to aspirate the specimen. The endometrial specimen was then aseptically removed from the syringe into a sterile petri dish and portions of the specimen were placed in appropriate media for transport to the Laboratory for Research in Women’s Health at the University of Alabama Birmingham. On arrival in the laboratory, all specimens were processed for culture by using appropriate culture media and atmospheric conditions.

Although the microbiologic focus of this investigation involved an evaluation of microbial colonization of the upper genital tract, specimen contamination by microorganisms residing in the lower genital tract was an important potential confounding factor. The technique used for collection of the endometrial specimen was designed to minimize such contamination. We selected several common lower tract microbial markers to confirm that the method of sampling alone did not result in positive endometrial cultures. These microbial markers included Neisseria gonorrhoeae, group B beta Streptococcus, Trichomonas vaginalis, and a ligase chain reaction test for Chlamydia trachomatis. Endometrial specimens were obtained (described later) and cultures for these same microorganisms were performed as well as for aerobic and anaerobic bacteria. Histopathology was also performed on the endometrial specimens as described later.

**Isolation and identification of mycoplasmas**

Media (10B, A8, and SP4 broth and agar) were prepared and quality controlled as described previously for cultivation of mycoplasmas. All swab-inoculated specimens and tissue homogenates were thoroughly mixed on a vortex mixer. Five 10-fold dilutions were made from a 100 µL aliquot of each specimen type in 10B and SP4 broths. An aliquot (20 µL) of the undiluted specimen and each dilution was inoculated onto A8 and SP4 agar. All plated media were incubated at 37°C under 5% CO2 in a humidified incubator. A8 plates were held for 2 weeks and SP4 plates for 6 weeks before being designated negative. Plates were evaluated microscopically for growth every 1 to 3 days. All broths were incubated at 37°C under appropriate atmospheric conditions. Ten B broth were held for 2 weeks and SP4 broths were held for 8 weeks and blind passaged between days 14 and 21. Any broth tube showing color change suggestive of mycoplasmal growth was subcultured to solid media and incubated further.

Colonies of U urealyticum were identified on A8 agar by urease production in the presence of CaCl2 indicator. Mycoplasma species were identified as M hominis by polymerase chain reaction. Positive and negative controls were run at the same time as the clinical isolates to ensure that there were no cross reactions or false-positive or false-negative results.

**Isolation and identification of aerobic and anaerobic bacteria**

All endometrial samples were placed into appropriate transport media used for the recovery of both aerobic and anaerobic bacteria, and the samples were processed within 4 hours of collection. The following media were inoculated for aerobic bacterial isolation: thioglycolate broth, chocolate agar, trypticase soy agar containing 5% sheep blood, and human bilayer with Tween (HBT) agar. The thioglycolate broth was held for 5 days and the plated media for 48 hours before being designated as negative. All plated media were incubated at 35°C in 5% CO2. Colonies were identified on the basis of Gram stain reaction and standard biochemical procedures. Identification procedures were those described in the Manual of Clinical Microbiology, and Bailey and Scott’s Diagnostic
Microbiology. In addition, anaerobic media used for primary isolation included Brucella blood agar, and chopped meat carbohydrate broth. All media were pre-reduced before inoculation. Additional pre-reduced media used when needed were kanamycin-vancomycin laked blood agar and Columbia CNA agar. Cultures were examined for growth after 48 to 72 hours of incubation in an anaerobic chamber atmosphere of 90% N₂, 5% CO₂, and 5% H₂ at 35°C. Negative primary isolation plates were discarded after 5 days. Liquid media were observed for up to 7 days, and any broth showing evidence of growth at any time was Gram stained and subcultured to solid media. In addition, any anaerobic growth was subcultured and incubated aerobically for determination of the organism’s atmospheric requirements. Organisms that were confirmed to be obligate anaerobes were identified by standard laboratory procedures as outlined in the *Wadsworth Anaerobic Bacteriology Manual*, and *Principles and Practices of Clinical Anaerobic Bacteriology*. These techniques included rapid enzymatic biochemical reactions, antibiotic disc susceptibility testing, and additional testing as required.

**Trichomonas cultures**

InPouch TV (Biomed Diagnostics) trichomonas culture pouches were used for the cultivation of *T vaginalis*. The pouches were incubated at 37°C for 5 days and were examined microscopically each day for the presence of characteristic motile trichomonads before being designated as negative.

**Chlamydia ligase chain reaction assay**

Swab specimens of the cervix and of the endometrial tissue were stored in buffer at −70°C for batched analysis. Detection of *C trachomatis* was accomplished with the ligase chain reaction (LCR) for DNA amplification (Abbott Laboratories, Chicago, Ill). The technique for LCR *Chlamydia* assay in both cervical and endometrial specimens was a modification of the procedure as previously described. We have reported that this procedure is superior to culture for the identification of this organism.

**Gonorrhea cultures**

All sample sites were directly inoculated at the time of collection (endocervical) or processing (endometrium) onto a selective plate containing GC-Lect Agar (Becton-Dickinson, Franklin Lakes, NJ) and which used the Jembeck System. This product contains a CO₂-generating system that provides an appropriate environment of carbon dioxide required for the growth of *Neisseria gonorrhoeae*. Within 1 hour of collection or processing, all plates were placed into an incubator containing 5% CO₂ at 35°C. The plates were held for 48 hours before being designated as negative for *N gonorrhoeae*. Growth exhibiting typical colonial and microscopic morphology was identified by using standard biochemical and enzymatic reactions as described in the *Manual of Clinical Microbiology*.

**Group B streptococcal cultures**

Endocervical and endometrial swabs were each used to inoculate a Todd-Hewitt broth containing gentamicin (8 µg/mL) and nalidixic acid (15 µg/mL). After a minimum 24-hour incubation at 35°C, the broth was subcultured onto a 5% sheep blood agar plate. The plate was held for 48 hours and was examined for the typical colonial morphology and hemolysis of group B beta *Streptococcus (S agalactiae)* before being designated as negative. Positive cultures were confirmed with Gram stain and standard Lancefield grouping techniques.

**Endometrial histopathology**

Histopathology was performed by a single pathologist. Endometrial tissue sections for light microscopic examination were fixed in 10% neutral buffered formalin, paraffin-embedded, and 5 µm sections stained with hematoxylin and eosin. Histologic endometritis was characterized by identification of plasma cells under 40× magnification.

**Statistical analyses**

All statistical analyses were performed with SAS version 8.2 software (Cary, NC). Categorical variables were compared with the use of χ² tests. Means for the 3 groups were compared by using analysis of variance with a Duncan multiple range test for multiple comparisons. Comparisons of means between 2 groups were performed with a 2-tailed unpaired Student *t* test or Wilcoxon rank sum test where indicated. We calculated adjusted odds ratios for endometrial colonization for previous spontaneous and indicated deliveries compared with term by using logistic regression analysis adjusting for possible confounding variables. To compare matched cervical and endometrial culture results, McNemar’s test for paired data was performed. An alpha level of .05 was used to determine statistical significance. The sample size allows for a statistical power of 99% for an effect size of 50% ranging down to a power of 82% for an effect size of 15% (with alpha = .05).

**Results**

A total of 820 women were enrolled in this study including 375 (45.7%) in the prior spontaneous preterm birth group, 142 (17.3%) in the prior indicated preterm...
Table I  Selected characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall</th>
<th>Spontaneous Delivery</th>
<th>Indicated Delivery</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>71%</td>
<td>70%</td>
<td>58%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>White</td>
<td>32%</td>
<td>34%</td>
<td>49%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Unmarried</td>
<td>69%</td>
<td>66%</td>
<td>57%</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>≤12 y of education</td>
<td>31%</td>
<td>28%</td>
<td>24%</td>
<td>.002</td>
</tr>
<tr>
<td>Smoking*</td>
<td>23%</td>
<td>27%</td>
<td>24%</td>
<td>.024</td>
</tr>
</tbody>
</table>

* Self reported.

Table II  Most common endometrial bacterial isolates

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Overall</th>
<th>SPTD</th>
<th>IPTD</th>
<th>STD</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gardnerella vaginalis</td>
<td>46</td>
<td>45</td>
<td>42</td>
<td>47</td>
<td>.539</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>37</td>
<td>40</td>
<td>39</td>
<td>32</td>
<td>.084</td>
</tr>
<tr>
<td>S viridans</td>
<td>15</td>
<td>17</td>
<td>8</td>
<td>16</td>
<td>.043</td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>species</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M hominis</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>.775</td>
</tr>
<tr>
<td>U urealyticum</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td>8</td>
<td>.023</td>
</tr>
<tr>
<td>S agalactiae</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>.480</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.663</td>
</tr>
<tr>
<td>(coagulase negative)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphtheroid spp.</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>.804</td>
</tr>
<tr>
<td>Gemella morbillorum</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>.941</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>3</td>
<td>2</td>
<td>5</td>
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<td>.130</td>
</tr>
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<td>Propionibacterium acnes</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>.001</td>
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<td>Prevotella bivia</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>.196</td>
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<td>Mobiluncus species</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>.213</td>
</tr>
<tr>
<td>C trachomatis</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>.883</td>
</tr>
<tr>
<td>S aureus</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>.551</td>
</tr>
</tbody>
</table>

SPTD, Spontaneous preterm delivery; IPTD, indicated preterm delivery; STD, spontaneous term delivery.

* P value for comparison of the 3 study groups (SPTD, IPTD, and STD).

Birth group, and 303 (37.0%) in the prior term delivery group. The mean gestational age at delivery in the preceding pregnancy was 26.4 ± 4.7 weeks in the spontaneous preterm delivery group, 27.2 ± 4.8 weeks in the indicated preterm birth group, and 39.6 ± 1.2 weeks in the term delivery group (P < .001). Mean delivery gestational ages in the 2 prior preterm birth groups were not significantly different. The interval from delivery to sampling for cultures and histology was similar in the spontaneous preterm birth group (83 days; mean ± SD), the indicated preterm birth group (82 days ± 16.7 days), and the spontaneous term group (82 days ± 15.7 days; P = .980). Characteristics of the study population are provided in the Table I. In this study population, women with a term delivery were more likely to be black, were less educated, and were more likely to be single (Table I).

Endometrial cultures were positive for at least 1 microorganism in 82% of the women. The most common endometrial isolates are listed in Table II. The median number of different bacterial species isolated from the endometrium in the spontaneous preterm delivery group was not significantly greater than the indicated preterm delivery group (2, range 0-9 vs 2, range 0-6 microorganisms, P = .132) but was significantly greater than the term delivery group (2, range 0-9 vs 1, range 0-7 microorganisms, P = .015). However, no significant difference in endometrial cultures positive for any microorganism were observed among women after a spontaneous versus an indicated preterm delivery (85% vs 79%, P = .102), or a spontaneous preterm versus a spontaneous term delivery (85% vs 81%, P = .123). The distribution of the commonly isolated microorganisms from the endometrium was largely similar among the 3 groups (Table II). In a logistic regression analysis controlling for race, marital status, maternal education, smoking, and maternal body mass index, the odds ratio for having a positive endometrial culture for any organism was 1.49 (95% CI 0.96-2.31) in the previous spontaneous preterm birth group and 1.06 (95% CI 0.61-1.84) in the previous indicated preterm birth group.

Plasma cell endometritis was present in 39% of the 506 specimens adequate for histologic evaluation and endometrial cultures were positive for 1 or more microorganisms in 83% of the women who had plasma cell endometritis. Plasma cells were present in 41% of cases in which the corresponding endometrial culture was negative. Combining the results of the cultures and histology, 86% of the women had evidence of either endometrial microbial colonization or histologic inflammation. Plasma cell endometritis was higher in the spontaneous preterm delivery (43%) and the indicated preterm delivery (40%) groups compared with the spontaneous term delivery group (33%) but this difference was not statistically significant (P = .160).

To address the issue of potential microbial contamination of the endometrial specimen during its acquisition by bacteria from the cervix and vagina, cervical cultures were obtained in all women for 3 microorganisms that are commonly present in the cervix (U urealyticum, M hominis, and S agalactiae). Although frequently isolated from the cervix, isolation of these 3 microorganisms was significantly less common from the endometrium (Table III).

Comment

These results do not support the original hypothesis that microbial colonization of the endometrium and plasma cell endometritis would be higher among women with a recent spontaneous preterm delivery compared with women with a recent indicated preterm or spontaneous induction of delivery.
term birth. Instead, the frequencies of microbial colonization and plasma cell endometritis assessed at 3 months’ postpartum were similar in all 3 groups of women. Therefore, these results do not explain the increased risk of subsequent preterm birth among women with a prior early spontaneous preterm birth before 34 weeks’ gestational age. A striking and unexpected finding in this study was the extraordinarily high number of positive endometrial cultures and the high frequency of plasma cell endometritis in all 3 groups. Plasma cell endometritis was present in nearly 40% of the specimens that were adequate for histologic evaluation and four fifths of all women in the study had endometrial bacterial colonization. Combining the results of the cultures and histology, 86% of the women had culture or histologic evidence of endometrial microbial colonization or inflammation.

One potential explanation for the high frequency of endometrial microbial colonization observed in this study is the possibility of microbial contamination of the endometrial tissue during acquisition of the specimen with bacteria from the cervix or vagina. Such microbial contamination could occur despite the precautions taken to limit this complication (see Methods). However, we do not believe that microbial contamination of the endometrial specimen is the explanation for the observed results for several reasons. First, such contamination would not explain the high frequency of plasma cell endometritis that was observed in all 3 groups. Additional compelling support for the conclusion that the high observed rate of microbial endometrial colonization is not due to gross contamination is derived from our data of matched cervical and endometrial cultures for specific microorganisms (Table III). If the high rate of positive endometrial cultures resulted from frequent contamination of the endometrial specimen with cervical bacteria, then we would have expected to find a high rate of positive endometrial cultures for these three organisms (U urealyticum, M hominis, and S agalactiae) when the corresponding cervical culture was positive. In fact, this was not the case. Although frequently isolated from the cervix, isolation of U urealyticum, M hominis, and S agalactiae was much less common from the endometrium. On the basis of results depicted in Table III, even in the unlikely event that 100% of the positive endometrial cultures for these 3 microorganisms were the result of contamination of the endometrial specimen with cervical bacteria when the concomitant cervical culture was positive for the same microorganism, the estimated rate of contamination would range only between 4.1% and 7.5%. Therefore, we believe that the extremely high rate of endometrial microbial colonization observed in this study largely represents a true picture of the microbiologic status of the endometrium in these women.

Another potential explanation for the high rate of positive endometrial cultures and plasma cell endometritis observed in this study is ascertainment bias caused by the limitation of the upper genital tract assessment to 1 time point at 3 months’ postpartum. This time frame was chosen for several reasons. First, genital tract changes resulting from hormonal, mechanical, and other influences of pregnancy are believed to resolve by 3 months’ postpartum. Second, recruitment and retention of subjects for this study, because of the necessity of voluntary submission to an endometrial biopsy, was anticipated to be challenging. We believed that our success in this regard would be improved if the interval between first contact immediately after delivery and ultimate enrollment in the study were shorter rather than longer. Third, we believed that serial endometrial biopsies would be unacceptable to women and would potentially have untoward effects. Therefore, we agreed to limit the number of endometrial biopsies performed on a given subject. Performing a single biopsy on groups of women at different time intervals also would have markedly increased the expense of the study and the number of women required for meaningful analyses. Nevertheless, we acknowledge that our endometrial culture and histologic observations are limited to a single investigation at approximately 3 months’ postpartum. Lower frequencies of endometrial colonization or plasma cell endometritis might have been observed at later postpartum time intervals in any or all of the 3 study groups as has been reported for plasma cell endometritis. Indeed, plasma cell endometritis is a common occurrence for several months after birth.

In summary, the results of this study indicate that differences in endometrial microbial colonization or inflammation do not explain the high rate of repeat preterm delivery among women with a prior spontaneous preterm birth. However, the high frequency of positive endometrial cultures and histologic endometritis observed in otherwise asymptomatic nonpregnant women (without signs or symptoms of endometrial infection) do call for a reevaluation of current assumptions about the microbiologic status of the endometrium in nonpregnant women. A prevailing assumption is that the endometrium in nonpregnant women is largely devoid of bacterial colonization. The previously described results argue otherwise, at least 3 months after delivery. If, in fact, endometrial colonization is a common condition in

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Cervix/endometrium culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U urealyticum</td>
<td>1.1</td>
</tr>
<tr>
<td>M hominis</td>
<td>0.1</td>
</tr>
<tr>
<td>S agalactiae</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table III: Culture results for selected microorganisms isolated from the cervix and endometrium

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Cervix/endometrium culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U urealyticum</td>
<td>49.6</td>
</tr>
<tr>
<td>M hominis</td>
<td>22.4</td>
</tr>
<tr>
<td>S agalactiae</td>
<td>15.6</td>
</tr>
</tbody>
</table>

P < .001 in each case (McNemar’s test).
nonpregnant women, why then are some women who have such colonization destined to have subsequent infection-related spontaneous preterm births while most women are not? The answer may be that there are differences in the host response to the presence of these bacteria rather than the mere presence of the bacteria themselves. One example might be the presence of certain proinflammatory cytokine gene polymorphisms that genetically program for an enhanced immune response in the presence of bacteria resulting in an increased release of bioactive factors that unfortunately lead to a spontaneous preterm birth. In any case, elucidation of this question remains an unanswered challenge and an important subject for future research.

References

Variation in microbiologic profiles among pregnant women with bacterial vaginosis

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KEY WORDS
Bacterial vaginosis
Mobiluncus
Pregnancy

Objective: The purpose of this study was to determine if clinical findings and sociodemographic variables among bacterial vaginosis (BV)-positive pregnant women are associated with different microbiologic profiles.

Study design: Pregnant women were assessed for BV by Nugent criteria. BV+ women were separated into 6 mutually exclusive microbiologic groups. In unadjusted analyses, we compared (1) sociodemographic and behavioral characteristics, and (2) 3 clinical characteristics among BV+ women with and without Mobiluncus (M+ vs M−). Unadjusted data were analyzed using the chi-square test. Multiple logistic regression was used to assess the likelihood of having clinical signs of BV in women with and without Mobiluncus spp while controlling for confounders.

Results: A total of 1756 BV+ pregnant women were followed. The M+ group (n = 702) was significantly more likely than the M− group (n = 1054) to be non-Hispanic black (80.9% vs 66.2%; P < .0001), older than 21 years (61.7% vs 48.7%; P < .0001), and to have had more than 3 lifetime sexual partners (66.4% vs 54.9%; P < .0001). The M+ group was also more likely to have clue cells on wet mount (63.9% vs 47.2%; P < .0001) and a positive amine odor after addition of KOH (57.2% vs 45.0%; P = .001). There was no difference in other demographic variables or physician diagnosis of abnormal vaginal discharge. In the adjusted analyses for each clinical outcome, all findings were consistent with the unadjusted analyses.

Conclusion: BV+ pregnant women with Mobiluncus spp are more likely to have clue cells present on wet mount, a positive amine odor after KOH preparation, and to be older, non-Hispanic black, and have had more lifetime sexual partners compared to BV+ women without any Mobiluncus species.

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Bacterial vaginosis (BV) is a condition in which the normal lactobacillary flora are replaced by an overgrowth of Gardnerella vaginalis, Prevotella/Bacteroides species, Mobiluncus species, and other organisms.1-3 The association between BV and preterm birth (PTB) has been reported by several authors.4-8 However, studies aimed at prevention of PTB by treatment of BV have

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yielded differing results. This discrepancy has led the American College of Obstetricians to take the position that “current data do not support the use of BV screening as a strategy to identify or prevent preterm birth.”

As our clinical recognition of BV has become standardized by adopting Nugent criteria, we can now appreciate that BV is not the same disease in all women. Microbiologic variation exists among BV-positive individuals and can be characterized by Gram-stain analysis. All women with a Nugent score of 7 to 10 are diagnosed as BV positive, but among these individuals, there is a wide variety of microflora. The purpose of this study was to determine if the microbiologic variation among BV positive individuals could explain variations in clinical signs of BV and differences in the prevalence of BV among different race/ethnic groups.

Material and methods

This study is part of a larger, ongoing clinical prevalence study assessing the association between maternal stress, BV, and risk of preterm birth. Pregnant women were recruited from public health centers in Philadelphia, Pennsylvania, between February 1, 1999 and April 20, 2003. Women were recruited for participation in this study at the time of their first prenatal care visit, at a mean gestational age of 14 ± 6 weeks. All English or Spanish speaking women with singleton pregnancies were eligible.

Practitioners collected 2 vaginal smears from all study participants, one for Gram-stain and one for wet mount. Practitioners also assessed the following clinical signs: the presence of clue cells on wet mount, amine odor after addition of potassium hydroxide (KOH), and vaginal discharge.

Air-dried vaginal smears were assessed for BV according to Nugent criteria. Each Gram stain was evaluated for the following morphotypes under oil immersion (100×) magnification: large uniform gram-positive rods (corresponding to Lactobacillus spp), small pleomorphic gram-variable rods (G vaginalis morphotypes), small gram-negative rods (Prevotella/Bacteroides morphotypes), and curved gram-variable rods (Mobiluncus morphotypes). After scanning the slide for representative areas, each morphotype was quantified from 1 to 4 with regard to the average number of morphotypes seen on 5 nonadjacent fields, using the scoring system listed in Tables I and II. Each morphotype was given a number and added to yield a score of 0 to 10 using the following equation: Lactobacillus + G vaginalis/Prevotella/Bacteroides + Mobiluncus = total score. In this scoring system, Lactobacillus is inversely scored, compared to G vaginalis/Prevotella/Bacteroides and Mobiluncus. A score of 0 to 3 was considered normal, a score of 4 to 6 corresponded to intermediate BV status, and a score of 7 to 10 was defined as positive BV status.

Based on the individual components of this classification scheme, all smears were categorized into 1 of 14 mutually exclusive microbiologic groups. Based on the microbiologic distribution, it was impossible for 8 of the groups to achieve a Nugent score above 6. Therefore, all BV-positive women (Nugent score 7-10) were classified into one of 6 mutually exclusive microbiologic groups. Three microbiologic groups contained Mobiluncus species (M+), and 3 did not (M−).

Gram-stain scoring was performed by a microbiologist (KA) who was blinded to the clinical findings and demographics of subjects. The investigator (LP) who designated individuals into respective microbiologic groups was similarly blinded. The following outcomes were compared between BV+, M+ women and BV+, M− women: clue cells identified on wet mount, positive amine odor after addition of KOH, and physician diagnosis of vaginal discharge. The same comparisons were carried out for BV intermediate women.

The statistical significance of all categorical data was determined using the chi-square test. To adjust for potential confounding variables, and to derive maximum likelihood estimates of adjusted relative odds with 95%
CI, multivariate logistic regression analyses were performed. Potential confounders were identified a priori by reviewing the literature and based on theoretical considerations. The following confounders were included in all of the multivariate models: race, maternal age, age at sexual debut, number of lifetime sexual partners, recent smoking, douching, parity, marital status, education, maternal annual income, and foreign-born status. Type I error was set at 0.05 (two-sided). All analyses were conducted using STATA 8.0 (College Station, Tex).

Results

A total of 4361 women met inclusion criteria and were eligible to participate in this study. Of these, 3881 (89.0%) consented to participate, and 3765 (86.3%) completed the study. There were 1756 (46.6%) BV+ women, and 553 (14.7%) BV-intermediate women. The largest subgroup of BV+ women (57.8%) demonstrated *G. vaginalis* and *Prevotella/Bacteroides* on Gram stain, and an absence of Lactobacillus and *Mobiluncus* species. The second largest subgroup (39.7%) had the same profile, except that *Mobiluncus* species were present on Gram stain. The other 4 possible classifications were rarely identified, accounting for just 2.5% of subjects. Because of this distribution, women with *Mobiluncus* spp (40.0% combined 3 groups, M+) were compared to women without *Mobiluncus* spp (60.0% combined 3 groups, M−) (Figure 1).

In unadjusted analyses, BV+ women with *Mobiluncus* were significantly more likely to be non-Hispanic black (80.9% vs 66.2%; χ²(1) = 45.2; *P* < .0001), to be above the median age of 21 (61.7% vs 48.7%; χ²(1) = 28.7; *P* < .0001), and to have had more than 3 lifetime sexual partners (66.4% vs 54.9%; χ²(1) = 22.9; *P* < .0001) compared with BV+ women without *Mobiluncus*. However, the presence of *Mobiluncus* spp was not associated with age at sexual debut, recent smoking, or douching (Figure 2). Furthermore,
in adjusted analyses, women with *Mobiluncus* spp were more likely to have clue cells on wet mount (63.9% vs 47.2%; $\chi^2_{(df)} = 23.2(1); P < .0001$) and a positive amine odor after addition of potassium hydroxide (57.2% vs 45.0%; $\chi^2_{(df)} = 11.6(1); P = .002$). However, there was no difference in the physician finding of an abnormal vaginal discharge (64.6% vs 61.5%; $\chi^2_{(df)} = 1.18(1); P = .28$). In the adjusted analysis for each clinical outcome, all findings were consistent with those from the unadjusted analyses. M+ women were 1.7 times more likely to have clue cells present on wet mount (95%CI: 1.3–2.3), and 1.5 times more likely to have amine odor after the addition of KOH to the wet mount compared with M− women. In addition, there was no difference between the 2 groups with regard to physician finding of abnormal vaginal discharge (OR 1.1; 95% CI 0.8–1.4) (Table III).

When we conducted this same analysis in BV-intermediate women, all of the demographic distributions and clinical outcomes followed identical trends when comparisons were made between M+ (n = 35) and M− (n = 518) BV-intermediate women (data not shown). However, none of the findings were statistically significant.

This study had sufficient power (>80%) to detect a minimum odds ratio of 1.5 for the association between the presence of *Mobiluncus* spp and each of the 3 clinical outcomes. Nonetheless, we did not find an association between the presence of *Mobiluncus* and physician diagnosis of abnormal vaginal discharge (Table III), suggesting that this null finding is not likely to be the result of a type II error.

**Comment**

In the past 20 years of obstetric research, few topics have been as widely studied as BV. A MEDLINE search using the keywords “bacterial vaginosis,” and “pregnancy” produced more than 500 corresponding articles since 1984. In fact, the association between BV and preterm birth (PTB) has been so consistently reported that there is little doubt in today’s medical community that a true relationship exists between them.4-8 Despite this body of evidence, however, an understanding of the exact nature of the relationship between BV and PTB remains elusive.

Strong association does not equal causation, and perhaps this explains, at least in part, why treatment trials aimed at eradication of BV to decrease the rate of PTB have yielded differing results.9-10 Another possibility is that although the relationship with BV and PTB may in fact be causative, it may be causative only for certain individuals. This may be due to differences in host response to BV, confounding risk factors for PTB,
or variations in the profile of pathogens constituting BV among different women.

The importance of the Nugent scoring system in providing standardized diagnostic criteria for BV cannot be overstated. However, the diagnosis of BV by Nugent score alone is limited because it clusters women with different vaginal flora into one group of BV-positive individuals. The limitations of Nugent criteria have already been recognized by some authors who have reported subsets of women with intermediate BV scores among different women.17 In a nonpregnant population of women seeking treatment at an STD clinic, Hillier et al also reported race/ethnic differences in the presence of Mobiluncus spp identified by Gram stain, with black women 2.5 times as likely to be M+ compared with white patients.18 Why black women exhibit more colonization of Mobiluncus is not known. To date, sexual transmission does not seem to be the primary method of acquisition of Mobiluncus spp, and a gastrointestinal reservoir has been suggested.19 It is possible that race/ethnic differences in host factors that modulate bacterial binding to vaginal and gastrointestinal cells may account for the observed race/ethnic differences in Mobiluncus carriage rates. For example, Lewis antigens are thought to inhibit the binding of infectious organisms to human cells. Lewis antigen deficiency has been associated with increased rate of urinary tract infections in women,20 recurrent vaginal candidiasis,21,22 and Helicobacter pylori infections.23 Some data suggest that blacks are more likely to be Lewis antigen-negative, and differences in Lewis antigen status or a similar host factor could potentially explain the race/ethnic variations in Mobiluncus colonization rates.24

In conclusion, different BV morphotypes exist among individuals. Demographic variations (age, race/ethnicity, etc) are observed when comparing BV+ women by Mobiluncus spp status. Microscopy also demonstrates that Mobiluncus spp is associated with cellular variation (more clue cells) and diagnostic variation (a positive amine odor after KOH preparation). However, this distribution of BV morphotypes does not seem to explain why some BV-positive individuals are diagnosed with abnormal vaginal discharge while others are not. Differential expression of Lewis antigens may provide a genetic mechanism for the increased prevalence of Mobiluncus among BV-positive non-Hispanic blacks and warrants further study. In the future, we plan to investigate whether the BV morphotypes characterized

| Table III | Multivariate logistic regression of the association between the presence of Mobiluncus species in BV-positive women and 3 clinical signs* |
|------------------|------------------|------------------|------------------|------------------|
| **Dependent variable** | **Mobiluncus species present**<sup>1</sup> OR (95% CI) | **Mobiluncus species present (%)** | **Mobiluncus species absent (%)** | **P value** |
| Clue cells on wet mount | 1.7 (1.3, 2.3) | 63.9 | 47.2 | < .00001 |
| Amine odor after KOH addition | 1.5 (1.1, 2.0) | 57.2 | 45.0 | .002 |
| Physician diagnosis of abnormal vaginal discharge | 1.1 (0.8, 1.4) | 64.6 | 61.5 | .28 |

* All models adjusted for the following variables: race, maternal age, number of lifetime sexual partners, recent smoking, douching, parity, marital status, education, maternal annual income, and foreign-born status.

<sup>1</sup> Mobiluncus species negative women are the reference group for all models.
in this study are associated with different vaginal levels of hydrolytic enzymes and proinflammatory cytokines and, ultimately, with different risks of preterm birth. As our understanding of BV evolves, it is important to recognize that microbiologic and clinical variation exists among BV-positive women, and our thinking of BV-positive women as a homogeneous population harboring the same disease must evolve as well.

References

Use of DNA hybridization to detect vaginal pathogens associated with bacterial vaginosis among asymptomatic pregnant women

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Department of Obstetrics and Gynecology, a and Center for Oral and Systemic Diseases, b University of North Carolina at Chapel Hill, Chapel Hill, NC; Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Women’s Hospital of the University of Pittsburgh Medical Center, Pittsburgh, PA c

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KEY WORDS
Bacterial vaginosis
Diagnosis
Pregnancy

Objective: The purpose of this study was to determine whether microbial DNA hybridization is a useful method to study bacterial vaginosis in asymptomatic pregnant women.

Study design: Vaginal specimens were collected at <26 weeks’ gestation from 230 women, and analyzed for bacterial vaginosis by both Gram stain using Nugent criteria and DNA hybridization for Gardnerella vaginalis, Prevotella bivia, Bacteroides ureolyticus, and Mobiluncus curtisi. Results were analyzed using McNemar’s paired test and chi-square test for trend, with significance set at $P < .05$.

Results: By Gram stain, 60 (26.1%) of 230 were positive for bacterial vaginosis, and 134 (58.3%) were negative. By DNA hybridization, 99 (43%) were positive for at least 1 pathogen, and DNA results were significantly associated with Gram-stain results ($P < .01$). As the Nugent score progressed from normal to abnormal flora, the proportion with $>1$ pathogen detected by DNA hybridization increased significantly ($P < .001$).

Conclusion: DNA hybridization may be a useful method to study shifts in vaginal flora during pregnancy.

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Bacterial vaginosis has been reported in 16% to 50% of pregnant women. 1,2 Bacterial vaginosis has been associated with numerous adverse outcomes, including post-cesarean section endometritis, spontaneous miscarriage, prematurity, and fetal growth restriction. 1,3,4 Despite its prevalence and impact, some important biologic questions remain unanswered. The etiology of this complex microbiologic syndrome characterized by a shift from normal vaginal flora to other organisms (Prevotella spp and Bacteroides spp, Gardnerella vaginalis, Mobiluncus spp, anaerobic Gram-positive cocci, and genital mycoplasmas) is unknown. 4-7 Studies of treatment...
of bacterial vaginosis to prevent preterm birth have reported conflicting results, but a recent meta-analysis affirmed the benefit of 1 week of oral antimicrobial therapy in women with asymptomatic bacterial vaginosis who have previously delivered preterm. These unresolved issues emphasize that further research on the biology of bacterial vaginosis is needed.

The diagnosis of bacterial vaginosis is usually based on clinical criteria, including homogeneous vaginal discharge, an elevated vaginal pH, the presence of clue cells, and an amine odor, but these clinical criteria are imprecise for various reasons. Recent intercourse, douching, menstruation, or contamination by cervical mucus can affect vaginal pH. In addition, both amine odor and characteristics of the vaginal discharge are observer dependent and subjective criteria. Determination of clue cells on wet mount is also prone to variability depending on the skill and training of the reader, the quality of the microscope, and the adequacy of the specimen.

To address these difficulties, Nugent et al proposed a scoring system for the diagnosis of bacterial vaginosis based on a Gram-stained vaginal smear. Nugent’s criteria are based on visual recognition of bacterial morphotype. A score is assigned to the vaginal flora as a weighted continuum from 0 to 10 based on large Gram-positive rods, small Gram-negative, or variable rods, or curved rod morphotypes. The sensitivity of Gram stain is significantly higher than clinical assessment, and is a reliable means to diagnose bacterial vaginosis. However, microscopic evaluation by Gram stain requires special skills that might not be available to all practitioners or researchers, and does not yield information about specific pathogens. Checkerboard DNA hybridization identifies presence of microbial DNA by using hybridizing labeled probes. The sensitivity of the technique is 10^4 CFU/mL. Digoxigenin-labeled, whole genomic DNA-probes were developed for Gardnerella vaginalis, Prevotella bivia, Bacteroides ureolyticus, and Mobiluncus curtisi by extracting chromosomal DNA from pure cultures grown from American Type Culture Collection strains 14018, 29303, 33387, and 35241. Probes were labeled using the High-Prime labeling kit (Boehringer Mannheim, Indianapolis, Ind).

Vaginal samples were thawed, boiled for 5 minutes, and neutralized with 0.8 mL 5 mol/L ammonium acetate. The samples were then deposited in parallel lanes onto nylon membranes (Boehringer Mannheim) using a Minislot device (Immunetics, Cambridge, Mass), and immobilized by exposure to ultraviolet light. After 2 hours of prehybridization, the DNA probes were placed on the membrane at right angles to the sample lanes by means of a Miniblotter device (Immunetics), and allowed to hybridize with the vaginal sample DNA overnight at 42°C. After a series of stringency washes, hybrids were detected by incubation with an anti-digoxigenin

### Material and methods

As part of the Oral Conditions and Pregnancy Study, 1115 pregnant women were prospectively enrolled to characterize the inflammatory and infectious components of periodontal disease. Institutional Review Board approval was granted to conduct the study, and written informed consent obtained from subjects. All women enrolled had vaginal specimens collected. A Dakron swab was placed in the posterior vaginal fornix, smeared on a glass slide, and allowed to dry. A second swab of vaginal fluid was placed in a microcentrifuge tube containing 0.15 mL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.6) and 0.15 mL of 0.5 mol/L NaOH, and frozen at −80°C for microbial DNA analysis. Checkerboard DNA hybridization was compared to Gram stain using Nugent criteria for the diagnosis of bacterial vaginosis among a random subset of 230 (20%) enrolled women.

### Table

<table>
<thead>
<tr>
<th>Gram-stain interpretation</th>
<th>Normal flora (Nugent score 0–3)</th>
<th>Intermediate flora (Nugent score 4–6)</th>
<th>Bacterial vaginosis (Nugent score 7–10)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA hybridization results*</td>
<td>Negative</td>
<td>18</td>
<td>5</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>18</td>
<td>55</td>
<td>99</td>
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<tr>
<td></td>
<td>Total</td>
<td>134</td>
<td>36</td>
<td>60</td>
</tr>
</tbody>
</table>

* Positive defined as detection of at least 1 vaginal pathogen at 104 CFU/mL.
antibody conjugated with alkaline phosphatase and the addition of a chemiluminescent substrate (Boehringer Mannheim). Signals were detected and quantitated with the help of a Lumi-Imager™ workstation (Boehringer-Mannheim) by comparing the unknown signals to the ones generated by pooled bacterial standards containing $10^6$ of each of the 4 species. In this analysis, the signal of the high standard ($10^6$) was used as reference to calculate the bacterial load of the vaginal samples. Any sample generating a signal equivalent to $10^4$ CFU/mL or greater was considered positive for the species.

Gram-stain methodology and interpretation using Nugent’s criteria was applied to stored vaginal smears by a single trained reviewer who was blinded to the DNA hybridization results. Results were analyzed using McNemar’s paired test and chi-square test for trend, with statistical significance set at $P < .05$.

**Results**

Of the 230 women studied, 55% were black, 53% unmarried, 23% reported tobacco, and 24% alcohol use during pregnancy, and 62% were nulliparous. Bacterial vaginosis was detected by Gram stain in 60 (26.1%) of women. Vaginal pathogens were detected by DNA hybridization in 99 (43.0%) of women. Gram-stain results were significantly associated with DNA hybridization detection of vaginal pathogens (Table, chi-square test for trend, $P < .001$).

Of the 99 specimens positive for vaginal pathogens by DNA hybridization, 53 (53.5%) were positive for at least 1 vaginal pathogen, 40 (40.4%) for 2 pathogens, 4 for 3 pathogens, and 2 for all 4 vaginal pathogens tested. The most common pathogen detected by DNA hybridization was *G vaginalis* (35.1%), followed by *P bivia* (22.6%), then *B ureolyticus*, and *M curtisi* (3% each). Specimens with a Nugent score of 7 to 10 were more likely to have *G vaginalis* detected by DNA hybridization than those with a score of 0 to 3 (90% vs 9%, $P < .001$). The proportion of specimens demonstrating 0, 1, or $>2$ vaginal pathogens by DNA hybridization was significantly associated with Nugent score (Figure, $P < .001$). Defining bacterial vaginosis as the presence of *G vaginalis* and 1 other anaerobe by DNA hybridization and comparing with Nugent score detection of bacterial vaginosis (score 7–10), the sensitivity and specificity of the DNA test were 60% and 89%, respectively.

**Comment**

Based on our findings, checkerboard DNA hybridization is a useful method to detect multiple vaginal pathogen targets. This technique allows detection of bacterial vaginosis by specific morphotype, which may assist in documenting the pathogen most often associated with perinatal complications. The sensitivity of Gram stain is $10^5$ organisms. A commercial DNA hybridization test (Affirm™ VP III, Microbial Identification Test, Becton-Dickinson, Sparks, Md) has been developed and compared to Gram stain for the diagnosis of bacterial vaginosis among symptomatic pregnant women. The automated probe system detects $>2 \times 10^5$ CFU/mL *G vaginalis*. However, the DNA probe was positive in 43% of women with lower organism counts. If presence of microbial DNA at low

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**Figure** Graph demonstrating proportion of vaginal specimens with 0, 1, or $>2$ target vaginal pathogens detected by DNA hybridization stratified by Nugent score category.
levels prove to be associated with clinical symptoms or adverse reproductive outcomes, use of DNA hybridization may be useful in studying treatment response and understanding treatment failures. Furthermore, in our population of asymptomatic women, a significant proportion of women with intermediate flora by Nugent’s criteria did not have 1 of the pathogens detected, and as the Nugent score increased the proportion of specimens with 2 or more pathogens also increased, emphasizing the intricate microbiology of asymptomatic bacterial vaginosis in pregnancy and the potential utility of DNA testing to study shifts in vaginal flora.

We believe that this method may prove useful to study vaginal flora shifts typical of bacterial vaginosis but caution interpretation for clinical utility. This study was not designed to test the DNA hybridization test as a clinical diagnostic test. Reporting the sensitivity and specificity of the DNA test implies that it was compared with a ‘gold standard’ test. The only results that we have on the study women are Gram-stain results because women were asymptomatic, and vaginal pH was not determined. There are many different criteria used and accepted for the diagnosis of bacterial vaginosis, and in the absence of a gold standard to compare the DNA test, the sensitivity and specificity may be misinterpreted. It would be preliminary to suggest that the DNA test is appropriate for clinical uses as the clinical implications of presence of DNA of vaginal pathogens in absence of symptoms remains to be determined. The intent of our study was to demonstrate that vaginal pathogens typical of bacterial vaginosis are present at low levels, which may be underappreciated by the Nugent score.

Socransky et al developed the checkerboard method in 1994 to simultaneously hybridize large numbers of DNA samples. DNA segments are fixed in separate lanes on a membrane, which are then exposed at right angles to multiple labeled whole genomic DNA probes against specific bacterial species. Hence, the multiplex assay allows for simultaneous detection of multiple organisms, which is the greatest advantage. However, checkerboard DNA-DNA hybridization results take 3 days to return, and an experienced laboratory is required to perform this test and, thus, is more costly than Gram stain. With these limitations the use of DNA hybridization may be best applied to research protocols aimed at determining the natural history of bacterial vaginosis during pregnancy, response to therapy, or reasons for conflicting results with treatment to prevent adverse pregnancy complications.

While our findings are encouraging and suggest usefulness of this DNA hybridization test for studying asymptomatic bacterial vaginosis during pregnancy, our study has limitations. Vaginal smear specimens were randomly selected, and it is possible that our sample is not representative of the entire study population, or not generalizeable to all pregnant women. However, the detection rate of bacterial vaginosis by Gram stain was comparable with other reports in pregnant women. There is no follow-up information regarding development of symptoms suggestive of bacterial vaginosis later in the pregnancy, to determine if detection by DNA hybridization predicts transition from asymptomatic to symptomatic infection. We have not examined pregnancy outcome data stratified by method of detection, and it is possible that detection by DNA hybridization does not correlate with clinically important infection.

We recognize that these issues warrant further study on a larger sample of women, and conclude that DNA hybridization may be a useful technology to apply to the study of bacterial vaginosis and its complications during pregnancy.

References


Is zygosity or chorionicity the main determinant of fetal outcome in twin pregnancies?

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KEY WORDS
Twin pregnancy
Zygosity
Chorionicity

Objective: The purpose of this study was to examine whether fetal outcome in twin pregnancies is dependent on zygosity or chorionicity.

Study design: This was a prospective observational study comprised of women with twin pregnancies who attended the fetal medicine unit at St Michael’s Hospital, Bristol, Ireland, during the years 1998 to 2000 and who were delivered in hospitals in south west England. After delivery, zygosity was determined with umbilical cord blood with the use of microsatellite markers that were amplified by polymerase chain reaction. Placentae were examined histologically for chorionic type. The perinatal outcomes of 3 groups of monozygotic monochorionic, monozygotic dichorionic, and dizygotic pregnancies were compared with the use of the Mann-Whitney U test and the Fisher’s exact test.

Results: All 92 dizygotic and 15 monozygotic dichorionic pregnancies resulted in live births. In 7 of the 39 cases in the monozygotic monochorionic group, either both twins were not live born or delivery occurred <24 weeks of gestation. The gestational age at delivery and birth weight were significantly lower, and there were a greater number of cases with birth weight discordancy of >25% in the monochorionic pregnancies compared with the other 2 groups (P < .05). There were no significant differences in any of the study parameters between the monozygotic dichorionic and dizygotic groups.

Conclusion: Fetal outcome in twin pregnancies is related to chorionicity rather than zygosity.

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Two thirds of twin pregnancies are dizygotic, and one third is monozygotic. All dizygotic twins are dichorionic. Monozygotic twins may have monochorionic or dichorionic placentation, depending on the timing of mitotic division after fertilization of the single egg. Approximately 30% of monozygotic twins are dichorionic; all twins with monochorionic and 10% with dichorionic placentae are monozygotic.1,2 It has been shown that fetal outcome is worse for monochorionic than dichorionic twins, which has been attributed mainly to complications that are caused by placental vascular anastomoses.3,4 However, it is unclear whether
absolute outcome is related to monochorionicity or monozygosity. The very few studies that have examined outcome in relation to zygosity by comparing the 3 groups of dizygotic, monozygotic monochorionic, and monozygotic dichorionic pregnancies were retrospective and have produced conflicting results.\(^6\)\(^7\) Furthermore only one of the studies determined zygosity by neonatal DNA examination,\(^8\) which is a method that is more accurate than testing by infant blood group.\(^5\)\(^7\)

The purpose of this prospective study was to examine whether outcome is dependent on zygosity or chorionicity with zygosity being determined by DNA analysis with the use of highly polymorphic markers.

**Material and methods**

This was a prospective study that examined fetal outcome in relation to zygosity and chorionicity in women with twin pregnancies who attended the fetal medicine unit during the years 1998 to 2000. The women were referred from St. Michael’s Hospital, Bristol, and others throughout the south west of England for ultrasound assessment of chorionicity in the first trimester, which was the subject of a previous publication in this journal.\(^9\) The patients gave informed consent to enter the study that had been approved by the Hospital Ethics Committee. After delivery, umbilical cord blood and placentae were collected and sent from the place of delivery to the analytic centers: Cord blood went to the Department of Molecular Genetics, Southmead Hospital, Bristol, for DNA extraction and zygosity determination; and the placentae went to the Department of Pathology, St Michael’s Hospital, for diagnosis of chorionicity.

Zygosity was determined with microsatellite markers that were amplified with the polymerase chain reaction. Microsatellites are di-, tri-, or tetranucleotide repeat sequences that show variability in their repeat copy number. These sequences are scattered throughout the genome and are found in non-coding regions. In this study, 12 markers from 12 different chromosomes were amplified in 4 multiplex reactions. Individual markers were chosen because of their high degree of heterozygosity and were combined in multiplex reactions based on similar reaction conditions for amplification, non-complementarity of primer sequences and non-overlapping repeat lengths in amplified products. Amplified maternal and twin DNAs were run in consecutive lanes. Dizygosity was established when twins had inherited opposite maternal (or paternal) alleles at a minimum of 2 independent markers. Monozygosity was established with Bayes calculation to a likelihood of 95% with 5 informative markers and to >99% with ≥8 informative markers. Calculations were based on a previous probability of 0.3 that single sex twins are monozygous. The placentae were examined macroscopically, and microscopic sections of the membranes were analyzed for the determination of the chorionic and amniotic type. Pathologic placental examination was not necessary in twins who were discordant for sex because these were dizygotic. The 3 groups of monozygotic monochorionic, monozygotic dichorionic, and dizygotic pregnancies were compared.

In pregnancies in which delivery occurred after 24 weeks of gestation and both twins were live born, the gestational age at delivery and birth weight distributions were compared by the Mann-Whitney U test. Birth weights were also expressed as a multiple of the SD of birth weight for a given gestational age and sex.\(^9\) The Fisher’s exact test was used to compare proportions (ie, percentages) between the groups. A probability value of <.05 was considered significant.

**Results**

There were 146 sets of twins of a total of 205 twins for whom complete delivery details on outcome, gestation at delivery, birth weight, zygosity, and chorionicity were available; these twins were used for final analysis. In 44 of the 59 excluded cases, zygosity was not performed. In another 2 cases, although cord blood was examined, it was not possible to ascertain zygosity because of maternal contamination of the cord blood DNA. In 9 cases for which zygosity testing in same sex twins showed all to be monozygotic, placent al histologic condition was not determined; in 5 of these cases, histologic examination was impaired because of placental autolysis or damage to the membranes. In 4 cases of twins with different sex, delivery details were not available. There were 5 cases with congenital malformations; 4 of these cases were in monozygotic pregnancies, which included 2 cases of gastrochisis and 1 case each with ventriculomegaly and anencephaly. Three of these 4 cases had a monochorionic placenta. The fifth case was of a fetus with hydrocephalus in a dichorionic pregnancy with undetermined zygosity.

There were 92 dizygotic twin pregnancies. In 51 of these cases in which the twins were the same sex, classification was based on DNA microsatellite analysis; in all cases the placentae were classified histologically as dichorionic. In 41 cases, the twins were different sex and therefore dizygotic. All 39 monozygotic monochorionic pregnancies had histologic placental examination. In 23 cases, histologic results were concordant with DNA microsatellite results. In a further 16 cases in which microsatellite analysis was not performed, the diagnosis of monzygosity was based on the histologic diagnosis of monochorionicity. There were 15 monzygotic dichorionic sets that were diagnosed after microsatellite analysis and histologic placental examination.
In the study population, all 92 dizygotic and 15 monozygotic dichorionic pregnancies resulted in live births. In the monozygotic monochorionic group, 7 of the 39 cases were excluded from statistical analysis of gestation at delivery and birth weight distributions because either both twins were not live born or delivery occurred at <24 weeks of gestation; outcome data in these cases are summarized in Table I. Statistical analysis of outcome in the remaining 139 pregnancies is detailed in Table II.

**Comment**

These data show that fetal outcome in twin pregnancies is related to chorionicity rather than zygosity because there was a greater number of deaths in the monochorionic group compared with the monozygotic dichorionic and dizygotic groups. Additionally, the gestational age at delivery and birth weight were significantly lower, and there were a greater number of cases with birth weight discordancy of >25% in the monochorionic pregnancies. There were no significant differences in any of the study parameters between the monozygotic dichorionic and dizygotic groups.

The hypothesis that chorionicity rather than zygosity determines fetal outcome is supported by the finding in our study that all 10 fetal losses were in the monochorionic group; 6 of these losses were related to twin-twin transfusion. In another study that involved 300 pregnancies, the perinatal mortality rate was found to be 16% in the monochorionic group, compared with 11% and 1% in the dizygotic and monozygotic dichorionic group.
groups, respectively; 88% of the deaths in the monochorionic group were related to twin-twin transfusion, and 3 of the 16 deaths in the dizygotic group were related to congenital anomalies. In a much earlier study of 293 sets of twins, the perinatal mortality rate was also higher in the monochorionic group at 11.2%, compared with 3.5% in the dizygotic and 7.7% in the monzygotic dichorionic groups; only 1 of the 15 deaths in the monochorionic group was attributed to vascular anastomoses in the placenta. However, it is difficult to make definitive statements relating to outcome in this series because it was published in the 1960s and 6 of the 9 deaths in the monzygotic dichorionic group were related to low birth weight. The finding in our study that the perinatal outcome is similar in dizygotic and monzygotic dichorionic pregnancies is supported by a study that involved 1008 twin pregnancies. In that series, the perinatal mortality rate was 6.5% in the monochorionic group, compared with rates of 2.2% and 2.6% in the dizygotic and monzygotic dichorionic pregnancies, respectively; the causes of deaths were not given.

The relation between chorionicity and outcome is strengthened by the finding that the monochorionic group had a higher proportion of twin pairs with birth weight discordancy of >25% compared with the other 2 groups. This is in agreement with another study that showed that the incidence of weight discordancy was 8.6% in the monochorionic group and 7.3% and 5.4% in the dizygotic and monzygotic dichorionic groups, respectively. In our study, there was also a trend for an increased rate of fetal growth restriction below the third percentile in the monochorionic group, compared with the other 2 groups; however, this did not reach statistical significance. This finding was also reported in a previous study that examined the differences among the 3 groups.

Another study that compared monochorionic with dichorionic pregnancies found that the incidence of fetal growth restriction was significantly higher in monochorionic pregnancies. Therefore, it is reasonable to assume that significant fetal growth discordance results either from interfetal transfusion or placental insufficiency. The finding that the median birth weight in the monochorionic group was lower also reflects the earlier gestational age at delivery in this group; this trend is shown in Table II. Indeed, earlier gestation at delivery in monochorionic pregnancies has been reported in other studies.

The number of cases in our report is not large enough to determine whether the incidence of congenital abnormality is related to zygosity or chorionicity. The incidence of structural malformations is reported to be higher in monzygotic than dizygotic twins, and some anomalies may be explained partly by the process of embryo cleavage itself or secondary to an hypoxic insult that is related to the vascular anastomoses. Further research to examine the association between fetal defects and zygotic and chorionic type is required.

Our method demonstrates a reliable way of assessing zygotic type. Examination by blood group analysis has been used for many years. However, this method is less accurate because the segregation of numbers into the monzygotic dichorionic group is based on population probabilities of same sex twins with the same blood groups, which may lead to incorrect classification in some cases. Additionally, some blood group antigens may be expressed weakly on some fetal cells, which could lead to errors in interpretation. Indeed, the most recent and largest study to assess outcome in relation to zygosity and chorionicity used infant blood group analysis. In another study that analyzed outcome according to zygosity with extracted DNA from umbilical cord or membranes and testing by restriction fragment length polymorphism analysis, it was not clear whether chorionicity had been determined by histologic analysis.

In monzygotic pregnancies, heterokaryotypy may very rarely occur where a chromosomal abnormality occurs in 1 of the fetuses because of a post-zygotic non-disjunction. Therefore, the finding of 2 different karyotypes does not necessarily indicate dizygosity, and DNA studies are recommended whenever there is doubt about the chorionic status of the twins. Although ultrasound examination in the first trimester can reliably assess chorionicity, there may be doubt in a few cases, particularly if the diagnosis of chorionicity is based on a second-trimester scan. This is important because, if there is doubt, DNA or microsatellite analysis can be used to indicate a dizygotic pregnancy and thus exclude monochorionicity. Selective termination of the affected fetus can be carried out more safely if the twins are dizygotic and, by definition, have 2 separate placentae. Indeed, molecular genetic prenatal determination of twin zygosity with the use of DNA from chorionic villi and amniocytes has been reported previously.

**Acknowledgments**

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Sonographic myometrial thickness predicts the latency interval of women with preterm premature rupture of the membranes and oligohydramnios

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KEY WORDS
PPROM
Myometrium
Oligohydramnios
Ultrasound preterm birth

Objective: Term labor is associated with global thinning of the myometrium. We hypothesized that a thickened myometrium at the time of preterm premature rupture of membranes (PPROM) predicts less myometrial wall stress and, consequently, a longer latency interval.

Study design: Myometrial thickness was measured prospectively in 76 pregnant women enrolled in the following groups: PPROM (n = 28, mean [range], gestational age [GA]: 29.5 weeks [w] [21.0 w-33.0 w]), preterm nonlabor control group (P-CTR), (n = 21, GA: 27.5 w [23.0 w-32.0 w]) and term nonlabor control (T-CTR) (n = 27, GA: 38.6 w [37.0 w-41.6 w]). All PPROM women had oligohydramnios (AFI: 1.4 cm [0.0 cm-5.1 cm]). MT was measured ultrasonographically at the midanterior, fundal, posterior, and lower uterine segment wall in cases and controls with an intraoperator variability <10%.

Results: Women in the PPROM group displayed uniform thickness of the uterine body (mean ± SEM, anterior: 10.6 ± 0.6 mm, fundal: 10.7 ± 0.7 mm, posterior: 8.9 ± 0.5 mm, P = .078). At midanterior site the myometrium of the PPROM group was thicker compared to both P-CTR (P < .001) and T-CTR (P = .025) groups. This difference was preserved at the fundus (PPROM vs P-CTR, P < .001; PPROM vs T-CTR, P = .015). There was a positive correlation between fundal MT and latency period (r = 0.43, P = 0.02) that persisted after adjusting for GA (P = .04). A fundal MT less than 12.1 mm was 93.7% sensitive and 63.6% specific for the identification of women whose latency period was less than 120 hours.

Conclusion: Significant thickening of the anterior and fundal walls of the uterus follows PPROM. A thick myometrium in nonlaboring patients with PPROM is associated with longer latency interval. Sonographic evaluation of MT may represent an alternative clinical tool for the prediction of a short latency interval in women with PPROM.

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Spontaneous rupture of the membranes is a normal component of labor and delivery. Membrane rupture before the onset of labor is considered premature (PPROM), and induction of labor is common if the patient is at or close to term. Patient management becomes more challenging when membrane rupture occurs preterm (PPROM), and in the absence of labor. The incidence of PPROM ranges from 2% to 20%, and occurs preterm (PPROM), and in the absence of labor. The incidence of PPROM ranges from 2% to 20%, and is associated with 18% to 20% of perinatal deaths.1-3 Most women with PPROM deliver within 48 hours of rupture, but the neonatal impact and general outcome depend largely on the gestational age (GA) at rupture.4,5 Though the physiologic explanation is obscure, the interval from PPROM to delivery varies inversely with GA at rupture.4 At less than 25 weeks' (w) gestational age (GA), the average interval from rupture to delivery (latency) is 11 days (d).6

Although scientists have long investigated “the timing of birth,” our understanding of the biological mechanisms regulating the events that prevent and initiate labor remains limited.7 Not surprisingly, any prediction of the latency interval for women with PPROM is imprecise. Amniotic fluid volume, GA, cervical length, and presence of intra-amniotic markers of inflammation have various prognostic values.8-10 Indeed, oligohydramnios is a risk factor for earlier delivery because abruption and infection are each more common when amniotic fluid volume is diminished.11,12 Women with PPROM and oligohydramnios at less than 25 w deliver earlier compared to those with adequate amniotic fluid volume (pocket > 2 cm).8 It is thus not surprising to find that 85% of women with adequate amniotic fluid deliver beyond 25 w, and have much lower neonatal morbidity and mortality rates.8 Nevertheless, prophylactic therapy with broad-spectrum antimicrobial treatment (but no tocolytic therapy) is also associated with longer latency interval than placebo.13

Similar to the myocardium, the force of labor is uterine wall tension opposed to the resistance of the cervix, perineum, and pelvis.14,15 Mathematical modeling reveals that uterine wall stress (defined as applied force per unit cross-sectional area of material) is directly proportional to both the intracavitary pressure and the radius of the curvature, but inversely proportional to the thickness of the myometrium.16 Thus, the thicker the myometrium, the lower the uterine wall stress. We hypothesized that a thick myometrium at the time of PPROM would be associated with less myometrial wall stress and, consequently, longer latency interval. We tested this hypothesis by measuring MT by ultrasound scanning in patients with PPROM immediately following rupture.

### Material and methods

#### Patients and protocol

Myometrial thickness (MT) was measured prospectively in 76 pregnant women: PPROM (n = 28), preterm non-labor control (P-CTR, n = 21), and term nonlabor control (T-CTR, n = 27). The Institutional Review Boards of Yale University and Wayne State University approved the study. We approached women admitted to the Labor and Delivery ward and to the antepartum inpatient High Risk service at both institutions. All women solicited for enrollment agreed to participate and provided written informed consent. All women in P-CTR group were recruited from the Ultrasound Unit at the Labor and Delivery ward and to the antepartum High Risk service at Yale University. Women were enrolled based on the availability of one of the investigators (CSB), and all enrolled women were included in the final analysis. For the PPROM group, inclusion required PPROM with singleton from 22 to 34 w GA. Exclusion criteria included: fetal anomalies, suspected fetal growth restriction (IUGR) (sonographic fetal weight < 10% percentile for GA), abnormalities of placentation (low lying placenta, abruptio placenta), uterine structural abnormalities, cervical cerclage, previous uterine scar. Management of the patients was left up to the treating team. All patients except one (22 w GA) received corticosteroids for lung maturity and antibiotics per PPROM protocol.

<table>
<thead>
<tr>
<th>Maternal variables</th>
<th>PPROM (n = 28)</th>
<th>P-CTR (n = 20)</th>
<th>T-CTR (n = 27)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean ± SEM</td>
<td>29.2 ± 1.2</td>
<td>25.3 ± 1.2</td>
<td>25.1 ± 1.2</td>
<td>.0281</td>
</tr>
<tr>
<td>Gravidity, median (range)</td>
<td>3 (0-11)</td>
<td>3 (1-8)</td>
<td>3.5 (1-7)</td>
<td>.9121</td>
</tr>
<tr>
<td>Parity: median (range)</td>
<td>2 (0-5)</td>
<td>1 (0-4)</td>
<td>1 (0-6)</td>
<td>.8871</td>
</tr>
<tr>
<td>Maternal weight (kg), mean ± SEM</td>
<td>90.8 ± 5.9</td>
<td>82.1 ± 3.9</td>
<td>92.3 ± 4.3</td>
<td>.3471</td>
</tr>
<tr>
<td>Gestational age (wk), median (range)</td>
<td>29.5 (21.0-33.0)</td>
<td>27.5 (23.0-32.0)</td>
<td>38.6 (37.0-41.6)</td>
<td>&lt; .0011</td>
</tr>
</tbody>
</table>

Data was analyzed by one-way ANOVA (†), Kruskal-Wallis ANOVA (‡). SEFW, Sonographically estimated fetal weight; AFI, amniotic fluid index.
In the absence of signs or symptoms of chorioamnionitis (fever over 100.4°F, abdominal tenderness, fetal tachycardia), and/or abnormalities of fetal heart rate (variable or late decelerations), and/or abruption, PPROM was managed expectantly. The diagnosis of PPROM was confirmed by visualization of amniotic fluid “pooling” through the cervical os during speculum examination, “nitrazine,” “ferning,” or amniocentesis-dye positive tests. Tocolysis and/or digital exams were not permitted. Patients received corticosteroids for lung maturity if less than 32 w GA, and antibiotic therapy (ampicillin/erythromycin or clindamycin in the event of allergy to penicillin). Women were monitored by cardiotocography at least twice daily for the presence of fetal heart abnormalities and/or uterine contractions.

The ultrasound examination was performed within 12 hours of PPROM. An abdominal ultrasound survey (Acuson, Sequoia 512, Acuson Corporation, Mountain View, Calif [WSU] and/or Voluson 730 Expert, General Electric, Milwaukee, Wis [Yale]) was performed using a 5.0 or 7.5 MHz transabdominal probe. The amniotic fluid index (AFI) was measured using the 4-quadrant technique. Oligohydramnios was defined as an AFI less than 5 cm. The myometrium was sonographically identified as the echo homogeneous layer between the serosa and the decidua. The MT was measured at 4 different sites: lower segment (LUS) (approximately 2 cm above reflection of the urinary bladder), midanterior wall (with the scan probe 1 cm above the maternal umbilicus), fundus, and posterior walls of the uterus. Thickness of the fundus was measured by placing the scan probe above the uterine fundus so that the entire curvature of the uterus was visualized. To assure consistency in the anatomic site, aortic pulsations were identified before evaluation of fundal MT and used as a reference for all subsequent measurements. Measurement of the posterior uterine wall was technically the most challenging. We demarcated the posterior wall using pulsations of the maternal abdominal aorta as anatomic marker. Each measurement was made from separate scan images. At least 3 measurements were obtained at each site and averaged. The investigator (CSB) was not aware of the previous numeric evaluation of MT in between measurements. Previous experience demonstrated no differences in MT among uterine wall sites. One investigator (CSB) conducted all ultrasound examinations and made all measurements. The intraobserver coefficient of variation for assessment of MT varied from 8% to 10% at each uterine site.

PPROM women were managed expectantly, and underwent serial evaluations of fetal well-being up to delivery (spontaneously or for clinical indications consistent with chorioamnionitis or abruption). The latency interval was defined as the time period (days or hours) from the time of membrane rupture reported by the patient to delivery. None of the PPROM women were delivered for topics unrelated to PPROM (elective induction at 34-35 w, preeclampsia, or other medical complications of pregnancy).

Statistical analysis

All data sets were subjected to normality testing using the Kolmogorov-Smirnov method. The data are reported.
as mean and standard error of the mean (SEM) (for normally distributed data), or as median and range (for skewed data). Continuous normally distributed data were compared using one-way analysis of variance (ANOVA) or one-way repeated measures ANOVA followed by post-hoc Student-Newman–Keuls tests as appropriate. Categorical data sets or data without normal distribution were compared using Kruskal-Wallis ANOVA on ranks followed by Dunn’s tests. Statistical analysis of all MT data sets was completed after logarithmic transformation to obtain a normal distribution (one-way ANOVA). The effect of PPROM on MT at different uterine sites was determined using two-way repeated measures ANOVA. Multivariate analysis with linear regression model was applied to identify any significant associations between maternal, fetal, or labor characteristics as independent variables and MT as the dependent variable. A Pearson product moment correlation was used to measure colinearity between the selected independent variables, as well as other relevant correlations between dependent and independent variables. Receiver operating characteristics (ROC) curve analysis was performed using MedCalc (Broekstraat, Belgium) statistical software. Survival analysis was performed using GraphPad Software (San Diego, Calif).

A \( p < .05 \) was considered to indicate statistically significant difference.

### Results

#### Characteristics of women at enrollment

Table I presents a series of demographic and ultrasonographic variables assessed at enrollment. Women with PPROM were significantly older compared with those in the P-CTR and T-CTR groups (Student-Newman–Keuls \( p < .05 \)). There were no significant differences among groups in terms of gravidity (Kruskal-Wallis ANOVA, \( p = .912 \)), parity (\( p = .887 \)), or maternal body weight (\( p = .347 \)).

Women with PPROM and P-CTR had similar GA (median [range] PPROM: 29.5 w [21.0 w-33.0 w] vs P-CTR: 27.5 w [23.0 w-32.0 w], Dunn’s \( p > .05 \)) and sonographic estimated fetal weights (SEFW) (mean \( \pm \) SEM, PPROM: 1287.6 \( \pm \) 524 g vs P-CTR: 1192.7 \( \pm \) 505 g, Student-Newman–Keuls, \( p = .500 \)). The GA and SEFW were significantly different from T-CTR group (GA: 38.6 w [37.0-41.6], Dunn’s \( p < .001 \); SEFW: 3270.0 \( \pm \) 81.4 g; Student-Newman–Keuls \( p < .001 \)). PPROM
women had significantly lower AFIs compared to both control groups (one-way ANOVA $P < .001$). There was no significant difference in the AFI between P-CTR and T-CTR groups (Dunn’s $P > .05$). Oligohydramnios was present in 96.4% (27/28) of PPROM patients vs 0% of P-CRL (0/21) and 22.2% of T-CTR (6/27) (chi-square $P < .001$). The sonographic estimated abdominal wall thickness did not differ among groups (one-way ANOVA, $P = .06$), and correlated directly with maternal body weight (Pearson $r = 0.371$, $P = .002$).

Sonographic estimated myometrial thickness (MT)

Figure 1 illustrates representative ultrasound images of the anterior uterine wall of a woman in the P-CTR (Figure 1A) and PPROM (Figure 1B) groups. Both women had similar GA at MT assessment (27 w).

Sonographic evaluation of the myometrial wall at term (T-CTR) demonstrated that MT for each patient was uniform between uterine body sites (mean ± SEM, anterior: 8.8 ± 0.5 mm, fundal: 8.6 ± 0.4 mm, posterior: 8.2 ± 0.3 mm, one-way ANOVA, Student-Newman–Keuls, $P = .557$). At term all uterine body sites were significantly thicker than LUS (4.7 ± 0.5 mm, Student-Newman–Keuls, $P < .001$). Similarly, MT assessment in the PPROM group revealed uniform thickness at each site of the uterine body (anterior: 10.6 ± 0.6 mm, fundal: 10.7 ± 0.7 mm, posterior: 9.0 ± 0.6 mm, Student-Newman–Keuls, $P = .078$), although the LUS was thinner in PPROM women compared to the other sites (7.7 ± 0.7 mm, Student-Newman–Keuls, $P < .001$) (Figures 2 and 3). In the P-CTR group the differences in MT between sites including LUS did not reach statistical significance (anterior: 7.2 ± 0.4 mm, fundal: 7.7 ± 0.4 mm, posterior: 7.4 ± 0.4 mm, LUS: 6.2 ± 0.5 mm, one-way ANOVA, one-way repeated measurements ANOVA, $P = .06$).

Among groups, MT was significantly thicker at midanterior site in the PPROM group compared with both P-CTR (one-way ANOVA, $P < .001$) and T-CTR ($P = .02$) (Figure 3). This difference was maintained at the fundal site (PPROM vs P-CTR, $P < .001$; PPROM vs T-CTR, $P = .01$). The posterior wall site was only marginally thicker in PPROM women compared with both control groups ($P = .05$). LUS was significantly thicker in PPROM compared with P-CTR ($P = .04$) and T-CTR ($P = .003$) women. MT of the LUS at term was not different from P-CTR ($P = .07$).
To evaluate global differences of MT, we performed a two-way repeated measures ANOVA and found a significant difference in MT among groups (\( P < .001 \), power = 0.975) and sites (\( P < .001 \), power = 0.999). There was a significant interaction among groups and sites (\( P = .049 \), power = 0.445).

**Outcome characteristics**

Women with PPROM had a median GA at delivery of 30.6 w (range: 24.3 w-34.4 w) with a median latency of 4 days (d) (range: 0.16 d-31.2 d). Twenty-nine percent of PPROM women were delivered by cesarean section. One of the PPROM women was induced with misoprostol at 22 w following neonatology consult, and was excluded from the latency analysis. There was a direct correlation between fundal MT and latency period (\( r = 0.52, P < .005 \), \( n = 27 \)) (Figure 4A). The median GA at delivery for the P-CTR group was 39 w (range: 36.2 w-41.1 w). The median time between MT assessment and delivery for the P-CTR group was 68 d (range 41 d-103 d) and for the T-CTR group was 8 d (range: 1 d-23 d).

We modeled MT as dependent variable against AFI, gestational age, placental thickness, SEFW, gravidity, parity, maternal weight, maternal abdominal wall thickness, and membrane status (ruptured = 1, intact = 0) as independent variables to determine the possible impact of external factors on the MT measurement. This analysis was limited to the patients in the PPROM and P-CTR groups (\( n = 49 \)). Regression analysis suggested fundal MT is significantly related (\( r = 0.533, P = .001 \)) to maternal weight (\( P = .01 \)) and membrane status (\( P = .02 \)), while the anterior and posterior MT were best determined using a combination of membrane status and GA (anterior: \( r = 0.597, P < .001 \), posterior: \( r = 0.418, P = .02 \)). There was no significant colinearity among the final parameters in the model.

Table II lists the sensitivities and specificities of fundal MT measurement for the prediction of the latency interval with the optimum cut-offs (value corresponding with the highest accuracy, i.e., minimal false-negative and false-positive results as reported by MedCalc) in predicting delivery from 48 to 168 hours. A MT less than 12.1 mm was 93.7% sensitive for the identification of women whose latency period would be less than 120 hours (h), and 63.6% specific for the identification of women whose latency period would be higher than 120 h. A survival analysis of the delivery interval for PPROM group demonstrated that women with MT above 12.1 mm had longer latency intervals than those with MT less than 12.1 mm at enrollment (MT \(< 12.1 \) mm: latency 72 h vs MT \( \geq 12.1 \) mm: latency 228 h) (log rank chi-square = 8.412, \( P = .003 \)) (Figure 4B).

**Comment**

We demonstrate in the present investigation that uterine wall thickness is altered in women with PPROM, and correlates with latency interval. This finding has both clinical and physiologic implications.

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**Figure 4** (A) Scattergram of the myometrial thickness versus latency period in women whose pregnancies were complicated by preterm premature rupture of membranes (PPROM). \( R \), regression line; \( PI \), prediction interval (confidence interval for the population). (B) Survival analysis of labor duration in women whose pregnancies were complicated by PPROM according to a cut-off in fundal MT above 12.1 mm.
PPROM and associated preterm delivery are considered the leading causes of perinatal morbidity and mortality in the US. Clinically, the GA at PPROM, SEFW, fetal presentation, fetal lung maturity, absence of intra-amniotic inflammation, degree of cervical dilatation, and state of myometrial contractility are carefully evaluated before deciding on a course of management. In the absence of clinical symptoms or laboratory signs of chorioamnionitis, the management of a pregnancy with PPROM is usually expectant, based on the assumption that even a minor delay in the interval to delivery will be beneficial to the fetus.

Even though investigators have searched for factors that predict the onset of preterm labor, thickness of the myometrium following PPROM has never been tested. Digital cervical examination, home uterine monitoring of uterine contractility, and thickness of LUS have each been studied. The digital cervical examination and frequency of uterine contractions have weak prognostic values. Not only are digital cervical examination of women with PPROM and frequency of uterine contractions poorly predictive, but a digital exam may actually increase the risk of ascending infection. Conversely, vaginal bleeding, risk scoring schemes, and fetal breathing activity are also predictive of the onset of labor, but either have poor sensitivity and specificity, or are accurate only at late stages in the pathogenic process. Despite being ineffective, many of the previously listed prediction strategies are widely used in the clinical practice. The most recent efforts to estimate the predictive value of LUS thickness in women with intact membranes also proved to be unsuccessful.

There has been much attention focused on the sonographic assessment of cervical length since shortening is associated with an increased risk of preterm delivery in both nulliparous and multiparous women. The preterm delivery prediction study conducted by the NICHD Maternal Fetal Medicine Unit Network concluded that the most powerful factors associated with preterm birth before 32 w are a positive fetal fibronectin test and a cervical length less than 10th percentile either alone or in combination with other maternal serum biochemical tests. Cervical length measurement after PPROM may also be useful for predicting preterm birth, as the risk of ascending infection remains low. Unfortunately, the modest sensitivity with high specificity of cervical length evaluation may reflect the fact there are several different patterns of “normal” change in cervical length. These patterns may vary from a gradual to an accelerated change or even a precipitous decrease in cervical length at term.

The clinical management after PPROM is complicated by the absence of a gold standard method to predict pathogenic processes leading to parturition. Our understanding of the mechanisms that determine the length of the latency interval after PPROM is hindered by the fact that the human myometrium and cervix appear to have redundant and parallel mechanisms to ensure adequate length of gestation. Furthermore, the impact of pregnancy and labor on the uterus and cervix differ greatly. The prevailing theories surrounding PPROM latency interval may well overestimate the importance of the cervix, leaving the role played by myometrial activation largely unexplored. Markers with prognostic value in predicting the latency interval (chorionic-decidual and myometrial cell activation) would be beneficial as aides to clinical management, as well as to enhance our understanding of the mechanisms triggering preterm labor contractions and PPROM.

Our previous sonographic observation that the myometrium thins symmetrically during active labor with the least amount of thinning at the uterine fundus stimulated us to rethink the mechanisms responsible for the uniform dispersion of the contractile forces that ensure efficient fetal expulsion. Consistent with our previous report, we now demonstrate that women with spontaneous PPROM and in the absence of myometrial activation have a thicker anterior and fundal wall compared with women who have intact membranes. Sudden decompression of the uterine sac, which had been filled with a minimally compressible fluid that normally opposed thickening, is the most likely physiologic explanation. We assume that women with a long latency interval after spontaneous PPROM are in a state of myometrial quiescence or incomplete myometrial activation, and demonstrate that the long latency and presumed myometrial quiescence are associated with a greater thickness of the anterior and fundal wall myometrium. These observations are consistent with previous interpretations that the mechanisms underlying physical disruption of amniochorion integrity are complex, and collagenolytic activation of matrix metalloproteinases can occur in the absence of uterine contractility (myometrial activation). It is possible that those women with PPROM and thin myometrium already experienced functional complete myometrial activation that allows for coordinated tone, contractions, and shorter latency interval.

### Table II

<table>
<thead>
<tr>
<th>Latency</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>MT cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;48 h</td>
<td>55.6%</td>
<td>88.9%</td>
<td>&lt;8.1</td>
</tr>
<tr>
<td>48-72 h</td>
<td>80%</td>
<td>58%</td>
<td>&lt;9.7</td>
</tr>
<tr>
<td>&lt;96 h</td>
<td>91.7%</td>
<td>53.3%</td>
<td>&lt;10.3</td>
</tr>
<tr>
<td>&lt;120 h</td>
<td>93.7%</td>
<td>63.6%</td>
<td>&lt;12.1</td>
</tr>
<tr>
<td>&lt;144 h</td>
<td>93.7%</td>
<td>63.6%</td>
<td>&lt;12.1</td>
</tr>
<tr>
<td>&lt;168 h</td>
<td>89.5%</td>
<td>62.5%</td>
<td>&lt;12.6</td>
</tr>
</tbody>
</table>

![Table II](image-url)
Sonographic evaluation of cervical length in women with PPROM is reported to have maximum sensitivities and specificities of 63% and 81%, respectively.29 We find that the sonographic measurement of fundal MT less than 8.1 mm has a similar sensitivity and specificity (55.6%, respectively, 88.9%). However, we further determined that a MT 12.1 mm or more is 93.7% sensitive and 63.6% specific for the prediction of a latency period longer than 120 h. Unfortunately, no cervical length data are currently available for comparison at 120 h. As a corollary to these findings, survival analysis revealed that a thickened myometrium in nonlaboring women with PPROM was associated with latency longer than 120 h. This is consistent with our previous report demonstrating that only active myometrial contractility is associated with widespread thinning of the myometrium independent of ROM,14 and explains why nonactive laboring women (thick myometrium) have longer latency periods than those with MT less than 12.1 mm.

Given the likely heterogeneity in the causes of preterm labor, our present and previous reports raise more questions. We have insufficient data at this time to determine how MT changes longitudinally over the course of the latency period in women who will undergo spontaneous onset of uterine contractions. Further, we still do not know the appropriate method to predict latency in women with PPROM. Studies combining cervical length and MT sonography, fetal fibronectin, proteomic analysis of the amniotic fluid at the time of PPROM, and development of highly sensitive noninvasive uterine contraction monitoring methods are warranted. Transabdominal ultrasound evaluation of MT and surface electromyographic analysis of uterine contractions remain the only noninvasive methods to evaluate choriodecidual myometrial activation.26,31 While transabdominal sonography is unsatisfactory for cervical evaluation, it is well accepted by the patients for management. Obstet Gynecol 1988;71:558-62.

Sensitive biochemical assays for β-human chorionic gonadotropin (β-hCG) hormone, cytokines, and corticotrophin releasing hormone (CRH), as well as serial evaluation of vaginal amniotic fluid combined with cervical length and MT sonography, may provide the context required for a reassessment of the mechanisms responsible for early or delayed delivery of the fetus.

References
Use of over-the-counter medications during pregnancy

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Objective: The most common medications used in pregnancy are nonprescription or over-the-counter medications, although there has been little research on their risks or safety. We describe the patterns of over-the-counter medication use among pregnant women.

Study design: Data were collected in 2 case-control studies of birth defects: the Slone Epidemiology Center Birth Defects Study (BDS) and the National Birth Defects Prevention Study (NBDPS).

Results: Among 7563 mothers of malformed and nonmalformed offspring in the Slone Epidemiology Center Birth Defects Study and 2970 mothers of nonmalformed offspring in the National Birth Defects Prevention Study, acetaminophen, ibuprofen, and pseudoephedrine were used by at least 65%, 18%, and 15%, respectively. Among women in the Slone Epidemiology Center Birth Defects Study, the use in pregnancy of aspirin and chlorpheniramine decreased from 1976 to 2004 and of ibuprofen, pseudoephedrine, diphenhydramine, dextromethorphan, and guaifenesin increased. Among women in the National Birth Defects Prevention Study, the use of acetaminophen, pseudoephedrine, diphenhydramine, and guaifenesin was higher during pregnancy than before pregnancy.

Conclusion: Findings show that over-the-counter medications are used by most pregnant women. Studies that examine specific over-the-counter medications in relation to specific birth defects are necessary to better inform pregnant women about risks and safety.

Ever since the thalidomide tragedy, there has been concern that medication use in pregnancy can cause adverse fetal outcomes. Indeed, other medications have since been identified as teratogens, such as the anticoagulant warfarin,^1^ the anticonvulsant valproic acid,^2^ and the acne medication isotretinoin.^3^ Because each of these medications requires a prescription, the identification of associated fetal effects was facilitated by medical record or pharmacy documentation. For over-the-counter (OTC) medication use in pregnancy, the only confirmed association is between late pregnancy aspirin use and intracranial hemorrhage in the newborn infant.^4^ There is a dearth of studies on OTC drugs, in part because approaches to the study of their potential fetal effects are complicated by the lack of a paper trail. In
granting OTC approval, the US Food and Drug Administration might take available data on pregnancy exposures and fetal outcomes into consideration, but adequate evidence, particularly on safety for specific birth defects, typically is not available. Hence, although the availability of a drug as an OTC product may reflect safety for its use by the nonpregnant population, it does not necessarily extend to safety for use during pregnancy. Nonetheless, the general perception that OTC medications are safe for the general public and therefore safe for pregnant women may have led, in part, to large proportions of pregnant women taking these products. Conversely, this ignorance about the safety of OTCs can raise concerns about risks to the fetus among the many women who take an OTC medication before recognizing that they are pregnant.

We describe the extent of OTC medication use during pregnancy and discuss the potential public health implications.

**Material and methods**

Since 1976, the Boston University Slone Epidemiology Center Birth Defects Study (BDS) has been interviewing the mothers of infants with a range of birth defects. During this time, the study has interviewed >23,000 mothers of offspring with and without birth defects from the greater metropolitan areas of Boston (1976-present), Philadelphia (1977-present), Toronto (1978-present), and San Diego (2001-present). Cases with major structural birth defects are identified from birth, and tertiary care hospitals and control subjects without birth defects from the same catchment areas are identified within 5 months after delivery. Mothers are interviewed by trained nurses within 6 months after delivery in person (1976-mid 1998) or by telephone (mid 1998-present). Standardized questions are asked about demographic, reproductive, and medical factors; behaviors (eg, smoking, alcohol use); diet; and medication use. Information on medication use is obtained by questions on drugs taken for a list of illnesses (eg, cold, flu, cough, sinus infection, or congestion), on categories of medications (eg, nasal sprays), and on selected specifically named medications (eg, Tylenol, Sudafed, Advil, Aleve). For reported medications, women are asked to retrieve the package, if possible. In 1999, a medication identification booklet was introduced, with color images of >350 specific OTC products. Women who reported the use of a cough, cold, or analgesic product were asked to refer to the booklet to help them identify which specific product they had taken. The use of generic forms of medications also were recorded.

The National Birth Defect Prevention Study (NBDBPS) is an on-going population-based case-control study of birth defects. Case subjects with major structural malformations and a random sample of control infants (live births with no major birth defects) whose estimated date of delivery was between November 1997 onward are identified in Arkansas, California, Iowa, Georgia, Massachusetts, New Jersey, New York, North Carolina, Texas, and Utah. Standardized interviews of case and control mothers are conducted by trained nurses within 6 months after delivery in person (1976-mid 1998) or by telephone (mid 1998-present). Standardized questions are asked about demographic, reproductive, and medical factors; behaviors (eg, smoking, alcohol use); diet; and medication use. Information on medication use is obtained by questions on drugs taken for a list of illnesses (eg, cold, flu, cough, sinus infection, or congestion), on categories of medications (eg, nasal sprays), and on selected specifically named medications (eg, Tylenol, Sudafed, Advil, Aleve). For reported medications, women are asked to retrieve the package, if possible. In 1999, a medication identification booklet was introduced, with color images of >350 specific OTC products. Women who reported the use of a cough, cold, or analgesic product were asked to refer to the booklet to help them identify which specific product they had taken. The use of generic forms of medications also were recorded.

### Table

<table>
<thead>
<tr>
<th>Medication</th>
<th>BDS* Pregnancy (%)</th>
<th>NBDBPS Trimester (%)</th>
<th>Prepregnancy (%)</th>
<th>Trimester (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>First</td>
</tr>
<tr>
<td>Analgesic</td>
<td>76.1</td>
<td>70.4</td>
<td>56.9</td>
<td>59.3</td>
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<tr>
<td>Acetaminophen</td>
<td>69.8</td>
<td>65.5</td>
<td>47.6</td>
<td>54.2</td>
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<tr>
<td>Ibuprofen</td>
<td>24.8</td>
<td>18.4</td>
<td>21.1</td>
<td>16.0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>8.0</td>
<td>4.3</td>
<td>4.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Naproxen</td>
<td>4.3</td>
<td>4.0</td>
<td>5.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Decongestant</td>
<td>27.7</td>
<td>16.0</td>
<td>5.8</td>
<td>8.1</td>
</tr>
<tr>
<td>Pseudoephedrine</td>
<td>25.1</td>
<td>15.4</td>
<td>5.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Other decongestant</td>
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<td>0.9</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Antihistamine</td>
<td>14.8</td>
<td>7.5</td>
<td>4.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>4.3</td>
<td>3.0</td>
<td>1.2</td>
<td>1.5</td>
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<tr>
<td>Diphenhydramine</td>
<td>7.8</td>
<td>2.9</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Loratadine</td>
<td>2.9</td>
<td>1.9</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Doxylamine</td>
<td>1.1</td>
<td>1.4</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Brompheniramine</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>.02</td>
</tr>
<tr>
<td>Cough medication</td>
<td>12.9</td>
<td>8.8</td>
<td>2.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Guaifenesin</td>
<td>9.2</td>
<td>6.2</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>8.0</td>
<td>3.4</td>
<td>1.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* A total of 7563 mothers of offspring with and without birth defects were interviewed between 1998 and 2004.

† A total of 2970 mothers of offspring without birth defects whose estimated date of delivery was between 1997 and 2001.

‡ Prepregnancy period is the 3 months before the estimated date of conception.
Figure 1  Secular trends of specific OTC medication use in the first trimester among 20,251 Birth Defects Study mothers of offspring with and without birth defects.
interviewers by telephone within 24 months after the estimated date of delivery about a range of factors that are similar to those in the BDS. Questions are asked about medications that were taken for specific illnesses (eg, cold or flu) and about specifically named products (eg, Tylenol, Advil, Aspirin, Aleve).

For women in both the BDS and the NBDPS, reported products were linked to their active ingredients by the Slone Epidemiology Center Drug Dictionary. This tool allowed us to examine all agents that are typically taken as OTC medications. For example, a report of Tylenol Allergy medication would be considered exposure to acetaminophen, pseudoephedrine, and chlorpheniramine, which are its 3 active components. We included all exposures to all agents that are available OTC, even when such an agent may have been prescribed. Information on dose was not collected. In the BDS, pregnancy is defined as the period beginning with the last menstrual period and ending at delivery. In the NBDPS, pregnancy is defined as the period beginning 2 weeks after the last menstrual period and ending at delivery. BDS data were restricted to participants from the Boston and Philadelphia centers because, in Canada, overall use tends to be less common and different products are available and because San Diego became part of the study only recently (2001). Mothers of offspring with and without birth defects are combined for this analysis, because rates of use of specific medications were not appreciably different between the 2 groups. For an examination of recent rates of medication use during pregnancy, data on 7563 BDS participants who were interviewed between 1998 and 2004 were included. For an examination of secular trends of medication use, data on 20,251 BDS participants who were interviewed between 1976 and 2004 were included. NBDPS data were restricted to Arkansas, California, Iowa, Georgia, Massachusetts, New Jersey, New York, and Texas because North Carolina and Utah became part of the study only recently (2003). Interviews of 2970 mothers of non-malformed live births who were delivered between October 1997 and June 2001 were included for this analysis. For both studies, OTC medications excluded vitamin, mineral, and herbal products.

Results

In the 1998 to 2004 BDS data, the top 10 medications that were taken in pregnancy, in rank order, were acetaminophen, ibuprofen, pseudoephedrine, aspirin,
naproxen, diphenhydramine, guaifenesin, albuterol, amoxicillin, and dextromethorphan. In the 1997 to 2001 NBDPS data, the corresponding medications were acetaminophen, ibuprofen, antibiotics (not otherwise specified), amoxicillin, pseudoephedrine, naproxen, guaifenesin, aspirin, diphenhydramine, and chlorpheniramine. In both the BDS and NBDPS, 8 of the top 10 products were available OTC. We compared the prevalence of use in pregnancy of OTC categories and specific agents for overlapping periods of the BDS and NBDPS (Table). In both studies, acetaminophen was the most commonly taken product, with at least 65.5% of women taking it at some point during pregnancy. Ibuprofen and pseudoephedrine were the next most commonly used products, with at least 15% of women exposed in pregnancy. Aspirin and naproxen were used less commonly, but they were nonetheless used by at least 4% of women. Cough medicines and antihistamines were used by at least 7% of women.

Also included in Table is OTC medication use among NBDPS women by trimesters of pregnancy and the 3-month period before the onset of pregnancy. Among analgesic products, there was a 5% decrease in ibuprofen use and a 2% decrease in naproxen use between prepregnancy and the first trimester of pregnancy. These decreases in use were countered by a 7% increase in the use of acetaminophen during the same time frame. The use of all 4 analgesic agents (acetaminophen, ibuprofen, naproxen, and aspirin) decreased from the first to the second and third trimesters. Among the cold, allergy, and cough medications, pseudoephedrine and guaifenesin use increased from prepregnancy to the second trimester, then decreased in the third trimester. With the exception of diphenhydramine, specific antihistamines showed minor decreases across trimesters, as did the antitussive dextromethorphan. The increase in diphenhydramine use from 1.0% during the prepregnancy interval to 1.8% in the third trimester was primarily due to the use of Benadryl.

BDS data reveal secular trends of selected specific OTC products for a 28-year period (Figure 1). As aspirin use decreased during the 1980s, acetaminophen use increased; as acetaminophen use leveled off in the 1990s, ibuprofen use increased after it became available OTC (1984), followed by an increase in naproxen use after it became available OTC (1994). Pseudoephedrine use increased up until the early 1990s, when it stabilized at prevalences of 15% to 18%. Among antihistamines, chlorpheniramine use has decreased, while diphenhydramine use has increased. Also, the use of loratadine, a
nonsedating antihistamine, increased from 0.2% when it first became available by prescription to 3.7% in 2003 after it became available OTC. Both guaifenesin and dextromethorphan use also increased over the past 2 decades. Ranitidine also increased in use from 0.6% in 1996 to 2000 to 1.6% in 2001 to 2004, after OTC availability in 1995 (data not presented).

We examined demographic and regional patterns of OTC medication use in the NBDPS data. In Figure 2, rates of analgesic, decongestant, and antihistamine use in pregnancy are presented for categories of maternal race or ethnicity, years of education, and age. Rates of analgesic and decongestant use were higher for white women, women with at least a high school education, and women who were at least 20 years of age. Antihistamine use showed the same pattern for maternal education and age, but the rates were similar for white, black, and Asian American women and lower for Hispanic women.

We adjusted the rates of medication use by standardizing each state to the racial or ethnic, age, and education distributions of all states combined. Figure 3 shows adjusted rates of analgesic, decongestant, and antihistamine use by state. Adjustment for these 3 demographic factors accounted for much of the variability of analgesic and antihistamine use across states, whereas the adjusted rates for decongestant use showed more variability.

Comment

OTC medication use during pregnancy is extremely common, as observed in the present findings and in several earlier studies in the United States.6-8 Both the BDS and NBDPS show that approximately two-thirds of women take acetaminophen and that approximately 1 in 6 women takes a decongestant or ibuprofen during pregnancy. Although the use of some medications, such as aspirin and chlorpheniramine, has decreased over the years, most usage has increased during the past 2 decades; ibuprofen, naproxen, diphenhydramine, dextromethorphan, and loratadine have continued to increase in the most recent years. Further, rates of use for acetaminophen, pseudoephedrine, chlorpheniramine, diphenhydramine, doxylamine, and guaifenesin in the first, second, or third trimester of pregnancy are actually higher than during the 3 months before pregnancy. Although such increases in use may be due to actual increases in upper respiratory symptoms during pregnancy, it is more likely that these changes may reflect a more relaxed attitude regarding the use of OTC drugs in pregnancy. Indeed, health care providers and Internet access could be partially responsible for this pattern. Some health care providers supply lists, both as handouts to their patients and on the Internet to the public, of medications that they deem to be safe to take during pregnancy. Interestingly, products that contain acetaminophen (eg, Tylenol), pseudoephedrine (eg, Sudafed), chlorpheniramine (eg, Dristan), diphenhydramine (eg, Benadryl), and guaifenesin (eg, Robitussin) often are included on these lists.9,10 Women might interpret the receipt of a list of “safe” medications as an encouragement for use should symptoms occur, whereas before pregnancy, they might have gone untreated.

The reported use of analgesics, decongestants, and antihistamines was higher for white, non-Hispanic women, women with more than a high school education, and women who were at least 20 years of age. Similar demographic patterns were observed in 2 separate studies that were conducted in the eastern United States in the 1980s.6,7

When these demographic factors were taken into account, the rates of analgesic and antihistamine use were similar across the states. For decongestants, the adjusted rates were lowest for the 3 northeastern states (Massachusetts, New York, and New Jersey).

For reported use of OTC medication during the first trimester, prevalences in the NBDPS are generally similar to those in a study of women who were delivered in 1995 and were interviewed within 96 hours of delivery.8 However, the reported uses of some medications were lower in the third trimester in the NBDPS, possibly because of the longer interval between pregnancy and interview. Differences in data collection methods might also account for the slightly higher prevalences of specific OTC products any time in pregnancy in the BDS rather than in the NBDPS. For example, at the beginning of the interview, the NBDPS asks questions about occurrences of cold and flu and the medications taken to treat them, whereas the BDS asks questions about illnesses and medication use toward the end of the interview. The reporting of cough, cold, or allergy medications might be more accurate in the NBDPS relative to the BDS because some women may grow weary of reporting exposures toward the end of the lengthy interview. Conversely, BDS interviews are conducted closer to the time of delivery and include more prompts for specific OTC products. Also, a medication identification booklet is used to help women to identify the product that was taken in a product line, which results in, for example, a larger proportion of acetaminophen-exposed women reporting the use of a combination product in the BDS (23%) than in the NBDPS (12%). The enhanced reporting of combination products results in more exposures to pseudoephedrine, antihistamines, guaifenesin, and dextromethorphan, which are available in various combinations with acetaminophen in a single product.

The potential for inaccurate recall is problematic in retrospective studies. For OTC medications, accurate recall can be more difficult because their use during pregnancy tends to be viewed more casually than does
the use of prescription drugs, the use tends to be of shorter duration than for prescription products, and the use tends to be “as needed” rather than on a particular schedule, which makes it more difficult to recall the precise exposure period. Recall of OTC medication use can be enhanced by prompting in multiple ways within the interview, such as asking for products by indication and by product name. Women can be asked to retrieve the medication package if they still have it, so that the exact name can be recorded.

Many OTC products are marketed according to the symptoms they treat and frequently contain more than 1 active ingredient (for example, TYLENOL PM, TYLENOL Allergy & Sinus, TYLENOL Cold and Flu, and TYLENOL Cough), each contain acetaminophen in combination with various antihistamines, decongestants, and anti-tussives). One way to help women recall the exact products that were taken is to ask questions about or provide pictures of specific products.

Specialized studies are necessary to generate information about the risks and safety of OTC medication use during pregnancy. Structural birth defects include many types of malformations that are heterogeneous with respect to their cause. Because known teratogenic medications tend to affect the development of specific types of birth defects rather than birth defects overall, it is important to assess the use of specific medications in relation to specific outcomes. For example, isotretinoin embryopathy includes certain types of characteristic brain, ear, and heart defects, and valproic acid increases the risk of neural tube defects (NTDs). If OTC medications were high-risk teratogens (like thalidomide or isotretinoin), they would most likely have come to attention. However, without careful and directed study, it is possible that smaller risks of specific defects are going undetected. From a public health perspective, it is useful to compare the impact of a teratogenic prescription drug with a putative teratogenic OTC drug. If valproic acid increases the risk of NTDs approximately 10-fold and 1% of pregnancies are exposed, the drug would be responsible for an estimated 360 NTD cases in the United States annually. For comparison, consider a hypothetic OTC drug that is taken by 20% of women and that increases NTD risk only 2-fold; although the risk is lower than that of valproic acid, its far greater prevalence of use would result in that OTC drug being responsible for approximately 800 NTD cases per year. The common use of OTC medications in pregnancy necessitates further studies to establish safety or to identify risks.

In conclusion, because most pregnant women ingest at least 1 OTC medication, it is imperative that we obtain empiric evidence of whether such exposures are safe. The methodologic challenges to the study of OTC medications in pregnancy must be overcome so that the risks and safety of specific products can be identified, critical information can be provided to drug manufacturers and public health authorities and, most importantly, both clinicians and women can be allowed to make more informed treatment decisions. Further, when risks are identified, that new knowledge can lead to insights into causal pathways.

Acknowledgments

We thank the Centers for Birth Defects Research and Prevention in Arkansas, California, Georgia, Iowa, Massachusetts, New Jersey, New York, and Texas for their data; Kathy Kelley, Research Pharmacist at Slone Epidemiology Center, for her assistance with drug classification; and the mothers who participated in the BDS or NBDPS.

References

Comparison of the TDx-FLM II and lecithin to sphingomyelin ratio assays in predicting fetal lung maturity

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Objective: We evaluated the usefulness of the TDx-FLM II and lecithin to sphingomyelin (L/S) ratio assays in predicting fetal lung maturity.

Study design: We retrospectively reviewed 218 consecutive paired TDx-FLM II and L/S ratio results. Women who delivered viable infants within 72 hours of amniotic fluid collection (n = 109) were included in the analysis of sensitivity and specificity. Concordance between tests was determined for all women tested during the study period, and in the subset of women who delivered viable infants within 72 hours of amniotic fluid collection.

Results: There were 9 respiratory distress syndrome (RDS)-affected infants born during the study period. Both the TDx-FLM II and L/S ratios had 100% sensitivity in detecting RDS at their best apparent cut-offs. There was a trend towards increased specificity of the L/S ratio compared with the TDx-FLM II (80% for L/S vs 73% for FLM II). The overall concordance between the TDx-FLM II and L/S ratio was approximately 75%.

Conclusion: The TDx-FLM II and L/S ratios are both sensitive tests for RDS; however, there is not good concordance between the two. The results provide new insight into the optimal use, in sequential or reflex cascade testing, of the TDx-FLM II and L/S ratio.

Respiratory distress syndrome (RDS), or hyaline membrane disease, is a major cause of death in newborns. RDS is caused by insufficient surfactant production by the neonatal lung, and can be predicted in part by laboratory measurements of fetal lung maturity. Several laboratory measurements of fetal lung maturity have been developed, all of which rely on the effect of fetal surfactant phospholipid in changing the biochemical properties of maternal amniotic fluid.

Of the tests for fetal lung maturity, two have emerged as the most common tests performed to assess fetal surfactant production. The lecithin to sphingomyelin (L/S) ratio is considered by many the gold standard for assessing fetal lung maturity; however, this test relies on time-consuming and technically difficult thin layer chromatography. The TDx-FLM II assay by Abbott Laboratories (Abbott Park, Ill) is an automated fluorescence polarization assay that is easy to perform, fast, and quantitatively measures the surfactant phospholipid...
present in amniotic fluid reported as milligrams surfactant present per gram of albumin.\textsuperscript{1-5} Previous studies of the TDx-FLM II have found that the test is a reliable predictor of fetal lung maturity. One recent study found that the TDx-FLM II test, using a cut-off value of 45 mg surfactant per gram albumin, was a better indicator of fetal lung maturity than the L/S ratio.\textsuperscript{6} Another study also suggested that the TDx-FLM test (in this case the first generation TDx-FLM, not the TDx-FLM II currently) may have better sensitivity in predicting fetal lung maturity than the L/S ratio, but the L/S ratio may have better specificity.\textsuperscript{4} In this context, the sensitivity of a test for fetal lung maturity refers to the probability of an immature result or index in an infant with RDS, while the specificity refers to the probability of a mature result in an infant without RDS. Others have suggested that TDx-FLM II values must be interpreted with regard to the gestational age of the neonate. In one such study, a polytomous regression program for probability estimation was used to combine risks for RDS based on gestational age and TDx-FLM II values. Although the optimal cut-off for detecting RDS (not considering gestational age) was 45 mg surfactant/g albumin, the authors found that the regression model still predicted significant risk for RDS (even with TDx-FLM II values $\geq 45$ mg/g) at gestational ages less than 34 weeks.\textsuperscript{7}

The aims of the present study were to compare the TDx-FLM II and L/S ratio in predicting fetal lung maturity. Because previous similar studies were limited by the small number of RDS-affected infants born, we did not attempt in this study to define the optimal cut-offs of the TDx-FLM II or L/S ratio tests using ROC analysis or other means. Rather, we compared the sensitivity and specificity of the TDx-FLM II test using its best apparent cut-off determined from previous studies (45 mg surfactant/g albumin) with the L/S ratio at cut-offs used commonly in clinical practice (2.0 or 2.5). In addition, we sought to determine the overall concordance between TDx-FLM II and L/S ratio results (ie, how often a mature prediction on one test corresponded to a mature prediction on the other). This is important because laboratories often perform the TDx-FLM II result first, and subsequently reflex an L/S ratio if the result falls outside of a defined range. To date, no study has compared a large series of TDx-FLM II and L/S ratio results. Because 90% of TDx-FLM II orders during the study period were accompanied by an order for L/S ratio, we were able to obtain the first large concordance data set to compare the results of consecutive paired TDx-FLM II and L/S tests. This concordance data will be useful for both laboratories and clinicians in establishing protocols for reflex L/S testing.

### Material and methods

This study was a retrospective evaluation of consecutive physician-ordered TDx-FLM II results that included an order for L/S ratio, collected over a 41-month period between August 2000 and December 2003 at a single site (Sunrise Hospital). Over this period, 90% of all physician orders for TDx-FLM II included an order for L/S ratio. During the study period, 218 paired TDx-FLM II and L/S tests were performed on 207 women. Samples tested included amniotic fluid from amniocentesis on women presenting with signs of preterm labor, vaginal pool samples from women presenting with preterm labor, and samples from asymptomatic women before scheduled cesarean section. Samples visibly contaminated with bilirubin, blood, or meconium were rejected from analysis.

Data from women who delivered infants within 72 hours of paired FLM II and L/S ratio tests ($n = 109$) were included in the analysis of fetal outcomes. The sensitivity and specificity of the FLM II at its apparent best cut-off (45 mg/g) was compared with the L/S ratio at the two cut-offs commonly used in clinical practice (2.0 and 2.5), both including and excluding women who received steroids at the time of amniotic fluid collection or between the time of fluid collection and delivery. Because the number of affected infants (infants with RDS) was expected to be small, we did not design the present study to define best cut-offs for the FLM and L/S ratio tests. Rather, we sought to evaluate the performance of the two tests in our population using best apparent cut-offs based on previous studies and clinical practice.

Infants who were treated with surfactant, or were placed on a ventilator more than 24 hours, or required continuous positive airway pressure for more than 24 hours, were given the diagnosis of RDS, provided that no other congenital anomalies were present that could explain the need for respiratory support. This clinical definition of RDS is identical to that used in the study by Fantz et al\textsuperscript{6} in order to make the data sets comparable. Twin gestations were included in the study but were counted as one data point because both twins had the same outcome in all cases of twin gestation. The study design was within guidelines provided by the Institutional Review Board at Sunrise Hospital.

All TDx-FLM II analysis was performed on two Abbott TDx-FLX analyzers (Abbott Laboratories) at Sunrise Hospital according to manufacturer’s instructions. The L/S ratio was determined by an outside laboratory (Quest Diagnostics, Las Vegas, Nev). The cut-off for fetal lung maturity was a ratio of 2.0 at the beginning of the study period; however, it changed during the study period to its present value of 2.5. Results of L/S ratio were typically available 6 to 8 hours after amniotic fluid collection, whereas TDx-FLM II results were typically available 6 to 8 hours after amniotic fluid collection.
available within 1 to 2 hours after fluid collection. The
distribution of gestational ages at time of amniotic fluid
collection ranged from 30 to 37 weeks. Over half the
women were tested at 34 to 35 weeks gestational age,
while less than 5% were tested at less than 32 weeks.

Statistical analysis was performed using GraphPad
InStat version 3 for Windows 2000 (GraphPad Soft-
ware, San Diego, Calif). Sensitivity refers to the prob-
ability of an immature test result in a patient with RDS.
Specificity refers to the probability of a mature result in
a patient without RDS. The predictive value of a mature
result is the percentage of patients with a mature result
who did not have RDS. The predictive value of an
immature result is the percentage of patients with an
immature result who did have RDS.

Results

A total of 218 paired TDx-FLM II and L/S ratio results
were performed on 207 women over the study period. Of
these 207 women, 109 delivered infants within 72 hours
testing. There were 9 infants who met the criteria for
RDS, and 100 unaffected infants born. All of the women
who delivered RDS-affected infants had TDx-FLM II
values on amniotic fluid less than 45 mg surfactant/g
albumin, and L/S ratios less than 2.0. Thus, the
sensitivity of the TDx-FLM II test at a cut-off of 45
mg/g, and the L/S ratio at a cut-off of 2.0, was 100%
(Table I). The range of TDx-FLM II values among
women who delivered affected infants was 20-38 mg/g,
and the range of L/S ratios was 0.9 to 1.9. The
gestational age, TDx-FLM II, and L/S ratio values of
the RDS-affected infants are listed in Table II.

Among the 100 women who delivered unaffected
infants, a TDx-FLM II value of ≥45 mg/g was obtained
in 73/100 cases (73% specificity). For the L/S ratio, 80/100
women had a value ≥2.0 (80% specificity), while 62/100
women had a value ≥2.5 (62% specificity). This repre-
sents a trend toward increased specificity of the L/S ratio
(at a cut-off of 2.0) vs the TDx-FLM II test (at a cut-off
of 45 mg/g), although the difference in specificity did not
reach statistical significance. The specificity of the L/S
ratio at a cut-off of 2.0 was significantly better than at
a cut-off of 2.5 (P < .05). The sensitivity, specificity,
predictive value of a mature result, and predictive value
of an immature result are listed in Table I.

Excluding women who received antenatal steroids at
the time of amniotic fluid collection or between the time
of amniotic fluid collection and delivery, there were 96
women who delivered infants within 72 hours of
amniotic fluid analysis. Among this population there
were 8 RDS-affected infants born. All 8 women who
delivered RDS-affected infants had TDx-FLM II amni-
otic fluid values less than 45 mg/g, and L/S ratios less
than 2.0 (sensitivity 100%). Among the 88 women who
delivered unaffected infants, 68/88 (77%) had a TDx-
FLM II value on amniotic fluid ≥45 mg/g, while 74/88
(84%) had an L/S ratio ≥2.0.

We also sought to determine the concordance be-
tween the TDx-FLM II test at its apparent best cut-off
(45 mg/g) and the L/S ratio at its apparent best cut-off
(2.0). Among the 109 test results evaluated on women
who delivered infants within 72 hours of TDx-FLM II
and L/S ratio tests, the overall concordance rate
(percentage of the time that a mature result on one
test correlated with a mature test on the other, and
immature results correlated) was 79%. In 7% of samples
tested in this population, the TDx-FLM II predicted
fetal lung maturity (≥45 mg/g), while the L/S ratio
predicted immaturity (<2.0). In 14% of samples tested,
the L/S ratio predicted maturity (≥2.0), while the TDx-
FLM II predicted immaturity (<45 mg/g) (Table III).

Among all 209 samples tested, the overall concor-
dance between the TDx-FLM II (at a cut-off of 45 mg/g)
and the L/S ratio (at a cut-off of 2.0) was 76%. In 4% of
samples tested in this group, the TDx-FLM II test
predicted fetal lung maturity (≥45 mg/g), while the L/S
ratio predicted immaturity (<2.0). In 19% of samples

### Table I

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tbody>
<tr>
<td>TDx-FLM II (≥45 mg/g)</td>
<td>100 (66-100)</td>
<td>73 (63-81)</td>
</tr>
<tr>
<td>L/S ratio (≥2.0)</td>
<td>100 (66-100)</td>
<td>80 (71-87)</td>
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<tr>
<td>L/S ratio (≥2.5)</td>
<td>100 (66-100)</td>
<td>62 (52-71)</td>
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### Table II

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>TDx-FLM II</th>
<th>L/S ratio</th>
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<tr>
<td>32</td>
<td>22.0</td>
<td>1.5:1</td>
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<tr>
<td>33</td>
<td>21.7</td>
<td>1.6:1</td>
</tr>
<tr>
<td>34</td>
<td>22.3</td>
<td>1.6:1</td>
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<td>35</td>
<td>29.3</td>
<td>1.9:1</td>
</tr>
<tr>
<td>36</td>
<td>26.7</td>
<td>1.6:1</td>
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<td>37</td>
<td>26.2</td>
<td>0.9:1</td>
</tr>
<tr>
<td>38</td>
<td>20.5</td>
<td>1.5:1</td>
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<td>39</td>
<td>22.2</td>
<td>1.6:1</td>
</tr>
<tr>
<td>40</td>
<td>38.2</td>
<td>1.5:1</td>
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</table>

### Table III

<table>
<thead>
<tr>
<th>TDx-FLM II</th>
<th>L/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;45 mg/g</td>
<td>&lt;2.0</td>
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</table>

Winn-McMillan and Karon
tested, the L/S ratio predicted maturity (≥2.0), while the TDx-FLM II predicted immaturity (<45 mg/g) (Table IV).

Comment

Three recent studies have examined the utility of the TDx-FLM II assay for the evaluation of fetal lung maturity.6-8 The studies by Fantz et al6 and Kesselman et al8 were retrospective reviews of consecutive TDx-FLM II values from women who delivered viable infants within 72 hours of amniotic fluid collection. Both studies included small numbers of RDS-affected infants (15 in the study by Fantz et al, 7 in the study by Kesselman et al) because of the low prevalence of RDS in the study populations (8.1% and 8.5%, respectively). Fantz et al6 found that no affected infants were born when the TDx-FLM II from maternal amniotic fluid was greater than or equal to 45 mg/g. In the study by Kesselman et al,8 2 RDS-affected infants were born to women whose amniotic fluid TDx-FLM II value was indeterminate (40-55 mg/g), while no affected infants were born when the FLM II value was ≥55 mg/g.

The study by Kaplan et al7 differed from the others in that (1) it was a case-finding study of RDS-affected infants that also had amniotic TDx-FLM II values available, and (2) the results were expressed as a probability model including both FLM II value and gestational age. Because a case-finding method was used in this study, the prevalence of RDS (15%) and number of affected infants (46) were greater than observed in Fantz et al6 and Kesselman et al.8 It should be noted that the majority of women in our study had amniotic fluid collection performed at 34 to 35 weeks gestational age, while less than 5% of women were tested at less than 32 weeks gestational age. Risk for RDS exists at less than 34 weeks gestational age.

In comparing results of various studies, however, a few cautionary notes are necessary. First, studies have used different clinical definitions of RDS, which can affect the prevalence of the disease. We used the same clinical definition of RDS as used in Fantz et al,6 and the prevalence of RDS in our study was nearly identical to that observed in Fantz et al6 and Kesselman et al.8 Second, Kaplan et al7 demonstrated that even with “mature” TDx-FLM II values of ≥45 mg/g, significant risk for RDS exists at less than 34 weeks gestational age. It should be noted that the majority of women in our study had amniotic fluid collection performed at 34 to 35 weeks gestational age, while less than 5% of women were tested at less than 32 weeks gestational age. Gestational age is clearly an important consideration in interpreting the results of the TDx-FLM II and L/S ratio tests.

Our study is the first to compare a full set of corresponding TDx-FLM II and L/S ratio values on women who delivered viable infants within 72 hours of amniotic fluid collection. We did not observe a difference in sensitivity between the TDx-FLM II and L/S ratio at their apparent best cut-offs (both had a sensitivity of 100%). However, we did observe a trend towards increased specificity of the L/S ratio compared to the TDx-FLM II, which did not reach statistical significance. The specificity of the L/S ratio at a cut-off of 2.0 was significantly better than at a cut-off of 2.5 (Table I).

Similar to other studies on the TDx-FLM II test, we were limited by the small number of RDS-affected infants born during the study period. In addition, our study was retrospective so clinician bias in establishing the diagnosis of RDS (based on either TDx-FLM II or L/S results or both) cannot be eliminated. However, our study adds significantly to the available information comparing the performance of the TDx-FLM II and L/S ratio tests.

In comparing results of various studies, however, a few cautionary notes are necessary. First, studies have used different clinical definitions of RDS, which can affect the prevalence of the disease. We used the same clinical definition of RDS as used in Fantz et al,6 and the prevalence of RDS in our study was nearly identical to that observed in Fantz et al6 and Kesselman et al.8 Second, Kaplan et al7 demonstrated that even with “mature” TDx-FLM II values of ≥45 mg/g, significant risk for RDS exists at less than 34 weeks gestational age. It should be noted that the majority of women in our study had amniotic fluid collection performed at 34 to 35 weeks gestational age, while less than 5% of women were tested at less than 32 weeks gestational age. Gestational age is clearly an important consideration in interpreting the results of the TDx-FLM II and L/S ratio tests.7,9

Our study is the first to evaluate the concordance between the TDx-FLM II and L/S ratio tests using a large series of consecutive samples. There was concordance of results (mature or immature prediction on both tests) on 79% of tests performed on women who delivered within 72 hours of amniotic fluid collection, and on 76% of results overall. The most common

<table>
<thead>
<tr>
<th>Table III</th>
<th>Concordance between TDx-FLM II and L/S results in women who delivered infants within 72 hours of amniotic fluid collection</th>
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</thead>
<tbody>
<tr>
<td>Number (%) in each category</td>
<td>FLM II &lt; 45 mg/g</td>
</tr>
<tr>
<td>L/S ≥2.0</td>
<td>15 (14%)</td>
</tr>
<tr>
<td>L/S &lt;2.0</td>
<td>21 (19%)</td>
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</tbody>
</table>

<table>
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<tr>
<th>Table IV</th>
<th>Concordance between TDx-FLM II and L/S results in all women who had both FLM II and L/S results performed (represents 90% of FLM II tests performed during study period)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%) in each category</td>
<td>FLM II &lt; 45 mg/g</td>
</tr>
<tr>
<td>L/S ≥2.0</td>
<td>42 (19%)</td>
</tr>
<tr>
<td>L/S &lt;2.0</td>
<td>71 (33%)</td>
</tr>
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</table>
A discrepant result was for the TDx-FLM II to predict fetal lung immaturity, while the L/S test predicted fetal lung maturity. Given that in most situations the TDx-FLM II result will be available first, our results suggest that there is value in performing the L/S ratio when the results of the TDx-FLM II suggest fetal lung immaturity. This conclusion is based on the available data (including ours), which suggest equal or better specificity of the L/S test compared with the TDx-FLM II. Our data do not support a role for reflex L/S testing when the TDx-FLM II suggests fetal lung maturity. This conclusion is based on the available data suggesting equal sensitivity between the 2 tests, and the relatively uncommon finding (4%-7% of samples, Tables III and IV) of an immature L/S result occurring with a mature TDx-FLM II result. Because of the limitations inherent in all studies of this type (small number of RDS-affected infants), further studies are necessary to determine the optimal use of the TDx-FLM II and L/S ratio tests.

References


Increasing maternal parity predicts neonatal adiposity: Pune Maternal Nutrition Study

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Objective: This study was undertaken to study the effect of parity on maternal and neonatal characteristics.

Study design: Maternal anthropometry, diet, micronutrient status, biochemistry, and physical activity were measured during pregnancy and detailed neonatal size recorded in 770 pregnancies in rural Maharashtra, India.

Results: Increasing parity was associated with larger offspring birth weight, skinfold thicknesses, and abdominal circumference, but not head circumference and length. Compared with primiparous women, multiparous women were older, less adipose, and more physically active but had similar education, socioeconomic status, nutritional intake, and weight gain during pregnancy. They had lower circulating concentrations of hemoglobin, albumin, ferritin, glucose, and insulin and lower total leucocyte counts at 18 and 28 weeks’ gestation. There was no difference in their husbands’ body size. The relationship between maternal parity and neonatal weight and adiposity was significant independent of the difference in maternal characteristics.

Conclusion: Increasing maternal parity predicts increasing adiposity in the newborn infant. This may result from maternal nutritional, cardiovascular, or immunologic factors.

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Material and methods

The PMNS methodology has been described previously.4-6 In brief, 2675 married women of child-bearing age (excluding sterilized couples), living in 6 villages (total population 35,000) were identified by a house-to-house survey. Of these, 2466 women agreed to take part in the study. The majority were illiterate, belonged to subsistence farming families, and worked on the farm as well as doing household work. Field workers visited the women monthly to record their menstrual dates, and as doing household work. Field workers visited the women monthly to record their menstrual dates, and every 3 months to record anthropometry (weight, height, waist and hip circumferences, and head circumference; and triceps, biceps, subcapular, and subscapular skinfold thicknesses). Maternal prepregnant fat mass was calculated by using the 4 skinfolds.7 Women who missed 2 successive periods were examined by ultrasound at 15 to 18 weeks to confirm pregnancy and assess gestational age.8 Gestational age was derived from the last menstrual period unless it differed from the sonographic estimate by more than 2 weeks, in which case the latter was used. Women entered the study if a singleton pregnancy of less than 21 weeks’ gestation was confirmed. Enrollment of pregnant women (n = 814) began in June 1994 and ended in April 1996. Their socioeconomic status was assessed with a standardized questionnaire, which derives a composite score based on occupation and education of the head of the household, caste, type of housing, and family ownership of animals, land, and material possession.9 Ethical permission for the study was granted by the KEM Hospital Ethical Committee, and by the local village leaders. All participants gave signed informed consent.

For all pregnant women, additional data were collected in a standardized manner at 18 ± 2 weeks’ and 28 ± 2 weeks’ gestation. These included the anthropometry of the women themselves (same measurements as prepregnancy) and their husbands (weight and height, waist and hip circumferences). A total of 21 trained observers made the measurements over the period of the study, with an average of 11 observers at any given time. A total of 9 interobserver variation (IOV) studies were performed during the study. The median (IQR) coefficient of variation (CV) for triceps skinfold was 8.4% (7.2-11) and for subscapular skinfold 2.7% (1.8-12.1).

Maternal dietary intakes were measured with a semiweighed 24-hour recall method and a food frequency questionnaire.4 Physical activity was assessed with a structured questionnaire that recorded the time spent in a range of activities and derived a total daily score, based on the relative energy expenditure of the different activities.6 Fasting blood samples were taken at both time points in pregnancy; in addition, at 28 weeks’ gestation an oral glucose tolerance test was carried out, with blood samples taken fasting and 120 minutes after a standard 75-g oral glucose load. Hemogram, glucose, lipids, and albumin were measured at Diabetes Research Labora-

tory at KEM Hospital, Pune. Assays for insulin, vitamin C, red cell folate, and ferritin were performed in Southampton and Cambridge, UK. Maternal hemoglobin concentrations and total leucocyte, red blood cell, and platelet counts were measured with a Coulter T450 analyser (Beckman Coulter UK Ltd, Buckinghamshire, UK). Serum albumin and plasma glucose and triglycride concentrations were measured with Randox kits (Randox Laboratories Ltd, Crumlin, Northern Ireland) on an Abbott Spectrum autoanalyzer (Abbott Laboratories, Abbott Park, Ill). Erythrocyte folate and serum ferritin concentrations were measured with radioimmunoassays (Beckton Dickinson UK Ltd, Oxford, UK).4 Serum vitamin C concentrations were measured with an ascorbate oxidase-orthophenylene diamine assay on a Roche Cobas Bio Centrifugal analyzer with fluorescence attachment.4 Plasma insulin concentrations were measured on the Access Immunoassay System (Sanofi Pasteur Diagnostics, Chaska, Minn) with the use of a 1-step chemiluminescent immunoenzymatic assay. Insulin resistance was calculated from fasting glucose and insulin concentrations by using the homeostasis model assessment equation (HOMA).10

The babies were measured by 1 of 3 trained field workers within 72 hours of birth. IOV studies revealed a CV of 9.5% (range 7.5-10.8) for triceps and 7.5% (range 4.0-8.8) for subscapular skinfold. At the time of these measurements, all babies were exclusively breast fed. Birth weight was measured to the nearest 25 g by using a Salter spring balance and crown-heel length to the nearest 0.1 cm using a portable stadiometer (Pedobaby, ETS, JMB, Brussels, Belgium). Triceps and subscapular skinfold thicknesses were measured to the nearest 0.2 mm, on the left side of the body, with Harpenden skinfold calipers (CMS Instruments, London, UK). Occipito-frontal head circumference and midupperarm circumference (MUAC) were measured to the nearest 0.1 cm with fiberglass tapes (CMS Instruments). Abdominal circumference was measured at the level of umbilicus in expiration. Placental weight was recorded to the nearest 5 g with the use of Ishida scales, after trimming off the umbilical cord and membranes.

Effect of seasonality was investigated for maternal and neonatal measurements in a standardized manner: summer (April-July), rainy season (August-November), and winter (December-March).

Statistical methods

Mothers were divided into 3 parity groups, which we define as follows: women who were pregnant for the first (primipara), second (para 2), and third or more times (multipara). Data represented as mean ± SD unless otherwise specified. Variables with skewed distributions (subscapular and triceps skinfold thicknesses, and maternal ferritin and insulin concentrations) were
log transformed to satisfy assumptions of normality. The significance of the mean difference between groups was analyzed by analysis of variance. We also tested for linearity of trend for maternal and offspring characteristics against parity. Multiple linear regression was used to determine the effects of maternal and paternal characteristics on the size of the newborn infants. Analyses were carried out with SPSS for Windows (version 10.0) (SPSS, Chicago, Ill).

Results

Of 814 women with confirmed pregnancies, 10 did not have prepregnant anthropometry recorded, 4 were beyond 21 weeks’ gestation at recruitment, and 3 had major fetal anomalies detected on early ultrasound scan and subsequently terminated the pregnancy. Of the remaining 797 women, 12 spontaneously aborted, 14 underwent a medical termination of pregnancy, and 1 died from pregnancy-induced hypertension. Percentages of women who had spontaneous abortions were similar in all 3 parity groups, but a higher percentage of multiparous women underwent medical termination of pregnancy (n = 10; 3.8%) than primipara (0%) or para 2 (n = 4; 1.5%).

Of the 770 mothers who completed pregnancy, 245 (32%) were primipara, 268 (35%) were para 2, and 257 (33%) were multipara (Table I). Of the 257 multiparous mothers, 167 were para 3, 59 were para 4, 19 were para 5, 8 were para 6, 2 were para 7, and 1 each of parity 8 and 9. Mothers of higher parity were older, but the mean age in the multiparous group was still only 24 years. There were no associations between parity and education level or socioeconomic status. Before pregnancy, women of higher parity were shorter and lighter and had thinner skinfolds and lower fat mass. These associations remained significant after adjusting for maternal age, socioeconomic status, and observer. Maternal head circumference was similar in all parity groups. Paternal weight, height, and body mass index did not vary with maternal parity (data not shown). At 28 weeks’ gestation, women of higher parity had higher physical activity scores. Though physical activity was

<table>
<thead>
<tr>
<th>Table I</th>
<th>Mean (SD) maternal characteristics according to parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
<td>Primipara</td>
</tr>
<tr>
<td>N</td>
<td>245</td>
</tr>
<tr>
<td>Age (y)</td>
<td>18.9 (2.2)</td>
</tr>
<tr>
<td>Illiterate (%), College educated (%)</td>
<td>17.7, 10.7</td>
</tr>
<tr>
<td>Socioeconomic score</td>
<td>27.1 (6.7)</td>
</tr>
</tbody>
</table>

Before pregnancy

| Height (cm) | 152.5 (4.7) | 151.8 (5.2) | 151.3 (4.9) | < .01 | .04 |
| Weight (kg) | 42.3 (4.9) | 41.6 (5.1) | 41.1 (5.0) | < .01 | .04 |
| BMI (kg/m²) | 18.2 (1.9) | 18.0 (1.9) | 17.9 (1.9) | .09 | .06 |
| Head circumference (cm) | 52.1 (1.4) | 52.3 (1.5) | 52.3 (1.5) | .21 | .81 |
| Subscapular skin fold (mm)† | 11.3 (8.9-14.1) | 9.9 (7.7-12.3) | 9.3 (7.5-11.6) | < .001 | < .001 |
| Fat mass (kg) | 9.6 (2.6) | 8.8 (2.7) | 8.4 (2.6) | < .001 | < .001 |

At 28 wks’ gestation

| Physical activity score | 62.1 (25.9) | 64.9 (26.5) | 69.8 (25.4) | < .001 | < .001 |
| Weight gain (kg) | 3.2 (2.0) | 3.7 (2.4) | 3.5 (2.1) | .09 | .06 |
| Energy intake (kcal) | 1678 (485) | 1672 (499) | 1671 (506) | .89 | .71 |
| Hemoglobin (g/L) | 113.0 (16.0) | 112.0 (16.0) | 108.0 (15.0) | < .001 | < .001 |
| Total leucocyte count (×10⁹/L) | 9.6 (2.1) | 9.1 (2.0) | 8.7 (1.8) | < .001 | < .001 |
| Red blood cell count (×10¹²/L) | 3.9 (0.4) | 3.9 (0.5) | 3.8 (0.4) | .47 | .22 |
| Serum albumin (g/L) | 37.0 (3.0) | 36.0 (3.0) | 36.0 (3.0) | < .001 | < .001 |
| Plasma ferritin (pmol/L)† | 29.2 (15.7-53.9) | 22.5 (15.7-40.0) | 22.5 (15.7-43.1) | .84 | < .01 |
| Erythrocyte folate (nmol/L) | 1062.8 (407.9) | 1049.2 (396.6) | 1026.5 (407.9) | .389 | .09 |
| 120-min plasma glucose (mmol/L) | 4.6 (1.2) | 4.4 (1.1) | 4.2 (0.9) | < .01 | < .01 |
| 120-min plasma insulin (pmol/L)† | 95.0 (38.0-148.0) | 77.0 (36.8-143.5) | 60.5 (25.8-107.3) | < .001 | < .001 |
| Insulin resistance (HOMA-R) | 0.59 (0.37-0.87) | 0.55 (0.37-0.88) | 0.50 (0.34-0.75) | .45 | .83 |
| Plasma triglycerides (mmol/L) | 1.5 (0.5) | 1.5 (0.6) | 1.5 (0.4) | .25 | .05 |
| Systolic blood pressure (mm Hg) | 115.3 (9.4) | 112.3 (9.2) | 110.4 (9.0) | < .001 | < .001 |

At birth

| Gestational age (wk) | 38.9 (2.1) | 38.8 (1.9) | 38.8 (1.8) | .67 | .64 |
| Preterm (%) | 13.5 | 9.7 | 12.1 | .40 | — |

P values indicate the significance of linear trend using analysis of variance.

* P after adjusting for maternal age and socioeconomic status.
† Median and interquartile range for log transformed variables.
related to season (highest in winter, least in summer), the parity and physical activity association was unaffected by seasonality. Weight gain during pregnancy, dietary intakes (energy, protein, carbohydrate and fat intakes, and intakes of green leafy vegetables and dairy products) and erythrocyte folate and plasma vitamin C concentrations, insulin resistance, and diastolic blood pressure did not vary with parity. Findings were similar in all parity groups. These findings were similar for seasonality. Ponderal index and abdominal circumference were also larger in babies born to mothers of higher parity, and subscapular and triceps skinfolds increased strongly with increasing parity. A substantial component of this relationship was due to smaller skinfold thickness in babies of primipara women compared with those of multipara women, but there was also a continuous relationship between maternal parity and offspring adiposity. Offspring size, including skinfold thicknesses, were related to gestational age at delivery. However, the relationship between maternal parity and offspring size was unaffected by adjusting for gestation at delivery and observer. Season at birth was not a significant predictor of offspring adiposity, and the adjustment for seasonality did not alter the relationship between maternal parity and offspring adiposity. In contrast, neonatal length, head circumference, and MUAC were similar in all parity groups. These findings were similar for boys and girls, except that MUAC rose with increasing parity in girls, and placental weight was higher in boys but not girls born to mothers of higher parity. These results were unaffected by adjustment for seasonality of delivery and in para 2 and multiparous women for the interpregnancy interval.

Table III shows the relationships of parity, the infant’s sex and gestational age at delivery, and parity-related maternal nutritional and biochemical characteristics to
newborn weight, sum of skinfold thicknesses, and head circumference. Neonatal measurements were strongly related to gestational age at delivery and fetal sex (boys were heavier and had larger head circumferences than girls). As already described, birthweight and neonatal skinfold thickness, but not head circumference, were larger in babies born to mothers of higher parity. Several of the other maternal variables were also related to newborn size. Increased maternal height, larger prepregnant fat mass, and lower hemoglobin and serum albumin concentrations during pregnancy were all associated with a larger newborn size. In a multivariate analysis, which included all maternal variables and which differed in the 3 parity groups and paternal size (height and body mass index), the effects of maternal parity on birthweight and neonatal adiposity were little changed and remained statistically significant.

Comment

In a large population-based study of rural Indian pregnant women, we have shown that increasing maternal parity is associated with increasing offspring adiposity, there was increased neonatal soft tissue but not increased skeletal size (length and head circumference). Multiparous mothers in our study were lighter, thinner, and more physically active than primiparous mothers. They also had lower hemoglobin, serum albumin and plasma glucose, insulin and ferritin concentrations, and lower systolic blood pressure. However, the effect of maternal parity on neonatal adiposity remained significant after adjusting for these characteristics.

Our population was rural and younger than in most other studies; the mean age of the multiparous group was only 24 years compared with 28 or more years elsewhere. The lower adiposity of multiparous mothers in Pune contrasts with other published studies, all of which report higher body mass index and fat mass in multiparous women. Dietary intakes were similar in all parity groups, but multiparous women were more physically active. Their lower adiposity may be a result of a high physical workload associated with both caring for the family and working on the farm, combined with repeated childbearing at a young age. The parity association was independent of socioeconomic status which itself was quite homogeneous for this rural farming population and also independent of seasonality of birth. In contrast to findings in other populations, in which maternal insulin resistance and the risk of diabetes tend to increase with rising parity, multiparous mothers in our study had lower postload plasma glucose and insulin concentrations. This is likely to be because of their lower adiposity and higher levels of physical activity. Women of higher parity had lower blood hemoglobin, plasma albumin, and ferritin concentrations, which may result from greater plasma volume expansion described in multiparous women. Against this, however, was the lack of association between parity

Table III  Multiple linear regression analysis of maternal parity and other maternal characteristics, fetal sex and gestational age, and paternal size, as predictors of birth weight, neonatal subscapular skinfold thickness, and head circumference

<table>
<thead>
<tr>
<th></th>
<th>Birth weight (G)</th>
<th>Sum of neonatal subscapular and triceps skinfolds (mm)</th>
<th>Neonatal head circumference (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Univariate</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>Parity (3 groups)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td>47.42 &lt; .001</td>
<td>48.68 .02</td>
<td>0.27 &lt; .001</td>
</tr>
<tr>
<td>2nd</td>
<td>109.42 &lt; .001</td>
<td>100.05 &lt; .01</td>
<td>0.19 &lt; .01</td>
</tr>
<tr>
<td>1st</td>
<td>−116.94 &lt; .001</td>
<td>−103.25 &lt; .01</td>
<td>0.17 .19</td>
</tr>
<tr>
<td>Socioeconomic score</td>
<td>1.15 .58</td>
<td>3.92 .14</td>
<td>0.004 .67</td>
</tr>
<tr>
<td>Age (y)</td>
<td>12.19 &lt; .01</td>
<td>6.34 .33</td>
<td>0.07 &lt; .001</td>
</tr>
<tr>
<td>Mother’s height (cm)</td>
<td>11.81 &lt; .001</td>
<td>8.88 &lt; .01</td>
<td>−0.005 .97</td>
</tr>
<tr>
<td>Prepregnant fat mass (kg)</td>
<td>26.43 &lt; .001</td>
<td>15.19 .03</td>
<td>0.06 &lt; .01</td>
</tr>
<tr>
<td>Activity score</td>
<td>−1.10 .04</td>
<td>−1.75 .02</td>
<td>0.002 .40</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>−24.49 .02</td>
<td>−31.94 &lt; .01</td>
<td>−0.02 .11</td>
</tr>
<tr>
<td>Leucocyte count (×10^9/L)</td>
<td>−13.83 .09</td>
<td>1.27 .89</td>
<td>−0.02 .67</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>−147.04 &lt; .01</td>
<td>−96.97 .10</td>
<td>−0.78 &lt; .01</td>
</tr>
<tr>
<td>Plasma ferritin (pmol/L)</td>
<td>−2.15 .12</td>
<td>−1.18 .48</td>
<td>−0.01 .08</td>
</tr>
<tr>
<td>120 min glucose (mmol/L)</td>
<td>1.00 .22</td>
<td>0.33 .72</td>
<td>0.007 .04</td>
</tr>
<tr>
<td>120 min insulin (pmol/L)</td>
<td>0.15 .29</td>
<td>0.06 .67</td>
<td>−0.004 .48</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>1.03 .51</td>
<td>0.09 .96</td>
<td>−0.009 .16</td>
</tr>
<tr>
<td>Husband’s height (cm)</td>
<td>4.79 .04</td>
<td>1.87 .51</td>
<td>−0.006 .55</td>
</tr>
<tr>
<td>Husband’s BMI (kg/m²)</td>
<td>16.73 .01</td>
<td>7.56 .25</td>
<td>0.03 .18</td>
</tr>
</tbody>
</table>

BMI, Body mass index.
and other hematologic parameters that would be expected to fall with plasma volume expansion, such as red blood cell and platelet counts. Alternatively, lower hemoglobin, albumin, and ferritin concentrations could indicate poorer nutritional status in the multiparous group, perhaps as a result of successive pregnancies. Finally, this may be a result of reverse causality, larger fetuses extracting more nutrients from the mother. The lower leucocyte count in mothers of higher parity has not, to our knowledge, been reported elsewhere. This could, again, be attributed to greater plasma volume expansion or poorer nutritional status in multiparous women, or possibly a reduced immune response to pregnancy as a result of higher parity.\textsuperscript{17,18} We favor the latter explanation especially because there was no association of parity with red cell and platelet counts.

As reported consistently in many different populations, the babies born to multiparous women in the PMNS were heavier.\textsuperscript{1,3,19,21} There was no demonstrable effect of parity on neonatal length and head circumference, but the offspring of multiparous women had thicker triceps and subscapular skinfolds, and were thus both peripherally and centrally more adipose. The relationship between maternal parity and offspring adiposity was continuous across parity though the difference between babies of primiparous women and the rest was the most striking. Offspring of multiparous women also had larger abdominal circumferences, indicating either greater abdominal adiposity or larger visceral size. Few other studies have examined neonatal size other than birthweight in relation to parity, but the findings appear similar to ours. Among 4206 consecutive live births in Ethiopia, increasing parity predicted larger birth weight but not length and head circumference.\textsuperscript{5} Only a few studies have measured neonatal skinfold thicknesses: both showed, like us, that increasing parity was associated with larger skinfold thickness or fat mass in newborn infants.\textsuperscript{22,23} In the whole population of PMNS mothers, neonatal weight, skinfold thicknesses, and abdominal circumference were strongly positively related to maternal fat mass.\textsuperscript{24} However, maternal fat mass decreased with increasing parity in rural Indian mothers in contrast with other population.\textsuperscript{11,12} Thus, more adipose offspring born to (less adipose) multiparous mothers presents a paradox.

We can only speculate as to the reasons for this increased neonatal adiposity, and as to whether it is an advantage or disadvantage. We have already reported that as a population, the PMNS babies were considerably smaller at birth than white babies born in the United Kingdom, but were relatively adipose.\textsuperscript{5} We suggested that this may have survival advantages in the neonatal period, but could lead to increased obesity in later life.\textsuperscript{25} and thus partly explain the South Asian propensity for high percentage body fat, insulin resistance,\textsuperscript{26-28} and type 2 diabetes.\textsuperscript{29} The increased body fat of babies born to mothers of higher parity could indicate “better” fetal nutrition, perhaps as a result of “better” maternal physiologic adaptations to pregnancy (vasodilatation, plasma volume expansion, and immunologic changes) or to more effective placental transfer of nutrients. Equally, it could indicate fetal “malnutrition,” for example, the fetuses of mothers who are undernourished as a result of successive pregnancies may receive inadequate substrate for skeletal and/or lean mass growth, and thus incorporate more energy into fat stores. The maternal metabolic variables that we measured could be considered proxies for maternal adaptations: vasodilatation (blood pressure), plasma volume expansion (hemoglobin, serum albumin, and plasma ferritin) and immune status (leucocyte count and plasma ferritin), and/or markers of maternal nutritional status: (hemoglobin, serum albumin, and plasma ferritin). The vascular adaptation and nutritional deficiency interpretations would provide opposing explanations of our findings. These measurements, however, did not explain the relationship of parity to birth size, which may be due to the relative crudeness of our measures, or point to some other aspect of parity as the mechanism mediating larger newborn size.

The strengths of our study were that it was population based, monitored mothers from before conception and throughout pregnancy, recorded gestational age accurately, measured maternal nutritional and metabolic status, and recorded detailed neonatal anthropometry. The main limitation was that the data were cross sectional, and hence some unmeasured maternal factors may vary between parity groups. Even in longitudinal studies in the same mothers, it is difficult to separate the effects of parity from other confounders such as advancing maternal age and secular changes in lifestyle and diet.

In summary, the PMNS mothers were multiparous at a younger age than in most other populations. They were lighter, less adipose, and more physically active than primiparous mothers, but did not differ in their dietary intakes or circulating nutrient levels. Their babies were heavier and more centrally and peripherally adipose, independent of maternal and paternal body size, and measures of maternal nutritional and metabolic status. This appears to be therefore a direct effect of parity. More research is required into maternal physiologic changes associated with increasing parity.\textsuperscript{30} Future epidemiologic studies of long-term outcomes should examine the relationship of birth order to adult health outcomes, especially those related to adiposity such as insulin resistance and type 2 diabetes.

Acknowledgments

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References

Misoprostol induces cervical nitric oxide release in pregnant, but not in nonpregnant, women

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KEY WORDS
Cervical ripening
Induction of labor
Nitric oxide
metabolite in cervical fluid
Placebo-controlled
Pregnancy termination
Prostaglandin

Objective: The cells of the human uterine cervix synthesize nitric oxide, which may be a factor in cervical ripening. We studied the effect of misoprostol on cervical nitric oxide release in nonpregnant and pregnant women.

Study design: Seventy-two nonpregnant (n = 15) and pregnant (n = 57; 26 in early pregnancy, 31 in late pregnancy) women were treated with either vaginal misoprostol (n = 54) or vaginal placebo (n = 18). The dose of misoprostol was 400 µg in nonpregnant and early pregnancy group, and 25 µg in late pregnancy group. Serial cervical fluid samples, collected before and up to 3 hours after misoprostol/placebo, were assessed for the concentration of nitric oxide metabolites by means of the Griess reaction.

Results: Placebo had no effect on cervical fluid nitric oxide metabolite level. In 1 to 3 hours, misoprostol induced 4.3- to 5.2-fold elevations in cervical fluid Nox concentrations in early pregnancy (P < .01), and 4.4- to 18.2-fold elevations in late pregnancy (P < .01), but these responses did not differ significantly from each other. Misoprostol had no effect on cervical fluid nitric oxide metabolites in nonpregnant women. There was a trend towards a relationship between cervical nitric oxide stimulation after misoprostol and cervical ripening.

Conclusion: Vaginal misoprostol stimulates cervical nitric oxide release in pregnancy. This suggests a joint action of nitric oxide and prostaglandins in cervical ripening.

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early pregnancy and in induction of labor.15-18 Although it can be administered orally, rectally, or buccally, the vaginal route of administration is favored in clinical routine owing to its superior clinical efficacy and lack of gastrointestinal side effects.16,17 The sensitivity of the cervix to misoprostol is enhanced during pregnancy, and, therefore, smaller doses of misoprostol are given in late pregnancy (around 25–50 mg) than in early pregnancy (around 400–800 mg).16,17 It is not exactly known by which mechanism misoprostol ripens the cervix, but stimulatory effects on MMPs and/or on cytokines in the cervix may be involved.19,20

Nitric oxide is rapidly converted to stable nitrate and nitrite (NOx), the overall level of which in cervical fluid reflects cervical NO release.21,22 We studied here the effect of misoprostol, as used in clinical routine, on cervical NO release in nonpregnant and pregnant women.

Material and methods

With permission of the local committee on ethics, we studied 72 healthy nonpregnant and pregnant women (Table I). Each woman received written and oral information on the purpose and conduct of the study, and written consent was obtained from each of them. The nonpregnant group (n = 15) consisted of healthy women admitted to hospital for tubal sterilization within 5 to 14 days after the cessation of preceding menstruation (follicular phase). They were healthy and did not use intrauterine contraceptive devices or contraceptive pills. The early pregnancy group comprised women (n = 26) who sought termination of pregnancy between 7 and 12 weeks of gestation for socioeconomic reasons. They had not experienced any bleeding or other signs of threatened abortion, and on the basis of findings in transvaginal ultrasonography, pregnancy was viable. The late pregnancy group consisted of women (n = 31) requiring labor induction because of a gestational age of 41 weeks or more (based on early pregnancy ultrasonographic examination) (n = 25), or because of cholestasis of pregnancy (n = 6); the cervixes of these women were unripe (Bishop score <5). No study subject had any signs of cervical infection, abnormal Pap smear, or a positive Chlamydia test result. No pelvic or cervical palpation was allowed within 4 hours before, and during

<table>
<thead>
<tr>
<th>Study group</th>
<th>Nonpregnant</th>
<th>Early pregnancy</th>
<th>Late pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women</td>
<td>15</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Age (y) (range)</td>
<td>39.9 ± 8.4*1 (24–52)</td>
<td>28.9 ± 7.7* (20–43)</td>
<td>31.4 ± 4.2* (23–39)</td>
</tr>
<tr>
<td>Nulliparous n (%)</td>
<td>7 (47%)</td>
<td>10 (38%)</td>
<td>13 (42%)</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>–</td>
<td>8.8 ± 1.8</td>
<td>40.4 ± 1.7</td>
</tr>
<tr>
<td>Nitric oxide metabolite</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable n (%)</td>
<td>14 (93%)</td>
<td>17 (65%)</td>
<td>25 (82%)</td>
</tr>
<tr>
<td>Concentration (μmol/L)</td>
<td>18.6† (15.0–75.2)</td>
<td>11.3† (&lt;3.8–22.0)</td>
<td>52.6† (&lt;3.8–147.4)</td>
</tr>
</tbody>
</table>

*P = .0002.
†P = .0008.
‡P = .01, compared to early pregnancy.
§P = .02, compared to late pregnancy.

Table II The concentrations of nitric oxide metabolites (NOx, μmol/L) in cervical fluid of study groups before and after vaginally administrated misoprostol or placebo (median, 95% CI)

<table>
<thead>
<tr>
<th>Study group</th>
<th>Nonpregnant misoprostol 0.4 mg (n = 15)</th>
<th>Early pregnancy misoprostol 0.4 mg (n = 17)</th>
<th>Late pregnancy misoprostol 0.025 mg (n = 22)</th>
<th>Early and late pregnancy placebo (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 0 min</td>
<td>18.6</td>
<td>15.0-75.2</td>
<td>7.1</td>
<td>&lt;3.8-24.4</td>
</tr>
<tr>
<td>At 60 min</td>
<td>19.2</td>
<td>14.6-58.3</td>
<td>29.9*</td>
<td>17.3-34.8</td>
</tr>
<tr>
<td>At 120 min</td>
<td>22.0</td>
<td>12.9-52.1</td>
<td>24.4*</td>
<td>12.0-36.5</td>
</tr>
<tr>
<td>At 180 min</td>
<td>23.3</td>
<td>13.2-45.9</td>
<td>26.3†</td>
<td>12.8-41.9</td>
</tr>
</tbody>
</table>

All P values compared to group specific level at 0 min.
*P = .002.
†P = .01.
‡P = .003.
§P = .0005.
the first 3 study hours because even gentle palpation may cause a release of cervical NO.\textsuperscript{21}

Misoprostol (Cytotec, Pharmacia Ltd, Morpeth, UK) or placebo (Placebo, pregelatinized starch, 136 mg, cellulose microcrystalline [90 mol/L], 114 mg, Schering, Turku, Finland) was administered to the posterior fornix of the vagina under visual control. The dose of misoprostol was 400 mg in nonpregnant subjects and in the early pregnancy group, and 25 mg in the late pregnancy group; this dosage was selected on the basis of the results of previous trials.\textsuperscript{16-18} The study was conducted as a placebo-controlled trial as far as possible in our clinical setting. The misoprostol group consisted of 15 nonpregnant and 39 pregnant women (17 women in early pregnancy and 22 in late pregnancy), and the placebo group of 18 pregnant women (9 parous women with early pregnancies and 9 women with late pregnancies); no placebo was used in nonpregnant women.

Cervical fluid samples were collected before the application of misoprostol or placebo, and at 1, 2, and 3 hours after its application. This study period was judged to be sufficiently long because after vaginal application, misoprostol reaches its peak level in plasma in 80 minutes.\textsuperscript{23} The samples (288 in all) were collected by introducing a Dacron swab (DuPont, Wilmington, Del) into the cervix under visual control, and keeping it there for precisely 20 seconds, as described previously.\textsuperscript{21,22,24} The swab was then washed in 1.5 mL of physiologic saline for 2 minutes, and the saline samples were kept frozen at \(-21\degree C\) until assayed for NOx by means of the Griess reaction.\textsuperscript{21,22,24} Macroscopically bloody cervical fluid samples (\(n = 12\)) were discarded, leaving 276 samples for analysis. The detection limit of the assay was 3.8 \(\mu\)mol/L, and the intra-assay and interassay coefficients of variation were 1.6% and 2.4%, respectively. To reduce the impact of interassay variation, the samples were assayed in only 7 batches.

Cervical ripening was assessed by one investigator (M V-T). In the nonpregnant and early pregnancy groups, the cervix was judged to be softened if a Hegar dilator of size 7 could be introduced into the cervix without force 3 hours after the application of misoprostol or placebo; otherwise, it was tight. In the late pregnancy group, cervical ripening was assessed by means of Bishop scores at 4 hours before and 3 hours after the application of misoprostol or placebo.

After the study period of 3 hours, the women were treated according to routine principles in our hospital. The nonpregnant group underwent laparoscopic tubal sterilization, and the early pregnancy group vacuum curettage, both under general anesthesia. Women in late pregnancy received 1 to 6 additional doses of...
misoprostol at 4-hour intervals until initiation of labor; after the 3-hour study period, no additional cervical fluid samples were collected.

Categorical data were analyzed by Fisher exact probability test, and repeated measures analysis of variance (ANOVA). Medians with their 95% CIs were used to describe absolute Nox levels. The Nox values were analyzed using nonparametric tests, such as the Mann-Whitney U test, Kruskal-Wallis one-way ANOVA, and Spearman rank correlation tests. All tests were two-sided and carried out using NCSS 2000 software (NCSS, Inc, Kaysville, Utah). Values of \( P < .05 \) were considered statistically significant. A concentration below the detection limit (3.8 \( \mu \text{mol/L} \)) was given an arbitrary value of 3.7 \( \mu \text{mol/L} \). To better describe treatment-induced changes in cervical fluid Nox levels, we also present the Nox data as percentages of pretreatment values.

### Results

On average, the nonpregnant group was older than the pregnant groups (Table I). The baseline cervical fluid Nox level was detectable less often in the early pregnancy group than in the nonpregnant and late pregnancy groups, but the levels were higher in the late pregnancy group than in the nonpregnant or early pregnancy groups (Table I). Taking all subjects into account (n = 72), parous women (median 29.3 \( \mu \text{mol/L} \), 95% CI 15.0–48.9) had higher (\( P = .02 \)) cervical fluid Nox concentrations than nulliparous women (median 18.6 \( \mu \text{mol/L} \), 95% CI <3.8–28.0), whereas in no subgroup was parity a factor regarding Nox. The age of the subject, the duration of amenorrhea, or cholestasis of pregnancy were not determinants regarding Nox levels.

The administration of misoprostol was followed by significant elevations in cervical fluid Nox concentrations in 1 to 3 hours both in early (4.3- to 5.3-fold rise) and in late (4.4- to 18.2-fold rise) pregnancy, but these responses did not differ significantly from each other (Table II, Figure). The response of Nox to misoprostol in women with cholestasis of pregnancy was similar to that in women without it. Nor did the women with post-term pregnancy differ in Nox response to misoprostol from the women with term pregnancy. Parity did not affect the response of Nox to misoprostol in any group. Misoprostol had no effect on cervical fluid Nox concentrations in nonpregnant women (\( P = .68 \)), even if analyzed on a relative scale (Table II, Figure). Placebo had no effect on cervical fluid Nox levels (\( P = .50 \)) when analyzed in all pregnant women (Table II, Figure), or in those in early and late pregnancy separately.

After misoprostol, the cervix was softened in 5 of 15 nonpregnant women (33%), and in 10 of 17 women in early pregnancy (59%) (Table III). Parity was no factor in this regard because 2 of 7 nulliparous (29%) and 3 of 8 parous (38%) women in the nonpregnant group, and 5 of 10 nulliparous (50%) and 5 of 7 parous (71%) women in the early pregnancy group had experienced cervical softening after misoprostol application. However, use of placebo was also associated with a softened cervix (Hegar 7) in 3 of 9 women in early pregnancy (33%). The baseline levels of Nox, and their responses to misoprostol or placebo, showed no statistically significant differences between women with softened or tight cervices (Table III). However, in the early pregnancy group, elevation of Nox levels (median or more) tended to occur more often (\( P = .09 \)) in women with a softened cervix (70%) than with a tight cervix (29%) (Table III).

In the late pregnancy group, elevation of Nox levels following misoprostol was not related to changes in

<table>
<thead>
<tr>
<th>Table III</th>
<th>Changes in the cervical status and in nitric oxide metabolite (Nox) level to misoprostol or placebo in nonpregnant women and in women with early pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Misoprostol 0.4 mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonpregnant (n = 15)</td>
</tr>
<tr>
<td></td>
<td>Tight</td>
</tr>
<tr>
<td>Number of women</td>
<td>10</td>
</tr>
<tr>
<td>Cervical fluid Nox</td>
<td></td>
</tr>
<tr>
<td>Baseline level</td>
<td>17.6</td>
</tr>
<tr>
<td>Median (95%CI)</td>
<td>15.0-75.2</td>
</tr>
<tr>
<td>Change from the baseline at 3 hours (median)</td>
<td>-5</td>
</tr>
<tr>
<td>Women with group specific median Nox response or above (%)</td>
<td>50</td>
</tr>
</tbody>
</table>

* \( P = .09 \).
Bishop score ($P = .33$), but the median cervical fluid NOx concentration per 1 Bishop score rose 4-fold (97.7 vs 24.3; $P = .04$) after misoprostol. The change in cervical fluid NOx levels following misoprostol bore no relationship to the final dose of misoprostol ultimately needed for labor induction, to the interval between sampling and initiation of labor, or to the need of cesarean section (18% in the whole series) or vacuum extraction (9%).

Comment

We show here that cervical NO release is induced by a vaginally administered, routine dose of misoprostol in pregnant, but not in nonpregnant women. Cervical NO release was assessed by the levels of cervical fluid NOx, a method which is reproducible and reflects accurately the availability of NO. Nonpregnant women were slightly older than pregnant women, but because there was no relationship between the age and NOx levels in the present series, this bias in age was no determinant for the differences in NOx levels observed. The NO stimulation, which was relatively similar in early and in late pregnancy, could be accomplished with a much smaller dose (only 6%) of misoprostol in late pregnancy. This effect of misoprostol was specific because placebo had no effect on cervical NO release either in early or in late pregnancy. We could not use placebo in nonpregnant women, but in view of the lack of effect of placebo in the pregnant groups, and the lack of effect of misoprostol in nonpregnant women, a placebo effect on cervical fluid NOx in nonpregnant women seems unlikely. Our data do not allow us to deduce which isofrom of NOS was primarily responsible for misoprostol-induced NO stimulation. However, we believe that it is iNOS, since it is abundantly expressed in cervical cells, and is generally regarded as the most significant NOS in the cervix during pregnancy.

In one of our previous studies, fewer nonpregnant women (46%) showed detectable cervical fluid NOx than in the present work (93%), although we employed the same methods. Biological variation in NOS could be one explanation for this discrepancy, especially because the total number of nonpregnant women studied was modest ($n = 26$). Another explanation, and perhaps a more likely one, could be a different cycle phase at the time of the sampling; the luteal phase in our previous work and the follicular phase in the present study. There is ample evidence of the inhibitory role of progesterone in the release of cervical NO in pregnancy. Our data imply that a lack of progesterone made cervical fluid NOx more often detectable in nonpregnant women in the present study. However, despite the high detection rate, cervical fluid NOx levels failed to respond to misoprostol in 3 hours in these women. We cannot deduce if a higher dose of misoprostol, or a more prolonged study period, would have resulted in a release of NO, but we regard it as unlikely, although cervical ripening after misoprostol may take 10 hours in nonpregnant women.

In contrast to nonpregnant women, cervical NOX concentrations responded to misoprostol by showing significant and consistent rises as early as after 1 hour in women in early pregnancy. Thus, in early pregnancy, an agent appears in the blood, or in the cervix, or in both, which makes cervical NO release responsive to misoprostol. Our data do not allow us to identify this agent, but it may come from the decidua, placenta, corpus luteum, or from all of them. It is unlikely that this agent is progesterone because progesterone inhibits the release of cervical NO in pregnancy, and low progesterone and elevated cervical NOx levels have been observed to precede the onset of clinical abortion in women with failed early pregnancy. Other candidates could be human chorionic gonadotropin (hCG), or inhibins, which appear at high levels during pregnancy, and which have been shown to be involved in the synthesis of NO in various study models. Moreover, it is not known if this factor affects NO synthesis directly, or indirectly, through changes in cytokines and/or other regulators.

The sensitivity of cervical NO release to misoprostol became enhanced from early to late pregnancy because we observed relatively higher and more persistent NOx responses in late pregnancy, although the dose of misoprostol was only 6% of that used in early pregnancy. The causes of increased sensitivity of NO synthesis to misoprostol in late pregnancy are not known, but they may be, in part, the same as those responsible for the appearance of the sensitivity of NO in early pregnancy. A role of hCG in the increased release of NO appears unlikely in late pregnancy because circulating levels of hCG fall with advancing gestation. It can be possible that the number of progesterone receptors in the cervix becomes reduced already before the initiation of labor, and thus, local progesterone insufficiency may ensue. This could be one explanation for the stimulation of cervical NO in late pregnancy, and for its higher sensitivity to misoprostol. We should also note that women with established post-term pregnancy were characterized with reduced cervical NOx levels. Therefore, a possibility exists that these women had a lower NOx capacity than women delivering at term. Against this speculation speaks the fact that there was no relation between the levels/responses of NOx and gestational day, and that women with hepatosis and near-term pregnancy showed similar NOx responses as did women who were past their dates.

There is abundant evidence that prostaglandins can stimulate the release of NO. Our data on the significant elevations in NO release following misoprostol show that this mechanism may also operate in...
the human cervix, perhaps primarily through iNOS. On the other hand, it is also known that NO can induce the expression of cyclo-oxygenase 2,4 and thereby increase the release of endogenous prostaglandins. Thus, misoprostol could perhaps initiate a chain reaction in the cervix of pregnant women; the initial NO stimulation caused by misoprostol is followed by an endogenous release of prostaglandins triggered by NO. Both these responses facilitate the cervical ripening essential for safe delivery.

The significance of misoprostol-induced NO release remains open. However, we found that cervical ripening, as assessed by clinical tests, tended to be more pronounced in the presence of greater NO release. This may imply that NO release, per se, could have potentiated the effect of misoprostol. This is supported by the results of clinical studies showing that NO donors, such as isosorbide mononitrate and glyceryl trinitrate, result in NO release in cervical ripening in early and late pregnancy.5-7 It is also possible that misoprostol (labor)-induced release of cervical NO may not only facilitate cervical opening, but also protect the cervical canal against ascending infections during miscarriage or labor. This speculation is supported by the fact that high amounts of iNOS/NO have been reported to kill bacteria and parasites in various studies.4 A dual role of cervical NO release is physiologically meaningful in women before abortion or labor.

In conclusion, vaginal misoprostol stimulates cervical NO release in pregnancy. This suggests a joint action of NO and prostaglandins in cervical ripening.

Acknowledgments

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N-acetyl-transferase phenotype and risk for preeclampsia

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KEY WORDS
N-acetyltransferase
Biotransformation
Preeclampsia
HELLP syndrome

Objective: This study was undertaken to determine whether the N-acetyltransferase (NAT) phenotype contributes to the susceptibility for the development of preeclampsia.

Study design: The NAT acetylator status was determined by measuring urinary caffeine metabolites in 134 nonpregnant women with a history of preeclampsia and in 109 control women with uncomplicated pregnancy. The χ2 and logistic regression analyses were used for statistical evaluation of differences in acetylator status.

Results: Significantly more fast acetylators were found among the women with a history of preeclampsia (46.3%) than among the controls (25.4%). Fast acetylators showed an odds ratio of 2.5 (95% CI 1.4-4.3) for preeclampsia. No differences in the acetylator status were found between women with a history of preeclampsia only and those with the HELLP syndrome as well.

Conclusion: The fast NAT acetylator status, which may result in altered NAT detoxification capacity, is associated with preeclampsia.

Preeclampsia represents one of the most serious medical disorders complicating human pregnancies. However, factors responsible for the initiation and progression of the disease process remain elusive. There is growing evidence that an imbalance between toxic compounds, such as lipid peroxides and oxygen free radicals, and detoxifying and scavenging substances might contribute to the pathophysiology of preeclampsia.1,4 Many enzymes, including the cytochromes P450,5 glutathione S-transferases,6 epoxide hydrolases,5,7 and N-acetyltransferases (NATs)5 are involved in the metabolism of endogenous and exogenous compounds. Women with an altered enzyme activity for glutathione S-transferase Pl or epoxide hydrolase were shown to have an increased risk for preeclampsia.8,9 NAT has never been studied in this context.

NATs are phase 2 enzymes, and 2 distinct isoenzymes are known: NAT1 and NAT2. Both NAT enzymes are involved in bioactivation (N-acetylation) as well as bioactivation (O- and N,O-acetylation), and use coenzyme A as a cofactor. Reactions catalyzed by NAT generally result in less toxic metabolites (detoxification...
However, several derivatives, notably in concert with oxidative metabolism by cytochrome P450-mediated reactions, are substrates for additional metabolism by NAT and result in more reactive compounds. Known exogenous substrates of NAT isoenzymes include arylamines and arylhydroamines, found in cigarette smoke, whereas NAT2 is involved in the metabolism of caffeine. A slow NAT2 acetylator status has been associated with an increased risk for (smoking-related) lung or bladder cancer and endometriosis, whereas a fast acetylator status has been associated with an increased risk for colon cancer.

The objective of this study was to investigate whether the NAT2 acetylator status, as determined by measurement of caffeine metabolites in urine, was associated with the risk for preeclampsia.

Figure 1  Schematic overview of caffeine metabolism. CYP1A2, Cytochrome P450 1A2; NAT, N-acetyltransferase; XO, xanthine oxidase; ?, unknown substratum.
Material and methods

The study group consisted of 134 nonpregnant women who had been previously admitted for preeclampsia to the antenatal wards or intensive care units of the Radboud University Nijmegen Medical Center (median time interval from delivery to study 13 months, range 3-42), 85 of whom showing the hemolysis elevated liver enzymes, low platelets (HELLP) syndrome as well. Of these 134 women, 48 were also used in a study concerning the role of epoxide hydrolase in preeclampsia.9 Preeclampsia was defined as the occurrence after 20 weeks' gestation of a diastolic blood pressure greater than 90 mm Hg (phase 5 Korotkoff sound) on 2 or more occasions at least 4 hours apart, and concordant proteinuria (urinary protein greater than 0.3 g/L in a 24-hour urine collection period). The HELLP syndrome was defined as the simultaneous occurrence of a platelet count less than \(1 \times 10^9\)/L, serum aspartate aminotransferase and serum alanine aminotransferase concentrations greater than 70 IU/L, and lactic dehydrogenase more than 600 IU/L. A group of 109 nonpregnant women served as controls. They all experienced normotensive pregnancies only (data obtained by questionnaire). Seventy-two of these women were also used as controls in a study comparing the NAT2 acetylator status in women with a child with orofacial cleft or spina bifida in comparison with women having healthy children.15 The median time interval from the last delivery to the moment of study was 14 months (range 9-36). All women were Dutch white and not pregnant or breastfeed- ing at the time of study.

All women filled in a questionnaire at home that was checked for missing or unclear answers by an additional personal interview, and in this way data on number of pregnancies, smoking habits, use of oral contraceptives and education levels were obtained. The education level was categorized as low (lower vocational, intermediate secondary, or intermediate vocational education) or high (higher secondary, higher vocational, or university education).

The institutional review board approved the study and a written informed consent was obtained from each study subject.

Analysis of NAT2 acetylator status was performed by quantifying caffeine metabolites in urine without giving an extra caffeine load, by using a modification of the method as described by Tang et al.16 The NAT2 acetylator status was established by estimating the ratio of the caffeine metabolites 5-acetylamino-6-amino-3-methyluracil (AAMU) and 1-methylxanthine (1X) in urine. As can be seen in the reaction scheme of Figure 1, an unknown metabolite of caffeine is converted by NAT2 into 5-acetylamino-6-formyl-3-methyluracil (AMFU), which is converted in vitro into AAMU, which is quantified. Another pathway, not catalyzed by NAT2, leads to 1X. Therefore, the NAT2 acetylator status can be estimated from the ratio AAMU (NAT catalyzed product)/1X (non-NAT catalyzed product); ie, a high AAMU/1X ratio points at a fast NAT acetylator phenotype.

To determine the NAT2 acetylator status, all women collected a first-void urine sample, which was stored at \(-30^\circ\text{C}\) within 1 hour until analysis. The concentrations of AAMU and 1X in urine were determined by high-performance liquid chromatography (HPLC) (model 590 solvent delivery system coupled with a WISP autosampler/injector and a 440 fixed-wavelength ultraviolet detector) by using a Bio-Gel TSK-20 gel filtration column (Bio-Rad, Mississauga, Canada). Calibration curves were made by adding solutions containing known concentrations of AAMU and 1X to blank urine. Frozen urine samples were warmed up to room temperature and vibrated ultrasonically to dissolve coprecipi- tated 1X. Then, 50 \(\mu\text{L}\) of 0.15 mol/L NaOH was added.
to 50 μL urine to convert AFMU into AAMU. After 10 minutes, 50 μL of 0.15 mol/L HCl and 50 μL of a 0.05% acetic acid solution were added. The samples were vigorously shaken after each step. An aliquot of 20 μL of the mixture was injected, and elution was performed with 0.05% acetic acid at a flow-rate of 1.0 mL/min, and monitored by measuring absorption at 263 nm. The column was washed before each run. Quantification was performed by measuring the area under the curve at retention times belonging to AAMU and 1X. The cutoff point separating slow from fast acetylators was determined by comparison with the calibration curves. Quantitation was performed by measuring the area under the curve at retention times belonging to AAMU and 1X. The cutoff point separating slow from fast acetylators was determined graphically.

The Mann-Whitney U test or χ² test, as appropriate, was used to assess statistical significance of differences in the population characteristics between cases and controls. The difference between groups with respect to slow or fast NAT2 acetylator status was tested with χ² analysis with Yates’ correction in 2 × 2 contingency tables. Women with an increased risk for preeclampsia were assumed to have the fast acetylator status. This risk was estimated by calculating the odds ratio (OR) with 95% CI for fast acetyulators compared with slow acetylators. Furthermore, logistic regression analysis was performed. If necessary, the OR was corrected for confounders (level of education, smoking, and use of oral contraceptives at the time of urine sampling). Statistical significance was taken as P < .05.

### Results

General characteristics of women with a history of preeclampsia and controls are shown in Table I. Smoking habits (19% in cases vs 18% in controls), use of contraceptives (37% in cases vs 42% in controls), and education level (47% low education level and 53% high education level in cases vs 42% and 58% respectively in controls) did not differ between cases and controls. Women in the preeclamptic group delivered at a significantly lower gestational age than the controls (33 [range 26-38] vs 39 [range 36-40]; P < .01). There were significantly more primiparae among the women with a history of preeclampsia than among the controls (79% vs 33%; P < .005).

The distribution of the AAMU/1X ratio in both cases and controls appeared to be bimodal.16,17 The cutoff point separating slow from fast acetylators was graphically determined at a ratio of 2.2 (Figure 2). We found significantly more fast acetylators in the preeclampsia group than in the control group (46.3% vs 25.7%, respectively; P < .005) (Table II). Women with the fast acetylator status had an increased risk of 2.5 (95% CI 1.4-4.3) for preeclampsia. No differences were found in acetylator status between women with a history of preeclampsia who either did or did not concurrently have HELLP syndrome develop (46.9% vs 45.9% fast acetylators, respectively; P = .96).

No association was found between the acetylator status and smoking habits, use of oral contraceptives, or level of education. Although more primipara women were found in the preeclampsia group, the OR did not change when adjusting for number of pregnancies.

### Comment

Metabolism of endogenous and exogenous toxins normally requires phase 1 modification, followed by phase 2 conjugation reactions. Increasing evidence suggests an
important role for biotransformation enzymes in pre-eclampsia.\textsuperscript{2,18,20} The risk for preeclampsia may be modified by variation in individual detoxification activities, which may disturb the balance between phase 1 and 2 enzymes. NAT enzymes are phase 2 enzymes that are involved in both bioinactivation and bioactivation, depending on the substrate.\textsuperscript{8} However, because NATs are involved in both bioactivation as well as inactivation reactions, the use of coenzyme A as a cofactor, the a priori hypothesis that fast acetylators are less prone to develop preeclampsia may be too simple. Several derivatives, notably in concert with oxidative metabolism by cytochrome P450 catalyzed reactions are substrates for additional metabolism by NATs, resulting in more reactive compounds, which subsequently may harm important cellular functions. It is not yet known which reactive intermediates formed by NAT are biologically plausible in terms of increasing susceptibility to pre-eclampsia, but reactive oxygen species such as lipid peroxides might be among them. After now having established an association between preeclampsia and a fast acetylator phenotype, further research should be performed to identify possible relevant NAT substrates or factors involved, which may be relevant for future prevention or treatment of preeclampsia.

In this case-control study we found that women with the fast NAT2 acetylator status were more prone to have preeclampsia develop. Higher acetylation may lead to more bioactivation of certain, yet unknown, compounds leading to an imbalance between toxic compounds and detoxifying and scavenging substances. This finding is in agreement with previous results of our group on impaired detoxification in preeclampsia. We recently showed that polymorphisms in genes encoding for the biotransformation enzymes glutathione S-transferase P1 and epoxide hydrolase, both of which may lead to altered enzyme activity of the corresponding enzyme are associated with preeclampsia.\textsuperscript{8,9} Furthermore, we found reduced amounts of glutathione S-transferase P1 in both placental and decidual tissue of preeclamptic women compared with levels in healthy pregnant controls.\textsuperscript{20}

We determined the NAT2 acetylator status in non-pregnant women with a history of preeclampsia and in women with uncomplicated pregnancies only. As Cascorbi et al\textsuperscript{17} showed a correlation of at least 90\% between the NAT2 acetylator status and the NAT2 genotype, it seems unlikely that pregnancy itself will affect the NAT2 acetylator status.

No association was found between NAT acetylator status and smoking, use of oral contraceptives, age, or education, which was in agreement with other studies.\textsuperscript{14,15,21,22} We did not investigate dietary intake of caffeine, and we determined the NAT2 acetylator status by measuring urinary caffeine metabolites without prior extra caffeine load, because it has been demonstrated earlier by Tang et al\textsuperscript{16} and Le Marchant et al\textsuperscript{14} that the amounts of caffeine intake via coffee, tea, or soft drinks in almost all individuals investigated was sufficient to make a reliable and reproducible estimate of each individual’s NAT phenotype by using the method as also applied by us. Indeed, in all 243 individuals investigated in this study, without prior caffeine load, the urinary levels of caffeine metabolites were high enough to be measured accurately. Levels of caffeine intake do influence the urinary levels of the various caffeine metabolites; however, because changes in the levels of these metabolites, as a result of changes in caffeine load, will follow the same trend, there seems to be hardly any effect on the AAMU/1X ratio.\textsuperscript{14,16,21}

In conclusion, we demonstrated that the fast NAT status is associated with preeclampsia, which suggests that interindividual variations in the metabolism of exogenous or endogenous toxic compounds may influence the risk for preeclampsia. Clinical use of our findings here may be 2-fold. First, it may be wise to avoid an excessive intake of some NAT2 substrates, such as coffee, whereby stimulation of the NAT2 enzyme activity is limited during pregnancy. Second, early screening for the NAT2 acetylator status may be of additional value in the early recognition of individuals at high risk for preeclampsia.

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References


Profound hypotension and associated electrocardiographic changes during prolonged cord occlusion in the near term fetal sheep

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KEY WORDS
Asphyxia
Fetal electrocardiographic monitoring
T/QRS ratio
ST segment
Sheep

Objective: To determine whether the onset of fetal hypotension during profound asphyxia is reflected by alterations in the ratio between the T height, measured from the level of the PQ interval, and the QRS amplitude (T/QRS ratio) and ST waveform.

Study design: Chronically instrumented near-term fetal sheep received complete occlusion of the umbilical cord for either 8 (n = 6) or 15-min (n = 9).

Results: Cord occlusion led to sustained bradycardia and severe acidosis. Mean arterial blood pressure initially rose and then fell to a nadir of 32.6 ± 2.6 mm Hg in the 8-min group and 9.3 ± 1.0 mm Hg in the 15-min group (P < .001). The T/QRS ratio rose initially in parallel with mean arterial blood pressure and then reduced as mean arterial blood pressure fell but remained significantly above baseline. Biphasic ST waveforms during occlusion occurred in only 2 fetuses, but biphasic and negative waveforms occurred during reperfusion in the 15-min group, with a significant rise in troponin T levels (0.58 ± 0.46 versus 0.02 ± 0.01 ng/mL at 6 h, P < .01).

Conclusion: Elevation of the T/QRS ratio does not identify fetal hypotension during severe hypoxia, but abnormal waveforms in the recovery phase may indicate developing cardiac injury.

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of elevation of the ST segment of the fetal electrocardiogram (ECG), typically measured relative to the QRS complex (the ratio between the T height and the QRS amplitude [T/QRS ratio]), is a marker for fetal hypoxia in clinical and experimental studies. However, the relationship between ST waveform changes and the development of fetal compromise is complex. Indeed, the appearance of biphasic and negative ST waveforms between variable decelerations induced by cord occlusion in fetal sheep seems to be more predictive of fetal compromise than the changes in T wave height or shape during decelerations. It is unclear whether it was the extreme brevity of hypotension during each brief variable deceleration, which limited the apparent utility of the ST waveform hypoxic changes in that model, or whether they were related not to hypotension per se but to some other factor such as myocardial injury.

To dissect these factors, we used a model of sustained hypoxia induced by umbilical occlusion in near-term fetal sheep in which responses during initial compensation (hypertension) and later decompensation (hypotension) could be contrasted. This study examined the relationship between ST waveform and T/QRS ratio changes and the development of hypotension. We compared the effects of 8 min of occlusion, which was predicted to cause only mild hypotension, with 15 min of occlusion, which is associated with profound hypotension, on postasphyxial recovery of mean arterial blood pressure (MAP), ST waveform shape changes, and fetal levels of a marker of cardiac injury, troponin T.

Material and methods

Surgery

Romney/Suffolk fetal sheep were instrumented between 119 and 126 days of gestation (term = 147 days) under general anesthesia (2% halothane in O2) using sterile techniques. All procedures were approved by the Animal Ethics Committee of the University of Auckland. Food but not water was withdrawn 18 h before surgery. Ewes were given 5 mL of Streptopen (Procaine penicillin [250,000 IU] and Dihydrostreptomycin [250mg/mL], Pitman-Moore, Wellington, New Zealand) intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by intravenous (IV) injection of Alphaxalone and Alphadolone (3 mg/kg, Schering-Plough Animal Health Ltd, Wellington, New Zealand) and general anesthesia maintained using 2% to 3% halothane in O2. The depth of anesthesia and maternal respiration were constantly monitored by trained anesthetic staff. Under anesthesia a 20-gauge IV catheter was placed in a maternal front leg vein, and the ewes were placed on a constant infusion isotonic saline drip to maintain maternal fluid balance.

Using sterile techniques, catheters were placed in the right femoral artery and vein, left and right brachial artery and right vein, and the amniotic sac. Ultrasound blood flow probes (size 3S, Transonic Systems Inc, Ithaca, NY) were placed around the left femoral artery for measurement of femoral blood flow (FBF). ECG electrodes were placed subcutaneously over the right shoulder and chest at apex level and sewn across the chest to record the fetal ECG. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, Calif). All leads were exteriorized through the maternal flank, and a maternal long saphenous vein was catheterized to provide access for postoperative care and killing of the sheep. Eighty milligrams gentamicin (Rousell, Auckland, New Zealand) was administered into the amniotic sac prior to the closure of the uterus. Amniotic fluid lost during surgery was replaced using normal saline warmed to 37°C.

A period of 4 to 6 days postoperative recovery was allowed before experiments commenced, during which time antibiotics were administered daily for 5 days IV to the ewe (600 mg benzylpenicillin sodium [Crystapen] and 80 mg gentamicin). Fetal catheters were maintained patent by continuous infusion of heparinized saline (40 U.mL⁻¹ at 0.2 mL.hour⁻¹), and the maternal catheter was maintained by daily flushing.

Recordings

Fetal arterial and venous blood pressure, corrected for amniotic fluid pressure (Novatrans II, MX860, Medex Inc, Ohio), heart rate, ECG, and FBF (T208 Ultrasonic Flowmeter, Transonic Systems Inc) were recorded continuously from 24 h before the experiment until 72 h afterward. The MAP signal was collected at 64 Hz and low pass filtered at 30 Hz. The ECG signals were stored to disk using custom software (Labview for Windows, National Instruments Ltd, Austin, Tex). They were analog filtered between 0.05 and 80 Hz and digitized at 512 Hz.

Experimental procedures

Fetuses were randomly assigned to either the 8-min occlusion group (n = 6) or the 15-min occlusion group (n = 9). Fetal asphyxia was induced by a rapid inflation of the umbilical occluder with sterile saline of a defined volume (6-8 mL) known to completely inflate the occluder. The success of occlusion was confirmed by observation of an immediate sharp rise in MAP and a fall in fetal heart rate (FHR) to below 100 beats/min. On completion of the occlusion period, the occluder was deflated over 10 to 15 seconds to prevent excessively rapid changes in the circulating blood volume. When bradycardia persisted for more than 30 seconds or fetal blood pressure did not increase to over 25 mm Hg in
the first 60 seconds after release of occlusion, then a dose of 0.3 mL (0.1 mL/kg estimated weight) of 1:10 000 epinephrine was given by slow IV push.

Fetal arterial blood samples (0.3 mL) were taken at baseline (60 minutes prior to occlusion), after 2 and either 7 minutes (8-minute occlusion group) or 12 minutes (15-minute occlusion group) during occlusion and then 30 minutes and 1, 2, 3, 4, 6, 24, 48, and 72 hours after release of the occluder for pH, blood gas, hematocrit, and hemoglobin determination (845 blood gas analyzer and cooximeter, Ciba-Corning Diagnostics, Mass) and for glucose and lactate measurements (model 2300, YSI, Yellow Springs, Ohio). Samples (0.5 mL) were frozen at −80°C for later measurement of cardiac troponin T values using the Elecsys 2010 immunoassay system (Roche, Boehringer-Mannheim, Germany). On completion of the experiment, the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g IV to the ewe: Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Data analysis and statistics

Off-line analysis of data was performed using customized Labview for Windows (National Instruments Ltd). One-second averages of MAP were calculated for each fetus; these data were used to derive the baseline MAP before and after occlusions, and the minimum MAP at the end of each occlusion. The ECG waveform was averaged with respect to the S wave over 5-second intervals. For each averaged waveform, the ratio between the T height, measured from the level of the PQ interval, and the QRS amplitude was calculated (T/QRS ratio) to give a measure of T-wave amplitude. The raw ECG data for each averaged waveform were visually assessed by an experienced researcher (J. W.) to identify ST waveform shape changes and verify software identification of the T-wave.

The effects of occlusion on time sequence data were evaluated by repeated-measures 1-way analysis of variance (SPSS, version 10, SPSS Inc, Chicago, III). The baseline period was taken as the mean of the hour before occlusion. When an effect of time was found, data were compared with baseline using Dunnett’s post-test. Intergroup comparisons for selected data were performed by analysis of variance. Statistical significance was accepted when \( P < .05 \). Data are mean ± SEM.

Results

Table I shows the number of fetuses, mean survival time, and the range of survival in each group. There were no differences in mean gestational age at surgery in each group or fetal weight at postmortem. All fetuses had normal blood gases and glucose and lactate levels by the standards of our laboratory (Table II). There was a significant postinsult mortality in the 15-minute group but not the 8-minute group. Seven lambs required epinephrine i.v. for fetal resuscitation during reperfusion.

### Table I

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Weight (g)</th>
<th>SEM</th>
<th>Gestation (d)</th>
<th>SEM</th>
<th>Survival (hr)</th>
<th>SEM</th>
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<tr>
<td>8 min</td>
<td>6</td>
<td>2837.8</td>
<td>134.4</td>
<td>122.1</td>
<td>1.2</td>
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<td>15 min</td>
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<td>184.2</td>
<td>124.4</td>
<td>0.4</td>
<td>16.0</td>
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### Table II

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>2 min</th>
<th>7 or 12 min</th>
<th>30 min post</th>
<th>1 h post</th>
<th>2 h post</th>
<th>4 h post</th>
<th>6 h post</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>15 min</td>
<td>7.36 ± 0.01</td>
<td>7.22 ± 0.17</td>
<td>6.93 ± 0.01</td>
<td>7.2 ± 0.02</td>
<td>7.25 ± 0.07</td>
<td>7.20 ± 0.06</td>
<td>7.06 ± 0.10</td>
</tr>
<tr>
<td>PCO₂</td>
<td>8 min</td>
<td>7.38 ± 0.01</td>
<td>7.23 ± 0.04</td>
<td>7 ± 0.01</td>
<td>7.31 ± 0.01</td>
<td>7.36 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.37 ± 0.01</td>
</tr>
<tr>
<td>pO₂</td>
<td>8 min</td>
<td>45.2 ± 2.1</td>
<td>69.9 ± 3.7</td>
<td>116.7 ± 0.2</td>
<td>48.5 ± 2.28</td>
<td>49.0 ± 3.5</td>
<td>50.6 ± 3.0</td>
<td>54.8 ± 1.7</td>
</tr>
<tr>
<td>Lactate</td>
<td>8 min</td>
<td>21.5 ± 0.9</td>
<td>5.9 ± 0.9</td>
<td>8.8 ± 0.6</td>
<td>26.3 ± 1.25</td>
<td>22.6 ± 2.7</td>
<td>20.7 ± 1.5</td>
<td>23.2 ± 2.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>8 min</td>
<td>21.8 ± 1.1</td>
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<td>5.5 ± 0.6</td>
<td>22.8 ± 1.30</td>
<td>21.5 ± 1.6</td>
<td>21.8 ± 1.2</td>
<td>22.1 ± 1.9</td>
</tr>
</tbody>
</table>

* The final blood sample during occlusion was taken at 7 minutes in the 8-minute group and 12 minutes in the 15-minute group.

\( P < .05 \), analysis of variance versus 8-minute group. Data are mean ± SEM.
Three did not respond; 4 showed a brisk response but then progressively deteriorated and died within 15 hours after the end of occlusion.

**Changes in fetal blood gases, glucose, and lactate**

Umbilical cord occlusion was associated with metabolic acidemia, hypoxia, and hypercapnia, which were more severe in the 15-minute group (Table II). Following release of occlusion, the 8-minute group showed normalization of arterial pH values, PO₂, and pCO₂ within 30 minutes after release of the occluder. In contrast, the 15-minute group showed a continuing metabolic acidosis, with a mild but persistent hypercarbia (Table II).

**Hemodynamic changes during umbilical cord occlusion**

Figures 1 and 2 show the changes in fetal MAP, T/QRS ratio, FHR, and FBF during and immediately after umbilical cord occlusion. In both groups MAP initially rose after occlusion and then fell, reaching baseline values after 6.5 ± 0.2 minutes and 6.8 ± 0.2 minutes (N.S.) in the 8- and 15-minute groups, respectively. The nadir of MAP was 32.6 ± 2.6 mm Hg (versus 43.6 ± 1.6 baseline) in the 8-minute group and 9.3 ± 1.0 mm Hg in the 15-minute group (versus 44.2 ± 1.8 baseline). The initial hypertension was associated with profound peripheral vasoconstriction with very low FBF (Figure 2). However, this effect was only temporary: FBF increased again after 5 to 6 minutes, to approximately 25% of baseline values.

**Hemodynamic changes after occlusion**

After release of occlusion, the 8-minute group showed rapid spontaneous recovery of both MAP and FHR (Figures 1 and 2), whereas the 15-minute group showed slow recovery of MAP and FHR. Epinephrine administration was required in 7 of the 9 fetuses in the 15-minute group, starting at a mean of 2.6 minutes after release of occlusion. MAP returned to baseline values in 0.7 ± 0.2 minutes in the 8-minute group and 5.3 ± 0.9 minutes in the 15-minute group.
Figure 3  

A. With the onset of occlusion, most animals showed negative T waves, followed by a rapid inversion toward a positive T wave and progressive ST elevation. B. After failure of compensation, typically the ST segment flattened, with progressive reduction in height and lengthening of the Q-T interval. C. Example of the tombstone-like shape of the ST segment. D. Example of the fork-like shape of the QRS segment. E. Postasphyxial recovery of MAP was associated with a rapid, deep inversion of the T wave, accompanied by initially a negative and then a positive ST-waveform. F. Once MAP had recovered, 4 animals showed ST depression, with a negative T wave, followed by a progressive return to baseline appearance.
minutes in the 15-minute group. In the 8-minute but not the 15-minute group, this was followed by transient hypertension, lasting approximately 10 minutes. The 8-minute group showed immediate overshoot tachycardia after release of occlusion, lasting 2 minutes, followed by transient normalization and then a further transient rise (Figure 2). In contrast, the 15-minute group showed a slower rise, which followed epinephrine in all but 2 cases, and then a longer period of tachycardia.

FBF flow during recovery in the 8-minute group showed a pattern of temporary normalization lasting 4 minutes, followed by a rapid fall to approximately 50% of the baseline values (Figure 2). In contrast, FBF in the 15-minute group showed a much smaller initial increase, which did not reach baseline levels, followed by severe sustained hypoperfusion.

T/QRS ratio and ST waveform changes

The initial T-wave orientation was variable prior to occlusion. However, all fetuses, including those with initially negative T waves, showed rapid T-wave and ST (positive) elevation at the onset of occlusion (Figures 1 and 3, A). Peak values of MAP were reached after 2.5 ± 0.5 minutes versus 1.9 ± 0.1 minutes of occlusion (8- and 15-minute groups, respectively, N.S.), whereas peak values of T/QRS were reached at 2.9 ± 0.6 minutes versus 3.7 ± 0.4 minutes (N.S.). With continued occlusion, the onset of fetal hypotension was accompanied by a fall of T/QRS height back toward normal values (Figure 3, B). In a few cases, negative T waves were seen briefly. However, the average T/QRS ratio remained significantly greater than baseline levels in both groups until the end of occlusion (P < .05, Figure 1).

The ST waveform showed a typical sequence of changes, which is illustrated in Figure 3. With the onset of occlusion, all fetuses in the 2 groups rapidly developed ST elevation. One 15-minute fetus initially showed a tombstone-like shape of ST waveform elevation with heart block (Figure 3, C), which resolved after several minutes. Biphasic ST waveforms were seen only in 1 15-minute fetus, after 14 minutes of occlusion, and in 1 8-minute fetus intermittently (for a total of 225 seconds). In the 15-minute group, the ST waveform elevation gradually resolved toward the end of occlusion, as shown in Figure 3, B.

In the 15-minute group, the degree of ST waveform elevation gradually reduced as the occlusion continued, as shown in Figure 3, B. Three fetuses showed bizarre ST waveform shapes toward the end of the occlusion period, 1 with a tombstone appearance and heart block and 2 with fork-like QRS shapes similar to bundle branch block patterns (Figure 3, D). These fetuses did not respond to epinephrine after release of occlusion and died soon after reperfusion. ST depression was never seen during occlusion.

After occlusion, 1 fetus in the 8-minute group developed biphasic ST waveforms, for 225.0 seconds, whereas the remainder rapidly returned to baseline ST waveform appearances. In the 6 surviving 15-minute group fetuses postasphyxial recovery of MAP was associated with development of deeply negative T-waves and biphasic ST waveforms in 4 fetuses (Figures 3, E and F); all but one had received epinephrine. When seen, biphasic waveforms lasted for 206 ± 71 seconds. ST depression was seen in 2 fetuses (for 6 and 9 minutes, respectively). By 30 minutes after release of the occluder, all fetuses showed positive ST waveforms, and only 1 had a deeply negative T wave.

Troponin T

Postasphyxial levels of troponin T were significantly greater in the 15-minute group than in the 8-minute group. Troponin levels in the 15-minute group rose from 0.02 ± 0.01 ng/mL at baseline to 0.38 ± 0.12 at 3 hours and 0.58 ± 0.21 at 6 hours, (P < .01 versus the 8-minute group). In contrast, there was no significant change in the 8-minute group, with levels of 0.02 ± 0.01 ng/mL at baseline, 0.02 ± 0.01 at 3 hours, and 0.025 ± 0.006 at 6 hours.

Comment

This is the first study to report fetal ECG changes during and after a catastrophic insult. Consistent with 1 previous acute study, 14 15 minutes of cord occlusion in the late-gestation fetal sheep was associated with profound hypotension, requirement for resuscitation with i.v. epinephrine, and a subsequent high mortality. Such unpredictable acute, catastrophic events, such as abruptio placentae or umbilical cord prolapse, continue to be a significant cause of perinatal morbidity.17

Dynamic ECG changes, particularly biphasic or negative ST waveforms, are suggested to reflect anaerobic myocardial metabolism and depletion of myocardial glycogen reserve, augmented by β-adrenergic stimulation.5,18 Therefore, we hypothesized that the profound hypotension would be associated with ST waveform changes during sustained occlusion. Initially, there was a dramatic elevation in ST and T-wave height at a time of good fetal compensation, as demonstrated by the marked hypertension, mediated by increased peripheral vascular resistance. Once MAP began to fall, however, the T/QRS ratio also fell, although it remained mildly elevated on average despite the development of profound metabolic acidosis and hypotension, in part secondary to failure of femoral vasocostriction.19

Thus, the elevation of the T/QRS ratio was greatest at the time of maximal fetal compensation. We speculate that this early maximal elevation corresponds with the time of maximal anaerobic cardiac metabolism, and the
subsequent attenuation of ST waveform height reflects depletion of the key substrate, cardiac glycogen, leading to a progressive reduction in anaerobic cardiac metabolism. These results are consistent with the finding, in studies in fetal lambs of prolonged moderate asphyxia induced by reduced uterine perfusion and of brief repeated cord occlusion, that T/QRS elevation did not indicate the development of fetal compromise. Thus, these data suggest that elevation of the T/QRS ratio in isolation is a marker for anaerobic cardiac metabolism due to fetal hypoxia rather than for the development of hypotension.

There are some data to suggest that the onset of significant fetal acidosis and hypotension may be reflected by the appearance of negative and biphasic ST waveforms, possibly reflecting myocardial ischemia. In the present study, this hypothesis was not confirmed because the majority of fetuses showed a consistent elevation of the ST segment during occlusion, with brief, transient biphasic ST waveforms in only 2 fetuses. In contrast, however, during recovery from asphyxia, biphasic waveforms or ST depression was seen transiently in 5 of the 6 survivors in the 15-minute group (and 1 animal in the 8-minute group). It is likely that these ST waveform changes reflect at least in part the development of myocardial injury, as shown by a marked rise in troponin T values in the 15-minute group. The rise was markedly more than adult normal values of < 0.03 μg/L and was associated with progressive cardiovascular decompensation. Similarly, evidence of reversible cardiac injury associated with hypotension has reported in near-term fetal sheep after repeated umbilical cord occlusion and in newborn infants with hypoxic-ischemic encephalopathy.

In conclusion, in a model of sustained asphyxia, the T/QRS ratio was maximally elevated during the early phase of fetal compensation, with elevated arterial blood pressure and peripheral vasoconstriction. This elevation reduced as compensation failed. This pattern may reflect the timing of maximal cardiac anaerobic metabolism. Abnormal ST waveforms occurred in only a few fetuses and were also not helpful in discriminating the development of fetal hypotension. However, transient biphasic waveforms and ST depression were seen transiently in 5 of the 6 survivors in the 15-minute group (and 1 animal in the 8-minute group). It is likely that these ST waveform changes reflect at least in part the development of myocardial injury, as shown by a marked rise in troponin T values in the 15-minute group. The rise was markedly more than adult normal values of < 0.03 μg/L and was associated with progressive cardiovascular decompensation. Similarly, evidence of reversible cardiac injury associated with hypotension has reported in near-term fetal sheep after repeated umbilical cord occlusion and in newborn infants with hypoxic-ischemic encephalopathy.

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Hyperemesis gravidarum: Epidemiologic findings from a large cohort

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Objective: This study was undertaken to quantify the frequency, clinical course, charges, and outcomes of hyperemesis gravidarum.

Study design: California birth certificate data linked with maternal and neonatal hospital discharge data in 1999 were used (N = 520,739). Hyperemesis was defined by ICD-9 codes. The frequency, estimated charges, and demographic characteristics associated with hyperemesis patients were assessed. Maternal and neonatal perinatal outcomes were compared by maternal hyperemesis status.

Results: Hyperemesis complicated 2,466 of 520,739 births. The average length of stay was 2.6 days and the average charge was $5,932. Singleton hyperemesis infants were smaller (3,255 vs 3,380 g; P < .0001) and more likely to be small for gestational age (29.21% vs 20.8%; P < .0001).

Conclusion: Hyperemesis occurs in 473 of 100,000 live births and is associated with significant charges. Infants of mothers with hyperemesis have lower birth weights and the mothers are more likely to have infants that are small for gestational age.

Nausea and vomiting of pregnancy are a known and common symptom of pregnancy affecting 50% to 90% of pregnant women. Hyperemesis, intractable nausea, and vomiting of pregnancy, is rarer but more serious. Hyperemesis can lead to dehydration, metabolic disturbances, and nutritional deficits requiring hospitalization, and, if left untreated, death. Current estimates of the incidence of hyperemesis are poor, but range from 0.3% to 2% of pregnancies. Most of these estimates are from small cohort studies, many of which were performed in the 1930s through the 1950s. Although there has been 1 population-based study regarding hyperemesis in Sweden using 1970s data, there have been no population-based studies in the United States. Therefore, this study proposes to study the incidence of hospitalization for hyperemesis per 100,000 live births, the demographic characteristics of women hospitalized for hyperemesis, charges for treatment, maternal clinical course, and perinatal outcomes associated with this condition in the United States.

Methods

After obtaining Institution Review Board approval from MetroHealth Medical Center and the Committee for Human Protection in the State of California, we obtained the 1999 patient discharge data (PDD) linked birth cohort data set from the State of California. The
PDD linked data set links birth certificate data with maternal and neonatal hospital discharge records for 9 months before and 12 months after delivery. All live births are recorded in this file, including those that occur outside of a hospital setting. Data are also linked with infant and fetal death certificates. Fetal death certificates are issued when the deceased fetus was greater than 20 weeks gestational age.

We removed records with a high likelihood for data entry error. We removed maternal age younger than 11 or older than 55 years. We removed birth weight less than 100 g or greater than 7000 g and infants delivered after 45 weeks. To ensure that all hyperemesis admissions were during the current pregnancy, we calculated the due date from the gestational age at delivery and the delivery date. From this data, we calculated a conception date, and excluded all admissions before the conception date. We also excluded hyperemesis admissions that were more than 2 weeks after the due date.

Antenatal hospital admissions with the primary diagnosis of hyperemesis were identified from International Classification of Diseases, ninth edition, (ICD-9) codes. All ICD-9 codes for vomiting and malnutrition inside and outside of pregnancy were used to identify patients with hyperemesis, as 1 study has indicated that patients with hyperemesis had more infants under 2500 g (7.8% vs 5.1%; \(P < .0001\)) and under 1500 g (1.54% vs 1.1%; \(P = .04\)) than singleton patients without hyperemesis.

Demographic characteristics of women with and without hyperemesis are seen in Table I. Hyperemesis patients are more likely to be younger and nonwhite. They are less likely to be Hispanic or married. There are no differences in Medicaid coverage, nulliparity, or education level. Hyperemesis patients are more likely to have a multiple gestation and overall more likely to have a cesarean delivery.

The median gestational age for admission for hyperemesis is 1.1 weeks. There were a total of 3245 admissions with the primary diagnosis of hyperemesis. With 2466 patients who had the primary diagnosis of hyperemesis, this means that there were an average of 1.3 admissions per patient. The average length of stay for a hyperemesis admission is 2.6 days. Admissions for hyperemesis were no more likely to be on a weekend than admissions for other conditions (30.3% vs 31%; \(P = .37\)). The average charge for an antenatal hyperemesis admission was $5,932. Hyperemesis patients had slightly more infants anomalies (1.2% without hyperemesis vs 1.7% with hyperemesis \(P = .05\)).

The average age at delivery for singleton with hyperemesis was statistically but not meaningfully decreased (Table II). The average birth weight was decreased in singleton hyperemesis patients. Singleton patients with hyperemesis had more infants under 2500 g (7.8% vs 5.1%; \(P < .0001\)) and under 1500 g (1.54% vs 1.1%; \(P = .04\)) than singleton patients without hyperemesis. In addition, singleton hyperemesis patients had more

Table I: Demographic characteristics of women with and without hyperemesis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with hyperemesis (N = 2433*)</th>
<th>Patients without hyperemesis (N = 518,306)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y) (avg)</td>
<td>27</td>
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<tr>
<td>White (%)</td>
<td>74</td>
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<td>Hispanic (%)</td>
<td>38.6</td>
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<td>High school education (%)</td>
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<tr>
<td>Married (%)</td>
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<tr>
<td>Medicaid (%)</td>
<td>38.3</td>
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<td>.22</td>
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<tr>
<td>Nulliparity (%)</td>
<td>39.1</td>
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<td>.53</td>
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<tr>
<td>Cesarean rate overall (%)</td>
<td>26.2</td>
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<tr>
<td>Nulliparous cesarean rate (%)</td>
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<td>.62</td>
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<td>Multiparous cesarean rate (%)</td>
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<tr>
<td>Multiple gestations (%)</td>
<td>6.7</td>
<td>2.7</td>
<td>&lt;.0001</td>
</tr>
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</table>

* Demographic data were missing on 33 hyperemesis patients.

In 1999 there were 520,739 live births in the State of California that met our entry criteria. There were 2,466 patients who were admitted antenatally with a primary diagnosis of hyperemesis. The rate of hyperemesis was 473 per 100,000 live births. Thirty-three hyperemesis patients did not have matching birth records so that demographic data were not available on those patients. In addition, singleton hyperemesis patients had more infants anomalies (1.2% without hyperemesis vs 1.7% with hyperemesis \(P = .05\)).

Deaths were stratified by gestational age (24-30, 31-36, 37-40, and greater than 41weeks). All comparisons were made with Student t tests and \(\chi^2\) where appropriate. Lastly, diagnoses of hyperemesis infants who died were examined.
SGA infants (29.21 vs 20.8; \( P < .0001 \)) and fewer LGA infants (9.8 vs 11.2; \( P = .04 \)). There were more fetal deaths (0.71 vs 43; \( P = .05 \)), but overall no difference in neonatal death rates. There were slightly more infant deaths in the neonatal period for infants born between 24 and 30 weeks. Pregnancy hospitalization charges were significantly higher.

Multiple gestations with hyperemesis and without hyperemesis showed no difference in gestational age at delivery (Table III). Multiple gestations with hyperemesis have lower birth weights, more low birth weight infants, and higher hospitalization charges for pregnancy. Overall, they did not have higher rates of fetal or neonatal death.

Twenty-seven infants of mothers admitted for hyperemesis died. All but 3 died during the birth admission. Of the 27 infants, 13 were intrauterine demises. Of the 14 liveborn infants who died, 8 were premature and data on gestational age was missing on 1. Three of the liveborn infants were anomalous.

**Comment**

The rate of hyperemesis in pregnancy has been difficult to estimate. The appropriate denominator for this rate is hard to define. Databases of all pregnancies in a population do not exist. From studies of early pregnancy, we know that up to 31% of all conceptions are lost before 24 weeks and that 22% of those are not even clinically recognized. Population databases do exist for live births in the form of birth certificate data. Thus, we present a ratio of hyperemesis to live births rather than a hyperemesis in pregnancy rate.

The numerator for hyperemesis is also difficult to determine. Many women have nausea and vomiting during their pregnancy that is considered normal and is treated as an outpatient.

To capture clinically meaningful hyperemesis, we chose to look at hyperemesis that was severe enough to require hospital admission. We classified patients as having hyperemesis by looking at the primary diagnosis ICD-9 code. We did not describe patients who had a hyperemesis qualifying ICD-9 code as a secondary diagnosis. It is possible that medically complex patients admitted for another reason also had hyperemesis. Thus, our estimate of 473 per 100,000 deliveries should be considered conservative.

Previously published reports of hyperemesis have quoted a rate of 0.3% to 2%. The largest study on hyperemesis was from 1973 to 1981 Swedish registry data. They showed a hyperemesis rate of 0.3%. Our study has similar numbers of cases but needed only 1 year of data. The large number of cases in a short time increases our ability to study hyperemesis admissions without having to account for changes in practice over time. The 2 population-based studies, the Kallen study and ours, show a rate on the lower end of the published range.

Hyperemesis infants are born earlier and weight less, and in singletons, they have higher rates of being SGA.

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Although the 125 g difference between average weights is clinically meaningless, the increased number of SGA infants is more concerning. Fetal death rates are also higher in hyperemesis singletons. However, from this study, we are unable to determine the cause of fetal death or whether these are directly related to hyperemesis or not. Importantly, overall neonatal death rates do not appear different.

Although all live births in California require a birth certificate to be filed, it is likely that at previable gestational ages, data may not be as complete. However, because we were interested in fetal and neonatal deaths, we included all gestational ages less than 45 weeks. Fetal death certificates should include all fetuses that die over 20 weeks; however, it is possible that incomplete data for fetuses less than 20 weeks may affect our results. Thus, caution should be in interpreting the increased fetal death rate among singletons with hyperemesis. Our fetal death data should be hypothesis generating for future studies and it may not be appropriate to counsel patients with these numbers.

Our data did show demographic differences in who is admitted for hyperemesis. Interestingly, insurance did not seem to differ between the groups. This suggests that financial barriers to outpatient management may be less important than other types of barriers. Women with hyperemesis were less likely to be married suggesting that lack of social support at home may contribute to the risk of hospitalization. Future studies that incorporate outpatient and inpatient management of hyperemesis may be better able to discern characteristics that contribute to inpatient versus outpatient management decisions. Physician preference may also play a part in the decision to hospitalize; however, we were not able to assess that from our study design and data sources. To ascertain the true costs of hyperemesis, outpatient costs as well as inpatient costs should be included. Our study only examines inpatient costs and as such underestimates the dollars spent on the treatment of hyperemesis.

Charges for inpatient management of hyperemesis are substantial totaling more than $18 million per year in California alone. California performed 13% of all live births in the United States in 1999. Thus, nationally, we can estimate that approximately $200,000,000 is charged nationally for inpatient management of hyperemesis. In April 2004, the American College of Obstetricians and Gynecologists (ACOG) published guidelines for treating nausea and vomiting of pregnancy. These were evidence-based recommendations for efficacy. However, the first-line drugs recommended are far cheaper than those at the bottom of the algorithm. Charges for hyperemesis after ACOG recommendations should be investigated in the future to see if ACOG publishing recommendations decreases hospitalization charges associated with hyperemesis.

**Conclusion**

Hyperemesis occurs in 473 per 100,000 live births and is associated with significant charges. Infants of mothers with hyperemesis have lower average birth weights and are more likely to be SGA. Future studies should incorporate inpatient as well as outpatient management of hyperemesis to better characterize rates of hyperemesis and the true cost of hyperemesis.

**References**

The involvement of Rho-associated kinases in agonist-dependent contractions of human maternal and placental arteries at term gestation

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KEY WORDS
Human arteries
Rho kinase
Pregnancy

Objective: The purpose of this study was to assess the involvement of rho kinase (ROK) in agonist-dependent contraction of omental, myometrial, and placental arteries of pregnant women at term.

Study design: Wire myography was used to assess if contractions of intact or α-toxin-permeabilized arteries obtained from women at elective cesarean section were influenced by the ROK inhibitor Y-27632.

Results: Western blotting indicated the presence of ROKα in each of the 3 tissue types. In intact human omental, myometrial, or placental arteries, Y-27632 dose-dependently reduced contractions to the thromboxane-mimetic U46619. In permeabilized vessels, U46619 induced substantial Ca2+-sensitization of contraction that was inhibited by Y27632. The phosphatase inhibitor calyculin A induced a Ca2+-sensitization of contraction similar to that of U46619 in permeabilized omental arteries that was unaffected by Y-27632, suggesting that ROK may signal via myosin phosphatase in these vessels.

Conclusion: These results are the first report of the involvement of ROK in the receptor-coupled constriction of intact and permeabilized arteries from pregnant women.

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In human pregnancy many dynamic vascular adaptations occur to accommodate changing physiologic circumstances. Cardiac output and blood volume increase substantially, and alterations in receptor-mediated vascular responsiveness may, at least partly, underlie mechanisms evoked to cope with this.1,2 Moreover, several pregnancy complications, for example, preeclampsia and intrauterine growth restriction, are associated with in vivo increased resistance of the maternal and/or placental circulations, as well as postpartum increased risks of maternal thrombosis and cerebral hemorrhage.3,4
In addition, these pregnancy conditions are linked by enhanced vascular sensitivity to constrictor agents. As such, it becomes crucial to understand the mechanisms regulating agonist-mediated contraction of vascular tissues in normal human pregnancy.

Receptor-mediated contraction of smooth muscle involves an increase in intracellular calcium (Ca$^{2+}$) and subsequent phosphorylation of myosin regulatory light chains (MLC$_{20}$). An additional mechanism for force enhancement exists whereby agonists induce a sensitization of the contractile filaments to the activating calcium. Such Ca$^{2+}$ sensitization of contraction involves a sequelae of signalling events, including activation of heterotrimeric G-proteins, stimulation of the small G-protein rhoA, and subsequent activation of its downstream effector rho-associated kinase (ROK). A major effect reported of ROK is the downstream inactivation of the targeting subunit of myosin phosphatase (MYPT1), although this remains a point of some controversy. Inhibition of MYPT activity would be anticipated to result in a net increase in MLC$_{20}$ phosphorylation, a phenomenon that indeed accompanies Ca$^{2+}$ sensitization of smooth muscle contraction in animals.

In a wide variety of smooth muscles a water-soluble, cell-permeant inhibitor of ROK, Y27632, has been extremely effective in reducing agonist-dependent contractions in vitro of intact and permeabilized preparations. In addition, in various animal models involving hyperstimulation of smooth muscle, such as hypertension, coronary vasospasm, or atherosclerotic lesions, ROK inhibition has successfully ameliorated the complications.

There are relatively fewer studies investigating the role of ROK activation in human blood vessels. In atherosclerotic coronary vessels or internal mammary arteries obtained from bypass operations, or in omental vessels from elderly patients undergoing bowel surgery, Y27632 reduced agonist-dependent contractions. However, there is no information on the involvement of ROK in constriction of human maternal or placental arteries. In human myometrium itself, there are conflicting reports of the actions of ROK. Therefore, we investigated the contractile responses of arteries isolated from normal pregnant woman at term. We investigated arteries dissected from biopsies of omentum, myometrium, and placenta, as this allowed us to observe the contractile responses in arteries of the maternal (omentum and myometrium) and placental circulations. We assessed if ROK was expressed in these vessels, and examined whether the ROK inhibitor Y27632 affected thromboxane-stimulated contractions of intact arteries. We also determined the extent of sensitization at submaximal activating Ca$^{2+}$ in permeabilized human arteries evoked by thromboxane stimulation, or phosphatase inhibition, and investigated if Y27632 inhibited each of these phenomena to obtain information on any mechanism of action of ROK.

### Material and methods

All biopsies were obtained following written informed consent according to local ethical committee guidelines, in compliance with the World Medical Association Declaration of Helsinki. Myometrial and omental biopsies were obtained from women undergoing elective cesarean section at term (37-41 weeks) not in labor. Placental biopsies were obtained from women at term undergoing vaginal or elective cesarean section delivery.

Small arteries were dissected from human biopsies with the aid of a stereomicroscope, and placed in physiological salt solution (PSS) of composition: NaCl 119 mmol/L, KCl 4.7 mmol/L, MgSO$_4$·7H$_2$O 1.2 mmol/L, NaHCO$_3$ 25 mmol/L, KH$_2$PO$_4$ 1.17 mmol/L, K$_2$EDTA 0.03 mmol/L, glucose 5.5 mmol/L, CaCl$_2$·2H$_2$O, 1.6 mmol/L at pH 7.4. Placental arteries were identified from the point of insertion of the umbilical artery, and traced along the surface of the chorial plate. Small arteries (<500 µm) were identified from successive branching, and dissected free from surrounding tissue. Myometrial small arteries were identified where they entered into the myometrial surface of the biopsy and dissected free of smooth muscle/connective tissue. Omental small arteries were selected compared to veins by a visual assessment of their wall to lumen ratio, and their morphology at sites of branching before dissection free from adipose tissue. Some vessel segments (10-20 mg weight) were snap frozen in liquid N$_2$ and stored at −80°C until processed for SDS-PAGE and Western blotting (see below). Other fresh arterial segments were mounted on 240-µm wires in 5 to 7 mL PSS-containing chambers of a Danish Myotechnologies 610M myograph (Danish Myotech, Aarhus, Denmark). Myometrial and omental arteries were normalized as previously described (MyoDaq software version 2.2, Danish Myotech), which allows determination of the vessel stretch to be applied in order to give a tension equivalent to 0.9 of L$_{13.3}$kPa, where L$_{13.3}$kPa is vessel diameter at which the active effective pressure is 13.3 kPa. Placental arteries were normalized to a tension equivalent to 0.9 of L$_{5.1}$kPa, where L$_{5.1}$kPa is vessel diameter at which the active effective pressure is 5.1 kPa. The vessels were equilibrated for 20 minute (at 37°C for intact artery studies and 25°C for permeabilized artery protocols).

### Intact tissue contractile studies

Control 2-minute exposures to high K$^+$ solution (60 mmol/L KCl containing PSS isosmotically substituted for NaCl) were performed to test for contractile viability. Arteries were then constricted to the thromboxane receptor agonist U46619 (0.1 nmol/L-1 µmol/L) for up to 15 minutes and washed in PSS. Vessels were reconstituted to 1 µmol/L U46619 and, after 3 to 4
minutes, exposed to incremental doses of the ROK inhibitor Y27632 (0.1-10 μmol/L).

**Permeabilized arterial contractile studies**

In this set of experiments, PSS-equilibrated arteries were exposed to a mock intracellular relaxing solution (composition: sodium creatine phosphate 10 mmol/L, Na₃ATP 5.2 mmol/L, magnesium methanesulphonate 7.3 mmol/L, potassium methane sulphonate, 74 mmol/L, K₂EGTA 1 mmol/L, buffered to pH 7.1 with 30 mmol/L 7.3 mmol/L, potassium methane sulphonate, 74 mmol/L, Na₂ATP 5.2 mmol/L, magnesium methanesulphonate 10 mmol/L, sodium creatine phosphate 10 mmol/L, and addition of KOH) before permeabilization with α-toxin. The solution is termed because it contains no added CaCl₂. The remaining constituents of the solution mimic an intracellular environment. Vessels were then equilibrated for 10 to 15 minutes in external relaxing solution, which was then aspirated and a 25-μL droplet of activating solution of pCa 6.7, where pCa = −log₁₀[Ca²⁺⁺]. The activating pCa 6.7 solution was supplemented with 500 U/mL α-toxin plus 10 μmol/L A23187 (an ionophore that renders the intracellular stores permeable to Ca²⁺) carefully placed over the vessel. Permeabilization with α-toxin was assumed to be complete when the ensuing constriction had reached a plateau or, in cases of little constriction on permeabilization, up to 30 minutes. The artery was then returned to relaxing solution (pCa 9). The experimental protocol then proceeded as depicted in the raw tracing of Figure 1. The vessel was exposed to activating solution pCa 4.5, and the ensuing constriction monitored. The amplitude of this constriction is measured as a in Figure 1. On return to relaxing pCa 9 solution, the vessel relaxed. Agonist-induced Ca²⁺ sensitization of constriction was then examined as follows: vessels were exposed, in turn, to submaximal activating solution pCa 6.7, solution pCa 6.7 plus 10 μmol/L GTP and GTP pCa 6.7 solution plus 1 μmol/L U46619. The amplitude of the constriction recorded, above that of pCa 6.7/GTP solution, upon the addition of U46619 was noted—this is depicted as b in Figure 1. Ca²⁺ sensitization of constriction was then calculated as the change in tension observed by addition of U46619 to pCa 6.7/GTP solution as a percentage of the change in tension seen to pCa 4.5 solution ((b/a) × 100% in Figure 1). Once the constriction had reached a plateau, the effect of Y27632 (10 μmol/L) on U46619-induced sensitization of contraction (to GTP pCa 6.7 solution) was examined. For each patient, any observed effects of Y27632 addition were compared to a simultaneous time control U46619-constricted vessel from the same patient run in parallel. In some experiments on permeabilized omental arteries, the Ca²⁺-sensitizing action of the phosphatase inhibitor calyculin A (cal A, 2 μmol/L) was examined by addition to GTP pCa 6.7 solution and compared to the effect of 1 μmol/L U46619 in a parallel artery from the same patient. Activating solutions pCa 6.7 and pCa 4.5 contained 10 mmol/L EGTA, and were prepared by addition of the appropriate amount of CaEGTA.²⁷

**SDS-PAGE and Western blotting**

Frozen arterial segments were homogenized at 4°C until completion in clean Duall glass tissue grinders in ~250 μL of homogenization buffer (10% glycerol [v/v], 4% sodium dodecyl sulfate (SDS; w/v), 1% Triton X-100 (v/v), 20 mmol/L MOPS, pH 7.0, 10 mmol/L dithiothreitol, 20 mmol/L α-glycerophosphate, 5.5 μmol/L leupeptin, 5.5 μmol/L pepstatin, 0.0205 TIU/mL aprotinin, 20 μmol/L PMSF). Each homogenate was centrifuged at 13,000g for 5 minutes, the supernatants decanted, and the protein concentration of each sample estimated using Bio-Rad DC Protein Assay reagents (Bio-Rad, Hercules, Calif). Following the addition of ×2 Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 mol/L Tris-HCl, pH 6.8) in a 1:1 ratio, the samples were heated at 100°C for 10 minutes, and subsequently stored at −20°C until use. Tissue homogenates (10-20 μg) were loaded onto a 10% separating polyacrylamide gel (0.1% SDS [w/v], 0.05% ammonium persulfate, 1:2000 TEMED, 0.375 mol/L Tris-HCl, pH 8.8), and electrophoresed in Tris/glycine running buffer at 40 mA/gel for for 35 to 40 minutes. Electrophoretic transfer of proteins from the gels to PVDF membranes was performed at 50 V/gel for 1.5 hours at room temperature (blotting buffer composition: 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, pH 8.3). Blotted membranes (5% dried milk in Tris-buffered saline [TBS], 1 hour) were incubated overnight at 4°C in TBS with 1% dried milk with 1:500 anti-ROKα (BD Transduction Laboratories, Lexington, Ky). Washed membranes were then incubated with 1:2000 goat anti-mouse peroxidase conjugate (CN Biosciences, Nottingham, UK) secondary antibody for 1 hour at room
temperatures, and stained with enhanced chemiluminescence (ECL, Pierce Super Signal, Pierce, Rockford, Ill).

Consumables

Other than those materials mentioned above, chemicals were purchased from Sigma-Aldrich Company, Ltd (Poole, Dorset, UK) except the following: NaCl, CaCl₂, NaHCO₃, KH₂PO₄, K₂EDTA, acetic acid, and methanol were purchased from BDH Laboratory Supplies, (Poole, Dorset, UK), acrylamide/bis acrylamide and filter paper from Bio-Rad Laboratories, Ltd, PVDF membrane from Schleicher & Schuell UK Ltd (London, UK), Super Signal West Pico Chemiluminescent Substrate from Pierce, Ltd, A23187, α-toxin, and Y27632 from CN Biosciences. A 10 mmol/L stock solution of 2000 U/mL. 

Results

The normalized mean diameters of vessels used in this study were 363 ± 29 μm (N = 15 patients, n = 27 vessels), 265 ± 28 μm (N = 8, n = 13), and 216 ± 23 μm (N = 8, n = 10) for, respectively, omental, myometrial, and placental arteries. Western blotting of homogenates of each arterial type from 3 separate women indicated that ROKα was expressed in omental, myometrial, and placental arteries (Figure 2A).

U46619 produced maintained constrictions of similar magnitude in arteries from omental (active effective pressure 12.7 ± 3.1 kPa), myometrial (14.0 ± 3.2 kPa), and placental (12.1 ± 1.0 kPa) vascular beds (Figure 2B to D; MANOVA). The ROK inhibitor Y27632 dose-dependently reduced U46619 constrictions in each vessel type (Figures 2 and 3). At the highest concentration of Y27632 used (10 μmol/L), U46619 constrictions were significantly reduced to 61 ± 6% (N = 8, Figure 3A), 44 ± 8% (N = 4, n = 6, Figure 3B), and 79 ± 5.6% (N = 5, Figure 3C) of maximum in, respectively, omental, myometrial, and placental arteries (MANOVA).

The above experiments suggest that ROK activation contributes to the contractions of intact human arteries. The possible mechanism of action of ROK was further investigated in arteries permeabilized with α-toxin, which retains receptor-coupled signalling mechanisms while allowing the concentration of Ca²⁺ of the bathing solution surrounding the myofilaments to be clamped. After permeabilization with α-toxin, exposure to pCa 6.7 solution plus GTP induced submaximal contractions that were 10 ± 2% (N = 7, n = 19, omental, Figure 4A and B), 16 ± 6% (N = 3, n = 6, myometrial, Figure 4C and D), and 30 ± 11% (N = 3, n = 6, placental, Figure 4E and F) of those observed to pCa 4.5 solution. Subsequent addition of 1 μmol/L U46619 to the pCa 6.7/GTP solution induced further pronounced tonic contractions (Figure 4A to F). The magnitude of the U46619-induced constrictions was not significantly different in omental (active effective pressure 8.8 ± 0.9 kPa), myometrial (10.2 ± 2.1 kPa), and placental (6.8 ± 0.4 kPa) vascular beds (P < .05; ANOVA). The U46619-induced Ca²⁺ sensitization of contraction was 173 ± 47% (N = 7, n = 13, omental), 131 ± 19% (N = 3, n = 6, myometrial), and 54 ± 15% (N = 3, n = 6, placental) of contraction to pCa 4.5 solution. Ten μmol/L Y27632 significantly reduced this Ca²⁺ sensitization of contraction in all 3 artery types (Figure 4B, D, and E). In the presence of Y27632, the U46619-induced Ca²⁺ sensitization of contraction was reduced to 0.68- ± 0.06-fold (N = 7, n = 8, omental, Figure 5A), 0.75- ± 0.04-fold (N = 3, myometrial, Figure 5B), and 0.41- ± 0.02-fold (N = 3, placental, Figure 5C) of that in the absence of ROK inhibitor.

In other smooth muscle tissues, it has been proposed that ROK activation results in a reduction of MYPT activity. Therefore, we sought to establish if direct inhibition of phosphatase activity in permeabilized omental arteries, with application of calyculin A, induced a significant Ca²⁺ sensitization. As illustrated in Figure 6C, application of calyculin A to pCa 6.7/GTP solution induced substantial Ca²⁺ sensitization of contraction that, when compared with U46619-stimulated Ca²⁺ sensitizations in arteries from the same patient, was of a similar magnitude. On average, the U46619-induced Ca²⁺ sensitization of contraction was 256 ± 95% of pCa 4.5, whereas the calyculin A-mediated Ca²⁺ sensitization was 392 ± 217% of pCa 4.5. Furthermore, while 10 μmol/L Y27632 significantly reduced the U46619-induced Ca²⁺ sensitization to 0.57- ± 0.08-fold of that in the absence of ROK inhibitor, similar to reported above, it was without effect on calyculin A-dependent Ca²⁺ sensitization of contraction (N = 3, Figure 6E).

Statistics

Active effective pressure (kPa) was calculated from the wall tension (mN/mm) divided by the internal radius of the vessel (mm). Dose response curves were analyzed by multifactorial analysis of variance (MANOVA) followed by Bonferroni post hoc test to indicate significance between data points. A comparison of U46619-induced arterial vasoconstrictions was performed using one-way ANOVA followed by Bonferroni post hoc test in all other instances, differences between data points were analyzed by unpaired or paired Student t test as appropriate. Significance was indicated by P < .05. Values are quoted as mean ± standard error of the mean, with n = number of samples, and N = number of patients.
Figure 2  ROK expression in human arteries, and inhibition of contractions by the ROK inhibitor Y27632. Upper panel: ROKα expressions in each artery type (A). Labels 1 to 3 refer to arterial samples from separate women. Lower panels: Representative tracings of the tonic constrictions of human omental (B), myometrial (C), and placental (D) arteries to 1 μmol/L U46619 (left traces) together with the inhibitory effect of cumulative doses (0.1-10 μmol/L) of the ROK inhibitor Y27632 (right traces). Parallel lines within traces represent a break in the experimental recordings.
Comment

In this study we report that ROKα is expressed in human omental, myometrial, and placental blood vessels isolated from normal pregnant women at term. Y27632, a pharmacologic inhibitor of ROK, reduces the U46619-induced contractions of each of these 3 artery types, implicating a role of ROK activation in agonist-induced contractions of human adult and fetal arteries of pregnant women. The data presented here from biopsies of normal pregnant women, together with that published previously on other arteries obtained from patients with significant clinical conditions, suggest that ROK stimulation is important in regulating arterial contractility in humans in both physiologic and pathophysiologic circumstances, respectively.

ROK stimulation has been suggested to contribute to agonist-induced Ca\(^{2+}\) sensitization of force in a wide variety of animal smooth muscle tissues. This phenomenon can best be investigated in permeabilized tissues where the intracellular milieu, particularly the concentration of calcium surrounding the myofilaments, can be controlled by the experimenters. In addition, receptor-coupled signaling of these preparations remains intact due to the small pore size created in the plasma membrane by α-toxin. In the α-toxin-permeabilized human omental, myometrial, and placental artery types studied here, where the activating Ca\(^{2+}\) is clamped at a suprabasal submaximal level, we found that U46619 resulted in pronounced Ca\(^{2+}\) sensitizations of force. Furthermore, Y27632 significantly reduced these agonist-mediated Ca\(^{2+}\) sensitizations in all 3 human artery types when compared to time controls in the absence of Y27632. As far as we are aware, this is the first direct demonstration of agonist-mediated Ca\(^{2+}\) sensitization of permeabilized arteries from pregnant women, and that these are inhibited by Y27632. These data, thus, illustrate that arteries of the maternal and placental circulations utilize Ca\(^{2+}\) sensitization pathways for constriction and implicate such mechanisms as a means of governing arterial constriction in pregnancy.

One possible physiologic mechanism whereby ROK regulates Ca\(^{2+}\) sensitization of contraction is by inhibition of MYPT activity. In permeabilized human omental arteries we sought to find out if inhibition of phosphatase activity with calyculin A affected Ca\(^{2+}\) sensitization of force, and if Y27632 could inhibit any such contraction. Calyculin A was found to induce substantial Ca\(^{2+}\) sensitizations of force that were of similar magnitude to that induced in arteries from the same patients by U46619. This indicates that even at submaximal activating Ca\(^{2+}\) there is substantial phosphatase activity in these human arteries, in agreement with that observed previously in animal smooth muscles. Furthermore, Y27632 did not affect the calyculin A-dependent constrictions even though it did reduce by approximately 43% the U46619-induced Ca\(^{2+}\) sensitizations in parallel arteries from the same patients. These experiments, thus, indicate that even at submaximal activating Ca\(^{2+}\) there is substantial phosphatase activity in these human arteries, in agreement with that observed previously in animal smooth muscles. Furthermore, Y27632 did not affect the calyculin A-dependent constrictions even though it did reduce by approximately 43% the U46619-induced Ca\(^{2+}\) sensitizations in parallel arteries from the same patients. These experiments, thus, indicate that a likely mechanistic effect of the ROK inhibitor Y27632 on U46619-dependent contractions of human arteries is an enhancement of myosin phosphatase activity. Future experiments should elucidate if this is as a result of altered MYPT phosphorylation directly, or of an indirect action on the activity of the phosphatase inhibitor protein CPI-17.

In summary, we have reported that, in maternal and placental arteries from pregnant women, the ROK inhibitor Y27632 attenuates agonist-induced contractions...
Figure 4  Representative tracings of α-toxin permeabilized human omental (A), myometrial (C), and placental (E) arteries illustrating Ca\(^{2+}\)-induced constrictions on changing from relaxing pCa 9 solution to maximal activating pCa 4.5 solution, and also the Ca\(^{2+}\)-sensitizing constriction of 1 μmol/L U46619 in GTP pCa 6.7 submaximal activating solution. Ten μmol/L Y27632 significantly reduced the U46619-induced Ca\(^{2+}\)-sensitization in placental (B), myometrial (D), and omental (F) arteries. Filled bar for time scale (10 min). Ten μmol/L Y27632 significantly reduced the U46619-induced Ca\(^{2+}\)-sensitization in omental (B), myometrial (D), and placental (F) arteries.
Figure 5  Mean data indicating the significant reduction in 1 μmol/L U46619-dependent Ca^{2+} sensitization of contraction (normalized as 1.0) by 10 μmol/L Y27632 in permeabilized human omental (A), myometrial (B), and placental (C) arteries.
Figure 6  Data exhibiting the effect of phosphatase inhibition with calyculin A (cal A) in permeabilized human omental arteries. Representative tracings from arteries from the same patient, run in parallel, are shown of pCa 4.5 activating solution contractions (A–D) and 1 μmol/L U46619 (A, B) or 2 μmol/L cal A-induced (C, D) Ca\(^{2+}\)-sensitizing contractions in GTP pCa 6.7 submaximal activating solution. Ten μmol/L Y27632 significantly reduced the U46619-induced Ca\(^{2+}\)-sensitization (B, mean data in E), but did not affect the cal A-dependent Ca\(^{2+}\) sensitizations (D, mean data in E). Filled bar in panels A to D for time scale (10 min).
of intact and permeabilized vessels most likely via an action on myosin phosphatase activity. This places ROK activation as a key component of receptor-coupled signal transduction mechanisms regulating human arterial contractility during pregnancy. Y27632 has been shown to inhibit arterial contractility in in vitro and in vivo assessments of arterial function in animal models of vascular complications. Therefore, it will be of interest in the future to examine if clinical complications of pregnancy that are associated with elevated vascular resistances, for example preeclampsia and intrauterine growth restriction, are associated with increased ROK activation and attenuated by administration of Y27632.

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References

Novel peptides prevent alcohol-induced spatial learning deficits and proinflammatory cytokine release in a mouse model of fetal alcohol syndrome

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Alcohol
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Objective: Previously, the novel peptides NAPVSIPQ and SALLRSIPA were shown to prevent alcohol-induced fetal death and growth abnormalities in a mouse model of fetal alcohol syndrome. This study evaluated whether these peptides could prevent long-term alcohol-induced learning abnormalities. In addition, because specific cytokines are known to effect long-term potentiation, a model of learning at the molecular level, we studied the effect of these novel peptides on tumor necrosis factor-α, interleukin-6, and interferon-γ levels.

Study design: We used a well-characterized mouse model of fetal alcohol syndrome. Pregnant mice were injected on day 8 with alcohol (0.03 mL/kg) or placebo. Pretreatment with NAPVSIPQ + SALLRSIPA (20 μg) or placebo was given 30 minutes before alcohol. Embryos were removed after 6 hours, at which time cytokine, tumor necrosis factor-α, interleukin-6, and interferon-γ levels were measured with enzyme-linked immunoassays. To test spatial learning, adult offspring from litters that were treated with alcohol, control, NAPVSIPQ + SALLRSIPA then alcohol, or NAPVSIPQ + SALLRSIPA alone were evaluated for latency to find a hidden platform in the Morris water maze.

Results: Alcohol treatment increased tumor necrosis factor-α levels versus control levels (50.0 ± 3.5 pg/mL vs 32.7 ± 2.4 pg/mL; P < .001), NAPVSIPQ + SALLRSIPA pretreatment prevented this increase (39.9 ± 2.8 pg/mL; P ≤ .01), with levels similar to control (P = .1). Similarly, alcohol increased interleukin-6 levels versus control levels (22.6 ± 1.4 pg/mL vs 17.3 ± 0.6 pg/mL; P < .001), and NAPVSIPQ + SALLRSIPA prevented this increase (19.1 ± 1.0 pg/mL; P ≤ .02), with levels similar to control levels (P = .2). Interferon-γ levels were not different among the 3 groups (alcohol, 14.6 ± 4.9 pg/mL; control, 17.9 ± 6.6 pg/mL; alcohol + NAPVSIPQ + SALLRSIPA, 13.6 ± 4.9 pg/mL; P = .2). In the Morris water maze, alcohol-treated groups did not learn over the 7-day trial compared with the control group (P = .001). Groups that were pretreated with NAPVSIPQ + SALLRSIPA then alcohol learned significantly, which was similar to the control group. Groups that were treated with only NAPVSIPQ + SALLRSIPA learned significantly earlier, with the shortest latency once learning commenced.

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Fetal alcohol syndrome (FAS) is the most common nongenetic cause of mental retardation and includes prenatal alcohol exposure, growth restriction, distinct facial features, and neurodevelopmental problems, particularly life-long compromises in learning and memory. In the United States, it affects 0.5 to 3 babies per 1000 births each year; in the 1990s, the prevalence of maternal alcohol consumption during pregnancy, including binge drinking, has increased. Additionally, many fetuses are exposed to alcohol but do not meet the full criteria for FAS. Depending on a number of factors that are not well elucidated (including amount, timing, and genetic predisposition), these children may also have significant sequelae from alcohol exposure, which includes neurodevelopmental and learning difficulties. The mechanisms by which alcohol induces the characteristic physical and cognitive deficits are complex and not well understood. However, the effects of prenatal alcohol administration have been paralleled in animal models, which implies that dysfunctional mechanisms or neuronal plasticity are responsible for the persistent neurobehavioral sequelae from prenatal alcohol exposure.

Animal studies that used the Morris water-maze, which is a widely used test of spatial learning, have shown that alcohol exposure prenatally, or during the brain growth spurt results in adult spatial learning deficits. Because spatial learning is particularly affected in FAS, we used this model, which requires spatial “mapping” based on localization of extra-maze cues. Previous studies have also shown that proinflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) affect long-term potentiation, a model of learning at the molecular level. Thus, these cytokines were measured as markers for alcohol-induced damage, perhaps giving insight into the basis of FAS-related cognitive deficits.

Previously, we demonstrated that treatment with 2 novel peptides, SALLRSIPA (ADNF-9) and NAPVSIPQ (NAP), prevented alcohol-induced fetal growth restriction, microcephaly, and oxidative damage in the mouse model of FAS. These peptides (which are derived from the glial proteins, activity-dependent neurotrophic factor, and activity-dependent neuroprotective protein, respectively) have broad neuroprotective action evident at subfemtomolar levels and a unique pharmacologic makeup because they mimic the activity of their parent proteins. Given the neuroprotective properties of these peptides, the objective of this study was to evaluate the efficacy of peptide treatment in preventing the long-term cognitive deficits caused by prenatal alcohol exposure.

**Material and methods**

We used a well-characterized mouse model of FAS. C57Bl6/J female mice (Jackson Laboratories, Bar Harbor, Maine) were kept under a 12-hour light, 12-hour dark regimen, with food and water available at all times. The mice received humane animal care in compliance with the “Guideline for Care and Use of Experimental Animals.” Six-week-old females (21-24 g) were mated with C57Bl6/J males for 4 hours. On gestational day 8, we injected pregnant mice intraperitoneally with 25% ethyl alcohol in saline (vol/vol) or vehicle alone at 0.03 mL/g body weight. Pretreatment with the peptides ADNF-9 and NAP (20 μg in 0.2 mL) or placebo was given 30 minutes before the alcohol. NAP was diluted in 50 μL DMSO and diluted in filtered Dulbecco’s phosphate-buffered saline solution. ADNF-9 was dissolved and diluted in filtered Dulbecco’s phosphate-buffered saline solution. The doses of the peptides were based on the protective effects of these peptides in the prevention of alcohol-induced fetal death, growth restriction, and microcephaly. Because the animals that received the alcohol were incapacitated for approximately 6 hours after the injection, food and water were withheld from all groups for the initial 6 hours.

Adult male offspring (≥35 and not > 50 days of age) were then tested in the Morris water-maze to assess spatial learning. Male offspring from the litters that were treated with alcohol (n = 9 mice), vehicle (control, n = 15 mice), pretreatment with NAP + ADNF-9 and alcohol (n = 15 mice), or NAP + ADNF-9 alone (n = 8 mice) were tested for latency to find a hidden platform in the water maze. Each animal underwent 2 consecutive trials daily for 7 days. Each trial allowed the mouse a maximum of 1 minute to find the hidden platform. The latency for each trial was recorded, and the average of the 2 trials was calculated for each of the 7 days. The average latency for each of the 7 days was then analyzed by analysis of variance (StatView, version 5.0.1; SAS Institute Inc., Cary, NC); a probability value of < .05 was considered significant.

To further delineate how the peptides prevented the alcohol-induced learning deficits, 2 inflammatory cytokines (TNF-α and IL-6) and an immunosuppressive cytokine (interferon gamma [IFN-γ]) as an internal
control were measured in the embryo and surrounding decidua of each mouse fetus. At least 2 embryos from 6 different litters per cytokine were tested. Embryo/decidua were dissected 6 hours after treatment with alcohol, control, or alcohol plus peptide treatment. They were quick frozen on dry ice and stored at −80°C. All samples were analyzed for total protein content, which was not found to be different between the groups. With the use of an enzyme-linked immunooassay (R&D Systems, Minneapolis, Minn), samples were assayed in duplicates, and the mean value was used for analysis. Each enzyme-linked immunosorbent assay that was performed was specific for its respective cytokine without cross-reactivity or interaction with other cytokines. The sensitivities of the assays for all 3 cytokines (TNF-α, IL-6, and IFN-γ) were 1.95 pg/mL. The intra-assay coefficients of variation were <5%. Data were analyzed by Mann-Whitney U test (StatView 5.0.1); a probability value of <.05 was considered significant.

Results

In the learning paradigm, offspring from the alcohol-treated litters demonstrated no evidence of learning over the 7-day trial (Figure). In contrast, animals from the control litters decreased their latency 50% by the fifth day (P<.001). Males from the litters who were pretreated with NAP + ADNF-9 and then given alcohol also significantly learned, with a learning curve not different from that of the control at all time points that were tested. The offspring from litters that were treated with only NAP + ADNF-9 had a learning curve with 2 distinguishing features: The onset of learning was significantly earlier than all other groups (P < .01), and the latency period was the shortest of all groups after the onset of learning.

In the cytokine paradigm, embryo/decidua TNF-α levels were elevated significantly in the alcohol-treated group (50.0 ± 3.5 pg/mL) versus control (32.7 ± 2.4 pg/mL; P < .001). NAP + ADNF-9 pretreatment prevented the alcohol-induced increase in TNF-α (39.9 ± 2.8 pg/mL; P ≤ 0.01), with levels not different from control (P = .1). Similarly, embryo/decidua IL-6 levels were elevated in the alcohol-treated group (22.6 ± 1.4 pg/mL) versus control (17.3 ± 0.6 pg/mL; P < .001), and NAP + ADNF-9 prevented the alcohol-induced increase in IL-6 (19.1 ± 1.0 pg/mL; P ≤ .02), with levels similar to control (P = .2). IFN-γ levels were not different among the 3 groups (alcohol, 14.6 ± 4.9 pg/mL; control, 17.9 ± 6.6 pg/mL; alcohol + NAP + ADNF-9, 13.6 ± 4.9 pg/mL; P = .2).

Comment

This study showed that alcohol treatment increases proinflammatory cytokine levels and produces spatial learning deficits in affected offspring. The data also imply that the peptides, NAP + ADNF-9, prevent alcohol-induced increases in proinflammatory cytokines and protect against alcohol-induced spatial learning deficits. In addition, this study suggests that treatment with NAP + ADNF-9 alone enhanced learning in normal mice, when compared with control. The data are consistent with previous work in which neonatal mice that were treated with intraperitoneal NAP alone exhibited increased performance in the Morris water maze.14 Cumulatively, these data suggest that peptide treatment that is initiated in utero significantly influences spatial learning, possibly through cytokine-mediated mechanisms.

This study confirmed previous studies that showed that alcohol administration to pregnant mice produces impairment of spatial learning and memory performance15,16 and that prenatal combined peptide treatment effectively prevented alcohol-induced learning and memory deficits in mature mice that were exposed in utero to alcohol. Although these data unveil some clues to the mechanism responsible for alcohol-induced loss-of-function, the complete picture remains to be delineated. Previous studies suggest the loss of brain neurons caused by alcohol toxicity may be produced by multifactorial mechanisms, which include increases in cytokines, decreased glutamate neurotransmission,18 interference with neurotrophic factor signaling or expression,19 and increases in oxidative stress through...
free radical generation. Therefore, previous studies regarding NAP and ADNF-9 action that involve these factors are relevant to understanding the effectiveness of treatment with these peptides.

Regarding the protective effects of the peptides on learning and memory, recent studies of hippocampal cultures demonstrated that the peptides alter glutamate release and N-methyl-D-aspartate (NMDA) receptors, both of which are important in learning and memory. Particularly, hippocampal neurons that were treated with ADNF-9 exhibited increased frequency of miniature postsynaptic currents, which suggests a presynaptic site as a target of the peptide-induced signaling system. Additionally, ADNF-9 treatment controls NR2A and NR2B subunit stability of the NMDA receptor in neurons that have yet to establish efficient synaptic connections. Although the exact mechanism by which ADNF-9 influences the synaptogenesis and neurotransmission used by glutamate remains unknown, in vitro studies strongly suggest that the peptide interacts with and regulates the glutaminergic synapses in developing neural systems. In addition, NMDA antagonists are known to produce apoptosis in the developing brain, and NMDA protects cerebellar neurons from alcohol toxicity. This protection was correlated with increases in nitric oxide and cyclic guanosine monophosphate levels. Thus, peptide-induced enhancement of glutamate neurotransmission/synaptogenesis may have a protective effect on alcohol-induced decreases in NMDA receptor stimulation.

Support for a relationship between alcohol and cytokine production was observed in a trophoblast cell line, which exhibited increases in IL-6, RANTES, and granulocyte colony-stimulating factor after ethanol treatment. Importantly, astrocyte cultures that were prepared from rats that were treated prenatally with alcohol were more sensitive to the cytotoxic effects of TNF-α than cultures that were obtained from control rats. Previous studies have shown that NAP prevented increases in TNF-α after closed head injury and that NAP treatment prevented TNF-α–induced toxicity in PC12 cells. Thus, the inhibition of proinflammatory cytokine production is another plausible mechanism through which the peptides could prevent ethanol-induced toxicity. In our study, the effect of combined peptide treatment on cytokine levels is consistent with this action, which accounts for the observed protective effects.

Another attractive mechanism is the link between ethanol toxicity and oxidative stress. Although evidence for such a relationship in the central nervous system is not yet apparent, the generation of free radicals has been proposed as a likely mechanism for alcohol-induced cytotoxicity. Indeed, antioxidant-mediated protection from ethanol-induced toxicity has been observed. Recent studies have implicated NADPH oxidase as a source of ethanol-related free radical generation. In addition, the generation of 1-hydroxyethyl radicals and acetaldehyde from xanthine oxidase metabolism of ethanol has been detected; this pathway could play a role in toxic effects that are associated with alcohol. Both NAP and ADNF-9 have been shown to provide protective action against oxidative stress that is produced by a number of agents, including ferrous sulfate, hydrogen peroxide, glutathione, and beta amyloid peptide. These studies suggest that the peptides have effects that prevent oxidative damage and therefore provide an additional mechanism to protect from alcohol-induced damage.

In summary, there is no single mechanism that can account for all the toxic effects that are produced by ethanol or the protective action of the peptides. As shown in the present study, the breadth and duration of the protective action that is provided by the peptides strongly supports broad overlap between the multifactorial mechanisms that produce damage that is associated with alcohol toxicity and the effective multifactorial mechanisms that are elicited by NAP and ADNF-9.

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Neutrophils from pregnant women produce thromboxane and tumor necrosis factor-α in response to linoleic acid and oxidative stress

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KEY WORDS
Preeclampsia
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Lipid peroxides

Objective: Preeclampsia is associated with oxidative stress, neutrophil activation, neutrophil infiltration into systemic vasculature, and elevated plasma levels of linoleic acid, the fatty acid precursor to arachidonic acid and its metabolite, thromboxane. In this study we evaluated whether linoleic acid under conditions of oxidative stress would stimulate neutrophil production of thromboxane and tumor necrosis factor-α.

Study design: Neutrophils were isolated from 14 normal pregnant women. Western blot demonstrated cyclooxygenase-2 expression at 18 hours of incubation, so this incubation time was used for experiments. Neutrophils (2 × 10⁶ cells/mL) were incubated in Dulbecco’s modified Eagle’s medium/F-12 with: (1) linoleic acid control; (2) an oxidizing solution enriched with linoleic acid; (3) oxidizing solution enriched with linoleic acid plus indomethacin; (4) oxidizing solution enriched with linoleic acid plus aspirin; (5) oxidizing solution enriched with linoleic acid plus NS-398, a specific inhibitor of cyclooxygenase-2; or (6) oxidizing solution enriched with linoleic acid plus pinane thromboxane, a thromboxane synthase inhibitor and receptor blocker.

Results: Oxidizing solution enriched with linoleic acid significantly increased oxidative stress in neutrophils. Compared with linoleic acid, oxidizing solution enriched with linoleic acid significantly increased neutrophil production of thromboxane and tumor necrosis factor-α. Indomethacin and aspirin inhibited oxidizing solution enriched with linoleic acid stimulation of thromboxane, but NS-398 was equally effective implicating cyclooxygenase-2 in the thromboxane response. Indomethacin inhibited oxidizing solution enriched with linoleic acid stimulation of tumor necrosis factor-α, but did not inhibit thromboxane stimulating thromboxane in the tumor necrosis factor-α response.

Conclusions: These data demonstrate that exposure of neutrophils from normal pregnant women to conditions present in preeclamptic women results in neutrophil activation with release of thromboxane and tumor necrosis factor-α. Newly synthesized thromboxane is cyclooxygenase-2 dependent and plays a role in the tumor necrosis factor-α response. Our data suggest a mechanism for maternal vasoconstriction and vascular inflammation in preeclampsia because activated,
thromboxane-secreting neutrophils migrate across endothelium into the microenvironment of the vasculature in which they could promote vasoconstriction, whereas release of tumor necrosis factor-α could cause vascular inflammation.

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Preeclampsia is associated with neutrophil activation^1; oxidative stress^2; and elevated plasma levels of linoleic acid (LA),^3 the fatty acid precursor to arachidonic acid and its inflammatory, vasoconstrictor metabolite, thromboxane. To date, no satisfactory explanation for maternal hypertension or vascular dysfunction has been offered and substantiated. Recently we demonstrated inflammation and infiltration of neutrophils in systemic vascular tissue in women with preeclampsia. Studies on isolated perfused placental lipid peroxides are primarily secreted into the placenta. The placental production rate of lipid peroxides is abnormally increased in preeclampsia, and the placenta. The placental production rate of lipid peroxides is abnormally increased in preeclampsia, and the placenta.

Neutrophil activation may be initiated in the intervillous space by increased secretion of lipid peroxides by the placenta. The placental production rate of lipid peroxides is abnormally increased in preeclampsia, and placental lipid peroxides are primarily secreted into the intervillous space. Studies on isolated perfused placental cotyledons have demonstrated that peroxides stimulate cyclooxygenase (COX) to increase placental thromboxane production, which is abnormally increased in preeclampsia. Monocytes also produce increased amounts of thromboxane in preeclampsia. Lipid peroxides have been shown to stimulate monocyte/macrophage release of TNFα, and thromboxane has been implicated in monocyte TNFα release. Elevated levels of lipid peroxides in preeclampsia may similarly activate neutrophils to release thromboxane and TNFα.

We hypothesized that exposure of neutrophils to conditions present in preeclamptic women would result in increased production of thromboxane and TNFα and that these effects would be mediated by increased activity of the inducible form of COX-2. To test this, neutrophils were isolated from the blood of normal pregnant women and incubated with an oxidizing solution enriched with linoleic acid in the presence and absence of inhibitors of various steps in the arachidonic acid pathway to thromboxane.

### Material and methods

#### Neutrophil isolation

Blood samples were obtained from 14 normal pregnant women during routine blood draws between 26 and 30 weeks of gestation by vein puncture into sodium heparin tubes. The study population consisted of both primigravid and multigravid patients between the ages of 19 and 40 years. The racial/ethnic composition was 8 black women, 4 white women, 1 Hispanic woman, and 1 Asian woman. This study was approved by the Office of Research Subjects Protection, Virginia Commonwealth University. The blood was processed in the laboratory within an hour following sample collection. Neutrophils were isolated by dual-histopaque density gradient centrifugation (Sigma Chemical Company, St Louis, MO). Neutrophils were aspirated from the histopaque-1077/histopaque-1119 interface and washed with phosphate-buffered saline, followed by centrifugation for 10 minutes at 200 × g. To lyse contaminating red blood cells, the cell pellet was suspended in ice-cold double-distilled water (3 mL) for 30 seconds with agitation on a vortex mixer. Ice-cold 0.6 M potassium chloride (1 mL) was then added to restore toxicity. The cells were pelleted, the supernatant was discarded, and the cells were resuspended for counting in 1 mL of Dulbecco’s modified Eagles’s medium (DMEM)/F-12 (Gibco, Invitrogen Corp, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum. Cell viability was greater than 90% as assessed by trypan blue exclusion staining.

### Western blot analysis

To determine the time course for COX-2 expression, neutrophils were divided into 15 × 10^6 mm nonadherent Teflon tubes (Minisorp, Nunc, Rochester, NY) (2 × 10^6 cells/tube). COX-2 expression was assessed at baseline (0 hours) and after 2, 4, 6, and 18 hours of incubation in a final volume of 1 mL with either LA (90 μM) or an oxidizing solution containing LA (OxLA). The oxidizing solution was composed of hypoxanthine (1.8 mM) + xanthine oxidase (0.005 units/mL) + ferrous sulfate (50 μM) plus LA (90 μM). After incubation, cells were rinsed with phosphate-buffered saline and then lysed with buffer (100 μL) consisting of 50 mM TRIS (pH 7.5), 1% Nonidet P-40, 100 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetra-acetic acid, 1 mM EDTA, 1 mM aminothalzyl benzene sulfonyl fluoride hydrochloride, 5 mM NaF, and 10% glycerol. Cell lysates were diluted with buffer (0.5 mol/L TRIS-HCl, 0.1% glycerol, 10% sodium dodecyl sulfate, 0.05% 2-methanol) to equalize protein concentrations and loaded on a 9% polyacrylamide gel for electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane for 1 hour at 65 V. After overnight blocking with a 5% solution of dry milk in Tris-buffered saline, the membrane was
incubated for 2 hours with mouse COX-2 monoclonal antibody (1:1000, Cayman Chemical, Ann Arbor, MI). Blots were then incubated for 1 hour with secondary antibody, a goat monoclonal antimouse IgG antibody conjugated to horseradish peroxidase (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Western Lightning Chemiluminescence Reagent Plus enhanced luminol (PerkinElmer Life Sciences, Boston, MA) was used to develop horseradish peroxidase using Kodak film (Hyperfilm MP, Amersham Pharmacia, Piscatawy, NJ).

**Experimental conditions**

To assess neutrophil production of thromboxane and TNFα in response to oxidative stress, neutrophils were diluted in DMEM/F-12 with 10% heat-inactivated fetal bovine serum to a final concentration of 8×10^6 cells/mL. Aliquots of the cell suspension (250 μL) were added to 15×100 mm nonadherent Teflon tubes (Minisorp, Nunc, Rochester, NY) containing 750 μL of DMEM/F-12 media with experimental treatments. The number of neutrophils isolated from each blood draw limited the number of treatments that could be done, so experiments were divided as follows. For assessment of oxidative stress, treatments were LA control and OxLA. For assessment of thromboxane B2 (TXB2) production, treatments were: (1) LA control; (2) OxLA; (3) OxLA plus indomethacin at a dose sufficient to inhibit phospholipase A2 (100 μM); (4) OxLA plus aspirin (100 μM) to inhibit COX-1 and COX-2; and (5) OxLA plus NS-398 (300 nM), a specific COX-2 inhibitor. For assessment of TNFα production, treatments were: (1) LA control; (2) OxLA; (3) OxLA plus indomethacin; and (4) OxLA plus pinane thromboxane (10 μM), a thromboxane synthase inhibitor and thromboxane receptor blocker.

LA, indomethacin, and aspirin were purchased from Sigma. Pinane thromboxane was purchased from Cayman. Treatments were run in duplicate. The tubes were placed in a slant rack that was then secured to an orbital mixer (VXR-510, Teckmar, West Germany). The assembly was placed in an incubator, and the tubes were incubated overnight with agitation at 37°C in an incubator gassed with 5% CO2. After 18 hours the tube contents were removed and centrifuged at 200 × g for 5 minutes. The supernatant was aliquoted and stored at −20°C until analysis.

**Assays**

**Lipid peroxide assay**

Lipid peroxides were estimated by an improved analysis of thiobarbituric acid reactive substances (TBARS), which primarily reflects malondialdehyde, a breakdown product of lipid peroxides. Tetramethoxypropane was used to generate malondialdehyde for the standard curve. Butylated hydroxytoluene was added to prevent oxidation during the heating step with thiobarbituric acid. Freshly thawed samples were analyzed. Serially diluted samples were parallel to the standard curve. Assay of media alone resulted in a zero dose response. Addition of increasing sample

**Thromboxane assay**

Thromboxane concentrations were estimated by specific radioimmunoassay of its stable metabolite, TXB2. TXB2 standard was purchased from PerSeptive Diagnostics, Inc (Cambridge, MA), and TXB2 antibody was purchased from Oxford Biomedical Research, Inc (Oxford, MI). Tritiated TXB2 was purchased from New England Nuclear (Dupont Research, Wilmington, DE). Dextran-coated charcoal was used to separate the bound from the free fraction. Serial sample dilutions were parallel to the standard curve. Assay of media alone resulted in a zero dose response. Addition of increasing sample
volumes resulted in a linear dose response. Within-assay variation was 4.5% and between-assay variation was 14.3%.

**TNF-α assay**

TNF-α concentrations were determined by enzyme-linked immunosorbent assay using a commercially available reagent kit (OptEIA Human TNF-α Set, PharMingen, San Diego, CA). Assay of varying volumes of cell homogenate resulted in a linear response, $Y = 0.739x - 4.225$, $r^2 = 0.991$. Within-assay variation was 2.5%.

**Statistical analyses**

Experimental data were analyzed by 1-way analysis of variance with Newman-Keul’s post hoc test. A statistical computer software program was used for analysis (GraphPad Prism 4.0 for Macintosh, GraphPad Software, Inc, San Diego, CA, www.graphpad.com). A probability level of $P < .05$ was considered to be statistically significant. Data are presented as mean ± SE.

**Results**

Western blot analysis was done to assess the time course for COX-2 protein expression when neutrophils were exposed to oxidative stress. Figure 1 shows that there was very slight expression of COX-2 protein at baseline (0 hours) and after 2, 4, and 6 hours of incubation, probably resulting from the isolation procedure. By 18 hours, there was some expression of COX-2 protein for cells exposed to LA but marked expression of COX-2 for cells exposed to OxLA. An 18-hour incubation time period was used for the TXB2 and TNF-α experiments.

To verify that OxLA induced oxidative stress, we incubated neutrophils with LA or OxLA for 18 hours and assayed the media for TBARS. TBARS were significantly higher in the media of cells exposed to OxLA (529 ± 85 nM per 1 million cells) as compared with those exposed to LA (233 ± 50 nM per 1 million cells) (Figure 2), indicating that treatment with OxLA induced oxidative stress.

OxLA significantly increased neutrophil production of TXB2 as compared with LA (542 ± 23 versus 421 ± 29 pg per 1 million cells, respectively) (Fig. 3). Both indomethacin and aspirin inhibited the ability of OxLA to stimulate TXB2 (443 ± 29 and 413 ± 22 pg per 1 million cells, respectively). NS-398 was equally as effective as indomethacin and aspirin at inhibiting TXB2 production in the presence of OxLA (435 ± 35 pg per 1 million cells).

OxLA significantly increased neutrophil production of TNF-α by neutrophils as compared with LA (99 ± 26 versus 20 ± 8 pg per 1 million cells, respectively) (Figure 4). Indomethacin abolished the ability of OxLA to stimulate TNF-α (2.3 ± 0.5 pg per 1 million cells). When neutrophils were exposed to OxLA plus pinane thromboxane, neutrophil TNF-α production was significantly inhibited (23 ± 8 pg per 1 million cells).

**Comment**

To date, no satisfactory explanation for maternal hypertension or vascular cell dysfunction in women...
with preeclampsia has been offered and substantiated. Recently we demonstrated inflammation and infiltration of activated neutrophils in the maternal systemic vasculature in preeclamptic women. Therefore, one possibility for hypertension and vascular cell dysfunction is release of thromboxane and inflammatory mediators, such as TNFα, by neutrophils that have infiltrated the vasculature. Because oxidative stress is a prominent feature in preeclampsia, we investigated whether oxidative stress stimulates neutrophils to produce thromboxane and TNFα. We used OxLA because the plasma concentrations of LA in preeclampsia are significantly elevated as compared with normal pregnancy, and LA is the dietary precursor for arachidonic acid, which is metabolized to thromboxane.

OxLA induced expression of COX-2 protein and caused a significant increase in neutrophil thromboxane production, compared with the LA control. Inhibiting phospholipase A2 abolished the OxLA-induced increase in thromboxane. These results suggest that neutrophils contribute to increased thromboxane levels in disorders marked by hyperlipidemia and oxidative stress, such as preeclampsia.

To resolve the mechanism whereby OxLA increased neutrophil thromboxane production, we focused on the activity of COX because the level of peroxide tone modulates this enzyme’s activity. COX exists in two main isoforms: COX-1, a constitutively active form, and COX-2, an inducible form during inflammation. To examine the potential roles of these enzymes, we used aspirin, an inhibitor of COX-1 and COX-2, and NS-398, a specific COX-2 inhibitor. Aspirin completely blocked the OxLA-induced increase in thromboxane. NS-398 was equally as effective as aspirin in abolishing thromboxane production. These results suggest that of the 2 COX isoforms, COX-2 plays the predominant role in thromboxane production induced by OxLA. Our findings are in agreement with recent studies showing selective COX-2 inhibitors can attenuate prostaglandin E2 and TNFα release by activated neutrophils and block thromboxane production stimulated by various neutrophil activators (fMet-Leu-Phe, phorbol myristic acid, lipopolysaccharide, TNFα, opsonized zymosan).

A study by Caughey et al implicated thromboxane in TNFα production by human monocytes from non-pregnant patients. In preeclampsia TNFα is abnormally increased in the maternal circulation and placenta. We hypothesized that OxLA would stimulate neutrophil TNFα release through a thromboxane-dependent mechanism. We found that OxLA markedly stimulated TNFα production as compared with LA in neutrophils isolated from pregnant women. In nonpregnant patients, Jovinge et al showed that oxidized low-density lipoprotein increased the expression of TNFα messenger ribonucleic acid and stimulated release of TNFα in human monocytes. Gorog found that oxidized lipids produce a long-lasting activation of monocytes in non-pregnant subjects. Our finding that OxLA stimulates neutrophils from pregnant women to release TNFα suggests a critical link between oxidative stress and immune system dysfunction in preeclampsia.

To investigate whether the OxLA-induced increase in neutrophil TNFα required thromboxane, we used inhibitors of enzymes that lead to thromboxane synthesis. Indomethacin completely abolished TNFα production, and pinane thromboxane, a thromboxane receptor antagonist and thromboxane synthase inhibitor, significantly reduced OxLA-induced neutrophil TNFα production. Thromboxane may mediate increased TNFα by enhancing the effect of nuclear factor-κB.

Numerous reports indicate that leukocytes are activated in preeclampsia. Activated neutrophils produce TNFα, and the activities of this cytokine may have important effects on the progression of preeclampsia. TNFα interacts with fatty acids to induce oxidative stress and dysfunction in endothelial cells. TNFα could also contribute to the pathophysiology of preeclampsia by stimulating expression of endothelial intracellular cell adhesion molecule-1, causing adherence of neutrophils to endothelial cells. We recently demonstrated increased endothelial expression of intracellular cell adhesion molecule-1 coincident with neutrophil adherence to the endothelium in preeclamptic women.

The results of this study show that exposing neutrophils from normal pregnant women to conditions present in preeclamptic women results in expression of COX-2 and increased thromboxane production. The newly synthesized thromboxane plays a role in mediating neutrophil TNFα production. These results, combined with our recent finding that activated neutrophils infiltrate the maternal systemic vasculature in preeclampsia, link oxidative stress and immune dysfunction in this disorder and offer mechanistic explanations for maternal vasoconstriction and vascular dysfunction.

References

Heat shock protein-70 and 4-hydroxy-2-nonenal adducts in human placental villous tissue of normotensive, preeclamptic and intrauterine growth restricted pregnancies

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Objective: The purpose of this study was to compare immunohistochemical expression of heat shock protein-70 (hsp70), a marker for oxidative stress, and 4-hydroxy-2-nonenal adducts (HNE), a marker for lipid peroxidation, in placental villous tissue of normotensive, preeclampsia, and intrauterine growth restricted (IUGR) pregnancies.

Study design: Placentas were collected and flash frozen in liquid nitrogen after delivery from normotensive pregnancies (n = 5), and pregnancies complicated by preeclampsia (n = 5), IUGR (n = 5), and preeclampsia plus IUGR (n = 4). Cryosections were cut and immunostained with polyclonal anti-hsp70 and monoclonal anti-HNE antibodies using Vectastain Elite ABC kit. Normal rabbit serum or mouse IgG were used as negative controls. Three independent observers, blinded to identity of tissue, examined each slide to identify cellular localization and intensity of the immunostaining. Western blot analysis and scanning densitometry were used to quantify and compare the amount of hsp70 and HNE adducts present in tissue homogenates.

Results: Positive immunostaining for both antibodies was observed in cytoplasm of syncytiotrophoblasts, extravillous trophoblasts, vascular smooth muscle, and endothelial cells for all groups. Expression of hsp70 and HNE adducts was reported as observers’ mean stained intensity. Overall, kappa showed good agreement between observers. Immunostaining intensity was similar in all tissue types for each group with the exception that immunostaining was significantly more intense in the vascular endothelium of the preeclamptic group for HNE adducts (P = .02) and significantly less intense in the IUGR group for hsp70 (P = .013). Scanning densitometric analysis of the Western blots showed no significant difference in total hsp70 and HNE adducts expression in all 4 tissue groups.
Preeclampsia remains one of the unsolved problems in obstetrics. Evidence is accumulating that oxidative stress and lipid peroxidation play a role in the pathogenesis of preeclampsia and may account for its clinical manifestations. Articles dealing with a specific marker of oxidative stress or reviewing several ones are evident in the literature.\textsuperscript{1-12} Maternal serum and placental levels of lipid peroxides are noted to be increased in preeclampsia when compared to normal pregnancies.\textsuperscript{2,3,8,11,12}

Various substances are linked to oxidative stress and lipid peroxidation. Inducible heat shock protein 70 (hsp70) has been shown to be up-regulated during oxidative stress.\textsuperscript{13-15} In addition, 4-hydroxy-2-nonenal (HNE), an aldehydic end product of lipid peroxidation, has been observed to be a mediator of toxic effects elicited by oxidative stress.\textsuperscript{16} Both substances have been shown to play a role in the pathophysiology of various human diseases. Their role in preeclampsia and IUGR has not been completely defined. Therefore, we proposed that expression of hsp70 and HNE adducts is increased in placental villous tissue of preeclampsia and IUGR pregnancies compared with those from normotensive and uncomplicated pregnancies.

**Material and methods**

All procedures were performed under protocols approved by the Institutional Review Board of the University of Cincinnati Medical Center and the Ethical Committee of Glasgow Royal Infirmary. Placental villous tissues (1 \times 1 \times 1 \text{cm}) were collected at the Glasgow Royal Infirmary immediately after delivery from normotensive pregnancies (n = 5) and from pregnancies complicated by either preeclampsia (n = 5), intrauterine growth restriction (IUGR) (n = 5) or preeclampsia plus IUGR (n = 4). Villous tissue was immediately flash-frozen in liquid nitrogen and stored at −80°C until processed. Preeclampsia was defined as a blood pressure of 140/90 mm Hg on at least 2 occasions at least 6 hours apart occurring after 20 weeks’ gestation and accompanied by proteinuria (>300 mg/L in a 24-hr urine collection). IUGR was defined as a fetal weight less than the 5th percentile using standardized Scottish birth weight table.

Cryosections (7 \text{μm}) of villous tissue were cut just before staining. Serial sections were immunostained using the Vectastain ABC Elite Kit (Vector Laboratories; Burlingame, Calif) with either polyclonal anti-hsp70 (StressGen Biotechnologies Corp, Victoria, BC, Canada) or monoclonal anti-HNE antibodies (Genox Corp, Baltimore, Md). Tissues were fixed in acetone at −20°C for 10 minutes and then washed with phosphate-buffered saline (PBS). For hsp70, the tissue sections were blocked with 5% goat serum diluted in 0.2% Tween-20 in phosphate buffered saline (PBS-T) at room temperature for 1 hour, whereas, 5% horse serum diluted in 1% saponin was used as a blocking agent for HNE adduct samples. The tissues were then incubated in primary antibody (anti-hsp70, 1:500; anti-HNE, 1:5) at 4°C overnight. Tissue sections were washed and incubated with respective secondary antibody (1:1000) for 30 minutes at room temperature. Sections were blocked for endogenous peroxidase for 10 minutes using 3% H\textsubscript{2}O\textsubscript{2} in deionized water. To achieve better staining results for HNE adducts, 1% saponin was included in all washes and antibody solutions up to and including the secondary antibody incubation, after which it was excluded from all reagent mixtures. After washing, slides were incubated in the ABC complex for 30 minutes at room temperature, and then stained with 3-amino-9-ethyl carbazole (AEC) until optimal staining was obtained (12 minutes for hsp70 and 17 minutes for HNE). Sections were counterstained with hematoxylin (Biomeda Corp, Foster City, Calif) and mounted in PBS/glycerol (1:9). Preimmune rabbit serum and mouse IgG were used as negative controls for hsp70 and HNE adduct antibodies, respectively. Cellular localization and intensity of the immunostaining (none = 0, minimal = 1, moderate = 2, or heavy = 3) of all tissue sections were identified using 3 observers who were blinded to tissue group identity.

For Western blot analysis, placenta samples were homogenized 3 times for 5 seconds with a Tissue Tearor (Glen Mills, Clifton, NJ) (initial speed of 14 and final of 22) at 4°C in homogenizing buffer consisting of 250 mmol/L sucrose and 50 mmol/L HEPES, pH 7.4, containing the following protease inhibitors: 0.7 μg/mL pepstatin, 10 μg/mL leupeptin, 100 μmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 100 μmol/L Na-p-tosyl-L-lysine-chloromethyl ketone (TLCK), and 200 μmol/L sodium orthovanadate (Calbiochem, San Diego, Calif). The supernatant was collected after centrifugation at 1000g for 10 minutes. Total protein quantification was performed using BCA method.\textsuperscript{17} Tissue samples were diluted in 2× sample buffer containing 0.25 mol/L TRIS (pH 6.8), 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.02% bromophenol blue, and heated to 100°C for 5 minutes.

**Conclusion:** Immunohistochemistry showed local changes for oxidative stress and lipid peroxidation in the vascular endothelium from placentas of preeclamptic and IUGR pregnancies. However, these changes were masked when studying tissue homogenates. © 2005 Elsevier Inc. All rights reserved.
Results

Similar patterns of hsp70 and HNE adduct immunostaining were observed in normal, IUGR, and PE placental tissues. Positive staining for hsp70 and HNE adducts was observed in the following cell types: syncytiotrophoblast, extravillous trophoblast, vascular smooth muscle, and endothelial cells in normal placentas. Figure 1 represents a sample of the immunostaining of hsp70 and HNE adducts for the normotensive group. All staining was cytoplasmic. No immunostaining was apparent in the negative controls (Figure 1B and D). Expression and localization of the hsp70 and HNE adducts are reported in the Table as the observers’ mean stained intensity. Kappa statistic showed good agreement. As observed in the normotensive group, expression of hsp70 and HNE adducts was noted in various cell types, with the expression all tissue types of hsp70 and HNE adducts (mean ± SD) in preeclampsia and IUGR. It is believed that endothelial dysfunction plays a role in preeclampsia, IUGR; preeclampsia and IUGR were not significantly different than those pregnancies with preeclampsia, IUGR; preeclampsia and IUGR were not significantly different than those pregnancies with preeclampsia, IUGR. Like other studies, our study does not conclusively define the role of oxidative stress and various markers in preeclampsia and IUGR.1,3-7,18-21 It is believed that endothelial dysfunction plays a role in preeclampsia, and may account for the maternal manifestation of the disease.

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*P < .05.

Comment

Like other studies, our study does not conclusively define the role of oxidative stress and various markers in preeclampsia and IUGR.1,3-7,18-21 It is believed that endothelial dysfunction plays a role in preeclampsia, and may account for the maternal manifestation of the disease.

Table Location and immunostaining observation intensity of Hsp 70 and HNE adducts

For SDS-PAGE gel electrophoresis, 20 μg of each protein sample was loaded in a 12% TRIS-glycine gel (BioRad, Hercules, Calif), and run at 40 mA/gel. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk in TBS-T for 1 hour, and then probed with rabbit anti-hsp70 polyclonal antibody (1:1000) at 4°C overnight. The membrane was washed and incubated with donkey antirabbit secondary antibody (1:1000). ECL Western Lightning Chemiluminescence Reagent (Amer- sham) was applied to visualize the hsp70 band on radiographic film. Presence of hsp70 was confirmed at the 70 kD using a prestained low molecular weight marker (BioRad). Band density was normalized and relative intensity was determined using scanning densitometry (Alpha Imager 5.0, Alpha Innotech Corp, San Leandro, Calif). The membranes were then stripped and reprobed with anti-HNE monoclonal antibody (15 μg/mL) using sheep antimouse secondary antibody (1:1000).

Statistical analyses include the following: Fisher exact test was used to compare the mean intensity of observed immunostaining between the groups. The presence of IUGR and preeclampsia was compared using logistic regression. The degree of classification accuracy regarding immunostaining intensity between observers was evaluated by kappa statistic. The relative intensity of the protein bands was compared using analysis of variance and Newman-Keuls post-hoc test. Statistical significance was set at P < .05.
disease. Immunohistochemistry revealed a significant increase in staining intensity of HNE adducts only in the vascular endothelium of preeclamptic placentas when compared to the other groups, supporting a role of increased lipid peroxidation and endothelial involvement in the underlying pathogenesis of preeclampsia. However, the decrease in staining intensity of hsp70 seen in placent al dysfunction in IUGR placentas, representing a decrease in oxidative stress, would not support a role for oxidative stress in the placental manifestations of IUGR.

Western blot analysis did not show a difference in hsp70 or HNE adduct present in total tissue homogenates the groups. Immunostaining intensity of hsp70 and HNE adducts in the various groups reflects local changes in the tissue, whereas Western blot analysis reflects total protein changes in the tissue homogenate, which would obliterate subtle cellular changes. This can explain why the immunohistochemical staining and Western blot results did not concur. The local increases in protein expression represented as immunohistochemical changes may not have been significant enough to be reflected as changes in the total amounts of protein or adduct seen in the homogenate used for the Western blot analysis.

For the Western blot analysis, a 70 kD band was observed in all samples probed with anti-hsp70, which is consistent with other literature reports. Two bands, at 56 and 61 kD, were noted on the HNE Western blot, again consistent with other investigators’ findings. The monoclonal antibody used for 4-hydroxy-2-nonenal immunostaining recognizes histidine and sulfhydryl adducts of 4-hydroxy-2-nonenal modified proteins. In some cases, 10 or more proteins have been detected by Western blot analysis using the 4-hydroxy-2-nonenal monoclonal antibody.

Abnormal placentation and inadequate maternal vascular response may also complicate pregnancies with preeclampsia or IUGR. Because changes were only seen in the vascular endothelium in this study and not generally throughout the placental tissue, a selective effect on the vascular reactivity of the placenta is a possibility. However, we cannot prove a cause and effect relationship that HNE adducts reflect changes in vascular reactivity. This can only be confirmed by placental perfusion studies. In addition, abnormal placentation and vasculature changes are mostly seen in placental bed biopsies in preeclampsia. Unfortunately, placental bed biopsies were not obtained in this study. This is a limitation of this study. Further investigation using placenta bed biopsies for hsp70 and HNE adduct concentration are needed to determine their role in preeclampsia.

The essential role of lipid peroxidation as the underlying pathogenesis of abnormal placentation leading to preeclampsia and IUGR was not completely demonstrated in this study. Various explanations may account for this. First, HNE adducts may not be a relative marker for lipid peroxidation. Placental HNE production, breakdown, and clearance may be rapid. Second, technical or system error could have resulted in the absence of significant differences between the groups. In addition, techniques for immunohistochemistry along with our antisera may not be sensitive enough to show a difference between the groups. Tissue collection and preparation may have confounded the findings for both hsp70 and HNE. For example, hypoxia that occurs in the uterus after separation of the placenta may induce expression of hsp70 and HNE adducts. However, this was not observed in studies performed in our lab with hsp70. We have observed immunostaining intensity for hsp70 was consistent among random sampling in the same placenta from pathologic pregnancies similar to these groups, and was not affected by increasing time from delivery to collection of placenta.

Our study, consistent with other studies previously mentioned, indicates that hsp70 and HNE adducts do not have a vital role in preeclampsia with or without IUGR, confirming that these markers should not be used in the analysis of oxidative stress in placentas from pregnancies complicated by these pathologic processes. However, before completely discarding these 2 markers, further studies should be undertaken on placental bed biopsies. In addition, other markers may be identified.
in the future that aid in the diagnosis of preeclampsia with or without IUGR. In closing, preeclampsia is a complicated pregnancy-related disease, and has eluded researchers as to its origin and cause, and will continually do so until we have better tools to aid in the understanding of the disease.

References

Cloning and cellular expression of aquaporin 9 in ovine fetal membranes

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KEY WORDS
Aquaporin 9
Amniotic fluid regulation
Intramembranous pathway
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Objective: Amniotic fluid (AF) absorption across fetal membranes is essential for AF volume homeostasis, balancing fetal swallowing, urine flow, and lung liquid production. In sheep, AF is absorbed primarily across the amniotic membrane into fetal vasculature situated between the amnion and chorion. Aquaporins (AQPs) are cell membrane proteins that serve as water channels. Recent studies have demonstrated the expression of AQP 1, 3, 8, and 9 in human chorioamniotic membranes and placenta. As AF dynamics continued to be explored primarily in the ovine model, we sought to clone and characterize the expression of ovine AQP9 in fetal membranes.

Methods: Ovine AQP9 gene was cloned with the use of homology reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR and Northern analysis were used to determine AQP9 gene expression, and immunohistochemistry (IHC) used to localize AQP9 protein expression in ovine fetal membranes.

Results: A 2085-base pair (bp) full-length complementary DNA (cDNA) sequence of ovine AQP9 was cloned. The ovine AQP9 cDNA is 86%, 82%, and 82%, and the predicted amino acid sequence (295 amino acids) is 77%, 71%, and 69% identical to human, rat, and mouse AQP9, respectively. RT-PCR and Northern analysis detected AQP9 messenger RNA expression in ovine amnion and allantois, but not in placenta, chorion, or umbilical cord. Immunohistochemistry localized AQP9 protein in epithelia of amnion and allantois.

Conclusion: The presence of significant AQP9 messenger RNA and protein expression in ovine fetal membranes suggests that AQP9 may be a major water channel for intramembranous AF resorption in sheep. The cloning of ovine AQP9 and the demonstration of AQP9 expression in amnion and allantois significantly enhances our understanding of ovine AF regulation and offers the potential for therapeutic approaches for the treatment of oligohydramnios and polyhydramnios.

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Aquaporins (AQP)s are cell membrane water channel proteins that have 6 transmembrane domains and 2 asparagine-proline-alanine (NPA) motifs. As demonstrated by studies of AQP1, AQP proteins organize in membranes as tetramers; however, each monomer forms a hydrophilic pore in its center and functions independently as a water channel. To date, at least 11 types of mammalian AQP have been identified with different AQP types expressed in different tissues. In vitro experimental models have demonstrated that AQPs substantially increase water permeability across the membranes in which they are expressed. Select AQP(s) (AQP 3, 7, and 9) are also permeable to neutral solutes such as glycerol and urea, in addition to water.

Recent advances have provided significant insight into the role of AQPs in the cause and pathophysiology of human diseases. For example, AQP2 mutation causes nephrogenic diabetes insipidus, whereas mutation of AQP0, the AQP expressed in epithelia of the lens, causes familial cataracts. More recent studies report that defective cellular trafficking of AQP5 in lacrimal and salivary glands explains the dry eyes and mouth in Sjogren’s syndrome. Studies in animal models have provided critical information regarding the essential functions of AQPs. AQP1 knockout mice demonstrate defective renal concentration ability, and transgenic mice lacking AQP3 develop diabetes insipidus, whereas AQP4 knockout mice exhibit defective gastric acid secretion, colon water transport, and altered skeletal muscle water permeability. These recent transgenic animal studies further highlight the important physiologic role of aquaporins in transmembranous fluid transport in various biologic systems.

Amniotic fluid (AF) volume is critical for normal fetal movement, growth, and development. Abnormalities of AF volume (ie, oligohydramnios, polyhydramnios) are common in human pregnancy. Polyhydramnios occurs in 0.2% to 1.6% of pregnancies, whereas oligohydramnios occurs in 8% to 38% of all pregnancies. Both poly- and oligohydramnios are associated with significant perinatal morbidity and mortality. AF is produced by fetal urine and lung liquid, and reabsorbed in part by fetal swallowing. The intramembranous pathway (ie, AF absorption across amniotic membranes) has been recognized as a critical regulatory path for AF volume changes with gestation. Therefore, AQP9 may be a critical water channel in fetal membrane fluid absorption.

To date, our knowledge of intramembranous AF regulation has been primarily obtained from the in vivo study of sheep. In sheep, though not humans, an extensive fetal vasculature network is situated between the amnion and chorion, potentially facilitating AF absorption across the amniotic membrane. In the current study, we sought to clone the full-length ovine AQP9 complementary DNA (cDNA) and examine the location of AQP9 messenger RNA (mRNA) in ovine fetal membranes and placenta. Cloning and characterization of AQP9 expression in ovine fetal membrane will establish an ideal animal model for the study of AQP9 in AF regulation and further explore the possibility of AQP9 as a therapeutic target for the treatment of oligohydramnios and polyhydramnios.

Material and methods

Ovine fetal membranes and other tissues

Animal study protocol was approved by the Animal Care and Use Committee at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Five ewes at 135 ± 2 days gestation (term = 145-150 days) were euthanized and amnion, chorion, allantois, and placenta were dissected and quick frozen in liquid nitrogen. Tissues were stored at −70°C until further analyses. Liver and kidney were obtained from one 1-month-old neonatal sheep as control tissues for the characterization of ovine AQP9 gene expression. Total RNA was isolated from these tissues with the use of Trizol reagent (Invitrogen, Carlsbad, Calif) for reverse transcriptase polymerase chain reaction (RT-PCR) and Northern analysis.

Homology RT-PCR cloning of ovine AQP9 cDNA

To clone ovine AQP9, we used the homology RT-PCR with primers derived from conserved sequences among human, mouse, and rat AQP9. The sequences of the sense and antisense primers were “ATCCTGAATTTGCTCTTG-GATGT” and “ACAGGAATCCACCAGAAGTT”, respectively. RT-PCR was performed with RNA isolated from ovine liver, as liver has been shown to express most abundant AQP9 in other species. RT-PCR methods were as detailed later, with the exception that only these 2 primers (10 pmols/reaction) were included. RT-PCR fragments were gel purified by using Qiagen gel purification kit (Qiagen Inc, Valencia, Calif) and the fragments were sequenced by dye terminator cycle sequencing with both forward and reversed primers in ABI 3100 DNA.
Analyzer (Applied Biosystems, Inc, Foster City, Calif). Sequence comparisons were performed with the NCBI BlastN program.

**Rapid amplification of cDNA end (RACE)**

On cloning of partial ovine AQP9 cDNA by homology RT-PCR, we cloned full-length ovine AQP9 cDNA by RACE procedures using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, Calif) with minor modifications of the manufacturer’s suggested protocol. To increase the success rate for RACE, total RNA isolated from sheep liver was used as template to synthesize the first strand 5' and 3' - RACE cDNA. The sequences of the gene specific primers for 5' and 3' - RACE PCR were “GAAACAGCTGGGTTGATGTGG” and “AGAGCCTGTTGTCATTGGC,” respectively. The RACE PCR fragments were gel purified by using Qiagen gel purification kit (Qiagen Inc), and the fragments were sequenced by dye terminator cycle sequencing in ABI DNA sequencer 3100 (Applied Biosystems, Inc). Nucleotide and deduced amino acid sequences comparison were performed with NCBI BlastN and BlastP program, respectively.

**RT-PCR**

Primers for AQP9 were “GGAGGGGTCATCACTATCAAT” and “ACAGGAATCCACCAGAAGTT,” and “ATCGTGATGGACTCCGGTGAC” and “GCTGATCCATCTGCTGGA” for β-actin, respectively. These primers are located in different exons, thus able to discriminate the PCR products from genomic DNA versus cDNA. These primers’ sequences are conserved in human, mouse, rat, and sheep genes. Thus, this multiplex RT-PCR can be used to study AQP9 gene expression in these species. The RT-PCR fragment size of sheep β-actin mRNA and AQP9 is 831 base pair (bp) and 581 bp, respectively. RT-PCR was performed as previously described.18

**Northern analysis**

For Northern analysis, biotin-labeled ovine AQP9 cDNA probe was prepared with the use of gel-purified RT-PCR-amplified ovine AQP9 fragment as a template for in vitro random-primed synthesis by Bioprime DNA Labeling Systems (Invitrogen). Fifteen micrograms of total RNA per sample were subjected to Northern analysis according to the standard protocol.18 Signals were analyzed with Bio-Rad MultiImager and Quantity One software (Bio-Rad Laboratories, Hercules, Calif). Membranes were stripped of AQP9 probes in solution containing 0.02 × SSC and 0.01% SDS at 95°C for 15 minutes and rehybridized with biotin labeled β-actin probe to normalize AQP9 mRNA level.

**Immunohistochemical staining**

Polyclonal primary antibody for AQP9 was purchased from Alpha Diagnostics (San Antonio, Tex). The AQP9 antibody was raised in rabbits immunized with a polypeptide of 16 amino acids derided from carboxyl terminal of rat AQP9 that has 60% homology to ovine AQP9. An immunohistochemical staining kit (Vector Laboratories, Burlingame, Calif) was used as previously described.18 After incubation of the primary antibody, the tissue was washed and incubated with the biotin-labeled secondary antibody. This was followed by a wash and then visualized with an avidin-biotin complex immunoperoxidase system (Vector Laboratories) by using 0.03% diaminobenzidine as the chromagen and hematoxylin as the counterstain. Protein expression signal was studied under light microscope. Negative controls were performed in parallel by using the primary antibody that was preabsorbed with the polypeptide used to immunize the rabbit for the production of the anti-AQP9 antibody.

**Results**

**Cloning of ovine AQP9**

We cloned a full-length 2085-base ovine AQP9 cDNA. The ovine AQP9 cDNA encodes for a protein with 295 amino acids. The nucleotide and the deduced amino acid sequences are shown in Figure 1. As expected, the deduced protein contains 2 NPA motifs, a characteristic of AQP protein. We performed sequence similarity analysis between this gene with known AQP9 cDNA of other species. The nucleotide sequence of this fragment is 86% identical to human and 82% identical to mouse and rat AQP9 cDNA (data not shown). The predicted amino acid sequence of this cloned ovine gene is 78%, 73%, and 72% homologous to human, mouse, and rat AQP9 protein (Figure 2), respectively. The extensive homology to AQP9 of other species is consistent with our conclusion that this 2085-base cDNA is ovine AQP9.

**RT-PCR**

To determine AQP9 gene expression in ovine fetal membranes, we carried out RT-PCR analysis of RNA isolated from tissues of 135-day-old ovine fetuses. Liver and kidney RNA from 1-month-old lamb were also studied as positive and negative controls, respectively. As expected, RT-PCR detected AQP9 in liver. In all fetal membrane tissues of the 5 fetuses studied, AQP9 mRNA was detected in amnion and allantois, but not in chorion, placenta, umbilical cord, or kidney (Figure 3).

**Northern analysis**

We further studied the AQP9 mRNA expression in ovine fetal membranes using Northern analysis.
Figure 1  Nucleotide sequence and deduced amino acid sequence of the cloned ovine AQP9. A polyadenylation signal consensus sequence is underlined. The conserved NPA motifs are in bold face.
Consistent with the RT-PCR results, AQP9 mRNA expression was detected in amnion, allantois, and liver, but not in chorion, placenta, umbilical cord, or kidney (Figure 3). As expected, AQP9 mRNA expression was much stronger in liver, with lesser though similar AQP9 gene expression levels detected in amnion and allantois. There was no alternative spliced AQP9 transcript detected in the Northern analysis. The AQP9 mRNA expression in amnion and allantois detected by Northern analysis was consistent among the 5 fetuses studied.

**Immunohistochemistry**

To determine the cellular expression of AQP9 protein, immunohistochemical staining of ovine amnion and allantois with anti-AQP9 antibody were performed. As shown in Figure 4, immunohistochemical staining with anti-AQP9 antibody detected positive signals in both apical and basal membranes of epithelial cells of amnion and allantois. In addition, slight intracellular staining in amniotic and allantoic epithelia was observed. To the contrary, negative controls performed with AQP9 antibody preabsorbed with the AQP9 polypeptide showed no staining, demonstrating that the antibody recognizes specifically the AQP9 protein. Consistent with the Northern analysis, the AQP9 protein expression level was similar between amnion and allantois, and consistent among all 5 fetal tissues studied.

**Comment**

AF absorption across chorioamniotic membranes and/or the placenta surface plays a critical role in AF volume regulation. In sheep, the chorioamniotic membranes are highly permeable to water as well as urea. The microscopic structure of ovine amnion and allantois reveal typical membrane characteristics of transporting epithelia. In humans, water transport across the chorionic plate occurs at a much higher rate than glucose. However, the molecular mechanism(s) for such elevated fetal membrane permeability to water is poorly understood. Over the past decade, it has been increasingly recognized that aquaporins are important water channels that permit high-cellular membrane permeability to water. AQP9 is highly permeable to water and urea. We previously reported that among 11 AQP5s studied, the AQP9 is the 1 with highest level of
expression in human fetal membranes and placenta, suggesting AQP9 may be an important water channel mediating intramembranous human AF resorption. In the current study, we aimed at cloning ovine AQP9 cDNA and characterizing its expression in fetal membranes. Because human, mouse, and rat AQP9 cDNA have been cloned and found to be highly conserved among these species, we first took the homology RT-PCR approach to clone a partial ovine AQP9 cDNA sequence. We then used 5' and 3' RACE strategies to attain and sequence the 5' and 3' end of the ovine AQP9 cDNA. By using these highly efficient methods, we have cloned a full-length 2085-base ovine AQP9 cDNA that is predicted to encode for a protein of 295 amino acids. The predicted ovine AQP9 protein contains 2 NPA motifs, a characteristic of AQP protein. Furthermore, the nucleotide sequence and the predicted amino acid sequences of the cloned ovine sequence are highly homologous to AQP9 of other species. On the other hand, the nucleotide sequence of the cloned fragment is only 30% to 50% identical to the 6 known ovine AQPs, AQP 1 through 5 and 824-28 (data not shown). Therefore, the 2085-base cDNA we cloned is indeed the ovine AQP9.

AF volume changes dramatically during human pregnancy. Average AF volume increases progressively from approximately 20 mL at 10 weeks to a peak volume of 750 to 1000 mL at 30 to 37 weeks' gestation and decrease sharply postterm. Recognizing that the AF volume is relatively stable during the near-term period of human pregnancy, we selected to study AQP9 expression at approximately 0.9 gestation (135 ± 2 days) ovine fetal membranes. In this study, the RT-PCR and Northern analysis both detected AQP9 mRNA expression in near-term ovine amnion and allantois. We further determined that AQP9 protein is expressed in epithelial cells of amnion and allantois by immunohistochemistry. In our most recent study, AQP9 gene expression was detected in human amnion, chorion, and placenta. The findings of only amnion and allantois AQP9 expression in sheep may represent the species variation. In humans, the amniotic membrane is avascular with no significant vascularity between the amnion and chorion. Thus, AF water and solutes must cross both amnion and chorion (or chorionic plate of placenta) to be absorbed into the circulation. Conversely, in sheep there is an extensive fetal microvessel network underneath the amniotic membrane. Therefore, water and solutes may be absorbed into fetal vaculature after crossing the ovine amnion epithelial cells. In sheep, fetal urine is excreted into both amniotic and allantoic sacs. As there is no other fetal route of fluid resorption, AQP channels in the allantoic membrane likely contribute to resorption of water and urea from the allantois. The observations that AQP9 is expressed in ovine and human amnion, ovine allantois, and human chorion and placenta, are consistent with the notion that AQP9 is important for both AF and allantoic fluid water and solute absorption across fetal membranes.

Despite the critical importance of intramembranous pathway in AF volume homeostasis, there have been limited reports delineating the molecular mechanism for water resorption through fetal membranes. Previously, AQP3 expression was demonstrated in cytotrophoblasts of ovine chorion and placenta, as well as fibroblasts of amnion and allantois. These authors also reported AQP1 in vascular endothelia of chorion and placenta. Whereas AQP1 expression was detected in human amnion and chorion, AQP3 was only found by RT-PCR and Western blotting analysis of chorion, but not in amnion, suggesting lower levels of AQP3 expression. Our recent study demonstrated AQP9 expression in human chorioamniotic membranes. The consistency of AQP9 gene expression in both human and ovine fetal membranes suggests that AQP9 may be a critical water channel regulating AF resorption through the intramembranous pathway.

Previous reports have suggested that AF volume changes during gestation may be dependent on alterations in solute as well as water permeability. The permeability of ovine amnion to urea decreases markedly with advancing gestation, correlating inversely with the increased AF urea concentration. As AQP9 functions as both a urea and water channel, one may postulate that amnion AQP9 expression is reduced near term, resulting increased AF urea concentration; whereas increased expression of alternative AQPs may enhanced intramembranous water flow and contribute

Figure 3 RT-PCR (A) and Northern analysis (B) analysis of AQP9 gene expression in ovine amnion, chorion, allantois, placenta, and umbilical cord.

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to reduced AF volume postterm. Further ontogenic study of AQP9 expression in ovine fetal membranes and correlation of the AQP9 level with the AF volume and composition changes throughout gestation will provide insight to this hypothesis.

In summary, we have cloned the full-length cDNA sequence of ovine AQP9 and demonstrated its mRNA and protein expression in ovine amnion and allantois. Together with the demonstrated AQP9 function as a water and neutral solute (including urea) channel, and our prior report of the high level of AQP9 expression in human fetal membranes, this study provides further evidence supporting our hypothesis that AQP9 is a major water and solutes channel mediating intramembranous AF fluid absorption. Cloning and identification of AQP9 expression in ovine fetal

Figure 4  Immunohistochemical analysis of AQP9 protein expression with anti-AQP9 antibody (left panel) and negative control (right panel) in ovine amnion and allantois. Brown stains are positive signals. Tissues were counter stained with hematoxylin (purple). (Original magnifications: ×200.)
membranes is a critical step toward establishing an animal model, allowing for future in vivo studies of the role of AQP9 in intramembranous AF transport. Although beyond the score of the current article, further studies are needed to (1) address the physiologic function of AQP9 in fetal membrane fluid transfer by determining whether amniotic fluid reduction and amniotic fluid volume loading will alter fetal membrane AQP9 expression in the sheep, (2) explore whether small molecule and endocrine factors (eg, vasopressin) can alter AQP9 expression in fetal membrane and change amniotic fluid dynamic, and (3) investigate the possibility of altering AQP9 expression as a therapeutic approach to oligohydramnios and polyhydramnios.

References

Limited differentiation to neurons and astroglia from neural stem cells in the cortex and striatum after ischemia/hypoxia in the neonatal rat brain

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Differentiation

Objective: We examined whether progenitor neural stem cells can differentiate successfully into mature neurons and astrocytes in a rat model of neonatal hypoxic-ischemic encephalopathy.

Study design: Seven-day-old Wistar rats were subjected to hypoxic-ischemic stress. At days 5 to 7 after hypoxic-ischemic stress, 5-bromodeoxyuridine (an early marker of cell proliferation) was injected, and the brains were retrieved at 14, 28, and 42 days after hypoxic-ischemic stress. Immunohistochemical and immunofluorescent studies were carried out for 5-bromodeoxyuridine, neuronal nuclear antigen (a marker protein of matured neuron), and glial fibrillary acidic protein (a protein marker of mature astrocytes).

Results: Only 1% of neuronal nuclear antigen–positive and 4.6% of glial fibrillary acidic protein–positive cells could be detected among the 5-bromodeoxyuridine-immunopositive cells in the peri-infarcted area of the cortex and the striatum, respectively, at 14 days after hypoxic-ischemic stress. There were no such double-staining cells at 28 and 42 days after hypoxic-ischemic stress.

Conclusion: The intrinsic ability for neurologic self-repair was limited at the maturation step after hypoxic-ischemic stress in the neonatal rat brain.

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Although there have been enthusiastic efforts to set up new therapeutic modalities for the treatment of perinatal hypoxic/ischemic (H/I) brain damage (such as brain hypothermia or special medication), there is no standard therapy that has been established for this disorder. Many infants with this problem have permanent neurologic deficits (such as cerebral palsy, seizure, sensory deficit, and mental retardation). Such chronic states of defect do not seem to be amenable to conventional therapy. Recent progress in regenerative medicine and in our understanding of neurogenesis may lead to the
recovery of neuronal functions that were lost by perinatal H/I-induced brain injury, with stem-cell therapy.

Neurogenesis comprises at least 3 processes: cell proliferation, migration, and differentiation.3,4 Recently, some research institutes, which include our laboratory, reported evidence of cell proliferation and migration during neurogenesis, with the use of a rat model of neonatal H/I encephalopathy that leads to unilateral brain damage. There were significant increases in proliferation of the neural stem cells in the subventricular region, which is the most important germinal matrix of the rat, not only on the injured side but also on the noninjured side.5 Immunohistochemistry for doublecortin, which is a marker of migrating neuronal precursors,6,7 showed significant migration of doublecortin-positive newly divided cells gathering around the infarction in the injured side, but not in the noninjured side (unpublished data).

These results encouraged us to test whether these progenitor neuronal cells could differentiate successfully into mature neurons. In the present study, we used a thymidine analog, 5-bromodeoxyuridine, as an early marker of cell proliferation in the normal adult rodent brain and antibodies against neuronal nuclear antigen (NeuN),8,9 which is a marker protein of mature neuron, and glial fibrillary acidic protein (GFAP), which is a marker protein of mature astrocytes.

**Material and methods**

**Animal model**

The Animal Research Committee of Okayama University approved this study. Pregnant Wistar rats were purchased from Japan Charles River (Shizuoka, Japan). On postnatal day 7, each pup was subjected to a modified Levine procedure to produce H/I brain injury.10 Briefly, the pups were anesthetized with ether, and the left common carotid artery was sectioned between double ligatures of 4-0 surgical silk. The pups were allowed to recover for 2 hours and were then exposed to an additional 2 hours of hypoxia (8% oxygen, 92% nitrogen) in a plastic container at 33°C. Sham-operated control animals were treated identically, except for the section of the left carotid artery and the subsequent hypoxia.

**Bromodeoxyuridine labeling and experimental design**

A cell proliferation marker, bromodeoxyuridine (Sigma Chemical Company, St. Louis, Mo), was dissolved in 0.9% saline solution. Bromodeoxyuridine (50 mg/kg) solution was injected intraperitoneally twice daily for 3 days from 5 to 7 days after the H/I treatment (ie, on postnatal days 12-14; Figure 1 ). We chose days 5 to 7 after H/I treatment because this time frame is within the maximal period of H/I-induced cell proliferation in our previous study and to avoid labeling proliferating glia.5 The animals were killed 14 days after H/I treatment (postnatal day 21), 28 days after H/I treatment (postnatal day 35), and 42 days after H/I treatment (postnatal day 49). Each group contained 6 pups. For each group of rats, a sham control group also received bromodeoxyuridine without the H/I insult (6 in each group).

**Tissue preparation**

The pups were killed under anesthesia with pentobarbital (50 mg/kg, intraperitoneally) and were then perfused transcardially with phosphate-buffered saline solution followed by 4% paraformaldehyde in phosphate-buffered saline solution (pH 7.4). The brains were removed, postfixed overnight, and rapidly frozen in 2-methylbutane that was chilled by liquid nitrogen after cryoprotection.
The brains were cut serially into 10-μm-thick coronal sections on a cryostat at −30°C, and the sections for caudate and dorsal hippocampus levels were mounted on glass slides and stored at −80°C until used for immunohistochemistry.

Immunohistochemistry

For immunohistochemical detection of bromodeoxyuridine labeling, the sections were incubated in 1N HCl at 65°C for 1 hour to denature DNA and then rinsed in 0.1 mol/L boric acid (pH 8.5) at 25°C for 10 minutes. Sections were incubated with mouse monoclonal anti-bromodeoxyuridine immunoglobulin G (IgG; 1:200; Oncogene Research Products, Boston, Mass) overnight at 4°C and were then incubated with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, Calif) for 1 hour at room temperature.

Immunoreactivities were developed in a horseradish peroxidase-streptavidin-biotin complex solution ( Vectastain ABC Kit; Vector Laboratories) for 30 minutes and were incubated for 5 minutes in a peroxidase reactive solution (0.02% diaminobenzidine, 0.02% H2O2). Peroxidase staining was examined with an Olympus microscope (BX-51; Olympus, Tokyo, Japan).

Double immunofluorescence

For double immunofluorescence detection of bromodeoxyuridine and NeuN, DNA-denatured sections were first incubated with sheep polyclonal IgG anti-bromodeoxyuridine (1:500; Biodesign, Saco, Mass) and mouse monoclonal IgG anti-NeuN (1:500; Chemicon, Temecula, Calif) overnight at 4°C and were then incubated with FITC-labeled donkey anti-sheep IgG (1:500; Molecular Probes, Eugene, Ore) and rhodamine-labeled goat anti-mouse IgG (1:500; Chemicon) for 1 hour at room temperature. We optimized the titration of 2 different antibodies for bromodeoxyuridine to obtain the equivalent sensitivity and specificity.

For double immunofluorescence detection of bromodeoxyuridine and GFAP, DNA-denatured sections were first incubated with mouse monoclonal IgG anti-bromodeoxyuridine (1:200; Oncogene Research Products) and goat polyclonal IgG anti-GFAP (1:200; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) overnight at 4°C and were then incubated with FITC-labeled horse anti-mouse IgG (1:500; Vector Laboratories) and Alexa Fluor 546-labeled donkey anti-goat IgG (1:500; Molecular Probes) for 1 hour at room temperature. The specificity of primary antibodies that we used was tested by omission of primary antibodies.

Sections were scanned with a confocal microscope that was equipped with argon and HeNe1 lasers (LSM-510; Zeiss, Jena, Germany). Sets of fluorescent images were acquired sequentially for the red and green channels to prevent crossover of color signals.

Cell counting

For single immunohistochemical staining, the numbers of bromodeoxyuridine-labeled cells in each side of the cortex and striatum were counted in 5 coronal sections (10 μm thick, spaced 50 μm apart) for each animal. Each 25 square areas of 660 × 660 μm were selected randomly from the cortex and striatum just adjacent to the infarcted area (that is, peri-infarcted areas). For cells that were immunofluorescently positive for both bromodeoxyuridine and NeuN or for bromodeoxyuridine and GFAP, the areas around the region of infarction were photographed at 460.6 × 460.6 μm for 5 of the coronal sections.

Statistical analysis

Cell numbers are given as means ± SDs. We compared the sham-operated controls with the ipsilateral and contralateral sides of the H/I-treated rats. Differences were tested with analysis of variance, followed by Tukey’s test for post-hoc analysis to evaluate intergroup differences. A probability value of <.01 was assumed to be statistically significant.

Results

All the rats survived from the time of H/I insult until the time of death. All brains of H/I-treated rats were damaged macroscopically on the left side.

Histologic changes

In the cortex, the H/I-treated rats showed a specific type of damage: Comb-like columnar degeneration appeared perpendicular to the cortical surface, with alternating regions of preserved neurons and gliosis. Bromodeoxyuridine-positive cells predominated in the areas of gliosis (Figure 2, A, B, and C). In regions with massive tissue infarction, bromodeoxyuridine-positive cells surrounded the infarcted area. In the striatum, the lateral-odorsal portion was predominantly affected, and bromodeoxyuridine-positive cells were identified mainly in the damaged areas where neuronal cells were sparse (Figure 2, D, E, and F).

Numbers of bromodeoxyuridine-positive cells

The number of bromodeoxyuridine-positive cells in the ipsilateral cortex of rats at 14 days after H/I treatment was significantly higher than that in the contralateral cortex of rats at 14 days after H/I treatment or in the cortex of the control rats (121.2 ± 24.8/mm2 vs 31.5 ± 6.9/mm2 and 26.6 ± 10.1/mm2, respectively [P < .01]; Figure 3). The numbers of bromodeoxyuridine-positive cells in the ipsilateral cortex decreased to 43.4 ± 20.9/mm2 and 37.2 ± 20.0/mm2 at 28 days after H/I.
treatment and 42 days after H/I treatment, respectively. On these days, the ipsilateral cortex still showed significantly more bromodeoxyuridine-positive cells compared with the contralateral cortex and the control cortex (43.4 ± 20.9/mm² vs 10.1 ± 4.4/mm² and 8.7 ± 2.8/mm², respectively [P < .01 at 24 days after H/I treatment]; 37.2 ± 20.0/mm² vs 6.2 ± 3.2/mm² and 4.1 ± 2.5/mm², respectively [P < .01 at 24 days after H/I treatment]).

The number of bromodeoxyuridine-positive cells in the ipsilateral striatum decreased to 103.8 ± 71.6/mm² at 28 days after H/I treatment and 83.3 ± 47.1/mm² at 42 days after H/I treatment. On these days after H/I treatment, the ipsilateral striatum still showed significantly more bromodeoxyuridine-positive cells compared with the contralateral striatum and the control striatum (103.8 ± 71.6/mm² vs 7.6 ± 5.1/mm² and 9.0 ± 6.0/mm², respectively [P < .01 at 24 days after H/I treatment]; 83.3 ± 47.1/mm² vs 3.7 ± 3.0/mm² and 6.2 ± 7.1/mm², respectively [P < 0.01 at 42 days after H/I treatment]).

**Double immunostaining for bromodeoxyuridine and NeuN or GFAP**

Cells staining for both bromodeoxyuridine and NeuN were observed sparsely in the ipsilateral cortex at 14 days after H/I treatment, but not at 28 days after H/I treatment or 42 days after H/I treatment. These cells resembled mature neurons and had unipolar or bipolar cell shapes (Figure 4, A-F). Such double-positive cells were never found in the contralateral cortex or other areas, including the striatum, at any days after H/I treatment. The concentration of such double-positive cells was 1.2 ± 2.2/mm²; thus on average, the bromodeoxyuridine-positive cells that were seen in the ipsilateral cortex of rats at 14 days after H/I treatment contained 1% of cells that were also positive for NeuN.

Cells that were immunopositive for both bromodeoxyuridine and GFAP were observed sparsely in the ipsilateral striatum at 14 days after H/I treatment, but

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**Figure 2** Immunohistochemistry for bromodeoxyuridine (BrdU) in the cerebral cortex (CTX; A to C) and striatum (ST; D to F) on the left (infarcted) side in coronal sections of H/I-treated rats at 14 days (A, D), 28 days (B, E), and 42 days (C, F) after H/I insult. Scale bar = 200 μm.
not at 28 days after H/I treatment or 42 days after H/I treatment. These resembled mature astroglia (Figure 4, C). Such double-positive cells were never found in the contralateral striatum or other areas, including the cortex, on any day after H/I treatment. The concentration of such double-stained cells was $9.4 \pm 7.7/\text{mm}^2$, or 4.6% of the bromodeoxyuridine-positive cells.

Comment

It was unexpected and rather disappointing that only 1% of mature neurons (NeuN immunopositive) could be detected among the bromodeoxyuridine-immunopositive cells in the infarcted and glial cell–replaced areas in the cortex of rats at 14 days after H/I treatment. The bromodeoxyuridine-positive cells represented those cells that would have divided 7 to 9 days previously (i.e., 5 to 7 days after the H/I insult), when cells would have been undergoing most proliferation in the subventricular zone. This cohort of cells must have migrated to the infarcted area in the cortex at 7 days after I/H insult. Our unpublished study showed that, at 7 days after H/I treatment, 108 of 456 bromodeoxyuridine-positive cells around infarcted area were immunopositive for double-cortin, which indicated that 23.7% of newly generated neural stem cells had become neural progenitor cells (Figure 5). At 14 days after H/I treatment, however, only a single cell differentiated into a mature neuron of 108 migrating neural progenitor cells. These few newly formed neurons could be found no more than 28 days after the H/I insult. This finding is consistent with the observation by Morshead and van der Kooy\textsuperscript{11} that most newly generated neurons in the adult appear to undergo programmed cell death rather than survive to make a neuronal network. Furthermore, the nuclear
morphologic condition of the cells that were doubly immunopositive for bromodeoxyuridine and NeuN suggested that they were some form of interneurons, not pyramidal neurons. Thus, it appears that these newly recruited neurons are not eligible to connect with other neurons to make a neural network or to contribute even partially to a functional recovery. This significantly poor ability to differentiate contrasts with the successful differentiation into neurons and astroglia of externally injected human neural stem cells in experiments with

Figure 4  A-F, Double immunofluorescence for bromodeoxyuridine (BrdU; green) and NeuN (red) of the left (infarcted) cortex 14 days after H/I treatment. Bromodeoxyuridine-positive cells in A and NeuN positive cells in B were overlaid in C. In this photograph, 4 double immunopositive cells (arrows) were identified. However, the concentration of such double positive cells was $1.2 \pm 2.2/\text{mm}^2$ (mean $\pm$ SD) on average. D-F, Three-dimensional reconstruction shows co-localization of bromodeoxyuridine and NeuN. Higher magnification of boxed area in C. Bromodeoxyuridine positive cells in D and NeuN positive cells in E were overlaid in F. G-I, Double immunofluorescence for bromodeoxyuridine (green) and GFAP (red) of the left striatum at 14 days after H/I insult. Bromodeoxyuridine positive cells in G and GFAP positive cells in H were overlaid in I. Scale bars are 50 $\mu$m in A-C and G-I and 10 $\mu$m in D-F.
normally developing animal brains. This poor ability to differentiate may be explained by the poor environment of the unilateral infarcted cortical area in this H/I brain-damage model with neonatal rats. For example, there may be poor neural input and output or a poor humoral milieu (such as low levels of neurotrophic factors). The poor self-repairing ability in this model seems to be associated with the large size of cell degeneration. Daval et al reported complete self-repair in the hippocampus region CA1 of neonatal rats. In their study, transient hypoxic stress resulted in a rather small decrease in neuronal cells in this region. Therefore, our present H/I insult seems to be too severe for successful neuronal self-repair.

The severely limited ability to differentiate from neuroblasts to mature neurons that are shown in the present study implies that attempts to produce functional recovery by stem cell transplantation will be unsuccessful for treating neonatal H/I encephalopathy, although exogenous stem-cell transplantation in this model has not been attempted. Additional manipulations (such as the administration of neurotrophic factors or an enriched environment surrounding the infants) may be required to facilitate cell differentiation. Bromodeoxyuridine-labeled cells from the subventricular zone that co-expressed doublecortin, and later NeuN, appear to have migrated into the ischemic cortex, but not into the ischemic striatum. This finding is contrary to the results of Arvidsson et al. Differences in brain maturity in the models may explain this regional discrepancy, and further studies clearly are needed.

As for astroglisis, it was also unexpected that GFAP-positive cells, which were abundant through the cortex and the striatum on the ipsilateral side, did not show bromodeoxyuridine staining, except in the striatum at 14 days after H/I insult. Zaidi et al injected bromodeoxyuridine at either postnatal day 21 or 22 and killed animals at postnatal day 35 to allow proliferating glial cells to express markers of differentiated cells. In that study, some of the GFAP-positive cells were bromodeoxyuridine-positive not only in the ipsilateral but also in the contralateral side. In the present study, bromodeoxyuridine was injected at an earlier time, so glial cell precursors that formed immediately after H/I insult might not have survived.

Figure 5 Schematic explanation of limited differentiation from neural progenitor cells that are doubly immunopositive for 5-bromodeoxyuridine (BrdU) and doublecortin (DCX) to mature neurons positive for bromodeoxyuridine and NeuN. Our unpublished study showed that at 7 days after H/I treatment, 108 of 456 bromodeoxyuridine-positive cells that were around the infarcted area were immunopositive for doublecortin. At 14 days after H/I treatment, however, only a single cell differentiated into mature neuron of 108 migrating neural progenitor cells. PND, Postnatal day.
In conclusion, we believe that successful stem-cell therapy for treating neonatal H/I encephalopathy is limited by poor differentiation from neural precursors to mature neurons, at least in this animal model. New strategies to overcome this limitation will be needed.

References

Intrauterine therapy of goitrous hypothyroidism in a boy with a new compound heterozygous mutation (Y453D and C800R) in the thyroid peroxidase gene. A long-term follow-up

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KEY WORDS
Fetal goiter
Congenital hypothyroidism
Intrauterine therapy
TPO gene

We report the results of intrauterine L-thyroxine therapy, and the long-term follow-up in a fetus who presented at 32 weeks’ gestation with goitrous hypothyroidism, hyperextension of the neck, and polyhydramnios. Spontaneous delivery was possible and hypothyroidism improved.

Molecular analysis revealed a new compound heterozygous mutation (Y453D/C800R) in the TPO gene.
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Case report

A 34-year-old woman, gravida 3, para 2, presented with polyhydramnios and premature labor during the 32nd week of gestation. Her thyroid function and thyroid antibodies were normal, history revealed no exposure to goitrogens.

Fetal ultrasound (with an Acuson 128, Acuson, Mountain View, Calif) demonstrated a bilobed symmetric anterior neck mass measuring 4.5 × 8.0 cm. It caused hyperextension of the head, esophageal obstruction, and polyhydramnios, making spontaneous delivery impossible. Fetal blood sampling (W.H.) at 32 + 6 weeks revealed severe hypothyroidism with a serum total thyroxine (TT4) of 2.4 µg/dL (reference values 3.2-16 µg/dL, measured by enzyme immunoassay ES 600, Böhringer, Mannheim, Germany) and a thyroid-stimulating hormone (TSH) of...
> 200 mIU/mL (reference values 3-14 mIU/mL, measured by enzyme immunoassay ES 600, Böhringer), so that 250 μg L-thyroxine were injected intraamniotically.

Hypothyroidism had improved at 36+5 weeks with a normal TT4 of 4.3 μg/dL, and a TSH of 80 mIU/mL. At this time 200 μg L-thyroxine were injected into the umbilical vein. The procedure was repeated at 37+6 weeks with another 100 μg L-thyroxine. Thyroid function had improved further. TT4 was 6.3 μg/dL, TSH 36.6 mIU/mL. The goiter was reduced to 4.2 × 5.1 cm so that a male infant with a small goiter was born by uncomplicated vaginal delivery at 39+6 weeks. Cord blood showed a TT4 of 5.2 μg/dL (11.4-22.4 μg/dL), and an elevated TSH above 200 mIU/mL. Therapy was started with 25 μg L-thyroxine and adjusted according to weight and TSH levels during the following 25 years. IQ tests showed median values of 112. Because of sensorineural and high tone hearing loss, hearing aids were prescribed.

The first child was born at 36 weeks with Apgar scores of 4/8/9. The initial TSH screening was negative. When hypotonia, macroglossia, and constipation developed, hypothyroidism was diagnosed with a TSH of 272 mIU/mL and a slightly enlarged thyroid gland. L-thyroxine treatment was started at 3 weeks. Developmental tests showed an IQ of 80. Hearing function was normal at 18 years.

Informed consent was obtained from all individuals for molecular analysis. Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp Blood Kit (Qiagen, Hilden, Germany). The human TPO gene was amplified and sequenced directly using an automated sequencing system (A 377, Applied Biosystems, Weiterstadt, Germany). The 2 brothers and the mother showed a heterozygous TPO gene mutation in exon 9 (Y453D). Both brothers and the father were heterozygous carriers of another mutation in exon 14 (C800R). Thus, the children were compound heterozygous, the parents carriers of 1 affected allele.

Comment

Only a few cases of prenatal intraamniotic thyroid hormone treatment for fetal goitrous hypothyroidism have been reported.1-3 Fetal goiter decreased rapidly in size in all patients shortly after L-thyroxine therapy was given in 1 or more doses varying from 10 μg/kg to 500 μg.1-3

To our knowledge, our patient is the first who received intraumbilical injections of L-thyroxine. We observed that hypothyroidism improved, and thyroid volume decreased, alleviating esophageal obstruction and polyhydramnios.

We had the unique opportunity to compare the long-term outcome in our patient with the outcome in his older brother affected by the same mutation in the TPO gene, but starting L-thyroxine therapy postnatally at 3 weeks of age. The prenatally treated patient had higher IQ values, even though sensorineural hearing loss could not be prevented.

Prenatal thyroid hormone treatment can be considered in carefully selected patients with goitrous hypothyroidism, especially when spontaneous delivery is impossible. Intrauterine therapy should only be done by experienced physicians. It is unclear whether intrauterine L-thyroxine therapy improves central nervous system development, intellectual outcome, and auditory function. This case report demonstrates definite short-term and possible long-term beneficial effects of this therapy.

In goitrous familiar hypothyroidism, molecular analysis should be performed to identify the molecular defect so that parents and physicians are aware of the genetic risk for further pregnancies.

References

Syndrome of hemolysis, elevated liver enzymes, and low platelet count: A severe consequence of hypertension in pregnancy

Louis Weinstein


Fig. 1. Burr cells—crevated, contracted red blood cells with spiny projections along the periphery.

Fig. 2. Schistocytes—small, irregularly shaped red blood cell fragments.
Commentary by Lawrence D. Longo, MD

Hemolysis, in conjunction with abnormal liver function tests and thrombocytopenia, has been recognized for many years as a complication of severe preeclampsia-eclampsia. Yet it remained until 1982 for Weinstein, on the basis of 29 patients with these complications, to coin the term HELLP Syndrome (“H” for Hemolysis, “EL” for elevated liver enzymes, and “LP” for low platelet count). Such patients often constitute a diagnostic dilemma for the physician, who may view the hemolysis, thrombocytopenia, hepatic disorder with possible hemorrhage, and/or uremic syndrome as separate and/or unrelated entities (many patients do not, in fact, have hypertension). Patients with this syndrome are subject to increased risk of adverse outcomes, including death and/or death of her fetus/newborn infant, such as the one mother with severe preeclampsia and microangiopathic hemolytic anemia and 3 infants in the present account. Since this original description, and as of this writing, there have appeared over a thousand reports of many thousands of patients with this life-threatening disorder. Figure 1 depicts crenated erythrocytes (Burr cells or spinocytes), while Figure 2 illustrates the irregularly shaped schistocytes (split cells, also called helmet cells) typical of microangiopathic hemolytic and other anemias.1 Experimental evidence suggests this latter cell conformation is a consequence of interaction between red cells and fibrin strands under arterial flow conditions.2 Weinstein described the HELLP Syndrome while at the University of Arizona, Tucson. Presently, he is Chair of the Department of Obstetrics and Gynecology at the Thomas Jefferson University, Philadelphia.

References

It has been a great ride: The history of HELLP syndrome

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“It is circumstances and proper timing that give an action its character and make it either good or bad.”

Agesilaus (444–400 BC)

This quote, written in approximately 400 BC, is as true today as it was when it was stated. Timing is everything. There are many examples of the value of timing and the importance of observation that are applicable to medicine. In 1846, Ignaz Philipp Semmelweiss (1818-1865) was appointed first assistant (lecturer) in Division I (the lying-in hospital) of Vienna’s Allgemeines Krankenhaus [general hospital], where the medical students practiced obstetrics. It was in the hospital’s Division II, where obstetrics was practiced by the midwives, that Semmelweiss observed a much lower mortality rate among the patients who were delivered, as related to puerperal infection. The importance of observation, timing, and being in the right place presented itself when Semmelweiss’s colleague, Jakob Kolletschka, cut his hand while performing an autopsy on a postpartum patient who had died of childbed fever. Semmelweiss observed that Jakob’s friend’s death was identical to that of the women who died from puerperal fever. Semmelweiss observed that Jakob’s friend’s death was identical to that of the women who died from puerperal fever and that the cause was likely to be from the transmission of the putrid organic material from examiner to patient. By the initiation of the simple task that was instituted by Semmelweiss of scrubbing one’s hands

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in a chlorinated lime solution between patients, a marked decrease in the mortality rate was noted in the postpartum patients on the medical student ward. A similar story exists about the discovery of penicillin by Sir Alexander Fleming (1881-1955). It was his observation of the inhibition of the growth of bacteria around the site of a mold that was growing in an open glass dish that had been left in his laboratory that led to the discovery of the antibiotic penicillin, which revolutionized the treatment of bacterial infections.

Both of these examples demonstrate the power of being in the right place at the right time and making some simple observations. This principle presented itself during my career and led to my discovery of the variant of preeclampsia now called hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome.

In 1979 after completing my obligatory military service, I was recruited to the University of Arizona to be their first maternal-fetal medicine fellow. At that time, fellows functioned in an independent manner and took call independently as a faculty member. In early 1980, I received a telephone call from an Indian Health Service physician in Tuba City, Ariz, about transferring a patient to the University hospital in Tucson. The history of the patient was that she was at 29 weeks of gestation, had minimal elevation of blood pressure, 1 to 2 + proteinuria, blood sugar of 25 mg/dL (patient was lucid), platelet count of 52,000, hematocrit level of 30%, and abnormal liver function test results. It was being the attending physician on call that started my process of being in the right (or wrong) place at the right time.

Obviously, it was apparent to me on the telephone that the laboratory workup of the patient was in error and that I would correct this when she arrived. The patient, who was brought by air transport, arrived late in the afternoon. Her history and physical examination were unremarkable, except that she stated that she had felt sick for approximately 1 week and that she had experienced right upper quadrant pain for several days. Her blood pressure was 130/84 mm Hg, and she had 2 + proteinuria. She was lucid and able to answer all questions but was quite stoic, as were most of the Native American patients. As I was not able to determine the presentation of the fetus, an ultrasound examination was performed that revealed the fetus to be anencephalic. Laboratory testing was performed and revealed a blood sugar of 30 mg/dL, platelet count of 35,000, hematocrit level of 25% with schistocytes and Burr cells on peripheral smear, liver function test results that were 10 times normal, and an elevated bilirubin level, with the majority being indirect. She had no evidence of any active bleeding or uterine activity.

The clinical picture was very confusing to me, so I consulted 2 of the best physicians I have ever known, my mentors, C. Donald Christian and William Droegemueller. Neither of them was sure of the clinical diagnosis; but, because of the concern for acute fatty liver, we all agreed to proceed with delivery and to support the patient metabolically. We administered intravenous fluids, and labor induction ensued. Six hours later she was delivered of an anencephalic infant. Over the next 18 hours, the patient’s platelet count continued to decrease; her hematocrit level decreased because of hemolysis with no active bleeding, and she remained profoundly hypoglycemic but lucid. A solution of 10% dextrose was administered intravenously with no change in blood sugar levels; the plan was to consider plasmapheresis. Approximately 24 after delivery, she became comatose, experienced cardiorespiratory arrest, and was unable to be resuscitated.

Postmortem examination revealed a massively swollen liver with multiple petechial hemorrhages, a large quantity of ascites, a pancreas with internal hemorrhage, and no obvious cerebral findings. Microscopic examination revealed disruption of both liver and pancreas cells with no obvious necrosis. A small amount of fatty deposits were present in the liver, but these were not consistent with the diagnosis of acute fatty degeneration. Although the patient probably had preeclampsia, we had no adequate explanation for her death.

This had a terrible personal impact on me. I had never experienced a maternal death, and I felt that it was my fault that the patient died. I then decided to devote much of my time to determine the cause of her demise and to try to educate myself as to how to treat the next patient with similar findings.

My initial thought was that the patient had some variant of preeclampsia. I started by doing an extensive literature search and looking for any pregnancy that was complicated by hemolysis, abnormal liver functions, thrombocytopenia, and/or hypoglycemia that was associated with maternal death. I was amazed by the number of articles, many of which were not in the obstetric literature and many of which described patients similar to the one I had treated. The key article that described a similar entity was the report of 3 cases in the New England Journal of Medicine in 1954 by Pritchard et al from Texas. Their 3 patients all experienced eclampsia with 1 survivor. McKay in 1972, Kitzmiller et al in 1974, and Killam et al in 1975, all reported patients similar to the patient that I had treated, each being linked to preeclampsia. There were many other isolated reports in the medical literature of the presence of hemolysis and thrombocytopenia with various diagnoses of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. The surgical literature described the entity of spontaneous rupture of the liver that occurred in pregnant patients who all appeared to have findings that were compatible with preeclampsia. The paper that impacted me the most was by Goodlin in 1976 (“Severe pre-eclampsia: Another great imitator”).
It was becoming more obvious to me, even early in my career, that preeclampsia was truly the great imitator. Now that I am a senior clinician, I am absolutely convinced of this.

After I had finished educating myself by my literature review, I sent a notice to the physicians who were practicing obstetrics in Arizona that I was looking for pregnant patients with unexplained thrombocytopenia, hemolysis, and elevated liver enzymes and that I would care for them if they were referred. Over the next 30 months, I had the opportunity to care for 29 patients and to learn much about this variant of preeclampsia. As I gathered the information I had received together, I appreciated that I was seeing a form of preeclampsia that often did not have hypertension of >140/90 mm Hg, proteinuria, or edema. I was able to determine that the most of the women who were affected had generalized malaise during the week before hospital admission that was out of proportion with her being in the late second or early third trimester. Many of the patients were experiencing nausea with or without vomiting and right upper quadrant pain. Looking at the natural progression of this entity, it appeared that the thrombocytopenia occurred first, elevated liver enzymes second, and hemolysis third. Also, the disease was progressive, and delivery was the only means of ending the process. However, approximately 25% of the patients had their worst manifestations during the postpartum period. What became apparent to me was that I was seeing a variant of preeclampsia.

As I started to put together the data for the 29 patients that would result in the 1982 American Journal of Obstetrics and Gynecology article,3 I wanted to help educate my fellow clinicians to assist them in recognizing these patients to prevent what happened to the first patient who I had seen with this entity. It was my belief that I needed to have a distinctive name for this “syndrome” so that most physicians would not forget it once they heard it. The problem with these patients, I realized, was not how to care for them when diagnosed but how to recognize that they were sick and needed care. It was then that the light dawned on me that what these women needed was what the entity should be called; therefore, the term HELLP syndrome was born. I was pleased that this term was both descriptive and reminded the clinician of what to give to the patient. I then collected data for 57 patients, which resulted in a second article in 1985.9 My colleague at the University of Tennessee, Baha Sibai, went on to publish several larger series, because his patient volume was much greater than mine.10,11 What has been most interesting and gratifying since the original 1982 article is the plethora of papers that have been published about patients with HELLP syndrome. Little has changed in the description: The entity is still a variant of severe preeclampsia, and delivery remains the prime therapy.

Over my academic career, I have been called numerous times to discuss the treatment of patients with HELLP syndrome and have reviewed many medical records to determine whether negligent medical care occurred. Several observations that I have made are clearly repetitive in nature.

The first observation is that the disease is progressive, and often the patients do not look very sick. The second observation is that the major marker for death is the presence of hypoglycemia and that the blood sugar should be checked frequently during the labor process and that every attempt should be made to keep the blood sugar at >60 mg/dL. Often the patient will need an infusion of 10% dextrose or a push of 50% dextrose to maintain the blood sugar level. I believe that the hypoglycemia is related to decreased glycogen stores in the liver and increased levels of circulating insulin from disruption of the acini of the pancreas from cellular edema. The third observation is that I cannot tell from either the history or physical examination the levels of the patient’s platelet count or liver enzymes. Therefore, I often order these tests with minimal clinical indications other than the possibility of preeclampsia. The fourth observation is that, of all the records that I have reviewed of patients who died from HELLP syndrome, the most common family of drugs that all patients have received in the days before death is not an antihypertensive or magnesium sulfate but an antacid. Most, if not all, patients with HELLP syndrome complain of “heartburn” and therefore have been advised to take an antacid. If a patient complains of heartburn or right upper quadrant pain during the third trimester, clinical and laboratory evaluations are warranted. The key to the treatment of any patient with suspected HELLP syndrome is simply recognition. Once the clinician has identified that the entity is present and that it is a variant of severe preeclampsia, delivery can be expedited, with the prolongation of pregnancy offered only to administer antenatal steroids for the benefits of the developing fetus.

I am somewhat pessimistic that any direct single cure will be found for preeclampsia. I believe that it is a disease of the placenta and microvascular system that has an immunologic basis. It is a form of rejection of the maternal half of the fetal genetic makeup and signifies that it is best for the baby to leave the hostile intrauterine environment and began life in a brave new world.

Currently, much research is being performed to evaluate the immunologic basis for the disease, but little progress in the clinical management has ensued. I believe that the cure for preeclampsia will remain elusive but that we shall improve in prolonging the pregnancy and safely inducing labor when necessary. I have done many things during my academic career and am proud of my accomplishments and articles. The question we all need to ask during and near the end of our careers is “Did we truly make a difference?” I believe that my experience
with this patient who died allowed me to identify this entity and to make other health care providers aware of the serious nature of this disease so that proper medical treatment can be offered and morbidity and death can be avoided. I believe that the time and effort that I put into learning, writing, and teaching about HELLP syndrome have resulted in better care and possibly the saving of life for other patients. I am truly grateful to my teachers and mentors who planted within me the seed for scientific curiosity and the desire to make a difference. I can truly say that it has been a great ride.

References

Recombinant human activated protein C treatment of septic shock syndrome in a patient at 18th week of gestation: A case report

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Recombinant human-activated protein C (rhAPC) has been suggested to treat sepsis. We present the case of a 19-year-old pregnant patient at the 18th week of gestation with septic shock syndrome that originated from urinary tract infection and was successfully treated with rhAPC.

Sepsis during pregnancy carries a substantial risk for both mother and fetus. Recombinant human-activated protein C (rhAPC) therapy has been suggested to treat severe septic shock syndrome in nonpregnant patients. RhAPC treatment in pregnancy has been recently reviewed by von Dadelszen et al. Pregnancy was 1 of the exclusion criteria of rhAPC treatment in the PROWESS trial. However, obvious contraindications of rhAPC treatment during pregnancy are not known. We report on a successful rhAPC treatment of severe septic shock syndrome in a pregnant patient.

Case report

A 19-year-old patient at the 18th week of gestation was registered to the Department of Obstetrics and Gynecology because of back pain and fever. In 4 hours the symptoms of severe sepsis developed: she became unconscious, tachypnoeic, tachycardic, anuric, and her blood pressure was hardly measurable. Some of the baseline parameters are summarized in the Table. Examination of the urine sample confirmed Escherichia coli greater than $10^5$. Septic shock syndrome originating from urinary tract infection was diagnosed with at least 2 major (respiratory and circulatory) organ system failures with rapid progression. The patient was transferred to the intensive care unit, where ventilation, infusions, norepinephrine and dobutamine, hydrocortisone, furosemide, meropenem, and transfusion were given. The APACHE II score was 27. Despite the therapeutic efforts, the patient’s condition further deteriorated, thus rhAPC treatment was decided at the recommended dose of 24 $\mu$g/kg$^{-1}$ h$^{-1}$ and continued for 96 hours. After the administration of rhAPC, the patient’s general condition rapidly improved (see T$_{24}$ values in the Table). There was no sign of any adverse
effects of rhAPC treatment. The fetus had normal heart rate throughout the assessment. The fetus and the quantity of amniotic fluid were found to be normal by ultrasound before, during, and after rhAPC treatment. On day 7, the patient was discharged from the hospital and 21 weeks later, at the 39th week of gestation, she gave birth to a healthy female infant weighing 2900 g (cesarean section was performed because of fetal distress probably caused by the umbilical cord wrapped around the neck).

### Table

Baseline characteristics on admission (T0) and after 24 hours (T24)

<table>
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<th>Parameters</th>
<th>Values</th>
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<tr>
<td>Central venous pressure (mm Hg)</td>
<td>18 12</td>
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<tr>
<td>Central venous oxygen saturation (%)</td>
<td>53 78</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>120 108</td>
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<tr>
<td>PaO2 (mm Hg)</td>
<td>48 92</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
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<tr>
<td>actHCO3(^{-}) (mmol/L)</td>
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<tr>
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<td>C reactive protein (mmol/L)</td>
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<td>APACHE II score</td>
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</table>

### Comment

Severe septic shock syndrome was diagnosed in a 19-year-old patient at the 18th week of pregnancy that originated from a urinary tract infection. According to the “Surviving Sepsis Campaign” guidelines\(^1\) aggressive treatment was started. RhAPC treatment has been suggested to treat severe sepsis in nonpregnant patients.\(^1,2\) Because of the deteriorating condition of the patient, we started rhAPC administration. She rapidly improved and was soon discharged from the hospital. At the 39th week of pregnancy a healthy female infant weighing 2900 g was delivered by cesarean section because of fetal distress.

At present little is known about rhAPC treatment during pregnancy.\(^3\) In our case no adverse effects of rhAPC therapy on mother or fetus were detected.

According to our knowledge, this is the first report on rhAPC treatment for severe septic shock syndrome during pregnancy, with a favorable outcome for both mother and fetus, suggesting the effectiveness and safety of rhAPC therapy during pregnancy.

### References

Clinicopathologic features of six cases of primary cervical lymphoma

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KEY WORDS
Cervix
Lymphoma

Objective: Primary lymphoma of the uterine cervix is rare, with less than 60 cases reported. We present a series of 6 patients with cervical lymphoma and review the literature.

Study design: Between 1988 and 2003, we identified 6 women with primary lymphoma of the uterine cervix treated at our institutions. Data for analysis were obtained from hospital charts, office records, and tumor registry files. We also reviewed 20 published reports on cervical lymphoma, providing information on 58 additional patients.

Results: The median age at diagnosis was 52 years (range 40-76). Three patients had an abnormal Papanicolaou test within 6 months of the diagnosis. Mean tumor size was 8.3 cm (range 3-14 cm). On the basis of the Ann Arbor system of staging where “E” denotes extranodal tumor origin, 2 patients had stage IE, 1 had stage IIE, and 3 had stage IVE disease. The median follow-up for these 6 women was 33 months (range 12-120). Adding the 6 patients in our series to the 58 patients obtained from published reports, 43 had stage IE, 14 had stage IIE, 2 had stage IIE, and 5 had stage IVE disease. There was no consistent pattern of treatment identified from our literature review.

Conclusion: Primary lymphoma of the uterine cervix is a rare malignancy. Most patients present with stage IE disease. Women with localized disease typically respond to various combinations of surgery, chemotherapy, and radiotherapy. Combination chemotherapy with tailored radiotherapy appears to be the preferred treatment option in women with advanced disease.

Primary malignant lymphoma of the genital tract is a rare disease accounting for only 1% of extranodal lymphomas.1 Of these genital tract lymphomas, only 0.6% arise from the uterine cervix.2-4 Freeman et al5 reported a series of 1467 patients with extranodal lymphomas and found only 3 cases of primary cervical
lymphoma. Over the last 25 years, less than 60 cases of cervical lymphoma have been reported in the literature. The clinical presentation of primary cervical lymphoma often is similar to that of squamous cell carcinoma of the cervix. Most patients experience abnormal bleeding and have a large, bulky cervix on pelvic examination. Because cervical lymphomas typically arise within the cervical stroma rather than the mucosa, cytology is not a sensitive screening tool. Histologically, cervical lymphoma appears similar to lymphomas arising in other sites. It is important to make the correct diagnosis of this uncommon disease because the treatment for cervical lymphomas differs from more common cervical cancers. We report a series of 6 patients and provide a review of the literature on primary lymphoma of the uterine cervix.

### Methods

Between December 1988 and January 2003, 6 women with primary malignant lymphoma of the cervix were diagnosed and treated at University of California, Irvine Medical Center and Long Beach Memorial Medical Center. After Institutional Review Board approvals from both institutions, these patients were identified from tumor registry databases. All patients underwent staging with bilateral trephine marrow biopsy and computer tomography of the thorax, abdomen, and pelvis. On the basis of the Ann Arbor staging classification for extranodal lymphomas, stage IE is defined as the involvement of a single extra-lymphatic organ or site, stage IIE as a localized involvement of extra-lymphatic organ or site and of 1 or more lymph node regions on the same

<table>
<thead>
<tr>
<th>Table I</th>
<th>International working formulation for classification of Non-Hodgkin’s lymphoma</th>
</tr>
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<tbody>
<tr>
<td><strong>Low grade</strong></td>
<td></td>
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<tr>
<td>A</td>
<td>Malignant lymphoma</td>
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<tr>
<td></td>
<td>Small lymphocytic</td>
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<td></td>
<td>Consistent with chronic lymphocytic leukemia</td>
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<td></td>
<td>Plasmocytoid</td>
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<tr>
<td>B</td>
<td>Malignant lymphoma follicular, predominantly small cleaved cell</td>
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<td></td>
<td>Diffuse areas</td>
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<tr>
<td></td>
<td>Sclerosis</td>
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<tr>
<td>C</td>
<td>Malignant lymphoma follicular, mixed small cleaved cell and large cell</td>
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<tr>
<td></td>
<td>Diffuse areas</td>
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<td></td>
<td>Sclerosis</td>
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<td><strong>Intermediate grade</strong></td>
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<td>D</td>
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<td></td>
<td>Predominantly large cell</td>
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<td>Sclerosis</td>
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<td>E</td>
<td>Malignant lymphoma, diffuse small cleaved cell</td>
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<tr>
<td>F</td>
<td>Malignant lymphoma, diffuse mixed-, small- and large-cell sclerosis</td>
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<tr>
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<td>Epithelioid component</td>
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<td>G</td>
<td>Malignant lymphoma, diffuse</td>
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<td></td>
<td>Large cell</td>
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<td>Cleaved cell</td>
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<td>Noncleaved cell</td>
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<td></td>
<td>Sclerosis</td>
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<tr>
<td><strong>High grade</strong></td>
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<td>Follicular area</td>
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side of the diaphragm, stage IIIE as a localized involvement of extra-lymphatic organ or site and of lymph node regions on both sides of the diaphragm, and stage IVE as a diffuse or disseminated involvement of 1 or more extra-lymphatic organs or tissues with or without associated lymph node involvement. Furthermore, lymphomas are classified histologically according to the International Working Formulation (IWF) for non-Hodgkin’s lymphomas (Table I). Specimens were reviewed by the pathologists at the institutions where the patients were treated. To confirm the diagnosis of lymphoma, all pathologic specimens were subjected to immunohistochemical and molecular studies to identify the cellular lineage, in particular, to differentiate B-cell and T-cell lymphomas. The clinical presentation, stage of disease, medical/surgical treatment, recurrence, and survival data were collected from hospital charts, office records, and tumor registry files. We performed a review of the literature with 58 additional patients from 20 published reports using a Medline search.

**Results**

We identified 6 women with cervical lymphoma among 1330 treated for cervical cancers at the 2 institutions from 1988 and 2003. The patients’ characteristics are summarized in Table II. The median age at diagnosis was 52 years (range: 40-76). Of the 6 women, the median parity was 2. One patient used tobacco in the past. Two women had a previous history of gynecologic tract infection; one with genital herpes and the other with syphilis. Both women were treated without sequelae. The most common presenting symptom was abnormal vaginal bleeding, occurring in 4 of 6 patients. Two women had 1 or more B symptoms typically associated with lymphomas, which include weight loss, weakness, night sweats, and fever. Three women also had history of an abnormal Papanicolaou (Pap) test within 6 months of diagnosis, 2 with atypical squamous cells of undetermined significance and 1 with cytologic features of carcinoma in situ. All 6 patients had either a cervical or cervicopelvic mass ranging from 3 to 14 cm. The diagnoses of lymphoma were made based on cervical punch biopsies in 3 of 6 patients. Of 2 women with cervical biopsy specimens not showing a malignancy, one had a computer tomography–guided biopsy of the cervicopelvic mass and the other underwent an exploratory laparotomy, bilateral salpingo-oophorectomy to make the diagnoses of primary cervical lymphoma. The remaining patient had a cervical biopsy showing a poorly differentiated carcinoma and underwent a radical trachelectomy and pelvic lymphadenectomy. The correct diagnosis of lymphoma was made on the excised cervix. All patients underwent a metastatic workup that included chest x-ray film, computer tomography or gallium scan of the abdomen and pelvis, and bone marrow biopsy. The median time from initial presentation to diagnosis was 5 months (range: 1 to 8 months).

<table>
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<tr>
<th>Patients no.</th>
<th>1</th>
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<th>4</th>
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<th>6</th>
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<td>62</td>
<td>40</td>
<td>41</td>
<td>49</td>
<td>76</td>
<td>52</td>
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<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
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<td>Asian</td>
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<td>Menopausal status</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
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<td>HRT</td>
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<td>No</td>
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<td>Presenting complaints</td>
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<td>Vaginal bleeding</td>
<td>Severe weakness</td>
<td>Vaginal bleeding</td>
<td>Vaginal bleeding</td>
<td>Vaginal bleeding, weight loss and fatigue</td>
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<td>Abdominal pain</td>
<td>Abdominal pain, weight loss, fever, night sweats</td>
<td>Abdominal pain</td>
<td>Abdominal pain</td>
<td></td>
<td></td>
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<tr>
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<td>Not available</td>
<td>ASCUS</td>
<td>Normal</td>
<td>ASCUS</td>
<td>CIS</td>
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<td>6 cm cervical mass</td>
<td>12 cm pelvic mass</td>
<td>12 cm barrel-shaped cervix</td>
<td>6 cm cervical mass</td>
<td>3 cm cervical mass</td>
<td>14 cm pelvic mass</td>
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<td>IEA</td>
<td>IVEA</td>
<td>IIIEB</td>
<td>IVEA</td>
<td>IEA</td>
<td>IVEB</td>
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<td>J (high-grade, small cell, non-Burkitt)</td>
<td>G (intermediate, large cell, diffuse)</td>
<td>G (intermediate to high, large cell, diffuse)</td>
<td>I (high, lymphoblastic)</td>
<td>G (intermediate, large cell, diffuse)</td>
<td>D (intermediate, predominantly large cell)</td>
</tr>
</tbody>
</table>

HRT, Hormone replacement therapy; ASCUS, atypical cells of undetermined significance; CIS, carcinoma in situ.

**Table II** Clinicopathologic features and outcome of 6 cases of primary cervical lymphoma
<table>
<thead>
<tr>
<th>Author</th>
<th>No. patients</th>
<th>Ann Arbor and histology group</th>
<th>Therapy</th>
<th>Surgery</th>
<th>radiation</th>
<th>Chemotherapy</th>
<th>Recurrence</th>
<th>Survival</th>
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<td>Steinfield et al</td>
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<td>H BSO</td>
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<td>None</td>
<td>NED</td>
<td>9 mo</td>
</tr>
<tr>
<td>Tunca et al</td>
<td>1</td>
<td>IEA G</td>
<td>None</td>
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<td>Vincristine</td>
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<td>NED</td>
<td>2.5 y</td>
</tr>
<tr>
<td>Volpe et al</td>
<td>1</td>
<td>IIEA E</td>
<td>None</td>
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<td>6 mo</td>
<td>DOD</td>
<td>3 y</td>
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<tr>
<td>Harris et al</td>
<td>17</td>
<td>4 IEA B</td>
<td>H BSO</td>
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<td>None</td>
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<td>14 y</td>
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<td>C H BSO</td>
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<td>None</td>
<td>None</td>
<td>NED</td>
<td>13 y</td>
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<td></td>
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<td>None</td>
<td>NED</td>
<td>4 y</td>
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<td></td>
<td></td>
<td></td>
<td>D H BSO</td>
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<td>Yes</td>
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<td>NED</td>
<td>&lt; 1 y</td>
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<td></td>
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<td></td>
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<td>None</td>
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<td>9 y</td>
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<td>Pelvis + PA</td>
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<tr>
<td>Komaki et al</td>
<td>3</td>
<td>3 IIEA F</td>
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<tr>
<td></td>
<td></td>
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<td>G None</td>
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<td>3 y</td>
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<td>Pelvis, abdomen</td>
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<td>NED</td>
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<td>NED</td>
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<td></td>
<td></td>
<td>G None</td>
<td>Pelvis, inguinals</td>
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<td>IEA G</td>
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<td></td>
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<td>Biswal et al</td>
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<td>Never</td>
<td>NED (NA)</td>
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</table>
Of the 6 patients, 2 had stage IE, 1 had stage IIIE, and 3 had stage IVE disease. Two patients, with stage IIIE and IVE disease, presented with B symptoms. According to the IWF classification, 4 women had intermediate grade B-cell lymphomas, 3 with group G (diffuse, large cell), and 1 with group D (large cell). Moreover, 2 patients had high-grade B-cell lymphomas, 1 with group I (lymphoblastic) and 1 with group J (small cell, non-Burkitt). Of the 2 patients with stage IE disease, one had a cervical biopsy specimen that revealed a poorly differentiated carcinoma. Because she had a previous subtotal hysterectomy for leiomyoma, she underwent a trachelectomy with bilateral pelvic lymphadenectomy. The final diagnosis was consistent with a high-grade, small cell, non-Burkitt B-cell lymphoma. Adjuvant chemotherapy was delayed secondary to postoperative complications, including a distal colonic obstruction requiring a transverse loop colostomy and an acute cholecystitis for which she underwent a cholecystectomy. Three months after the primary surgery for her lymphoma, she experienced an intra-abdominal recurrence to the liver and spleen. She received 6 courses of bleomycin, doxorubicin, cyclophosphamide, and vincristine (BACOD) and remained free of disease for more than 6 years. She subsequently died of causes unrelated to her lymphoma. The other patient with stage IE lymphoma underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymphadenectomy. She received adjuvant pelvic radiotherapy and remains free of disease 14 months after her diagnosis.

The patient with stage IIIEB disease underwent 8 cycles of cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP). She had a disease-free interval of 29 months and recurred in the abdominal retroperitoneal lymph nodes. She was treated with CHOP and rituxan for her recurrence but died 11 months later. All 3 patients with stage IVE disease received combination chemotherapy. One was diagnosed with an intermediate grade (group G) disease and received 8 courses of CHOP with pelvic radiotherapy for a 12-cm pelvic tumor. She is disease free 10 years after her diagnosis. The other one with intermediate grade (group D) disease received 8 cycles of CHOP with rituxan and is alive without disease 1 year after her treatment. The remaining patient had high-grade (group I) disease for which she received 5 cycles of CHOP with rituxan. Because of persistent, bulky, cervical disease, she underwent a total abdominal hysterectomy. Postoperatively, she was treated with an additional 3 cycles of chemotherapy and is alive without disease 3 years after her diagnosis.

Immunohistochemical data were available in all 6 patients. All cases stained positive for CD-20. BCL-2 and monotypic immunoglobulin lambda light chain was found to be positive in 4 specimens and CD-19 in 3 specimens. In addition, 1 tumor sample was positive for CD-10, CD-23, and CD43. We obtained information on 58 additional patients from 20 published reports using a Medline search. Including the 6 patients in our series, 43 patients had stage IE, 14 had stage IIE, 2 had stage IIIE, and 5 had stage IVE disease. There was no consistent
pattern of treatment identified from the published reports (Table III).

Comment

Lymphoma of the uterine cervix is an uncommon disease that provides a diagnostic challenge for the clinician and the pathologist. Because the majority of patients present with abnormal vaginal bleeding, a gynecologist usually performs the initial evaluation and treatment. Because cervical lymphomas typically originate from the cervical stroma, the superficial squamous epithelium is often preserved, leading to a false-negative cytologic smear. Of 5 patients who had cytologic smears in our series, 2 were normal, 2 had atypical cells of undetermined significance, and the remaining 1 showed carcinoma in situ. Similarly, Harris et al found that only 5 of 10 patients in their study had a positive cytologic smear showing malignant cells. Although the Pap smear test can show false-negative results in patients with cervical lymphoma, 3 of 6 patients presented with abnormal vaginal bleeding, a clinician and the pathologist. Because the majority of case reports provided in this extensive literature review used the Ann Arbor staging system, we recommend a deep incisional or excisional biopsy to establish a definitive diagnosis when the initial biopsy is nondiagnostic. In such instance, the initial biopsy usually contains atypical epithelial cells coexisting with lymphoid infiltrates. The authors recognize that the Ann Arbor staging system has been replaced by the World Health Organization classification. Because the majority of case reports provided in this extensive literature review used the Ann Arbor staging system, we reported our series as such to provide a standard for comparison of the therapeutic outcome.

Primary cervical lymphomas have a rapid growth pattern. Six patients had a normal pelvic examination within the past year but presented with a 6-cm or greater cervical lesion. Muntz et al reviewed a series of patients with stage IE cervical lymphomas and found that half of these women had tumors larger than 4 cm at presentation.

Because cervical lymphoma is an uncommon malignancy, the standard treatment has not been established. Some authors have recommended primary surgical treatment for stage IE disease, whereas others have suggested combination chemotherapy with or without radiotherapy. Muntz et al advocated primary surgery, followed by individualized radiation therapy for those with stage IE disease localized in the pelvis. For women with stage IE diffuse bulky disease, they recommended high-dose radiotherapy with brachytherapy. On the other hand, others have suggested combination CHOP chemotherapy with radiation, particularly in patients with bulky stage I to II disease. Because the survival rates in patients with earlier stage cervical lymphomas are excellent with either surgery, chemotherapy, or radiotherapy, young women who desire to retain their fertility will benefit from combination chemotherapy.

On the basis of previous studies of patients with extranodal non-Hodgkin’s lymphoma, combination chemotherapy has been used in treating patients with disease that is more advanced than stage IE, with CHOP as the most common regimen used. All 4 women with stage IIIIE to IV disease in our series received the CHOP regimen, with or without surgery or radiotherapy. Three are alive without any evidence of disease at 1, 2.5, and 10 years after their diagnosis. The patient who died from her disease received CHOP after a disease-free interval of 29 months and died 11 months after her recurrence despite further treatment. Lastly, chemotherapy alone may not be sufficient in the treatment of stage II to IV disease with bulky tumors in the pelvis. Some authors have advocated surgery or radiotherapy in addition to combination chemotherapy to decrease the risk of central recurrence.

Malignant lymphoma of the uterine cervix is a rare disease. The diagnosis is typically made with a histologic analysis of a deep cervical biopsy. Patients with localized stage IE disease with nonbulky lesions often will respond to primary surgery, chemotherapy, or radiotherapy alone; however, combined therapy seems to be favored in many centers. Combination chemotherapy with tailored radiotherapy is the preferred treatment option in women with more advanced disease.

References

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Port site ischemic necrosis: An unforeseen complication of laparoscopic surgery

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KEY WORDS
Laparoscopy
Port site
Necrosis

A case of necrosis of the 10-mm umbilical port site after laparoscopic closure of enterocele in a 66-year-old woman is reported. Ischemic necrosis of the superficial tissue with cellulitis of the umbilical wound was seen during debridement. This highlights a hitherto unforeseen complication of laparoscopic surgery.

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Laparoscopic techniques have become the forefront of gynecologic operating in the past decade. In parallel with the increase in the number of procedures performed, we are also seeing an increase in different techniques and methods. In conjunction with the above mentioned rise, we are observing an expansion in the types of complications that could arise.

A hitherto unreported complication of ischemic necrosis of the tissue at the site of suturing of the port incision is reported.

Case report

A 66-year-old woman was referred in June 2003 for laparoscopic closure of enterocele. A 10-mm umbilical port was introduced for the laparoscope. An open laparoscopic entry (Hasson) was performed for entry into the abdominal cavity, and adhesions were found in the upper abdomen. There were no intra-operative complications. The 10-mm port incision was closed with 2 stitches of no. 1 vicryl.

She was discharged home as asymptomatic, on the third postoperative day. On the fifth postoperative day, the patient noticed pain and discoloration of the wound, which worsened over the next 48 hours. She was readmitted on the seventh postoperative day and the scar appeared to be tender, with areas of skin discoloration and erythema. Intravenous antibiotic therapy was commenced. She was seen by the surgeons on the tenth postoperative day and examination showed the wound to be necrotic with cellulitis and abscess formation.

Debridement of the wound was performed, no communication was seen intra-abdominally and the skin and superficial tissues, which were found to be necrotic, were excised. The postoperative period was uneventful.

Histology showed skin and subcutaneous tissue necrosis of 1.8 x 1 cm area, with abscess and granulation tissue formation.

Comment

An infective cause for the initiation of the wound necrosis was not considered during the management,
as the symptoms started with pain and discoloration of the skin. The clinical course suggested that infection was superimposed on to the necrosed tissue by the tenth postoperative day. Also, the patient was not diabetic and did not give a previous history of poor wound healing. Hence, an ischemic cause for the wound necrosis was considered.

The causative factors for postoperative ischemic necrosis would be difficult to ascertain, but it could be due to an inherent defect of the anterior abdominal wall anatomy and blood supply or it could be due to problems arising during the suturing. In the light of the above considerations, it might be more pertinent to use rapidly absorbable sutures in view of the fact that anatomic approximation for only a minimal number of days is necessary to facilitate complete healing. Also, techniques of closure could be modified with devices to enable approximation under better vision to ensure only the wound edges and not a wide area of tissue is taken in the suture.

Complications occurring at port sites are not uncommon and have been described mainly as infections, bleeding, haematoma formation, and also long-term complications such as hernia formation.\(^1\) So far the question of obstructing the blood supply to a confined area of skin and subcutaneous tissue caused by suturing has not been raised. To the best of our knowledge, this rare complication has not been previously reported.

**Conclusion**

The rapidly expanding field of laparoscopic surgery has resulted in a greater frequency and variety of complications.\(^2\) Modifications of the technique of closure of port site incisions may be required to prevent obstruction of blood supply to the skin and subcutaneous tissues, which could be the primary causative factor in the above case of necrosis. Reviews of the current closure techniques may be necessary to avoid further similar complications.

**References**

Spontaneous epidural hematoma of the spine in pregnancy

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Spontaneous spinal epidural hematomas are uncommon. Progressive neurologic deficits that are associated with epidural hematomas can develop rapidly and require prompt treatment. We present a case of spontaneous epidural hematoma of the thoracic spine that complicated a term pregnancy.

Spontaneous spinal epidural hematomas (SSEHs) are exceedingly rare, with an incidence of approximately 1 per 1,000,000 individuals. Clinical manifestations include the acute onset of back or neck pain with subsequent rapid and progressive loss of sensory and motor function. Prompt surgical decompression remains the optimal treatment. We present a case of SSEH of the thoracic spine that complicated a term pregnancy.

Case report

A healthy 30-year-old woman (para 1001) was seen at 37 weeks 6 days of gestation with a 10-hour history of back pain and acute progressive lower extremity paresthesias, weakness, and sensory loss. On examination she was afebrile with stable vital signs aside from elevated blood pressures and new onset proteinuria that was consistent with preeclampsia. Neurologic examination was remarkable for lower extremity paraplegia with 0/5 motor strength, absent lower extremity reflexes, no proprioception, and complete sensory loss bilaterally to the level of T7. Magnetic resonance imaging (MRI) revealed a 4-cm intraspinal mass with an intensity that was consistent with acute hemorrhage that extended from T6 to T9 spinal levels anterior to the cord with extrinsic compression noted (Figure).

Emergent cesarean delivery was performed and followed by a T7-T9 laminectomy with evacuation of an epidural hematoma. Follow-up MRI on postoperative day 1 revealed decompression and partial re-expansion of the spinal cord. By postoperative day 6, she had regained slight lower extremity sensation with some movement in her distal lower extremities. She underwent intensive rehabilitation and regained ambulatory status by the fourth month after her delivery. By 6 months, she had regained all of her strength and sensation. At the 1-year follow-up evaluation, she continued to have excellent neurologic recovery with no residual morbidities.

Comment

We have presented a case of SSEH that complicated pregnancy. A review of the English literature...
(MEDLINE 1966-2000; keywords: spontaneous spinal epidural hematoma and pregnancy) revealed only 4 previous reported cases of SSEH in pregnancy.\textsuperscript{2-5} SSEH, although rare, can cause rapidly evolving neurologic deficits within minutes to hours. The onset is typically acute, with an onset of sudden back or neck pain followed quickly by signs of cord compression. Complete and permanent neurologic deficits can occur; death has been reported rarely.\textsuperscript{1} The extent of the symptoms is typically dependent on the spinal level and degree of compression. Less commonly, patients may present in a subacute manner.

SSEHs are thought to result from low pressure within the epidural space relative to venous pressure, which
predisposes to rupture of a pre-existing pathologic venous system.\textsuperscript{1,5} Factors such as hypertension, hemophilia, vasculitis, anticoagulants, and Valsalva maneuver may also predispose to bleeding and hematoma formation.\textsuperscript{1} During pregnancy, a combination of the aforementioned factors and the changing venous dynamics of pregnancy may accentuate the potential for rupture.\textsuperscript{2-5} Preeclampsia may have contributed significantly to the development of SSEH in this patient, which is a finding that has never been reported.

MRI is the diagnostic imaging modality of choice. The exact location and size of the lesion must be ascertained as accurately as possible to prepare for surgical treatment. Computerized tomography can be falsely negative, and myelography is less specific and more invasive. The presence of spinal cord edema or ischemia is especially worrisome and substantially reduces the potential for functional recovery. Prompt surgical decompression by laminectomy with hematoma evacuation is the standard of care in most circumstances. Nonoperative treatment has been reported with good outcomes in patients with mild symptoms. Measures such as immobilization, the administration of steroids, and the treatment of coagulopathy may result in rapid improvement. Before laminectomy, measures such as hyperventilation and minimization of crystalloid and glucose levels improve spinal cord perfusion. However, surgery is recommended if progressive neurologic symptoms develop or recovery is not rapid.\textsuperscript{3} Treatment decisions in pregnancy depend on the gestational age. Decompression can be performed adequately and successfully intrapartum, with the patient in the lateral position.\textsuperscript{2,3} For women at term, cesarean delivery may be advantageous, followed by surgical spinal decompression, especially because the early delivery of the fetus decreases epidural venous engorgement. Functional recovery after surgery is related directly to the interval between symptom onset and surgical decompression and the rate of development and size of the hematoma.\textsuperscript{1} Although uncommon, SSEH is an acute neurologic emergency that mandates prompt recognition and treatment.

References

Incisional hernia on the 5-mm trocar port site and subsequent wall endometriosis on the same site: A case report

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KEY WORDS
Incisional hernia
Wall endometriosis
Trocar

A 26-year-old nulliparous woman underwent a laparoscopy to remove a 10-cm endometrial cyst on the left ovary (type II Nezhat). The cyst was extracted from the 10-mm umbilical incision; the other 2 trocars were inserted through 5-mm incisions. One year later, in correspondence to the previous 5-mm incision site, a hernia occurred that contained omentum and was reduced easily with a local anesthetic. After 2 years of good health, an aching nodule occurred on the same trocar site; vaginal ultrasound examination showed another left ovarian cyst. A second laparoscopy was performed; the cyst was very adherent and was removed in fragments. The wall nodule was removed, and the histologic examination classified it as endometriosis.

A 26-year-old nulliparous patient, with recurrent pelvic pain and dysmenorrhea, was given a gynecologic examination. The examination revealed a 10-cm cyst on the left ovary, probably endometriosis, at ultrasound examination. A laparoscopy was performed to remove the cyst, which was very easily cleavable (type II Nezhat). The cyst was extracted from the 10-mm umbilical incision; the other 2 trocars were put through 5-mm incisions. Histologic evaluation confirmed the diagnosis. Subsequently, the patient was treated with gonadotropin-releasing hormone analogues for 6 months.

One year later, after a physical strain, the patient noticed a tumefaction in correspondence to the previous 5-mm incision site; the clinical examination suggested the existence of a hernia, which contained omentum, which was reduced easily with a local anesthetic.

After 2 years of good health, an aching nodule arose on the same trocar site; vaginal ultrasound examination showed another left ovarian cyst. A second laparoscopy was performed; the cyst was very adherent and was removed in fragments. The wall nodule was removed, and the histologic examination classified it as endometriosis (Figure).

The patient has been treated with gonadotropin-releasing hormone analogues and oral contraceptives and is experiencing complete remission.

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Comment

This clinical case raises 2 main questions: What caused the incision hernia on the 5-mm trocar site? How could endometriosis wall implantation happen without direct contact during laparoscopic surgery?

Although the incidence of incision hernias on the trocar incision site was largely documented in literature (incidences, 21/100,000),1 with regard to the first question it is interesting to consider that, at the moment on the basis of available data, the recommendation is to repair iatrogenic fascial damages that are >10 mm. In fact, cases of incisional hernia on trocar incision sites that are <5 mm are described on occasion.2

To comprehend the reason that an incisional hernia could occur on a 5-mm incision site, we formulate the following hypothesis: The existence of an intrinsic parietal defect is made worse by the trocar’s insertion. The creation of an iatrogenic defect, followed by trocar movements during surgery, could transform a 5-mm breach in a major fascial defect, not well repaired during abdominal suture.

As regard to the second question, we recall that, for a long time, essentially 2 theories were formulated to explain the presence of ectopic endometrial tissue; this case could provide another cause of discussion in addition to the controversial and partly unknown pathogenesis of this disease.

Coming back to our clinical case, a question remains about a possible explanation about the cause of the endometriosis nodule on the 5-mm incision site, which again highlights the fact that no endometrial lesions were extracted from this site.

At last, making clear that the comprehension of pathogenetic mechanisms of this disease is determined by immunologic evaluation, we could exhort surgeons to evaluate carefully the entity of parietal damages because of the trocar’s passing and to repair the iatrogenic defect if required. Moreover, a careful disease’s staging should be done to identify the additional

Figure  Wall endometriosis nodule sectioned after removal.
implantations in addition to main ovarian lesion so that we could give considerable diagnostic and prognostic help for the clinical follow-up evaluation of these patients. We are waiting for a more efficient comprehension of the factors that are involved in the disease’s development and recognize the possible use of specific drugs.

References

Prenatal diagnosis of amniotic sheets by magnetic resonance imaging

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KEY WORDS
Amniotic sheets
Uterine synechia
Pregnancy
Magnetic resonance imaging (MRI)
Ultrasound

We report prenatal diagnosis of an amniotic sheet by magnetic resonance imaging (MRI) at 28 weeks’ gestation. The amniotic sheet divided the uterine cavity into 2 compartments, with the fetus and placenta located in the upper portion of the amniotic cavity. Although prenatal diagnosis of amniotic sheets can be performed with ultrasonography, the wider field of view and excellent tissue contrast provided by MRI may allow better spatial visualization of the amniotic sheet and, therefore, improve the diagnostic accuracy.

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Case report

A 27-year-old woman, gravid 1, para 1, was referred to our hospital at 28 weeks of gestation for a work-up and the management of abnormal placentation. Her previous pregnancy had been uneventful, but she received a postpartum curettage because of an adherent placenta. The amount of menstrual flow after the first pregnancy was normal. The present pregnancy was confirmed at a previous clinic at 7 weeks’ gestation (Figure 1A). At 13 weeks’ gestation, ultrasonography showed protruded anterior and posterior walls with a normal fetal heart beat and normal fetal growth (Figure 1B). At 22 weeks’ gestation, ultrasonography revealed the presence of another amniotic cavity with polyhydramnios beneath the “original” amniotic cavity, which carried a normal-looking fetus (Figure 1C). She was then referred to our hospital at 28 weeks’ gestation. On vaginal examination, the cervix was dilated 3 cm and 70% effaced. Because she complained of frequent uterine contractions, an
The intravenous administration of ritodrin hydrochloride was started. MRI (1.5-T scanner system, Gyroscan Intera T15, Philips) showed that the uterine cavity had 2 amniotic cavities, i.e., upper and lower, which were interconnected. Between the 2 amniotic cavities a septum-like structure was noted. The fetus and placenta were located in the upper amniotic cavity, and the placenta attached from the anterior through posterior wall across the septum-like membrane (Figure 2). There were no direct connections between the fetus and septum-like structure. Although the edge of septum was not evident, we concluded that the septum was an amniotic sheet secondary to uterine synechia with a part of placenta on it. Based on these findings, the patient was diagnosed as having a third-trimester pregnancy complicated by an amniotic sheet.

An ultrasound scan showed that the fetus was in the left acromiodorsoanterior position with remarkable polyhydramnios. The fetal growth was normal with no apparent gross anomalies. The pulsed and color Doppler imaging demonstrated a fine blood flow between the posterior wall and placenta. On the second day in hospital, an administration of MgSO₄ was commenced in addition to ritodrine because of the increased frequency of uterine contractions. At 34 weeks’ gestation, uterine contraction was further augmented with a complaint of severe abdominal fullness, and the forebag eventually protruded into the vaginal cavity. An ultrasound examination at 34 weeks’ gestation indicated that the estimated fetal body weight was 2721 g with no detectable anomalies, and there were no apparent morphologic changes in the septum-like structure. Because of the possibility of abruption or umbilical cord prolapse, a cesarean delivery was performed at 35 weeks’ gestation and a live 2744 g male with Apgar scores of 8/9 at 1/5 minutes was delivered. The amount of amniotic fluid was approximately 1000 mL. After the delivery, a membranous septum was found in the right side of the uterine cavity, and the placenta occupied the lower part of the upper lumen. The backside of the septum was whitish and thickened with a fibrous texture. There was a route between the upper and lower cavities on the left side of the placenta. The placenta was spontaneously removed from the anterior wall with the septum; however, the placenta attachment on the posterior wall was removed manually without notable bleeding. Pathologic examination revealed that the septum was made of a thin layer of myometrium containing invading trophoblasts. The patient’s puerperal course was uneventful.

**Comment**

In general, ultrasonography is suitable for the observation of localized areas of the uterus or fetus. MRI is more fitted to understand whole intrauterine structures by the 3-dimensional observation, and to observe the posterior side of uterine cavity, especially in the third trimester, when the ultrasound scanning is hampered by the fetus. Therefore, we consider that the use of MRI in combination with ultrasonography can be a potent strategy in the diagnosis of amniotic sheets.

The MRI scan of the present case was peculiar, i.e., the uterine cavity was divided into 2 components separated...
by the amniotic sheets with a part of placenta. Korbin et al reported that the placenta implants around the synechia were observed in approximately one fourth of the cases. The most important abnormality for differential diagnosis is amniotic band syndrome. Amniotic band syndrome was excluded by the lack of direct connections between the septum and fetus, and the lack of apparent fetal anomalies. We also suspected congenital uterine anomalies, such as a septate uterus. However, a septate uterus was not likely because the septum in a septate uterus is usually in the fundus and oriented in a sagittal plane, whereas the septum in the present case was located midportion of the uterus with an axial plane. Therefore, to our knowledge, this is the first report of MRI detecting an amniotic sheet in the third trimester of pregnancy. The pathogenetic process of the present case is totally unknown. However, based on Figures 1 and 2, we speculate that this rare situation was caused by the implantation of an ovum near the adhesion site, and the zygote grew encompassing the adhesion site without disruption of the string-like septum.

Uterine synechia (Asherman’s syndrome) during pregnancy has been reported to be associated with an adherent placenta. Jewelewicz et al reported that the ratio of placenta accreta in patients with uterine synechia was 9%. In the present case, we also predicted the presence of an adherent placenta because we observed blood flow between the posterior uterine wall and placenta. Therefore, we prepared an autologous blood transfusion before the operation. With regard to the mode of delivery, we selected an elective cesarean delivery because of the risk of placental abruption and umbilical cord prolapse during labor.

In conclusion, we reported a case with an amniotic sheet in the third trimester detected by MRI. It is very important that the amniotic sheets not be confused with the bands of amniotic band syndrome; while amniotic band syndrome is associated with multiple fetal anomalies, amniotic sheets appear to be a totally benign process unassociated with fetal deformation. Misinterpretation of the finding of amniotic sheets could lead to unwarranted abortion of normal fetuses. Therefore, the use of MRI, in addition to ultrasonography, can improve the diagnostic accuracy in earlier gestational weeks and, thus, contribute for proper managements of amniotic sheets secondary to uterine synechia.

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Posterior reversible leukoencephalopathy in a case of postpartum eclampsia

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KEY WORDS
Late onset eclampsia
Posterior reversible leukoencephalopathy
Postpartum headache

This case report describes an atypical presentation of eclampsia. A 26-year-old lady presented 5 days’ postpartum with a series of grand mal seizures after an uneventful pregnancy and delivery. An MRI scan of the brain showed areas of low signal involving cerebral white matter and right cerebellum. Within 2 weeks, all symptoms and radiologic abnormalities had resolved. © 2005 Mosby, Inc. All rights reserved.

Case report

A 26-year-old Bengali lady living in the UK booked routinely at 12 weeks of pregnancy. She had no significant medical or family history, and was not taking any regular medication. A previous pregnancy in 1996 was uneventful, and resulted in the spontaneous vaginal delivery of a baby girl. During this pregnancy her antenatal period was also uneventful. At 38 weeks’ gestation, labor was induced 36 hours after prelabor spontaneous rupture of membranes. After a normal vaginal delivery, she was discharged home the following day. She had been normotensive throughout the pregnancy, and before discharge her blood pressure was 110/75 mm Hg.

Five days later she presented to the accident and emergency department feeling generally unwell, complaining of a sudden onset of severe headache and flashing lights. On admission, she had a generalized grand mal seizure, which terminated spontaneously. Her blood pressure was noted to be 159/86 mm Hg compared with her booking blood pressure of 110/70 mm Hg. Urinalysis revealed a trace of protein. A full blood count showed no major abnormalities, with a platelet count of 282. Her electrolytes were normal. Aspartate transaminase and uric acid levels were marginally elevated at 48 and 0.39, respectively. Detailed neurologic examination showed no abnormalities. A CT scan of the head was performed immediately after admission and was normal. Lumbar puncture showed an elevated opening pressure at 40 cm. Protein was elevated at 0.94 g/L with other CSF constituents normal.

She was started on magnesium sulphate, given low-molecular-weight heparin and high-dose acyclovir. The differential diagnosis at this stage was late onset eclampsia, venous sinus thrombosis, and encephalitis. She had multiple grand mal seizures over the next 3 days and was started on phenytoin. An MRI scan of the brain 6 days after presentation showed confluent areas of high signal on FLAIR sequences, and low signal on T1-weighted images involving the cerebral white matter and superior part of the right cerebellum (Figure). Within a

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few days her fits had stopped. A subsequent repeat MRI of the brain showed a complete resolution of the previous abnormalities. A repeat lumbar puncture was also normal with negative oligoclonal bands. All other microbiological, autoimmune, and thrombophilia studies showed no abnormalities. Two weeks after her first grand mal seizure she had made a full recovery with no neurologic sequelae, and was discharged from hospital. A diagnosis of reversible posterior leukoencephalopathy syndrome was made. She has since had no recurrence of her seizures.

Postpartum eclampsia is categorized as early (<48 hours) or late (>48 hours) eclampsia. A recent study revealed that up to 33% of eclampsia occurred postpartum, with 79% of these arising 48 hours after delivery. Interestingly, only 22% of those with late onset eclampsia had been previously diagnosed with preeclampsia. The majority had at least 1 prodromal symptom, with headache and epigastric pain being the most common. In cases of late onset postpartum eclampsia edema, proteinuria and hypertension are often not present until just before the onset of seizures, making early diagnosis difficult.

Posterior reversible encephalopathy is an infrequent diagnosis in day-to-day obstetric care but is known to be part of a spectrum of conditions associated with eclampsia. It is characterized by headache, altered mental status, cortical blindness, seizures, and a characteristic MRI picture showing high signal lesions in the posterior cerebral white matter. Although the name suggests that it is usually reversible, early diagnosis and treatment is essential because irreversible neurologic deficits or death may occur. Treatment involves lowering mean arterial blood pressure to below 125 mm Hg and anticonvulsant therapy. The syndrome is not unique to pregnancy. A similar clinical and radiologic picture may occur in a number of conditions, including malignant hypertension and cyclosporine toxicity, and is believed to be caused by a loss of integrity of the blood brain barrier and vascular leaks.

It is crucial to consider late onset postpartum eclampsia in any woman who presents after delivery with severe headache, epigastric pain, or visual disturbance, even in those without a previous diagnosis of preeclampsia. Eclampsia will continue to present us with diagnostic challenges, and efforts need to be directed to educate patients and health care workers to report symptoms early and to investigate them appropriately.

References

Twin-to-twin transfusion syndrome at 11 weeks of gestation

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Second-trimester twin-to-twin transfusion is well known, but first-trimester cases rarely have been described. We present the case of a monochorionic twin at 11 + 0 weeks of gestation with single increased nuchal translucency and normal karyotypes. At 12 + 5 weeks of gestation, double intrauterine death was diagnosed, followed by delivery of a strikingly red and white fetus.

A 33-year-old woman, gravida 3, visited our unit at 11 + 0 weeks of gestation for ultrasound examination. A monochorionic-diamniotic twin pregnancy was seen. Both fetuses showed a crown-rump length appropriate for gestational age (twin A, 4.71 cm; twin B, 4.70 cm) and a normal amount of amniotic fluid. Nuchal translucency thickness in fetus A was normal (0.16 cm); nuchal translucency of fetus B was markedly increased (0.68 cm, far above the 95th percentile). No other fetal anomalies were seen. There were no anatomic or Doppler signs that were suggestive of impending cardiac failure. The differential diagnosis included a chromosomal defect in 1 or both twins, cardiac abnormality of twin B (not yet visible sonographically), and extreme early onset twin-to-twin transfusion syndrome (TTTS).

At 12 + 5 weeks of gestation, double intrauterine death was diagnosed. At that time, the crown-rump length of twin A and twin B both measured 6.56 cm. Twin B’s nuchal translucency was still increased, and the amniotic fluid was normal for both. We sampled amniotic fluid from each sac, which revealed normal female karyotypes. Two days later, a very pale fetus and a dark red fetus, both with a body weight of 20 g, were delivered (Figure). Because of a retained placenta, dilatation and curettage was performed. Therefore, dye injection into chorionic vessels could not be performed. Autopsy was refused.

Comment

Chronic TTTS develops as a result of a small and chronically unbalanced blood flow between monochorionic twins through vascular anastomoses on the placenta. The diagnosis is based on ultrasonography showing a combination of polyuric polyhydramnios in the recipient’s sac and oliguric oligohydramnios in the
Quintero et al developed a staging system to determine the severity of the circulatory imbalance between the twins. Thus defined, TTTS complicates approximately 15% of monochorionic-diamniotic twin pregnancies in the second and early third trimester. Acute TTTS can occur after the intrauterine death of one twin and involves a sudden loss of a substantial amount of blood. The survivor then exsanguinates through vascular anastomoses, presumably an arterioarterial one, into the low-pressure circulation of the dying or dead twin, followed by hypovolemia, ischemic brain injury, or death.

In our patient, one of the earliest TTTS cases to be reported thus far, the first and only symptom was an increased nuchal translucency in 1 twin. Although this finding is related strongly to chromosomal defects, both twins had normal karyotypes. Increased nuchal translucency has been reported previously to be associated with an increased risk (odds ratio, 3.5) of the subsequent development of TTTS. Sebire et al suggest that the recipients’ hypervolemia causes heart failure with a subsequent accumulation of fluid behind the neck in the first trimester. Nuchal translucency thickness tends to normalize after 14 weeks of gestation. Fetal urine production then commences. We speculate that only then does volume overload in the recipient result in the excessive urine production that causes polyhydramnios, which is the main indicator of second-trimester TTTS. Because fetal diuresis contributes little to the amniotic fluid in the first trimester, it seems plausible that the classic oligo/polyhydramnios sequence cannot yet occur. However, as possibly happened in this case, severe first-trimester TTTS may worsen so rapidly that single or double fetal death occurs before the second trimester is reached. Benirschke and Maslia described the 2 earliest cases of first-trimester TTTS. Those were strongly suggestive of such long-lasting circulatory imbalance, as was revealed by the high cardiac-to-body ratio in recipient twins. Another possibility is that fetus B had a lethal malformation. It is well known that severe cardiac and other malformations can cause subcutaneous edema in the nuchal region. Fetal anomalies were not seen on ultrasound, but autopsy was not performed. Thus, both early chronic TTTS and acute TTTS after single fetal death should be considered here. Whether severe first-trimester TTTS or a lethal malformation of fetus B was the primary cause of intrauterine death will remain unknown. Either way, inter-twin vascular connections, which are present invariably in all monochorionic placentas, enabled the second twin to exsanguinate into the dead fetus, which caused the co-twin death because of acute TTTS. This would imply, in our case, the red twin to be the fetus with increased nuchal translucency who died first, and the pale twin to be the co-twin who died consequently through exsanguination.

In conclusion, TTTS can be seen in various ways at different gestational ages. Besides the well-known risks of severe second-trimester TTTS, we believe that TTTS can cause fetal death or neurologic damage, even in the first trimester of pregnancy. The only presenting symptom may be a single increased nuchal translucency.

References
Spontaneous closure of the hymen during pregnancy

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KEY WORDS
Spontaneous closure of hymen
Pregnancy

The hymen marks the distal most extent of the vagina. Imperforate hymen is a rare genital disorder, which is diagnosed generally during adolescence consequent to hematocolpos. The case presented here, however, describes spontaneous closure of hymen during pregnancy.

Case report
A 23-year-old pregnant woman was seen at our clinic with uterine contractions. On initial examination, regular intensive contractions, with a duration of 30 seconds, were observed. Vaginal examination was not possible; however, a fully intact and bulging hymen with no papillomatosis was noted (Figure 1). She immediately was evaluated by ultrasonography, which showed a 30-week fetus that was concordant to the gestational age. An amniotic index of 250 mm indicated hydramnios. Sonography revealed funneling, and a dense fluid collection in the vagina was seen. A surgical hymenotomy was performed. A stellate incision was made with local anesthesia. On incision, purulent material was discharged from vagina. A sample was taken for microbiologic assay. In the pelvic examination, the vagina and cervix were found to be structurally normal with a cervical dilation of 4 cm and 80% effacement with bulging amnion. Despite attempted tocolysis, a 2350-g male fetus was delivered with an Apgar score of 2/4 at 1 and 5 minutes. The fetus had no spontaneous respiration and a heart rate of 60 beats/minute and was cyanotic. Intubation was performed promptly by the pediatricians. Physical examination of the newborn infant revealed no esophageal atresia. The baby was transferred to the intensive care unit. After a few hours, the baby died of respiratory distress.

In her detailed history, we learned that menstruation began normally without any problems. She had no complaint related with menstrual activity. She also
experienced no coital abnormalities after her marriage. She had coital activity until she learned that she was pregnant.

A hymenal biopsy specimen was taken during delivery to evaluate the ultrastructure of hymen by electron microscopy (Carl Zeiss EM 900; Zeiss, Jena, Germany). The ultrastructure of the hymen showed metabolically active connective tissue cells. Mitotic figures were especially evident in the basal epithelial cells. The connective tissue cells contained abundant rough endoplasmic reticulum (RER) and ribosomes that indicated cell activity. Increased vascularization and widespread distribution of collagen fibers under epithelial tissue were strong evidence of accelerated hymenal tissue reorganization (Figure 2, A and B).

Although she had not had intercourse, the surgically created hymenal orifice was still patent at postpartum week 8. The couple began coital activity and have not reported any coital or menstrual problem until now.

Comment

The hymen marks the distal most extent of the vagina and the most proximal boundary of the vulvar vestibule. It may be imperforate, round, annular, septate, cribiform, or parous. On the vaginal surface, there is a nonkeratinized stratified squamous epithelium, which is more or less glycogenated in response to estrogen exposure, as seen in women of reproductive age, newborn infants, pregnant women, and postmenopausal women who receive estrogen therapy. On the vulvar surface, the vestibular epithelium appears similar to the vaginal epithelium in women of reproductive age.

Imperforated hymen is a rare genital disorder. Generally, it is diagnosed during adolescence consequent to hematocolpos. These patients may demonstrate other lower genital tract abnormalities. None of these problems were associated with the present case. Secondary closure of hymen has been reported in 2 cases. Berkowitz et al documented a 5-year-old abused child with distortion of hymen, laceration of perineal body, and loss of normal anal tone. Seven months after surgical correction, the hymen was replaced by a thick and opaque scar with no orifice. Chao-Hsi and Ching-Chung reported a 32-year-old pregnant woman who experienced closure of the hymen during week 37 of gestation of her second pregnancy. The baby was delivered by cesarean delivery, but a thick fibrotic membrane at the vaginal orifice blocked the passage of lochia. Therefore, a surgical incision was made to enable
drainage. She had been subjected to a hymenotomy at age 13 years because of imperforate hymen and hematocolpometra. At age 18 years, after cessation of normal menstruation because of significant weight loss, a thin membrane was observed and excised surgically. No problem occurred during her first pregnancy. Although spontaneous closure of a hymen-like structure occurred in these 2 cases, there were mitigating circumstances that suggest the formations were related to extensive scar formation. The current case, by contrast, involves spontaneous closure of the hymen in the absence of previous surgical procedures. In our opinion, after the cessation of coital activity, a healing process that was possibly related to the pregnancy may explain the closure. Active growth was demonstrated by electron microscopy. The connective tissue cells demonstrated markedly increased rough endoplasmic reticulum and free ribosomes. Between these cells, vascularization and connective tissue collagen fibers were obvious. These ultrastructural appearances indicate reorganized hymenal tissue.

References

Small bowel obstruction due to adhesive disease observed after uterine fibroid embolization

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KEY WORDS
Uterine fibroid embolization
Adhesion
Bowel obstruction

After uterine fibroid embolization (UFE), the development of intra-abdominal adhesions, especially those involving the bowel, is a very rare complication. Seven months after UFE, a patient had a complete small bowel obstruction develop that was caused by an adhesive band between the posterior fibroid and cul-de-sac. She underwent an exploratory laparotomy, lysis of adhesion, and myomectomy. No bowel resection was needed. Inflammation after UFE may cause the development of intraperitoneal adhesions. We report an unanticipated case of a complete small bowel obstruction caused by an adhesion observed after UFE.

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Uterine fibroid embolization (UFE) is an increasingly used, effective, and minimally invasive treatment for symptomatic leiomyoma. UFE is a treatment alternative to hysterectomy or myomectomy, 2 surgeries with high postsurgical rates of adhesive disease, often affecting the bowel. The development of adhesions, especially those involving the bowel, after UFE is a very rare complication.¹ ² We present an unusual case of a delayed complete small bowel obstruction caused by adhesions observed after UFE.

Case report

A 32-year-old nulligravid woman with menorrhagia and anemia had a fibroid uterus diagnosed on clinical examination. She denied a history of prior surgeries, pelvic pain, endometriosis, or pelvic infections. A pelvic ultrasound verified a uterus measuring 13.9 × 10.8 × 13.5 cm with multiple myomas, the largest, 9 × 8 × 6.5 cm, arising posteriorly. She elected to undergo a uterine fibroid embolization performed via a 4-French sheath in the right common femoral artery. Coaxial catheterization with a 3-French catheter was used to deliver 500 to 700 μm Embospheres (Biosphere Medical, Inc, Rockland, Mass) selectively into both right and left uterine arteries under fluoroscopic guidance until near-stasis of flow was achieved. Both embolizations were performed with the catheter tip in the transverse portion of the uterine artery. No reflux of particles out of either uterine artery was observed. The patient had no acute postoperative complications and returned to work in approximately one week. Her prior symptoms soon thereafter improved.

Seven months after the UFE, she experienced an acute onset of crampy abdominal pain, accompanied by
nausea and vomiting. In an emergency department, her abdominal examination revealed diffuse tenderness and rebound. A computed tomographic scan had findings consistent with a distal complete small bowel obstruction. An exploratory laparotomy found a dense adhesive band running between a 2-cm subserosal posterior fibroid and the cul-de-sac, trapping a dilated loop of small bowel. The location of this fibroid corresponded with the preembolization 9 × 8 × 6.5-cm fibroid. No other abdominal disease, including other adhesions or endometriosis, was visualized. The adhesion was lysed, releasing the obstructed loop of bowel. This posterior fibroid was then removed. A careful inspection of the previously obstructed area of small bowel revealed no discoloration or nonviable areas, allowing the avoidance of a resection. Postoperatively, the patient did well. After slowly advancing her diet, she was discharged on the fourth postoperative day.

Comment

A MEDLINE search using the MeSH terms uterine artery embolization, uterine fibroid embolization, adhesion, and bowel obstruction, identified only 2 articles that potentially attributed adhesion formation as a result of UFE.1,2 One of these articles also reported a partial small bowel obstruction caused by the adhesion disease.2

Intrauterine adhesions causing infertility were reported by Honda et al1 in 4 patients after UFE. In their discussion, a hypothesized etiology of adhesion formation was infection and inflammation resulting from sloughed necrotic fibroids obstructing the cervix and interfering with the passage of intrauterine discharge.1 They did not report any intra-abdominal adhesions in their series.

Payne and Haney2 reported a patient who had a partial small bowel obstruction develop 2 weeks after UFE. On laparotomy, the patient underwent extensive adhesiolysis, including separating small bowel adherent to the uterus. Also found was a large ovarian adenocarcinoma. No mention was made of whether the patient had previously undergone any surgical proce-

dures, which could have also been an cause of her adhesive disease.

The proposed mechanism of action of adhesion formation in our case is similar to that encountered in surgical patients. The large posterior fibroid, in direct contact with the cul-de-sac, underwent devascularization and ischemia after UFE. The resulting inflammatory reaction set off a cascade of intra-abdominal adhesion formation between the peritoneal surfaces. Inflammation of the adjacent peritoneal surfaces initiated adhesion formation with the formation of a fibrin matrix in the presence of suppressed fibrinolysis. Local ischemia allowed persistence of the fibrin matrix. Vascular granulation tissue, containing fibroblasts, macrophages, and giant cells, then gradually replaced the matrix. As the adhesion band slowly matured, it was covered by mesothelium and connective tissue fibers.

Although it is possible that the adhesion could have preceded the UFE, given her lack of previous intra-abdominal surgeries, or any evidence of prior pelvic infections or endometriosis, our patient’s adhesion and subsequent complete bowel obstruction is most plausibly a result of the UFE. Although adhesion formation is a common occurrence after myomectomy and hysterectomy, it may also occur when fibroid disease is treated with UFE, which may subsequently lead to a small bowel obstruction, in this case 7 months postembolization. In patients presenting with abdominal pain who have previously undergone UFE, small bowel obstruction should be included in the differential diagnosis. In our case, prompt recognition of and intervention for the obstruction prevented the need for a small bowel resection.

References

LETTERS TO THE EDITORS

Selective fetocide reverses preeclampsia in discordant twins

To the Editors: We read with interest the article by Heyborne and Porreco,1 who reported on 2 cases of resolution of preeclampsia after selective fetocide in discordant twin pregnancies. We recently reported on a similar case,2 in which a 32-year-old nulliparous woman with a dichorionic discordant twin pregnancy had preeclampsia at 28 weeks of gestation. Twin A was developing normally; twin B showed severe intrauterine growth restriction. Expectant management was favored initially, but the maternal condition worsened, with hypertension necessitating high doses of nifedipine and labetalol and proteinuria increasing up to 6.5 g per day. Selective fetocide of the compromised twin was presented as an alternative to delivery, and the patient accepted this option. After selective termination at 32 weeks of gestation, we observed a dramatic improvement in the clinical and biologic condition of the woman, who eventually was delivered of a healthy infant who weighed 2560 g at 38 weeks of gestation and of a stillborn second twin who weighed 330 g. The postpartum course was uneventful.

These concordant reports provide further clinical evidence that preeclampsia is caused by placental factors. Heyborne and Porreco1 hypothesize that placental involution might be necessary for the resolution of the disease. However, placental involution is known to occur after several weeks, whereas preeclampsia resolved within several days in our patient and in the 2 cases described by the authors. We would suggest rather that the interruption of placental blood flow in the pathologic placenta may have been enough to reverse the disease. This mechanism is consistent with the historic observations of resolution of preeclampsia after spontaneous death of 1 twin. We believe that the interruption of placental blood flow may act as a mechanism of placental exclusion, thus leading to decrease or arrest of the release of the substances that are involved in the maintenance of the disease. These observations open prospects for further research on the mechanisms of this complex disease and to offer an acceptable alternative therapy in selected cases.

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References

Reply

To the Editors: We thank Audibert et al for their comments regarding our manuscript and apologize for failing to reference their case report that was published while our manuscript was in preparation. We agree
that their similar experience strengthens the link between a pathologic placenta and the disease process that we recognize as preeclampsia and further supports selective termination as a reasonable management strategy in a few carefully selected cases.

Inasmuch as the underlying disease processes that results in the clinical syndrome of preeclampsia remain largely hypothetical, the mechanisms by which selective termination reverses these processes are doubly so. We offered placental involution as a potential mechanism because, in our cases, the time course of disease resolution, which was possibly aided by the administration of corticosteroids, accorded reasonably well with the course of placental involution that was known to us from the somewhat analogous situation of delayed-interval delivery.

Audibert et al suggest a more rapid mechanism, although their paper indicates a 2-week interval for their patient’s 24-hour urinary protein excretion to diminish to approximately 2 g, which is an elevated level that persisted for the remainder of the pregnancy; and the continued administration of antihypertensive agents was required. We are not certain of the precise meaning of placental exclusion. Certainly the fetal circulation within the placenta ceases immediately with selective termination, but there is no a priori mechanism to immediately “exclude” the placenta from the maternal circulation.

Another potential mechanism for disease resolution is the removal of the fetal metabolic “load” from the placenta, which presumably improves the placenta’s ischemic condition. In any case, it seems likely that whatever mechanism is involved, it does not entirely correct the underlying disease process immediately but simply reduces it below some threshold beneath which the signs and symptoms of preeclampsia resolve sufficiently to allow the safe continuation of the pregnancy.

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Is circulating extracellular VEGF increased in preeclamptic women?

To the Editors: I read with interest the article of McKeeman et al.1 Although the authors should be congratulated by their observations on sFlt-1 levels in normotensive and preeclamptic women, I would like to discuss some issues concerning the measurement of vascular endothelial growth factor (VEGF) concentrations.

The authors found that there is a trend for serum VEGF levels to be higher in preeclamptic, compared with normotensive, women at 12, 20, and 30 weeks of gestation and that this increase becomes statistically significant at 37 weeks of gestation and before delivery.

It is well known that VEGF is stored in α granules of circulating platelets and is released in serum during blood clotting in a time-dependent manner.2,3 Although the authors reported that serum was “separated by centrifugation after clotting,” they did not provide evidence of standardization of the interval between venipuncture and separation of serum from blood cells; this limit of the study may have contributed to reduce the difference in VEGF levels between preeclamptic and normotensive women. Importantly, it has been proved that allowing whole blood samples to clot for between 2 and 6 hours before serum is collected reduces the time-dependent, non-uniform release of VEGF4; this approach should be chosen when serum VEGF is measured.

Furthermore, it would be interesting to know whether the force that was used to centrifuge blood samples and the length of centrifugation were standardized during the study period because they may affect platelet activation by mechanical stress and, consequently, may have an impact on serum VEGF levels.3

Considering that serum VEGF concentrations are correlated to platelets counts,4 it would also be interesting to evaluate whether the differences in VEGF levels between preeclamptic and normotensive women are confirmed after correcting for platelet count.

In light of the fact that serum VEGF concentrations reflect blood platelets counts rather than VEGF synthesis by peripheral tissues,2 it has been suggested that plasma and not serum should be used for the determination of circulating extracellular VEGF. In plasma, platelet degranulation is minimized by adding anticoagulants to blood samples; CTAD (citrate, theophylline, adenosine, dipyridamole) tubes should be used for sample collection when plasma VEGF is measured.
I believe that a meticulous processing of serum blood samples and the use of plasma may improve the value of VEGF measurement throughout the whole pregnancy.

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References

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Cytokines, preeclampsia, and uterine denervation?

To the Editors: McKeeman et al1 have found increased maternal circulating levels of cytokines and their receptors (vascular endothelial growth factor and vascular endothelial growth factor receptor-1) at different gestational ages in preeclampsia. Several explanations may account for these observations that include a prior or continuing injury to the placental bed. Variable uterine denervation at the deciduomymetrial interface may prevent appropriate endovascular trophoblast invasion and generate a maternal cytokine response.2 Possible sources of regional uterine denervation include curettage, back injury, or, most commonly, a low residue diet that results in sustained constipation. The clinical “phenotype” of the preeclamptic syndrome may vary with the extent of the injury to the placental bed (inversely proportional to gestational age of onset), the placental site (dennervated placental site, recurrent hypertensive syndrome), the concomitant loss of uterine afferent nerve fibers (intrauterine growth restriction, no maternal hypertension), and the increases in maternal systemic vascular compliance (HELLP [hemolysis, elevated liver enzymes, and low platelet count] syndrome). Denervation-reinnervation in other tissues has been associated with different cytokine responses that include vascular endothelial growth factor.3 In this detailed longitudinal dataset, does the maternal cytokine response vary with the gestational onset of preeclampsia? Does it vary with the other possible clinical “phenotypes”? Are other cytokines (of neural origin) involved?

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References

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Reply

To the Editors: We are pleased with the interest our study has received, and we thank Drs Ferrero and Quinn for their comments. We hope that this response will clarify a number of issues regarding the
importance of circulating VEGF and sFlt-1 in preeclampsia.

First, we would like to address the queries from Dr Ferrero regarding the treatment of blood samples. A standard method was implemented for the collection of all serum samples throughout the study. After venipuncture, blood samples were left at room temperature for 4 to 6 hours until clotted and serum was separated by centrifugation at 1200g (4°C) for 20 minutes. These standardized methods were enforced for each sample. We did not consider it necessary to correct the VEGF data for platelet counts, although this is something that could be investigated in future studies.

Before this work, a small preliminary study was performed to assess the optimum specimen for VEGF measurement throughout pregnancy. VEGF concentrations were analyzed in serum, EDTA plasma and lithium heparin plasma collected from both pregnant (n = 4) and nonpregnant (n = 4) subjects. There was little variation in VEGF concentrations that were measured in serum and plasma from either the pregnant or nonpregnant volunteers. Therefore, on the basis of these data and the fact that the original VEGF radioimmunoassay method was optimized in serum, we opted for the use of serum for the measurement of VEGF throughout pregnancy.

Although plasma recently has been suggested as the optimum medium for VEGF measurement, the use of serum is important in cases in which platelets are activated and in which platelet-derived VEGF is important. Because of this, serum has been suggested as being the more useful specimen for the assessment of VEGF in cancer patients. In the case of preeclampsia, some women can go on to experience HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, which involves platelet activation and the additional release of VEGF. Therefore, it could be important to assess total VEGF concentrations under such circumstances because excess circulating VEGF has been linked to the endothelial cell damage that is seen in these patients. Furthermore, recent investigations have also confirmed the involvement of elevated VEGF serum concentrations in the onset of preeclampsia.

Although we do not eliminate the use of plasma for VEGF analysis, the use of heparinized containers should be avoided because of the binding interactions that can occur between VEGF, sFlt-1, and heparin. We agree that it is important to reduce the release of VEGF from platelets during processing (by implementing stringent sample handling procedures) so that any changes in VEGF concentrations are representative of events that occur in vivo.

Second, we find the views of Dr Quinn on the reasons for preeclampsia interesting. Our study was an observational study that demonstrated that, at the time of onset, circulating VEGF and sFlt-1 concentrations are increased in a preeclamptic group. Although these cytokines may not be the root cause of the syndrome, they could act as influential mediators. There is not enough data to correlate with gestational age of onset and not sufficient patients to correlate for a phenotypic factor. With regard to other cytokines of neural origin, this is beyond the scope of this paper but certainly raises questions for future research.

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References


Accidental fetal lacerations during cesarean delivery: Experience in an Italian level III university hospital

To the Editors: We read with interest the article by Dessole et al concerning accidental fetal laceration during cesarean delivery. Several methods are used during cesarean delivery to minimize the risk of fetal accidental...
laceration occurrence. Nonetheless, those procedures do not ensure sufficient reliability. We recommend transabdominal or transvaginal ultrasonographic examination by the surgeon immediately before the initiation of the operative procedure. In only a few minutes, ultrasonography can provide important information: the uterine wall thickness, the fetal presentation or orientation, and the amniotic fluid volume. Those findings can help the surgeon characterize the inherent risk of fetal laceration injury at the cesarean delivery. Careful consideration of the possibility of accidental fetal laceration at the instant of uterine incision is the most important safety device.

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References

Reply

To the Editors: We appreciate the interest and the comments of Nishijima et al about our article concerning accidental fetal lacerations during cesarean delivery.1 Nishijima et al recommend transabdominal or transvaginal ultrasonographic examination by the surgeon immediately before the initiation of the operative procedure. We agree with this consideration and think that ultrasonographic findings (such as uterine wall thickness, fetal presentation or orientation, and the amniotic fluid volume) can help the surgeon to characterize the inherent risk to the fetal laceration injury at the cesarean delivery also. Our medical management in the operating theater states always to perform obstetric examination before the performance of cesarean delivery. In cases of doubt about fetal presentation and/or orientation, we do transabdominal and transvaginal ultrasonography. Then, if we observe an abnormal presentation, we use the precautions that we have reported. But, despite this management, the risk of accidental fetal lacerations during cesarean delivery may be reduced but not eliminated. Thus, before the performance of cesarean delivery, the patient always should be informed about this risk of accidental fetal lacerations.

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Reference
Correction


Table I  Inhibitory activity on oxytocin-induced contractions in rat myometrium in vitro and binding affinity for PXR for metabolites of progesterone

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<th>Steroid</th>
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<td>DMSO (control)</td>
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<td>4-pregnen-3,20-dione (progesterone)&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>RU486 (mifepristone, n = 1)</td>
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<sup>*</sup> Concentrations 1-100 μM were used but only the 100 μM dose is shown (n = 3 – 10 experiments for each metabolite except mifepristone).

<sup>1</sup> Calculated as the EC<sub>50</sub> concentration (μM) for activation of mouse PXR from the transient transfection assays (see text).

<sup>2</sup> Denotes statistically significant inhibition by ANOVA (P < 0.05). The bold font indicates the metabolite used in the present studies.
The Following lists contains Diplomates Passing the 2004 Ob/Gyn Oral Examination From 11/12/2004 to 01/14/2005

Certificates valid through 12/31/2010

Report Date: 02/02/2005

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Carley, Michael E., MD, Dallas, TX
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Carmona-Keller, Diana Patricia, MD, Venetia, PA
Carney, Michele C., MD, Aurora, IL
Carrera-Leal, Benito, MD, Brownsville, TX
Carroll, C. Shannon, DO, Jackson, MS
Carter, Andrew W., MD, Scottsdale, AZ
Caruthers, Thomas Jefferson Jr., MD, Atoka, TN
Cassagnol, Hans, MD, Mountain Top, PA
Cha, Ae Seon, MD, Troy, OH
Chadwick, Tuesday L., MD, Paris, TX
Chalmers, Robert W., MD, Ft. Wainwright, AK
Chan, John K., MD, Foster City, CA
Chan, Kent C., MD, Astoria, NY
Chancellor, Jeff D., MD, Waco, TX
Chang, Joe S., MD, Irving, TX
Chang, Lisbeth H., MD, Encino, CA
Charles, Hillary H., MD, Phoenix, AZ
Chauhan, Subodhsingh Rambahsingh, MD, Charleston, WV
Chauhdry, Tahir Akram, DO, Hornell, NY
Chavez, Martin R., MD, Hillsborough, NJ
Chen, Dehan, MD, Paramus, NJ
Cheshir, Kimberly Ann, MD, Dallas, TX
Cheung, Mon-Lai, MD, Bakersfield, CA
Chismar, Steven, MD, North Lima, OH
Chmait, Ramen H., MD, Tampa, FL
Choi, Janet Mee-Kyung, MD, New York, NY
Chong, Deborah, MD, Sacramento, CA
Christie, Heath DiMaio, MD, Gainesville, FL
Chu, Christina Shuwai, MD, Mooröstown, NJ
Chu, Kristine, MD, Englewood Cliffs, NJ
Chung, Judith Hyunsuk, MD, Long Beach, CA
Chung, Susie Noel, MD, Baltimore, MD
Clayton, Esther R., MD, Minneapolis, MN
Cly, Geoffrey Charles, MD, Fort Wayne, IN
Cobb, Kelly A., MD, Spartanburg, SC
Cockrum, Holly, MD, North Little Rock, AR
Cohn, Jennifer Lynn, MD, Novi, MI
Cole, David Scott, MD, Ardsley, NY
Cole, Karen F., MD, Jackson, MS
Cole, Nichole Fleming, MD, Houston, TX
Coleman, Jenell S., MD, Berkeley, CA
Coleman, Joseph I., MD, Lake Wylie, SC
Coley, Katherine Price, MD, Hilton Head Island, SC
Collin, Jennifer Wiley, MD, Niskayuna, NY
Connell, Kathleen Anne, MD, Shelton, CT
Connelly, Diane A., MD, San Bernardino, CA
Cooper, Jason Edward, MD, Houston, TX
Copenhaver, Catherine S., MD, Bethesda, MD
Corbett, Anna Rachel, MD, Denver, CO
Cordts, Vicki Lynne, MD, Cudahy, CA
Corovessis, Catherine C., MD, Katy, TX
Courban, Daniella, MD, Cambridge, MA
Cox, Carol S., MD, Tampa, FL
Crandall, Blane, MD, Clinton, OK
Crawford, Janel, MD, El Dorado Hills, CA
Crews, James H., MD, Jackson, MS
Cropper, Stephanie, MD, Pomona, CA
Crowley, Kelli B., MD, Schererville, IN
Crute, Kimberly A., MD, Savannah, GA
Curber, James M., MD, Charlottesville, VA
Cummings, Allegra, MD, New York, NY
Currenc, Andrea F., MD, Asheville, NC
Cutler, Wendy, MD, Austin, TX
Cwiak, Carrie, MD, Atlanta, GA
Daftary, Gaurang Shirish, MD, New Haven, CT
dalloul, Mudar, MD, Brooklyn, NY
dalton, Vanessa, MD, Ann Arbor, MI
davidson, Aaron, MD, Bayside, NY
davis, Daniel Heckel, MD, Columbus, IN
davis, Kent Stuart, MD, Seattle, WA
Davis, Michael, MD, Portland, OR
dayal, Molina Bhatnagar, MD, Rockville, MD
de Riese, cornelia, MD, lubbock, TX
de Vita, Laura, MD, Sandy Hook, CT
decesare, julie A. Zemaitis, MD, Gulf Breeze, FL
deFalco, Lisa, DO, Fredon, NJ
defranco, Emily, DO, Lexington, KY
dekay, Peter B., MD, Reno, NV
delong, Gregory A., MD, Millersburg, OH
deevers, Kimberly M., DO, apo, AE
dean, Gillian, MD, New York, NY
deev, adrienne M., MD, Ft. Worth, TX
deli, Barbara, MD, New York, NY
demasio, Kafui Alfreda, MD, MPH, North Castle, NY
dennard-Hall, Keisha, MD, Pittsburgh, PA
dennehy, Daniel Thomas, MD, Wallingford, CT
devlin, Jeanine Grillo, MD, Huntington Valley, PA
diluigi, Andrea, MD, West Hartford, CT
dietrich, Yvonne M., MD, Ocean Springs, MS
dillard, Naima Aleijah, MD, Greensboro, NC
dillard, Tiiffany Clark, MD, Champaign, IL
dinnall, Vanessa N., MD, New York, NY
dizon, Jennifer J., MD, South Pasadena, CA
dobay, Kristin Josef, MD, Clarksville, TN
doll-Pollard, Anne Elizabeth, MD, breeise, IL
Donnelly, Jennifer H., MD, Tulsa, OK
dougherty, John Joseph, MD, Franklin, VA
downs, Levi Stanford, Jr., MD, Minneapolis, MN
Drake, Brian E., MD, Portland, OR
Draper, Joy, MD, Richmond, VA
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Duncan, Kelly D., MD, Renton, WA
Dunn, Jennifer Colleen, MD, Elmhurst, IL
Duplantier, Noel Marie, MD, Bay St. Louis, MS
Durbin, Mary A., MD, Bay City, MI
Durfee, Mary Kay, MD, Forest Lake, MN
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Dyoco, Lorree L., MD, Pewaukee, WI
Dzanic-Cemalovic, Naida, MD, Roosevelt Island, NY
Eble, Amy Catherine Wiedo, MD, Manvel, TX
Echols, Karolynn Teresa, MD, Lafayette, LA
Eckert, Cynthia A., MD, Overland Park, KS
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Eckman, Troy Douglas, MD, Macomb, IL
Eddie, Abeer A., MD, East Amherst, NY
El Gammal, Nahed Z., MD, Decatur, GA
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Fahmy, Farris, MD, Rye Brook, NY
Famuyide, Abimbola O., MD, Rochester, MN
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Fiore, Joseph, MD, Hampton Cove, AL
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Flora, Stefanie, MD, Bellevue, MI
Flowers, Coy A., MD, Jacksonville, NC
Flubacher, John Scott, DO, Winter Harbor, ME
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Hennesy, Michael S., MD, Centennial, CO
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Herbolsheimer, Heather, DO, Bowie, MD
Hernandez, Carmelo A., MD, Brevard, NC
Hernandez-Parkhurst, Annette M., MD, Tucson, AZ
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Hill, James Bernard, MD, Fayetteville, NC
Hillebrand, Linda, DO, Laverne, CA
Hinton, Emily, MD, Dallas, TX
Hoe, Eleanor Meng-Yao, MD, Atlanta, GA
Ho, Phoebe Fei, MD, Puyallup, WA
Ho, Michael, MD, Atlanta, GA
Hogue, Thomas C., MD, Fayetteville, NC
Hoilett-Barrett, Althea, MD, Watertown, NY
Holcroft, Cynthia Jean, MD, Baltimore, MD
Hollingsworth, Jill A., MD, Indianapolis, IN
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Hou, Dennis, MD, Hillborough, CA
Hough, Tobi M., MD, Madison, IN
Householder, JeanMarie, MD, Fort Smith, AR
Hu, Michael Pao-Chun, MD, Chesterton, IN
Huang, Jennifer, MD, Holliswood, NY
Huang, Louise, MD, Novato, CA
Huang, Melinda, MD, Eastchester, NY
Huang, William, MD, New York, NY
Huang, Wilson H., MD, Henderson, NV
Huebert, Allison L., MD, Jefferson City, MO
Huertas, Otoniel, MD, Woodland Hills, CA
Icatar, Julianne Yantachka, MD, Norwalk, CT
Illuzzi, Jessica L., MD, Fairfield, CT
Imhoff, Anna L., MD, Winston-Salem, NC
Iskandar, Sammy R., MD, Easley, SC
Isom, Matthew J., DO, Harker Heights, TX
Ivester, Thomas Steven, MD, Chapel Hill, NC
Ivie, Jocelyn Q., MD, Las Vegas, NV
Jabara, Sami Issam, MD, Harrisburg, PA
Jacobson, Rebecca Eve, MD, Highland Park, IL
Jacobstein, Julie M., MD, Lafayette Hill, PA
Jain, Manish, MD, West Bloomfield, MI
Jarrell, April, MD, Spartanburg, SC
Jarvis, Nicole, MD, Norman, OK
Javernick-Hodges, Necole Ann, MD, Meridian, ID
Jazbec, Andrea M., MD, Denver, CO
Jebelli, Babak, MD, Redlands, CA
Jeffries, Lisa R., MD, Boulder, CO
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Jennings, Christopher, MD, Anderson, SC
Jensen, Teresa G., MD, Fremont, CA
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Jin, Wen Hui, MD, New York City, NY
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Johnson, Kim, DO, Trenton, MI
Johnson, Kristi A., MD, Wooster, OH
Johnson, Malinda K., MD, Andover, MN
Johnson, Melissa, MD, Sturbridge, MA
Johnson, Temeka L., MD, Pearl, MS
Johnson, Traci C., MD, Lilburn, GA
Jones, Andrea D., MD, Naperville, IL
Jones, Karin D.S., MD, Riverside, CA
Jones, Monica Brown, MD, Rochester, MN
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Jones, Rachel Zloczower, MD, Wilmington, NC
Jones-Monte, Kathleen R., MD, Henderson, NV
Jordan, Maureen O., MD, Colorado Springs, CO
Judge, Karen Olson, MD, Seattle, WA
Jurema, Marcus W., MD, Providence, RI
Ka, Hysoo, MD, New Lenox, IL
Kagumba, Ada A., MD, Quincy, IL
Kahen, Tanaz, MD, Encino, CA
Kakarla, Nirupama, MD, Bellaire, TX
Kalish, Robin Beth, MD, New York, NY
Kamat, Aparna, MD, Houston, TX
Kamelle, Scott Ahmed, MD, Santa Rosa, CA
Kang, Cambria, MD, Santa Ana, CA
Kang, Katherine Eunhwa Lee, MD, Fort Lee, NJ
Kaplan, Chaim E., MD, Brooklyn, NY
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Katz, Simone L., MD, Hayward, CA
Kaufman, Leah, MD, Glen Oaks, NY
Kaufman, Leesa Ann, MD, San Antonio, TX
Kavanagh, Colleen M., MD, Oakland, CA
Keeley, Christopher Courtney, MD, Roanoke, VA
Kelly-Layton, Tammy Rose, MD, Las Vegas, NV
Kerner, Nicole P., MD, Niskayuna, NY
Kerr, Mary Campbell, MD, Los Angeles, CA
Kessel, Allan D., MD, Ferndale, MI
Kesselman, Erica, MD, Pomeret Center, CT
Kessler, Michael, MD, Hartsdale, NY
Khabele, Dineo, MD, Nashville, TN
Khoudary, Maryann, MD, Warren, NJ
Kickham, Jennifer Moore, MD, Cambridge, MA
Kim, Eleanore S., MD, Oakland, CA
Kim, Martha B., MD, Portland, OR
Kim-Ashchi, Sunwook, MD, Jacksonville, FL
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King, Amy L., MD, Myrtle Beach, SC
King, Kathy A., MD, Oconomowoc, WI
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Kleiss, Kimberly McCann, MD, Atlanta, GA
Klingele, Christopher Joseph, MD, Rochester, MN
Knight, Teresa L., MD, Clayton, MO
Knutson, Christina M., MD, Boise, ID
Koellermeier, Michelle Marie, MD, Neenah, WI
Kohanowski, Erika Perl, MD, Mexico, MO
Kohl-Thomas, Belinda M., MD, Temple, TX
Kolberg, Amy M., MD, Yankton, SD
Kongkasuwan, Kimberly R., MD, Gent, WV
Koscica, Karen Lynn, D.O., Neptune, NJ
Koutoulas, Antigoni, MD, Boxford, MA
Kovac, Christine, MD, Georgetown, TX
Kovacs, Peter, MD, Budapest
Kramer, Susan L.J., MD, Highland Park, IL
Kruger, Janine K., MD, Middleton, WI
Kruskol, Bryan Mitchell, DO, South Elgin, IL
Kulsakdinun, Pamorn, MD, South Barrington, IL
Kusic, Michael B., MD, Falls Church, VA
Kwan, Mindy, MD, New York, NY
Kwiecien, Marni S., MD, Tigard, OR
Kwon, Christina Hyun Sook, MD, New York, NY
LaCour, Delese E., MD, Baltimore, MD
Laasch, Cassie, MD, Royal Oak, MI
Lachance, Deborah L., MD, Grand Forks, ND
Lai, Amy Y., MD, New Hyde Park, NY
Lalwani, Sasmira, MD, Cambridge, MA
Lam, Garrett Ka Keung, MD, Scottsdale, AZ
Lambrou, Nicholas Constantine, MD, Coral Gables, FL
Lamvu, Georigne M., MD, Durham, NC
Landes, Jennifer Mae, DO, Pennington, NJ
Lane, Felicia L., MD, Newport Beach, CA
Larkin, Molly A., MD, Chadds Ford, PA
Lashbrook, Daphne L., MD, Norman, OK
Lathi, Ruth Bunker, MD, Palo Alto, CA
Lawrence, Debra Celeste, MD, Conway, AR
Lawrence, Melissa, MD, Kailua, HI
Lawson, Glasine O., MD, St. Clair, MI
Lawton, Robyn Denise, MD, Placentia, CA
Le, Khanh Nha, MD, Fairfax, VA
LeBlanc, Joy Paul, MD, Pearlard, TX
Lee, Alice, MD, New York, NY
Lee, Angie Y., MD, Pompton Lakes, NJ
Lee, Ding-Ding Kelly, MD, Brooklyn, NY
Lee, Dorothy, MD, Great Falls, MT
Lee, Katrina L., MD, Plano, TX
Lee, Lily L., MD, Manhattan Beach, CA
Lee, Lydia K., MD, Roslindale, MA
Lee, Nina Y., MD, Antioch, CA
Lee, Sondra B., MD, Los Angeles, CA
Leff, Ricky Phillip, MD, Melbourne, FL
Leigh, Melanie A., MD, Abingdon, VA
Leonard, Caroline J., MD, St. Paul, MN
Leonard, Stacy Laraine, MD, Texarkana, TX
Leung, Michael P., MD, Houston, TX
Levi, Andrew J., MD, Easton, CT
Levin, Daniel Emil, MD, Hazlehurst, MS
Levy, Chanah, MD, Lutherville, MD
Lewis, Andrew J., MD, Winston-Salem, NC
Lewis, Arlene D., MD, Fredricksburg, VA
Lewis, Dawnette Ann-Marie, MD, MPH, Bronx, NY
Lewis-Boardman, Mary Beth, MD, Carmel, NY
Li, B. Charles, MD, Memphis, TN
Lin, Kathleen, MD, Philadelphia, PA
Lin, Nancy, MD, Portland, OR
Lin, Suzanne H., DO, Lewis Center, OH
Linares, Claudio E., MD, Oregon, OH
Lindemann, Matt, MD, Anchorage, AK
Lindley, Elisa M., MD, Rancho Mirage, CA
Lipschitz, Lisa, MD, San Diego, CA
Lipscomb, Lewis D., Jr., MD, Southern Pines, NC
Littles, Xercerla Adrenna, MD, Mesquite, TX
Lock, W. Scott, MD, Palo Alto, CA
Lockett, Tammy M., MD, Brooklyn, NY
Lockey, Renee, MD, Austin, TX
Loewen, Natalie K., MD, Salt Lake City, UT
Lograno, Paul, MD, Smithtown, NY
Lopez, Sandra, MD, Chula Vista, CA
Loudon, Holly C., MD, New York City, NY
Louis, Martha, MD, Glen Cove, NY
Loveland, Alecia, MD, Atlanta, GA
Loveland, Joan Elizabeth, MD, Washington, DC
Lowder, Laura Hines, MD, Charlotte, NC
Lowre, Cheri A., MD, Studio City, CA
Lu-Ferguson, Ming X., MD, Metuchen, NJ
Lucas, Maureen C., MD, Augusta, ME
Luke, Emily Spencer, MD, La Jolla, CA
Lyell, Deirdre Judith, MD, Palo Alto, CA
Lynch, Sean M., MD, Belmont, NC
Lyons, Karen S., MD, Glasgow, KY
MacLaurin, Nancy A., MD, Durham, NC
Maddox, Jennifer Mills, MD, Birmingham, AL
Mahoney, Mary K., MD, Minneapolis, MN
Mahr, Dominique, MD, Los Angeles, CA
Mainguy, Sarah Byfield, MD, Minneapolis, MN
Mann, Sylvia L. X., MD, Stevenson Ranch, CA
Marave, Jerome Sambrano, MD, Cockeysville, MD
Mark, Alice G., MD, Jamaica Plain, MA
Marks, Nicholas R., MD, Littleton, NH
Marquillin, Bridget M., MD, Timberlake, NC
Marshall, Kimberly A., MD, West Burlington, IA
Martin, Amy G., MD, Addison, TX
Martin, Jerry K., Jr., MD, Oxford, MS
Martin, Julie Ann, MD, Olathe, KS

2004 Ob/Gyn Oral Examination Diplomates
<table>
<thead>
<tr>
<th>Name</th>
<th>City, State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin, Todd D.</td>
<td>Lincoln, NE</td>
</tr>
<tr>
<td>Marvin, Judy L.</td>
<td>Spokane, WA</td>
</tr>
<tr>
<td>Marzano, David A.</td>
<td>Ann Arbor, MI</td>
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<tr>
<td>Mason, Romy E.</td>
<td>Denver, CO</td>
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<tr>
<td>Massa, Bonni Stacy</td>
<td>San Francisco, CA</td>
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<tr>
<td>Mathis, Robert T.</td>
<td>Columbus, GA</td>
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<tr>
<td>Mathison-Ezieme, Linda Joy</td>
<td>Harvey, LA</td>
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<tr>
<td>Mattern, Shannon E.T.</td>
<td>High Point, NC</td>
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<td>Matthews, Louise Suzanne</td>
<td>Winnetka, IL</td>
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<td>Mazarei, Nahid</td>
<td>Reston, VA</td>
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<tr>
<td>McCarthy, Lizbeth</td>
<td>Louisville, CO</td>
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<tr>
<td>McCartin, Richard T.</td>
<td>Kapolei, HI</td>
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<td>McClure, Kathryn A.</td>
<td>Gulfport, MS</td>
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<td>McCullough, Michael</td>
<td>Clarksville, TN</td>
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<tr>
<td>McDonald, Tedd Mikel</td>
<td>North Richland Hills, TX</td>
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<tr>
<td>McElrath, Thomas Frederick</td>
<td>Sherborn, MA</td>
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<td>McElroy, Tara M.</td>
<td>Mayfield Heights, OH</td>
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<td>McGee, Carmen</td>
<td>Atlanta, GA</td>
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<td>McGhee, Vida L.</td>
<td>Munster, IN</td>
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<td>Mcintosh, Elizabeth</td>
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<td>McIntosh, Kimberly Jaye</td>
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<tr>
<td>McKinney, Gisele</td>
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<td>McKeight, Erica J.</td>
<td>Hudson, OH</td>
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<tr>
<td>McLawhorn, Netasha D.S.</td>
<td>Henderson, NC</td>
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<tr>
<td>McNeive, Daniel F.</td>
<td>St. Louis, MO</td>
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<tr>
<td>Medley, Tamara C.</td>
<td>Grants Pass, OR</td>
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<td>Mehta, Minal G.</td>
<td>Irvine, CA</td>
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<td>Meigs, Amory H.</td>
<td>Philadelphia, PA</td>
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<td>Meinig, Martin L.</td>
<td>Fairfax, VA</td>
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<td>Mele, Michele M.</td>
<td>Philadelphia, PA</td>
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<td>Melnik, Marc David</td>
<td>Whittier, CA</td>
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<td>Memarzadeh, Sanaz</td>
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<td>Mendelsohn, Andrea R.</td>
<td>Castro Valley, CA</td>
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<tr>
<td>Merhi, Nahla O.</td>
<td>New Lenox, IL</td>
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<td>Merideth, Melissa A.</td>
<td>Bethesda, MD</td>
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<td>Mestad, Renee E.</td>
<td>Gresham, OR</td>
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<td>Metherell, James F.</td>
<td>Greenville, SC</td>
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<td>Meyer, Leslie S.</td>
<td>Fredericksburg, VA</td>
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<td>Michael, Kristi C.</td>
<td>Shreveport, LA</td>
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<td>Miguel, Breno Loureiro</td>
<td>Houston, TX</td>
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<td>Minto, Oleta R.</td>
<td>Aiken, SC</td>
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<td>Miser, Alyson Deas</td>
<td>Alexandria, VA</td>
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<td>Misra, Suman L.</td>
<td>Bloomfield Hills, MI</td>
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<td>Mitchell, Christopher Ray</td>
<td>Rome, GA</td>
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<td>Mitchell, Leah S.</td>
<td>Lexington, KY</td>
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<td>Moein, Sudabeh</td>
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<td>Mohamed, Fazil A.</td>
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<td>Mohammed, Decca</td>
<td>Parsippany, NY</td>
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<td>Molina-Millet, Leonardo</td>
<td>San Juan, PR</td>
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<td>Monela, Paul Richard</td>
<td>Henderson, NV</td>
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<td>Mondestin, Myriam A.</td>
<td>Monroe, NJ</td>
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<td>Morgan, Natalie A.</td>
<td>Omaha, NE</td>
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<td>Morgan, Sandra L.</td>
<td>Alpena, MI</td>
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<td>Morrison, Jeffrey D.</td>
<td>Newport News, VA</td>
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<td>Moseman, Cher P.</td>
<td>Colorado Springs, CO</td>
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<td>Moulton, James Richard, II</td>
<td>Atlanta, GA</td>
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<td>Moylan, Laura Beth</td>
<td>Dover, DE</td>
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<td>Mozayeni, Pantea</td>
<td>Azusa, CA</td>
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<td>Muench, Michael V.</td>
<td>Point Pleasant, NJ</td>
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<td>Mullin, Patrick Michael</td>
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<tr>
<td>Murdock, Tammy J.</td>
<td>San Antonio, TX</td>
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<tr>
<td>Murray, Catrina</td>
<td>South Bend, IN</td>
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<td>Murray, Kristin M.</td>
<td>Anchorage, AK</td>
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<td>Murthy, Kamaljeet Purhar</td>
<td>Lafayette Hill, PA</td>
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<tr>
<td>Murthy, Rachel M.</td>
<td>Athens, GA</td>
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<tr>
<td>Naim, Arjang</td>
<td>Los Angeles, CA</td>
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<tr>
<td>Nazareth, Sonja F.</td>
<td>Irvine, CA</td>
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<tr>
<td>Neal, Laura</td>
<td>Dubuque, IA</td>
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<tr>
<td>Nelson, Erin L.</td>
<td>Fayetteville, NC</td>
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Zoneraich, Nathaniel, MD, Vienna, VA

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