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⇒ indicates articles with a Clinical Perspective; ★ indicates articles available online only.
Dr Stephen Westaby attributes his professional approach to his having trained with John Kirklin, MD, at the department of surgery at the University of Alabama, Birmingham, and to a long-standing relationship with Denton Cooley, MD, and Bud Frazier, MD, of the Texas Heart Institute, Houston. Dr Westaby’s detailed knowledge of the history of cardiac surgery culminated in the publication of his book, *Landmarks in Cardiac Surgery*.1

After hearing of Dr Denton Cooley’s implantation of a totally artificial heart in July 1981, Dr Westaby travelled to Houston to meet the surgeon and see the device. Thus began his enduring interest in mechanical circulatory-support technology and his relationship with the centre. The Texas Heart Institute and the Oxford Centre collaborated with Robert Jarvik, MD, already widely known for his work on the development of artificial hearts, to introduce his miniaturised axial flow pump, the Jarvik 2000 left ventricular assist device (LVAD) (Figure 1). They are currently developing an even smaller device for infants and children.

Dr Westaby says, “There are several thousand patients in the United Kingdom with New York Heart Association stage III/IV heart failure who suffer severely debilitating symptoms of fatigue and breathlessness, who would be suitable for LVAD treatment.” These include patients with ischaemic heart disease or idiopathic dilated cardiomyopathy who do not have comorbidities that would terminate their lives within 2 years; for these patients, an off-the-shelf solution could offer good quality of life. “I see mechanical circulatory support not restricted to bridge to transplantation but offered as a widespread alternative to those without access to a donor heart,” he says.

Figure 1. The team celebrate the first Jarvik 2000 heart implantation: From the left, Dr Stephen Westaby, Dr Bud Frazier, Dr Denton Cooley, and Dr Robert Jarvik.

**Pioneers of Cardiology: Stephen Westaby, BSc, MS, PhD, FRCS, FESC**

**A Leader in the Use of Artificial Heart Technology Says It Is a Key, Cost-Effective Process**

Dr Stephen Westaby is a consultant cardiac surgeon and professor of biomedical science at the Oxford Heart Centre, United Kingdom. He has performed more than 10 000 heart operations and has an international reputation. A pioneer in artificial heart technology, he explains to Mark Nicholls how he sees this technology as an important element for the future of cardiac surgery.

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**European Society of Cardiology Working Groups**

Mario Marzilli, MD, chair of Working Group 6 on coronary pathophysiology and microcirculation explains how his group tries to raise awareness among cardiologists of small-vessel dysfunction in the pathogenesis of coronary disease. Page f117

**Cardioelectrophysiology in Poland**

Andrzej Lubinski, MD, and Andrzej Bissinger, MD, discuss the state of cardioelectrophysiology in Poland, where the number of implanted defibrillators has increased by 300% in the last 5 years. Page f119
Because of cost restrictions—each device costs £60 000 (€84 000) plus around £50 000 (€70 000) in hospital costs—the number of operations has been limited in the United Kingdom. Because of the restricted funds for research and development in the United Kingdom’s National Health Service, the clinical trials planned by Dr Westaby and his colleagues have not moved ahead. “All the destination therapy implants of the Jarvik system have been funded by charitable donations or peer review grants,” he says. “The United Kingdom could be at the forefront of applying new LVAD technology, but we have not been able to move it forward for economic reasons.” Although small, Dr Westaby’s programme has achieved good results. He points out, “The majority of patients have not died from cardiac failure but from other problems, such as cancer or lung disease.”

Dr Westaby also believes that heart failure patients receive unfair treatment within the National Health Service. “My argument is that renal failure patients are offered dialysis irrespective of age, with or without the possibility of transplantation. We have now developed an alternative for advanced heart failure that has proven reliable up to 7 years and that could be applied to probably 20 000 patients a year in this country. Most renal failure patients are treated, but palliation of advanced heart failure is by transplantation alone.” He believes that such operations will become cost-effective, particularly for patients who require multiple hospital admissions for stabilisation and who receive expensive devices such as cardiac resynchronisation and implantable defibrillators. He says, “I predict that the cost of LVADs will fall because of competition with newer and more innovative rotary blood pumps that will eventually be implanted without a major open operation. You only have to avoid 2 or 3 hospital admissions to pay for the device.”

Dr Westaby’s first permanent Jarvik implant has lasted far longer than any other type of artificial heart, and the patient, Peter Houghton has not had a single device-related adverse event. Mr Houghton was breathless at rest, with pitting oedema to the thighs, ulcerated legs, and ascites when he had the device implanted in June 2000. He has since spent less than 10% of his time in hospital; without the device, he would have died within weeks. “The tradeoff has been 7 years of life that is ongoing, with a substantial reduction in hospital costs,” says Dr Westaby. He adds, “The important thing for people who receive these LVADs is that they should not sit home and do nothing.”

Mr Houghton has been able to travel to the United States on several occasions (Figure 2) to give lectures about life with an artificial heart. He emphasises that one could not consider life with an LVAD normal. “You have to change the batteries twice a day and carry the equipment around continuously, but it is much better than being severely symptomatic with heart failure,” he says.

The Jarvik 2000 is implanted within the failing left ventricle via sternotomy or left thoracotomy. The development group addressed the life-threatening problem of power-line infection by bringing electricity through a titanium pedestal screwed onto the skull behind the ear (Figure 3). The development, based on the success of cochlea implant technology, was successful because scalp skin is highly vascular, lacks fat, and heals well. All external components of the system are exchangeable, so cables, the controller, and batteries can be exchanged periodically. This is particularly useful for permanent LVAD implants. Its robustness was once demonstrated when Mr Houghton was at a supermarket. A thief snatched the bag containing the device controller and the battery, disconnecting the cable from the skull pedestal. When the built-in alarm sounded, the assailant dropped the bag. When the cables were connected again, Mr Houghton’s LVAD restarted.

“The rate of recovery after receiving an LVAD is not dissimilar to that of transplantation,” says Dr Westaby, “though the titanium of the device is completely inert, so there is no rejection and little risk of infection.” Both in the laboratory and from clinical experience, the Oxford Group has shown that patients with continuous flow devices and attenuated pulse pressure in the circulation have normal organ function indefinitely. “The only major change is a predictable thinning
of the aortic medial layer in response to reduced mean blood pressure.” Dr Westaby says, “and I consider the attenuation of pulse pressure as a potential therapeutic option for patients with severe arterial disease.”

He also hopes to use LVADs to promote recovery in the failing left ventricle, and he believes the combination of LVADs with adjuvant therapy such as stem cells, genetic manipulation, or drug therapy will eventually provide an alternative to transplantation for most heart failure patients.

“We will use the LVAD to rest the native myocardium and remove elevated left ventricular end-diastolic pressure. This improves endocardial blood flow and may provide a platform on which stem transplantation rather than whole heart transplantation will be based,” he believes. “With smaller, more reliable devices, the American healthcare system expects LVADs to be a common treatment by 2010, with more than 100,000 implantations per year at a cost of $10,000,000."

Recently, Dr Westaby has used short-term LVADs to increase the safety of very-high-risk conventional heart failure operations. The blood pump sustains the patient through the period of myocardial stunning. Patients with left ventricular ejection fraction of <15% and elevated left ventricular end-diastolic pressure are identified preoperatively and are then weaned directly from cardiopulmonary bypass onto the LVAD for a period of 4 to 5 days. “Patients who might otherwise have died in cardiogenic shock have been sustained in this way and have subsequently recovered,” says Dr Westaby, “and I think that interventional cardiology, together with statins and lifestyle changes, will substantially reduce the amount of coronary bypass surgery worldwide, and heart failure surgery will increase in scope and become a speciality in itself.” He concludes, “The use of both short- and long-term circulatory support technology will play an important part in this programme.” In 2004, Dr Westaby won recognition for his efforts when he received the prestigious Ray C. Fish award for scientific achievement for his work with continuous-flow LVADs and the pulseless circulation.

Mark Nicholls is a freelance medical writer.

References
working group involves correcting the widespread but mistaken belief that coronary artery disease affects solely the major coronary vessels. Dr Marzilli explains, “Most cardiologists will look for a blockage and, if they don’t find one, will rule out coronary artery disease as a diagnosis. But a number of patients with ischaemic heart disease are not suffering from a single blocked artery at all. And this is not a small minority—it’s around 30% of cases!” He warns, “We need to look at ways to improve outcomes in these patients and to not discard them as being false positives just because no blockage is visible.”

The working group has 2 manuscripts in preparation that will draw attention to this problem. The first will make the case for considering coronary microcirculation in the pathophysiology of heart disease (see Figure), and the other will set out clinical methods to help patients whose disease stems from microcirculatory problems. “In the long run, we want to have the cardiology world fully aware that the problem is not just concentrated at 1 level but that it is spread around all the cardiovascular tree,” Dr Marzilli says.

But he recognises the challenge the working group faces in persuading the cardiology community as a whole to see coronary artery disease as a more complicated problem than the standard model suggests, and to see that simple interventions such as revascularisation and stenting may not provide the optimal solution for all patients.

“I’m fully confident that sooner or later we will achieve this aim,” he says, “but unfortunately, the weight of the individual ESC working groups depends on the financial potential of the research they support, and there is not a lot of money in our field. We are basically fighting a very unfair battle.” He comments, “We are a small group of people with limited, if any, support from industry, which means there is no money available. Yet, we’re trying to prove concepts that are not in the interest of the majority of cardiologists. In fact, our ideas may be perceived as negative by those people who just aim to increase the number of procedures of any kind because of the economics involved.”

Dr Marzilli says that the working group hopes to expand its numbers and attract new members, especially among young people. He adds, “I would like to see better communication between the leaders of the ESC and the working groups, and between the leaders of the working groups and the national societies of cardiologists they represent.”

In particular, he opposes the idea of the working group withdrawing from the ESC and forming an independent European society of specialists in microcirculation and coronary pathophysiology. He believes that this would lead to poorer communication with other cardiologists and to less valuable research.

He says, “When a working group becomes an independent society, it limits its interaction with other sections of cardiology. It focuses on its own interests exclusively and intensifies certain procedures and interventions that generally are associated with increasing cost, preventing a fully critical appraisal of the cost–benefit ratio.” Dr Marzilli continues, “This limits the critical comparison and discussion and prevents objective evaluation. And, of course, people who are involved in doing the procedure eventually also become those who say that this procedure is needed. It becomes sort of a scientific label that is not justified.”

He also acknowledges a more straightforward reason for why his working group will probably not follow the example of other specialist societies and split off from the ESC. “The pressure for the working groups to separate out from the ESC mostly comes from industry and the money involved, so we just don’t have that pressure!” he concludes.

Emma Baines is a freelance medical writer.
S
ome details of the health system in Poland and its financ-
ing system provide the context in which the country’s car-
dioelectrophysiologists work. The population of Poland is
about 38.5 million, and in 1997 the country had about 91 000
doctors (2.4 physicians per 1000 people), 214 000 nurses, and
about 679 general hospitals, with 6.2 beds per 1000 people.1

The health system in Poland is based on a central national
budget: the Narodowy Fundusz Zdrowia, or National Health
Fund. Those in employment pay social security and health
insurance contributions. Employers also contribute social
security and national insurance taxes for each employee. Those
insured under the Narodowy Fundusz Zdrowia have the right
to health services to maintain their health, prevent diseases,
have their injuries treated, and have diseases diagnosed and
treated. During hospital stays, patients receive all operations,
diagnostic tests, and medicines free of charge.

The Narodowy Fundusz Zdrowia has a limited budget;
this, in turn, limits access to medical procedures, including
 electrophysiology. Nevertheless, much progress has been
made. In the last 5 years, the number of implantable cardiac
defibrillators (ICDs) placed in patients increased by 300%,
and the number of ablations increased by >200%. Poland
currently has 77 pacemaker-implantation centres, 45 ICD-
implantation centres, and 26 ablation centres.

The number of new pacemaker implantations in Poland
is 548 per million/population, and 9 per million/population
receive biventricular-pacing pacemaker implantations. Fifty-
one per million/population receive ICD implantations and 91
per million/population receive ablation procedures. For com-
parison, about 300 ablations per million/population are per-
formed in Germany.

Dr Bissinger (pictured left) says, “Despite the increases
in electrophysiological pro-
cedures in recent years, the
numbers are still too small to
meet the demand for them.”
He explains, “For example, the
estimated number of patients
with Wolff-Parkinson-White
syndrome in our country is
about 180 000, but we are only doing ablations at the rate
of about 3000 per year. That means we cannot even keep
up with the population growth of patients with Wolff-
Parkinson-White syndrome.” He continues, “As a result, there
is a long line of people on the waiting lists. For example, in
our department, the waiting time for ICD implantation is
about 14 days; for Wolff-Parkinson-White ablation, it is about
2 months.”

Dr Andrzej Lubinski is chair of the Heart Rhythm
Working Group in Poland. This organisation began as a part
of the Polish Cardiac Society in 1973. Among its various
aims, the working group establishes standards and guidelines
of management and treatment of patients with cardiac
arrhythmias, lobbies government departments to improve the
development and financial support for the treatment of
cardiac arrhythmias, and cooperates with heart rhythm
organisations in other countries.

Dr Lubinski is also the chief of clinic of a new develop-
ment: the department of invasive cardiology and cardio-
diabetology at the Medical University of Lodz. Dr Lubinski
says, “The department was created just 2 years ago, and it is
composed of 3 parts,” and explains that these involve invasive
cardiology, both elective and in acute coronary syndromes;
electrophysiology, with 2 electrophysiology laboratories for
pacemakers and ICD implantations, radiofrequency abla-
tions, and CARTO mapping; and cardiodiabetology, where
cardiologists cooperate with diabetologists to treat patients
with diabetes mellitus and cardiovascular problems.

“In Poland, nearly 1 in 10 people have diabetes, and the
number having the disease is expected to double in 20 years,”
says Dr Lubinski. Patients with diabetes account for about
25% of all hospitalised patients with cardiovascular disease.
Diabetic patients with coronary artery disease have a mortal-
ity rate some 2- to 3-fold that of patients without diabetes.
Cardiovascular diseases are responsible for 70% to 80% of
deaths among diabetic patients. Dr Lubinski says, “Therefore,
it is very important to protect patients with diabetes and to
have this as a specific focus of our department’s work.”

Dr Lubinski points out, “The new department’s team has
presented results of research on cardiodiabetology problems
at several congresses in Poland and abroad.” He cites the
example of Dr Bissinger’s work on the effect of diabetic autonomic neuropathy on P-wave dispersion and recurrences of atrial fibrillation in patients with diabetes mellitus type 2. Dr Bissinger presented his findings during the World Congress of Cardiology in Barcelona, Spain, in September 2006.

When asked to identify the most satisfying aspect of his work, Dr Lubinski replies, “It is radiofrequency ablation—especially complicated arrhythmias like atrial fibrillation or ventricular arrhythmias. The procedures take a long time, but one is rewarded by the cure of the patient. It improves not only a patient’s quality of life; it often reduces mortality.”

Dr Bissinger considers successful biventricular pacing implantation to be the most satisfying part of his work. He says, “I enjoy this procedure, not only as a technical success, but for the clinical improvement of the patient. Biventricular pacing is not for all heart failure patients, so those who can potentially benefit need to be carefully identified. It’s gratifying to hear patients who had severe heart failure tell me after implantation just how much better they feel.”

Dr Bissinger also obtains great satisfaction from selecting the right patients for ICD implantation. “I am very satisfied when a patient who has had an ICD implantation for primary prevention of sudden cardiac death subsequently has a ventricular fibrillation incident properly detected and interrupted by the implanted device.” The Figure above demonstrates such an event.

Dr Bissinger concludes, “More information about electrophysiology in Poland will shortly be published in English as well as Polish.” This will be available on the Heart Rhythm Working Group Web site.2

Robert Short is a freelance medical writer.

References

This ECG recording shows an implantable cardiac defibrillator detecting ventricular fibrillation. A 20-J defibrillation shock then terminates the arrhythmia. The latter part of the tracing shows the restoration of sinus rhythm, which saves the patient’s life.

European Meetings Update

June 2007

15–19 June
17th Scientific Meeting of the European Society of Hypertension
Milan, Italy
For further information, contact info@eshmilano.org

19–23 June
Mayo International Vascular Symposium
Reykjavik, Iceland
For more information, contact cme@mayo.edu

21–23 June
2nd International Symposium Integrated Biomarkers in Cardiovascular Diseases
Berlin, Germany
For further information, contact biomarkers@lorenzinifoundation.org

24–27 June
Europace 2007
Lisbon, Portugal
For further information, contact europace@escardio.org
Ibanez et al. RESPONSES, ASSOCIATIONS OF EXERCISE TREADMILL TEST HERITABILITY, LINKAGE, AND GENETIC ANALYSIS OF ISCHEMIC MYOCARDIUM AT RISK USING CARDIAC MAGNETIC RESONANCE, EARLY METOPROLOL ADMINISTRATION BEFORE CORONARY REPERFUSION RESULTS IN INCREASED MYOCARDIAL SALVAGE: ANALYSIS OF ISCHEMIC MYOCARDIUM AT RISK USING CARDIAC MAGNETIC RESONANCE, by Ibanez et al.

The use of early β-blockade in the setting of acute myocardial infarction is recommended by guidelines (oral, class I and intravenous, class IIA) and is widely practiced. Nonetheless, the underlying mechanism of effect has not been firmly established, particularly for early intravenous therapy. Translational models have reported inconsistent results on infarct size, perhaps partly because infarct size has been measured indirectly using enzymatic release. In this issue of Circulation, Ibanez and colleagues use a porcine occlusion/reperfusion model and cardiac magnetic resonance imaging to examine the effect of intravenous metoprolol given during coronary occlusion on infarct size in a randomized, placebo-controlled format. Using recently validated cardiac magnetic resonance techniques, they show that given a similar volume of myocardium at risk, metoprolol was associated with greater myocardial salvage and smaller initial infarct size, as well as greater recovery of left ventricular function over 3 weeks. These interesting data shed light on at least one of the potential underlying mechanisms of effect of early β-blockade on infarct size using contemporary methodology. In an accompanying editorial, Bates examines these data within the perspective of the evolution of the use of β-blockade early in the course of acute myocardial infarction, with focus on contemporary trials and guidelines. See p 2909 (editorial p 2904).

RELATIONSHIP BETWEEN BLOOD PRESSURE AND STROKE RECURRENT IN PATIENTS WITH INTRACRANIAL ARTERIAL STENOSIS, by Turan et al.

To protect the brain against hyperperfusion, it is a common belief among clinicians that blood pressure should not run too low in patients with intracranial stenosis. On the basis of long-term data in >500 patients with angiographically verified stenosis (50–99%) of an intracranial artery, Turan et al investigated whether such a therapeutic approach is, indeed, justified. In the Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) trial, ischemic stroke during follow-up increased with increasing blood pressure before and after adjusting for other risk factors. Specifically, this was also the case in the territory supplied by the stenotic artery, both in patients with moderate as well as those with severe narrowing of an intracranial blood vessel. The risk of stroke was particularly pronounced in patients with the highest systolic blood pressure. Thus, in patients with known intracranial stenosis, higher blood pressure is associated with a higher risk of stroke, a conclusion that contradicts commonly held beliefs. The findings of Turan et al, therefore, suggest that the current practice in the management of such patients should be reconsidered. Interventional trials should be performed in this patient population to determine whether appropriate blood pressure lowering is as protective in this patient population as it is in hypertensive patients at large. See p 2969 (editorial p 2907).

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Images in Cardiovascular Medicine
Eosinophilic Heart Disease in Acute Myeloproliferative Disorder. See p e614.
Changes in Left Atrial and Pulmonary Venous Anatomy During Respiration: A 4-Dimensional Computed Tomography-Based Assessment and Implications for Atrial Fibrillation Ablation. See p e617.
Rapid Formation of Left Ventricular Giant Thrombus With Takotsubo Cardiomyopathy. See p e620.

Correspondence
See p e622.
Role of Intravenous β-Blockers in the Treatment of ST-Elevation Myocardial Infarction
Of Mice (Dogs, Pigs) and Men

Eric R. Bates, MD

Interestingly, the article that immediately followed the Reimer et al article in the November 1977 issue of Circulation was by the same group and was entitled “Infarct Size Reduction by Propranolol Before and After Coronary Ligation in Dogs.” Using the same animal model, they randomized dogs to intravenous propranolol before coronary occlusion, intravenous propranolol 3 hours after coronary occlusion, or saline infusion and euthanized the dogs at 24 hours without restoring reperfusion. Pretreatment with propranolol decreased transmural infarct size from 85% to 52%, whereas delayed treatment was about half as effective, decreasing infarct size to 71%.

Whether other pharmacological or mechanical interventions added to reperfusion therapy can further reduce myocardial infarct size has been a major area of preclinical and clinical investigation for 3 decades. There have been dozens of preclinical studies investigating the effect of intravenous β-adrenergic receptor blocking agents (β-blockers) on infarct size and left ventricular remodeling in ischemia-reperfusion models with rats, rabbits, dogs, and pigs. Benefit has been inconsistent, influenced by drug, dose, timing of initiation, ischemic time, measurement technique, and other variables.

In this issue of Circulation, Ibanez and colleagues subjected 12 Yorkshire pigs to balloon occlusion of the left anterior descending artery for 90 minutes followed by reperfusion. Compared with placebo, intravenous metoprolol administered 15 minutes after the onset of ischemia resulted in a 27% reduction in infarct size and was associated with improved regional and global left ventricular function as measured by high-resolution cardiac magnetic resonance imaging. Two of the placebo-treated pigs died before completing the protocol.

The use of cardiac magnetic resonance imaging to measure infarct size is an exciting research application of a new imaging modality, as illustrated by this elegant study. However, the clinical relevance of infarct size reduction with intravenous β-blocker therapy seen in this study is less obvious, especially when one considers that treatment was initiated only 15 minutes after interruption of coronary blood flow and total ischemic time was only 90 minutes. Moreover, abrupt ligation of a normal dog artery or balloon occlusion of a normal pig artery is different from thrombotic occlusion of an inflamed atherosclerotic human artery with distal embolization after reperfusion. In addition, human infarct size is modulated by preconditioning, intermittent or persistent infarct artery occlusion, collateral circulation, oxygen demand, and completeness of reperfusion. It has been known since the beginning of the fibrinolytic era that reperfusion must be accomplished within 3 hours of symptom onset to achieve human enzymatic infarct size reduction. Unfortunately, delays in time to treatment often prevent achievement of that goal. The major explanation for the failure of new interventions (drugs, thrombectomy devices, distal protection devices, cooling) to reduce infarct size in recent clinical trials has been that treatment usually was initiated within a later time frame.

Guideline Recommendations and Performance Measures
The 2004 European Society of Cardiology expert consensus document on β-blockers states that “intravenous administra-

—Robert Burns, “To a Mouse” (1785)
tion should be considered in patients with ischemic pain resistant to opiates, recurrent ischemia, and for the control of hypertension, tachycardia, and arrhythmias7 (class I indication). Intravenous β-blockers to limit infarct size have a class II recommendation. The 2004 American College of Cardiology/American Heart Association STEMI guidelines8 state that “it is reasonable to administer intravenous β-blockers promptly to STEMI patients without contraindications, especially if a tachyarrhythmia or hypertension is present” (class IIa recommendation). Furthermore, “β-blockers should not be administered to patients with frank cardiac failure evidenced by pulmonary congestion or signs of a low-output state” (class III recommendation). For the past 5 years, one of the Joint Commission core measures for STEMI has been the percentage of patients without contraindications who receive a β-blocker within 24 hours after hospital arrival (http://www.coreoptions.com/new_site/cahocore.html). Contraindications include β-blocker allergy, bradycardia (heart rate < 60 bpm), heart failure, shock, and second- or third-degree atrioventricular block.

### Clinical Trials

The evidence base for these guideline recommendations was heavily influenced by 3 trials from the early 1980s in which intravenous followed by oral β-blockers were used as monotherapy for STEMI.9–11 For instance, fibrinolytic therapy was not given in the First International Study of Infarct Survival (ISIS-1) trial, and only 5% of patients were discharged on an antplatelet agent.11 Patients treated within 12 hours of symptom onset had a 17% reduction in enzymatic infarct size in the Goteborg Metoprolol Trial,12 whereas enzymatic infarct size was lower in patients treated within 7 hours of symptom onset in the Metoprolol in Acute Myocardial Infarction (MIAMI) trial.10 A meta-analysis of 28 trials suggested that treatment of 1000 patients with β-blockers would lead to the avoidance of 6 deaths, 6 reinfarctions, and 4 cardiac arrests.11 A later meta-regression analysis found no mortality benefit with intravenous initiation of β-blocker therapy.13

In the reperfusion era, it has been difficult to prove any added benefit of intravenous β-blocker therapy. Van de Werf and coworkers14 randomized 292 patients receiving alteplase within 5 hours of STEMI onset to early intravenous and continued oral atenolol, alindine (a bradycardic agent lacking negative inotropism), or placebo. No differences could be observed in enzymatic or scintigraphic infarct size, left ventricular ejection fraction, or regional wall motion. Clinical events were similar except for a greater incidence of nonfatal pulmonary edema in the atenolol group (6% versus 1% in the alindine group and 0% in the placebo group; P=0.02). In the Thrombolysis in Myocardial Infarction II-B (TIMI-IB) study,15 1434 patients treated with alteplase within 6 hours of STEMI onset were randomized to immediate (within 2 hours of initiating lytic therapy) intravenous followed by oral metoprolol or deferred (day 6) oral metoprolol. No differences were detected in left ventricular ejection fraction or regional wall motion at hospital discharge. Mortality was not different at 6 weeks, but a lower incidence existed of reinfarction (2.7% versus 5.1%; P=0.02) and recurrent chest pain (18.8% versus 24.1%; P<0.02) at 6 days in the immediate intravenous group. This study was done in an era before routine treatment with enoxaparin, clopidogrel, and coronary stent implantation, interventions that also have been shown to reduce postreperfusion ischemic events.

In a post hoc analysis of the Global Utilization of Streptokinase and TPA for Occluded Arteries (GUSTO-1) trial16 early intravenous atenolol was associated with more death, heart failure, shock, recurrent ischemia, and pacemaker use than early oral use, despite the exclusion of patients with preexisting hypotension, bradycardia, or signs of heart failure. In the Clopidogrel and Metoprolol in Myocardial Infarction Trial (COMMIT),17 45 852 patients within 24 hours of suspected STEMI were randomized to intravenous and continued oral metoprolol or placebo. For every 1000 patients on treatment (mean, 15 days), metoprolol was associated with 1 less death (7.7% versus 7.8%; P=0.69), 5 fewer reinfarctions (2.0% versus 2.5%; P=0.001), and 5 fewer episodes of ventricular fibrillation (2.5% versus 3.0%; P=0.001). In contrast, 11 more patients had cardiogenic shock (5.0% versus 3.9%; P<0.0001), occurring mainly during the first 24 rates. Rates of cardiogenic shock were greater for those ≥70 years of age, with systolic blood pressure < 120 mm Hg, with a heart rate > 110 bpm, or with Killip class > 1. In addition, an excess of 14 patients experienced heart failure requiring treatment (14.1% versus 12.7%; P<0.0001), 31, persistent hypotension (6.0% versus 2.9%; P<0.0001), and 32, bradycardia (5.4% versus 2.2%; P<0.0001). The conclusion from both of these studies was that early intravenous β-blocker therapy had limited value and that later initiation of oral β-blocker therapy in stable patients was more prudent.

### Conclusions

Robert Burns, the Scottish poet, earned his living by farming. His sadness and despair at destroying a field mouse nest while plowing his fields, at a time (December) when it was impossible to rebuild, led to his poem “To a Mouse.” The famous lines quoted above and often by others in different human experiences serve as a metaphor 30 years after the seminal observations by Reimer et al for the mostly unsuccessful clinical attempts at further reducing myocardial infarct size with several agents, despite best intentions and great promise in preclinical studies and even phase II clinical trials. Similarly, one could conclude that frustration has been associated with a variety of cell therapy attempts in clinical trials to rebuild myocardial scar with functioning myocytes, despite preclinical promise, although it is hoped that goal will not be impossible to achieve.

Intravenous β-blockers have inconsistently reduced infarct size in animal models and have not reduced mortality rates in recent clinical trials. They also have been associated with some harm in those trials. It has been > 2 years, after presentation of the COMMIT trial results, that the American College of Cardiology/American Heart Association/Agency for Healthcare Research and Quality/Centers for Medicare and Medicaid Services/Joint Commission on Accreditation of Healthcare Organizations Practice Advisory was issued on the use of intravenous then oral β-blockers in the early stages of STEMI (http://www.ahrq.gov/clinic/commitadvisory.htm).
No final statement on performance measures has been released. It may be time to remove routine intravenous β-blocker therapy from our acute treatment protocols for STEMI and instead focus on initiating oral β-blocker (and angiotensin-converting enzyme inhibitor) therapy the next day when hemodynamic stability has been established. This technique would remove the early risk associated with β-blockers while retaining their hospital benefit on reinfarction and ventricular fibrillation rates. Unfortunately, despite the best laid schemes of mice (dogs, pigs) and men with regard to intravenous β-blocker therapy, early reperfusion therapy remains the best (and maybe only) strategy for limiting myocardial infarct size in patients with STEMI.

Disclosures

None.

References


Key Words: Editorials, myocardial infarction, receptors, adrenergic, beta reperfusion
Cerebroprotection by Hypertension in Ischemic Stroke
The Crumbling of a Hypothesis

Franz H. Messerli, MD

May not the elevation of systemic blood pressure be a natural response to guarantee a more normal circulation to the heart, brain and kidneys? These words, taken from a renowned textbook of medicine, clearly illustrate that in the 1940s the teaching doctrine was to consider elevated blood pressure a compensatory mechanism serving to force blood through sclerotic arteries to the ischemic target organs. Hypertension was regarded as “essential” and therefore “should not be tampered with, even were it certain that we could control it.”  We have since learned that hypertension is a powerful risk factor for stroke, heart attacks, and renal failure and that lowering blood pressure dramatically reduces the risk of these events. The only clinical situation in which blood pressure elevation often still is considered protective is in the sequence of an acute ischemic stroke. Indeed, authoritative voices such as that of Adams and Victor have warned and continue to warn against lowering blood pressure in this setting with statements such as, “We agree with Britton and colleagues that it is prudent to avoid antihypertensive drugs in the first few days unless...the blood pressure is high enough to pose a risk to other organs.” This statement can be found in the 1989 edition of this venerable neurology textbook and is repeated verbatim in every single subsequent edition until 2005. It thus has taught numerous neurologists that elevated blood pressure in the sequence of an ischemic stroke was a “noli me tangere” and that lowering blood pressure should be avoided. Because Adams and Victor obviously considered the referenced study to be definitive enough to be taught for many years, I took the liberty to look at it carefully. In their article, Britton et al reported on a series of 6 patients presenting with acute onset of neurological symptoms and extremely high blood pressure who had either a hypertensive crisis or a stroke. Five of 6 patients were comatose before admission, and in 4 of the 6 patients, a hemorrhagic (not an ischemic) stroke was documented. With prompt institution of antihypertensive therapy, systolic pressure was lowered precipitously to <100 mm Hg. Not unexpectedly, of the 6, only 1 patient survived. On the basis of their few cases, the authors concluded that convincing evidence of a beneficial effect of blood pressure reduction in the setting of an acute stroke was lacking but also considered that “the deterioration might have been the natural terminal cause in these patients with severe brain lesions.” Clearly, from this meager study, no conclusion can be drawn on the management of blood pressure in patients with ischemic stroke.

The article by Turan et al in the present issue of Circulation throws some light on this contested issue. The authors reported that in patients with intracranial stenosis, the risk of ischemic stroke was increased rather than decreased with higher blood pressure and that this also was true in the territory of the stenotic vessel. Whether stenosis was moderate (<70%) or more severe, increased blood pressure, diastolic more than systolic, increased the risk of stroke in the territory of the stenotic vessel. Although the risk of a subsequent stroke was driven mainly by systolic blood pressure elevations >160 mm Hg, no evidence existed that maintaining systolic pressure in the stage I hypertensive range (between 140 and 159 mm Hg) was cerebroprotective. Of note, this study is a post hoc analysis based on average follow-up blood pressures and therefore does not allow any conclusions on the relationship between ischemic stroke and blood pressure at the time of the acute event. However, the findings argue strongly against the common clinical wisdom of leaving high blood pressure untreated in patients with intracranial stenosis. Lowering blood pressure was not associated with an increased stroke risk after 4 months. Moreover, the cerebrovascular benefits of a decrease in blood pressure continued to grow for several years. Both the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) and the Individual Analysis of Antihypertensive Intervention Trials (INDANA) database have clearly shown that in patients with cerebrovascular disease, blood pressure remains the most important risk factor for a recurrent event.

Recent experimental data fully support the view of Turan et al. Neurovascular protection was conferred by lowering blood pressure with antihypertensive therapy 3 hours after middle cerebral artery occlusion during reperfusion after experimental cerebral ischemia in rats. Although enalapril or dihydralazine caused a decrease in infarct size without influencing neurological outcome, the angiotensin receptor blocker (ARB) candesartan caused a similar decrease in blood pressure and infarct size but also resulted in improved neurological outcome. We have previously suggested that antihypertensive medications that increase angiotensin II levels such as thiazide diuretics, calcium antagonists, and ARBs could be more cerebroprotective than agents that lower angiotensin II levels such as β-blockers and angiotensin-converting enzyme inhibitors. This hypothesis was based on experimental findings but also was supported by a recent meta-analysis of 206,632 patients in 26 prospective randomized clinical trials. Angiotensin II–decreasing drugs proved to be less stroke protective than antihypertensive drugs.
that increased angiotensin II levels. Thus, ARBs may well have stroke protective effects beyond blood pressure lowering. In the recent Morbidity and Mortality After Stroke, Eprosartan Compared With Nitrendipine for Secondary Prevention (MOSES) trial, eprosartan, for a similar blood pressure reduction, reduced cerebrovascular events better than nitrendipine in hypertensive stroke patients.\textsuperscript{14} Whether these stroke protective effects occur in the arterial tree of the brain or in the brain tissue itself is unknown. However, if indeed the brain tissue is involved, the effect may depend on the ability of the ARB to cross the blood-brain barrier. Conceivably, not all ARBs are created equal in this regard.\textsuperscript{15}

Most patients with an acute ischemic stroke experience a transient increase in blood pressure regardless of whether they were hypertensive or normotensive before the event. The pathogenesis of stroke-associated hypertension is likely to be multifactorial, possibly related to stress and anxiety, intracerebral pressure, reactive bradycardia, increased activity of the sympathetic nervous system, etc. The penumbra is an underperfused but viable zone surrounding the infarcted area in the cerebrum. Current dogma teaches that survival of the penumbra depends on an increase in blood pressure and that any fall in blood pressure could possibly threaten survival of the penumbra and increase the infarcted area.\textsuperscript{16} Indeed, in patients with ischemic stroke, injections of epinephrine have been recommended as a means of raising the systemic blood pressure above the usual levels to guarantee adequate perfusion of the penumbra.\textsuperscript{3} Not surprisingly, no outcome data are available to support the clinical use of this heroic procedure.

Conversely, we should consider that elevated blood pressure in the sequence of an ischemic stroke may not only increase the risk of cerebral hemorrhage into the infarcted area but also give rise to ischemic edema. Thus, at the present, no consensus exists on how to best treat patients with elevated blood pressure after ischemic stroke. Even a thorough Cochrane review was unable to produce conclusions, finding the data “wholly inadequate” to guide clinical practice.\textsuperscript{17} Fortunately, 2 trials—Continue or Stop Post-Stroke Antihypertensives Collaborative Study (COS-SACS)\textsuperscript{18} and Controlling Hypertension and Hypotension Immediately Post-Stroke (CHIPPS)\textsuperscript{19}—are underway that may throw some light on the risks and benefits of blood pressure lowering in patients with acute stroke.

Where does this leave the consulting physician who has to deal with an acute blood pressure elevation in a poststroke patient? The blood pressure in such patients may be exquisitely sensitive to antihypertensive therapy, and a gingerly approach is patient? The blood pressure in such patients may be exquisitely deal with an acute blood pressure elevation in a poststroke

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**Disclosures**

Dr Messerli reports having served as an ad hoc consultant/speaker for the following organizations: Abbott, GlaxoSmithKline, Novartis, Pfizer, AstraZeneca, Bayer, Boehringer Ingelheim, Merck, Bristol-Meyers Squibb, Forest, Sankyo, and Sanofi.

**References**

Early Metoprolol Administration Before Coronary Reperfusion Results in Increased Myocardial Salvage
Analysis of Ischemic Myocardium at Risk Using Cardiac Magnetic Resonance

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Background—β-Blockers improve clinical outcome when administered early after acute myocardial infarction. However, whether β-blockers actually reduce the myocardial infarction size is still in dispute. Cardiac magnetic resonance imaging can accurately depict the left ventricular (LV) ischemic myocardium at risk (T2-weighted hyperintense region) early after myocardial infarction, as well as the extent of necrosis (delayed gadolinium enhancement). The aim of this study was to determine whether early administration of metoprolol could increase myocardial salvage, measured as the difference between the extent of myocardium at risk and myocardial necrosis.

Methods and Results—Twelve Yorkshire pigs underwent a 90-minute left anterior descending coronary occlusion, followed by reperfusion. They were randomized to metoprolol (7.5 mg during myocardial infarction) or placebo. Global and regional LV function, extent of myocardium at risk, and myocardial necrosis were quantified by cardiac magnetic resonance imaging studies performed 4 and 22 days after reperfusion in 10 survivors. Despite similar extent of myocardium at risk in metoprolol- and placebo-treated pigs (30.9% of LV versus 30.6%; P=NS), metoprolol resulted in 5-fold-larger salvaged myocardium (32.4% versus 6.2% of myocardium at risk; P=0.015). The LV ejection fraction significantly improved in metoprolol-treated pigs between days 4 and 22 (37.2% versus 43.0%; P=0.037), whereas it remained unchanged in pigs treated with placebo (35.1% versus 35.0%; P=NS). The extent of myocardial salvage was related directly to LV ejection fraction improvement (P=0.031) and regional LV wall motion recovery (P=0.039) at day 22.

Conclusions—Early metoprolol administration during acute coronary occlusion increases myocardial salvage. The extent of myocardial salvage, measured as the difference between myocardium at risk and myocardial necrosis, was associated with regional and global LV motion improvement. (Circulation. 2007;115:2909-2916.)

Key Words: imaging ■ magnetic resonance imaging ■ metoprolol ■ myocardial infarction
rately quantify the extent of myocardial necrosis in vivo. It also has been shown that CMR can depict the area of myocardium at risk, which displays high signal intensity on T2-weighted images early after MI as a result of the presence of edema. Therefore, it is feasible to noninvasively evaluate with CMR the extent of myocardial salvage as the difference between myocardium at risk and myocardial necrosis. The aim of this study was to analyze the therapeutic benefit of early intravenous β-blocker administration on the ischemic myocardium at risk in a swine model of acute coronary occlusion.

**Methods**

**Study Design**

Acute MI was experimentally induced in Yorkshire Albino pigs (n=12; weight, 33±3 kg) by closed-chest, 90-minute left anterior descending coronary artery occlusion. Animals were randomized 1:1 to intravenous metoprolol or placebo (sodium chloride). CMR studies were performed 4 and 22 days after MI to quantify LV global and regional functional parameters, area of edema, and MI size. Animals were euthanized within 1 hour after the last CMR study for histopathological validation. The study protocol was approved by an institutional animal research committee.

**Experimental Procedures**

Twelve hours before the experimental MI, a loading dose of clopidogrel (150 mg) was administered. Subsequently, clopidogrel (75 mg/d) was maintained for 5 days. Anesthesia for the intervention was induced by intramuscular injection of ketamine (30 mg/kg), xylazine (2.2 mg/kg), and atropine (0.05 mg/kg). Animals underwent endotracheal intubation, and anesthesia was maintained by isoflurane inhalation. Continuous infusions of amiodarone (300 mg, 75 mg/h) and lidocaine (150 mg, 37.5 mg/h) were initiated before the procedure in all pigs as prophylaxis for malignant ventricular arrhythmias. Cardiac rhythm and arterial oximetry were monitored continuously during the procedure. MI was induced by catheter-based 90-minute balloon occlusion of the left anterior descending coronary artery immediately after the origin of the first diagonal branch. Approximately 15 minutes after balloon inflation, intravenous metoprolol (three 2.5-mg injections every 3 to 5 minutes for a total of 7.5 mg) was infused into the pigs assigned to the β-blocker arm. After balloon deflation, patency of the left anterior descending artery was angiographically confirmed by contrast injection. Buprenorphine (0.03 mg/kg) and ceftazoline (25 mg/kg) were administered every 12 hours for 5 days in all animals.

For the CMR studies, pigs were anesthetized by intramuscular injection of ketamine, xylazine, and atropine. Anesthesia was maintained by continuous intravenous propofol infusion. Animals were kept under mechanical ventilation. After the last CMR, animals were heparinized (100 IU/kg) and euthanized with pentobarbital (Sleepaway 75 mg/kg, Fort Dodge, Wyeth, Overland Park, Kan), and the heart was excised for histopathological analysis.

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.

**Noninvasive CMR Protocol**

CMR studies were performed with a 1.5-T magnet (Magneton Sonata, Siemens Medical Solutions, Erlangen, Germany) using a phased-array cardiac coil by investigators blinded to the treatment arm. All images were acquired with ECG gating and during suspended respiration. First, contiguous short-axis cine images covering the LV from base to apex were acquired using a standard steady-state free-precession sequence (repetition time, 3.5 ms; echo time, 1.5 ms, flip angle, 60° to 90°; field of view, 200×150 mm; phase oversampling, 80%; generalized autocalibrating partially parallel acquisitions (GRAPPA) factor, 2; matrix, 192×115; slice thickness, 6 mm; no gap; bandwidth, 930 Hz per pixel; lines per segment, 11). Subsequently, edema imaging was performed with a T2-weighted, triple inversion-recovery fast spin-echo sequence (repetition time, 2 to 3 heartbeats; echo time, 65 ms; time interval, 100 ms; field of view, 300×225 mm; matrix, 256×125; slice thickness, 6 mm; bandwidth, 349 Hz per pixel; echo-train length, 17). Finally, DE imaging was performed 15 minutes after the administration of 0.2 mmol/kg gadopentate dimeglumine using an inversion-recovery fast gradient-echo sequence (repetition time, 8 ms; echo time, 4 ms; time interval optimized to null normal myocardium; gating factor, 2 to 3; field of view, 300×225 mm; matrix, 256×144; slice thickness, 6 mm; bandwidth, 160 Hz per pixel; lines per segment, 23). The slice positions for both T2-weighted and DE acquisitions matched those of the cine images.

**CMR Data Analysis**

All CMR images were analyzed by researchers blinded to the study arm or histopathology data. LV function analysis was performed with dedicated software (Argus, Siemens Corporate Research, Princeton, NJ). After manual tracing of epicardial and endocardial contours, a large region of interest was drawn within a remote normal myocardial segment. Abnormal areas for each sequence, defined as those with a signal intensity 3 SD above the mean signal intensity of normal myocardium, were automatically highlighted and quantified (Figure 1). Myocardial necrosis was defined by the extent of abnormal DE; myocardium at risk was defined by the extent of edema (high signal intensity on T2-weighted images) in the day 4 CMR study; and salvaged myocardium was defined as the difference between myocardium at risk and myocardial necrosis. All measurements were expressed as percentage of the total LV myocardial volume; the absolute MI size also was quantified in grams (calculated as volume multiplied by myocardial density [1.05 g/cm³]). The transmural extent index of MI within each segment was calculated as a percentage of the total segment area as previously described. Global transmural extent index of MI was calculated as the mean of all segmental transmural extent indexes of MI in the DE-positive segments. In addition, 3 consecutive short-axis slices containing both edema and DE were selected in each animal for the segmental (regional) analyses of edema and DE distribution.

In the T2-weighted and DE images, the signal-to-noise ratios of both normal and abnormal myocardium were quantified as the average of the mean signal intensity within a region of interest divided by the mean value of noise (obtained from a region of interest in the air). Contrast-to-noise ratios of abnormal versus normal myocardium were defined as the difference of their signal-to-noise ratios.

**Histological Infarct Size Analysis**

After the animals were sacrificed, the hearts were perfused with cold PBS and stiffened by overnight immersion in isotonic agar solution at 4°C. After stiffening, hearts were washed with cold PBS, and the LV was sliced (short-axis, 6-mm-thick slices without gap) with a commercial meat slicer. Slices were incubated for 5 to 7 minutes in warm 1% trimethyl tetrazolium chloride (TTC) solution at 37°C. After TTC incubation, the slices were immersed in 4% paraformaldehyde for 12 hours. After paraformaldehyde fixation, high-resolution digital images from all slices were acquired, and areas of infarction (negative for TTC staining) and normal myocardium (positive TTC staining) were quantified with ImageJ software (National Institutes of Health, Bethesda, Md) (Figure 2, top). The MI volume was expressed as a percentage of the total LV myocardium.
Statistical Analysis

Continuous variables are expressed as mean±SEM. Statistical comparisons of means were made by Student’s paired and unpaired t tests. To calculate the correlation of variables, Pearson’s coefficients were used. The limits of agreement between infarct size in DE CMR and histology were analyzed by the Bland-Altman plot. Two multivariate linear regression models were performed to predict the change of global LVEF and segmental systolic thickening, respectively. Baseline (day 4 CMR) variables that either had a clinically plausible relation to improvement of function or appeared to be associated with an increase in LVEF or regional systolic thickening, indicated by a value of \( P<0.20 \) in univariate analysis, were used as independent variables. For the global and regional functional improvement, the independent variables were LVEF, volume of noninfarcted myocardium, global transmural extent index of MI, and extent of salvaged myocardium (global) and percentage of systolic thickening within each segment, segmental volume of noninfarcted myocardium, transmural extent index of MI, and the extent of segmental salvaged myocardium (regional). A value of \( P<0.05 \) (2 tailed) was considered statistically significant. All statistical analyses were performed with the statistical software package SPSS 11.0 (SPSS Inc, Chicago, Ill).

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Successful left anterior descending coronary artery occlusion was achieved in all 12 cases. Two animals died of refractory ventricular fibrillation during the procedure. Therefore, a total of 10 pigs (6 in the metoprolol arm, 4 in the placebo group) made up the final study. Both groups showed a similar mean heart rate during the procedure (66±2 and 65±3 bpm for the metoprolol and placebo groups, respectively; \( P=\text{NS} \)). The metoprolol group showed a significantly lower incidence of ventricular fibrillation than the placebo group (33% versus 66%, respectively; \( P=0.047 \)) and a similar rate of ventricular tachycardia (33% versus 33%; \( P=\text{NS} \)) during the procedure.

CMR Analysis

The average signal-to-noise ratio for the T2-weighted images was 9.94±0.5 in the edematous myocardium and 3.77±1.7 in the remote normal myocardium (\( P<0.001 \)). The corresponding values on the DE images were 6.68±0.3 for infarcted myocardium and 1.57±0.1 for normal myocardium, respectively (\( P<0.001 \)). The contrast-to-noise ratio between abnormal and normal myocardium was similar for both sequences (6.17±0.5 for T2-weighted images, 5.11±0.3 for DE images: \( P=\text{NS} \)).

The results of CMR-derived parameters at days 4 and 22 after MI are presented in the Table. At day 4, no significant differences were observed in LVEF between study groups. From day 4 to 22, the LVEF significantly improved in the metoprolol arm (\( P=0.037 \)), whereas it remained unchanged in the placebo arm. Change in the LVEF from day 4 to 22 was higher in metoprolol animals (5.8 versus −0.1; \( P=0.079 \)). Metoprolol treatment resulted in a significantly smaller extent of MI in terms of both absolute infarct mass and percentage of the LV myocardium, noticeable at day 4. The extent of myocardium at risk (volume of edema at day 4 CMR) did not differ between the 2 groups. As a result, the percentage of salvaged myocardium (the primary comparison of the study)
was significantly larger in metoprolol animals (32.4±6.0%) than in the placebo group (6.2±6.8%; \( P=0.015 \); the Table and Figure 3).

In all cases, the transmural extent index of MI was 100% in \( \geq 4 \) segments. Regional analysis of DE-positive segments showed a nonsignificant difference in the transmural extent index of MI (73±3% in metoprolol versus 68±3% in the placebo group; \( P=\text{NS} \); Figure 4).

Overall, at day 4, edema-positive segments showed a statistically significant lower percentage of systolic thickening than nonedematous segments (20±2% versus 38±3%; \( P<0.001 \)). In addition, we found a statistically significant inverse correlation (\( R=-0.42, P<0.001 \)) between the presence of edema and percentage systolic thickening at day 4.

To examine which CMR parameters at day 4 predict global or segmental functional improvement over time, multivariate regression models were used. The basal LVEF (\( \beta=0.979, P=0.004 \)) and the extent of salvaged myocardium at day 4 (\( \beta=0.897, P=0.039 \)) were strongly associated with improvement of LVEF over time. The global transmural extent index also showed a nonstatistically significant (\( \beta=0.854, P=0.065 \)) association with LVEF improvement.

At a segmental level, the regional size of salvaged myocardium at day 4 was the only variable associated with the improvement in percentage of wall thickening between days 4 and 22 (\( \beta=0.333, P=0.036 \)).

**Histopathology–CMR Correlation**

Excellent correlation (\( R=0.844, P=0.008 \)) and agreement (mean bias, −2%; limits of agreement, 4.1% and −8%) were observed between infarct volume in histology (TTC staining) and the volume of DE in the last CMR (Figure 2). No correlation was observed between infarct volume on histology and volume of edema in the last CMR study.

**Discussion**

In this study, we describe the benefits associated with early administration of metoprolol on LV function and myocardial salvage in an experimental model of acute coronary occlusion. The porcine model of acute MI was selected because of the anatomophysiological similarities with humans. The functional and structural LV performance was evaluated in a reproducible swine model of anterior wall MI over a 3-week period. The reproducibility of the experimental model is highlighted by the small dispersion values of the volume of myocardium at risk (30.7±1.6% of LV). We exploited the versatility and the noninvasive characteristics of high-resolution CMR, validating the results with the most conventional histopathological analysis. The main findings of the present study are that (1) intravenous metoprolol during coronary occlusion and before mechanical reperfusion is a highly effective cardioprotective agent, resulting in a 27% smaller MI than placebo, despite an initially equivalent amount of myocardium at risk, a cardioprotective effect that was independent of its negative chronotropic effects, and (2) the extent of myocardial salvage was an independent predictor of LV functional recovery, both global and regional wall motion.

To the best of our knowledge, this is the first in vivo, noninvasive evaluation of the effect of \( \beta \)-blockade on MI size with high-resolution CMR. In addition, we could perform detailed in vivo characterization of the entire ischemic region, not only of the MI size but also of the salvaged myocardium (noninfarcted myocardium at risk). A controlled model of experimental MI enabled us to evaluate the independent effects of intravenous metoprolol administration on MI size. This may be more difficult to achieve in a clinical environment, where many other factors such as duration and degree of coronary occlusion, completeness of reperfusion, and prior medication use play a role in final MI size.
The efficacy of β-blockers as cardioprotective agents has been widely studied. Preclinical animal studies have shown contradictory results: Experimental models of reperfused\textsuperscript{15,19} and nonreperfused\textsuperscript{16,18,20} MI showed either a reduction in\textsuperscript{15,16} or no effect on the final MI size.\textsuperscript{18–20} Most of the animal studies analyzed the MI size ex vivo (postmortem) early after the MI induction without follow-up.

In clinical practice, β-blockers have unquestionably demonstrated to be beneficial in the setting of acute MI, resulting in reduced mortality when administered early.\textsuperscript{4–6} As a result, current practice guidelines recommend early β-blockade in subjects after an acute MI,\textsuperscript{11} although no general consensus exists on the optimal timing of administration. In this clinical scenario, the effect of β-blockade on MI size is controversial. In the prethrombolytic era, several clinical trials investigated the impact of β-blockade on MI size. In the Multicenter Investigation of the Limitation of Infarct Size trial, intravenous propranolol followed by oral treatment failed to reduce MI size.\textsuperscript{24} Comparable results were found with similar regimens of propranolol administration by other investigators.\textsuperscript{33} Contrarily, other studies have demonstrated a significant reduction in MI size in patients receiving β-blockers compared with control subjects.\textsuperscript{4,21,23} In the thrombolytic era for MI reperfusion, the results were also inconclusive. Van de Werf et al\textsuperscript{34} showed that the intravenous administration of atenolol followed by oral therapy to MI patients receiving...

### CMR-Derived Parameters

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<th>Metoprolol</th>
<th>Placebo</th>
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<tr>
<td>Day 4 CMR</td>
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<tr>
<td>LVEDV, mL</td>
<td>96±6</td>
<td>123±12</td>
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<td>82±15</td>
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<td>LVEF, %</td>
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<td>Myocardium at risk,* % of LV with edema</td>
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<td>30.6±1.0</td>
<td>0.6</td>
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<tr>
<td>Infarct volume, % of LV</td>
<td>20.9±1.6</td>
<td>28.7±2.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Infarct mass, g</td>
<td>12.7±0.7</td>
<td>21.9±2.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Salvaged myocardium,† % of LV</td>
<td>10.0±2.3</td>
<td>1.9±1.2</td>
<td>0.028</td>
</tr>
<tr>
<td>Percent salvaged myocardium‡</td>
<td>32.4±6.0</td>
<td>6.2±6.8</td>
<td>0.015</td>
</tr>
<tr>
<td>Day 22 CMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDV, mL</td>
<td>109±8</td>
<td>132±9</td>
<td>0.1</td>
</tr>
<tr>
<td>LVESV, mL</td>
<td>62±5</td>
<td>86±10</td>
<td>0.09</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>43.0±2.8$§</td>
<td>35.0±3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>LV with edema, %</td>
<td>15.2±2.5$§</td>
<td>20.0±2.5$§</td>
<td>0.2</td>
</tr>
<tr>
<td>Infarct volume, % of LV</td>
<td>16.6±1.3</td>
<td>20.6±1.7$§</td>
<td>0.1</td>
</tr>
<tr>
<td>Infarct mass, g</td>
<td>11.5±1.2</td>
<td>16.4±1.4$§</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are mean±SEM where appropriate. LVEDV indicates LV end-diastolic volume; LVESV, LV end-systolic volume.

*Edematous myocardium.
†The volume of LV showing edema but not DE in the day 4 CMR.
‡Obtained as follows: 100×extent of salvaged myocardium/extent of edematous myocardium in the day 4 CMR.
§Significant differences between day 4 and 22 CMR.

![Figure 3](image-url). Percent salvaged myocardium (salvaged myocardium normalized to myocardium at risk). The y axis corresponds to “100×extent of salvaged myocardium/extent of myocardium at risk.” A, Mean and SE of the mean of both treatment arms; B, the individual data.

![Figure 4](image-url). Distribution of the transmural extent index of MI in DE-positive segments. Center line represents the 50th percentile; box plots illustrate the 25th and 75th percentiles.
alteplase did not reduce MI size. In the Thrombolysis Early in Heart Attack Trial,35 patients from the recombinant tissue plasminogen activator plus metoprolol arm had smaller MIs than those in the recombinant tissue plasminogen activator alone arm. In the age of percutaneous interventions for coronary revascularization, the effect of β-blockade has been analyzed in a limited and nonrandomized fashion. Although several observations have confirmed the beneficial clinical effect of early β-blockade after MI with this invasive reperfusion modality,36–38 the effect of β-blocker administration in MI size remains unclear. The administration of β-blockers before elective percutaneous coronary interventions also has been associated with significant discrepancies; although intracranial propranolol resulted in less myocardial damage,39 the oral administration of metoprolol failed to demonstrate any evidence of less myocardial injury.40 Finally, prior chronic treatment with β-blockers was associated with smaller MIs after primary percutaneous intervention.41 One limitation in the interpretation of the clinical results is that the MI size measurement was done mostly by indirect methods such as ECG changes or creatine kinase-MB fraction release.

Our study represents a model of mechanical MI reperfusion closely mimicking the human scenario. The findings reported here suggest that initiation of this therapy while the artery is still occluded results in significant cardioprotection, a finding that might have significant clinical implications. We started the metoprolol infusions 75 minutes before reperfusion to mimic a hypothetical human scenario in which the intravenous β-blocker agent could be initiated at MI diagnosis (in patients without contraindications). In addition, metoprolol injection was associated not only with smaller MI size at day 4 but also with significant LVEF recovery at day 22. These observations are in agreement with the results from the Controlled Abciximab and Device Investigation to Lower Late Angioplasty Complications trial in which early intravenous β-blockade was associated with greater improvements in LVEF over time.37

The exact mechanism(s) of action by which β-blockers could result in reductions in MI size remain to be fully elucidated. It has been widely suggested that β-blockers lessen the magnitude of the MI by decreasing oxygen consumption secondary to slow heart rate during or early after MI.11 However, in our study, the reduction in MI size was independent of the heart rate achieved during the MI induction.

Early after a coronary occlusion, the ischemic area at risk of necrosis is characterized by substantial interstitial and intracellular edema, which may be further increased by reperfusion.42,43 In addition, reperfusion may further increase the production of edema.44 Postischemic edematous area can be visualized with the use of T2-weighted “black-blood” CMR, and the use of this approach to depict the ischemic myocardium at risk has been validated by different groups using different experimental models.26,42 In addition, the extent of myocardial necrosis can be depicted accurately with the use of DE CMR as validated with histopathology in our and other studies,25 enabling noninvasive visualization of both the infarcted tissue and the myocardium at risk. An important finding of our study is that the extent of salvaged myocardium at day 4 was identified as an independent predictor of LV functional recovery. This is in agreement with the study by Aletras et al,26 who showed improvement in contractility in edematous areas early after experimental MI in a canine model. Coronary occlusion in dogs usually leads to subendocardial MI as a result of a well-developed net of collaterals.45 In such cases, it is difficult to ascertain whether the presence of edema provides incremental information over the transmural extent of MI for the prediction of contractile function recovery. In our study, we found that the extent of salvaged myocardium was a strong predictor of regional and global functional improvement independently of total volume of noninfarcted myocardium,46 transmural extent of MI,47,48 or global size of edematous area,26 providing valuable further comprehensive information.

This novel predictor of LVEF improvement highlights the value of visualizing both the final size of necrosis and the extent of salvaged myocardium. This may be important in evaluations of the efficacy of cardioprotective and regenerative therapies.49

Study Limitations

Given the small sample size, a relatively large number of statistical tests were performed. Despite this potential source of statistical bias, all the results in this work point the same direction; therefore, we believe that the totality of the evidence is strong enough to support the results reported here.

We administered metoprolol in a single time point. Thus, our investigation does not allow conclusions regarding the potential additive gains associated with maintained β-blockade in the post-MI period. Similarly, whether chronic use of β-blockers before the MI lessens the beneficial effect of intravenous therapy, as suggested by some studies,37,41 requires further investigation. In our protocol, we used continuous infusion of amiodarone during the entire procedure as prophylaxis for malignant arrhythmias. Amiodarone also exerts a small β-blocker activity, which probably explains the similar heart rate in both groups, and thus could have mitigated the differences between the metoprolol and placebo groups. Although the use of a different antiarrhythmic drug without β-blocker properties would have been desirable, in our experience, the mortality of MI induction without amiodarone infusion is very high in this animal model. Nevertheless, because both study arms received the same dose of medication, the potential benefits associated with the use of amiodarone should be identical in both groups. Thus, the significant differences seen in our study should be associated exclusively with the administration of metoprolol.

Conclusions

In a swine MI model closely mimicking human cardiac anatopathology, a single dose of metoprolol during ongoing MI results in 5-fold-larger salvaged myocardium (27% reduction in MI size). This increase is independent of decreases in heart rate with the administration of the drug. Our results suggest that, in the setting of acute MI, β-blockers should be administered as early as possible, while the
coronary artery is still occluded. The cardioprotective effect was demonstrated by the smaller infarct size and larger area of salvaged myocardium. In addition, this study shows the predictive value of the quantification of salvaged myocardium on regional and global LV function recovery at 3 weeks.

Acknowledgments

We acknowledge the great help with the animal care by the veterinarians and their group at the Center of Comparative Medicine and Surgery. We want to thank Thomas O’Donnell (Siemens Corporate Research) for providing us with the postprocessing imaging tools. We are indebted to Noemi Escalera, M. Urooj Zafar, Jose Rodriguez, Boris Cortes, and Frank Macaluso for their great work.

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Disclosures

None.

References


2. Reimer KA, Jennings RB. The “wavefront phenomenon” of myocardial ischemic cell death, II: transmural progression of necrosis within the ischemic bed size (myocardium at risk) and collateral flow. Lab Invest. 1979;40:634–644.


34. Roberts R, Croft C, Gold HK, Hartwell TD, Jaffe AS, Muller JE, Mullin SM, Parker C, Passamani ER, Poole WK. Effect of propranolol on

CLINICAL PERSPECTIVE

Beyond time to reperfusion (the major determinant for myocardial salvage in acute myocardial infarction), interventions to reduce myocardial death (cardioprotection) are strongly needed to move ahead in this field. β-Blockers have been shown to reduce mortality in the acute myocardial infarction setting, but early intravenous administration before mechanical reperfusion is not widely adopted. In fact, ST-segment–elevation myocardial infarction practice guidelines catalogue oral β-blocker administration as a class I indication; the intravenous route is a class IIA indication. In the era of mechanical reperfusion for ST-segment elevation myocardial infarction, the reperfusion injury is a frequently observed phenomenon. It has been suggested that some cardioprotective therapies may act by reducing this reperfusion-related incident. If this were the case, effective circulating levels of the eventually cardioprotective drug at reperfusion would be crucial. Whether the cardioprotection observed in this study is related to a reduction in reperfusion-related myocyte loss is not addressed, but it is plausible and therefore deserves to be fully elucidated. Cardiac magnetic resonance imaging allows direct visualization of cardioprotection early after acute myocardial infarction, as shown here. This provides an accurate tool to explore the effect of certain interventions in humans. In this work, the extent of salvaged myocardium, as directly assessed by cardiac magnetic resonance imaging 4 days after acute myocardial infarction, correlated with the local and global left ventricular motion recovery. This novel predictor of left ventricular recovery may be used in the clinical area as a surrogate end point for early assessment of cardioprotective-regenerative therapies.
Heritability, Linkage, and Genetic Associations of Exercise Treadmill Test Responses

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Background—The blood pressure (BP) and heart rate responses to exercise treadmill testing predict incidence of cardiovascular disease, but the genetic determinants of hemodynamic and chronotropic responses to exercise are largely unknown.

Methods and Results—We assessed systolic BP, diastolic BP, and heart rate during the second stage of the Bruce protocol and at the third minute of recovery in 2982 Framingham Offspring participants (mean age 43 years; 53% women). With use of residuals from multivariable models adjusted for clinical correlates of exercise treadmill testing responses, we estimated the heritability (variance-components methods), genetic linkage (multipoint quantitative trait analyses), and association with 235 single-nucleotide polymorphisms in 14 candidate genes selected a priori from neurohormonal pathways for their potential role in exercise treadmill testing responses. Heritability estimates for heart rate during exercise and during recovery were 0.32 and 0.34, respectively. Heritability estimates for BP variables during exercise were 0.25 and 0.26 (systolic and diastolic BP) and during recovery, 0.16 and 0.13 (systolic and diastolic BP), respectively. Suggestive linkage was found for systolic BP during recovery from exercise (locus 1q43–44, log-of-the-odds score 2.59) and diastolic BP during recovery from exercise (locus 4p15.3, log-of-the-odds score 2.37). Among 235 single-nucleotide polymorphisms tested for association with exercise treadmill testing responses, the minimum nominal probability value was 0.003, which was nonsignificant after adjustment for multiple testing.

Conclusions—Hemodynamic and chronotropic responses to exercise are heritable and demonstrate suggestive linkage to select loci. Genetic mapping with newer approaches such as genome-wide association may yield novel insights into the physiological responses to exercise. (Circulation. 2007;115:2917-2924.)

Key Words: blood pressure ■ exercise ■ genetics ■ heart rate

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Exercise treadmill testing (ETT) is a well-established method to detect signs of ischemic heart disease in symptomatic patients.1 More recently, ETT response measures have been shown to predict a range of cardiovascular events such as new-onset hypertension,2–3 cardiovascular morbidity and mortality,4–9 sudden death,10 and all-cause mortality in asymptomatic patients.5,8,11–13 Specifically, chronotropic incompetence,6,12 blood pressure (BP) and heart rate (HR) response during exercise,2,3,5,10 and BP and HR during recovery after exercise3,5,7–10 are some of the variables that have been associated with adverse outcomes. Further, several studies have indicated that ETT characteristics could improve cardiovascular disease risk prediction beyond that of the global Framingham risk score or the European counterpart, Systematic Coronary Risk Evaluation (SCORE).14–16

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Interindividually in ETT response measures may be caused by genetic influences, environmental determinants, or a combination of both. In particular, definition of the
genetic determinants of ETT traits may yield novel insights into the physiological response to exercise and the pathological conditions predicted by ETT. In a report from the HERITAGE family study (HEalth, RiSk factors, exercise Training And GEnetics), several chromosomal regions with potential genetic linkage for hemodynamic exercise characteristics were found. Previous genetic association studies have been limited by a focus on 1 or a limited number of candidate genes, by small selected study samples, or by unconventional exercise testing protocols. At present, the genetic determinants of ETT measures with a standard Bruce protocol are largely unknown.

Accordingly, using a large community-based sample, we evaluated the heritability and linkage of ETT measures and performed comprehensive association analyses to examine if variation in 14 candidate genes from the neurohormonal pathways influences interindividual variation in ETT measures.

Methods

Study Sample

The design and selection criteria of the Framingham Offspring study have been previously described. The second examination (1978 to 1982) comprised 3863 participants. This examination included an ETT in addition to a physician-obtained medical history, routine physical examination, 12-lead ECG, and biochemical tests (such as glucose and lipid profile). Subjects were excluded from the present study for the following reasons: prevalent cardiovascular disease, valvular disease, resting ST-segment abnormality, use of cardiac glycoside or β-blocking agents, age <20 years at index examination, inadequate or missing ETT data, inability to complete the first stage of the Bruce protocol, interrupted ETT as a result of ischemic response, hypotension, chest pain, and arrhythmia during exercise. After these exclusions, 2982 participants (1586 women) remained eligible for the present study. The heritability analyses were based on 2053 participants (1068 women) from 949 extended families with at least 2 members. The largest 291 families (1068 individuals, 543 women) were genotyped with a 10-cM-density genome scan by the Mammalian Genotyping Service laboratory at the Marshfield Clinic (Marshfield, Wis; marker set 8A, average heterozygosity 0.77; http://research.marshfieldclinic.org/genetics), as previously described. The association analyses were based on 1227 unrelated participants (ie, 1 person randomly selected from each family or biologically unrelated to any other participants, and selection was designed to include equal numbers of men and women. The Institutional Review Board at Boston University Medical Center approved the study, and all participants gave written informed consent.

Exercise Testing Protocol

The participants underwent a submaximal ETT according to the standard Bruce protocol while their ECGs were continually monitored and recorded (simultaneous V1 and V5; Clinical Data Inc, Newton, Mass) during exercise and for 4 minutes into the recovery period after exercise. Exercise was terminated when the participants reached their target HR (85% of their age- and sex-predicted maximal HR), and the participants immediately got off the treadmill and rested in a supine position. Exercise testing was terminated prematurely for the following reasons: limiting chest discomfort, dyspnea, fatigue, or leg discomfort; hypotension or a severe hypertensive response; or the development of significant ECG abnormalities such as an ischemic ST-segment response.

The Bruce protocol ETT phenotypes examined in the study were defined as follows:

- Systolic BP during the second stage of exercise (measured once at the middle of the stage)
- Diastolic BP during the second stage of exercise (measured once at the middle of the stage)
- HR during the second stage of exercise (measured once at the middle of the stage)
- Systolic BP at the third minute of the recovery phase
- Diastolic BP at the third minute of the recovery phase
- HR at the third minute of the recovery phase

With the definition of the exercise phenotypes at the second stage of the Bruce protocol, we standardized the duration of exercise before assessment of BP and HR. Also, most participants reached this level of exercise, which leads to enhanced generalizability. BP and HR were assessed at the third minute of the recovery phase to maintain consistency with previous studies.

Tag Single-Nucleotide Polymorphism Selection and Genotyping Methods

The 14 genes in the association analyses were selected from the CardioGenomics project (http://cardiogenomics.med.harvard.edu/pga-overview), the objective of which was to examine genetic factors associated with echocardiographic left heart structure and function. Genes from the neurohormonal pathways were selected a priori for their potential involvement in hemodynamic and chronotropic responses to exercise and included the following genes: ADRA1A, ADRA1B, ADRA1D, ADRB1, ADRB2, ACE, AGTR1, AGTR2, AGT, NPPA, NPPB, NPR1, NPR2, REN (Table I in the online-only Data Supplement). The rationale for gene selection from these pathways was that the neurohormonal systems are known to be important in the regulation of BP and HR, and that most of the prior genetic association studies of hemodynamic response to exercise have focused on single genes in these pathways with conflicting results. In a reference DNA panel, we characterized the linkage disequilibrium structure for common single-nucleotide polymorphisms (SNPs) at each locus and selected tag SNPs as previously described. With the Sequenom MassARRAY platform (Sequenom, Inc, San Diego, Calif), we genotyped the tag SNPs in the Framingham Heart Study sample, which consisted of 1227 unrelated participants (randomly selected to include only 1 participant from each family), who provided blood samples for DNA extraction during the sixth clinical examination (1995 to 1998). Redundant SNPs were genotyped to help assess for linkage disequilibrium block structure similarity between reference panel and Framingham Heart Study sample and in the event of genotype failures. Linkage disequilibrium plots for each of the 14 genes are available at http://cardiogenomics.med.harvard.edu genes/gene-list. Any SNPs genotyped that were not in Hardy–Weinberg equilibrium (P<0.01) were not included in the analyses.

Statistical Analyses

Data were presented as means (SDs) or percentages. First, we performed multivariable linear regression models in all 2982 participants to assess the contribution of clinical covariates to the ETT variables, separately for each of the 6 ETT variables. The covariates were selected on the basis of prior studies, and included age, sex, body mass index, diabetes mellitus, smoking, ratio of total to high-density lipoprotein cholesterol, and treatment for hypertension for all ETT variables. Additionally, systolic BP during exercise was also adjusted for systolic BP at rest; diastolic BP during exercise for diastolic BP at rest; HR during exercise for HR at rest; systolic BP during recovery for systolic BP at rest; systolic BP during second stage of exercise, and peak systolic BP during exercise; diastolic BP during recovery for diastolic BP at rest; diastolic BP during second stage of exercise, and peak diastolic BP during exercise; and HR during recovery for HR at rest, HR during second stage of exercise, and peak HR during exercise. Standardized residuals (mean 0, SD 1) constructed after covariate-adjustment served as the primary pheno-
type for the heritability, linkage, and association analyses. These analyses were performed with SAS 8.2 (SAS Institute, Cary, NC).

**Heritability Analyses**

Diastolic BP and HR during recovery had skewed distributions and were therefore modeled as Winsorized variables in the heritability and linkage analyses. Heritability estimates for the ETT variables were obtained in 2053 participants (1068 women) from 949 extended families with at least 2 members by variance-components methods with the Sequential Oligogenic Linkage Analysis Routines package (Southwest Foundation for Biomedical Research, San Antonio, Tex). With this approach, maximum-likelihood estimation was applied to a mixed-effects model that incorporated fixed covariate effects, additive genetic effects, and residual error. The additive genetic effects and residual errors were assumed to be normally distributed and to be mutually independent. The analyses were performed with residuals from the multivariable models mentioned above.

**Linkage Analyses**

Multipoint quantitative trait linkage analyses were conducted in the largest 291 families that underwent a 10-cM–density genome scan (1068 individuals, 543 women) with the residuals from multivariable-adjusted models with use of GENEHUNTER software (Ward Systems Group, Inc, Frederick, Md). Linkage was assessed by use of polygenic models that incorporated genetic marker data (ie, identical-by-descent status) and comparison with models that did not incorporate genetic marker information across the chromosome (multipoint analysis). The log (base 10) of the ratio of the likelihoods of the polygenic models (ie, the log-of-the-odds (LOD) score, the traditional measure of genetic linkage) was calculated.

**Association Analyses**

With a general model of inheritance, we constructed multivariable linear regression analyses to test the null-hypothesis that the level of ETT variables did not differ by candidate SNP genotype. With a sample size of 1000 unrelated individuals (accounting for up to 10% missing genotypes) and a significance level 0.01, we had 80% and 90% power to detect a quantitative trait locus that accounted for 1.3% and 1.6% of the residual variance. False discovery rates were calculated for the associations with the lowest nominal probability values to account for multiple testing.

**Secondary Analyses**

In secondary analyses, we performed heritability, linkage, and association analyses with alternative residuals without trait-specific adjustments. These alternative residuals were created with multivariable linear regression models in all 2982 participants, separately for each of the 6 ETT variables, and the covariates included age, sex, body mass index, diabetes mellitus, smoking, ratio of total to high-density lipoprotein cholesterol, and treatment for hypertension for all ETT variables. In further post hoc analyses, we iterated the primary analyses (heritability, linkage, and association analyses) with the original residuals in a subsample with exclusion of individuals with antihypertensive treatment at baseline (n=180).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

The mean age of the participants was 43 years (range 20 to 70). The clinical characteristics are presented in Table 1.

**ETT Heritability**

Heritability is the proportion of the unexplained phenotypic variance (ie, after covariates were accounted for) explained by additive genetic effects (ie, additive familial effect, because shared early environment cannot be distinguished from pure genetic effect with this family structure). The heritability analyses demonstrated a highly significant genetic component for all ETT traits. The highest estimates were found for HR, both during exercise and during recovery after exercise, with respective heritability estimates of 0.32 and 0.34 (Table 2). The heritability estimates for systolic and diastolic BP during exercise were higher (0.25 and 0.26) than those for systolic and diastolic BP during the recovery phase (0.16 and 0.13).

**Linkage Analyses**

The linkage analyses resulted in several LOD scores >1.5 (Table 3). Of these only 2 genomic segments reached a level of suggestive linkage (LOD=2.2) as proposed by Lander and Kruglyak. The first of these peaks was located at 1q43-44 (LOD 2.59) and was linked to systolic BP during recovery phase. The other was located at 4p15.3 (LOD 2.37) and was linked to diastolic BP during recovery phase.

**Associations Between ETT Phenotypes and Candidate Gene SNPs**

Ten associations between the examined SNPs and ETT phenotypes reached a nominal significance level of P<0.01 (Table 4). Eight of the associations included genes encoding adrenergic alpha-receptor proteins. Among 235 SNPs tested for association with ETT responses, the minimum nominal probability value was 0.003. The false discovery rate for that
association was 99%, if adjusted for all genotype-phenotype association tests performed.

Secondary Analyses

The results from the secondary analyses with alternative residuals without trait-specific covariates are shown in Tables II, III, and IV of the online-only Data Supplement. The heritability estimates were generally higher than those from the primary analyses (online Data Supplement Table II). The linkage analyses that used these alternative residuals resulted in several LOD scores >1.5 (online Data Supplement Table III). The 2 genomic segments that reached a level of suggestive linkage in the primary analyses showed high LOD scores also in these secondary analyses. The highest of these peaks (locus 1q43-44 for systolic BP during recovery phase), demonstrated a LOD score of 3.47 at the same locus for the corresponding trait (without adjustment for resting and exercise systolic BP). The association analyses that used these alternative residuals duplicated 2 of the associations from the primary analyses (the associations of rs544215 and rs3787441, and HR during exercise) (online Data Supplement Table IV).

When all participants with antihypertensive treatment were excluded, the results from the heritability, linkage, and association analyses that used the original residuals were similar to those of the primary analyses, although the point estimates were generally slightly lower and the probability values slightly higher (data not shown).

Discussion

Principal Findings

In this large community-based study with a familial structure, we examined the genetic determinants of hemodynamic and chronotropic responses to exercise. We observed moderate heritability for each of 6 ETT traits examined, and we found 2 peaks of

<table>
<thead>
<tr>
<th>Table 2. Heritability Estimates for the Different ETT Phenotypes (n=2053)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETT Phenotype†</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Systolic BP during exercise</td>
</tr>
<tr>
<td>Diastolic BP during exercise</td>
</tr>
<tr>
<td>HR during exercise</td>
</tr>
<tr>
<td>Systolic BP during recovery</td>
</tr>
<tr>
<td>Diastolic BP during recovery</td>
</tr>
<tr>
<td>HR during recovery</td>
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</tbody>
</table>

*The heritability estimates are based on residuals from multivariable models adjusted for age, sex, body mass index, diabetes mellitus, smoking status, ratio of total to high-density lipoprotein cholesterol, and treatment for hypertension for all ETT variables. Additionally, systolic BP during exercise was also adjusted for systolic BP at rest; diastolic BP during exercise for diastolic BP at rest; HR during exercise for heart rate at rest; systolic BP during recovery for systolic BP at rest, systolic BP during second stage of exercise, and peak systolic BP during exercise; diastolic BP during recovery for diastolic BP at rest, diastolic BP during second stage of exercise, and peak diastolic BP during exercise; and HR during recovery for HR at rest, HR during second stage of exercise, and peak HR during exercise. ETT indicates exercise treadmill test.

†For definitions of the phenotypes, see Table 1.

| Table 3. Maximum Multipoint LOD Scores >1.5 for the Different ETT Phenotypes (n=1068) |
|----------------------------------------|-----------------|-----------------|
| ETT Phenotype* and Chromosome Location | Map Distance (cM) | Closest Marker(s) | Multivariable-Adjusted LOD Score† |
| Systolic BP during exercise            |                 |                 |                                 |
| 1q32.1                                 | 196.7           | AFMA132YC9      | 2.02                            |
| 5q13.2                                 | 78.8            | GATA138B05      | 1.57                            |
| 10p23.3                                | 106.0           | rs1887922       | 1.61                            |
| Diastolic BP during exercise           |                 |                 |                                 |
| 19q13.1                                | 50.8            | GATA156F11      | 1.63                            |
| HR during exercise                     |                 |                 |                                 |
| 1p32.1-31.1                           | 78.4            | GATA165C03/GATA152F05 | 1.91                      |
| 5q14.3                                | 99.1            | GATA89G08       | 2.09                            |
| 7p15.1-14.3                           | 37.6            | GGA43F06/GATA13G11 | 1.73                      |
| 7q21.1                                | 81.1            | GATA73D10/GATA87D11 | 1.67                      |
| 14q24.1                                | 52.4            | GGA44A12        | 1.91                            |
| Systolic BP during recovery            |                 |                 |                                 |
| 1q43-44                                | 269.9           | GATA4A09/AFMC013WC9 | 2.59                      |
| 2p12                                  | 93.7            | GATA71604       | 1.68                            |
| Diastolic BP during recovery           |                 |                 |                                 |
| 4p15.3                                | 27.8            | AFM157XG3/ATT015 | 2.37                            |
| 4q28.2                                | 117.1           | ATA26B08        | 1.93                            |
| HR during recovery                     |                 |                 |                                 |
| 5q35.3                                | 194.1           | 164X88          | 1.60                            |
| 21q21.1                                | 0               | GATA11C12       | 1.66                            |

*For definitions of the phenotypes, see Table 1.

†LOD scores were calculated from residuals from multivariable models. For covariates included in the models, see Table 2.
suggestive linkage for systolic and diastolic BP during recovery from exercise (LOD 2.6 and 2.4, respectively). In addition, we performed analyses of potential associations between ETT variables and 235 SNPs in 14 candidate genes from the neurohormonal pathways. These genes were selected a priori because the neurohormonal systems are known to be important in the regulation of BP and HR, and because most prior genetic association studies of the same traits have focused on single genes in these pathways. The association analyses rendered nonsignificant results after adjustment for multiple statistical testing. In secondary analyses that used alternative residuals without adjustments for trait-specific covariates (such as HR at rest, HR during second stage of exercise, and peak HR during exercise for the HR during recovery trait), the heritability estimates were generally higher, and the linkage peaks from the primary analyses were reproduced, whereas only 2 of the associations from the association analyses were duplicated. The fact that the results differed somewhat between these analyses was expected because exercise response phenotypes adjusted for corresponding resting and exercise covariates are physiologically and genetically different phenotypes from those without adjustment for corresponding covariates. Without adjustment for these trait-specific covariates, the analyses are more influenced by the resting phenotypes (for exercise phenotypes) and resting and exercise phenotypes (for recovery phenotypes).

**Previous Studies of Genetic Determinants of Exercise Hemodynamics**

The HERITAGE family study has previously reported on the genetic determinants of response to exercise.17 Some impor-

| TABLE 4. Association of Selected Candidate Gene SNPs* and Hemodynamic Response to Exercise (n=1227) |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ETT Phenotype† | Gene Symbol‡ | SNP | Genotype | No. | Trait Value Mean (SEM)§ | Nominal P¶ | |
|----------------|-------------|-----|----------|-----|--------------------------|------------| |
| Systolic BP during exercise | ADRA1A | rs489223 | AA | 920 | 0.047 (0.033) | 0.004 | |
| | | | AG | 212 | −0.059 (0.068) | | |
| | | | GG | 13 | −0.802 (0.276) | | |
| Diastolic BP during exercise | AGT | rs2493136 | GG | 397 | 0.100 (0.051) | 0.003 | |
| | | | AG | 567 | −0.109 (0.043) | | |
| | | | AA | 217 | 0.076 (0.069) | | |
| Diastolic BP during exercise | ADRA1D | rs835873 | CC | 735 | 0.002 (0.038) | 0.008 | |
| | | | CT | 277 | −0.092 (0.062) | | |
| | | | TT | 37 | 0.466 (0.169) | | |
| HR during exercise | ACE | rs4305 | GG | 311 | −0.059 (0.056) | 0.010 | |
| | | | AG | 556 | 0.093 (0.042) | | |
| | | | AA | 256 | −0.114 (0.062) | | |
| HR during exercise | ADRA1A | rs544215 | TT | 332 | −0.019 (0.054) | 0.005 | |
| | | | CT | 577 | 0.068 (0.041) | | |
| | | | CC | 249 | −0.176 (0.062) | | |
| HR during exercise | ADRA1D | rs3787441 | AA | 511 | 0.061 (0.043) | 0.007 | |
| | | | AG | 372 | −0.094 (0.051) | | |
| | | | GG | 69 | −0.264 (0.118) | | |
| Systolic BP during recovery | ADRA1A | rs483392 | CC | 309 | −0.022 (0.057) | 0.005 | |
| | | | CT | 436 | 0.050 (0.048) | | |
| | | | TT | 185 | −0.236 (0.073) | | |
| Systolic BP during recovery | ADRA1A | rs7820633 | TT | 440 | 0.032 (0.047) | 0.005 | |
| | | | CT | 561 | −0.062 (0.042) | | |
| | | | CC | 171 | −0.256 (0.075) | | |
| Diastolic BP during recovery | ADRA1A | G2286a1 | TT | 1098 | 0.031 (0.029) | 0.009 | |
| | | | GT | 20 | −0.541 (0.216) | | |
| | | | TT | 0 | NA | | |
| HR during recovery | ADRA1B | rs11953285 | AA | 783 | −0.013 (0.034) | 0.010 | |
| | | | AC | 234 | −0.095 (0.063) | | |
| | | | CC | 20 | 0.583 (0.215) | | |

NA indicates not applicable.

*Only SNPs associated to ETT variables with a nominal P<0.01 are presented.
†For definitions of the phenotypes, see Table 1.
‡Official gene symbol according to Human Gene Organisation nomenclature (http://www.gene.ucl.ac.uk/nomenclature/).
§Trait values are means of standardized residuals multivariable-adjusted for covariates, described in Table 2.
¶P for genotype–phenotype association by general model of inheritance.
tant differences exist between the HERITAGE family study and the present investigation. First, the overall objective of the HERITAGE study was to examine responses to 20 weeks of aerobic exercise training in subjects from a selected sample of sedentary but healthy individuals without hypertension or chronic disease, whereas the present study examined the genetic determinants of response to exercise in a cross-sectional community-based study without any interventions. Second, the HERITAGE study used an exercise protocol with cycle ergometry and hemodynamic measurements at different loads and different percentages of maximal oxygen uptake, whereas we performed ETT with the standardized and widely used Bruce protocol. Third, with 2982 participants, efforts to search for genetic factors that influence ETT traits. The HERITAGE study has reported the heritability for maximal oxygen uptake to be \( \sim 50\% \) and that the heritability for training response after 20 weeks of training is \( \sim 30\% \) for HR and lower for BP response. Further, the heritability estimates for systolic BP, diastolic BP, and HR during submaximal exercise at 50 W have been reported as 45%, 55%, and 59%, respectively, in the HERITAGE study. These data have not been published to date but were summarized in another report by the same group of investigators. Also, other studies have demonstrated a significant genetic component in hemodynamic and chronotropic response to exercise, although none of these studies have reported heritability estimates. To our knowledge, estimates of heritability of hemodynamic responses to exercise with a standard Bruce protocol have not been published.

Notably, the 2 highest linkage peaks were found for the 2 traits with the lowest heritability, systolic and diastolic BP during recovery. This might seem contradictory at a first glance, but one should remember that these traits are polygenic, which means that many quantitative trait loci of modest effect exist, which contribute to the genetic variability of the traits, and that the linkage analyses does not necessarily pick up signals from all of these.

**Heritability of Hemodynamic Response to Exercise**

Our study demonstrated the heritability of hemodynamic response to exercise to be significant, which supports efforts to search for genetic factors that influence ETT traits. The HERITAGE study has reported the heritability of maximal oxygen uptake to be \( \sim 50\% \) and that the heritability for training response after 20 weeks of training is \( \sim 30\% \) for HR and lower for BP response. Further, the heritability estimates for systolic BP, diastolic BP, and HR during submaximal exercise at 50 W have been reported as 45%, 55%, and 59%, respectively, in the HERITAGE study. These data have not been published to date but were summarized in another report by the same group of investigators. Also, other studies have demonstrated a significant genetic component in hemodynamic and chronotropic response to exercise, although none of these studies have reported heritability estimates. To our knowledge, estimates of heritability of hemodynamic responses to exercise with a standard Bruce protocol have not been published.

**Genetic Linkage of ETT Phenotypes**

We are aware of only 1 previous study that examined genetic linkage of exercise hemodynamics, and that was also a report from the HERITAGE family study. The only locus that reached the level of suggestive linkage in this study was detected on chromosome 8q21 (LOD 2.36) for systolic BP training response (ie, the difference in systolic BP at 50 W load before and after 20 weeks of training). Additionally, some evidence of linkage was detected on 10q23-24 (LOD 1.84) for systolic BP during exercise (ie, systolic BP at 80% of maximum oxygen uptake). This peak for systolic BP during exercise was supported by the findings in the present study. Even though the LOD score of 1.61 at the same locus did not reach the level of suggestive linkage, this might still be interesting because it is a replication of a finding from the only previous linkage study on hemodynamic response to exercise. One gene in this region that merits further consideration is the retinol binding protein 4 (RBP4) gene, which recently has been associated with insulin resistance in repeated studies.

The highest LOD score in our study was found for systolic BP during the recovery phase at locus 1q43 – 44. A potential candidate gene in this region is the CHRM3 (acetylcholine receptor M3) gene. Acetylcholine receptors play an important role in the regulation of the cardiovascular system through vagal mediation of the autonomic nervous system. Another acetylcholine receptor subtype, CHRM2, has recently been suggested to be associated with HR recovery after exercise.

**Association Studies of Candidate Genes and ETT Phenotypes**

Many of the previous studies that examined associations between candidate genes and hemodynamic response to exercise have focused on individual SNPs in 1 or a limited number of candidate genes. These studies have rendered different results, which in part can be explained by the use of small and selected samples. In contrast, the present study is based on a large community-based cohort with unselected participants from the community. We comprehensively characterized the underlying genetic variation in 14 candidate genes in a reference sample, selected tag SNPs to capture common variation, and genotyped tag SNPs in the Framingham Heart Study cohort. Notably, most of the associations with the lowest nominal probability values were found for SNPs from genes that code adrenergic alpha-receptor proteins. Most previous genetic associations studies of hemodynamic and chronotropic response to exercise have examined genes from the renin-angiotensin-aldosterone system or beta-adrenergic systems. To our knowledge, no previous studies have been conducted of genes that code adrenergic alpha-receptor proteins in relation to exercise physiology. However, a recently published study demonstrated genetic variation in the ADRA1A gene to be associated with essential hypertension in a Chinese population. Nevertheless, although some nominal probability values in the present study were low, no findings resulted that were statistically significant after adjustment for multiple statistical testing. Testing in additional samples will be required to validate these putative associations.

**Strengths and Limitations**

The strengths of the present study are the large community-based sample, routine assessment of the ETT, and the use of standardized clinical covariates in multivariable models. Further strengths include the simultaneous consideration of heritability, genetic linkage and genetic association in the same study, and the comprehensive characterization of common variation in each examined gene. The present study also
has some limitations. First, because our sample consisted mainly of whites of European descent, the generalizability of our findings to other ethnic groups is unknown. Second, we may have failed to detect SNP-phenotype associations because of insufficient statistical power.

Conclusions

In summary, in our large community-based cohort we found modest heritability for several exercise responses. Further, we found evidence suggestive of genetic linkage to select loci for systolic and diastolic BP during recovery from exercise. Finally, comprehensive analyses of potential associations between ETT variables and 235 SNPs in 14 candidate genes from the neurohormonal pathways rendered results that were nonsignificant after adjustment for multiple testing. However, our findings indicate that the genes that code adrenergic alpha-receptor proteins might be plausible targets for future candidate gene-based studies. Alternatively, genetic mapping of TRH-alpha-receptor proteins might be plausible targets for future candidate gene-based studies. Nevertheless, genetic mapping by newer approaches such as genome-wide association may yield novel insights into the physiological response to exercise.

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Disclosures

None.

References


The blood pressure and heart rate responses to exercise treadmill testing predict incidence of cardiovascular disease, but the genetic determinants of hemodynamic and chronotropic responses to exercise are largely unknown. The present study demonstrated that blood pressure and heart rate responses to exercise were moderately heritable. Furthermore, we found suggestive genetic linkage for systolic blood pressure during recovery from exercise at chromosome 1 and for diastolic blood pressure during recovery from exercise at chromosome 4. Finally, comprehensive analyses of potential associations between exercise response and 235 single-nucleotide polymorphisms in 14 candidate genes from the neurohormonal pathways rendered results that were nonsignificant after adjustment for multiple testing. However, our findings indicate that the genes that code adrenergic alpha-receptor proteins might be plausible targets for future candidate gene-based studies. Alternatively, genetic mapping with newer approaches such as genome-wide association may yield novel insights into the physiological response to exercise.
Heart Failure

Loss of \textit{Mdm4} Results in \textit{p53}-Dependent Dilated Cardiomyopathy

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\textbf{Background}—Although several loci for familial dilated cardiomyopathy (DCM) have been mapped, the origin of a large percentage of DCM remains unclear. Mdm2, a \textit{p53}-negative regulator, protects cardiomyocytes from ischemic and reperfusion-induced cell death. Mdm4, a homolog of Mdm2, inhibits \textit{p53} activity in numerous cell types. It is unknown whether Mdm4 plays a role in the inhibition of \textit{p53} in fully differentiated tissues such as adult cardiomyocytes and whether this role is associated with DCM.

\textbf{Methods and Results}—The conditional knockout of \textit{Mdm4} in the heart by use of cardiomyocyte-specific \textit{Cre} (\textit{aMyHC-Cre}) allele does not result in any developmental defects. With time, however, mice with deletion of \textit{Mdm4} in the adult heart developed DCM and had a median survival of 234 days. More interestingly, the onset of DCM occurs significantly earlier in male mice than in female mice, which mimics human DCM disease. DCM in \textit{Mdm4} mutant mice was caused by loss of cardiomyocytes by apoptosis, and it was \textit{p53}-dose dependent.

\textbf{Conclusion}—Activity of \textit{p53} was inhibited by Mdm4 even in the fully differentiated cardiomyocyte. Elevated apoptosis mediated by the \textit{p53} pathway in cardiomyocytes may be a mechanism for DCM. (\textit{Circulation}. 2007;115:2925-2930.)

\textbf{Key Words:} apoptosis \textbullet{} myocytes, cardiac \textbullet{} cardiomyopathy \textbullet{} genetics \textbullet{} survival

Dilated cardiomyopathy (DCM) is one of the leading causes of heart failure in the United States, with an annual incidence estimated to be 5 to 8 cases per 100 000 population. DCM is characterized by ventricular chamber dilation with normal or decreased wall thickness and impaired systolic function.\textsuperscript{1-3} DCM has both hereditary and acquired forms. Two sarcomere structural genes have been identified to be involved with DCM, the cardiac actin and phospholamban genes.\textsuperscript{4,5} Additionally, several loci for familial dilated cardiomyopathy (DCM) have been mapped to 1p1\textsubscript{1}/H11002,\textsuperscript{1q1,6 3p25−3p22,7 1q32,8} and 9q13−9q22,\textsuperscript{9} which suggests genetic heterogeneity in DCM.\textsuperscript{10} Despite efforts over many years to identify the causative genes in DCM, the underlying mechanism(s) of a large percentage of DCM remains elusive. Mice have also been used as models for different types of cardiomyopathies, such as DCM.\textsuperscript{5,11} Mdm2, a negative regulator of the \textit{p53} tumor suppressor, was recently shown to protect murine cardiomyocytes from ischemic/reperfusion-induced cell death, which implicates the \textit{p53} pathway in cardiac cell survival\textsuperscript{12} and cardiomyopathy diseases.

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Mdm2 is an E3 ubiquitin ligase and negative regulator of \textit{p53}. Mdm4 is a homolog of Mdm2, which also inhibits \textit{p53} activity by masking its transcriptional activation domain.\textsuperscript{13} During embryonic development, \textit{p53} activity is suppressed by both Mdm2 and Mdm4. Loss of \textit{Mdm2} in mice results in embryonic lethality by \textit{p53}-dependent apoptosis.\textsuperscript{14,15} Loss of \textit{Mdm4} also causes \textit{p53}-dependent embryonic lethality by initiation of cell cycle arrest and apoptosis.\textsuperscript{16-18} Additionally, Mdm2 and Mdm4 synergize to inhibit \textit{p53} in the developing central nervous system during embryogenesis.\textsuperscript{19,20} These data demonstrate that \textit{p53} activity is inhibited by Mdm2 and Mdm4 in proliferating cells. In postmitotic cells in the central nervous system, both Mdm2 and Mdm4 are also required to inhibit \textit{p53}.\textsuperscript{20} However, when \textit{Mdm2} and \textit{Mdm4} are deleted in the adult smooth muscle cells, loss of \textit{Mdm2}-induced \textit{p53}-dependent apoptosis, but acute loss of \textit{Mdm4} does not show obvious defects,\textsuperscript{21} which suggests that, in quiescent or fully differentiated cells, Mdm4 is not required to inhibit \textit{p53} activity. It remains unclear whether Mdm4-mediated \textit{p53} inhibition in differentiated cells is tissue specific.

To determine whether Mdm4-mediated \textit{p53} inhibition is important in another differentiated cell type, we chose to delete \textit{Mdm4} in cardiomyocytes in mice. Previously, we crossed the \textit{a}-myosin heavy chain promoter–driven \textit{Cre} mouse (\textit{aMyHC-Cre}) to a \textit{Mdm4}-conditional allele to generate cardiomyocytes that lack \textit{Mdm4}. Mice with deletion of \textit{Mdm4} in cardiomyocytes did not show obvious defects during development and perinatal stages,\textsuperscript{22} which provides an
excellent opportunity to study whether Mdm4 is required to inhibit p53 activity in the adult cardiomyocytes. Adult mouse cardiomyocytes do not have regenerative capacity, although they do proliferate during fetal development. Shortly after birth, positive cell cycle factors such as cyclin A and cdk2 are downregulated and the cell cycle inhibitors p21 and p27 are upregulated, which allows cardiomyocytes to become quiescent. Mice with deletion of Mdm4 in cardiomyocytes showed severe edema and heart failure as early as 3 months of age. Mutant mice developed DCM and exhibited apoptosis in adult cardiomyocytes, which indicates a role for Mdm4 in differentiated cardiomyocytes.

To test whether the DCM phenotype was p53-dependent, we used p53-conditional and null alleles to generate Mdm4 mutant mice with 1 or no p53 alleles. Survival studies of these mice clearly showed the phenotype was dependent on p53 dose, which demonstrates that elevated p53 activity may be one of the causes for DCM.

Methods

Mice
A conditional allele of Mdm4, Mdm4loxPlox, has 2 lox P sites that surround exon 2, which contains the ATG start codon. Deletion of exon 2 results in a null allele, designated Mdm4Δ2,22 Mdm4loxPloxΔMyHC-Cre mice were crossed to Mdm4loxPlox mice to generate the cohorts of Mdm4loxPloxΔMyHC-Cre and Mdm4loxPloxΔMyHC-Cre mice. The breeding and maintenance of mice were performed in a specific pathogen-free mouse facility under institutional guidelines.

X-Gal Staining of Adult Heart
Frozen cross-sections of ΔMyHC-Cre, Rosa26-lacZ adult hearts were stained according to a previous protocol.25

TUNEL Assays/Immunohistochemistry Staining
Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays carried out on paraffin-embedded sections were modified with avidin biotin complex and diaminobenzidine kits from Vector Laboratories (Burlingame, Calif).26 Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays carried out on paraffin-embedded sections were modified with avidin biotin complex and diaminobenzidine kits from Vector Laboratories (Burlingame, Calif).19

Statistical Analysis
Two-way ANOVA and Kaplan-Meier survival analysis were performed with Prism 4 software (GraphPad Software, San Diego, Calif). Differences were considered significant at a value of P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Loss of Mdm4 in the Adult Heart Caused Dilated Cardiomyopathy
A conditional allele of Mdm4, Mdm4loxPlox contains lox P sites that surround exon 2, and deletion of exon 2 in the germline results in a null allele designated Mdm4loxPloxΔMyHC-Cre transgene25 were crossed to mice homozygous for the Mdm4-conditional allele (Mdm4loxPloxlox) to generate mice with different combinations of alleles, such as those that lack Mdm4 in cardiomyocytes. Deletion of Mdm4 in the embryonic heart does not cause any developmental defects.22 We maintained mice for >1 year to examine the possible role of Mdm4 in fully differentiated adult cardiomyocytes. Cohorts of mutant Mdm4loxPloxΔMyHC-Cre and control Mdm4loxPloxΔMyHC-Cre mice were monitored. To make possible examination of Cre-specific recombination, some mice contained the ROSA26-lacZ reporter, as Cre-recombination at the ROSA26 locus allows expression of β-galactosidase (these mice were not part of the cohort study). Robust and specific LacZ staining was observed in the adult heart with frozen sections from 5-month-old mice with both ΔMyHC-Cre and Rosa26-lacZ transgenes (Figure 1A). Obvious blue staining in the cardiomyocytes was present in both right and left ventricles, as well as in the septum between the 2 ventricles (Figure 1A; other data not shown), which was consistent with previous studies that showed tissue-specific expression of Cre from the ΔMyHC promoter.26 Specific recombination at the Mdm4 locus was previously observed in the adult mice with PCR primers that distinguish conditional and recombinant alleles.22 Because mice inherit an Mdm4-conditional and a null allele, every recombination event leads to a cell that completely lacks Mdm4.

The earliest abnormality was observed in Mdm4loxPloxΔMyHC-Cre mutant mice at 3 months of age. By 8 to 10 months of age, most of the mutant mice had swollen bodies, showed difficulty moving, and were out of breath. Mutant mice eventually died as a result of heart failure. The survival of Mdm4loxPloxΔMyHC-Cre mice was significantly shorter than
Mdm4^−/FX αMyHC-Cre control mice, with median survival of 234 and 318 days, respectively (P<0.0001) (Figure 1B). αMyHC-Cre mice survived up to 1 year of age,^30^ and Mdm4^−/FX^ mice survived even up to 2 years of age, although some Mdm4^−/FX αMyHC-Cre mice died before 1 year of age, perhaps as a result of toxicity of Cre expression plus the loss of 1 allele of Mdm4. Interestingly, the mutant male mice died significantly earlier than the female mice, with median survival at 208 and 243 days, respectively (P<0.007) (Figure 1C). To examine the cause of the death in more detail, Mdm4^−/FX αMyHC-Cre mutant mice were euthanized and dissected when they were moribund. The mutant mice had obvious edema in the lung and/or abdomen. The mutant hearts were enlarged, and all 4 chambers were dilated and paler than the hearts of control mice (Figure 2A). Cross-sections of the heart showed that the ventricular walls in mutant hearts were thinner and obviously hypertrophic (Figure 2B and 2C). These observations indicated severe dilated cardiomyopathy in the mutant mice. Mason trichrome staining was also performed to detect collagen deposition, a marker of fibrosis in the heart. Positive blue staining, which indicated fibrosis, was clearly evident in mutant hearts in comparison to hearts from control mice (Figure 2B and 2D). Atrial natriuretic peptide is a molecular marker widely used to characterize cardiomyocyte hypertrophy and heart failure. Immunochemistry staining showed atrial natriuretic peptide was also prominent in mutant but not control hearts (Figure 2E). Together, these data indicated that loss of Mdm4 induced dilated cardiomyopathy, which led to heart fibrosis and eventually heart failure in the mutant mice.

**Loss of Adult Cardiomyocytes in Mdm4 Mutant Mice**

Hearts from Mdm4^−/FX αMyHC-Cre mice showed thinner walls in the both left and right ventricles and were positive for atrial natriuretic peptide and Mason trichrome staining as compared with controls, which suggests loss of cardiomyocytes in the mutant hearts. To test whether loss of Mdm4 in the adult heart caused loss of fully differentiated cardiomyocytes, the cell number from cross-sections of both mutant and control hearts at 3 and 8 months of age was determined.

Although the number of cardiomyocytes at 3 months of age was similar in the control and mutant hearts (P=0.14), strikingly, the hearts of Mdm4^−/FX αMyHC-Cre mice had half the number of cardiomyocytes at 8 months of age as compared with Mdm4^−/FX αMyHC-Cre control mice (Figure 3A and 3B) (P=0.015). To investigate whether loss of cardiomyocytes in the heart was caused by the apoptosis, TUNEL assays were performed. TUNEL-positive cells were clearly evident only in mutant mice at 3 months of age when the cardiomyocytes are terminally differentiated (Figure 3B). In line with the differentiated and quiescent nature of adult cardiomyocytes, bromodeoxyuridine labeling indicated a lack of proliferation in both mutant and control mice (data not shown). These experiments demonstrated that loss of Mdm4
in adult cardiomyocytes induced apoptosis, which indicates that Mdm4 is still required in these differentiated and quiescent cells.

**DCM Caused by Loss of Mdm4 Was Dependent on p53 Dose**

To understand whether the DCM phenotype caused by loss of Mdm4 is p53-dependent, the p53-null and p53-conditional alleles were introduced into Mdm4<sup>Δ2FX</sup>/αMyHC-Cre mutant mice. On loss of 1 p53 allele, Mdm4<sup>Δ2FX</sup>αMyHC-Cre mice showed an extended median survival to 274 days compared with 234 days for Mdm4<sup>Δ2FX</sup>/αMyHC-Cre mice with 2 wild-type p53 alleles (P<0.0001). These data indicated that loss of a single p53 allele alleviated the severity of the phenotype. Because >90% of p53-null mice die by 6 months of age as a result of the development of lymphomas,27,32 we could not examine the survival advantage in Mdm4<sup>Δ2FX</sup>αMyHC-Cre mice null for p53. We therefore used a p53-conditional allele<sup>28</sup> and combined it with the Mdm4 mutant alleles to generate Mdm4<sup>Δ2FX</sup> p53<sup>lox/lox</sup>αMyHC-Cre mice to determine whether loss of both p53 alleles specifically in the heart could actually rescue the DCM phenotype. The median survival for Mdm4<sup>Δ2FX</sup> p53<sup>lox/lox</sup>αMyHC-Cre mice lengthened to 403 days, which was significantly longer (P<0.0005) than Mdm4<sup>Δ2FX</sup> p53<sup>-/-</sup>αMyHC-Cre mice (Figure 4A). Trichrome staining of the hearts of Mdm4<sup>Δ2FX</sup> p53<sup>lox/lox</sup>αMyHC-Cre mice at about 6 months of age showed a significant reduction in staining as compared with the mutant mice with a single p53 allele (Figure 4B). Mdm4<sup>Δ2FX</sup> p53<sup>lox/lox</sup>αMyHC-Cre mice were not edematous nor out of breath and died as a result of the development of various tumors (data not shown). These data demonstrated that the DCM phenotype caused by loss of Mdm4 was dependent on p53 dose.

**Discussion**

Loss of Mdm4 in terminally differentiated cardiomyocytes led to a p53-dependent lethal DCM. Mice that lacked Mdm4 in cardiomyocytes gradually lost these cells by apoptosis, and eventually died of heart failure. These data demonstrate that Mdm4-mediated inhibition of p53 in adult cardiomyocytes was essential to normal heart function. The rescue of the DCM phenotype by concomitant loss of p53 indicated that abnormal elevated p53 activity may induce DCM. It will be important to determine whether loss of Mdm4 and/or elevated p53 activity actually is a mechanism that leads to heart failure in humans. Another interesting observation in this mouse model is that the phenotype of Mdm4<sup>Δ2FX</sup>αMyHC-Cre mice recapitulates the gender differences of human heart failure. Women with heart failure survive substantially longer than men.33,34 This genetically defined mouse model with loss of Mdm4 in the adult heart may provide a relevant model to understand the gender differences of DCM-induced heart failure.

Recently, the concept of stem cell therapy has attracted many clinicians to test heart repair with a variety of stem cells. Although the cardiac transfer of stem and progenitor cells shows a favorable impact on tissue perfusion and contractile performance of the injured heart, the mechanism of stem cell therapy is still unclear, and it is essential to determine the right stem cell type in the right clinical setting.35 This genetically defined mouse model with loss of Mdm4 in the adult heart may provide a good model to test stem cell therapies in DCM.

Activity of p53 is high in proliferating progenitor cells before and after birth (G.L., unpublished observations, 2006). Loss of Mdm2 and Mdm4 in these cells results in p53-mediated cell-cycle arrest and apoptosis.19,20 Additionally, p53 activity is inhibited by Mdm2 in differentiated smooth muscle cells in the small intestine, and loss of Mdm2 in these cells results in p53-dependent apoptosis. The role of Mdm4 in the inhibition of p53 in smooth muscle cells seems unimportant, as deletion of Mdm4 does not cause any obvious defects.21 The difference between our study and loss of Mdm4 in smooth muscle cells is either a result of tissue specificity of Mdm4 function, or that Mdm4 loss requires a longer period to develop a severe phenotype. The second possibility is consistent with the notion that Mdm4 loss causes less severe phenotypes in all tissues examined thus far in comparison to loss of Mdm2.19–22

The importance of understanding the role of the p53 pathway in differentiated tissues is underscored by a strategy to treat cancer patients with molecular drugs to disrupt the binding of p53 to Mdm2.36,37 This strategy is widely accepted as a treatment option in cancer patients with wild-type p53. Recently, Nutlin-3 and Rita, 2 small molecules that disrupt the p53–Mdm2 interaction, have been shown to be effective in cancer cell lines and in a xenograft model.41 Because Mdm4 is another p53-negative regulator, and Mdm4 is overexpressed in many tumor cell lines and primary tumors such as tumors with wild-type p53,42,43 it is also an attractive choice for the design of drugs to target Mdm4 interaction with p53. Additionally, because Mdm2 and Mdm4 bind the same domain of p53,44 it is also possible that future drugs will disrupt both p53–Mdm2 and p53–Mdm4 interaction. In consideration of all possibilities for this therapeutic strategy, it is very important to know whether these drugs will affect the normal functions of human tissues. Recent data demonstrate that p53 activity is inhibited by Mdm2 even in adult mouse tissues.45 The present study implicates the importance of the Mdm4–p53 interaction in normal heart function, which sug-

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**Figure 4.** DCM phenotype in Mdm4<sup>Δ2FX</sup>αMyHC-Cre mutant heart depends on p53 dose. A, The survival of Mdm4<sup>Δ2FX</sup>αMyHC-Cre mutant mice with 0, 1, or 2 p53 alleles. B, Trichrome staining of Mdm4<sup>Δ2FX</sup>αMyHC-Cre mutant mice with or without p53 at 6 months. Scale bar=100 μm.
gests that drugs that disrupt this interaction in patients may have unwanted side effects.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Although several loci for familial dilated cardiomyopathy have been mapped, the underlying mechanism(s) of a large percentage of dilated cardiomyopathy remains unclear. Mdm4 is an inhibitor of the p53 tumor suppressor, and mice with deletion of Mdm4 in the adult heart developed dilated cardiomyopathy with significantly earlier onset in male than in female mice, which thus recapitulates the gender differences observed in humans. The cause of dilated cardiomyopathy in this mouse model is a gradual loss of cardiomyocytes by p53-dependent apoptosis. Thus, this genetically defined mouse may provide a good model to test stem cell replacement therapies. The present study also has important implications for cancer treatment with specific drugs to disrupt the interaction between p53 and its negative regulators, Mdm2 and Mdm4. Although 2 small molecules, Nutlin-3 and Rita, have been shown to be effective in cancer cell lines and in a xenograft model, our study suggests the importance of the Mdm4–p53 interaction in normal heart function, which indicates that drugs that disrupt this interaction in patients may have unwanted side effects.
Genetic and Shared Environmental Factors Do Not Confound the Association Between Birth Weight and Hypertension
A Study Among Swedish Twins

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Background—Studies have found associations between low birth weight and increased risks of cardiovascular diseases in adulthood. However, these associations could be due to confounding by genetic or socioeconomic factors.

Methods and Results—We performed a study on Swedish like-sexed twins with known zygosity who were born from 1926 to 1958. First, to obtain an overall effect of birth weight on risk of hypertension, we performed cohort analyses on all twins (n=16 265). Second, to address genetic and shared environmental confounding, we performed a nested co-twin control analysis within 594 dizygotic and 250 monozygotic twin pairs discordant for hypertension. Birth characteristics, including birth weight, were obtained from original birth records. Information from adulthood was collected from a postal questionnaire in 1973 (body mass index, height, smoking, and alcohol use) and from a telephone interview conducted from 1998 to 2002 (hypertension and socioeconomic status). Hypertension was defined as reporting both high blood pressure and treatment with antihypertensive medication. In the cohort analysis, the adjusted odds ratio for hypertension in relation to a 500-g decrease in birth weight was 1.42 (95% confidence interval, 1.25 to 1.61). In the co-twin control analyses, the corresponding odds ratios were 1.34 (95% confidence interval, 1.07 to 1.69) for dizygotic and 1.74 (95% confidence interval, 1.13 to 2.70) for monozygotic twins.

Conclusions—In the largest twin study on the fetal origins of hypertension, we found that decreased birth weight is associated with increased risk of hypertension independently of genetic factors, shared familial environment, and risk factors for hypertension in adulthood, including body mass index. (Circulation. 2007;115:2931-2938.)

Key Words: hypertension • birth weight • twins

Numerous studies have reported associations between measures of fetal growth restriction and increased risks of cardiovascular diseases later in life.1 It has been suggested that restricted fetal growth may reflect insufficient energy supply for organ development, which in turn may increase disease susceptibility later in life.2 However, it also has been argued that the association may be confounded by socioeconomic and genetic factors.3,4

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Twin studies provide opportunities to study the association between fetal growth and hypertension, controlling for shared (familial) environmental and genetic factors.5 Twin siblings share genes (half if dizygotic and all if monozygotic), intrauterine exposures, maternal factors, and early environment. Furthermore, twin siblings often differ in birth weight.6 Because twin siblings have identical gestational age, differences in birth weight within twin pairs reflect differences in fetal growth.

We analyzed prospectively collected information on birth characteristics on 16 265 Swedish like-sexed twins with known zygosity to investigate whether the association between birth weight and hypertension is confounded by shared (familial) environmental or genetic factors.

Methods

Study Population

Eligible participants in the present study were like-sexed twins born in Sweden from 1926 to 1958, who are included in the Swedish Twin Registry (n=37 392).7 In 1973, 30 305 twins (81%) from intact pairs and residing in Sweden responded to a postal questionnaire on health.8

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and lifestyle habits. In 1998, twins born in 1958 or earlier who were alive and residing in Sweden were invited to participate in a telephone interview called the Screening Across the Lifespan Twin (SALT) Study. Among the 32,905 like-sexed twins born from 1926 to 1958 invited to participate in SALT (regardless of their participation in the 1973 questionnaire), the response rate was 74% (n = 24,295). Nonresponse was due largely to refusal (55%) and inability to make contact (39%). In this study, we restricted the cohort to twins with known zygosity (n = 23,547), as determined by questions on childhood resemblance. Self-reported zygosity has been validated with DNA markers in a subsample of 199 twin pairs and was proved correct in 99% of the twin pairs. Among the 23,547 individuals in the defined study population, 21,125 (90%) had previously responded to the postal questionnaire in 1973.

Outcome

In SALT, all participating twins were asked questions about their medical history and current use of prescription medications. In the present study, individuals were diagnosed with hypertension if they answered yes to both of the following 2 questions: “Do you have or have you had high blood pressure?” (question 1) and “Do you take any medication daily?” (question 2) and named an antihypertensive drug. An antihypertensive drug was identified as a medication having an Anatomic Therapeutic Chemical Classification System code of C02, C03, C07, C08, or C09 and being listed in the Swedish drug. An antihypertensive drug was identified as a medication having an Anatomic Therapeutic Chemical Classification System code of C02, C03, C07, C08, or C09 and being listed in the Swedish Drug Compendium during the years 1997 to 2002. An individual was classified as nonhypertensive if he or she answered “no” to question 1 and, when asked question 2, either stated that he or she did not take prescription medications or reported medications that were not listed as antihypertensive drugs.

In the defined study population, 3232 (13.7%) were classified as having hypertension, 17,381 (73.8%) as nonhypertensive, and 2934 (12.5%) as nonclassifiable. Among those not classified, 29.4% reported a hypertensive medication in question 2 but answered “no” to question 1, 64.8% answered yes to question 1 but did not name a hypertensive medication in question 2, and 5.8% did not answer both questions. To test the sensitivity of our definition of hypertension, we included in additional analyses those who reported hypertension but did not use antihypertensive drugs as antigens (64.8% of those originally assessed as being nonclassifiable).

Exposures

Information about maternal and birth characteristics is routinely documented at birth by the attending midwife, and birth records are kept at local delivery archives throughout Sweden. The recording of information on birth records and the preservation of these records are required by law. Correct birth identification of each twin within a pair was ensured by restriction to twin pairs who were both baptized and named at birth or who agreed on birth order in SALT. Information about birth order was validated in a sample of 2713 like-sexed twin pairs who were both baptized at birth and responded to the question on birth order in SALT. A 95% agreement existed between birth order as stated in SALT and in the birth records. For the 23,547 like-sexed twins with known zygosity, medical birth records, with correct identification of individual twins, were obtained for 18,572 (79%) individuals, among whom 16,265 (in 5791 complete twin pairs) had information both on birth weight and hypertension in adulthood.

Information on parental socioeconomic status (SES) and obstetric and birth characteristics was collected from original medical birth records. Gestational age was based on date of the last menstrual period. SES was classified according to recommendations by Statistics Sweden. SES at birth was defined as the highest SES of the parents using information from mothers’ and fathers’ occupations. SES in adulthood was based on self-report of occupation in the SALT interview. Those currently employed were asked what occupation they had during the last 12 months; those not currently employed were asked what their last occupation was; and retirees were asked about their primary occupation in adulthood. Information on adult weight, height, smoking, and alcohol consumption was collected through the 1973 postal questionnaire. Body mass index (BMI) was calculated as the ratio between weight and squared height (kg/m²). Smoking status was dichotomized into those who had ever smoked (current and previous smokers) and those who had never smoked. Alcohol consumption was classified according to recommendations by the World Health Organization as low consumption (women, 0 to 19 g alcohol/d; men, 0 to 39 g alcohol/d), medium consumption (women, 20 to 39 g alcohol/d; men, 40 to 59 g alcohol/d), and high consumption (women, >40 g alcohol/d; men, >60 g alcohol/d).

Statistical Methods

The association between birth weight and hypertension was initially analyzed in the twin cohort, including 16,265 twins with information on birth weight and hypertension. Generalized linear mixed models were used to correct for the fact that we have correlated data, with random intercepts that vary from pair to pair and assuming a logit link function fitted with PROC NLMIXED (SAS Institute, Cary, NC).

The effect of birth weight on risk of hypertension, controlling for common genetic and shared environmental factors, was estimated in a nested co-twin control analysis. To infer confounding by genetic and shared environmental factors on the association between birth weight and hypertension, the co-twin control analysis was stratified by zygosity. Whereas the cohort analysis uses the entire cohort of twins, the co-twin control analysis was restricted to the 594 dizygotic and 250 monozygotic twin pairs discordant for hypertension. The paired effects in the co-twin control analysis were estimated by conditional logistic regression in SAS with PROC PHREG. In the co-twin control analysis, healthy co-twins were used as matched controls for the cases. Because twins share intrauterine exposures, maternal factors, 50% (dizygotic) or 100% (monozygotic) of their segregating genes, and generally childhood and adolescent environment (97% of the twins responded that they lived with their co-twin until 15 years of age), the matched nature of the co-twin control design minimizes confounding by these factors. If the estimated paired effects are smaller than the effect seen in the cohort or null, the association between birth weight and hypertension in the cohort analysis is probably confounded by factors shared by co-twins. Because dizygotic twins share on average 50% of their segregating genes, the estimated paired effect of birth weight on risk of hypertension in dizygotic twins only partly accounts for confounding by shared genetic factors, which are fully controlled for in monozygotic twins. Thus, if the paired effect of birth weight on risk of hypertension is smaller in monozygotic than in dizygotic twins, the association is confounded by genetic factors. If the paired effect is similar in monozygotic and dizygotic twins but smaller than the cohort effect, the association is confounded by shared environmental factors.

The study was approved by the research ethics committee of the Karolinska Institutet. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Rates of hypertension by birth, maternal, parental, and adult characteristics are shown in Table 1. Rates of hypertension were substantially influenced by year of birth, and women had higher rates of hypertension than men. Rates of hypertension consistently decreased with increasing birth weight, and the highest rate was found among individuals born with low birth weight (<1999 g). More than half of the twins who were obese (BMI ≥30.0 kg/m²) in 1973 had hypertension in 1998 to 2002. Rates of hypertension were higher among twins born to parents with low SES than among those born to parents with higher SES. Similarly, twins with low SES in adulthood had higher rates of hypertension. Smokers and high alcohol consumers in 1973 had higher rates of hypertension compared with nonsmokers and low alcohol consumers.
Those lacking information on gestational age and parental SES were, at the time hypertension was diagnosed, 3 and 7 years older than those with information. In addition, those lacking information on gestational age had 116-g-lower mean birth weight than those with information.

Table 2 displays frequencies of hypertension among twins with lower versus higher birth weight in a twin pair in relation to risk factors of hypertension in adulthood. In contrast to genetic, maternal, and shared environmental factors, risk factors in adulthood cannot be controlled for by design. By assessing differences in the distribution of such risk factors, we sought to see whether such risk factors were found more often among twins born with low birth weight compared with those with high birth weight. Risk factors in adulthood...
TABLE 2. Within-Pair Distribution of Risk Factors for Hypertension in Adulthood Among Twin Pairs Discordant for Birth Weight*

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Twins With Lowest Birth Weight in a Twin Pair (2477 ± 460 g)</th>
<th>Twins With Highest Birth Weight in a Twin Pair (2813 ± 462 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>BMI in 1973, kg/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18.5</td>
<td>601</td>
<td>12.5</td>
</tr>
<tr>
<td>18.5–24.9</td>
<td>3765</td>
<td>78.5</td>
</tr>
<tr>
<td>25–29.9</td>
<td>398</td>
<td>8.3</td>
</tr>
<tr>
<td>≥30</td>
<td>32</td>
<td>0.7</td>
</tr>
<tr>
<td>Height in 1973, cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥163</td>
<td>1216</td>
<td>25.2</td>
</tr>
<tr>
<td>164–169</td>
<td>1185</td>
<td>24.6</td>
</tr>
<tr>
<td>170–176</td>
<td>1224</td>
<td>25.4</td>
</tr>
<tr>
<td>≥177</td>
<td>1200</td>
<td>24.9</td>
</tr>
<tr>
<td>Smoking status in 1973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>2103</td>
<td>46.8</td>
</tr>
<tr>
<td>Ever smoker</td>
<td>2393</td>
<td>53.2</td>
</tr>
<tr>
<td>Alcohol consumption in 1973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2640</td>
<td>58.4</td>
</tr>
<tr>
<td>Medium</td>
<td>546</td>
<td>12.1</td>
</tr>
<tr>
<td>High</td>
<td>1336</td>
<td>29.5</td>
</tr>
<tr>
<td>SES in adulthood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue-collar worker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unskilled</td>
<td>1346</td>
<td>25.2</td>
</tr>
<tr>
<td>Skilled</td>
<td>876</td>
<td>16.4</td>
</tr>
<tr>
<td>White-collar worker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low level</td>
<td>846</td>
<td>15.9</td>
</tr>
<tr>
<td>Intermediate level</td>
<td>1286</td>
<td>24.1</td>
</tr>
<tr>
<td>High level</td>
<td>671</td>
<td>12.6</td>
</tr>
<tr>
<td>Self-employed worker</td>
<td>314</td>
<td>5.9</td>
</tr>
</tbody>
</table>

*The analysis was restricted to twins from intact twin pairs discordant in birth weight.

generally were associated with rates of hypertension among those with both lower and higher birth weight in a pair. However, only height and SES were distributed differently between the 2 birth weight groups (P = 0.01 and 0.02, respectively); those with lower birth weight in a pair tended to be shorter and were more likely to be blue-collar workers in adulthood, factors that were associated with increased rates of hypertension.

Table 3 shows unadjusted and adjusted odds ratios (ORs) from the cohort analysis of birth weight and risk of hypertension. The risk of hypertension increased with decreasing birth weight. Using birth weight as a continuous variable, we found that a 500-g decrease was associated with a 24% increase in risk of hypertension in the unadjusted model and 42% in the fully adjusted model. However, the models had varying sample sizes as a result of missing information on covariates. We therefore also restricted all the models to subjects without missing values on any covariates (n = 9294). Adjusting for covariates did not substantially alter the association between birth weight and risk of hypertension. No significant interactions were found between birth weight and birth year, sex, or any of the risk factors for hypertension in adulthood (data available on request).

Table 4 displays the results from the co-twin control analyses of the association between birth weight and risk of hypertension stratified by zygosity. The “unadjusted model” in a co-twin control analysis (by default) controls for all factors shared by the twins. Among both dizygotic and monozygotic twins, rates of low birth weight (≤1999 g) were higher among cases compared with their healthy co-twin controls. Among dizygotic twins, a 500-g difference in birth weight within a twin pair was associated with a 34% increased risk of hypertension. Contrary to the hypothesis of genetic confounding, we found that a 500-g difference in birth weight within monozygotic pairs was associated with a 74% increased risk of hypertension. To confirm our interpretation of the effect of adjustment for selected covariates, we fitted an unadjusted model that was based on the same sample as that used in the adjusted model. Within both dizygotic and monozygotic twin pairs, we found similar estimates in the unadjusted and adjusted models, indicating that the included risk factors for hypertension...
did not confound the association between birth weight and risk of hypertension.

Finally, we repeated the cohort and co-twin control analysis after reclassifying those subjects who reported hypertension but not antihypertensive medication as being hypertensive instead of nonclassifiable. Including the additional hypertensive twins attenuated the effect of a 500-g decrease in birth weight on risk for hypertension, both in the cohort

<table>
<thead>
<tr>
<th>TABLE 3. Unadjusted and Adjusted ORs of Hypertension in Relation to Birth Weight in the Cohort Analyses of Swedish Like-Sexed Twins Born From 1926 to 1958</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varying models¶</td>
</tr>
<tr>
<td>Included individuals, n</td>
</tr>
<tr>
<td>Birth weight, g</td>
</tr>
<tr>
<td>≤1999</td>
</tr>
<tr>
<td>2000–2499</td>
</tr>
<tr>
<td>2500–2999</td>
</tr>
<tr>
<td>3000–3499</td>
</tr>
<tr>
<td>≥3500</td>
</tr>
<tr>
<td>Per 500-g decrease</td>
</tr>
<tr>
<td>Nested models¶</td>
</tr>
<tr>
<td>Included individuals, n</td>
</tr>
<tr>
<td>Per 500-g decrease</td>
</tr>
</tbody>
</table>

Values are expressed as OR (95% CI) where appropriate.
*Adjusted for gestational age and sex.
†Adjusted for variables above and mother’s age at birth, maternal parity, parental SES at birth, and birth year.
‡Adjusted for variables above and SES in adulthood, BMI, height, and smoking status and alcohol consumption in 1973.
§Reference group.
¶Number of twins in each model depends on missing values in the selected covariates.
All models were restricted to twins with information on all covariates used in model 3.

<table>
<thead>
<tr>
<th>TABLE 4. Unadjusted and Adjusted ORs of Hypertension in Relation to Birth Weight in the Co-Twin Control Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case Control OR (95 % CI)</td>
</tr>
<tr>
<td>No. % No. % Unadjusted* Nested Unadjusted*† Adjusted‡</td>
</tr>
<tr>
<td>Dizygotic twins, n</td>
</tr>
<tr>
<td>Birth weight, g</td>
</tr>
<tr>
<td>≤1999</td>
</tr>
<tr>
<td>2000–2499</td>
</tr>
<tr>
<td>2500–2999</td>
</tr>
<tr>
<td>3000–3499</td>
</tr>
<tr>
<td>≥3500</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Per 500-g decrease</td>
</tr>
<tr>
<td>Monozygotic twins, n</td>
</tr>
<tr>
<td>Birth weight, g</td>
</tr>
<tr>
<td>≤1999</td>
</tr>
<tr>
<td>2000–2499</td>
</tr>
<tr>
<td>2500–2999</td>
</tr>
<tr>
<td>3000–3499</td>
</tr>
<tr>
<td>≥3500</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Per 500-g decrease</td>
</tr>
</tbody>
</table>

*Twin pairs are matched for shared environmental and common genetic factors.
†Unadjusted model restricted to twins with values on all covariates used in the adjusted model.
‡Adjusted for SES in adulthood, BMI, height, and smoking status and alcohol consumption in 1973.
§Reference group.
analysis (fully adjusted model: OR, 1.28; 95% confidence interval [CI], 1.17 to 1.40) and within dizygotic and monozygotic twins in the co-twin control analysis (adjusted OR, 1.16; 95% CI, 0.98 to 1.38; and adjusted OR, 1.24; 95% CI, 0.95 to 1.62, respectively).

Discussion
We found that the risk of hypertension increased with reduced birth weight both in a cohort of twins and within dizygotic and monozygotic twin pairs. Thus, our results, based on the largest twin study to date, support the hypothesis that fetal growth per se influences the risk of hypertension later in life.

Twin studies provide control for environmental and genetic factors shared by twins. A recent meta-analysis of twin studies supported the possibility that factors shared by twins confound the association between birth weight and blood pressure.4 However, no convincing evidence was found to support genetic, as opposed to shared environmental, confounding. In contrast to studies on twins, studies of full siblings found stronger relationships between birth weight and blood pressure within than between families, arguing that factors that are fixed between consecutive pregnancies, including maternal genotype, do not confound the association.13,14 Sibling studies provide control for familial (genetic and shared environmental) factors. However, full siblings share only half of their genes, and maternal factors and intrauterine environment may differ between siblings.

Differences between our results and those reported in previous twin studies may be partly attributable to differences in sample size and definition of outcome. A meta-analysis of twin studies included 7336 twins with known zygosity from 10 studies,4 whereas our study included 9731 dizygotic (3332 twin pairs and 3067 single twins) and 6534 monozygotic (2459 twin pairs and 1616 single twins) twins, of whom 594 dizygotic and 250 monozygotic twin pairs were discordant for hypertension. All twin studies included in the meta-analysis compared mean blood pressure measurements among young and middle-aged individuals, whereas the present study included middle-aged and elderly twins with hypertension.

In addition to the large sample size, our study also benefits from several other strengths. Information on perinatal and parental sociodemographic characteristics was retrieved from original birth records, and data on exposures in adulthood, except SES, were collected at least 25 years before ascertainment of the outcome (hypertension). Our exposure and covariate information should preclude recall bias.

The prevalence of self-reported hypertension in our study population (23%) is lower than the prevalence of hypertension for the Swedish population with the same age distribution (34%).15 The primary cause of the low prevalence of hypertension in our study is likely the fact that some hypertensive individuals were incorrectly classified as not having hypertension. The sensitivity of self-reported hypertension is low (ranging from 43% to 82%), whereas the specificity is reported to be high (ranging from 80% to 95%).16 Women are up to twice as likely to correctly report hypertension as men,16 which may explain the obtained increased rate of hypertension among women compared with men. In the present study, we used, in addition to self-reported high blood pressure, daily use of antihypertensive prescription drugs as a prerequisite to be classified as having hypertension. This resulted in a prevalence of hypertension of 16%, but rates of hypertension increased as expected with increasing age, BMI, smoking, and high alcohol consumption. Given the high specificity and probably low sensitivity of the diagnosis of hypertension in our study and assuming nondifferential misclassification, we have, if anything, underestimated the effect of birth weight on hypertension.16

When we also included those subjects who reported hypertension but not antihypertensive medication as having hypertension, the association between birth weight and hypertension was attenuated. Nevertheless, birth weight was still significantly associated with hypertension in the cohort analysis. In the co-twin control analyses, risks within monozygotic twin pairs, although slightly attenuated relative to those found in the entire cohort, were larger than within dizygotic pairs, implying that genetic factors do not confound the association. We speculate that the attenuation of risk is due to the following 2 causes. First, some of those reporting hypertension but not antihypertensive medication may have falsely reported hypertension and thus diluted the hypertensive group with nonhypertensive individuals. Second, a recent Swedish study found that the willingness of doctors to initiate or give further antihypertensive drug treatment increased with severity of hypertension.17 Thus, those reporting hypertension but not antihypertensive medication may have a milder form of hypertension than those also reporting antihypertensive medication and hence a weaker association with birth weight.

The appropriateness of adjusting for BMI when studying associations between birth weight and cardiovascular diseases later in life has been questioned.18 However, in the present study, we found no evidence that BMI or other factors in adulthood influenced the association between birth weight and hypertension.

Twin studies may be considered almost ideal in studying associations between fetal growth and subsequent risks of cardiovascular diseases. First, analyses taking zygosity into account allow for varying degrees of control for genetic factors.5 Second, twins are generally brought up together, and analyses within twin pairs provide control for unmeasured environmental and socioeconomic factors during childhood.5 Third, differences in birth weight within twin pairs reflect differences in fetal growth.

Generalizability of our findings in twins to the general population may be a concern. Because twins generally are more growth restricted in utero than singletons,19 they may have higher rates of coronary heart disease and hypertension later in life. However, twins do not differ from singletons with respect to risk of cardiovascular mortality and blood pressure.20 21 In our cohort analyses, results were consistent with those previously reported in unrelated singletons.22 In the co-twin control analyses, we found that the association between birth weight and hypertension was not confounded by genetic or shared environmental factors. If anything, our results indicate that the effect of birth weight differences on
risk of hypertension is stronger within monozygotic than dizygotic twins, indicating that the association may well be explained by factors affecting fetal nutrition and growth.

As with singletons, birth weights of twins are influenced by environmental insults and genetic and placental factors. The fetal origins of adult diseases hypothesis states that fetal malnutrition during the third trimester increases the risk of cardiovascular diseases later in life.2 Although weight gain during the third trimester is less pronounced among twins compared with singletons,19 intertwin disparity in fetal size within twin pairs increases with gestation.23

All dizygotic twins and a third of monozygotic twins are dichorionic (ie, they have separate placentas). In dichorionic twins, discordant fetal growth is related to both differences in genetic influences (dizygotic twins) and placental factors (dizygotic and monozygotic twins).24-23 Monochorionic twins share placentas and are always monozygotic. In monochorionic twins, discordant fetal growth is related to the vascular architecture of the shared placenta, including umbilical cord insertions.26-27 A recent study found that unequal placental sharing appears to be the primary contributor to birth weight discordance in monochorionic twin pairs.28 Thus, birth weight discordance within dizygotic and monozygotic twins is related mainly to placental factors that influence fetal nutrition. We cannot see any major differences in being subjected to inadequate placental nutrition between monozygotic twins, dizygotic twins, or singletons.

It has been proposed that the fetus, in response to undernutrition in utero, makes physiological adaptations that may be beneficial in the short term but may lead to increased risk of hypertension later in life.1 Influences on organs and mechanisms regulating blood pressure during the prenatal period have been studied as potential mechanisms for the fetal origins of hypertension. One proposed mechanism is alterations in the structure and function of the kidney, leading to reduced numbers of nephrons and resulting glomerular hyperfiltration.29 Others have proposed structural changes in the vasculature tree, leading to impaired endothelial function and arterial stiffness, as a potential mechanism.30 Most studies of mechanisms behind fetal origins of hypertension, however, have focused on fetal exposure to glucocorticoids.31 Excessive prenatal exposure to glucocorticoids is deemed important in the programming of hypertension in that it is both growth inhibitory32 and suggested to be a common cause of many structural and physiological changes related to regulation of blood pressure, including low nephron number,29 activation of the renin angiotensin system,33 and alterations in the hypothalamic-pituitary-adrenal axis.34

Conclusion
Whatever the underlying mechanisms, our findings suggest that the association between fetal growth and hypertension is independent of genetic factors and environmental factors during childhood and adulthood.

Sources of Funding

Disclosures
None.

References

CLINICAL PERSPECTIVE

It has been suggested that associations between measures of fetal growth and cardiovascular disease in adulthood are confounded by socioeconomic and genetic factors. The present study refutes such claims and suggests that the association between birth weight and hypertension is not confounded by genetic or environmental factors shared among twin siblings. This implies that the fetal environment and intrauterine growth have an independent influence on risk of cardiovascular disease in adulthood. Therefore, clinical investigations of birth weight–discordant monozygotic twins on established risk factors for cardiovascular disease such as blood lipids, hemostasis, insulin-like growth factors, and signs of atherosclerosis should give new insights into the fetal origins of cardiovascular disease and the identification of preventive mechanisms.
Signal Transducer and Activator of Transcription 1 Is Required for Optimal Foam Cell Formation and Atherosclerotic Lesion Development

Sudesh Agrawal, PhD; Maria Febbraio, PhD; Eugene Podrez, MD, PhD; Martha K. Cathcart, PhD; George R. Stark, PhD; Guy M. Chisolm, PhD

Background—Signal transducer and activator of transcription 1 (Stat1) potently regulates gene expression after stimulation by certain cytokines involved in tumorigenesis and host defenses. The present study investigated a novel role for Stat1 in foam cell formation and atherosclerosis.

Methods and Results—Inhibition of Stat1 activity by a Stat1-specific DNA “decoy” oligomer transfected into differentiated human THP-1 cells, and deficiency of stat1 in mouse macrophages significantly inhibited foam cell formation assessed by lipid staining and cholesteryl ester accumulation compared with control cells. The mechanism of Stat1 regulation of foam cell formation was uniquely dependent on the scavenger receptor CD36. Blunted Stat1 activity and stat1 deficiency significantly decreased expression of CD36 but not of scavenger receptor-A compared with controls, as assessed by immunoblotting and flow cytometry. Deficiency of CD36 but not scavenger receptor-A in mouse macrophages removed any dependency of foam cell formation on Stat1. In an intraperitoneal model of foam cell formation in which foam cells form in vivo independently of the model ligands used in vitro, stat1 deficiency significantly inhibited foam cell formation and CD36 expression. Transplantation of bone marrow from apolipoprotein e−/−xstat1−/− mice into lethally irradiated, atherosclerosis-susceptible apolipoprotein e−/− recipients significantly reduced both en face aortic lesion coverage and aortic root lesions compared with recipients of bone marrow from genetically matched apolipoprotein e−/− mice.

Conclusions—Stat1 regulates CD36 expression and foam cell formation in macrophages in vitro; the Stat1 regulation of foam cell formation requires CD36. The regulation of CD36 expression by Stat1 may be important in other pathophysiological CD36-dependent events. Stat1 deficiency reduces atherosclerosis in an apolipoprotein e−/− atherosclerosis-susceptible bone marrow transplantation model. (Circulation. 2007;115:2939-2947.)

Key Words: atherosclerosis • CD36 antigens • foam cells • lipoproteins • macrophage • signal transduction • STAT1 transcription factor

The janus kinase-signal transducers and activators of transcription (Stat) signaling pathway regulates certain biological events in development, differentiation, apoptosis, proliferation, and immune responses.1,2 Stat1 is a transcription factor that is a downstream target of interferon (IFN)−α and -γ and certain other cytokines.3 Binding of IFN-γ to its receptor evokes a cascade of events that involves phosphorylation of the receptor and associated janus kinase proteins. The activated receptor–janus kinase complex serves as a docking site for Stat1, which leads to its phosphorylation at tyrosine-701. Homodimers of activated Stat1 can translocate to the nucleus where they bind consensus DNA γ-activated sites, which leads to activation or repression of the transcription of numerous genes.1,2 Although IFN-γ has been shown to be proatherogenic,4−6 a role for Stat1 in atherosclerosis has not been previously reported.

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In early fatty streak lesions of human and mouse atherosclerosis, lipoprotein accumulation and oxidation occur. This is followed by activation of the endothelium, recruitment of circulating monocytes and their adhesion to the endothelium, diapedesis into the intima, and differentiation to macrophages.7,8 Macrophage uptake of oxidized lipoproteins via scavenger receptors such as CD36 and scavenger receptor-A (SR-A)9,10 is hypothesized to be an early event in atherosclerotic lesion formation,11 which leads to the formation of lipid-engorged “foam cells.”

The studies herein tested the hypothesis that Stat1 is proatherogenic. The role of Stat1 was studied with in vitro and in vivo foam cell formation model systems and an
atherosclerosis-susceptible mouse model. The results revealed that Stat1 positively influences lesion formation in vivo and is required for optimal progression of foam cell formation in macrophages in vitro and in vivo. In addition, Stat1 regulation of foam cell formation in vitro was mediated by CD36. These roles of Stat1 may be important not only for the understanding of atherosclerosis, but also of other CD36-dependent biological events.

Methods

THP-1 Cell Differentiation and Foam Cell Formation
THP-1 cells obtained from American Type Culture Collection (ATCC, Manassas, Va.) were differentiated by stimulation with 15 nM phorbol 12-myristate 13-acetate (PMA) for 2 hours and then washed with PBS and cultured in RPMI-1640 with 10% fetal bovine serum for 10 days. Foam cell formation was induced by incubation of differentiated THP-1 cells (grown on glass coverslips) for 48 hours with cupric ion-oxidized low-density lipoprotein (LDL) (Cu-oxLDL; 50 μg/mL) or other forms of modified LDL. Cells were stained with Oil Red O as described. Photographs of foam cells were taken with a phase-contrast microscope, and at least 10 microscopic fields were counted from 4 different slides for the same treatment for quantification of foam cells.

Flow Cytometry
Washed THP-1 cells were incubated with phycoerythrin-conjugated anti-human CD11b or fluorescein isothiocyanate-conjugated anti-human CD36 antibodies for 60 minutes on ice in the dark. Mouse bone marrow macrophages (BMMs) were incubated first with a mouse anti-mouse CD36 polyclonal antibody and second with a phycoerythrin-conjugated anti-mouse IgG, and mouse IgG was used as an isotype control (all antibodies were from BD Biosciences, San Jose, Calif). At least 25,000 cells were analyzed on a Becton-Dickinson FACScan with Cell-Quest software.

Transfection of a Stat1 Decoy and Missense Oligodeoxyribonucleotides Into THP-1 and Mouse Primary Macrophages
A double-stranded DNA oligomer, used successfully as a DNA “decoy” in previous studies, was used to specifically inhibit Stat1 activity. The phosphorothioated oligodeoxyribonucleotides, the decoy and missense control oligomers, were purchased from Sigma-Genosys Biotechnologies, Inc (Woodlands, Tex).

Gel Shift Assay
For detection of Stat1 DNA binding to protein, a gel shift assay was performed as described. The probe used for Stat1 DNA binding was the consensus binding site for Stat1, double-stranded DNA 5'-CAT GTT ATG CAT ATT CCT GTA AGT G-3', purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Isolation of Mouse Peritoneal Macrophages and Bone Marrow-Derived Macrophages
Elicited mouse peritoneal macrophages (MPMs) were harvested by peritoneal lavage with ice-cold PBS 2 to 3 days after intraperitoneal thioglycollate stimulation in female C57BL/6 mice. Primary cultures were prepared at a density of 10^6 cells/16 mm diameter well in RPMI-1640 that contained 10% fetal bovine serum and used 48 hours after plating. For BMMs, femurs were dissected under sterile conditions as described. BMMs were cultured for 24 hours, washed with PBS, and incubated for at least 4 days before experiments in Dulbecco modified Eagles medium with 30% conditioned media from L-929 cells (media rich in granulocyte-macrophage colony-stimulating factor), supplemented with 10% fetal bovine serum and 50 U/mL of both penicillin and streptomycin.

Immunoblot Analysis
BMMs were lysed with radioimmunoprecipitation buffer (Roche Diagnostics, Penzberg, Germany). Protein was transferred to PVDF membrane for immunoblotting using a polyclonal anti-mouse CD36 antibody generated and characterized by M.F. (unpublished observations, 2006).

LDL Preparation, Oxidation, Uptake and Binding
LDL was isolated from human plasma as described. LDL was alternatively oxidized with myeloperoxidase-generated nitrating intermediates (NO2-oxLDL), or modified by acetylation (Ac-LDL), as described. Lipoprotein iodination and their binding, uptake, and degradation by cultured cells were performed as described previously.

Intraperitoneal Foam Cell Formation
Sixteen-week-old apolipoprotein e-/- (apo e-/-) or apo e-/-stat1-/- male mice were fed a high-fat diet (42% calories from fat; Harlan Teklad TD.88137, Madison, Wis) for 4 weeks, and peritoneal cells were harvested and counted according to published protocols. Cells were washed with ice-cold PBS and plated for protein expression or foam cell formation measurements.

Bone Marrow Transplantation
The stat1-/- mouse (SV129 background) was bred into the apo e-/- mouse (C57B16 background) for 5 generations, and genetically matched littermates that were either apo e-/-stat1-/- or apo e-/- were used in intraperitoneal foam cell formation experiments or as bone marrow donors (4 males each of apo e-/-stat1-/- and apo e-/-) by previously described methods. Eight-week-old apo e-/- male mice (C57 Bl/6, Jackson Laboratory, Bar Harbor, Me) (12 to receive apo e-/- bone marrow and 12 to receive apo e-/-stat1-/- bone marrow) were lethally irradiated as per animal facility guidelines and previously published protocols. Polymerase chain reaction (PCR) for stat1 alleles was performed on DNA isolated from whole blood samples from the recipient mice posttransplantation (see Animal Genotyping and PCR below). PCR analysis performed as previously described revealed 75% to 100% engrafment of apo e-/- or apo e-/-stat1-/- bone marrow into lethally irradiated apo e-/- recipient mice (2 mice that received stat1-/- bone marrow did not show significant engrafment of the stat1-/- allele; data from these 2 mice were excluded).

Animal procedures were approved by the Cleveland Clinic Institutional Animal Care and Use Committee and performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Animal Genotyping and PCR
Genotyping was performed on ear clip-derived DNA. For apo e genotype, the PCR protocol described on the Jackson Laboratory Web site was used. For stat1 genotype PCR analysis, 2 different reactions were used: one that used the stat1 sense primer 5'-CTACCAAGATATCTGCTAGAC-3' and antisense primer 5'-CTCTACACCTTCTGACACC-3' to detect the wild-type allele, and one that used the stat1 sense primer 5'-CTACCAAGATATC-TGCTAGAC-3' and neoprimer 5'-CCGCCATTTCCGGTCGAC-3'. The reaction contained 3 μL of genomic DNA and 0.5 μL of the pooled primers at 50 pmol/μL each. The Expand High Fidelity PCR system kit (Roche Diagnostics) was used. The reaction mixture was preheated to 94°C for 3 minutes, then run for 31 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes, and then again at 72°C for 3 minutes at the end of the reaction. The products were run on a 2% agarose gel to distinguish the 285-bp wild-type apo e allele product from the 570-bp knockout allele product.

Atherosclerosis Lesion Measurements
Atherosclerotic lesions were quantified in bone marrow transplanted mice by 2 independent and blinded assessments: en face aortic coverage measured by computer-assisted planimetry, as described by...
Guy et al.\textsuperscript{24} and analysis of lesion area at the aortic root, performed as described by Guy et al.\textsuperscript{24} under the supervision of Dr Jonathan D. Smith (Cleveland Clinic, Cleveland, Ohio). The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Blunting Stat1 Binding to DNA Inhibited Foam Cell Formation in Differentiated Human Monocytoid THP-1 Cells

To determine if Stat1 can regulate foam cell formation, a frequently studied in vitro foam cell model system was used: the human monocyte-like leukemic cell line THP-1. Stat1 had been shown to be involved early in cell differentiation;\textsuperscript{25} therefore, the effects of Stat1 on foam cell formation were only tested after differentiation. The time course and degree of Stat1 activation were determined after differentiation was initiated with PMA. Differentiation was monitored by CD11b/MAC-1 expression\textsuperscript{26} with use of fluorescence-activated cell-sorting analysis. CD11b reached a maximum and a plateau at 3 days (Figure 1A). Stat1 activation was assessed by immunoblot analysis with use of antibodies specific for Stat1 phosphorylation at serine-727 and tyrosine-701 and by measurement of binding of a radiolabeled DNA Stat1 consensus binding element to proteins in nuclear extracts. In both assays, adherent THP-1 cells expressed a relatively low but detectable basal Stat1 activity. This increased markedly in response to treatment with PMA; however, after 2 days Stat1 activation returned to basal levels (data not shown). Thus, to test the effect of Stat1 on foam cell formation independently of the effect of Stat1 on differentiation, experiments were conducted after 3 days.

Foam cell formation was induced postdifferentiation by exposure of THP-1-derived macrophages to Cu-oxLDL for 2 days. A double-stranded DNA decoy oligomer was transfected into differentiated THP-1 cells to block Stat1 binding to its target genes.\textsuperscript{14} The effectiveness of this decoy was assessed by measurement of the binding of a radiolabeled DNA Stat1 consensus binding element to proteins in the nuclear extract. Transfection of the Stat1 decoy reduced Stat1 binding markedly (80% to 85%) compared with differentiated THP-1 cells that were untreated, treated with Superfectin alone, or transfected with missense double-stranded DNA (Figure 1B). Foam cell formation, as identified by Oil Red O staining, was readily apparent in cells treated with Cu-oxLDL alone, or transfected with missense double-stranded DNA (Figure 1C). Cholesteryl ester accumulation, also used to quantify foam cell formation, was significantly decreased by \textasciitilde50% in Cu-oxLDL–incubated cells treated with the Stat1 decoy compared with the above-mentioned control cells (Figure 1D).

Stat1 Deficiency Inhibited Foam Cell Formation in Mouse Bone Marrow Macrophages

In an independent approach, BMMs from wild-type and \textit{stat1}\textsuperscript{−/−} mice (SV129 genetic background) were obtained by harvesting of marrow cells and inducing differentiation.\textsuperscript{27} After 5 days, macrophages were treated with Cu-oxLDL for 48 hours to induce foam cell formation.\textsuperscript{28} Lipid accumulation, assessed with Oil Red O (see Methods), was 60% to 70% less in BMMs from \textit{stat1}\textsuperscript{−/−} mice than in those from wild-type mice (Figure 2A). Cholesteryl ester accumulation after Cu-
oxLDL treatment was also attenuated in BMMs from stat1−/− mice compared with those from wild-type mice (5.8±0.5 μg/mg versus 9.8±1.7 μg/mg of protein; P<0.008) (Figure 2B). Similar results were obtained with use of MPMs from stat1−/− and wild-type mice (data not shown).

CD36 Expression Was Inhibited in THP-1 Cells After Stat1 Activity Was Blunted

Macrophages recognize and selectively ingest modified lipoproteins through multiple cell surface scavenger receptors to become foam cells. Deficiencies of either of 2 such receptors, SR-A and CD36, have been reported to reduce lesions in atherosclerosis-susceptible mouse models.29,30 We hypothesized that the mechanism of decreased foam cell formation after interference with Stat1 could be a result of Stat1 regulation of 1 or both receptors. Immunostaining for CD36 by flow cytometry revealed marginally reduced basal and significantly reduced Cu-oxLDL–induced CD36 expression on Stat1-decoy–treated and differentiated THP-1 cells compared with sham-treated control cells (Figure 3A).

CD36 Expression Was Inhibited in Bone Marrow Macrophages From stat1−/− Mice Compared With Those From Wild-Type Mice

BMMs from stat1−/− mice exposed to Cu-oxLDL showed significantly reduced basal expression of CD36 compared with those from wild-type mice by flow cytometry (Figure 3B) and Western blot analysis (Figure 3C). Cu-oxLDL enhanced CD36 protein expression in a time-dependent manner in BMMs from stat1−/− mice and wild-type mice, but the level of induction was significantly less in stat1−/− macrophages. Expression of SR-A was not affected by the absence of Stat1, as analyzed by immunoblotting and flow cytometry (data not shown). These data showed that at least a correlation existed between reduced Stat1 activity or Stat1 deficiency, reduced CD36 expression, and reduced foam cell formation.

To test whether decreased CD36 expression after interference with Stat1 resulted in reduced CD36 function (ie, decreased binding and cell association of CD36 ligands), particular forms of modified LDL that have been shown to be recognized by distinct scavenger receptors were used.12 It has been shown that LDL does not bind SR-A or CD36; Ac-LDL is a preferential ligand for SR-A; Cu-oxLDL is a ligand for both SR-A and CD36; and NO2-oxLDL is a preferential ligand for CD36.12 Thioglycollate-elicited MPMs from stat1−/− or wild-type mice were incubated with 75 μg/mL of the 125I-labeled ligands, LDL, Ac-LDL, or NO2-oxLDL, or with 50 μg/mL 125I-labeled Cu-oxLDL. Ligand binding (incubation at 4°C) and cell association (ie, binding and uptake...
after incubation at 37°C) were quantified as described. Binding of 125I-labeled Ac-LDL did not differ between wild-type and stat1/H11002/H11002 macrophages. In contrast, binding of 125I-labeled NO2-oxLDL and binding of 125I-labeled Cu-oxLDL were both significantly decreased in macrophages from stat1/H11002/H11002 mice compared with macrophages from wild-type mice (Figure 4A). Analogous results were obtained with BMMs from stat1/H11002/H11002 and wild-type mice (data not shown). The relationship between Stat1 regulation of CD36 expression, CD36 ligand recognition, and foam cell formation was probed further by treatment of BMMs from stat1/H11002/H11002 and wild-type mice with 50 μg/mL unlabeled Cu-oxLDL, NO2-oxLDL, or Ac-LDL for 24 hours. Oil Red O staining revealed that the number of lipid-engorged foam cells was similar in Ac-LDL treated wild-type and stat1/H11002/H11002 macrophages. In contrast, a 40% to 45% (P<0.025) decrease was observed in the number of foam cells in stat1/H11002/H11002 macrophages treated with Cu-oxLDL and a 60% to 65% (P<0.003) decrease in stat1/H11002/H11002 macrophages treated with NO2-oxLDL compared with macrophages from wild-type mice (Figure 4B). These data are consistent with the interpretation that Stat1 regulates the expression of CD36 but not SR-A, and that the altered expression has functional impact through binding and uptake of CD36-recognized lipoproteins.

**Decreased Foam Cell Formation in Macrophages From stat1/H11002/H11002 Mice Was Caused by Inhibition of CD36, but not SR-A; Deficiency of CD36 Abolished Dependency of Foam Cell Formation on Stat1**

To determine whether regulation of CD36 by Stat1 was required for the Stat1 effect on foam cell formation, and to determine whether Stat1-regulated proteins other than CD36 markedly influenced foam cell formation, thioglycollate-elicited MPMs were isolated from background-matched sr-a/H11002/H11002, cd36/H11002/H11002, sr-a/H11002/H11002/H11003 cd36/H11002/H11002, and wild-type mice, and replicate cultures were treated with the Stat1 DNA oligomer “decoy.” Foam cell formation was unchanged by decoy treatment of macrophages from cd36/H11002/H11002 or cd36/H11002/H11002 sr-a/H11002/H11002 mice. Specificity of the decoy was evaluated as shown in Figure 1B. Results shown are expressed as cholesteryl ester:protein ratios and are means±SD of data combined from 2 experiments.

**Figure 4.** CD36 deficiency diminished the Stat1 dependency of foam cell formation; Stat1 dependency of foam cell formation occurred for lipoprotein ligands recognized by CD36 (Cu-oxLDL, NO2-oxLDL), but not those recognized by SR-A (Ac-LDL). A, Specific ligand binding of 125I-labeled LDL, Ac-LDL, Cu-oxLDL, or NO2-oxLDL was assessed in MPMs from wild-type and stat1/H11002/H11002 mice. Ac-LDL is a preferential ligand for SR-A, Cu-oxLDL is a ligand for both SR-A and CD36, and NO2-oxLDL is a preferential ligand for CD36. LDL does not bind either SR-A or CD36. Results are expressed as means±SD of triplicate measurements of specific μg 125I-labeled lipoprotein bound per mg cell protein after 5 hours. Asterisks indicate statistically significant differences compared with wild-type mice (P<0.001). B, MPMs from wild-type and stat1/H11002/H11002 mice were treated with unlabeled LDL, Ac-LDL, Cu-oxLDL, or NO2-oxLDL for 48 hours and stained with the neutral lipid stain Oil Red O to visualize cytoplasmic lipid accumulation. Scale bar=10 μm. C, Foam cell formation was measured by cholesteryl ester accumulation, was reduced in MPMs from wild-type or sr-a/H11002/H11002 mice compared with their respective missense-treated control cells after transfection of the cells with the Stat1 DNA oligomer “decoy.” Foam cell formation was unchanged by decoy treatment of macrophages from cd36/H11002/H11002 or cd36/H11002/H11002 sr-a/H11002/H11002 mice. Specificity of the decoy was evaluated as shown in Figure 1B. Results shown are expressed as cholesteryl ester:protein ratios and are means±SD of data combined from 2 experiments.
formation was significantly inhibited by the Stat1 decoy in MPMs from wild-type and sr-α mice (Figure 4C). In contrast, no significant inhibition of foam cell formation was observed in Stat1 decoy-treated MPMs from cd36−/− or double-knockout mice (Figure 4C); the absence of CD36 removed the Stat1 dependency of foam cell formation. Similar results were observed in Stat1 decoy-treated BMMs from these animals (data not shown). These data demonstrated a requirement for CD36 in Stat1 regulation of foam cell formation and revealed that, in the absence of CD36, blockade of Stat1 activity did not inhibit foam cell formation by CD36-independent means.

**Foam Cell Formation and CD36 Expression Were Inhibited In Vivo by Stat1 Deficiency**

To determine whether foam cell formation in vivo was limited by stat1 deficiency, and whether CD36 expression was reduced in vivo by stat1 deficiency, a variation of the technique reported by Li et al.31 was adapted (see Methods). Six days after peritoneal thioglycollate injection into either apoε−/− mice or apoε−/−×stat1−/− mice, peritoneal cells were harvested and assessed with Oil Red O staining and cholesteryl ester:protein measurement after allowing in vivo foam cell formation. Cell surface CD36 expression and plasma cholesterol were also quantified. Cholesteryl ester accumulation was significantly reduced in peritoneal cells from apoε−/−×stat1−/− mice compared with those from apoε−/− mice (1.29±0.1 μg/mg cell protein (n=12) versus 2.65±0.1 μg/mg (n=11); P<0.003) (Figure 5A), as was CD36 cell surface expression (546.3±79.2 relative units of intensity (n=12) versus 1102±164.0 (n=11); P<0.005) (Figure 5B). No significant difference existed in the total number of peritoneal cells harvested (27±1.7×10⁶ versus 28.2±2.0× 10⁶; P<0.15) or plasma cholesterol levels (1869±239 μg/mL [n=12] versus 2194±120 μg/mL [n=11]; P<0.25) between the apoε−/− and apoε−/−×stat1−/− mice. Thus, Stat1 deficiency is linked to reduced CD36 expression on foam cells formed in vivo, analogous to the results obtained in vitro. In addition, the role of Stat1 in foam cell formation that was observed with the model of lipoproteins oxidized in vitro was recapitulated when foam cells were produced from ligands in vivo.

**Atherosclerotic Lesion Formation Was Inhibited in apoε−/− Mice That Received Bone Marrow From apoε−/−×stat1−/− Compared With apoε−/− Mice That Received Bone Marrow From apoε−/− Mice**

Eight-week-old apoε−/− mice were irradiated and received bone marrow transplants. After 4 weeks, they were fed a high-fat diet for an additional 14 week-old. apoε−/− mice that received bone marrow from apoε−/−×stat1−/− mice had significantly reduced en face aortic lesion area coverage compared with recipients of bone marrow from apoε−/− mice (1.41±0.4% (n=10) versus 4.33±0.8% (n=12), which is a 69% decrease (P<0.004) (Figure 6A to C). Aortic root lesions were reduced from 274 400±24 360 μm² (n=12) in apoε−/− recipients to 165 100±18 740 μm² (n=10) in apoε−/−×stat1−/− recipients (Figure 6D through 6F), which is a ≈45% decrease (P<0.0026). Surprisingly, compared with apoε−/− mice that received apoε−/− bone marrow, mice that received apoε−/−×stat1−/− bone marrow were statistically significantly heavier (Figure 6H) and had significantly higher plasma cholesterol levels (Figure 6G). Immunohistochemistry with anti–phospho-Stat1 revealed that phosphorylated Stat1 was present in aortic root lesions from apoε−/− mice but not in aortic root lesions from apoε−/−×stat1−/− mice (data not shown).

**Discussion**

Our results reveal 2 novel findings. First, in both in vitro and in vivo macrophage cell systems, interference with Stat1-dependent pathways downregulated CD36 expression. Reduced foam cell formation in vitro by Stat1 pathway disruption depended on CD36. Second, our in vivo results showed that Stat1 deficiency reduced foam cell formation in an intraperitoneal inflammation model and reduced atherosclerosis in an atherosclerosis-susceptible bone marrow transplantation mouse model.

Our discovery that Stat1 helps regulate CD36 and foam cell formation is reinforced by the consistency of the findings in 3 distinct in vitro models (differentiated human THP-1 cells, BMMs, and MPMs), with use of different inducers of macrophage differentiation (PMA, granulocyte-macrophage colony-stimulating factor–rich L929-conditioned media, and thioglycollate recruitment and differentiation in vivo) and use of different means of Stat1 pathway interference (blockade of Stat1 binding to DNA and Stat1 deficiency). Consistent results were also obtained in an in vivo peritoneal inflammation model of foam cell formation, in which foam cell formation occurred in vivo, independent of in vitro–modified model lipoproteins.
Our results show by multiple approaches that the mechanism by which Stat1 regulates foam cell formation involves CD36 and that CD36 was a required element in the regulatory pathway. The effect of Stat1 on foam cell formation was absent in macrophages deficient in CD36, but not in macrophages deficient in SR-A. This effect of Stat1 on CD36 is likely indirect. The CD36 promoter does not contain a known consensus Stat1 binding motif. Such a binding motif is present in the SR-A promoter\(^{22}\); however, we did not find evidence of Stat1 regulation of SR-A expression.

The discovery of the regulation of CD36 by Stat1 has potential far-reaching implications that are independent of atherosclerosis. CD36 plays a role in a number of important pathological and physiological processes, such as long-chain fatty acid transport, recognition and clearance of apoptotic cells, sequestration of the malarial parasite, and immune responses to infections.\(^{9,33,34}\) CD36 has been implicated in the pathogenesis of diabetes mellitus and the metabolic syndrome by virtue of its effect on fatty acid uptake and utilization, and is critical in the ability of tissues to meet their energy needs during stress or exercise.\(^{9,33,34}\) If CD36 is regulated by Stat1 in contexts other than atherosclerosis, their elucidation could lead to enhanced understanding of these physiological and pathophysiological processes and may provide alternative approaches to development of targeted drug therapy.

A well-established role for Stat1 has been demonstrated in tumorigenesis and host-defense mechanisms,\(^{35,36}\) but its role in atherosclerosis is a novel observation. Our findings are of particular interest in light of multiple prior observations. Stat1 is a downstream target in IFN-\(\gamma\) signaling, and several studies have identified a role for IFN-\(\gamma\) in atherosclerosis, such as in atherosclerosis-susceptible mice.\(^{4–6}\) Our data could be construed to suggest that Stat1 represents a step in the intracellular pathway by which IFN-\(\gamma\) or other cytokines worsen fatty streak formation and that CD36 might mediate this process. However, in vitro reports of the effect of IFN-\(\gamma\) on CD36 are inconclusive. IFN-\(\gamma\) has been shown to enhance\(^{37}\) and to decrease\(^{38}\) CD36 in various monocyte and macrophage-like cell systems. In addition, there are other possible explanations. Stat1 activity was recently shown to contribute to 15-lipoxygenase expression,\(^{14}\) and 15-lipoxygenase expression is believed to be atherogenic.\(^{39,40}\)

On the basis of our in vitro findings that CD36 mediates the reduced foam cell formation caused by interference with Stat1 pathways, it is tempting to speculate that the reduction in atherosclerosis we observed in Stat1 deficiency was also mediated by CD36. However, this would be premature. Although reduced foam cell lesions in atherosclerosis-susceptible mice deficient in either CD36 or SR-A have been reported, a recent paper has questioned the role of scavenger receptors in atherosclerosis.\(^{41}\) In that study, in the absence of CD36 en face aortic analysis showed significant protection from atherosclerosis in female mice, but only a...
trend toward reduced lesion area in males. Furthermore, the aortic root lesion data were discordant; females that lacked CD36 had larger lesions, and no significant difference was found in males.41 Thus, our data could reflect other CD36-independent and antiatherogenic influences exerted by the deficiency in stat1.

Our data highlight the idea that Stat1 inhibition could represent a target to reduce inflammation. Certain substances known to inhibit Stat1, albeit nonselectively, namely the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibiting statins42 and epigallocatechin-3-gallate,43 have also been shown to inhibit atherosclerosis in humans and animals.44,45 Statins are known to have antiatherosclerotic effects in excess of that predicted from their effects on LDL lowering; our results invite speculation that a part of these pleiotropic effects of statins could be linked to Stat1 inhibition.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Signal transducer and activator of transcription 1 (Stat1) is a very well-studied transcription factor in the janus kinase–Stat signaling pathway. It is a downstream target of interferon-γ and other specific cytokines and regulates numerous genes involved in immune responses, tumorigenesis, and inflammation. The results reported here reveal for the first time that Stat1 regulates the conversion of macrophages to lipid-engorged “foam cells,” an early cellular event in the pathological sequence of atherosclerotic lesion development. Furthermore, mice deficient in Stat1 developed significantly less atherosclerosis than did control mice in a bone marrow transplantation model of atherosclerosis. The in vitro data presented here show, also for the first time, that Stat1 regulates the scavenger receptor CD36, a protein linked to fatty acid transport, apoptotic cell recognition, metabolic syndrome, and responses to infection. The potential significance of these findings includes the possibility that further studies of Stat1 regulation of CD36 and its influence on atherosclerosis will identify novel therapeutic targets in pathological Stat1-dependent signaling pathways. Interestingly, certain substances known to inhibit Stat1, albeit nonselectively (eg, 3-hydroxy-3-methylglutaryl coenzyme A reductase-inhibiting statins and epigallocatechin-3-gallate) have also been shown to inhibit atherosclerosis in humans and animals. Because statins are known to have antiatherosclerotic effects in excess of that predicted from their effects on lowering low-density lipoprotein, it is tempting to speculate that a part of the pleiotropic effects of statins could be through Stat1 inhibition.
Delta-Like 4 Induces Notch Signaling in Macrophages
Implications for Inflammation

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Background—Activated macrophages contribute to the pathogenesis of inflammatory diseases such as atherosclerosis. Although Notch signaling participates in various aspects of immunity, its role in macrophage activation remains undetermined.

Methods and Results—To explore the role of Notch signaling in inflammation, we examined the expression and activity of Notch pathway components in human primary macrophages in vitro and in atherosclerotic plaques. Macrophages in culture express various Notch pathway components including all 4 receptors (Notch1 to Notch4). Notch3 selectively increased during macrophage differentiation; however, silencing by RNA interference demonstrated that all receptors are functional. The ligand Delta-like 4 (Dll4) increased in macrophages exposed to proinflammatory stimuli such as lipopolysaccharide, interleukin-1β, or minimally-modified low-density lipoprotein in a Toll-like receptor 4– and nuclear factor-κB–dependent fashion. Soluble Dll4 bound to human macrophages. Coincubation of macrophages with cells that expressed Dll4 triggered Notch proteolysis and activation; increased the transcription of proinflammatory genes such as inducible nitric oxide synthase, pentraxin 3 and Id1; resulted in activation of mitogen-activated protein kinase, Akt, and nuclear factor-κB pathways; and increased the expression of Dll4 in macrophages. Notch3 knockdown during macrophage differentiation decreased the transcription of genes that promote inflammation, such as inducible nitric oxide synthase, pentraxin 3, Id1, and scavenger receptor-A. These in vitro findings correlate with results of quantitative immunohistochemistry, which demonstrated the presence of Dll4 and other Notch components within macrophages in atherosclerotic plaques.

Conclusion—Dll4-triggered Notch signaling may mediate inflammatory responses in macrophages and promote inflammation. (Circulation. 2007;115:2948-2956.)

Key Words: atherosclerosis ♦ DLL4 protein, human ♦ inflammation ♦ macrophages ♦ receptors, Notch

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Proinflammatory macrophages contribute importantly to a wide variety of pathological states including cancer, neurologic disorders such as Alzheimer’s disease, and cardiovascular diseases ranging from atherosclerosis, in-stent stenosis, and arterial and valvular calcification to heart failure.1–5 Macrophages adapt to the local microenvironment and acquire various functions associated with physiological and pathological processes. In the context of atherosclerosis, activated macrophages participate critically in every stage of lesion progression, from fatty streak formation to the onset of acute thrombotic complications. Matrix-degrading enzymes and prothrombotic molecules elaborated from activated macrophages may promote plaque disruption and subsequent thrombosis.1,6–9 Moreover, macrophage proliferation may contribute to development of the inflamed plaque.10 Additionally, macrophages secrete various proinflammatory cytokines such as interleukin-1β (IL-1β) that induce atherothrombosis-associated molecules and the activation of endothelial cells and smooth muscle cells. Thus, macrophages participate in an amplification cascade that sustains...
inflammatory responses in the atherosclerotic plaque and promote its structural instability and thrombogenicity.

Clinical studies have established that lipid-lowering therapy reduces the onset of acute coronary events, possibly in part through attenuation of inflammation and macrophage activation. However, despite effective lipid lowering, cardiovascular events remain a significant clinical problem. Further understanding of mechanisms that trigger macrophage activation could lead to more effective therapeutic strategies for atherosclerosis and its acute complications.

The Notch pathway mediates juxtaocrine signaling that requires cell-to-cell contact and critically determines the growth, differentiation, and survival of various cell types in diverse tissues. The Notch family members (Notch1 to Notch4) are large type I transmembrane receptors that undergo proteolytic processing by a furin-like convertase during transit to the cell surface. Binding of a ligand (e.g., Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged1, or Jagged2) triggers sequential receptor cleavage by ADAM and metalloproteinase domain (ADAM)-type metalloproteinases and γ-secretase, which results in the liberation and nuclear translocation of Notch intracellular domain (NotchICD). Association with the sequence-specific DNA-binding factor RBP-Jκ leading to the formation of a transcriptional activator complex that induces the transcription of Notch target genes.

Previous studies of the role of the Notch pathway in immune cells have focused mainly on lymphocytes. Notch signaling participates in lymphocyte development, maturation, activation, and transformation. However, Notch signaling can also influence myeloid cell differentiation, and its expression and role remain undetermined in macrophages, a key cell type in inflammation and many other diseases. Previous immunohistochemical and ultrastructural studies clearly demonstrated direct membrane contact between adjacent macrophages, which supports a role for homotypic juxtaocrine communication between macrophages in inflamed tissues. Here we provide the evidence that Dll4 expression increases in activated human macrophages and that Dll4 binding induces proinflammatory responses. Our findings suggest that the Dll4-Notch pathway participates in inflammatory states characterized by macrophage activation.

Methods

Cell Cultures

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation and cultured in RPMI-1640 that contained 5% human serum. In stimulation assays, confluent macrophages were treated with Ultra-pure lipopolysaccharide (LPS; InvivoGen, San Diego, Calif), cytokines, or minimally modified LDL (mmLDL). Coculture experiments used a murine stromal cell line (MS5-Dll4) or GFP (MS5-GFP). Resuspended MS5 cells were stably transfected with a construct that expressed human Dll4-GFP (mmLDL). Coculture experiments used a murine stromal cell line (MS5-Dll4) or GFP (MS5-GFP). Resuspended MS5 cells were stably transfected with a construct that expressed human Dll4-GFP (mmLDL). Coculture experiments used a murine stromal cell line (MS5-Dll4) or GFP (MS5-GFP).

Reverse Transcription and Quantitative Polymerase Chain Reaction

TaqMan quantitative polymerase chain reaction (PCR) was performed on GeneAmp 5700 (Applied Biosystems, Foster City, Calif). Quantitative PCR detection of human Dll4, toll-like receptor 4 (TLR4), inducible nitric oxide synthase (iNOS), and pentraxin 3 (PTX3), and Id1 was performed on Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, Calif) (see Table in the online Data Supplement for oligonucleotide sequences). Quantitative PCR values were normalized to GAPDH. Relative fold changes were calculated by the comparative threshold cycles (Ct) method, 2^ΔΔCt.

Transfection and RBP-Jκ/CBF-1 Luciferase Reporter Assay

200 nM of small interfering RNA (siRNA) was applied to human macrophages with cationic lipid-mediated transfection. Plasmids that contained a RBP-Jκ/CBF-1 firefly-luciferase reporter gene and a TK-Renilla luciferase internal control reporter gene were cotransfected into RAW264.7 cells by nucleofection (amaxa, Gaithersburg, Md). After coculture of RAW264.7 cells with MS5-Dll4 or MS5-GFP cells for 48 hours, luciferase activities were determined in whole cell lysates by use of the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis).

Dll4.Fc Binding Assay

Dll4.Fc protein was generated from human full-length Dll4 cDNA subcloned into human IgG1 fusion protein vector, pEd.Fc (M.J. Tavares, PhD, et al., unpublished observations, 2006). Dll4.Fc binding assay was assessed on human macrophages. After blockade of nonspecific binding, macrophages were incubated for 30 minutes at 4°C with 1 μg Dll4.Fc or control Fc fragment prebound to 0.5 μg biotinylated anti-human goat IgG at 15° to 20°C, followed by Streptavidin-phycocerythrin (2.5 μg/mL) for 45 minutes at 4°C.

Immunohistochemistry and Western Blotting

Immunohistochemistry was performed on fresh frozen sections of discarded human carotid endarterectomy specimens, collected in accordance with a protocol approved by the Institutional Review Board of the Brigham and Women’s Hospital. For Western blotting, 80 μg of sample protein was loaded into each lane. After incubation with primary antibodies, blots were incubated with horseradish peroxidase–tagged secondary antibodies and stained with an ECL detection kit (Perkin Elmer, Waltham, Mass).

Statistical Analysis

GAPDH-normalized Ct values of control and various treatment groups were compared statistically with the Mann-Whitney U test. Individual relative fold changes were calculated with the Equation 2^ ΔΔCt and illustrated in figures as mean relative fold changes ± SEM. Pearson’s correlation coefficient (R) with 2-tailed test of significance was used to determine bivariate correlations.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Notch3 Increases During Monocyte-Macrophage Differentiation

To explore the possible role of the Notch pathway in macrophages, we used real-time reverse transcription–PCR to examine the expression of Notch pathway components during the differentiation of human monocytes to macrophages in culture. Differentiation was gauged by the expression levels of macrophage scavenger receptor A, a macrophage marker (Figure 1A). At day 10 in culture, macrophages (n=4) expressed mRNAs for multiple Notch receptors and ligands (mean PCR Ct: Notch1, 30.77; Notch2, 26.89; Notch3, 28.45; Notch4, 33.91; Dll1, 34.79; Dll3, 36.45; Dll4, 40.00; Jagged1, 28.38; Jagged2, 35.75). Notably, expression levels of Dll4 were lower than those of other Notch ligands.
changed. Jagged2, Manic Fringe, and Radical Fringe also increased during macrophage differentiation. Western blots showed increased expression of full-length Notch3 protein at day 10 (Figure 1B), which corroborated the mRNA findings. Relative to their intrinsic GAPDH expression, human primary macrophages expressed more Notch3 mRNA than human aortic smooth muscle cells and radial artery endothelial cells (both \( P < 0.05 \) versus macrophages) (Figure 1C).

Proinflammatory Stimuli Induces Dll4 Expression in Macrophages

We used LPS (Ultra-pure LPS, InvivoGen) to broadly ascertain the effects of a proinflammatory stimulus on the Notch pathway in human primary macrophages. LPS stimulation (100 ng/mL) for 3 hours led to a dramatic induction of Dll4 mRNA in 24 different macrophage donors (3776.3 ± 1717.1 fold increase, \( P = 3.08 \times 10^{-7} \)) (Figure 2A). Dll4 expression was triggered by LPS in a time- and dose-dependent manner (Figure 2B and 2C). The expression of Notch receptors did not change substantially with LPS treatment (Figure 2D). LPS increased mRNA levels of Jagged1 (6.1 ± 1.2 fold, \( P < 0.01 \)) and ADAM17 (3.0 ± 0.7 fold, \( P < 0.05 \); \( n = 5 \)) (Figure 2D).

We also examined the effects of other proinflammatory stimuli implicated in atherogenesis. mmLDL and IL-1\( \beta \) increased Dll4 mRNA expression (68.7 ± 36.3-fold and 130.9 ± 61.7-fold, respectively, at 3 hours; \( P < 0.01 \) for both) in macrophages, whereas tumor necrosis factor \( \alpha \), interferon \( \gamma \), and granulocyte macrophage-colony stimulating factor had no significant effect (Figure 3).

Western blot analysis showed that LPS (Figure 4A) and IL-1\( \beta \) (Figure 4B) also increased expression of Dll4 protein. Furthermore, although the mRNA and protein levels of Notch3 were not increased, in Western blots stained with an antibody specific for the intracellular domain of Notch3, we observed that LPS induced a shift in the Notch3 polypeptides from 280 kDa (the size of newly synthesized, unprocessed Notch3) to 100 kDa (the size of furin-processed Notch3) (Figure 4C). These findings suggest that LPS increases the furin-processing of Notch3, an event that is predicted to enhance both the surface expression of Notch3 and therefore its availability to ligand.\(^{15}\)

**TLR4 Silencing and Nuclear Factor-\( \kappa B \) Inhibition Limits Dll4 Induction by LPS**

TLR4 serves as a receptor for LPS,\(^{24,25}\) TLR4 siRNA treatment silenced TLR4 mRNA expression in human macrophages (\( P < 0.05 \) versus control siRNA) (Figure 5A), and decreased LPS-induced Dll4 mRNA expression (\( P < 0.05 \) versus LPS + control siRNA) (Figure 5A). To examine the possible role of the nuclear factor-\( \kappa B \) (NF-\( \kappa B \)) pathway downstream of TLR4 in LPS-induced Dll4 expression, we used a cell-permeable peptide, SN50, that inhibits nuclear translocation of the active NF-\( \kappa B \) complex that contains the p50 subunit.\(^{26}\) SN50 substantially reduced Dll4 expression at 100 \( \mu g/mL \) (95.8 ± 3.3%; \( P < 0.05 \) versus LPS only group) (Figure 5B), whereas SN50M, the control peptide, did not affect Dll4 expression.

Macrophages also expressed ADAMS that participate in receptor cleavage, and Fringe proteins that modulate ligand-mediated signaling (mean PCR \( C_T \): ADAM10, 25.31; ADAM17, 28.43; Lunatic Fringe, 33.87; Manic Fringe, 27.62; Radical Fringe, 26.59). Differentiation was accompanied by a marked rise in Notch3 mRNA, which increased 10.1 ± 5.0 fold and 16.4 ± 11.4 fold by days 7 and 10, respectively (\( P < 0.05 \) for both). In contrast, Notch1 and Notch4 mRNA expression decreased at days 7 and 10 (\( P < 0.05 \) for both), whereas Notch2 expression was un-

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**Figure 1.** Notch3 expression increases preferentially during macrophage differentiation in culture (real-time reverse transcription-PCR). A. Increased expression of macrophage SR-A mRNA (mean 12.8 ± 9.4-fold and 30.0 ± 23.0-fold increases at days 7 and 10, respectively, relative to day 0) indicated differentiation of human peripheral blood-derived monocytes into macrophages (inset). Notch3 mRNA increased mean 10.1 ± 5.0-fold and 16.4 ± 11.4-fold at days 7 and 10, respectively, in parallel with SR-A. Notch1 and Notch4 mRNA at days 7 and 10 reduced slightly, whereas Notch2 did not change. Jagged2, Manic Fringe, and Radical Fringe mRNAs increased during macrophage differentiation. Lunatic Fringe and other Notch components did not change significantly. Bars indicate mean mRNA expression levels at days 5, 7, and 10, relative to day 0, in 4 macrophage donors. All probability values were calculated with Mann-Whitney test for PCR \( C_T \) values, and asterisks indicate \( P < 0.05 \) versus day 0. Error bars = SEM. B. Western blot shows increased Notch3 protein expression at day 10 versus day 0; \( \alpha \)-tubulin serves as a loading control. The data represent 2 of 4 macrophage donors that provided similar results. C. Human primary macrophages express more Notch3 mRNA than human primary aortic smooth muscle cells or radial artery endothelial cells. Histogram shows mean Notch3 mRNA levels in macrophages, smooth muscle cells, and endothelial cells in 4 donors normalized to GAPDH mRNA of each cell type. Probability values were calculated with Mann-Whitney test for PCR \( C_T \) values, and asterisks indicate \( P < 0.05 \) versus macrophages. SR-A indicates scavenger receptor A; MFng, Manic Fringe; RFng, Radical Fringe; LFng, Lunatic Fringe; EC, endothelial cells; and SMC, smooth muscle cells.

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**Figure 2.** Inhibition of Notch3 expression during macrophage differentiation. A. Western blot shows increased expression of full-length Notch3 protein at day 10 (Figure 1B), which corroborated the mRNA findings. Relative to their intrinsic GAPDH expression, human primary macrophages expressed more Notch3 mRNA than human aortic smooth muscle cells and radial artery endothelial cells (both \( P < 0.05 \) versus macrophages) (Figure 1C). B. Inhibition of Notch3 expression during macrophage differentiation. A. Western blot shows increased expression of full-length Notch3 protein at day 10 (Figure 1B), which corroborated the mRNA findings. Relative to their intrinsic GAPDH expression, human primary macrophages expressed more Notch3 mRNA than human aortic smooth muscle cells and radial artery endothelial cells (both \( P < 0.05 \) versus macrophages) (Figure 1C).
Confirmation of Notch Signaling in Macrophages

Dll4 Binding to Macrophages Triggers Notch Signaling

To examine whether Dll4 binds to macrophages and triggers Notch signaling, we performed 4 assays. First, we detected significant binding of Dll4-Fc-biotinylated IgG complex to human macrophages as compared with the control Fc-biotinylated IgG complex or Streptavidin-phycocerythrin alone (Figure 6A). Second, other experiments were conducted with feeder cell lines stably transfected with a vector that expressed GFP alone (MS5-GFP) or Dll4-GFP (MS5-Dll4); these feeder cells are much less adherent to culture dishes than are macrophages, which makes it possible to remove these cells before harvesting macrophages for analysis (see Figure in the online Data Supplement). Human primary macrophages cocultured with MS5-Dll4 generated Notch1ICD (Figure 6B), the activated form of Notch1. Accumulation of Notch1ICD was sensitive to compound E, a potent γ-secretase inhibitor (Figure 6C), which suggests that Dll4 activates the canonical Notch signaling pathway. Third, the Dll4-Fc-IgG complex, but not Fc-IgG, also induced Notch1ICD production (Figure 6D). Fourth, when cocultured with MS5-Dll4 cells, the RAW264.7 macrophage cell line showed a >10-fold increase in the activity of a Notch-sensitive luciferase reporter gene that contains multiple binding sites for Notch1.
Dll4-Notch Binding Induces Inflammatory Pathways and Genes in Macrophages

Of further interest,Dll4 binding increased phosphorylated extracellular signal-regulated kinases 1 and 2 and Akt in human primary macrophages (Figure 7A), which indicates that Notch signaling induces mitogen-activated protein kinase (MAPK) and Akt pathways in this cell type. Coculture with MS5-Dll4 also increased the intracellular signal-regulated kinases 1 and 2 and Akt in human primary macrophages (Figure 7A). Further-more, Dll4 binding increased phosphorylated extracellular signal-regulated kinases 1 and 2 and Akt in human primary macrophages (Figure 7A). Additionally, Dll4 binding increased phosphorylated extracellular signal-regulated kinases 1 and 2 and Akt in human primary macrophages (Figure 7A).}

Dll4 Colocalizes With Macrophages in Human Atherosclerotic Plaques

Staining for Dll4 and Notch3, as examples of ligand and receptor expression, co-localized with CD68 (a macrophage marker) in the tunica intima of human atherosclerotic plaques (Figure 8A). Neither nonimmune IgG nor PBS showed positive staining (data not shown). Computer-assisted color image quantification followed by statistical regression analysis demonstrated that immunoreactivity for Dll4 correlated positively with CD68 staining (Figure 8B). Although immunostaining did not demonstrate clearly whether subpopulations of macrophages express Dll4, Notch3, or both, quantitative analysis correlated strongly Dll4 and Notch3 staining (Figure 8B). Staining for Notch3 and other ligands (ie, Dll1, Jagged1, and Jagged2) also correlated positively with CD68 staining (Figure 8B). Taken together, these data indicate the presence of multiple Notch signaling pathway components in macrophages found in atherosclerotic plaques.
The present study affirms our hypothesis that the Notch pathway plays an important role in macrophages, a key cell type in inflammation and atherosclerosis. Evidence that supports this idea includes the expression of multiple Notch receptors and ligands in human macrophages; markedly increased Dll4 expression in human macrophages stimulated with LPS, mmLDL, or IL-1β, an event that likely involves TLR4 and NF-κB; the

![Graphs and images showing experimental results]

**Figure 7.** Dll4-triggered Notch signaling increases proinflammatory properties in human primary macrophages. A, Coculture with MS5-Dll4 cells, but not MS5-GFP, induced phosphorylation of extracellular signal-regulated kinases 1 and 2 and Akt and decreased accumulation of IκBα, which indicates activation of the MAPK, Akt, and NF-κB pathways in macrophages. These data represent cultures from 3 different macrophage donors that produced similar results. B, 6-hour coculture of MS5-Dll4 cells with human differentiated macrophages increased mRNA expression of iNOS (mean fold increase of 971.20 ± 396.00, PCR Ct, P < 0.00001; n = 12), PTX3 (6.16 ± 1.86-fold, PCR Ct, P < 0.00001; n = 12), and Id1 (183.5 ± 48.00-fold, PCR Ct, P < 0.001; n = 5), compared with MS5-GFP control cells. C, Dll4-induced macrophage expression of iNOS, PTX3, and Id1 diminished with siRNA knockdown of Notch1, Notch2, Notch3, or Notch4 when compared to control siRNA oligonucleotides treatment (100%). Representative data from 2 of 4 different donors are shown. D, Notch3 siRNA applied to day 5 macrophages resulted in reduced expression of iNOS, PTX3, Id1, and SR-A when harvested at day 10 and analyzed in comparison with control siRNA (100%). Representative data from 2 of 4 different donors are shown. E, MS5-Dll4 induced expression of Dll4 mRNA in differentiated macrophages (mean fold increase of 19,528.80 ± 3815.70, PCR Ct, P < 0.0005). These data represent cultures from 6 different macrophage donors.

**Discussion**

The present study affirms our hypothesis that the Notch pathway plays an important role in macrophages, a key cell type in inflammation and atherosclerosis. Evidence that supports this
Two seminal reports elaborated on the pathological role thatDll4 plays in tumor angiogenesis, whereupon blockade ofDll4 signaling to Notch by therapeutic anti-Dll4 antibody led to uncoupling and deregulation of the vascular supply required by the tumor for proliferation and survival.27,30 The pathological participation of macrophages in tumorigenesis, tumor angiogenesis, and invasion,3 reminiscent of intralecular cell phenotypic modulation, plaque angiogenesis, remodeling, and instability in atherosclerosis,1,6–11 is increasingly recognized, and it remains to be seen whetherDll4 is involved.

Our finding that LPS, mmLDL, or IL-1β, but not tumor necrosis factor α, interferon-γ, or granulocyte macrophage-colony stimulating factor, induces early expression of Dll4 concurs with current knowledge that members of the toll/IL-1 receptor superfamily share common adaptor proteins.25 TLR4 siRNA knockdown and pharmacological inhibition of NF-κB suggest that TLR4 and NF-κB mediate, at least in part, LPS-induced Dll4 expression (Figure 5). LPS and mmLDL are TLR4 ligands,24,25,37 and TLR4 may participate in various inflammatory diseases such as atherosclerosis and acute coronary events.38,39 Human primary macrophages from 24 donors displayed a wide variation in the magnitude of Dll4 induction by LPS (Figure 2A), which may be explained by genetic variants at TLR4.40 A more complete understanding of the role of the Toll/IL-1 receptor superfamily in Dll4 transcription of iNOS, PTX3, Id1, and Dll4 itself; and the presence of Notch pathway components, such as Dll4 and Notch3, in human atherosclerotic plaques rich in macrophages.

Dll4 expression induced in human primary macrophages by proinflammatory stimuli (LPS, IL-1β, and mmLDL) (Figures 2 through 4) and the detection of immunoreactive Dll4 in human atherosclerotic plaques (Figure 8) indicate possible homotypic and heterotypic roles for Dll4 in activated macrophages. Dll4 expressed on neighboring macrophages within atherosclerotic plaques could have important homotypic functions, such as a role in macrophage activation. In addition to homotypic cell-cell interactions, proinflammatory heterotypic interactions can be surmised from previous studies that suggested roles for Dll4 in angiogenesis,27–30 the proliferation of hematopoietic cells,31–33 and induction of Dll4 mRNA by LPS in dendritic cells.34,35 Dorsch et al reported that adoptive transfer of constitutively active Dll4 had negligible effects on monocytes, but did not determine its effects on differentiated macrophages.35 Recent evidence suggests that Dll4 plays a vital role in the cardiovascular system. Haploinsufficiency of the mouse Dll4 gene is embryonic lethal with resulting gross vascular developmental abnormalities.36 Two seminal reports elaborated on the pathological role that Dll4 plays in tumor angiogenesis, whereupon
other signaling pathways like MAPK, Akt, and NF-κB that regulate cell growth and inflammation.41–43 The present study demonstrates that Dll4 binds to and activates Notch in macrophages (Figure 6A through 6E) and promotes the activation of the MAPK, Akt, and NF-κB pathways (Figure 7A). Dll4 also triggered the transcription of genes such as iNOS, PTX3, and Id1 (Figure 7B and 7C) that may enhance plaque burden, progression, and thrombogenicity by contributing to a proinflammatory macrophage phenotype.44–46 Furthermore, whereas stimulation through the Toll/IL-1 receptor pathway (eg, by LPS or IL-1β) induces Dll4 expression in macrophages (Figures 2 and 3), Dll4-triggered Notch signaling increases expression of Dll4 itself (Figure 7E), which is reminiscent of a Dll4-induced positive-feedback mechanism that links vascular endothelial growth factor signaling to Notch in endothelial cells.47

The initiation of Notch signaling requires receptor cleavage by γ-secretase.16 The present study shows that Dll4 binding to Notch on macrophages (Figure 6A) induces Notch (Notch1) cleavage that is suppressible by γ-secretase inhibition (Figure 6B and 6C). Dll4 can bind to Notch1, Notch3, and Notch4 in vitro,40 and Dll4 and Notch3 colocalized to macrophages within human atherosclerotic plaques (Figure 8). Notch3 also plays a critical role in vascular smooth muscle cell maturation and function,47,48 and, in other cell types, promotes the activation of MAPK and NF-κB pathways through uncertain mechanisms.41,43,46 Among Notch receptors, Notch3 expression preferentially increased during monocyte differentiation into macrophages (Figure 1), and Notch3 knockdown on day 5 of macrophage differentiation led to reduced expression of iNOS, PTX3, and Id1 in differentiated macrophages on day 10 (Figure 7D). However, siRNA silencing of each Notch receptor attenuated downstream expression of iNOS, PTX3, and Id1 (Figure 7C), and further work will be needed to delineate the contribution of each Notch receptor. The complexity of this question is heightened by the ability of each NotchICD to form higher order homodimers and possibly heterodimers with other NotchICDs on downstream target genes that contain paired RBP-Jκ/CFB1 binding sites,49 a response element that is found in a subset of Notch target genes, as well as genetic data that indicate that individual Notch receptors have both distinct and overlapping functions.13,50 Therefore, exploration of the functional evidence for the role of each receptor in macrophage activation and inflammation will require further investigations that use loss- and gain-of-function approaches in vitro and in vivo.

Taken together, our results support the idea that Notch signaling participates in juxtacrine homotypic communication between macrophages and also in the amplification of the proinflammatory milieu in inflamed tissues. Thus, further understanding of the Notch pathway in the context of macrophage biology likely will provide novel insights into the mechanisms of inflammation and new opportunities for rational therapeutic intervention.

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Disclosures

None.

References

Accumulating clinical and preclinical evidence suggests that activated macrophages contribute critically to the pathogenesis of a wide variety of cardiovascular diseases such as atherosclerosis and its acute thrombotic complications, arterial and valvular calcification, in-stent stenosis, vein graft failure, and heart failure. This list extends to other incurable diseases such as cancer and Alzheimer’s disease. Therefore, further understanding of molecular and cellular mechanisms of macrophage activation will provide important insights into prevention and treatment in various disease contexts. The present study tested the novel hypothesis that the Notch signaling pathway mediates macrophage activation. Our findings indicate that proinflammatory stimuli induce macrophage Delta-like 4 (Dll4), a Notch ligand previously thought to be endothelium-specific. Dll4 then promotes various proinflammatory responses in macrophages through 4 receptors (Notch1 to Notch4), which implicates the Dll4-Notch axis in inflammation. Interestingly, Dll4 binding also induces expression of Dll4 itself in macrophages, which suggests a Dll4-Notch positive feedback loop. Therefore, inhibition or modulation of Notch signaling may retard or reverse inflammation in diseases such as atherosclerosis and prevent devastating acute or chronic complications. The role of Notch signaling in macrophage biology as a therapeutic target deserves translational research with a multidisciplinary approach.
Receptor for Activated C-Kinase 1, a Novel Interaction Partner of Type II Bone Morphogenetic Protein Receptor, Regulates Smooth Muscle Cell Proliferation in Pulmonary Arterial Hypertension

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Background—Pulmonary arterial hypertension (PAH) is characterized by selective elevation of pulmonary arterial pressure. The pathological hallmark of PAH is the narrowing of pulmonary arterioles secondary to endothelial cell dysfunction and smooth muscle cell proliferation. Heterozygous mutations in \( \text{BMPR2} \), encoding the type II bone morphogenetic protein receptor (BMPRII), were identified in PAH, suggesting that alterations to BMPRII function are involved in disease onset and/or progression.

Methods and Results—We identified the receptor for activated C-kinase (RACK1) as a novel interaction partner of BMPRII by yeast 2-hybrid analyses using the kinase domain of BMPRII as a bait. Glutathione-S-transferase pull-down and coimmunoprecipitation confirmed the interaction of RACK1 with BMPRII in vitro and in vivo. RACK1–BMPRII interaction was reduced when kinase domain mutations occurring in patients with PAH were introduced to BMPRII. Immunohistochemistry of lung sections from PAH and control patients and immunofluorescence analysis of primary pulmonary arterial smooth muscle cells demonstrated colocalization of BMPRII and RACK1 in vivo. Quantitative reverse-transcription polymerase chain reaction and Western blot analysis showed significant downregulation of RACK1 expression in the rat model of monocrotaline-induced PAH but not in pulmonary arterial smooth muscle cells from PAH patients. Abrogation of RACK1 expression in pulmonary arterial smooth muscle cells led to decreased Smad1 phosphorylation and increased proliferation, whereas overexpression of RACK1 led to increased Smad1 phosphorylation and decreased proliferation.

Conclusions—RACK1, a novel interaction partner of BMPRII, constitutes a new negative regulator of pulmonary arterial smooth muscle cell proliferation, suggesting a potential role for RACK1 in the pathogenesis of PAH. (Circulation. 2007;115:2957-2968.)

Key Words: cardiovascular diseases ▪ hypertension, pulmonary ▪ remodeling

Pulmonary arterial hypertension (PAH) is a progressive and ultimately fatal disease defined by selective elevation of the mean pulmonary arterial pressure by at least 25 mm Hg at rest or >30 mm Hg during exercise.1,2 The underlying cause of this sustained elevation is an increased pulmonary vascular resistance, resulting in progressive right heart hypertrophy, reduced right heart function, and heart failure caused by increased right ventricular afterload.1,3-5 A key event in the development of PAH is pulmonary vascular remodeling, a complex process involving all layers and cells of the vessel wall.6,7 The pathological hallmark of vascular remodeling in PAH is the progressive narrowing and obstruction of small pulmonary arteries as a result of changes in the structure and function of cells located within the vessel wall (including endothelial and smooth muscle cells [SMCs], as well as adventitial fibroblasts).8,9 Structural changes that are observed routinely in PAH include vascular cell hypertrophy, hyperplasia, and an increased deposition of extracellular matrix proteins (including collagen and elastin). Although the pathological changes typical in PAH...
have been well defined, the origin of this disease remains unclear. In 2000, positional cloning revealed that patients affected by familial PAH exhibited germ-line mutations within the BMPR2 locus, which encodes the type II bone morphogenetic protein receptor (BMPRII).11-14 A ubiquitously expressed member of the transforming growth factor (TGF)-β receptor superfamily. To date, direct sequence analysis has identified multiple heterogeneous germ-line mutations in BMPR2 exons in ≈50% of familial PAH and 10% to 25% of idiopathic PAH (IPAH) patients.15,16 Most of these mutations represent missense, nonsense, or frame-shift mutations in BMPRII and are predicted to lead to a loss of function of BMPRII protein.16,17

The BMP ligands exhibit pleiotropic effects in different cell types, including the regulation of cell proliferation, apoptosis, and differentiation, as well as tissue patterning and organogenesis in the developing embryo.18,19 BMP signaling is induced on ligand binding to the high-affinity type I BMP receptors BMPRIA (ALK3) and BMPRIB (ALK6). Type I receptors then form a heterotetrameric complex of type I and type II receptors, which phosphorylates the intracellular signaling proteins Smad1 and Smad5. Smad1 and Smad5 form complexes with Smad4, translocate to the nucleus, and regulate the transcription of BMP-responsive genes.20,21 BMP-dependent signaling has been demonstrated to modify the proliferative response of SMCs because BMP2, BMP4, and BMP7 have been reported to inhibit vascular SMC proliferation.22-24 In families with BMPR2 mutations, this mutation causes PAH, but the exact molecular mechanism of this genotype-to-phenotype axis remains to be elucidated. It is currently hypothesized that mutations in the gene encoding BMPRII generate dysfunctional receptors that may induce proliferation of pulmonary artery SMCs (paSMCs), promoting an increase in pulmonary vascular resistance and ultimately pulmonary hypertension. Although these genetic studies have assigned a causative role for BMP receptors in the development of PAH, our understanding of the functional contributions and expression of this system in the lung in general, and PAH in particular, is still evolving.

Methods

Plasmid Construction

Full-length mouse BMPRII cDNA was amplified by polymerase chain reaction (PCR) using primers containing built-in Apal restriction sites and ligated into pCMV-HA (Invitrogen, Carlsbad, Calif). A yeast 2-hybrid construct encoding the BMPRII kinase domain (residues 209 to 530) was created using primers containing built-in EcoRI and BamHI restriction sites and inserted into pBKK7 (BD Biosciences, San Jose, Calif). Full-length receptor of activated C kinase (RACK)-1 was amplified by PCR and ligated into pCMV5A-Myc (Invitrogen). All primers sequences used for cloning are given in Table I of the online Data Supplement.

Yeast 2-Hybrid Screen

To identify novel BMPRII-interacting proteins, a yeast 2-hybrid screen was performed using the Matchmaker3 GAL4 2-hybrid system (BD Biosciences). The bait plasmid containing the BMPRII kinase domain was transformed into Saccharomyces cerevisiae strain AH109 and mated with strain Y187, which was pretransformed with an 11-day mouse embryonic cDNA library constructed in the yeast 2-hybrid vector pACT2. Diploid yeast cells were grown on high-stringency selection media (lacking the essential amino acids Leu, Trp, His, and Ade supplemented with X-Gal). Plasmids from positive yeast colonies were isolated and sequenced. All sequences obtained were compared with known transcripts in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (www.ncbi.nlm.nih.gov/BLAST).

Glutathione-S-Transferase Pull-Down Assay

A prokaryotic expression vector expressing the BMPRII kinase domain fused to glutathione-S-transferase (GST) was overexpressed in Escherichia coli BL21. Recombinant BMPRII-GST was recovered by lysis of the cells in 20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L EGTA, 0.1% (vol/vol) β-mercaptoethanol, and 1× protease inhibitors (Complete; Roche, Mannheim, Germany). GST-BMPRII was incubated with glutathione-sepharose beads (Amersham Biosciences, Uppsala, Sweden) (1.5 hours, 4°C) and, to avoid nonspecific binding, washed (3 times) with 20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, EGTA, and 0.5% (vol/vol) Triton X-100 supplemented with 1× of Complete protease inhibitors. GST-BMPRII was incubated with lysates from NIH3T3 cells overexpressing Myc-tagged RACK1 (1.5 hours, 4°C) in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 0.5% [vol/vol] NP-40, and 1× Complete protease inhibitors). After extensive washing (3 times, 1.5 mL in lysis buffer, samples were boiled for 5 minutes in 2× Laemmli sample loading buffer (60 mmol/L Tris-Cl [pH 6.8], 10% [vol/vol] glycerol, 2% SDS, 5% [vol/vol] β-mercaptoethanol, and 0.025% [vol/vol] bromophenol blue) and resolved on 12% SDS-PAGE gels.

Site-Directed Mutagenesis

Truncated GST-BMPRII kinase domain fusion proteins were prepared by site-directed mutagenesis of the wild-type GST-BMPRII kinase domain fusion protein using the Quick-Change site-directed mutagenesis system (Stratagene, La Jolla, Calif). Mutagenic primers carried a single nucleotide substitution identified in IPAH patients, which generated a premature stop codon at positions 1483, 1397, 1348, and 994. The primers used are listed in Table II in the online Data Supplement. All point mutations were verified by direct sequencing.

Immunoprecipitation

Protein G-agarose beads (50 μL of a 1:1 suspension in lysis buffer; Amersham Biosciences) were preincubated with anti-Myc IgG (2 μg; Cell Signaling Technology, Beverly, Mass). NIH3T3 cells overexpressing HA-tagged BMPRII and Myc-tagged RACK1 were lysed in 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 0.5% (vol/vol) NP-40, and 1× Complete protease inhibitors. Cell extracts were then incubated with antibody-bead complexes (2 hours, 4°C). The immunoprecipitates were washed (3 times, 0.5 mL lysis buffer), resuspended in 2× Laemmli sample loading buffer, boiled (5 minutes), and resolved on 12% SDS-PAGE gels.

Human Tissues and paSMCs

Lung tissue samples were obtained from 12 patients with IPAH (mean age, 34.5±10.5 years; 8 women, 4 men) and 9 control subjects (organ donors; mean age, 37.8±14.1 years; 5 women, 5 men). None of the IPAH patients exhibited BMPR2 mutations. Samples were placed in 4% (wt/vol) paraformaldehyde within 30 minutes after explantation. The study protocol was approved by the ethics committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol. Primary paSMCs were generated from lobar pulmonary arteries from donors or IPAH patients known to harbor a mutation in BMPR2 (n=3 for each) as described.26

Cell Cycle Analysis by Flow Cytometry

To determine DNA content, cells were harvested by trypsinization 24 hours after transfection, fixed overnight at 4°C with 75% (vol/vol)
ethanol, washed, and incubated in PBS containing 10 μg/mL propidium iodide and 100 μg/mL RNase for 1 hour at 37°C. Data were acquired using a fluorescent-activated cell sorter (FACS) Canto flow cytometer and analyzed by FACS DiVa software package (BD Biosciences). A minimum of 10 000 cells were analyzed per sample. Gates based on forward and side scatter were set to eliminate cellular debris and cell clusters.23

**Immunofluorescence and Immunohistochemistry**

Cells were seeded in 9-well chamber slides and processed for immunofluorescence analysis as described.23 Immunohistochemical analysis of paraffin-embedded lung sections from healthy transplant donors or IPAH patients was performed as outlined.27

**Western Blot Analysis**

Cell extracts (20 μg) were resolved on 10% reducing SDS-PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, Calif). Protein expression was analyzed using antibodies against the following epitopes: Myc (Cell Signaling, Danvers, Mass), HA (Sigma-Aldrich, Saint Louis, Mo), GST, pSmad1/3, or Smad1 (all from Cell Signaling). Immune complexes were visualized with horseradish peroxidase–conjugated secondary antibodies (Pierce, Rockford, Ill) using the ECL Plus system (Amersham Biosciences).23

**Reverse-Transcription PCR**

Total RNA was extracted from fresh-frozen lung samples using the Roti-Quick RNA extraction procedure according to the manufacturer’s instructions (Roth, Karlsruhe, Germany). RNA samples were reverse transcribed using ImProm II reverse transcriptase (RT; Promega, Mannheim, Germany). Real-time PCR was performed by the Sequence Detection System 7700 (PE Applied Biosystems, Austin, Tex). Cells were transfected with siRNA (100 nmol/L) using the Basic Nucleofactor Kit (Amaxa Biosystems, Cologne, Germany).28,29 Signals were normalized to porphobilinogen deaminase. All primers sequences for RT-PCR are given in Table III in the online Data Supplement.

**Transfection With Small Interference RNA**

Four small interference RNA (siRNA) sequences directed against human RACK1 were used to attenuate RACK1 expression in paSMCs (siRNA sequences shown in Table IV in the online Data Supplement). To control for nonspecific gene inhibition of the siRNAs, a negative-control siRNA sequence was used (Ambion, Austin, Tex). Cells were transfected with siRNA (100 nmol/L) using the Basic Nucleofactor Kit (Amaxa Biosystems, Cologne, Germany). The siRNA-mediated downregulation of target genes was assessed 24 hours after transfection in the RNA analysis and 48 hours after transfection for protein analysis.

**A Monocrotaline Rat Model of PAH**

Samples from the monocrotaline-induced rat model of PAH were obtained as described previously.30,31

**Assessment of Cell Proliferation**

Cell proliferation of paSMCs was assessed by direct cell counting and [3H]-thymidine incorporation analysis as described previously.23

**Luciferase Reporter Assay**

Luciferase assays were performed with the pID120 reporter construct containing a BMP-responsive element upstream of a firefly luciferase gene as previously described.24

**Statistical Analysis**

Values are presented as mean±SEM. The means of indicated groups were compared using 2-tailed Student t test or a 1-way ANOVA with Tukey’s highest-significant-difference post hoc test for studies with >2 groups. A level of P<0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Identification of RACK1 as a Novel BMPRII Interaction Partner**

To identify novel interaction partners of BMPRII, a yeast 2-hybrid screen was performed using the BMPRII kinase domain as a bait. This screen identified 3 overlapping clones (clones 42, 58, and 97; Figure 1A), which exhibited >97% sequence identity with RACK1, a molecule containing 7 WD domains (Figure 1A). To verify the interaction of BMPRII with these 3 clones, as well as full-length RACK1, by reverse 2-hybrid analysis, all constructs were subsequently expressed as Gal4 activation domain (AD) fusion proteins (designated AD-clone 42, AD-clone 58, AD-clone 97, and AD-RACK1). These clones were transformed into yeast pretransformed with the BMPRII kinase domain or the complete BMPRII cytosolic region fused to the Gal4–DNA–binding domain (BD). We were able to verify the interaction of BMPRII with RACK1, demonstrated by colony growth on high-stringency selective plates when DB-BMPRII kinase or DB-BMPRII total were expressed alongside AD-clone 42, AD-clone 58, AD-clone 97, or AD-RACK1 (Figure 1B).

**RACK1–BMPRII Interaction In Vivo**

We next sought to independently verify the BMPRII–RACK1 interaction in mammalian cells. Figure 2A presents the results of a GST pull-down assay, illustrating the specific interaction of the kinase domain of BMPRII with Myc-tagged RACK1, whereas GST alone did not interact with RACK1. Similar results were obtained in coimmunoprecipitation experiments, which further demonstrated a BMP2-independent interaction of BMPRII with RACK1 (Figure 2B). We then asked whether BMPRII mutations that have been found in IPAH patients would affect the interaction of RACK1 with BMPRII and generated 4 different BMPRII constructs containing the mutation Q495X, W466X, Q450X, or R332X (Figure 2C).

Interestingly, all BMPRII variants did interact with RACK1, but their interaction was significantly weaker than wild-type BMPRII (Figure 2D), indicating that the full-length BMPRII kinase domain was required for maximal binding to RACK1. These results were obtained in coimmunoprecipitation experiments, which further demonstrated a BMP2-independent interaction of BMPRII with RACK1 (Figure 2B). We then asked whether BMPRII mutations that have been found in IPAH patients would affect the interaction of RACK1 with BMPRII and generated 4 different BMPRII constructs containing the mutation Q495X, W466X, Q450X, or R332X (Figure 2C).

Interestingly, all BMPRII variants did interact with RACK1, but their interaction was significantly weaker than wild-type BMPRII (Figure 2D), indicating that the full-length BMPRII kinase domain was required for maximal binding to RACK1. We next elucidated the effect of the shortest truncated variant of BMPRII (R332X) on paSMC proliferation by cell cycle analysis. We observed that cell proliferation was decreased when wild-type BMPRII was transfected into paSMCs, whereas it was increased if the BMPRII mutant R332X was expressed (Figure 2F). Transfection efficiency was monitored by FACS analyses of enhanced green fluorescent protein (EGFP) expression and was routinely ~50% in paSMCs (Figure 2E).

**RACK1–BMPRII Colocalization in paSMCs In Vivo and In Vitro**

To further investigate the expression and function of RACK1 and its interaction with BMPRII in the lung, we analyzed RACK1 protein localization in the lung. Localization of BMPRII, RACK1, and smooth muscle actin (SMA) was...
determined by immunostaining of donor and IPAH lung tissues. As depicted in Figure 3A, BMPRII staining was demonstrated within endothelial cells and paSMCs. Similarly, RACK1 expression was localized predominantly to paSMCs and, to a lesser extent, endothelial cells (Figure 3B). SMA staining served as a marker for paSMC localization (Figure 3C). At the single-cell level, RACK1 and BMPRII exhibited regions of colocalization within the cytoplasm and at the cell surface, as demonstrated by immunofluorescence costaining of BMPRII and RACK1 (Figure 3D through 3F). Notably, intense staining for BMPRII and RACK1 was observed in plexiform lesions of IPAH patients, as depicted in Figure 4A through 4F. Although SMA staining in plexiform lesions was observed routinely in nonluminal cells, strong staining for BMPRII and RACK1 was observed in luminal cells and, to a lesser extent, in surrounding paSMCs (Figure 4B, 4D, and 4F).

**RACK1-BMP Receptor Expression in paSMCs**

To quantitatively analyze the expression of RACK1 and BMP receptors in paSMCs, we performed real-time RT-PCR of RACK1 and all BMP receptors using mRNA derived from paSMCs cultured from lobar pulmonary arteries of control and IPAH patients exhibiting BMPR2 mutations. We detected a significant reduction in BMPRII but not BMPRIA, BMPRIB, or RACK1 expression levels in paSMCs derived from IPAH patients (Figure 4G). Of note, most of the BMPRII expressed in paSMCs can be attributed to the full-length molecule, whereas expression of the short isoform is significantly lower, as detected using isoform-specific primers (Figure 4G).

**RACK1 Expression in Monocrotaline-Induced Pulmonary Hypertension**

To further explore the regulation of RACK1 in a nongenetic model of pulmonary hypertension, we chose the rat model of monocrotaline-induced PH. Using semiquantitative and quantitative RT-PCR, we found that the expression of RACK1 mRNA was significantly downregulated 4 weeks after monocrotaline administration compared with control rats or rats 2 weeks after monocrotaline administration (Figure 5A and 5B). Similarly, RACK1 protein expression was significantly downregulated after 4 weeks but not 2 weeks of monocrotaline administration, as depicted by Western blot analysis and densitometry (Figure 5C and 5D). The reduced BMPRII expression also was observed in this model further argues for a dramatic reduction of RACK1–BMPRII interaction and an effect thereof on PAH pathogenesis.
Figure 2. Interaction of wild-type and mutated BMPRII constructs with RACK1 in mammalian cells in vivo. A, Interaction of GST-tagged BMPRII with Myc-tagged RACK1. Myc-tagged RACK1 was associated with glutathione-sepharose in the presence of GST-tagged BMPRII (lane 1, top blot) but not in the presence of GST alone (lane 2, top blot). B, Coimmunoprecipitation of HA-tagged BMPRII and Myc-tagged RACK1 in a ligand-independent manner. NIH-3T3 cells were transiently transfected with a plasmid encoding BMPRII-HA (lanes 3 and 4) and Myc-RACK1 (lanes 2 through 4). Myc-tagged RACK1 was immunoprecipitated from cell lysates with an anti-Myc antibody, and immunoprecipitates were probed with an anti-HA antibody to detect BMPRII. HA-tagged BMPRII was detected in RACK1 immunoprecipitates prepared with an anti-Myc antibody, regardless of BMP stimulation (lanes 3 and 4, top blot). Proper and equal protein expression is illustrated in the middle and lower blots. Lysates from untransfected NIH cells served as a negative control (lane 1). C, Overexpressed BMPRII variants carrying amino acid substitutions were visualized by Coomassie Blue staining. D, GST pull-down of Myc-tagged RACK1 using the indicated GST-tagged BMPRII variants. Pull-down of Myc-tagged RACK1 is illustrated in the top; proper expression and equivalent input of Myc-tagged RACK1 are illustrated in the bottom. Band intensities were quantified by densitometry and are illustrated in the histogram underneath the blots. *P<0.05. E, Evaluation of plasmid transfection into human paSMCs. Either empty vector (EV) or an EGFP expression vector was transfected into primary paSMCs and cultured for 24 hours. Cells were then trypsinized and analyzed for EGFP expression by FACS. F, Synchronized paSMCs transfected with cDNA encoding wild-type BMPRII or a truncated BMPRII variant as a result of a premature stop codon (R332X) were harvested after 24 hours, fixed, stained, and analyzed for DNA content by flow cytometry. Percentages of cells in the G0/G1 phase (green), S phase (pink), and G2/M phase (blue) are indicated. Cells transfected with EV served as a negative control for the assay.
Functional Effects of Alterations in RACK1 Expression on paSMC Proliferation

Because paSMC proliferation is a key event in the development of PAH and because RACK1 expression was significantly altered in PAH, we continued by investigating the effect of RACK1 knockdown, observed in the monocrotaline model, on paSMC proliferation. For this purpose, we initially designed 4 different siRNAs directed against RACK1. Using this approach, we were able to knock down RACK1 expression by 72%, as assessed by real-time RT-PCR and Western blot analysis (Figure 6A and 6B). As depicted in Figure 6C, RACK1 knockdown by siRNA treatment resulted in a signif-

Figure 3. Localization of BMPRII and RACK1 in healthy lungs and isolated paSMCs. A through C, Immunohistochemical analysis of endogenous BMPRII (A), RACK1 (B), and SMA (C) expression in the normal human lung. D through F, Immunofluorescence analysis of BMPRII and RACK1 expression in primary human paSMCs. BMPRII was visualized with an FITC-labeled antibody (green; D); RACK1 was visualized with an Alexa 594–labeled antibody (red; E). Colocalization results in a yellow cellular staining (F). DAPI staining (blue) facilitated visualization of the nuclear compartment of the cells.

Figure 4. Localization of BMPRII and RACK1 in lungs of patients with IPAH. Localization of BMPRII (A and B), RACK1 (C and D), and SMA (E and F) was assessed in large vessels (left column) and in plexiform lesions (right column). The mRNA expression of BMP receptors, including total BMPRII, its long form (LF), its short form (SF), BMPRIA, BMPRIB, or RACK1, was analyzed by real-time PCR (data reflect the mean±SEM). *P<0.05.
significant increase in paSMC proliferation compared with mock siRNA-transfected cells. These data further support a role for RACK1 in the regulation of paSMC proliferation and suggest that perturbations to RACK1 expression and/or function may lead to enhanced paSMC cell growth. Next, the effect of RACK1 overexpression on paSMC proliferation was assessed by cell cycle analysis and [3H]-thymidine incorporation. Cell cycle analysis demonstrated that overexpression of RACK1 led to an arrest of cell proliferation, as evident by an increase in the G0/G1 population, and a decrease in the number of cells in the S and G2/M phases (Figure 7A). Importantly, thymidine incorporation revealed a >50% decrease in paSMC proliferation compared with cells transfected with empty vector (Figure 7B). Platelet-derived growth factor was used as a positive control.

**Effect of RACK1 on BMP Signaling**

Finally, we investigated the effect of modulating RACK1 expression on Smad signaling by analyzing BMP2-dependent Smad1 phosphorylation under conditions of RACK1 overexpression or knockdown (Figure 8A and 8B). Although RACK1 overexpression led to increased Smad1 phosphorylation, RACK1 downregulation resulted in reduced Smad1 phosphorylation. In agreement with this, RACK1 overexpression also induced a 2-fold increase in pID120 luciferase expression after BMP2 stimulation compared with cells transfected with empty pcDNA vector (Figure 8C).

**Discussion**

The present study tested the hypothesis that novel interaction partners of BMPRII are essential regulators of SMC proliferation, a key finding in PAH. The basis of this hypothesis is the observation that up to 50% of patients with familial PAH exhibit germ-line mutations in \textit{BMPR2}, the gene locus encoding BMPRII. Despite this overwhelming genetic evidence for a causal involvement of \textit{BMPR2} mutations in PAH pathogenesis, we are still unclear about the precise mechanisms that give rise to such a localized disease in the setting of a germ-line mutation, which leads to the expression of the BMPRII protein variants throughout the body. Because most mutations described thus far are localized to the kinase domain and predicted to lead to a truncation of BMPRII protein,\textsuperscript{16} a loss and/or gain of function resulting from the specific mutations is proposed to be involved in PAH pathogenesis. Indeed, several recent publications have indicated that \textit{BMPR2} mutations can alter intracellular signaling by p38 kinase or Smad proteins,\textsuperscript{26,32–35} although considerable discussion exists as to whether BMPRII mutations or its allelic loss increases or decreases Smad signaling.

Furthermore, truncations of BMPRII could result in the gain/loss of interaction of an as-yet unknown binding partner of BMPRII in a ligand-dependent or -independent manner. Therefore, the aim of our study was to uncover novel BMPRII-interacting proteins, to elucidate their function in...
paSMCs, and to investigate their localization and expression in healthy and diseased lungs. Here, we have identified RACK1 as a novel interaction partner of BMPRII using the yeast 2-hybrid system. This novel interaction was confirmed by GST pull-down and coimmunoprecipitation in mammalian cells and occurred in a BMP2-independent manner, suggesting a constitutive interaction involved in basal maintenance of the smooth muscle phenotype.

Figure 6. Effect of RACK1 depletion on proliferation of primary paSMCs. A, RNA levels of RACK1 in paSMCs transfected with 4 different siRNA (si 1 through 4) were assessed by quantitative RT-PCR. Expression differences between siRNA- and mock-treated cells are depicted as log fold-change (ΔΔCt) values. B, Western blot of protein extracts obtained from paSMCs after siRNA treatment. β-Actin served as a loading control. C, Proliferation of paSMCs after transfection with siRNA against RACK1 was assessed by [3H]-thymidine incorporation. Values represent the mean±SEM of 3 independent experiments. *P<0.05.

Figure 7. Effect of RACK1 overexpression on proliferation of primary human paSMCs. A, Synchronized paSMCs transfected with empty vector (EV) or a RACK1-expressing vector were harvested after 24 hours, fixed, stained, and analyzed for DNA content by flow cytometry. Percentages of cells in the G0/G1 phase (green), S phase (pink), and G2/M phase (blue) are indicated. B, paSMC proliferation was assessed by direct [3H]-thymidine incorporation analysis (n=3). Platelet-derived growth factor (PDGF) stimulation was used as a positive control for the assay. *P<0.05.
RACK1 is a 36-kDa cytosolic protein that is composed of 7 WD40 motifs, which are predicted to form a 7-bladed propeller structure important in protein-protein interactions. These WD repeats are highly conserved among species, including plants, Drosophila melanogaster, higher mammals, and humans. RACK1 is expressed ubiquitously in most tissues such as brain, heart, kidney, liver, pancreas, spleen, or lung, suggesting an important homeostatic function in different cell types. RACK1 was originally identified on the basis of its ability to bind the activated form of protein kinase C, described to stabilize the active form of protein kinase C, and to facilitate its protein trafficking within the cell. RACK1 binds to and inhibits Src family kinases, which also were recently described to interact with BMPRII. Through its interaction with protein kinase C or Src kinases, RACK1 can function as a critical adaptor protein mediating cross-talk between serine/threonine and tyrosine kinase signaling pathways. In addition, RACK1 has been described to directly interact with a specific protein at a time, offering the intriguing possibility of alternative signaling in the presence and/or absence of functional BMPRII such as in patients with BMPR2 mutations.

In light of the BMPR2 mutations that occur in PAH patients, we determined whether BMPRII truncations affected their interaction with RACK1. To do so, we genetically engineered 4 different nonsense mutations derived from PAH families, which generated a premature stop codon within the BMPR2 cDNA at position 1483, 1397, 1348, or 994, respectively. All of these mutants led to the expression of a truncated receptor that lacked the long intracellular tail, along with different stretches of the BMPRII kinase domain (Figure 2). All of these BMPRII variants still interacted with RACK1, but their ability to do so was significantly decreased compared with the wild-type receptor. Expression constructs encoding the shortest of these truncated BMPRII variants led to increased cell proliferation, as measured by cell cycle analysis, when transfected into primary paSMCs. In contrast, wild-type BMPRII led to decreased paSMC proliferation.
BMPR2 mutations generally exhibit antiproliferative properties on paSMCs, this effect is lost in paSMCs from patients with IPAH who harbor mutations in the gene encoding BMPRII.22 Similarly, we also have demonstrated that paSMCs from monocrotaline-treated rats, which exhibited reduced BMPRII levels, are insensitive to the antiproliferative effects of BMP ligands.29 Thus, our data indicate that loss of interaction between BMPRII and RACK1 results in less BMP signaling and hence loss of the antiproliferative effect of BMP on paSMCs and finally increased paSMC proliferation. Although we would hesitate to propose that this is a dominant mechanism in all PAH patients, our data suggest that this may contribute to PAH pathogenesis in those patients with BMPR2 mutations that lead to a truncation and/or loss of its kinase domain.

Conclusions

Genetic associations and functional genomics strongly point to BMPR2 mutations as a causal factor in PAH. In addition, genetic, somatic, and/or environmental factors such as appetite suppressants, hypoxia, survivin, or serotonin undoubtedly also play a role in the pathobiology of PAH. In recent years, BMPRII function was studied intensively, and with the yeast 2-hybrid assay, 3 novel interaction partners have been reported. Tetex 1, a light chain of dynein, was identified as a novel BMPRII interacting molecule.48 This molecule binds to and is phosphorylated by BMPRII and was suggested to interact with downstream mediators of BMPRII (eg, Smads). Src kinase was reported as another BMPRII interaction partner.50 The interaction between BMPRII and Src inhibited Src kinase activity by reducing its phosphorylation at tyrosine 418 in a ligand-dependent manner and further inhibited downstream cell cycle regulators suggested to influence cell proliferation. In addition, BMPRII was identified in a recent screen for LIM kinase–interacting proteins, all clones identified as encompassing the tail region of BMPRII.49 Further analyses revealed that the interaction between LIMK1 and BMPRII inhibited the ability of LIMK1 to phosphorylate cofilin, which was alleviated by the addition of BMP4. Most interestingly, LIMK1-BMPRII interaction altered the actin cytoskeleton dynamics. Although these investigations have led to an increased understanding of BMPRII functions, we are now at a stage where novel BMPRII interaction partners play a role in pathological SMC functions such as cell proliferation with direct relevance to disease.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by elevated blood pressure in the pulmonary circulation resulting from a progressive increase in pulmonary vascular resistance. Pathogenetic mechanisms of PAH include vasoconstriction, in situ thrombosis, and remodeling of the pulmonary arterial vessel wall that results in medial hypertrophy as a consequence of augmented proliferation of endothelial and pulmonary artery smooth muscle cells. Patients suffering from familial PAH exhibit heterogeneous germ-line mutations in the *BMPR2* locus, a gene encoding the type II bone morphogenetic protein receptor (BMPRII). Although genetic studies have assigned a causal role for *BMPR2* mutations in the onset and/or development of PAH, our knowledge of the functional contribution of these mutations is still evolving, and PAH remains an incompletely understood and essentially incurable disease. In the present study, we have identified the receptor of activated C-kinase (RACK)-1 as a novel interaction partner of BMPRII using a 2-hybrid screen. RACK1 colocalized with BMPRII in pulmonary arterial smooth muscle cells in vitro and in vivo and was a negative regulator of pulmonary arterial smooth muscle cell proliferation. Therefore, our studies have identified a novel signaling intermediate downstream of BMPRII, which essentially controls pulmonary arterial smooth muscle cell proliferation. Modifying RACK1 expression or enhancing RACK1–BMPRII interaction represents a novel option in reverse vascular remodeling, a therapeutic goal for patients with PAH.
Relationship Between Blood Pressure and Stroke Recurrence in Patients With Intracranial Arterial Stenosis

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Background—Many clinicians allow blood pressure to run high in patients with intracranial stenosis to protect against hypoperfusion. We sought to determine whether higher blood pressure decreases the risk of stroke in these patients.

Methods and Results—Data on 567 patients in the Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) trial were analyzed. Time to ischemic stroke and stroke in the same territory of the stenotic vessel was compared in patients grouped by mean systolic blood pressure (SBP) and mean diastolic blood pressure (DBP) during the study. Additional analyses were based on severity and location of stenosis. Ischemic stroke risk increased with increasing mean SBP and DBP on univariate analysis ($P<0.0001$, $P<0.0001$) and after adjustment for risk factors ($P=0.0008$, $P<0.0001$). Elevated mean SBP and DBP also resulted in increased risk of stroke in the territory in univariate ($P=0.0065$, $P<0.0001$) and adjusted ($P=0.0002$, $P=0.0005$) analyses. The increased risk of stroke with increasing SBP was driven largely by patients in the highest SBP group. Patients with moderate (<70%) stenosis had increased risk of stroke ($P<0.0001$, $P=0.003$) and stroke in the territory ($P=0.0002$, $P=0.010$) with increased SBP and DBP. Patients with severe ($\geq 70\%$) stenosis had increased risk of stroke and stroke in the territory with elevated DBP ($P=0.004$, $P=0.004$).

Conclusions—In patients with intracranial stenosis, higher blood pressure is associated with increased (not decreased) risk of ischemic stroke and stroke in the territory of the stenotic vessel. These findings argue strongly against the common clinical practice of maintaining high blood pressure in patients with intracranial stenosis. (Circulation. 2007;115:2969-2975.)

Key Words: blood pressure ■ cerebral infarction ■ cerebrovascular circulation ■ hypertension ■ intracranial arteriosclerosis ■ prevention ■ stenosis

The optimal target blood pressure level in patients with symptomatic stenosis of a major intracranial artery (middle cerebral artery, internal carotid artery, vertebral artery, or basilar artery) is unknown. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7) does not provide target blood pressure levels for stroke patients specifically but recommends a blood pressure $<140/90$ mm Hg in general to decrease stroke and cardiovascular risk.¹ Clinical trials and epidemiological studies of stroke patients have demonstrated that lowering blood pressure reduces the risk of stroke.²–⁶ However, it is common practice to allow blood pressures $\geq 140/90$ mm Hg in patients with intracranial stenosis in the United States. This practice is based on expert opinion⁷–⁹ and studies that suggest that lowering blood pressure may increase the risk of stroke in some patients with severe carotid stenosis.¹⁰

The recently completed Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) trial, which compared aspirin and warfarin in patients with symptomatic 50% to 99% stenosis of a major intracranial artery, provided a unique opportunity to evaluate the relationship between blood pressure level and risk of stroke in these patients.

Methods

Study Design

Patients enrolled in the WASID trial were included in this post hoc analysis. The WASID trial was an investigator-initiated, randomized, double-blind, multicenter clinical trial conducted at 59 sites in North America to compare aspirin with warfarin in patients with symptomatic intracranial stenosis.¹¹ Two of the 569 patients enrolled in WASID had no blood pressure information and therefore were excluded. Details of the study design have been published.¹²

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been published previously. All patients had a transient ischemic attack or nondisabling stroke within 90 days that was attributable to angiographically verified 50% to 99% stenosis of a major intracranial artery (carotid, middle cerebral, vertebral, or basilar). Patients with tandem extracranial carotid stenosis, cardiac source of embolism, or uncontrolled severe hypertension (systolic blood pressure [SBP] >180 mm Hg or diastolic blood pressure [DBP] >115 mm Hg) were excluded.

During follow-up, patients were contacted monthly by phone and seen in person every 4 months to determine whether any strokes had occurred. All patients had blood pressure measured at baseline and every 4-month visit. Blood pressure was measured from the right arm with the patient sitting at rest with the arm supported at the level of the heart. At each visit, if the initial blood pressure reading was >140/90 mm Hg, a second reading was taken at the end of the visit, and the lower of the 2 readings was used.

Elevated blood pressure was managed by the study neurologist and the patient’s primary care physician, who were initially requested to follow the guidelines established in the National High Blood Pressure Education Program (1993) consensus document and subsequently the JNC7 guidelines when they were released in 2003.

Patients were followed up to the time of a stroke, death, or a common termination date (final follow-up visits for patients who were alive without a stroke were done during August 2003). Ischemic stroke was defined as a new focal neurological deficit of sudden onset lasting ≥24 hours and not caused by hemorrhage. Ischemic stroke that was considered “definitely” in the same territory of the symptomatic stenotic intracranial artery when the neurological signs correlated with a new infarct on computed tomography or magnetic resonance imaging in an area of the brain supplied by the symptomatic stenotic artery. Ischemic stroke was considered “probably” in the territory of the symptomatic stenotic artery when the neurological signs localized to an area of the brain supplied by the symptomatic stenotic artery but without new infarct on brain imaging. In this analysis, ischemic strokes that were definitely or probably in the territory of the stenotic artery were considered in the territory. The territory of ischemic stroke was determined by the local investigator and independently by a central investigator. In cases when disagreement existed as to whether the stroke was in or out of the territory, a second central investigator independently adjudicated the location, and the classification made by 2 of the 3 investigators was used.

Statistical Analyses
Because no difference was found between the 2 treatment arms with respect to ischemic stroke prevention, patients assigned to warfarin or aspirin were combined in this analysis. Summary statistics for continuous variables are reported as mean ± SD. All analyses were done with SAS/STAT (9.1.3) software (SAS Institute, Cary, NC).

Primary Analysis
Blood pressure was averaged over the course of the entire study period for each patient. For the primary analysis, the risk of ischemic stroke in any vascular territory and the risk of stroke within the territory of the stenotic vessel were compared in groups of patients stratified by mean SBP (≤119, 120 to 139, 140 to 159, ≥160 mm Hg) and mean DBP (≤79, 80 to 89, ≥90 mm Hg). These blood pressure categories were chosen on the basis of the JNC7 classification of normal blood pressure, prehypertension, stage 1 hypertension, and stage 2 hypertension. The DBP categories of JNC7 stage 1 (90 to 99 mm Hg) and 2 (≥100 mm Hg) hypertension were combined because of the small number of patients with stage 2 hypertension (n = 3).

The cumulative probability of an ischemic event was estimated with the product-limit method. A log-rank trend test was used to test whether an increasing category of mean SBP or DBP was associated with increased risk of any ischemic stroke or ischemic stroke in the territory of the stenotic artery. Cox proportional-hazards regression was used to estimate hazard ratios for increasing category of mean SBP or DBP, with the lowest category as the reference, both unadjusted and adjusted for other risk factors potentially related to stroke.

The assumption of proportional hazards was tested by fitting a model that included terms for the blood pressure categories and the interaction of these categories with the logarithm of follow-up time. The statistical significance of the interaction terms was assessed with a simultaneous test; a nonsignificant test result (P > 0.05) indicated that the proportional-hazards assumption was met. The assumption was met for SBP and DBP for any ischemic stroke and for SBP for stroke in the territory. The assumption was not met for DBP for stroke in the territory (P = 0.0018). However, a graph of the hazard functions for the blood pressure categories suggested that the functions were parallel until later follow-up times when relatively few events occurred (ie, the majority [83%] of the events occurred during the first year, and the interactions were not significant when times to event or last follow-up after 1 year were censored at that time [P = 0.089]). Therefore, we proceeded as if the assumption were met.

Subgroup Analyses
The analyses comparing categories of mean blood pressure described above were done for subgroups of patients with moderate (<70%) and severe (≥70%) intracranial stenosis and by arterial location of intracranial stenosis (ie, anterior circulation [carotid or middle cerebral] and posterior circulation [vertebral or basilar]). These analyses were done separately within each subgroup. In addition, in an effort to determine the effect of blood pressure on stroke risk early in the follow-up period, an analysis was done in which events and last follow-up visits occurring after 4 months were censored at 4 months.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Patient Characteristics
At baseline, the mean age of the 567 patients was 63.6 ± 11.4 years; 38% were female, 30% were black, 38% were diabetic, 71% had a lipid disorder, and 84% were hypertensive. The average blood pressure throughout the follow-up period of all patients was 140 ± 13/77 ± 8 mm Hg. Two hundred sixty-seven patients (47%) had a mean SBP during follow-up that was greater than the JNC7-recommended target SBP of <140 mm Hg (stage 1 and 2 hypertension), and 34 (6%) had mean DBP that exceeded the JNC7 target DBP of <90 mm Hg (stage 1 and 2 hypertension). The mean follow-up time was 1.8 ± 1.3 years. The average number of blood pressure readings during that time was 5.8 ± 3.6. With regard to antihypertensive therapy, 18% of patients were taking 1, 25% were taking 2, 22% were taking 3, and 24% of patients were taking ≥4 antihypertensive medications. The percentage of patients taking no antihypertensive medications was 11%. Table 1 shows the percentage of patients taking antihypertensive medications from various drug classes throughout the course of the study.

Relationship of Blood Pressure and Stroke Risk
When the data were analyzed using the JNC7 categories for blood pressure, the risk of any ischemic stroke was found to increase with increasing mean SBP and DBP (P < 0.0001 and P < 0.0001, respectively) using a log-rank trend test (Figure, A). Increasing mean SBP and DBP also were associated with increased risk of ischemic stroke in
the territory of the stenotic artery ($P=0.0065$ and $P<0.0001$, respectively) (Figure, B). Hazard ratios and 95% CIs are presented in Table 2. The increase in stroke risk with increasing SBP appears to be driven largely by events in the highest blood pressure group. When analyzed without the highest group (SBP $\geq 160$ mm Hg), no significant difference existed in stroke rates among the blood pressure groups. This may be due to the small sample size in some groups.

**Relationship of Blood Pressure and Stroke Adjusted for Risk Factors**

Elevated mean SBP ($P=0.0008$) and DBP ($P<0.0001$) were associated with increased risk of stroke after adjustment for age, type of qualifying event (transient ischemic attack versus stroke), gender, body mass index, time from qualifying event, history of diabetes mellitus, history of hypertension, race, percentage of intracranial artery stenosis at study entry, and hyperlipidemia (Table 2). Similarly, stroke in the territory also was increased in patients with elevated mean SBP ($P=0.0002$) and DBP ($P=0.0005$) after adjustment for the factors listed above.

**Blood Pressure Versus Stroke Risk Within Patient Subgroups**

**Moderate and Severe Stenosis**

There were 353 patients with moderate ($<70\%$) stenosis, 206 patients with severe ($\geq 70\%$) stenosis, and 8 patients missing a central reading of percent stenosis. Among the patients with moderate stenosis, increasing mean SBP and DBP were associated with a greater risk of any ischemic stroke ($P<0.0001$ and $P=0.003$) (Table 3) and stroke in the territory ($P=0.0002$ and 0.010), similar to the group at large.

In the patients with severe stenosis, only increasing mean DBP was significantly associated with increased risk of any ischemic stroke ($P=0.004$) or ischemic stroke within the territory ($P=0.004$). Although increasing mean SBP was not associated with an increased risk of stroke ($P=0.32$) or stroke in the territory ($P=0.43$) in patients

---

**TABLE 1. Percentage of Patients Taking Hypertensive Agents From Each Class at Any Point Throughout the Follow-Up Period**

<table>
<thead>
<tr>
<th>Antihypertensive Class</th>
<th>Patients on Medication in Class at Any Point, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin-converting enzyme inhibitor</td>
<td>61</td>
</tr>
<tr>
<td>$\beta$-Blocker</td>
<td>48</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>45</td>
</tr>
<tr>
<td>Diuretic</td>
<td>45</td>
</tr>
<tr>
<td>Vasodilator</td>
<td>17</td>
</tr>
<tr>
<td>Angiotensin receptor blocker</td>
<td>16</td>
</tr>
<tr>
<td>$\alpha$-Agonist</td>
<td>11</td>
</tr>
</tbody>
</table>

---

A. Cumulative incidence of ischemic stroke according to mean SBP and DBP. B. Cumulative incidence of ischemic stroke in the territory of the stenotic artery according to mean SBP and DBP.
with severe stenosis, no evidence existed for a reduction in stroke risk with higher blood pressures (Table 4).

**Anterior and Posterior Circulation**

In analyses of data from the subgroup of patients with stenotic posterior circulation arteries (vertebral and basilar, n=243), the risk of ischemic stroke was significantly increased with increasing mean SBP and DBP (P=0.0012 and P=0.0009). Ischemic stroke within the territory of the stenotic artery also was significantly increased in patients with posterior circulation stenosis with increasing mean SBP and DBP (P=0.0093 and P=0.008). In patients with anterior circulation stenosis (carotid and middle cerebral, n=306), the risk of ischemic stroke also was significantly increased with increasing mean SBP and DBP (P=0.0092 and P=0.0023). Ischemic stroke within the territory of the stenotic artery was not significantly increased in patients with anterior circulation stenosis with increasing mean SBP (P=0.24) but was associated with increasing mean DBP (P=0.0075).

**History of Hypertension**

There were 475 patients with a history of hypertension at study entry. These patients demonstrated an increased risk of ischemic stroke (P=0.0003 and P<0.0001) and an increased risk of ischemic stroke in the territory (P=0.015 and P<0.0001) with increasing mean SBP and DBP, similar to the group at large.

**Early Events**

Fifty-five patients had an ischemic stroke within the first 4 months of the follow-up period. In analyses of only these early strokes, increasing mean SBP and DBP did not have a significant reduction on the risk of stroke overall or stroke in the territory.

**Discussion**

The practice of maintaining higher blood pressure in patients with symptomatic stenosis of a major intracranial artery to protect against hypoperfusion and stroke is not

---

**TABLE 2. Hazard Ratios and 95% CIs According to Average SBP and DBP for Any Ischemic Stroke and Stroke in Territory***

<table>
<thead>
<tr>
<th>Blood Pressure, mm Hg</th>
<th>Sample Size, n</th>
<th>Events, n (%)</th>
<th><strong>HR</strong> (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤119</td>
<td>32</td>
<td>3 (9)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>120–139</td>
<td>268</td>
<td>42 (16)</td>
<td>1.6 (0.5–5.3)</td>
<td>0.046</td>
</tr>
<tr>
<td>140–159</td>
<td>218</td>
<td>40 (18)</td>
<td>2.0 (0.6–6.4)</td>
<td>0.252</td>
</tr>
<tr>
<td>≥160</td>
<td>49</td>
<td>21 (43)</td>
<td>5.8 (1.7–19.6)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>DBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤79</td>
<td>348</td>
<td>46 (13)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>80–89</td>
<td>185</td>
<td>49 (26)</td>
<td>2.1 (1.4–3.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>≥90</td>
<td>34</td>
<td>11 (32)</td>
<td>3.0 (1.6–5.8)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*The hazard ratios (HRs) and probability values are for comparisons between the lowest blood pressure category and each of the higher blood pressure categories.

<table>
<thead>
<tr>
<th>Blood Pressure, mm Hg</th>
<th>Sample Size, n</th>
<th>Events, n (%)</th>
<th><strong>HR</strong> (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤119</td>
<td>32</td>
<td>3 (9)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>120–139</td>
<td>268</td>
<td>42 (16)</td>
<td>1.4 (0.4–4.5)</td>
<td>0.590</td>
</tr>
<tr>
<td>140–159</td>
<td>218</td>
<td>40 (18)</td>
<td>1.1 (0.3–3.7)</td>
<td>0.881</td>
</tr>
<tr>
<td>≥160</td>
<td>49</td>
<td>21 (43)</td>
<td>4.7 (1.4–16.0)</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>DBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤79</td>
<td>348</td>
<td>46 (13)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>80–89</td>
<td>185</td>
<td>49 (26)</td>
<td>2.2 (1.4–3.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>≥90</td>
<td>34</td>
<td>11 (32)</td>
<td>3.5 (1.7–7.3)</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

---

**TABLE 3. Unadjusted Hazard Ratios and 95% CIs According to Average SBP and DBP for Any Ischemic Stroke and Stroke in Territory for Patients With Moderate (<70%) Intracranial Stenosis***

<table>
<thead>
<tr>
<th>Blood Pressure, mm Hg</th>
<th>Sample Size, n</th>
<th>Events, n (%)</th>
<th><strong>HR</strong> (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤119</td>
<td>23</td>
<td>1 (4)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>120–139</td>
<td>169</td>
<td>17 (10)</td>
<td>2.2 (0.3–16.9)</td>
<td>0.431</td>
</tr>
<tr>
<td>140–159</td>
<td>138</td>
<td>23 (17)</td>
<td>4.0 (0.5–29.5)</td>
<td>0.176</td>
</tr>
<tr>
<td>≥160</td>
<td>23</td>
<td>11 (48)</td>
<td>14.3 (1.8–111.2)</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>DBP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤79</td>
<td>229</td>
<td>24 (10)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>80–89</td>
<td>105</td>
<td>24 (23)</td>
<td>2.3 (1.3–4.0)</td>
<td>0.0046</td>
</tr>
<tr>
<td>≥90</td>
<td>19</td>
<td>4 (21)</td>
<td>2.5 (0.9–7.2)</td>
<td>0.090</td>
</tr>
</tbody>
</table>

*The hazard ratios (HRs) and probability values are for comparisons between the lowest blood pressure category and each of the higher blood pressure categories.
supported by the results of this study. In fact, higher SBPs and DBPs were associated with increased risk of ischemic stroke overall and an increase in stroke in the territory of the stenotic vessel. Although the high risk of stroke with increased SBP was driven largely by the SBP 160 mm Hg group, no evidence existed that maintaining SBP in a moderately hypertensive range (ie, 140 to 159 mm Hg), as is commonly done in clinical practice, was protective against stroke. Previous studies have demonstrated that patients with heterogeneous and often undefined causes of stroke have a lower risk of stroke recurrence with lowering of blood pressure, but this study is the first to demonstrate this finding among patients with symptomatic intracranial stenosis.

The increased risk of stroke in the same territory in patients with higher blood pressures may have been due to the effects of elevated blood pressure on the progression of atherosclerosis. The severity of intracranial atherosclerosis, which is the most important predictor of the risk of stroke in these patients, has been shown to be related to blood pressure in a multivariate analysis. This suggests that the association between higher blood pressure and increased risk of stroke in the same territory may be explained by progressive stenosis in patients with poorly controlled blood pressure. Ischemic stroke in territories other than that of the symptomatic intracranial artery in patients with higher blood pressures may have been related to penetrating artery disease or progression of previously asymptomatic large artery disease.

Another possible explanation for the lower rates of stroke among patients with lower blood pressures is that specific antihypertensive agents (eg, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and diuretics) may confer other benefits (so called “pleiotropic effects”) for stroke prevention besides blood pressure lowering. One of these benefits may be that angiotensin-converting enzyme inhibitors have a unique ability to maintain cerebral perfusion in patients with large-vessel atherosclerosis. Given that most patients in this study were taking multiple antihypertensive medications, we did not have sufficient power to evaluate the possible pleiotropic effects of the different classes of antihypertensives.

Our findings do not support previous recommendations that have advised against lowering blood pressure in patients with chronic hypertension and large-artery occlusive disease because of concern over stroke from hypoperfusion. Chronic hypertension is believed to shift the autoregulatory plateau of the cerebral pressure-flow relationship toward a higher pressure to maintain normal cerebral blood flow and thereby protect the brain against high intravascular pressure. This shift in autoregulation theoretically makes the brain more susceptible to ischemia at normal blood pressures, resulting in increased stroke risk at low normal blood pressures or a J-shaped curve for blood pressure and stroke risk. However, in our study, 475 patients (84%) with chronic hypertension did not have a decreased risk of ischemic stroke in the territory of the stenotic vessel when their blood pressure was elevated but did in fact demonstrate an increased risk of stroke in the territory. These findings are consistent with research demonstrating that chronically hypertensive patients readapt their cerebral blood flow autoregulation toward normal when their blood pressure is effectively treated. Our findings suggest that patients with intracranial atherosclerosis and chronic hypertension do not demonstrate a J-curve effect and argue strongly against the common clinical practice of maintaining high blood pressure in patients with intracranial stenosis.

We also sought to determine whether certain subgroups of patients with intracranial stenosis might require higher blood pressures to prevent stroke. One of these subgroups was patients with severe (70%) stenosis. On the basis of the hypoperfusion hypothesis, one might expect that patients with severe intracranial stenosis would be more susceptible to hypoperfusion with lower blood pressure. Although the subgroup of patients in our study with severe stenosis did not demonstrate a decreased stroke risk with

### Table 4: Unadjusted Hazard Ratios and 95% CIs According to Average SBP and DBP for Any Ischemic Stroke and Stroke in Territory for Patients With Severe (70%) Intracranial Stenosis

<table>
<thead>
<tr>
<th>Blood Pressure, mm Hg</th>
<th>Sample Size, n</th>
<th>Events, n (%)</th>
<th>HR (95% CI) P</th>
<th>Stroke in Territory</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥119</td>
<td>9</td>
<td>2 (22)</td>
<td>...</td>
<td>2 (22)</td>
</tr>
<tr>
<td>120–139</td>
<td>96</td>
<td>24 (25)</td>
<td>1.0 (0.2–4.4) 0.951</td>
<td>21 (22) 1.0 (0.2–4.1) 0.956</td>
</tr>
<tr>
<td>140–159</td>
<td>76</td>
<td>16 (21)</td>
<td>0.9 (0.2–3.9) 0.883</td>
<td>10 (13) 0.6 (0.1–2.6) 0.468</td>
</tr>
<tr>
<td>≥160</td>
<td>25</td>
<td>10 (40)</td>
<td>2.2 (0.5–10.2) 0.302</td>
<td>7 (28) 1.5 (0.3–7.4) 0.593</td>
</tr>
<tr>
<td>DBP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥79</td>
<td>115</td>
<td>21 (18)</td>
<td>...</td>
<td>15 (13)</td>
</tr>
<tr>
<td>80–89</td>
<td>76</td>
<td>24 (32)</td>
<td>1.8 (1.0–3.3) 0.045</td>
<td>19 (25) 2.0 (1.0–3.9) 0.044</td>
</tr>
<tr>
<td>≥90</td>
<td>15</td>
<td>7 (47)</td>
<td>3.0 (1.3–7.2) 0.0111</td>
<td>6 (40) 3.5 (1.4–9.0) 0.0096</td>
</tr>
</tbody>
</table>

*The hazard ratios (HRs) and probability values are for comparisons between the lowest blood pressure category and each of the higher blood pressure categories.*
lower SBP (as seen in the patients with moderate stenosis), no evidence existed of an increased risk of stroke with lower SBP in patients with severe stenosis. This finding suggests that although patients with severe intracranial stenosis might not benefit as much from lowering blood pressure for preventing a stroke in the territory of a stenotic intracranial artery (possibly because their disease is already so severe), it is still advisable to lower their blood pressure to reduce the risk of stroke in other territories and to reduce other medical complications from hypertension. Of note, our inability to demonstrate an increased stroke risk with elevated SBP in patients with severe stenosis might also have been due to a lack of power given the small number of patients in this group.

Another subgroup of patients commonly thought to benefit from higher blood pressures is patients with stenosis of the posterior circulation arteries. Our analysis, however, showed that patients with posterior circulation stenosis, when analyzed separately, demonstrated the same increased risk of ischemic stroke and ischemic stroke in the territory with increasing blood pressure seen in the group at large. This finding suggests that allowing patients with posterior circulation stenosis to have chronically elevated blood pressures does not appear to be beneficial.

Although it appears that lowering blood pressure in patients with symptomatic intracranial stenosis decreases the risk of stroke, the optimal timing of blood pressure reduction remains an issue. This study did not examine the effect of lowering blood pressure in acute, unstable patients with intracranial stenosis; thus, the findings are not applicable in that setting. However, our analysis of the temporal relationship between blood pressure levels and stroke risk indicates that lowering blood pressure soon after enrollment was not associated with an increased risk of stroke by 4 months and that the benefit of lower blood pressure may be achieved within 1 year and continues to grow for several years.

The main limitations of this study are that it is a post hoc analysis and was based on blood pressure measures that were averaged throughout the follow-up period. Therefore, we cannot provide a direct correlation between each ischemic stroke and the blood pressure levels at the time of the stroke. For example, a patient with initially severely elevated blood pressure who was brought under good control later in the follow-up period could have had a stroke while normotensive. Another limitation was the small number of patients in some blood pressure groups, which may have resulted in our inability to demonstrate a significant difference between each of the blood pressure groups. As can be seen in the Figure and Table 2, the largest differences are between the $\geq 160$ mm Hg category and the lower blood pressure categories. Finally, although some standardization existed for blood pressure measurement technique during follow-up, no standardization existed in the type of equipment used to measure each blood pressure. This variability may have limited the accuracy of the readings used in these analyses.

Despite these limitations, the data from this study show a strong association between elevated blood pressure and increased risk of ischemic stroke in patients with intracranial stenosis. These results argue against the common practice of allowing higher blood pressures in patients with intracranial stenosis to protect against hypoperfusion.

Sources of Funding

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Disclosures

G. Cotsonis and M.J. Lynn have received funding from a research grant (1 R01 NS36643) from the US Public Health Service National Institute of Neurological Disorders and Stroke for this trial. Dr Chaturvedi has received fees from Boehringer-Ingelheim and BMS/Sanoﬁ for participation in speakers’ bureaus and from Pfizer for consultant activities and has received grant support from Boehringer-Ingelheim and Pfizer. Dr Chimowitz reports being paid fees by the Bristol-Myers Squibb/Sanofi Pharmaceuticals Partnership, AstraZeneca, and the Sankyo Lilly Partnership for consulting on antithrombotic agents that were not evaluated in this trial and from Guidant Corp for consulting on a medical device (an intracranial stent) that was not evaluated in this trial. Dr Turan reports no conflicts.

References


CLINICAL PERSPECTIVE

Allowing blood pressures of ≥140/90 mm Hg (permissive hypertension) in patients with intracranial stenosis is common practice in the United States and is based on the theory that lowering blood pressure in these patients decreases perfusion through the stenotic artery, thereby causing ischemia in the territory of the stenotic artery. Few or no clinical data are available to support or refute this widely accepted theory. This study of patients with symptomatic intracranial stenosis finally provides some evidence to address this theory. The results of this study show that allowing chronically elevated blood pressures in patients with symptomatic intracranial atherosclerosis does not decrease the risk of stroke in the territory of the stenotic artery. In fact, permissive hypertension in patients with intracranial stenosis actually increases the risk of stroke in the territory of the stenotic vessel and stroke overall. Even patients with severe (>70%) stenosis or posterior circulation stenosis, patients thought to particularly benefit from elevated blood pressures, did not show any benefit with higher blood pressures. Therefore, the results of this study argue strongly against the common clinical practice of maintaining high blood pressure in patients with intracranial stenosis.

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Should aldosterone antagonists be considered as primary therapy for prevention of sudden cardiac death?

**Added Benefit of Mineralocorticoid Receptor Blockade in the Primary Prevention of Sudden Cardiac Death**

*Bertram Pitt, MD; Geoffrey S. Pitt, MD, PhD*

**S**udden cardiac death (SCD) is a major public health issue. In patients with heart failure (HF) of various origins, including ischemia post-myocardial infarction (MI), successful development of pharmacological therapies that target neurohormonal abnormalities and modulate disease progression has changed the major cause of death from progressive pump failure to SCD from cardiac arrhythmias. Conditions such as hypertension, hypertrophic cardiomyopathy, aortic stenosis, diabetes mellitus, and aging are accompanied by hypertrophy and fibrosis, increasing the risk of SCD. Also affected are patients with inherited arrhythmogenic disorders such as long-QT syndrome and Brugada syndrome (BrS). Although the mechanisms responsible for SCD, its epidemiology, and treatment have recently been reviewed,1–4 1 aspect of therapy that deserves further emphasis for the prevention of SCD is the role of aldosterone blockade (AB) or, more precisely, mineralocorticoid receptor blockade (MRB).

The Role of MRB in Reducing SCD in Patients With Severe Chronic HF Associated With SLVD and in Patients With SLVD and HF Post-MI

MRB with spironolactone 12.5 to 50 mg/d was shown to be effective in reducing SCD by 29% ($P=0.02$) and total mortality by 30% ($P<0.001$) in patients with severe HF due to SLVD associated with either ischemic heart disease or idiopathic dilated cardiomyopathy when added to standard therapy that included an angiotensin-converting enzyme inhibitor (ACEI), β-adrenergic blocking agent (BB), diuretics, and digoxin in the Randomized Aldactone Evaluation Study (RALES).5 Because

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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this study was initiated before the positive results of trials with BB in severe HF were published, only 10% to 11% of patients randomized in RALES were on BB.

In a subsequent study, the Eplerenone Post–Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS), the more selective MRB eplerenone, at 25 to 50 mg/d, was also associated with a 21% ($P = 0.02$) reduction in SCD and a 15% ($P = 0.008$) reduction in total mortality in patients with HF and SLVD post-MI. In EPHESUS, 83% of patients were on an ACEI and/or angiotensin receptor blocker (ARB) and 75% of patients on a BB before hospital discharge. Eplerenone was also effective in reducing SCD in patients on “optimal therapy” that included aspirin, reperfusion, a statin, an ACEI and/or an ARB, and a BB, suggesting that MRB adds to the efficacy of the standard therapy of HF in reducing SCD and total mortality. It should be noted that the definition of SCD in EPHESUS included death occurring within 1 hour of new symptoms, unwitnessed death with no new symptoms within the previous 72 hours, or cardiac arrest with death within 28 days thereafter; it is therefore possible that not all the deaths classified as SCD were due to an arrhythmia. Nevertheless, the significant reduction in total mortality in RALES and EPHESUS suggests a beneficial effect of MRB on SCD.

**Role of MRB in the First 30 Days After MI**

In patients with SLVD and/or HF, the first 30 days has been shown to be the period of highest risk for SCD. In EPHESUS, eplerenone 25 mg/d, equivalent to approximately 12.5 mg/d of spironolactone, was effective in reducing both SCD (by 37%; $P = 0.051$) and total mortality (by 31%; $P = 0.004$) during this high-risk period. It is possible that eplerenone may be even more effective in reducing SCD if therapy is initiated earlier. In EPHESUS, eplerenone was administered between 3 to 14 days post-MI (mean = 7 days). Elevated plasma aldosterone levels for patients with ST-segment elevation MI on admission to the hospital predicted an increase risk of death and resuscitated cardiovascular death independent of the patients’ age, reperfusion status, or the presence of HF. Of importance, 83% of the patients in this study did not have evidence of HF on admission, suggesting an important role for the early administration of an AB to patients post-MI regardless of the presence of clinical HF. In another study, Hayashi et al randomized patients with their first anterior ST-segment–elevation MI after primary percutaneous coronary intervention on day 1 post-MI to an AB strategy consisting of intravenous canrenone, an injectable MRB, followed by oral spironolactone for 30 days. They found that the AB strategy was well tolerated and was associated with a significant improvement in ventricular remodeling and collagen formation. The potential benefits of initiating AB therapy on day 1 post-MI will, however, require further testing, especially because of the negative experience with the early administration of intravenous ACEI post-MI.

AB during the first 30 days post-MI should be considered because early ICD implantation may be detrimental. The role of ICDs in reducing SCD in patients with SLVD after 30 days post-MI is not in dispute, as established by the results of the Second Multicenter Automatic Defibrillator Implantation Trial (MADIT-II). Yet in MADIT-II, in which the mean time of ICD implantation was 81 months post-MI, no reduction in SCD occurred during the first year post implantation. Moreover, The Defibrillator in Acute Myocardial Infarction Trial (DINAMIT), which tested the hypothesis that ICD placement during this early vulnerable period would be protective, found an excess of deaths due to an increase in noncardiovascular deaths during 1 year of follow up. Thus, while further exploration of the role of ICDs in the early post-MI setting continues, current guidelines do not recommend the insertion of an ICD in patients post-MI with SLVD before 30 days. The reason for the apparent failure of an ICD to reduce SCD during this early phase is unclear. On the basis of these observations, it would, however, appear prudent to treat patients with SLVD post-MI who otherwise qualify for implantation of an ICD with optimum pharmacological therapy, including a BB, ACEI and/or ARB, an MRB, and an ICD to provide both early and late protection from SCD.

In patients with chronic HF and SLVD associated with ventricular asynchrony, CRT has also been shown to be effective in reducing SCD. However, as yet no evidence exists for the effectiveness of CRT in reducing SCD early post-MI because such a study has not yet been performed. Given the beneficial effects of MRB on ventricular remodeling post-MI, we believe that the early administration of an MRB to prevent persistent SLVD and thus the need for an ICD and/or a CRT in many patients should be evaluated. The development of promising stratification algorithms to determine which patients are most likely to benefit from ICDs, such as microvolt T-wave alternans, presents opportunities to test this hypothesis.

**The Role of MRB in Reducing SCD in Patients With New York Heart Association Class II HF and SLVD**

The role of MRB in reducing SCD in patients with mild HF and SLVD is less certain than in those with severe HF (New York Heart Association class III and IV). The role of the MRB eplerenone in patients with New York Heart Association class II HF and SLVD is currently being evaluated in a large scale, prospective, blinded, multicenter trial, the Effect of Eplerenone versus Placebo on Cardiovascular Mortality and Heart Failure Hospitalization in Subjects With NYHA Class II Chronic Systolic Heart Failure (EMPHASIS-HF).

**The Role of MRB in Reducing SCD in Patients With HF and Preserved Left Ventricular Systolic Function**

Mounting evidence suggests that the incidence of HF associated with preserved left ventricular systolic function
(HFPSF) is rising as a result of the aging of the population and the increasing epidemic of obesity and diabetes mellitus.\textsuperscript{18} In contrast to patients with chronic HF and SLVD, patients with HFPSF have not been shown to receive a reduction in either total mortality or SCD from pharmacological therapy. The Candesartan in Heart Failure Assessment of Reduction in Mortality and Morbidity in Patients with Preserved Systolic function (CHARM–Preserved Trial)\textsuperscript{19} suggested that the ARB candesartan may provide a benefit in reducing hospitalization for HF in these patients, but not in reducing total mortality or SCD. The role of the ARB irbesartan in patients with HFPSF is currently being explored in the Irbesartan in Patients with Heart Failure and Preserved Systolic Function trial (IPRESERVE).\textsuperscript{20} Whereas no data currently exist on the effectiveness of an MRB in reducing SCD in patients with HFPSF, spironolactone has been shown to improve diastolic function in patients with HFPSF\textsuperscript{21} and is currently being evaluated in a large-scale, prospective randomized multicenter trial sponsored by the National Heart, Lung, and Blood Institute, the Treatment of Preserved Systolic function in Cardiac Failure with an Aldosterone Antagonist (TOPCAT).\textsuperscript{22} The resultant effects on SCD in this patient population will be eagerly awaited.

**The Role of MRB in the Prevention of SCD in Patients Without SLVD or HF**

The role of MRB in preventing SCD in patients without SLVD, such as those with essential hypertension accompanied by myocardial fibrosis and/or hypertrophy, is also promising but as yet unproven. Both myocardial fibrosis and hypertrophy predispose to SCD. MRB, as noted above, has been shown to be effective in reducing myocardial fibrosis and hypertrophy in experimental animal models as well as in patients with essential hypertension. Myocardial fibrosis increases electrical inhomogeneity of conduction and alters gap junction function. Ventricular hypertrophy as well as myocardial fibrosis is associated with a decrease in coronary flow reserve,\textsuperscript{23} which predisposes to myocardial ischemia and therefore SCD. Whereas, as yet, no large scale studies exist that demonstrate a benefit of MRB on SCD in patients with hypertension without SLVD or HF, an analysis of the EPHEUS data has suggested that almost all of the benefit of eplerenone in reducing SCD in patients with SLVD and HF post-MI may have occurred in those patients with a history of hypertension, even though they were not hypertensive at the time of their MI.\textsuperscript{24} The explanation for the benefit of MRB in these patients is uncertain but may relate to prevention of detrimental electrical remodeling associated with an increase in MR activity (see Mechanisms by Which MRB Reduces SCD below).

MRB may also play a role in preventing SCD in patients with obstructive coronary artery disease but without structural abnormalities of the ventricle. MRB has been shown to improve endothelial function in an experimental model of hyperlipidemia,\textsuperscript{25} and MRB has been shown to be effective in preventing the development of experimental atherosclerosis in the ApoE knock-out mouse as well as in a primate model of atherosclerosis.\textsuperscript{26,27} Thus, although not as yet proven, the effectiveness of MRB in the primary prevention of SCD can be postulated both for patients without SLVD but with structural abnormalities of the ventricle (such as fibrosis and hypertrophy) and for patients with obstructive coronary artery disease or ischemia without structural abnormalities of the ventricle.

**Mechanisms by Which MRB Reduces SCD**

Although MRB affects a number of mechanisms associated with SCD, it is unlikely that any single mechanism explains its benefits in reducing SCD in patients with chronic HF and SLVD, as well as in patients with SLVD and HF post-MI. Under certain circumstances, one or another of the mechanisms reviewed below may be of particular importance.

Aldosterone has been shown to increase tissue ACE expression and to upregulate the AT1 receptor.\textsuperscript{28} Thus, in HF or in the post-MI setting, an increase in serum aldosterone levels may lead to an increase in the concentration and effect of angiotensin II on the AT1 receptor, resulting in a vicious cycle, with activation of the renin-angiotensin-aldosterone system and a further production of aldosterone by the adrenal gland. Because angiotensin II is a major stimulus for the production of aldosterone from the adrenal gland, one might conclude that the use of an ACEI and/or ARB would decrease aldosterone levels and eliminate the need for an MRB. Other stimuli, such as the extracellular concentration of potassium, are also of importance. For example, in angiotensinogen knock-out mice, in which no angiotensin II is present, an increase in serum sodium with a consequent decrease in serum potassium significantly raises aldosterone levels.\textsuperscript{29} Although an ACEI and/or ARB may transiently suppress the production of aldosterone, over time aldosterone production “escapes” and the serum aldosterone level increases.\textsuperscript{30}

Whereas activation of MR is important in regulating renal sodium, potassium, and magnesium concentration in the distal renal tubule, it is also of importance in a number of other tissues that express MR, including the myocardium, vascular wall, glomerulus, and brain.\textsuperscript{31,32} In HF, expression of MR in the myocardium increases.\textsuperscript{33} Furthermore, cortisol may also activate the MR in several tissues, such as the renal tubule and vascular wall. These tissues, however, express the enzyme 11-\(\beta\)-hydroxysteroid dehydrogenase-2 that regulates the conversion of cortisol to cortisone, which cannot stimulate the MR.\textsuperscript{34,35} In situations in which reactive oxygen species increases, such as in HF and hypertension, 11-\(\beta\)-hydroxysteroid dehydrogenase-2 may be downregulated, thereby preventing the conversion of cortisol to cortisone. The consequent increase in cortisol may activate the MR. In the cardiomyocyte, which does not express 11-\(\beta\)-hydroxysteroid dehydrogenase-2, the MR may be normally occupied but not activated by cortisol. With increased reactive oxygen species generation, however, the cortisol-MR
complex is activated through unknown mechanisms.35 Aldosterone has also been shown to downregulate the enzyme glucose-6-phosphate dehydrogenase, resulting in a decrease in antioxidant reserve and an increase in reactive oxygen species,36 which could result in activation of the MR.

Activation of the MR also may directly affect the electrical properties of the ventricle, providing a substrate for arrhythmias and SCD. In a mouse model, conditional cardiac-specific overexpression of the MR led to fatal arrhythmias.37 Several factors may contribute. In these mice, the ventricular action potential was prolonged, a harbinger of arrhythmogenesis and an independent risk factor for SCD in HF patients.2 In a rat post-MI model, canrenone, the active metabolite of spironolactone, decreased the ventricular fibrillation threshold.38 At the tissue level, activation of the MR results in potassium loss, as well as apoptosis, fibrosis, hypertrophy, and central sympathetic nervous system activation.39–41 The increase in myocardial fibrosis and resultant ventricular remodeling could promote electrical inhomogeneity and a propensity to ventricular arrhythmias. At the cellular level, aldosterone has been demonstrated to alter the expression of several different ion channels that contribute to the regulation of the cardiac action potential. These resultant changes in the cardiac action potential may also be important contributors to arrhythmogenesis. For example, aldosterone has been shown to cause a dose-dependent increase in the expression of the gap junction protein connexin-43 in cultured rat ventricular myocytes and a concomitant change in conduction velocity.42 Aldosterone also affects the levels of several different ionic currents in a manner that consistently results in prolongation of the ventricular action potential. Activation of the MR has been shown to cause an increase in the inward calcium channel current, ICaL.43 These changes occur within 1 week of MI, before any morphological changes in the ventricle, and can be prevented by MRB. Aldosterone also decreases ICaL, the transient outward K+ current.43 Interestingly, a decrease in ICaL has consistently been found in myocytes from HF patients or in animal models of HF.2 Either of these changes, an increase in the calcium current or a decrease in ICaL—or both together—would prolong the cardiac action potential. Consistent with these findings, MR overexpression in cardiac myocytes causes ion channel remodeling, resulting in prolonged ventricular repolarization that is associated with an upregulation of ICaL and a downregulation of ICaL, resulting in severe ventricular arrhythmias.37 Administration of aldosterone also increases the expression of cardiac sodium channels.44 Whereas the mechanism by which this mode of regulation could contribute to arrhythmogenesis is not clear, it offers a hint of what might underlie the arrhythmogenesis of BrS, an inheritable disorder characterized in many individuals by a haploinsufficiency of SCN5A, the gene for the major cardiac sodium channel.45 Interestingly, these patients usually do not experience SCD until adulthood, suggesting the contribution of additional factors to arrhythmogenesis. Thus, the report that SCD in these individuals may be associated with the development of myocardial fibrosis46 and an animal model which showed that myocardial fibrosis can be associated with a loss of function of SCN5A raises the intriguing possibility that sodium channel expression and aldosterone levels may be linked in a feedback loop so that the decrease in sodium channels in BrS patients leads to a compensatory increase in aldosterone production and consequent fibrosis.48 Thus, although the effect is not as yet proven, it can be postulated that early administration of an MRB to an individual with BrS will protect against the subsequent development of myocardial fibrosis and thus SCD.

Activation of the MR has also been shown to block norepinephrine uptake into the myocardium, which is associated with an increase in circulating norepinephrine levels and ventricular arrhythmias.49 MRB, in contrast, improves the uptake of norepinephrine into the myocardium and decreases ventricular arrhythmias. MRB also improves parasympathetic activity, as indicated by improved heart rate variability, QT dispersion, and baroreceptor function.49,50,51 These changes are also associated with an increase in NO availability, which can affect the release of norepinephrine from sympathetic nerve terminals and parasympathetic activity as well as endothelial and platelet function.52,53 MRB has also been shown to decrease plasminogen activator inhibitor-1 levels, improve fibrinolysis, and prevent thrombosis.54,55 Depending on circumstances, one or another of these mechanisms may be of particular importance in preventing SCD. The benefits of MRB in terms of preventing SCD early post-MI are more likely due to their effects on electrical remodeling of the myocardium, whereas the effects on ventricular remodeling, collagen formation, and hypertrophy may be of equal or greater importance in preventing SCD in patients with HF and SLVD over the long term.

The Risk of MRB Causing Hyperkalemia

Whereas MRB has been proven to reduce SCD in patients with severe HF and SLVD, as well as in patients post-MI with SLVD and HF, many clinicians have been reluctant to use an MRB because of the fear of inducing serious hyperkalemia. In both the RALES4 and EPHESUS7 studies, patients randomized to an MRB had a reduction in SCD and total mortality without any deaths attributable to hyperkalemia. However, since publication of RALES,5 a number of reports have appeared showing that the use of spironolactone in clinical practice resulted in a marked increase in the incidence of hyperkalemia associated in some circumstances with renal failure and/or death.56–59 A review of these reports reveals that many of these episodes of hyperkalemia were due to the use of higher doses of spironolactone than the 12.5 to 50 mg/d used in RALES and that many deaths occurred in patients with severe renal dysfunction (estimated GFR <30 mL/min) and/or a serum potassium >5.0 mEq/L. A solution to this problem may be to encourage physicians to measure serum potassium either before starting a patient on spironolactone and/or during follow up. In both RALES and EPHESUS,7
serum potassium was measured before considering the use of an MRB, and if serum potassium was >5.0 mEq/L the MRB was withheld. Serum potassium was measured during the first week after starting an MRB, at 1 month, and every 3 to 6 months thereafter. In patients with chronic kidney disease, monitoring of serum potassium should be more frequent. Similarly, if a change in serum electrolyte status is suspected, such as after an episode of vomiting or diarrhea or after adding a drug that might affect potassium excretion, serum potassium should be remeasured. If serum potassium is >5.5 mEq/L, the dose of the MRB should be halved, and if >6.0 mEq/L in a nonhemoalyzed sample, the MRB should be withheld until the serum potassium returns to <5.0 mEq/L.

It should be pointed out that hyperkalemia (defined as a serum potassium >5.5 mEq/L) and especially “serious” hyperkalemia (serum potassium >6.0 mEq/L), although a matter of concern, may not necessarily be associated with any serious consequences. Recent data emphasize that serum potassium concentration is not a good indicator of myocardial tissue potassium levels. In patients with HF treated with drugs affecting the transport of potassium into the myocardial tissues such as digoxin, ACEI, ARBs, BBs, or non-steroidal antiinflammatory agents there may be a defect in transporting potassium into tissues. In some cases, a serum potassium concentration of >7.0 mEq/L may not be accompanied by any ECG or clinical manifestations of hyperkalemia if the tissue potassium concentration, as reflected by red blood cell potassium concentration and ionized calcium concentration, are normal. Because the determination of red blood cell potassium concentration is not yet routinely available, it would be prudent to minimize the potential risks of hyperkalemia associated with MRB by eliminating, if possible, any drugs such as potassium supplements or nonsteroidal antiinflammatory agents that could contribute to hyperkalemia, to prescribe a low-potassium diet, to monitor serum potassium and the ECG serially, and to discontinue the MRB if any ECG and/or clinical manifestations of hyperkalemia appear.

The situation with regard to the use of an MRB to prevent SCD and the risk of hyperkalemia may be analogous to the use of warfarin in a patient to prevent thromboembolism and the risk of serious bleeding. When the international normalized ratio is closely monitored during the initiation of warfarin and the dose adjusted periodically according to this ratio, the risk of serious bleeding can be minimized while preventing thromboembolism. One would consider it malpractice if a patient had a serious bleeding episode while on warfarin and the international normalized ratio had not been measured. Similarly, physicians who elect to prescribe an MRB should be obligated to monitor serum potassium and the ECG.

In conclusion, MRB has been shown to play an important role in the primary prevention of SCD in patients with severe chronic HF and SLVD, as well as in patients with SLVD and HF post-MI. It is likely, but not as yet proven, that MRB will also prevent SCD in patients with mild HF and SLVD or HFPSF, as well as in patients without SLVD or HF, including those with hypertension and myocardial fibrosis and/or hypertrophy and ischemic heart disease, as well as in other conditions associated with myocardial fibrosis and/or hypertrophy, such as aortic stenosis, diabetes mellitus, and BrS. Although hyperkalemia is a potential risk, with careful patient selection on the basis of renal function, serial monitoring of serum potassium and the ECG, and appropriate adjustment of the dose of the MRB in response to these factors, it is likely that the benefits of MRB in reducing SCD should far outweigh its potential risks. This hypothesis will, however, require further prospective evaluation in large scale randomized trials.

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Disclosures
Dr B. Pitt has received honoraria from Pfizer (>$10,000) and serves on the consultant/advisory boards for Pfizer and Novartis.

References


Response to Pitt and Pitt

Robert A. Kloner, MD, PhD, and David S. Cannom, MD

The 2 MADIT trials that we described in our article unequivocally show that patients with remote myocardial infarctions (>3 to 4 weeks) and reduced left ventricular ejection fraction demonstrated a reduction in total mortality and sudden death with automatic implantable cardioverter-defibrillators. In contrast, in the DINAMIT study patients with recent acute myocardial infarctions (6 to 40 days) and reduced left ventricular function did not demonstrate a reduction in total mortality but did demonstrate a decrease in sudden cardiac death with implantable cardioverter-defibrillator therapy. In the EPHESUS study, eplerenone given early after myocardial infarction (3 to 14 days after myocardial infarction, with an average of ≈7 days) reduced total mortality (but only by ≈15%) and decreased sudden cardiac death in patients with compromised ventricles. We agree with Pitt and Pitt that in the early post–myocardial infarction phase, patients with reduced cardiac function benefited from an aldosterone antagonist. However, existing data do not yet support the administration of this agent specifically to postinfarction patients with left ventricular dysfunction after 2 weeks of infarction with or without concomitant implantable devices. Thus, beyond this time, while it is known that implantable cardioverter-defibrillators reduce total mortality and can be considered first line therapy for preventing sudden death, information on administration of aldosterone antagonists starting during the later phase of myocardial infarction is missing, and therefore these agents cannot be considered primary therapy for preventing sudden cardiac death. It is clear that more research into the interaction (or lack of interaction) between implantable cardioverter-defibrillator and/or cardiac resynchronization devices and pharmacological agents such as aldosterone antagonists is needed, both in the early and late phases after acute infarction. Furthermore, the same type of research on device–drug interaction is needed for patients with congestive heart failure due to any cause, including nonischemic dilated cardiomyopathy.
Uncertainty on the Use of Aldosterone Antagonists for Primary Therapy for Sudden Cardiac Death in the Setting of Implanted Devices

Robert A. Kloner, MD, PhD; David S. Cannon, MD

In the early development of therapy for acute myocardial infarction, it was thought that once the necrotic process had been completed (usually within 24 hours of coronary artery occlusion), additional therapies could not affect outcome. However, after completion of the necrotic process, the myocardial infarction may thin and stretch (involving lengthwise slippage of myocytes), a phenomenon referred to as myocardial infarct expansion. This process causes local left ventricular cavity dilatation followed by gradual global left ventricular dilatation and lengthwise (eccentric) hypertrophy of the noninfarcted tissue. Apoptosis (programmed cell death) and some attempt of the myocardium to regenerate, especially at the infarct border zone, may also contribute to this remodeling process of the ventricle. If the left ventricle remodels in such a way that it becomes very dilated, then the prognosis is poor, and heart failure is more likely to occur. These later processes of myocardial infarct expansion and left ventricular remodeling became the target of therapies such as angiotensin-converting enzyme (ACE) inhibition that could be initiated after 24 hours of coronary occlusion. ACE inhibition, angiotensin receptor blockade, and long-term β-blockade have become standard pharmacological approaches for postinfarction left ventricular dysfunction and heart failure.

Response by Pitt and Pitt p 2989

Ventricular arrhythmias can occur in both the acute and chronic phases of acute myocardial infarction and can lead to sudden cardiac death (SCD). Reentrant arrhythmias may arise at the border zone of infarcts, causing monomorphic ventricular tachycardia that may occur years after the index infarction. Recurrent myocardial ischemia resulting in an unstable substrate may contribute to polymorphic ventricular tachycardia or ventricular fibrillation. Agents such as β-blockers that are anti-ischemic may reduce sudden death by quieting this unstable substrate. In the Multicenter Automatic Defibrillator Implantation Trial (MADIT) II, implantable defibrillators were shown to reduce mortality in post–myocardial infarction patients with left ventricular dysfunction entirely due to a reduction in SCD. It is likely that these devices did not primarily improve the arrhythmic substrate. However, long-term cardiac resynchronization will encour-
age reverse remodeling that might reduce the substrate for arrhythmia.

ACE Inhibitors and Angiotensin Receptor Blockers After Myocardial Infarction

In the now classic study by Pfeffer et al., captopril administered long-term, starting within about the first few weeks of myocardial infarction, decreased total mortality, congestive heart failure, and recurrent myocardial infarction. Echocardiographic analysis demonstrated that captopril reduced diastolic dilatation at 2 years, suggesting a decrease in deleterious left ventricular remodeling. Other studies confirmed that long-term administration of an ACE inhibitor improved cardiac outcome after myocardial infarction. Benefits of long-term therapy with the angiotensin receptor blocker valsartan were also reported to benefit post–myocardial infarction patients with left ventricular dysfunction. Valsartan was shown to result in similar but not superior effects on survival compared with captopril in the Valsartan in Acute Myocardial Infarction (VALIANT) study. Furthermore, treatment with captopril plus valsartan resulted in no advantage over treatment with either agent alone. In a head-to-head comparison of the ACE inhibitor captopril with the angiotensin receptor blocking agent losartan in patients with acute myocardial infarction and evidence of heart failure or left ventricular dysfunction, captopril was associated with a nonsignificantly lower all-cause mortality and a significantly lower cardiovascular mortality compared with losartan, but losartan was better tolerated than captopril. Some of these studies demonstrated less ventricular arrhythmias when an ACE inhibitor was used.

β-Blockers After Myocardial Infarction

Another class of drugs that has been shown to reduce mortality after acute myocardial infarction is the β-blockers. The landmark Beta-Blocker Heart Attack Trial (BHAT) tested the effect of long-term propranolol therapy in patients after a myocardial infarction. More than 3800 patients were randomized to either propranolol (180 to 240 mg/d maintenance dose) or placebo starting 5 to 21 days after myocardial infarction and were followed up for 15 months. Total mortality was 7.2% in the propranolol group and 9.8% in the placebo group (26% reduction). Sudden cardiac death occurred in 3.3% of the propranolol patients versus 4.6% of the placebo patients (28% reduction). A subset of 826 of these patients also had paired ambulatory ECG monitoring at baseline and after 6 weeks of therapy. An increase in ventricular arrhythmias over the 6-week period was blunted by propranolol. Some but not all studies in which other β-blockers were administered after myocardial infarction showed a reduction in SCD. Hjalmarson postulated that the more lipophilic β-blockers (timolol, metoprolol, propranolol) were more likely to demonstrate this benefit because they could penetrate the brain and maintain high vagal tone during stress, thus reducing ventricular fibrillation. Of course, it is also possible that the β-blockers are primarily reducing postinfarction ischemia, which would explain why they are antiarrhythmic.

Although these findings represented a major advance, as pointed out by Fonarow et al., many of the earlier studies with β-blockers did not include patients with heart failure. The Carvedilol Post-Infarct Survival Control in LV Dysfunction (CAPRICORN) study determined the effects of carvedilol added to standard therapy (including ACE inhibitors) for patients with an acute myocardial infarct who had an ejection fraction <0.40. More than 1900 patients with a mean ejection fraction of 0.33 were randomized to placebo or carvedilol and followed up for 15 months. Total mortality was reduced from 15.3% in the placebo group to 11.9% with carvedilol. SCD was reduced as well (Table 1). In the CAPRICORN study, sudden death occurred in 5% of carvedilol patients versus 7% of placebo patients (P=0.098).

Aldosterone Antagonists After Myocardial Infarction

What is known about the use of aldosterone blockers in patients with myocardial infarction and postmyocardial dysfunction? The crucial study is the Eplerenone Post–Acute Myocardial Infarction Heart Failure Efficacy and Survival (EPHESUS) study by Pitt et al. The EPHESUS trial was a double-blind, placebo-controlled study of the selective aldosterone blocker eplerenone, examining morbidity and mortality in post–myocardial infarction patients with heart failure and left ventricular dysfunction (left ventricular ejection fraction of ≤40%). Therapy was started 3 to 14 days after acute myocardial infarction, and patients received placebo (n=3319) or eplerenone (25 mg/d; n=3313) for 4 weeks.

### Table 1. Recent Key Randomized Controlled Trials of Nonantiarrhythmic Drugs and Effect on SCD

<table>
<thead>
<tr>
<th>Drug</th>
<th>Study Characteristics</th>
<th>No.</th>
<th>HR for SCD (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Blocker</td>
<td>CAPRICORN: acute MI (3–21 days), LVEF ≤:0.40</td>
<td>1959</td>
<td>0.24 (0.11–0.49)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>Meta-analysis of 15 randomized controlled trials (patients with CHF)</td>
<td>15 104</td>
<td>0.80 (0.70–0.92)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>HOPE population (patients without CHF or LV dysfunction)</td>
<td>9297</td>
<td>0.79 (0.64–0.98)</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>RALEs: CHF, LVEF ≤:0.35</td>
<td>1663</td>
<td>0.70 (0.54–0.95)</td>
</tr>
<tr>
<td>Eplerenone</td>
<td>EPHESUS: acute MI (3–14 days), LVEF ≤:0.40</td>
<td>6632</td>
<td>0.79 (0.64–0.97)</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction; HOPE, Heart Outcomes Prevention Evaluation; LVEF, left ventricular ejection fraction; and CHF, congestive heart failure.

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after which the dose was increased to 50 mg/d. If the potassium concentration was $>5.5$ mmol/L, the dose of study drug was reduced or treatment stopped temporarily. Patients were already on standard optimal medical therapy including ACE inhibitors, angiotensin receptor blockers, β-blockers, diuretics, and reperfusion therapy. Follow-up was for 16 months. Fewer patients died in the eplerenone group (478; 14.4%) than in the placebo group (554 patients; 16.7%; relative risk [RR]$=0.85$; 95% confidence interval [CI], 0.75 to 0.96; $P=0.008$). Death due to cardiovascular causes occurred in 407 patients in the eplerenone group versus 483 patients in the placebo group (RR$=0.83$; 95% CI, 0.72 to 0.94; $P=0.005$). Sudden death from cardiac causes occurred in 162 of the eplerenone group versus 201 in the placebo group (RR$=0.79$; 95% CI, 0.64 to 0.97; $P=0.03$). Death from cardiovascular causes or hospitalization for cardiovascular events, death from any cause or any hospitalization, and hospitalization for heart failure were also reduced by eplerenone. At 1 year, potassium levels increased by 0.2 mmol/L in placebo-treated patients versus 0.3 mmol/L in the eplerenone group (P$<0.001$). Serious hypokalemia (potassium $<3.5$ mmol/L) occurred in 8.4% of eplerenone patients versus 13.1% in the placebo group (P$<0.001$). Hyperkalemia, with a serum potassium level $\geq6.0$ mmol/L, occurred in 5.5% of eplerenone-treated versus 3.9% of placebo-treated patients (P$=0.002$). Twelve patients in the eplerenone group versus 3 in the placebo group were hospitalized for the condition; 1 patient in the placebo group died of the condition.

A number of proposed mechanisms for the reduction in mortality in the eplerenone group were suggested, including effects of eplerenone on plasma volume and electrolyte excretion, reductions in coronary vascular inflammation, improvements in endothelial function, attenuation of platelet aggregation, improvements in ventricular remodeling with a decrease in activation of matrix metalloproteinases, and a decrease in interstitial fibrosis. Besides these direct effects on the vasculature and myocardium, the authors pointed out that aldosterone blockade decreased sympathetic drive in experimental animal studies, improved norepinephrine uptake in heart failure victims, and improved heart rate variability.

One of the simplest explanations for the benefit of eplerenone in reducing sudden death is its prevention of hypokalemia, a known trigger of ventricular arrhythmias, especially in patients also taking digitalis preparations. In a letter to the editor, Coca and Buller$^{21}$ raised the issue that the 21% decrease in the rate of sudden death from cardiac causes associated with eplerenone in the EPHESUS trial may have been attributable to the reduction of hypokalemia. However, Pitt responded that “a preliminary analysis of data from EPHEUS reveals a significant reduction in the risk of sudden death from cardiac causes, which is independent of the effects of eplerenone in preventing hypokalemia.” It is still conceivable that some of the benefit of eplerenone in reducing sudden death was related to preventing hypokalemia. Subsequent analyses revealed that eplerenone reduced the risk of sudden death by 33%$^{22}$ in patients with baseline left ventricular ejection fraction of $\leq30\%$ and that eplerenone reduced the early incidence of sudden death by 37% within 30 days of randomization in this trial.$^{23}$

Although the results show that eplerenone reduced sudden death in the post–myocardial infarction patients with left ventricular dysfunction, many questions in this field remain unanswered. Was this benefit primarily due to a reduction in hypokalemia? Would eplerenone provide this benefit to patients with heart failure but not in the post–myocardial infarction setting? Would eplerenone benefit patients with heart failure who had automatic implantable defibrillators and/or patients with biventricular pacing for cardiac resynchronization therapy?

### Aldosterone Antagonists in Patients With Chronic Heart Failure

The RALES (Randomized Aldactone Evaluation Study)$^{24}$ was a double-blind, randomized study of 1663 patients with severe heart failure and a left ventricular ejection fraction $\leq35\%$ who were already on an ACE inhibitor, a loop diuretic, and in many cases digoxin. Patients were randomized to 25 mg of the aldosterone antagonist spironolactone (n=822) versus placebo (n=841). The study was stopped at 24 months with 386 deaths (46%) in the patients receiving placebo versus 284 deaths (35%) in the spironolactone group (RR of death$=0.70$; 95% CI, 0.60 to 0.82; P$<0.001$). Spironolactone was associated with a lower risk of death from progressive heart failure as well as a lower rate of sudden death. Sudden death due to cardiac cause occurred in 82 of 822 spironolactone-treated patients versus 110 of 841 placebo-treated patients (RR$=0.71$; 95% CI, 0.54 to 0.95; P$=0.02$). Spironolactone also reduced all cardiac causes for hospitalization as well as hospitalization for worsening heart failure. The median potassium concentration increased by 0.30 mmol/L in the spironolactone group but did not increase in the placebo group. Serious hyperkalemia was observed in 10 placebo patients (1%) and 14 spironolactone patients (2%; P$=NS$).

Again, although the exact mechanism by which the aldosterone antagonist reduced SCD in RALES is unknown, prevention of hypokalemia cannot be ruled out entirely, despite the rather small increase in potassium levels in the treated group. In addition, it is unknown whether spironolactone could also reduce sudden death in post–myocardial infarction patients with left ventricular dysfunction with or without clinical congestive heart failure or in patients already receiving automatic implantable cardioverter-defibrillators (ICDs) and/or biventricular pacing for resynchronization therapy.

A small study by Ramires et al$^{25}$ randomized 35 patients with class III congestive heart failure due to dilated or ischemic cardiomyopathy and mean ejection fraction of 33% to spironolactone in addition to standard medical therapy for
ACE inhibitors and aldosterone antagonists. proved electrolyte balance, including less hypokalemia with epithelial function, a reduction in sympathetic tone, and improvement in ventricular remodeling and endothelial function. Again, a host of mechanisms has been postulated, including improvements in ventricular remodeling. The studies with the aldosterone antagonists to date do not clarify which of these mechanisms is most likely.

Clinical trials are currently lacking of patients with chronic heart failure (not related to the postinfarct setting) who received eplerenone. Table 1 summarizes some recent key randomized trials of nonantiarrhythmic drugs and their effect on SCD. Several agents used for the treatment of heart failure (as well as hypertension) have demonstrated this benefit: β-blockers, ACE inhibitors, and aldosterone antagonists. Again, a host of mechanisms have been postulated, including improvements in ventricular remodeling and endothelial function, a reduction in sympathetic tone, and improved electrolyte balance, including less hypokalemia with ACE inhibitors and aldosterone antagonists.

For all the aforementioned studies, the assessment of whether the precise cause of death is arrhythmic or due to heart failure, recurrent infarction, or other causes is very difficult. The ICD randomized trials have used total mortality as the end point precisely because retrospective analysis of an individual death is so difficult. Therefore, studies with β-blockers, ACE inhibitors, angiotensin receptor blockers, and aldosterone antagonists that claim to demonstrate a reduction in sudden death need to be interpreted cautiously and with the realization that all deaths, in a sense, are sudden. It is feasible that the mechanisms of benefit of agents including eplerenone and spironolactone may be directly antiarrhythmic, indirectly antiarrhythmic (for example, preventing hypokalemia), or due to a change in the cardiac substrate (for example, an anti-ischemic effect or a reduction in ventricular remodeling). The studies with the aldosterone antagonists to date do not clarify which of these mechanisms is most likely.

**Aldosterone Antagonists in ICD and Cardiac Resynchronization Trials**

The primary prophylactic ICD trials were initiated in the early 1990s at the same time that new data were emerging on the importance of β-blockers and ACE inhibitors in the prevention of SCD in patients with low ejection fraction. As is noted in Table 2, use of β-blockers and ACE inhibitors increased over time but was quite low in both the MADIT I and Multicenter Unsustained Tachycardia Trial (MUSTT) when these therapies had not reached wide acceptance. The use of spironolactone antagonists was very sparing in the ICD trials except for the Comparison of Medical Therapy Pacing and Defibrillation in Heart Failure (COMPANION) trial, which was conducted by heart failure specialists. It is of interest that, as the medical therapy in these trials improved, the degree of superiority of the ICD over conventional therapy declined (from MADIT I to Sudden Cardiac Death in Heart Failure [SCD-HeFT]), suggesting that survival in both arms in these trials improves as a result of contemporary background medical therapy. In all the trials except 2 (Coronary Artery Bypass Graft [CABG] Patch and the Defibrillator in Acute Myocardial Infarction Trial [DINAMIT]), the ICD demonstrated a survival advantage over best medical therapy. In the Defibrillators in Non-Ischemic Cardiomyopathy Treatment Evaluation (DEFINITE) trial, implantation of ICDs in patients with nonischemic dilated cardiomyopathy and already on ACE inhibitors and β-blockers resulted

<table>
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<th>Digoxin</th>
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Values are percentage of patients. NA indicates not applicable.

*Spironolactone/potassium-sparing diuretics.

16 weeks. Spironolactone was initiated at 50 mg/d until week 12 and then was decreased to 25 mg/d until the end of 16 weeks. After 16 weeks, ambulatory ECG monitoring revealed a lower frequency of ventricular premature beats and episodes of nonsustained ventricular tachycardia in the spironolactone group compared with the control group. Spironolactone was also associated with an improvement in ventricular arrhythmias during treadmill exercise. The authors observed that before administration of spironolactone and after adjustment for baseline drug therapy, there was a reduction in serum sodium, potassium, and magnesium that was corrected after 16 weeks of spironolactone therapy. The authors postulated that “a possible explanation for the reduced frequency of ventricular arrhythmia could be related to electrolyte regulation promoted by spironolactone in combination with ACE inhibitors.” They described the concern that hypokalemia could have contributed to increased arrhythmias in the setting of digoxin, which was then corrected by the aldosterone antagonist.

The primary prophylactic ICD trials were initiated in the early 1990s at the same time that new data were emerging on the importance of β-blockers and ACE inhibitors in the prevention of SCD in patients with low ejection fraction. As is noted in Table 2, use of β-blockers and ACE inhibitors increased over time but was quite low in both the MADIT I and Multicenter Unsustained Tachycardia Trial (MUSTT) when these therapies had not reached wide acceptance. The use of spironolactone antagonists was very sparing in the ICD trials except for the Comparison of Medical Therapy Pacing and Defibrillation in Heart Failure (COMPANION) trial, which was conducted by heart failure specialists. It is of interest that, as the medical therapy in these trials improved, the degree of superiority of the ICD over conventional therapy declined (from MADIT I to Sudden Cardiac Death in Heart Failure [SCD-HeFT]), suggesting that survival in both arms in these trials improves as a result of contemporary background medical therapy. In all the trials except 2 (Coronary Artery Bypass Graft [CABG] Patch and the Defibrillator in Acute Myocardial Infarction Trial [DINAMIT]), the ICD demonstrated a survival advantage over best medical therapy. In the Defibrillators in Non-Ischemic Cardiomyopathy Treatment Evaluation (DEFINITE) trial, implantation of ICDs in patients with nonischemic dilated cardiomyopathy and already on ACE inhibitors and β-blockers resulted
in a nonsignificant trend toward a reduction in death from any cause and a significant decrease in sudden death.

To conclusively determine whether aldosterone antagonists confer additional benefit on reducing SCDs in patients with ICDs, one would need to design a study in which patients with ICDs (and preferably a group without ICDs as well) were randomized to aldosterone antagonists versus placebo in addition to the usual heart failure medicines. Unfortunately, and to the best of our knowledge, such a study has not been performed. We have been able to obtain some observational retrospective data from MADIT II and the COMPANION trial that address this issue to some extent. We briefly present our findings, realizing that limitations exist that must be kept in mind when these data are viewed. The limitations of this analysis include the following: a lack of randomization for the use of spironolactone; a relatively small number of patients who were assigned to spironolactone, which resulted in the studies not being powered to definitely answer the question about a benefit of aldosterone antagonists in patients already treated with ICD, cardiac resynchronization therapy (CRT), or both; the possible presence of a type II or β error; and the possibility of confounding biases. For example, patients assigned to spironolactone may have been sicker. The analyses below are retrospective and must be considered exploratory and hypothesis generating, not definitive. However, when we were assigned the topic of presenting the “con” side of the aldosterone antagonist argument by Circulation, we tried to find all available data on this concept. Therefore, we briefly present our findings below, and we are not aware of more definitive data at the time of this writing.

Although there has been little use of spironolactone in the ICD trials, the MADIT II Investigators have kindly provided new data on the issue of the possible effects of spironolactone in this trial (S. McNit, MS, and J. Hall, PhD, written communication, 2006). MADIT II was a study of 1232 patients with a prior myocardial infarction (>8 years) and a reduced left ventricular ejection fraction (<=0.30). Patients were randomized to receive an implantable defibrillator or conventional medical therapy.43 No attempt was made to risk stratify by invasive electrophysiological testing. The primary end point was death from any cause. During an average of 20 months of follow-up, the mortality rates were 14.2% in the ICD group versus 19.8% in the conventional medical therapy group, representing a 31% reduction in the risk of death in the ICD patients (hazard ratio [HR]=0.69; 95% CI, 0.51 to 0.93; P=0.016). The conclusion of these investigators was that prophylactic implantation of a defibrillator improved survival and should be considered as therapy for patients with prior myocardial infarction and poor left ventricular dysfunction.

At study commencement (in which patients were randomized in a 3:2 ratio to ICD versus conventional therapy), 101 patients (13.6%) randomized to ICD treatment were receiving spironolactone, and 57 (11.6%) in the conventional arm were receiving this drug. Spironolactone use was analyzed as a time-varying risk factor in proportional hazards regression analysis of the various end points in MADIT II. Because of the clinical suspicion that spironolactone may have been used as a result of a hospitalization for heart failure, the first occurrence of heart failure was also used as a time-dependent factor.40 The HR for all-cause mortality for patients while on spironolactone compared with patients and periods not on spironolactone was 1.13 (P=0.53). Thus, no overall effect of spironolactone use on all-cause mortality was found in MADIT II. The HR for spironolactone for all-cause mortality in the conventional medical arm was 1.43 versus 0.90 in the ICD arm (P=0.23 for difference).

The HR for spironolactone for sudden death in the conventional arm was 1.13 (P=0.76). The HR for spironolactone for first appropriate shock in the ICD arm was 1.51 (P=0.07). The HR for spironolactone for either first appropriate shock or death in the ICD arm was 1.20 (P=0.34). Most of the HRs for spironolactone exceeded unity, suggesting a trend toward an increased risk of the end point occurring when the patients were on the drug relative to being off the drug. However, drug usage could be a proxy for heart failure risk or severity of heart failure. In summary, on the basis of a retrospective analysis, the MADIT II trial produces no evidence that spironolactone provides a benefit.

Supporting evidence is provided by the COMPANION trial along similar lines. The COMPANION trial randomized >1500 New York Heart Association class III/IV heart failure patients with a prolonged QRS and ejection fraction ≤35% to optimal medical therapy, optimal medical therapy plus CRT, or optimal medical therapy plus CRT plus an ICD. Both CRT and CRT plus an ICD reduced combined all-cause mortality and hospitalization in heart failure patients. CRT plus an ICD reduced all-cause mortality; CRT alone had a trend toward reduced mortality.

In the COMPANION trial, 55% of patients were treated with spironolactone, and this was not associated with risk of death in any treatment group and did not protect against appropriate shocks in the patients with CRT plus an ICD. However, β-blockers and ACE inhibitors did afford such protection (L. Saxon, MD, written communication, 2006, and Saxon et al44).

**Summary**

In summary, recent studies have shown the usefulness of eplerenone for post–myocardial infarction patients with heart failure and spironolactone for patients with chronic congestive heart failure. In these 2 studies (which lacked use of ICDs), the aldosterone antagonists reduced SCD. There are a number of potential explanations for the mechanism of this benefit, including protection against hypokalemia. In recent retrospective analyses of MADIT II and COMPANION trials of patients with left ventricular dysfunction/heart failure in which ICDs were used, no evidence was provided that spironolactone afforded a survival benefit or reduced the need for appropriate ICD shocks. Aldosterone antagonists may still benefit heart failure patients who have ICDs independently of
reduction of arrhythmias, for example, by reducing heart failure symptoms and/or hospitalizations. Thus, if patients with heart failure on spironolactone receive an ICD, we do not suggest that the spironolactone be stopped. Should an aldosterone antagonist be added for patients with severe heart failure who already have a defibrillator? In this case, spironolactone may reduce heart failure symptoms, but whether spironolactone will further reduce total mortality or sudden death is uncertain. Prospective, adequately powered, randomized, blinded trials are needed to examine the interaction or possibly the lack of interaction between ICD, CRT, and both with the aldosterone antagonists. Specifically, a study is needed in which patients who are already on standard medical therapy plus ICD, CRT, or both are randomized to an aldosterone antagonist versus placebo to determine whether this class of drugs further reduces mortality, SCD, and hospitalizations for heart failure. Unfortunately, we think it is unlikely that any agency or industry would fund such a study, and therefore it is possible that a definite answer to whether aldosterone antagonists confer benefits in addition to those of implantable devices alone may never be known.

Disclosures

Dr Kloner is a consultant and speaker for Pfizer. Dr Cannom is a consultant and speaker for Medtronic and Boston Scientific.

References

15. Ramires FJ, Mansur A, Coelho O, Maranhao M, Gruppi CJ, Mady C, Ramires JA. Effect of spironolactone on ventricular arrhythmias in con-
gestive heart failure secondary to idiopathic dilated or to ischemic cardiomyopathy. Am J Cardiol. 2000;85:1207–1211.

Response to Klener and Cannom

Bertram Pitt, MD, and Geoffrey S. Pitt, MD, PhD

Drs Klener and Cannom suggest that reduction of sudden cardiac death (SCD) with mineralocorticoid receptor blockade is attributable to prevention of hypokalemia. Review of EPHEUSUS, however, does not show any relationship between reduction in total mortality or SCD and serum K⁺. Rather, as we noted, the reduction in SCD could be attributed to a mineralocorticoid receptor blockade (MRB)–induced increase in tissue K⁺, which may not be reflected by serum K⁺. We agree that the mechanisms by which MRB reduced SCD in RALES and EPHEUSUS have not been elucidated. As for β-blockers and angiotensin-converting enzyme inhibitors, these protective mechanisms are speculative. While Drs Klener and Cannom note that retrospective analyses of COMPARION and MADIT II did not show a benefit of MRB in reducing SCD or inappropriate shocks, we must point out that these trials were not powered to examine these effects. Furthermore, we propose that the major benefit of MRB is the primary prevention of SCD. But for MRB, which reduced total mortality within 30 days after myocardial infarction in EPHEUSUS, it is likely that many patients who would qualify for an implantable cardioverter-defibrillator would not have survived to receive it because implantable cardioverter-defibrillators do not reduce mortality when implanted <30 days after a myocardial infarction, nor for ≈1 year when they are implanted >30 days after a myocardial infarction. We agree that definitive demonstration of a role for MRB in reducing SCD in patients with implantable cardioverter-defibrillators can only be provided by a randomized clinical trial.
Drug-Eluting Stents “Deliver Heartburn”
How Do We Spell Relief Going Forward?

Mitchell W. Krucoff, MD; Ashley Boam, MSBE; Daniel G. Schultz, MD

"B"reakthrough" technologies may produce rare or unexpected performance issues in postmarket use, especially when rapid market penetration into large patient populations outpaces the development of clinical knowledge. Although high-profile meetings or news media coverage may help draw attention to such issues, ultimately it is careful scientific consideration of safety and effectiveness issues, with transparent public access, that will help physicians and patients make decisions about how and when these technologies are best used in the practice of medicine. To this end, a 2-day special advisory panel on drug-eluting stent (DES) thrombosis was convened in December 2006 by the US Food and Drug Administration (FDA), as has recently been summarized by Laskey et al and by Farb and Boam.

Since the first safety alerts in 2003 to 2004 raised concerns that DES might be associated with an increase in rare but often catastrophic thrombotic events, public, professional, and regulatory attention has been focused on delineating the basis for this concern. In the spring of 2006, new reports of increased stent thrombosis rates with DES at the European Society of Cardiology in Barcelona led to a cascade of published reports and opinions that were characterized in The Wall Street Journal headline as "Coated Stents Deliver Heartburn." The article did not specify whether the reference was to heartburn in already-stented patients, cardiologists, DES manufacturers, the FDA, or Wall Street itself. In fact, the issues surrounding DES touch on all of the above.

The FDA panel’s proceedings served a key purpose by publicly reviewing available information and concluding that DES “on-label” use is both safe and effective relative to bare metal stents (BMS), while pointing clinicians to the American College of Cardiology/American Heart Association/Society for Cardiovascular Angiography and Interventions guidelines for dual-antiplatelet (DAP) therapy out to 1 year in patients with low risk of bleeding. The panel also represented a uniquely international effort to focus on this topic. In addition, however, the panel crystallized many key questions that could not be clearly answered about higher event rates with off-label use and optimal duration of antiplatelet therapy.

In the aftermath of the panel, FDA, clinicians, and the industry have continued a dialogue on how best to interpret and apply the panel recommendations. Industry, research organizations, and academics are to be applauded for collaborative efforts to continue to collect and provide unbiased access to new and extended patient-level data, and several leading peer-reviewed journals have expedited publication to facilitate dissemination of these findings. For example, 7 articles on DES outcomes were included in the March 8, 2007, issue of The New England Journal of Medicine, including 5 original peer-reviewed scientific reports of analyses that included larger patient numbers with longer follow-up periods than previously available. Like the panel proceedings themselves, however, the nobility and utility of these efforts and the collaborations they represent also clarify the limitations imposed by ongoing retrospective analyses. These reports originate from studies designed on the basis of the best insights of the past—in many cases, insights that industry, academics, and regulatory experts shared at that time. It is clear that these studies simply do not contain all the information that is best suited to the differently informed needs of the present and future. For instance, very few data are presented in any of these reports on actual compliance with, or interruption of, DAP therapy or inclusion of many more complex “real-world” patients in randomized cohorts. Thus, although this issue of The New England Journal of Medicine represents a great deal of effort to revisit DES safety as an area of widespread interest, it also highlights some of the potential hazards of retrospective data mining, with 4 of these 5 reports including many of the same clinical trials, mixed together and reported differently.

Such a "going-forward" focus is the primary agenda for clinicians, academic researchers, regulatory authorities, and industry in 2007. Objectives for the future can be considered from 3 perspectives: (1) best care of patients who already have permanently implanted approved DES platforms, (2) best application of currently approved DES platforms in patients yet to be treated, and (3) best approaches to safety and effectiveness evaluation of new, investigational DES platforms.
No medical device is entirely safe, and the use of a medical device is routinely based on a clinical balance between its effectiveness and its safety-related risks. What constitutes acceptable equipoise with DES—how much less restenosis or target vessel revascularization might balance how much more stent thrombosis, or whether revascularization with stents is desirable compared with alternatives such as medical therapy or bypass surgery—is fundamentally a decision for practitioners, professional societies, and patients. Accurately determining the incidence of adverse clinical events, however, especially in the case of rare but catastrophic occurrences such as stent thrombosis, is a challenge and a concern for all constituencies, including regulatory authorities.

For current and immediate-future patient care using already-approved DES platforms, the FDA will begin with a careful reappraisal of DES labeling to ensure that the information provided to physicians and patients is as up to date, accurate, and informative as possible. Oversight of medical product labeling is one of the FDA’s strongest tools in ensuring that the information provided to users is data driven and comprehensive. Labeling also forms the basis for the agency’s evaluation and enforcement of promotion and advertising activities. Labeling can and should be updated to reflect new information that can be reviewed and verified.

Fundamental to the evaluation of new DES platforms is recognizing how difficult it is, from both a clinical and a regulatory perspective, to characterize rare adverse events associated with DES thrombosis. Such events often emerge for the first time in the postmarket environment, after the device has been used on a much larger and more diverse patient population than in the initial clinical studies. Additional complexities range from the consistency and quality of manufacture and the technique of clinical operators to the quality and utility of patient data that are collected. Stent thrombosis also may be affected by the biological characteristics of individual patients, the implantation techniques used, and the structure of the DES, including the polymer, the drug, and the metal stent platform. Late events also are complicated by the fact that, over time, it is more difficult to determine whether adverse outcomes result from stent thromboses per se or are related to the progression of underlying coronary disease or the comorbidities with which it is associated.

Addressing these complexities will be the challenge for all new DES evaluations that seek to determine how new DES platforms compare, in both safety and effectiveness, to existing therapeutic alternatives. DES platforms are complex and quickly iterating. They are classified as class III medical devices requiring (1) successful completion of a premarket approval application before marketing; (2) submission of annual reports, adverse event reports, and data from required postmarket studies after marketing; and (3) approved premarket approval supplements for significant changes to the device or its labeling. The FDA’s “total product lifecycle” approach to regulation of medical devices reflects the FDA’s expectation that, as new data and knowledge are acquired for a single device or type of device, this knowledge will be applied to future research and development and to updated regulatory considerations. Previous data requirements for bench, animal, and clinical studies for DES approval were developed using knowledge extrapolated from our experience with BMS supplemented with pharmacological data from systemic applications of the drug eluted. Future developments in this field will need to incorporate further what we are learning today about these combination products.

In addition, the FDA will reassess the kinds of data needed to ensure the safety and effectiveness of DES, both before they are marketed and after they are in widespread use. This does not mean that studies currently underway will be instantaneously rendered obsolete or that future studies will be held to a different regulatory threshold, but it does mean that relevant new questions must be addressed in a manner consistent with sound scientific principles and appropriate regulatory models. Careful consideration must be given to the need to better define the risks of stent thrombosis and the benefits of reduced restenosis in the populations in whom the devices are used over the time period needed to accurately assess these events. These issues can be addressed by judicious refinement of premarket and postmarket studies with the ultimate goal of designing a comprehensive clinical program that balances patient safety with product availability.

Comprehensive premarket approval evaluations generally require the use of randomized clinical trial (RCT) designs. RCTs of DES will require careful scientific and statistical consideration based on current knowledge. For instance, stent thrombosis comparisons between BMS and DES in early pivotal study designs censored patients after reintervention for restenosis. More recent reevaluations have noted that this analysis plan may bias perception of BMS safety (because of higher restenosis rates, with more censored patients) unfavorably relative to DES. Some experts have suggested that elimination of such censorship actually would produce a more theoretically rigorous “intent-to-treat” analysis plan.

Analyses of currently available data also should inform the selection, definition, and power calculation for primary safety and effectiveness end points in DES RCTs. The use of the Academic Research Consortium consensus end point definitions related to DES outcomes for the FDA panel presentations and most publications since represents an important lesson learned that can be leveraged going forward. The adoption of a single set of consensus definitions reflecting possible, probable, and definite stent thrombosis has been quite useful, even with the realization that limitations of these definitions include the variability of sensitivity/specificity, depending on how they are applied. Justifiable concerns about applying them to previously concluded studies through retrospective rejudications of events are not an issue for prospective planning of new studies going forward. Despite their limitations, the panel made clear the utility of defining clinical end points consistently across a wide range of clinical trials and patient reports. For rare or unexpected postmarket safety events, use of consistent definitions may be important both for detecting a safety signal and for mechanistically evaluating its source—or, as had recently been described, for appreciating the smoke compared with actually seeing what is burning.

Selection of appropriate primary efficacy end points is another key area for DES evaluations. The use of surrogate measures such as angiographic late loss may be less sufficient...
in light of current awareness of complex interactions of device features, procedural features, and patient characteristics. More recent analyses of all death and myocardial infarction (including subsets of cardiac/noncardiac death and Q-wave/non-Q-wave myocardial infarction), combined with landmark analyses to help understand whether certain time periods are more vulnerable for particular treatment cohorts, have provided helpful information in retrospective analyses and could be considered in future trial designs. Angiographic and intravascular ultrasound measures may be more useful as biomarkers of vessel response to DES implantation than as surrogate markers for DES efficacy.

Another lesson learned from previous DES studies is the influence of study design on reintervention rates, in particular, through use and timing of protocol-mandated angiography that encourages “occulostenotic” interventions. Although for existing studies the independence of such evaluations may not be possible, for future studies, the completion of clinical evaluations before protocol angiography or the separation of angiographic follow-up studies from clinical studies may be useful.

Retrospective comparisons with historical BMS data have been important for informing product labeling and clinical decisions; however, they may become less meaningful as practice patterns evolve. Pivotal DES RCTs have already shifted from superiority designs compared with BMS to active-control, “head-to-head” DES noninferiority studies. It is notable that in the recent studies in The New England Journal of Medicine, not 1 study, even the Swedish national registry, could provide substantive data comparing any 2 DES platforms. Enabling well-designed clinical trials to keep pace with expanding clinical practice will require discipline and commitment on the part of industry, government agencies, and DES users. Depending on the labeling issues sought in future DES RCTs, either approved BMS or DES platforms might serve as control groups, and superiority, noninferiority, or integrated designs may be appropriate. Confidence intervals used to define such analysis plans will require robust statistical justification based on updated current information.

Over time and as DES products evolve to further stages of the product lifecycle, evaluation of risks and benefits may ultimately be able to leverage larger databases or longitudinal analyses to lower the burden of RCTs or to answer specific DES safety and performance questions without RCTs per se. However, the informative use of registries or larger longitudinal data sets will need the development of formal analytic methods to translate that data into useful information for key clinical decision making and/or reasonable product evaluation.

Another important aspect of DES studies going forward is the recognition that the safety behavior of these devices is tied directly to the obligatory use of an adjunctive pharmacological regimen of DAP therapy with aspirin and thienopyridines such as ticlopidine or clopidogrel. Although this relationship early after stent implantation is well established, the optimal duration or utility of more extended DAP therapy is less clear and may well be different for different DES platforms. Not a single study analyzed in any of the reports in the March issue of The New England Journal of Medicine provided per-patient detailed DAP therapy information because these data generally were not collected. Going forward, patient education and careful documentation of compliance, need for interruption, and patient care strategies during interruption are now recognizable as important areas of interest.

On a more general level, as medical products become more complex and innovative, we can expect an ever-greater number to combine drugs and devices. This means that the FDA must enhance its own intramural collaboration to ensure that these combination products are evaluated smoothly and without duplication of effort. With the passage of the Food and Drug Modernization Act of 1997, the Office of Combination Products was created specifically to provide the administrative oversight for this very circumstance. The interaction of DES safety and DAP therapy steps beyond current intramural structure at the FDA because the safety of the device is driven in part by an obligatory drug regimen that is not actually part of the device per se. For these instances, the FDA will explore how information on the safety and effectiveness of the drug regimen can be incorporated more effectively into the evaluation of the device. Under its Critical Path Initiative, the agency, partnering with a group such as the Duke Clinical Research Institute, can engage in bringing together representatives from various device and drug companies, along with interested parties from the academic community, to develop the clinical trial models needed to answer the scientific questions and to address simultaneously the business and regulatory requirements that will ensue. The FDA also will explore ways in which international collaboration might be expanded to better incorporate data from other countries.

Finally, we now have the opportunity to leverage significantly from the many lessons learned and clarifications resulting from the combined efforts at the December 2006 FDA panel. It was crucial when evaluating the benefits and risks of DES for the panel to distinguish between on-label use of the device (in patients who meet the criteria specified in the product labeling) and off-label use (in patients who do not meet these criteria). This distinction was particularly important in view of the widespread off-label use of DES. Not only are thrombosis rates different in on-label compared with off-label patient populations, but the quantity and quality of data available for these 2 groups differ as well. Thus, in on-label use patients, concurrently randomized cohorts treated with BMS provided robust comparisons from pivotal studies. The use of these studies to characterize rare safety events by compiling larger cohorts through meta-analyses also clarified the criticality of using patient-level data, which supported different conclusions than previous, publicly reported meta-analyses constructed from summary tables. More recent publication of even longer-term follow-up pooling of patient-level data from on-label use studies adds support to the panel conclusion that on-label use is both safe and effective relative to BMS.
higher event rates overall might represent a population of “higher risk, higher benefit” from DES use compared with other treatment options or might represent a more vulnerable cohort at greater safety risk than on-label patients. The absence of definitive data today highlights the importance of acquiring such data in the future. For any individual DES program, at least 2 broad strategies could be identified. If more complex patients were incorporated into premarket pivotal DES studies, it would both add knowledge of investigational device performance in these patients versus concomitantly randomized controls and serve to expand the definition of on-label patients to better match real-world postmarket practice. Furthermore, such an “enriched” patient study cohort would increase the density of clinical end points, reducing the burden for the size of RCTs. The inclusion of more complex patients in premarket pivotal RCTs brings challenges, however, to both statistical analysis plans and other clinical trial operations. Another possibility is to develop more systematic approaches to data collection in the postmarket environment. In this setting, both randomized and nonrandomized studies could have potential utility for understanding specific patient populations, regional practice patterns, or other key information that might support extension of indications, enhance the quality and accuracy of safety information related to DES use in real practice, or both.

The range of issues pertinent to new study designs touched on above is summarized in the Table. Thoughtful, program-specific considerations along these lines going forward will help to answer important questions such as whether differences exist between various DES configurations that may provide advantages for specific target populations and lesion types, how different DES models will perform over time, and to what degree DES safety is related to other obligatory medical therapy. The application of these insights may provide more accurate estimates of event rates for new DES designs released for clinical use. It should also be recognized, however, that in the application of these concepts to specific product lines, we are likely to generate new questions and to leave some old questions unresolved. Nonetheless, these directions may well facilitate clarification and, to some degree, redefinition of how to establish reasonable assurance of safety and effectiveness of new DES therapies.

The FDA will continue to work toward a better understanding of the pathophysiology of coronary artery disease, the biological responses to DES and other treatment modalities, and the complex design, manufacturing, and usage issues associated with DES technology. Using tools such as the Critical Path Initiative and the Medical Device Surveillance Network program, the FDA will collaborate with its academic and industry partners to advance the science on which effective regulatory strategies must be based. In the face of limitations of preclinical and animal models, the advance of an “organ donor”–like approach to retrieval of autopsy DES specimens in both noncardiac and cardiac deaths, as is currently being discussed in conjunction with the FDA Critical Path Cardiac Safety initiative and the National Institutes of Health, could provide unique and critical histopathological understanding of DES implantation sites.

Clarifying the risks and benefits of DES and using them in the safest and most effective way possible cannot be accomplished by any single group. Professional societies, physicians, and the industry all have roles to play by conducting effective clinical studies, using devices wisely, reporting adverse events promptly, sharing clinical findings, and developing criteria for appropriate use. In addition, it will require both collaboration and creativity to review and to redefine the landscape of new DES evaluation to encourage the development of new therapeutics while ensuring that, over millions of human exposures, they are both safe and effective. If we all work together to meet this responsibility, it will spell relief from DES heartburn not only looking backward but also going forward.

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References
5. Harrington RA, Califf RM. Late ischemic events after clopidogrel cessation following drug-eluting stenting: should we be worried? J Am Coll Cardiol. 2006;48:2592–2595.


**Key Words:** device approval process • stents • thrombosis
Noninherited Risk Factors and Congenital Cardiovascular Defects: Current Knowledge

A Scientific Statement From the American Heart Association Council on Cardiovascular Disease in the Young

Endorsed by the American Academy of Pediatrics

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Abstract—Prevention of congenital cardiovascular defects has been hampered by a lack of information about modifiable risk factors for abnormalities in cardiac development. Over the past decade, there have been major breakthroughs in the understanding of inherited causes of congenital heart disease, including the identification of specific genetic abnormalities for some types of malformations. Although relatively less information has been available on noninherited modifiable factors that may have an adverse effect on the fetal heart, there is a growing body of epidemiological literature on this topic. This statement summarizes the currently available literature on potential fetal exposures that might alter risk for cardiovascular defects. Information is summarized for periconceptional multivitamin or folic acid intake, which may reduce the risk of cardiac disease in the fetus, and for additional types of potential exposures that may increase the risk, including maternal illnesses, maternal therapeutic and nontherapeutic drug exposures, environmental exposures, and paternal exposures. Information is highlighted regarding definitive risk factors such as maternal rubella; phenylketonuria; pregestational diabetes; exposure to thalidomide, vitamin A cogeners, or retinoids; and indomethacin tocolysis. Caveats regarding interpretation of possible exposure-outcome relationships from case-control studies are given because this type of study has provided most of the available information. Guidelines for prospective parents that could reduce the likelihood that their child will have a major cardiac malformation are given. Issues related to pregnancy monitoring are discussed. Knowledge gaps and future sources of new information on risk factors are described. (Circulation. 2007;115:2995-3014.)

Key Words: AHA Scientific Statements ■ heart defects, congenital ■ heart disease ■ risk factors

Congenital cardiovascular defects (CCVDs) represent some of the more prevalent malformations among live births¹ and remain the leading cause of death from congenital malformations.² Disease prevention has been hampered by a lack of information about modifiable risk factors for abnormalities in cardiac development. Over the past decade, there have been major breakthroughs in the understanding of inherited causes of CCVDs, including the identification of specific genetic abnormalities for some types of malformations.³ Although relatively less information has been available on noninherited modifiable factors that may have an adverse effect on the fetal heart, there is a growing body of epidemiological literature on this topic. The proportion of cases of CCVDs that are potentially preventable through changes in the fetal environment is currently unknown. One study suggests that the fraction of cases attributable to identifiable and potentially modifiable factors may be as high as 30% for some types of defects.⁴ The lack of reliable information on modifiable risk factors has made it difficult to create population-based strategies to reduce the burden of...
illness from CCVDs and for couples to make lifestyle choices to reduce the likelihood of having a child with a major cardiac malformation.

The purpose of this article is to review the current state of knowledge regarding noninherited risk factors for structural cardiac anomalies, to provide guidance to potential parents that could reduce the likelihood that their child will have a major cardiovascular malformation, and to provide guidance for pregnancy monitoring after known exposures. The current state of knowledge of inheritable causes of CCVDs is reviewed separately and is not included. Similarly, because this statement focuses on factors that influence cardiac development during weeks 2 to 7 of gestation, this review is limited to parental exposures during the first trimester of pregnancy and the 3 months before pregnancy (ie, periconceptional period) that could result in structural abnormalities; exposures that may cause other types of cardiac injury (eg, congenital heart block, myocardial damage) are not considered.

Methods
This statement summarizes the currently available literature, as of May 2006, on prenatal parental conditions and exposures and risk for CCVDs in offspring. English-language publications in scientific journals reporting data on risks of CCVDs in offspring after maternal or paternal diseases, conditions, or exposures were identified through Medline searches, bibliographies of individual articles, and reviews of scientific journals. The information about maternal drug exposure also includes information from the Teratogen Information System (http://depts.washington.edu/terisweb/teris/) and the online version of Shepard’s Catalog of Teratogenic Agents. Publications were assessed to determine the quality of information available (eg, consistency of findings and study design, including the ability to estimate magnitude of risk and exclude chance and bias as possible explanations) regarding a specific type of parental condition or exposure during pregnancy and the risk of having an infant with a major CCVD. Conditions or exposures for which only limited information was available such as a single published epidemiological study were included but generally considered insufficient for discussion. Exceptions were maternal conditions about which there has been some concern (ie, systemic lupus erythematosus and HIV-1 infection). Case reports and case series were not considered to be sufficiently reliable for discussion, unless confirmed by epidemiological studies. From the review of published epidemiological studies, parental conditions and exposures were classified into one of the following categories: factors possibly associated with a decreased risk of CCVDs, factors associated with an increased risk of CCVDs, factors not associated with risk of CCVDs, and factors that have been studied but for which the information about risk of CCVDs is inconclusive. Although some exposures may be risk factors for specific types of CCVDs and not others, quantified measures of overall relative risk such as relative risk (RR) or odds ratios (ORs) are given, if known, to attempt to quantify the increased risk of having a child with any CCVDs, as well as with a specific CCVD, after a specific exposure. Presentation of results for specific types of malformations is, of course, often limited by the different methods used to identify and group malformations in different reports. Confidence limits for the RR or ORs are given if available; confidence limits that contain the value 1.0 indicate that the RR or OR estimate does not differ statistically from the null value (ie, 1.0).

To date, there are no published reports of large prospective cohort studies examining environmental or other exposures associated with CCVDs. The best available information comes from large population-based case-control studies specifically designed to investigate possible risk factors for CCVDs. Two such studies deserve specific mention. The Baltimore-Washington Infant Study (BWIS) was prospectively conducted in the Baltimore, Washington, and northern Virginia area between 1981 and 1989 with a random sample of infants without CCVDs ascertained from the same birth cohort. The National Public Health Institute in Helsinki retrospectively conducted a study in Finland of cases and controls born during 1982 to 1984. In both of these case-control studies, information on potential exposures early in pregnancy was obtained by interview of the parents after the child was born. There were no available reliability or validation studies of the parental reports.

Results
The findings from this review are summarized in the tables. Table 1 summarizes the literature regarding 1 factor that may be associated with a decreased risk of CCVDs, specifically supplementation with a multivitamin containing folic acid. Table 2 summarizes the literature on factors that may increase the risk of a pregnancy resulting in an infant with any CCVDs and with a specific CCVD. Table 3 shows the same information, organized by CCVD phenotype rather than type of exposure. Table 4 shows factors that have been studied but for which no associations have been found thus far. Table 5 shows factors that have been studied but for which too little information is available to make a determination about risk.

Multivitamins and Folic Acid
One of the most important recent discoveries is the possibility that periconceptional intake of multivitamin supplements containing folic acid may reduce the risk of CCVDs in offspring, similar to the known risk reduction for neural tube defects seen with folic acid. This finding was first identified
after analysis of data from a Hungarian randomized trial on birth defects8,9 (Table 1). Findings from subsequent case-control studies have been generally supportive but not conclusive.8,10–12

Two of the studies examined a broad range of heart defects rather than any specific type.9,10 Use of multivitamins containing folic acid was associated with an ≈60% overall reduction in risk for congenital heart defects in the Hungarian randomized trial (RR, 0.42; 95% confidence interval [CI], 0.19 to 0.98)8,9 and an ≈25% reduction in risk in a population-based case-control study done in Atlanta (OR, 0.76; 95% CI, 0.60 to 0.97).10 These and other studies also examined specific types of CCVDs. Multivitamin use was associated with a reduced risk for conotruncal defects in 2 population-based case-control studies (54% and 30%, respectively).10,11 The Hungarian trial also provides suggestive data (no case of conotruncal defects in the supplemented group, 2 cases in the nonsupplemented), but the trial was too small to provide definitive results. A third study showed possible risk reduction for 1 but not all types of conotruncal heart defects.9 A fourth, a hospital-based case-control study,12 showed no evidence of reduction.

For ventricular septal defects (VSDs), 2 studies, a population-based case-control study and the Hungarian randomized trial, were consistent with a reduction in risk (40% and 85% reduction, respectively).9,10 The hospital-based case-control study again found no risk reduction.12

In addition to these studies directly testing the association between multivitamin use and risk for heart defects, other studies among high-risk groups present ancillary evidence supporting a protective effect of folic acid–containing multivitamin supplements. For example, 2 studies have shown that women who used medications that are folic acid antagonists had an increased risk of having babies with heart defects but that this risk was reduced for women who also took multivitamin supplements containing folic acid.13,14

In a third study,15 an increased risk for heart defects associated with maternal febrile illness (see below) appeared to be reduced among women using multivitamin supplements around the time of conception and during early pregnancy. Similar findings have been reported for other birth defects.16

The findings of a possible protective effect for CCVDs from folic acid–containing multivitamin supplements are encouraging but inconclusive given the limited number of studies and mixed results. Additional studies are warranted to determine whether the association of specific phenotypes with multivitamins can be corroborated in large population-based studies in which multivitamin intake can be validated, potential confounders such as maternal age and diabetes can be taken into account, and the components of the multivitamin supplements responsible for the association can be identified.

Maternal Illnesses and Conditions

Phenylketonuria

Untreated maternal phenylketonuria is associated with a >6-fold-increased risk of heart defects.17–20 The most frequent defects are tetralogy of Fallot, VSDs, patent ductus arteriosus (PDA), and single ventricle. Fortunately, with strict diet control before conception and during pregnancy, this excess risk can be reduced.18–22

Maternal Diabetes

CCVDs have been associated with maternal pregestational and, less consistently, with gestational diabetes.6,23–34 The associations with gestational diabetes are hypothesized to be due to inclusion of a group of women with previously undetected type 2 diabetes among women classified as having gestational diabetes.27–29 Specific types of cardiovascular malformations associated with maternal pregestational diabetes include laterality and looping defects,5 transposition of the great vessels,5,33 nonchromosomal atrioventricular septal defects,6 VSDs,6,33,35 hypoplastic left heart syndrome,6 conotruncal defects,36 outflow tract defects,33,35 cardiomyopathy,6 and PDA.33 Diabetes appears to induce malformation before the seventh week of gestation.37

Studies have shown a clear link between glycemic control during organogenesis and fetal malformations.38,39 Although strict glycemic control before conception and during pregnancy has been shown to reduce risk levels comparable to those of the general population,40 achieving and maintaining euglycemia early in pregnancy remains a challenge because many women with diabetes neither plan their pregnancies nor achieve adequate glycemic control before conception.39,41 Given the increasing prevalence of risk factors for diabetes,42–44 it is important to gain a better understanding of the current impact of both preexisting and gestational diabetes on CCVDs.

Although congenital anomalies associated with maternal diabetes are presumed to be related to abnormalities in maternal metabolic fuels essential for embryogenesis,45 precise pathogenic mechanisms remain unclear. One hypothesis is that abnormal glucose levels characteristic of diabetes mellitus disrupt expression of a regulatory gene in the embryo, leading to embryotoxic apoptotic cellular changes.46 The prevention of diabetic embryopathy by antioxidants in animal studies suggests that oxidative stress resulting from metabolic abnormalities and generation of free radicals is another possible mechanism.47–52 The increasing prevalence of type 2 diabetes among women of childbearing age in recent decades42–44,53,54 makes identifying and implementing effective prevention strategies a high priority.

Rubella, Febrile Illnesses, and Influenza

The potential association between maternal infections and birth defects was first suggested by the observation of the relation between maternal rubella infection early in gestation and the congenital rubella syndrome in offspring.55–57 It is now well known that maternal rubella infection during pregnancy can result in offspring with PDA, pulmonary valve abnormalities, peripheral pulmonary stenosis, and VSDs58–60 and that the risk of rubella embryopathy can be virtually eliminated by ensuring that women of childbearing age are immunized against rubella.61 More recent studies suggest that other maternal febrile illnesses during the first trimester of pregnancy also may be associated with an increased risk for certain heart defects.6,15,16,21,61 Mothers reporting any febrile illnesses during the first trimester of pregnancy have a 2-fold-higher risk of offspring with any heart defect in these
<table>
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<td></td>
<td>Laterality and looping</td>
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<td>d-TGA</td>
<td>3.8–27.2</td>
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<td></td>
<td>AVSD</td>
<td>10.6</td>
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<td>Septal defects</td>
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<td>Aortic coarctation</td>
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<td>VSD</td>
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<td>d-TGA with intact ventricular septum</td>
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<td>Tricuspid atresia</td>
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<td>PDA</td>
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<td>Pulmonary valve abnormalities</td>
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<td>Peripheral pulmonic stenosis</td>
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<td>Any defects</td>
<td>†</td>
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<td>Maternal nontherapeutic drug exposure</td>
<td>Outflow tract defects</td>
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<td>Cranial neural crest defects (cardiac and noncardiac)</td>
<td>0.7–4.8</td>
<td>168, 171, 172</td>
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<tr>
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<td>Pulmonic stenosis and other noncardiac defects</td>
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<td>Indomethacin tocolysis</td>
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<td>d-TGA</td>
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<td>AVSD (Down syndrome)</td>
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<td>VSD</td>
<td>1.9</td>
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<tr>
<td></td>
<td></td>
<td>Bicuspid aortic valve</td>
<td>4.1</td>
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<tr>
<td></td>
<td>Sulfasalazine‡</td>
<td>Any defects</td>
<td>3.4</td>
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<tr>
<td></td>
<td>Thalidomide</td>
<td>Any defects</td>
<td>†</td>
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<tr>
<td></td>
<td>Trimethoprim-sulfonamide‡</td>
<td>Any defects</td>
<td>2.1–4.8</td>
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</table>
studies. Specific groups of defects that have been shown to be associated with maternal febrile illness include pulmonic stenosis, tricuspid atresia, coarctation of the aorta, all left-sided obstructive defects, and VSDs. A case-control study in California found an association of maternal fever with conotruncal defects among offspring born to mothers who did not use multivitamins. In some of these studies, the febrile illness often was characterized as flu-associated fever or influenza; thus flu-associated fever also was a risk factor for any cardiac defect and for some specific malformations. The mechanism by which maternal febrile illnesses may result in malformations is unclear. One possibility is altered apoptosis. Apoptosis is known to be involved in cardiac morphogenesis, for example, in the development of the cardiac outflow tract, and can be altered by both fever and infection. Another possibility is a direct effect of the underlying infection, as with maternal rubella infection. Most studies to date have been unable to distinguish between independent and joint effects associated with maternal fever, maternal infection, and use of certain medications to control the fever or infection.

**Obesity**

A number of studies have examined the association between maternal prepregnancy obesity and CCVDs, although findings have been inconsistent. A study by Waller et al reported an association between maternal obesity, defined as a body mass index of >26 kg/m², and a grouped category of defects of the great vessels. Two additional studies found no statistically significant increased risks for any heart defect or conotruncal heart defects in relation to maternal obesity. A recent study reported a 6.5-fold risk elevation for aggregate cardiac defects among obese black women, and Watkins et al reported a 2-fold increase in risk of aggregate cardiac defects in relation to maternal obesity. Obesity is a complex condition that has to be studied carefully to minimize underreporting of body weight, especially in case-control studies, and the possibility of confounding by other factors associated with nutrition, such as the intake of micronutrients or use of multivitamin supplements, or with obesity, such as type 2 diabetes.

**HIV Infection**

Maternal infection with HIV can transmit the infection vertically to offspring. Children infected with HIV-1 in utero have an increased risk of dilated cardiomyopathy and inappropriate left ventricular hypertrophy. Such children also are more likely to have low left ventricular fractional shortening. Maternal HIV has not been associated with an increased risk of structural congenital cardiovascular malformations thus far.

**Systemic Lupus Erythematosus**

Although a high proportion of infants with congenital complete heart block are born to women with systemic lupus erythematosus, no published reports show an association between maternal connective tissue disease and an increased risk of structural congenital cardiovascular malformations.

**Epilepsy**

Offspring of women with epilepsy are at an increased risk for congenital malformations, including congenital heart de-
Because several therapy-related factors could account for this increased risk, including direct teratogenic effects of anticonvulsant drug therapy and an indirect effect of the drugs by interfering with folate metabolism, it has been difficult to determine whether maternal seizures are independently associated with an increased risk of heart defects.

**Maternal Therapeutic Drug Exposure**

The US Food and Drug Administration (FDA) has classified a number of medications according to risk for birth defects if ingested during pregnancy. Although this classification relates to birth defects in general and not specifically to congenital cardiac defects, when available, the FDA description of risk as defined in Table 6 is included in each of the therapeutic drug discussions that follow.83

**Thalidomide**

Thalidomide is known to be a cardiac teratogen and therefore contraindicated during pregnancy and among women planning a pregnancy. Thalidomide embryopathy includes cardiovascular malformations ranging from ventricular and atrial septal defects (ASDs) to complex conotruncal defects.84 No safe dose of thalidomide treatment during the critical period of gestation has been established, and cases of thalidomide embryopathy have been described after maternal ingestion of as little as one 50-mg capsule during this time (FDA category X).

**Vitamin A Cogeners/Retinoids**

Maternal intake of isotretinoin has been shown to cause congenital cardiac defects in addition to other malformations. Characteristic features of isotretinoin embryopathy include central nervous system malformations, micrognathia, cleft palate, thymic and eye anomalies, and cardiac and great vessel defects. The frequency of congenital anomalies does not appear to be increased among children of women who discontinue therapy before conception.85 These medications are contraindicated during pregnancy and among women planning a pregnancy (FDA category X).

**Etretinate**

Etretinate persists in the body for an extremely long time after administration, and the length of time that teratogenic effects may occur is currently not known. In case reports, congenital abnormalities possibly related to prior etretinate therapy have been seen as long as 45 months after therapy was stopped.86 No large studies examining the association of acitretin have been performed. Because acitretin can be

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### TABLE 3. Exposures With Reported Risk for Specific CCVDs

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Exposures That May Increase Risk</th>
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</thead>
<tbody>
<tr>
<td>All left-sided obstructive defects</td>
<td>Febrile illness</td>
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<tr>
<td>All right-sided obstructive defects</td>
<td>Febrile illness</td>
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<td>Aortic coarctation</td>
<td>Febrile illness</td>
</tr>
<tr>
<td>AVSD</td>
<td>Febrile illness</td>
</tr>
<tr>
<td>AVSD (Down syndrome)</td>
<td>Influenza</td>
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<tr>
<td>AVSD (nonchromosomal)</td>
<td>Influenza</td>
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<tr>
<td>Bicuspid aortic valve</td>
<td>Influenza</td>
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<tr>
<td>Conotruncal defects</td>
<td>Influenza</td>
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<tr>
<td>Cranial neural crest defects</td>
<td>Influenza</td>
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<td>(cardiac and noncardiac)</td>
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<tr>
<td>d-TGA</td>
<td>Influenza</td>
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<td>d-TGA with intact ventricular septum</td>
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<tr>
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<td>Organic solvents</td>
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<td>Laterality and looping</td>
<td>Organic solvents</td>
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<td>Membranous VSD</td>
<td>Vitamin A cogeners/retinoids</td>
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<td>Outflow tract defects</td>
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<td>Tetralogy of Fallot</td>
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<td>Transposition of the great arteries</td>
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**TABLE 3. Continued**

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<td>VSD</td>
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<td>Outflow tract defects</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>PDA (BTW &gt;2500 g only)</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Peripheral pulmonic stenosis</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Pulmonic stenosis</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Pulmonary valve abnormalities</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Septal defects</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>TAPVR</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
<td>Maternal rubella</td>
</tr>
<tr>
<td>Transposition of the great arteries</td>
<td>Maternal rubella</td>
</tr>
</tbody>
</table>

**Abbreviations and references as in Table 2.**
TABLE 4. Reported Exposures With No Evidence of an Association With Risk for CCVDs

<table>
<thead>
<tr>
<th>Maternal Illnesses/Conditions</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>74–76</td>
</tr>
<tr>
<td>Maternal therapeutic drug exposure</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>87–90</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>120, 121</td>
</tr>
<tr>
<td>Diazepam</td>
<td>87–89, 91, 118, 119</td>
</tr>
<tr>
<td>Oral contraceptives</td>
<td>138</td>
</tr>
<tr>
<td>Penicillin</td>
<td>91–95</td>
</tr>
<tr>
<td>Vaginal metronidazole</td>
<td>96–98</td>
</tr>
<tr>
<td>Maternal nontherapeutic drug exposure</td>
<td>6, 91, 147–150</td>
</tr>
</tbody>
</table>

converted to etretinate in the body, the length of time that acitretin may cause teratogenic effects may be longer than its half-life (50 to 60 hours).

Topical therapy with tretinoin in usual doses during pregnancy is unlikely to pose a substantial teratogenic risk, but data are insufficient to state that there is no risk.

**Antibiotics**

Rothman et al. observed an association with maternal ampicillin (FDA category B) treatment “about the time pregnancy began” in a case-control study of 390 infants with congenital heart disease, specifically transposition of the great arteries. Their follow-up study of similar design did not confirm these findings. Additionally, a separate case-control study failed to show an association between ampicillin use and congenital heart disease. Finally, in a large population-based (Hungarian) case-control study of maternal ampicillin use in the second or third month of pregnancy, no association was found among 4468 cases with cardiovascular malformations.

Multiple large studies have shown no association between the use of penicillin (FDA category B) and an increased risk of congenital anomalies in general. One Danish population-based record linkage study that examined the frequency of congenital heart defects in mothers given penicillin during the first trimester showed it to be no higher than expected.

The epidemiological data regarding maternal vaginal metronidazole (FDA category B) use early in pregnancy were summarized in 2 meta-analyses. In both instances, the risk of congenital anomalies in offspring was not increased. One of the studies included in these analyses specifically examined a large group (984) of infants with cardiovascular defects. In the BWIS, maternal use of metronidazole during pregnancy was found to be associated with an increased risk of outflow tract anomalies with normally related great arteries (OR, 6.0; 95% CI, 1.8 to 20.7) and an increased risk of membranous VSDs (OR, 12.2; 95% CI, 3.0 to 50.2).

An association was found with maternal trimethoprim-sulfonamide (FDA category C) treatment during the second or third month of gestation in a case-control study among 3870 infants with cardiovascular defects (OR, 4.8; 95% CI, 1.5 to 16.1). Similar findings were reported from the Hungarian case-control surveillance of congenital abnormalities (OR, 2.1; 95% CI, 1.4 to 3.3). The risks were reduced if the mother also took folic acid supplementation.

**Antiviral/Antiretroviral Agents**

An association was observed between major congenital anomalies, including congenital cardiovascular malformations, and a maternal prescription for zidovudine (FDA category C) during pregnancy in a Medicaid record linkage study. When the exposures were broken down by trimester of pregnancy, the significant association was seen among women who received the prescription in the third trimester, not in the first or second, a finding inconsistent with a teratogenic mechanism. The Antiretroviral Pregnancy Registry has been established and to date has not shown an increase in congenital defects in women receiving therapy in the second or third trimester.

**Antifungal Therapies**

In a UK cohort study and a Danish record linkage study, the frequency of congenital anomalies was not increased in infants of women who received prescriptions for a single oral dose of fluconazole (FDA category C) in the first trimester of pregnancy. Similarly, in a prospective study, the frequency of congenital anomalies was not increased in women receiving fluconazole with median doses of 200 mg. It should be noted that 4 children have been described with a similar and unusual pattern of congenital anomalies (including congenital heart disease) in offspring whose mothers were treated during most or all of the first trimester with a high dose (400 to 800 mg/d) of fluconazole for coccidioidomycosis meningitis. These observations suggest the need for further study of fluconazole treatment with consideration of possible threshold effects.

**Anticonvulsants**

Although many large epidemiological studies of the offspring of epileptic women have been published, currently available data are incapable of resolving the controversy as to whether the malformations are due to the epilepsy or the anticonvul- sant therapy. Additionally, the studies examining congenital malformations in infants of women who took anticonvulsant therapies are difficult to interpret because accurate assessment of the effects of the anticonvulsant treatment may be confounded by multiple other factors. Specifically, many women with seizures are treated with multiple therapies either serially or simultaneously, and most women with seizures are treated with an anticonvulsant drug (leaving no control group). There are characteristic anomalies associated with some of the anticonvulsants (eg, fetal hydantoin syndrome), which may involve cardiac abnormalities (phenytoin, FDA category D; valproic acid, FDA category D).

**Lithium**

An association has been observed between maternal treatment with lithium carbonate during pregnancy and the occurrence of Ebstein’s anomaly. In a voluntary reporting registry, serious congenital cardiovascular anomalies were observed in 8% of 225 infants born to mothers who had taken lithium during the first trimester of pregnancy. One third of these infants had Ebstein’s anomaly. Contradicting these
### TABLE 5. Exposures Studied but Insufficient Data to Determine Risk for CCVDs

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal illnesses/conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>6, 23–34</td>
</tr>
<tr>
<td>Obesity</td>
<td>6, 44, 70–73, 118</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>77–79</td>
</tr>
<tr>
<td>Nausea</td>
<td>146</td>
</tr>
<tr>
<td>Life event stress</td>
<td>36, 197</td>
</tr>
<tr>
<td><strong>Maternal therapeutic drug exposure</strong></td>
<td></td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>144</td>
</tr>
<tr>
<td>Amobarbital</td>
<td>6</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>6</td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>6</td>
</tr>
<tr>
<td>Aspirin</td>
<td>216</td>
</tr>
<tr>
<td>Barbiturates (except amobarbital)</td>
<td>6</td>
</tr>
<tr>
<td>Bendectin</td>
<td>88, 145</td>
</tr>
<tr>
<td>Clomiphene</td>
<td>5, 36</td>
</tr>
<tr>
<td>Daclomycin</td>
<td>143</td>
</tr>
<tr>
<td>Deoxyribucin</td>
<td>143</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>100–104</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>6</td>
</tr>
<tr>
<td>Lithium</td>
<td>108–117</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>6</td>
</tr>
<tr>
<td>Oral contraceptives</td>
<td>6</td>
</tr>
<tr>
<td>Narcotics</td>
<td>6</td>
</tr>
<tr>
<td>Parasympatholytics</td>
<td>6</td>
</tr>
<tr>
<td>Phenothiazines</td>
<td>6</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>87, 88, 91</td>
</tr>
<tr>
<td>Topical tretinoin</td>
<td>85, 86</td>
</tr>
<tr>
<td>Xanthenes</td>
<td>6</td>
</tr>
<tr>
<td>Ziduvin</td>
<td>99</td>
</tr>
<tr>
<td><strong>Maternal nontherapeutic drug exposure</strong></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>6, 36, 119, 151–154</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>6, 64, 217</td>
</tr>
<tr>
<td>Cocaine</td>
<td>6, 155–159</td>
</tr>
<tr>
<td><strong>Maternal environmental factors</strong></td>
<td></td>
</tr>
<tr>
<td>Air quality</td>
<td>178, 179</td>
</tr>
<tr>
<td>Herbicides/pesticides/rodenticides</td>
<td>175, 177, 214, 218</td>
</tr>
<tr>
<td>Proximity to hazardous waste site</td>
<td>187–189</td>
</tr>
<tr>
<td>Trichloroethylene in groundwater</td>
<td>180</td>
</tr>
<tr>
<td>Water chlorination byproducts</td>
<td>182–186</td>
</tr>
<tr>
<td><strong>Maternal sociodemographic characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>6, 191</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>36, 192–196, 211</td>
</tr>
<tr>
<td>Socioeconomic status</td>
<td>192</td>
</tr>
<tr>
<td><strong>Paternal factors/exposure</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>198, 202–204</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>6, 217</td>
</tr>
<tr>
<td>Cocaine</td>
<td>6, 204</td>
</tr>
<tr>
<td>Alcohol exposure (father)</td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>6</td>
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</table>

### TABLE 5. Continued

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greatest number of drinks on any occasion</td>
<td>6</td>
</tr>
<tr>
<td>Housing characteristics</td>
<td></td>
</tr>
<tr>
<td>Type of home (individual, townhouse, apartment)</td>
<td>6</td>
</tr>
<tr>
<td>Gas heating</td>
<td>6</td>
</tr>
<tr>
<td>Electric heating</td>
<td>6</td>
</tr>
<tr>
<td>Oil heater</td>
<td>6</td>
</tr>
<tr>
<td>Gas stove</td>
<td>6</td>
</tr>
<tr>
<td>Electric stove</td>
<td>6</td>
</tr>
<tr>
<td>Cooking with kerosene, coal, or wood</td>
<td>6</td>
</tr>
<tr>
<td>Medical exposures</td>
<td></td>
</tr>
<tr>
<td>Maternal dental x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Maternal chest x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Maternal skeletal x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Maternal abdominal x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Paternal dental x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Paternal chest x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Paternal skeletal x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Paternal abdominal x-rays</td>
<td>6</td>
</tr>
<tr>
<td>Maternal home and occupational exposures</td>
<td></td>
</tr>
<tr>
<td>Anesthetic gas (occupational)</td>
<td>6</td>
</tr>
<tr>
<td>Arsenic</td>
<td>6</td>
</tr>
<tr>
<td>Art dyes</td>
<td>6</td>
</tr>
<tr>
<td>Arts and crafts painting</td>
<td>6</td>
</tr>
<tr>
<td>Cadmium</td>
<td>6</td>
</tr>
<tr>
<td>Carpentry</td>
<td>6</td>
</tr>
<tr>
<td>Cold, extreme (occupational)</td>
<td>6</td>
</tr>
<tr>
<td>Drug manufacturing</td>
<td>6</td>
</tr>
<tr>
<td>Dry-cleaning solvents</td>
<td>6</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>6</td>
</tr>
<tr>
<td>Home tap water</td>
<td>181</td>
</tr>
<tr>
<td>Housekeeping cleaners</td>
<td>175</td>
</tr>
<tr>
<td>Jewelry making</td>
<td>6</td>
</tr>
<tr>
<td>Laboratory chemicals</td>
<td>6</td>
</tr>
<tr>
<td>Laboratory viruses</td>
<td>6</td>
</tr>
<tr>
<td>Lead score</td>
<td>6</td>
</tr>
<tr>
<td>Mercury</td>
<td>6</td>
</tr>
<tr>
<td>Pesticides, insecticides, rodenticides</td>
<td>175, 177</td>
</tr>
<tr>
<td>Plastics manufacturing</td>
<td>175</td>
</tr>
<tr>
<td>Propellants</td>
<td>6</td>
</tr>
<tr>
<td>Pyrolysis/combustion products</td>
<td>175</td>
</tr>
<tr>
<td>Stained glass crafts</td>
<td>6</td>
</tr>
<tr>
<td>Textile dyes</td>
<td>6</td>
</tr>
<tr>
<td>Welding</td>
<td>6</td>
</tr>
<tr>
<td>Paternal home and occupational exposures</td>
<td></td>
</tr>
<tr>
<td>Anesthetic gas (occupational)</td>
<td>6</td>
</tr>
<tr>
<td>Arsenic</td>
<td>6</td>
</tr>
<tr>
<td>Art dyes</td>
<td>6</td>
</tr>
<tr>
<td>Arts and crafts painting</td>
<td>6</td>
</tr>
<tr>
<td>Auto body repair work</td>
<td>6</td>
</tr>
</tbody>
</table>
reports, no association was seen in a case-control study of 10 698 children with congenital anomalies, but the number of exposures in the case and control groups was small.115 More recent retrospective, prospective, and meta-analysis studies suggest that lithium appears not to be a cardiac teratogen (FDA category D).116,117

**Benzodiazepines/Barbiturates**

An association with the maternal use of diazepam (FDA category D) or related drugs during the first trimester of pregnancy was observed in 2 case-control studies of almost 400 children each.87,118 Bracken89 reanalyzed these data and failed to find a significant association, and Zierler and Rothman88 found no association in a follow up-study. No association with maternal use of diazepam during the first trimester of pregnancy was seen in case-control studies of 150 children with VSDs.91,119

The frequencies of congenital anomalies were not significantly increased among infants of women occasionally treated with amobarbital (FDA category D) as a hypnotic. However, the frequency of cardiac malformations was increased (RR, 2.6; 95% CI, 1.0 to 5.2).91 The risk for chronic or high-dose maternal use is unknown.

**Sympathomimetics**

A case-control study by Rothman et al87 observed a slightly higher rate of exposure to phenylephrine (FDA category C) early in pregnancy in mothers of infants with congenital heart disease than in controls. This observation was not confirmed in a later and more rigorous study by the same authors.98 No association was seen between the first-trimester use of phenylephrine and congenital heart disease in a large cohort study.91

**Corticosteroids**

A possible association between maternal corticosteroid use and congenital cardiac malformations was identified in the BWIS by univariate analysis (OR, 1.71; 95% CI, 1.01 to 2.88). This finding was no longer significant after other variables were taken into account.120 Using data derived from a population based case-control study that included 207 cases of conotruncal heart defects, Carmichael and Shaw121 showed no association between maternal corticosteroid use and congenital cardiovascular malformations.

**Folate Antagonists**

Associations with maternal treatment with sulfasalazine (FDA category B) or another dihydrofolate reductase inhibitor during the second or third month of pregnancy were observed in a case-control study of 3870 infants with cardiovascular defects (OR, 3.4; 95% CI, 1.1 to 6.1). These associations were not seen among the subset of mothers who took supplemental folic acid. As mentioned, maternal use of trimethoprim-sulfonamide also has resulted in congenital heart defects in offspring,13,14 with risk reduction if mothers also took folic acid supplementation (see the Antibiotics section).

**Nonsteroidal Antiinflammatory Drugs**

Ericson and Kallen122 examined use of nonsteroidal antiinflammatory drugs in early pregnancy in a large registry study (n=2557) and found that the adjusted OR for any congenital malformation was 1.04 (95% CI, 0.84 to 1.29), but for cardiac defects, the OR was 1.86 (95% CI, 1.32 to 2.62). There was no drug specificity for cardiac defects.

Associations with maternal use of ibuprofen (FDA category B) during pregnancy have been reported in evaluations of infants with dextro-looped transposition of the great arteries (OR, 2.5; 95% CI, 1.2 to 4.9), membranous VSDs (OR, 1.9; 95% CI, 1.0 to 3.5), atrioventricular septal defects, Down syndrome (OR, 2.4; 95% CI, 1.1 to 4.2), and bicuspid aortic valve (OR, 4.1; 95% CI, 1.8 to 9.3).4 No association

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>6</td>
</tr>
<tr>
<td>Carpentry</td>
<td>6</td>
</tr>
<tr>
<td>Drug manufacturing</td>
<td>6</td>
</tr>
<tr>
<td>Dry-cleaning solvents</td>
<td>6</td>
</tr>
<tr>
<td>Extreme cold (occupational)</td>
<td>6</td>
</tr>
<tr>
<td>Hair dyes</td>
<td>6</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>6</td>
</tr>
<tr>
<td>Ionizing radiation</td>
<td>6</td>
</tr>
<tr>
<td>Laboratory chemicals</td>
<td>6</td>
</tr>
<tr>
<td>Lead score</td>
<td>6</td>
</tr>
<tr>
<td>Marijuana</td>
<td>6</td>
</tr>
<tr>
<td>Mercury</td>
<td>6</td>
</tr>
<tr>
<td>Pesticides</td>
<td>6</td>
</tr>
<tr>
<td>Plastics manufacturing</td>
<td>6</td>
</tr>
<tr>
<td>Solvents</td>
<td>6</td>
</tr>
<tr>
<td>Stained glass crafts</td>
<td>6</td>
</tr>
<tr>
<td>Textile dyes</td>
<td>6</td>
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<tr>
<td>Varnishes</td>
<td>6</td>
</tr>
</tbody>
</table>

**TABLE 6. FDA Categories of Risk for Birth Defects**

<table>
<thead>
<tr>
<th>Category</th>
<th>Description of Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No fetal risk shown in controlled human studies.</td>
</tr>
<tr>
<td>B</td>
<td>No human data available. Animal studies show no fetal risk or animal studies show a risk but not a fetal risk.</td>
</tr>
<tr>
<td>C</td>
<td>No controlled studies on fetal risk available for human beings or animals, or fetal risk shown in controlled animal studies but no human data available (benefit of drug use must clearly justify potential fetal risk in this category).</td>
</tr>
<tr>
<td>D</td>
<td>Studies show fetal risk in human beings (use of drug may be acceptable even with risks, such as in life-threatening illnesses or where safer drugs are ineffective).</td>
</tr>
<tr>
<td>X</td>
<td>Risk to fetus clearly outweighs any benefit from these drugs.</td>
</tr>
</tbody>
</table>
was seen in infants with atrioventricular septal defect without Down syndrome.

Two studies by Souter et al. and Hammerman et al. document the association between indomethacin tocolysis and persistent PDA. The magnitude of these effects appears to be greatest when indomethacin is administered within 48 hours of delivery. Additionally, there have been case reports of persistent pulmonary hypertension and premature closure of the ductus arteriosus in infants whose mothers took other forms of nonsteroidal antiinflammatory drugs, including naproxen, diclofenac, ketoprofen, indomethacin, and sulindac.

**Female Hormones**

A potential risk for congenital cardiac defects in offspring from maternal use of oral contraceptives was identified in 2 case-control studies. Wiseman and Dodds-Smith evaluated the case histories included in Heinonen et al. study and found that only half were exposed during the critical period of cardiogenesis. Oral contraceptive use was no longer significantly associated with congenital heart disease in an analysis restricted to early exposure. Ferencz et al. studied mothers of 110 children with heart disease and found no association with maternal hormone therapy. Additionally, a recent meta-analysis failed to document any associations between oral contraceptive use and CCVD; in general, the data are now thought to support their safety.

An association with maternal use of clomiphene was observed in a case-control study of 126 children with coarctation of the aorta (OR, 4.5; 99% CI, 1.0 to 19.9). No association with maternal use of clomiphene was seen in a case-control study involving 83 infants with conotruncal cardiac defects. In the BWIS, maternal use of clomiphene was found to be associated with an increased risk of tetralogy of Fallot (OR, 3.2; 95% CI, 1.6 to 6.3).

**Narcotics**

Two case-control studies, each involving 300 to 400 children with congenital heart disease, reported an association with maternal codeine (FDA category C) use during the first trimester of pregnancy, but methodological limitations raise doubt as to their validity. No association was observed in 2 other studies.

**Chemotherapy**

There have been no published studies examining the effect of chemotherapy treatment during pregnancy. The literature has been limited to studies of patients who have been treated with chemotherapeutic agents before becoming pregnant. A large case-control study investigating congenital anomalies in children of patients who received chemotherapy for cancer in childhood and adolescence identified structural congenital cardiac defects in 10.0% (2 of 20) of the offspring of women who had been treated in the past with dactinomycin compared with 0.6% (24 of 144) among the children in a multicenter survey of fetal anomalies (P = 0.01). Of note, studies examining the use of doxorubicin showed no fetal effects in human or animal experiments (antineoplastics, FDA category D).

**Angiotensin-Converting Enzyme Inhibitors**

A recent cohort of 29507 infants from a large database of Tennessee Medicaid patients was linked with vital records and hospitalization claims during the first year of life to study the risk of congenital malformations after maternal exposure to angiotensin-converting enzyme used to treat maternal hypertension. This study identified a prevalence of use of angiotensin-converting enzyme inhibitors in the first trimester of 0.7% and a higher risk of major congenital malformations, including malformations of the heart (OR, 3.72; 95% CI, 1.89 to 7.30), in offspring of mothers exposed to angiotensin-converting enzyme inhibitors in the first trimester of pregnancy. The prevalence of major malformations identified in the reference group (2.6%) was lower than expected in the general population (3.0% to 3.5%), raising questions about possible differences in ascertainment and classification of major malformations by exposure group. There is a need for further studies of this issue using standard methods of case ascertainment and classification and accounting for potential risk factors.

**Composite Drugs**

Bendectin, a combination of doxylamine and pyridoxine, is no longer available in the United States. Extensive studies provide no evidence that maternal use alters the risk of congenital anomalies in offspring. Specifically, case-control studies provide no consistent indication that maternal use of Bendectin during the first trimester of pregnancy increases the risk of congenital heart disease.

**Maternal Nontherapeutic Drug Exposure**

**Caffeine**

Caffeine is known to cross the placenta, and concern that caffeine may cause birth defects prompted the FDA to caution pregnant women to limit their caffeine intake. As illustrated below, there is no clear association between caffeine ingestion during human pregnancy and congenital heart disease.

A case-control study of 2030 malformed infants, including 277 with cardiac defects, evaluated risk associated with caffeine ingestion, including consumption of tea, coffee, and cola. No risk was identified for consumption of any of the 3 beverage types. Risk also was assessed in relation to amount of total daily caffeine ingestion in the categories of any ingestion per day, >200 mg/d, and >400 mg/d. Again, no risk was identified in doses equivalent to 4 cups of coffee per day. Too few mothers consumed as much as 1000 mg/d caffeine to assess the risk of very high consumption.

In a population-based cohort study of 850 mothers who drank ≥8 cups of coffee per day, the frequency of all congenital malformations, including heart disease, was not increased from expected. In another well-controlled cohort study, 595 women who drank ≥4 cups of coffee daily also did not produce offspring with congenital anomalies any more frequently than expected. In addition, in a study of 12696 women who took caffeine-containing medications in the first 4 months of pregnancy, the frequency of congenital anomalies, including heart disease, was no greater than expected. Caffeine also was evaluated as a potential risk
factor in the BWIS. Again, no association was observed between cardiac defects and caffeine consumption or caffeine dose. Other studies also have failed to identify an association. 

Alcohol

Ever since the first description of the fetal alcohol syndrome by Jones and Smith in 1973, several studies have documented a wide range of teratogenic effects of alcohol consumption during pregnancy, including cardiac defects. It has been suggested that ethanol may produce fetal tissue edema and affect the turgor of the primitive cardiac loop. Studies of this topic are especially difficult because of the notorious problem of obtaining reliable estimates of alcohol consumption during pregnancy in addition to other forms of recall bias. In a prospective study that collected information on maternal alcohol consumption during the first trimester of pregnancy, investigators noted no increased risk of major malformations among offspring of women who consumed 1 to 2 drinks per day compared with offspring of nondrinkers. In a case-control study of 90 patients with conotruncal abnormalities and 150 with VSDs born in Finland between 1982 and 1983, the effect of maternal alcohol use was compared with 756 controls. Although more mothers of infants with conotruncal malformations consumed any alcohol, consumed alcohol regularly every week, and consumed >1 drink per occasion, these results did not reach statistical significance. Maternal alcohol consumption during the first trimester of pregnancy was more common among the mothers of infants with VSDs (47%) than among those of controls (38%; \( P<0.05 \)). A case-control study of conotruncal defects in Atlanta showed no association with maternal reports of alcohol consumption (OR, 0.72; 95% CI, 0.49 to 1.06) or “binge” drinking (OR, 0.44; 95% CI, 0.13 to 1.46). A more recent case-control study that examined the risk of congenital anomalies with different sporadic and daily doses of alcohol consumption in Spain reported an increased risk of congenital heart defects as a group only with the highest level of maternal consumption of alcohol per day (ie, >92 g/d). In the BWIS, the only association between alcohol and cardiovascular malformations was limited to increased risk for small muscular VSDs with heavy consumption (5 drinks on a single occasion) during the period defined by the last menstrual period \( \pm 3 \) months. There was no evidence of a trend in the risk of any cardiac defect with increased exposure. A similar study from Finland also reported that maternal alcohol consumption during the first trimester appeared to double the risk of ASDs (OR 1.9; 95% CI, 1.0 to 3.4) but that the dose-response trends in risk were inconsistent with causal association.

Cocaine and Marijuana

A case report by Shepard et al suggested that single ventricle may result from maternal cocaine ingestion by inducing coronary occlusion in the developing fetal heart. Martin and Khoury used data from a case-control study, the Atlanta Birth Defects Case-Control Study, to investigate the role of maternal cocaine ingestion in the induction of single ventricles. None of the 27 case infants were reportedly exposed to cocaine during early pregnancy, and only 7 of the control infants (0.43%) were exposed during early pregnancy. These data suggest that in this population the use of cocaine was rare or underreported.

An increased frequency of cardiovascular malformations was observed among 214 infants with neonatal toxicology screens showing the prevalence of cocaine in 1 study, with peripheral pulmonic stenosis as the leading diagnosis and in far greater numbers than in the general population. A meta-analysis of 6 other epidemiological studies revealed no significant association between maternal cocaine use in pregnancy and fetal cardiovascular malformations. Subsequent case-control studies have reported an association of maternal reports of cocaine use with an increased risk of any cardiac defects (adjusted OR, 11.6; 95% CI, 0.89 to 151.5), heterotaxy (OR, 3.7; 95% CI, 1.3 to 10.7), and membranous VSDs (adjusted OR, 2.4; 95% CI, 1.3 to 4.4). The imprecise results in 2 of these studies are due to small numbers of cases with maternal reports and could reflect rare exposures, underreporting, or sampling variability.

In the Atlanta Birth Defects Case-Control Study, a 2-fold increase in risk of isolated simple VSDs was identified for maternal self- and paternal proxy-reported marijuana use. Risk of isolated simple VSDs increased with regular \( (\geq3 \) d/wk) marijuana use for both maternal self- and paternal proxy report, although the association was significant only for maternal self-report. Maternal use of marijuana was evaluated in the BWIS and was found to be associated with a slight increase in risk for Ebstein’s anomaly. Adams et al used a case-control design with sufficient power to identify a 2-fold increase in risk for conotruncal defects and did not find an association (FDA category C).

Cigarette Smoking

A number of studies have investigated maternal cigarette smoking and congenital heart disease. A meta-analysis of studies published between 1971 and 1999 (12 analyses of all heart defects combined and 7 analyses of heart defect groups or specific phenotypes separately) found no association for all heart defects combined (OR, 1.07; 95% CI, 0.98 to 1.17) and mixed results for analyses of specific groups or phenotypes. The latter probably reflects differences in methods, including case ascertainment, classification, control of confounding, and case group sample size, between the different studies. Some recent studies have reported associations of maternal smoking with heart defects combined (OR, 2.1; 95% CI, 1.2 to 3.5 in the Torf and Christianson study; OR, 1.56; 95% CI, 1.12 to 1.82 in the Woods and Raju study), but others such as the BWIS and a Swedish study have not. Some studies have reported associations between maternal smoking and heart defect groups, including ASDs (OR, 2.2; 95% CI, 1.1 to 4.3), atrioventricular septal defects (OR, 2.3; 95% CI, 1.2 to 4.5), and tetralogy of Fallot (OR, 4.6; 95% CI, 1.2 to 17.0). However, these associations were not corroborated by larger studies such as the BWIS and a study conducted in Sweden. Recent exploratory analyses of small case groups based on the BWIS data have identified associations of maternal smoking with single ventricle and L-transposition of the great arteries. Further research is needed to determine whether there is a relationship between...
maternal smoking and risk of heart defects based on large population-based studies using more standardized case ascertainment and classification methods.

**Vitamin A**
A number of studies have examined the association between high vitamin A in the diet and/or supplements and neural crest cell defects (ie, cardiac and noncardiac defects) or outflow tract defects. Some studies suggest that a high intake of vitamin A is associated with an increased risk of CCVDs, whereas others suggest no increased risk. One possible reason for the inconsistency of the findings may relate to differences in methods of assessing high exposure to vitamin A intake. Worth noting are 2 studies reporting an increased risk of CCVDs with an intake of >10 000 IU retinol in the form of supplements and animal studies reporting the occurrence of defects of the cardiac outflow tract and other neural crest–derived structures (FDA category X at dosages >18 000 to 25 000 IU/d).

**Maternal Environmental Exposures**

**Organic Solvents**
Studies of this topic can be difficult because organic solvents often comprise a mixture of chemicals, because the composition varies between different commercial preparations, and because of limitations in the way that exposure was defined in retrospective case-control studies. A few have reported associations of cardiac defects with reported maternal exposure to solvents and paints. Reports of exposure to degreasing or other solvents have been associated with an increased risk of hypoplastic left heart syndrome, coarctation of the aorta, pulmonic stenosis, transposition of the great arteries with intact ventricular septum, tetralogy of Fallot, total anomalous pulmonary venous return, nonchromosomal atrioventricular septal defects, and Ebstein’s anomaly. Maternal reports of occupational exposure to organic solvents have been associated with an increased risk of VSDs and dyes, lacquers, and paints with conotruncal malformations and mineral oil products with coarctation of the aorta.

**Herbicides, Pesticides, and Rodenticides**
A study suggesting an association of maternal employment in the agricultural industry with an increased risk of conotruncal defects suggested a possible association with chemicals used in agriculture. In the BWIS, maternal reports of potential exposure to herbicides and rodenticides were associated with an increased risk of transposition of the great arteries and of potential exposure to pesticides with total anomalous pulmonary venous return and membranous VSDs. A case-control study of various potential sources and numerous measures of maternal exposure to pesticides and congenital anomalies found mixed results for conotruncal defects. A more recent case-control study of various end-product uses reported an increased risk of conotruncal defects with maternal reports of exposure to insecticides.

**Air Quality**
Two recent studies have examined possible associations of ambient air pollutants with CCVDs. One study conducted in southern California reported possible increased risks of any heart defects and of VSDs with increased ambient levels of carbon monoxide, of aortic artery and valve anomalies with increased levels of ambient air levels of ozone during the second month of pregnancy, and possible decreased risks of these defects with increased air levels of these pollutants during the third month of pregnancy. Another study conducted in 7 Texas counties evaluating potential exposures during weeks 3 to 8 of pregnancy reported possible increased risks of tetralogy of Fallot with carbon monoxide, isolated ASDs with particulate matter <10 μm in aerodynamic diameter, and isolated VSDs with similar dioxide, as well as a possible risk of isolated ASD with carbon monoxide and isolated VSD with ozone. These findings underscore the need for further studies using standard heart defect classification systems to elucidate whether the associations are real or are due to chance or bias.

**Groundwater Contamination**
The risk of congenital cardiac defects was reported to be greater among children of parents who had contact with areas that had groundwater contaminated with trichloroethylene than among children of parents who had no such contact. This study did not evaluate the relation between maternal water consumption and risk of cardiac defects. Another study that did evaluate maternal consumption of home tap water during the first trimester of pregnancy found an increased risk of cardiac anomalies.

**Water Chlorination Byproducts**
A possible association between maternal exposure to chlorination byproducts that result from the interaction of residual chlorine and organic matter in tap water and cardiac defects in offspring has been the subject of several investigations. These studies evaluated information on the type of chlorination treatment at the water plant or on levels of trihalomethanes measured at sampling points in the water distribution but not on actual levels of contaminants in water consumed or used for showering. These studies found no associations with cardiac defects.

**Other Environmental Concerns**
Evaluations of possible associations of heart defects with maternal exposure to ionizing radiation have been limited. The BWIS examined possible associations of heart defects with maternal reports of exposure to ionizing radiation in occupational settings or as part of medical or dental evaluations and found few reports of such exposures and no evidence of any associations. Concerns have been raised about the risk of birth defects in communities situated near hazardous waste sites or other sources of environmental pollution. Large population-based studies have evaluated this issue with mixed results. One study found an increased risk of all heart defects as a group, but the results were imprecise because of the small number of exposed cases (n=3). Two studies found no associations with cardiac defects. Surveillance data from population-based congenital anomaly registers in 16 regions of Europe (mainly Western Europe) were analyzed to evaluate the impact of the Chernobyl accident on the prevalence of selected congenital anomalies. Chernobyl had no detectable impact on the prevalence of congenital anomalies in Western Europe.
Maternal Sociodemographic Characteristics

Age
In the BWIS, maternal age was not associated with non-heritable CCVDs as a group. Analysis by specific defects found that maternal age of ≥30 years was associated with an increased risk of transposition of the great arteries (OR, 1.7; 95% CI, 1.1 to 2.7) and Ebstein’s anomaly (OR, 2.6; 95% CI, 1.4 to 4.8), that more advanced maternal age (>34 years) was associated with an increased risk of bicuspid aortic valve (OR, 2.5; 95% CI, 1.3 to 4.8) and ASDs (OR, 1.6; 95% CI, 1.0 to 2.5), and that young maternal age (<20 years) was associated with an increased risk of tricuspid atresia (OR, 2.8; 95% CI, 1.3 to 6.4). An analysis of nonchromosomal birth defects of the Metropolitan Atlanta Congenital Defects Program from 1968 to 2000 found associations of advanced maternal age (35 to 40 years) with an increased risk of all heart defects (OR, 1.2; 95% CI, 1.0 to 1.4), tricuspid atresia (OR, 1.2; 95% CI, 1.02 to 1.39), and right ventricular outflow tract defects (OR, 1.28; 95% CI, 1.10 to 1.49).

Race/Ethnicity
Racial/ethnic variations in risk of a specific CCVD have been noted by a number of reports. Compared with black infants, white infants have been found to have an increased prevalence of Ebstein’s anomaly, aortic stenosis, atrioventricular septal defects, ASDs, coarctation of the aorta, truncus arteriosus, transposition of the great arteries, tetralogy of Fallot, pulmonary stenosis, hypoplastic left heart syndrome, and a decreased prevalence of pulmonary stenosis. In a population-based study of variations in prevalence of birth defects in offspring of Hispanic and black women in California between 1987 and 1997, no variations in prevalence were noted compared with the prevalence in offspring of non-Hispanic white women.

Reproductive History
A history of reproductive problems has been associated with an increased risk of tetralogy of Fallot (previous miscarriage: OR, 1.5; 95% CI, 1.0 to 2.2), nonchromosomal atrioventricular septal defects, ASDs, coarctation of the aorta, truncus arteriosus, transposition of the great arteries, tetralogy of Fallot, pulmonary stenosis, hypoplastic left heart syndrome, and a decreased prevalence of pulmonary stenosis. In a population-based study of variations in prevalence of birth defects in offspring of Hispanic and black women in California between 1987 and 1997, no variations in prevalence were noted compared with the prevalence in offspring of non-Hispanic white women.

Maternal Stress
Maternal stress as measured by maternal reports of job loss, divorce, separation, or death of a close relative or friend was found to be associated with an increased risk of conotruncal heart defects (OR, 2.4; 95% CI, 1.4 to 4.2) in a case-control study in Atlanta. A more recent case-control study in California obtained a similar result (OR, 1.4; 95% CI, 1.0 to 2.1) with a stronger effect among offspring of mothers who had not completed high school (OR, 2.4; 95% CI, 1.3 to 4.8).

Paternal Exposures
There is growing concern that paternal factors may play a role in the origin of congenital defects in general and of CCVDs in particular. New dominant mutations are more common in older fathers, and paternal age has been shown to be associated with birth defects such as achondroplasia and Alpert syndrome and in genetic conditions known to affect the cardiovascular system such as Marfan syndrome; the average age of fathers of children with sporadic or new mutation forms of Marfan syndrome was greater (37 years versus 30) than the general population. Paternal factors also have been shown to be important in diseases thought to have a combined genetic and environmental origin such as diabetes mellitus; children of a type 1 diabetic father have a greater likelihood of developing type 1 diabetes mellitus than children of a mother with diabetes. This section examines the evidence for various paternal factors.

Paternal Age
Several studies have focused on paternal age as a risk factor for congenital cardiac defects in offspring. Olshan et al evaluated the effect of paternal age on the risk of congenital heart defects in 4110 cases of congenital heart defects from the British Columbia Health Surveillance registry; matched controls were obtained from the birth files of British Columbia. The association of paternal age with 8 cardiac defects was examined after controlling for maternal age and other risk factors. A general pattern of increasing risk with increasing paternal age was found for ASDs, VSDs, and PDA. Offspring of men <20 years of age were also at higher risk for VSDs (OR, 2.0; 95% CI, 1.1 to 3.6) and possibly ASDs (OR, 1.9; 95% CI, 0.9 to 4.3). A separate study by Lian et al using data from the Metropolitan Atlanta Congenital Defects Program, also found an increased risk for ASDs and VSDs with increasing paternal age after adjustment for maternal age and race. In contrast, a Chinese study found no relationship between advancing paternal age and congenital heart defects. In fact, risk was higher for men <25 years of age compared with men ≥25 years of age at the time of the child’s birth (OR, 2.27; 95% CI, 1.85 to 2.79).

Risk for men ≥25 years of age also was increased for VSDs, PDA, and tetralogy of Fallot. Similarly, an analysis of data from the BWIS that focused on isolated membranous VSDs found no association with paternal age.

Other Paternal Exposures
Some studies have been conducted to evaluate the role of paternal exposures in the origin of congenital heart disease, but the number of studies is limited, and the results are inconclusive. The BWIS reported an association of paternal cocaine use with an increased risk of any CCVD in general and with VSDs and tricuspid atresia in particular. An analysis of data from the BWIS performed by Ewing et al found that reports of paternal marijuana use (OR, 1.36; 95% CI, 1.10 to 1.69) and use of cocaine among older fathers (OR, 3.92; 95% CI, 1.30 to 11.86) were associated with the occurrence of an isolated membranous VSD in offspring. Other authors suggested that 5% of cases of isolated membranous VSDs may be attributed to older fathers who used cocaine. The potential for recall bias associated with illicit drug use makes it difficult to interpret the conclusiveness of these findings.
Savitz et al.206 evaluated the influence of paternal factors on congenital cardiac anomalies using data from 1959 to 1966 Kaiser Health Plan members who participated in the Child Health and Development Study. The authors could not demonstrate any statistically significant relationships, although trends were identified for paternal cigarette smoking, alcohol intake, and older age.

Discussion

This statement provides a summary of well-known prenatal maternal conditions or exposures associated with an increased risk for cardiac defects (ie, definite risk factors) such as maternal rubella infection, phenylketonuria, diabetes, thalidomide, vitamin A congeners/retinoids, and indomethacin tocolysis. In addition, this article summarizes available information on several prenatal maternal and paternal factors that also may alter the risk for cardiac defects in the offspring (ie, possible risk factors). Particularly noteworthy is the suggestion that maternal use of multivitamin supplements containing folic acid during the periconceptional period may be associated with a reduced risk for some cardiac defects. This association and others reported in the literature warrant further evaluation because findings thus far are based on limited studies and/or tend to be mixed. This article also summarizes available information on a wide range of factors for which no associations have been noted or the evidence has been found to be insufficient to assess the risk for CCVDs.

Caveats

In interpreting findings on possible associations between nongenetic factors and CCVDs, we must remember that such associations from observational studies may be due to the exposures or factors of interest, but they may also be a result of chance, bias, or confounding. An observational study can yield an association as a result of sampling variation of the controls or multiple comparisons in an exploratory study. Recall bias is a potential concern because assessment of exposure to many factors (eg, first-trimester fever, medication use, consumption of vitamin supplements, solvents) often is based on parental recall after the birth of the child. Confounding is also of concern in that an apparent association between reported analgesic use and a heart defect might be due to confounding by the condition for which the analgesic was taken (eg, influenza or a febrile illness), and the apparent protective effect of multivitamin supplement use might be due not to the use itself but to the behavior of the user. Because some maternal illnesses can result in treatment with medications, uncertainty remains in some areas regarding independent effects of the disease or its treatment on fetal risk. A lack of an association between exposure and disease risk may be real, but it also may reflect effect dilution resulting from grouping of phenotypes with different inherent susceptibilities or errors in exposure assessment. In this review, most of the findings on risk factors come from case-control studies, and the best available information comes from 2 large population-based case-control studies specifically designed to investigate risk factors for congenital heart disease in an exploratory manner: the BWIS conducted in the Baltimore-Washington area between 1981 and 19896 and the study conducted in Finland by the National Public Health Institute in Helsinki of cases and controls born during 1982 to 1984.7 Although these larger, population-based studies used standardized methods for ascertaining and classifying cardiac defects, control selection, and methods to minimize potential biases and confounding, the above methodological issues may still be present. Therefore, the consistency of the findings from among multiple well-designed studies is particularly important.

Implications for Prevention

With these caveats in mind, the information presented here and the precautionary principle207–209 yield some guidelines that could be useful to prospective parents who wish to minimize their chances of having a baby with a CCVD. These guidelines are listed in Table 7. It is important to note that these guidelines are aimed at minimizing potential prenatal exposure to risk factors for congenital heart defects only, not other adverse health outcomes. Prospective parents should discuss important health behaviors that may affect a pregnancy such as nutrition, physical activity, lifestyle, and occupation with their primary care provider or obstetrician. Women of childbearing age should take multivitamins containing folic acid on a daily basis in the periconceptional period and should avoid certain types of behaviors such as exposure to organic solvents. Women of childbearing age also should obtain prenatal care, including testing for diabetes and past rubella exposure; should discuss any medication use with their obstetrician; and should avoid contact with ill people, especially those with rubella or influenzalike illnesses.

Recommendations also are possible for screening for possible cardiac defects using fetal echocardiography during pregnancy when warranted by reports of prenatal maternal illnesses or exposures. The need for screening any individual should be made on an individual basis from the type, likelihood, and level of potential exposure, as well as the time of gestation during which it occurred. This decision typically will be made as a result of the obstetrical history.

Ultimately, the aim of epidemiological studies is to provide information necessary for development of prevention policies and interventions. Because congenital heart defects represent

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**TABLE 7. Recommendations to Prospective Parents Based on Evidence and the Precautionary Principle**

<table>
<thead>
<tr>
<th>Recommendation</th>
<th>Details</th>
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<tr>
<td>Mothers who wish to become pregnant should:</td>
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<tr>
<td>Take a multivitamin with folic acid daily</td>
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<tr>
<td>Obtain preconception and prenatal care with specific attention to detection and effective management of phenylketonuria and diabetes and vaccination for rubella</td>
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<tr>
<td>Discuss any medicine use with your doctor, even over-the-counter medications</td>
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<tr>
<td>Avoid contact with people with flu or other febrile illnesses</td>
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<tr>
<td>Avoid exposures to organic solvents</td>
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*These are recommendations based on evidence available in the medical literature to reduce risk of offspring with a congenital heart defect only. Prospective parents should discuss other important health behaviors with their healthcare provider and/or obstetrician.*
some of the more prevalent birth defects, result in significant lifelong morbidity, and are an important cause of mortality attributed to birth defects, the development of effective prevention interventions is paramount from a public health perspective. However, the evidence base to support the development and implementation of effective prevention policies and interventions specifically directed at reducing the public health impact of congenital heart defects is somewhat limited.

Nevertheless, some strategies may be considered that may help to ameliorate risk for congenital heart defects on a population basis. In part, these must be based on concern regarding a broader set of risks for pregnancy outcomes other than heart defects alone. Preconception care and appropriate dietary management for women with phenylketonuria should be an important strategy. Detection and appropriate management of diabetes before and during pregnancy should be an important priority, given the increasing prevalence of type 2 diabetes and glucose intolerance in the general population. Guidelines for managing diabetes before and during pregnancy have been published by the American Diabetes Association.202,203 Ensuring that women of childbearing age are immunized against rubella is also an important and practical strategy. Medications that are suspected of causing congenital defects, including congenital heart disease, should have warnings about that risk to allow mothers and physicians to make informed decisions about the risks and benefits of use of the medication during pregnancy. One strategy that has already been implemented is the recommendation for use of prenatal vitamins. Continuing to emphasize the importance of using prenatal vitamins containing folic acid is practical and important.

Implications for Further Research
Information available regarding several potential noninheritable risk factors for congenital heart defects is limited because of few studies, few exposures of mothers or fathers to yield highly reliable findings, or possible methodological issues. A recent example of this problem of limited available information involves the drug paroxetine. The FDA has recently changed the pregnancy category of this drug from C to D because of concerns related to possible increased risk of congenital cardiac malformations in the fetus raised by preliminary results from epidemiological studies. A warning has been placed in the prescribing information for the drug and on the FDA Web site (http://www.fda.gov/medwatch/safety/2005/safety05.htm#Paxiil3). No evidence-based studies have been published in the scientific literature to date. Clearly, further research on many of the potential risk factors discussed in this statement is needed to expand the evidence base needed for the development of prevention strategies. The potential for expansion of the evidence base may be realized within the next few years, with the recent implementation of 2 large population-based studies in which standard methods for classification and grouping will be used. One of these is the National Birth Defect Prevention Study (NBDPS), a multicenter population-based case-control study of birth defects, which has been ascertaining and collecting clinical information on children with birth defects, including congenital heart defects, on an ongoing basis since 1997.212 This is the largest case-control study of birth defects conducted in the United States and will include one of the largest collections of cases of heart defects from several regions of the country. The NBDPS will facilitate evaluation of a wide array of known and suspected risk factors for subgroups of the population and will enable investigators to evaluate the relation between various types of heart defects and candidate genes, environmental factors, and gene-environment interactions. A number of data analyses have already been initiated, and some results should become available within the next few years. Another potential future source of information is the National Children’s Study (NCS).213 This study will explore a broad range of environmental factors that influence health and well-being of children. Because this study plans to examine ≈100 000 children across the United States and follow them during prenatal development, through birth, in childhood, and into adulthood, it will provide opportunities to evaluate prospectively the impact of prenatal exposures on some of the more common heart defects, as well as the developmental outcomes, other comorbidities, transition to adulthood issues, and the survival experience of children with heart defects.

Conclusions
In conclusion, this statement summarizes the current state of knowledge of noninherited risk factors for both mothers and fathers that may increase or, in some cases, decrease the likelihood that a congenital cardiac defect may occur in offspring. Much of the recent evidence is preliminary and may not ultimately prove to be causal. Nevertheless, some reasonable recommendations are offered to prospective parents and healthcare professionals that may reduce the risk of having a child with a congenital cardiac defect based on the current state of knowledge for the prevention of other birth defects and the precautionary principle. Similarly, pregnancies with some types of maternal exposures may warrant prenatal screening with fetal echocardiography. To date, no public policies or interventions are specifically directed at reducing the public health impact of congenital heart disease. However, new studies such as the NBDPS or the NCS may yield evidence needed to support the development of such policies or interventions in the future.214–218

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We gratefully acknowledge the assistance and research support provided by Dr Janine Politka and Jenny Nakahara from Teratogen Information System in compiling the sections on maternal therapeutic and nontherapeutic drug exposure. We would also like to thank Dawn England, MPH, Children’s Hospital Boston, and Ralphella Washington, Children’s Memorial Hospital, for their support during the writing of this statement.
Disclosures

Writing Group Disclosures

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<th>Writing Group Member</th>
<th>Employment</th>
<th>Research Grant</th>
<th>Other Research Fund</th>
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This table represents the relationships of writing group members that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all members of the writing group are required to complete and submit.

Reviewer Disclosures

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<td>None</td>
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<td>James H. Moller</td>
<td>University of Minnesota</td>
<td>None</td>
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<td>None</td>
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<tr>
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<td>California Birth Defects Monitoring Program</td>
<td>None</td>
<td>None</td>
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This table represents the relationships of reviewers that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all reviewers are required to complete and submit.

References


The goal of this review is to provide more information for clinicians on the expanding knowledge of the involvement of genetic contributions to the origin of congenital heart disease (CHD). There has been a long-standing clinical view that most CHD occurs as isolated cases. On the basis of studies of recurrence and transmission risks, a hypothesis of multifactorial etiology was proposed. In this type of inheritance, the genetic predisposition of the individual interacts with the environment to cause the congenital heart defect. In recent years, separate environmental and genetic causes have been identified. Classic mendelian transmission of congenital heart defects in some families has been described in the literature. In the past decade, molecular genetic studies have exploited these observations of families with multiple affected individuals and have provided insights into the genetic basis of several forms of CHD, such as atrial septal defect or patent ductus arteriosus. These initial discoveries demonstrate that the genetic contribution to CHD has been significantly underestimated in the past. This review includes descriptions of the currently available diagnostic tools and their applications. Some syndromes, including DiGeorge syndrome, Williams-Beuren syndrome, Alagille syndrome, Noonan syndrome (NS), and Holt-Oram syndrome, have been highlighted in the text for the purpose of illustrating some of these new technologies. For further clinical details, interested readers are referred to a genetics textbook such as Smith’s Recognizable Patterns of Human Malformation.

In reading this review, it is important to remember that human cardiovascular genetics is in the early phase of gene discovery; consequently, the field is changing rapidly. Ge-
nomic testing of embryos, fetuses, children, and adults, in both research and clinical settings, is expanding more quickly than are regulatory and surveillance programs. As part of these changes, clinically available genetic tests for various forms of CHD move from the research laboratory to the bedside or clinic at variable speeds. The pace of discovery is such that today’s state of the art quickly becomes outdated. As a means of keeping abreast of the latest genes and availability of testing, the reader is referred to online resources such as Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/) and GeneTests (http://www.genetests.org/), which are updated regularly.

Prevalence of CHD

Cardiac malformations present at birth are an important component of pediatric cardiovascular disease and constitute a major percentage of clinically significant birth defects, with an estimated prevalence of 4 to 50 per 1000 live births. For example, it is estimated that 4 to 10 liveborn infants per 1000 have a cardiac malformation, 40% of which are diagnosed in the first year of life.5,6 The true prevalence, however, may be much higher. For example, bicuspid aortic valve, the most common cardiac malformation, is usually excluded from this estimate. Bicuspid aortic valve is associated with considerable morbidity and mortality later in life and by itself occurs in 10 to 20 per 1000 in the general population.7 Recent studies are finding a high degree of heritability of bicuspid aortic valve, alone and with other cardiovascular anomalies, especially left ventricular outflow tract obstructive disorders.9-11 When isolated aneurysm of the atrial septum and persistent left superior vena cava, each of which occurs in 5 to 10 per 1000 live births, are taken into account, the incidence of cardiac malformations approaches 50 per 1000 live births.12 The incidence of ventricular septal defect (VSD) has also been demonstrated to be as high as 5% in 2 independent cohorts of 5000 serial newborns and 5000 serial premature infants in Israel.13,14 In light of the above considerations, an incidence of CHD of 50 per 1000 live births is a conservative estimate.15,16

In the year 2000, the prevalence of CHD in the pediatric population was estimated at approximately 623 000 (320 000 with simple lesions, 165 000 with moderately complex disease, and 138 000 with highly complex CHD).16 Tremendous advances in medical and surgical care of children with CHD over the past decade have made survival into adulthood a reality. At the time of the Bethesda Conference in 2000, an estimated total of 787 000 adults were living with CHD (368 800 with simple disease, 302 500 with moderately complex disease, and 170 700 with highly complex disease).17,18 This assessment of prevalence in the adult population is likely low, because many adult patients, particularly minorities, have been lost to follow-up. It has been estimated that the population of adults with CHD is growing by ~5% per year, which predicts that the total adult CHD population likely reached 1 million by 2005.19 This means that the number of adults living with CHD has for the first time surpassed the number of children with CHD. Clearly, it is imperative that many disciplines within the medical community, including adult cardiologists and thoracic surgeons, internists, obstetricians, family practitioners, and ancillary healthcare personnel, acquire an understanding of CHD and its inheritance so that proper lifetime care can be provided for this burgeoning patient population, which to date has been largely unfamiliar to all but pediatricians and pediatric cardiologists.

Importance of Identifying the Genetic Basis of CHD

Extraordinary diagnostic precision and definitive therapies with relatively low morbidity and mortality characterize the state of the art in the management of most CHD (eg, the arterial switch operation for transposition of the great arteries or device closure of intracardiac shunts). These types of therapies indicate that more and more individuals with CHD are going to live to adulthood and may have the opportunity to reproduce. Although there have been tremendous advances in diagnosis and treatment of CHD, our knowledge of the causes of CHD has been limited but has advanced in recent years. Despite the many advanced therapies currently available for a number of heart defects, significant morbidity and mortality are still associated with some types of CHD, for example, hypoplastic left heart syndrome. Improved understanding of possible causes will permit insight into the pathobiological basis of the congenital heart problem and allow definition of disease risk, 2 critical elements for disease prevention. For the clinician caring for a child with CHD, it is very important to determine whether there is an underlying genetic pattern (eg, deletions, duplications, or mutations), for the following reasons: (1) there may be other important organ system involvement; (2) there may be prognostic information for clinical outcomes; (3) there may be important genetic reproductive risks the family should know about; and (4) there may be other family members for whom genetic testing is appropriate. The following sections describe currently available techniques for evaluating infants and children with CHD.

Current Genetic Techniques for Evaluation of Congenital Heart Defects

Congenital heart defects often occur in the setting of multiple congenital anomalies, including abnormal facial features, or in association with limb anomalies, other organ malformations, developmental abnormalities, or growth abnormalities. We now have a number of genetic tests that can assist the clinician in diagnosing genetic alterations in the child with CHD. These include cytogenetic techniques, fluorescence in situ hybridization (FISH), and DNA mutation analysis. After discussion of these techniques, some syndromes that illustrate the use of these genetic techniques will be highlighted, and finally, a suggested approach for comprehensive assessment of these children is provided with an algorithm.

Chromosome Analysis

Before the availability of advanced cytogenetic techniques such as FISH, standard chromosome analysis revealed chromosomal aberration in 8% to 13% of neonates with CHD.20 With improved resolution in cytogenetic analysis and the availability of molecular techniques, the prevalence of chromosomal abnormalities in selected congenital heart defects is now estimated to be much higher.21 In contrast, of all children with chromosomal abnormalities, at least 30% have a con-
genital heart defect, with the incidence varying from that of the general population to nearly 100%, as in trisomy 18. Therefore, chromosomal analyses in children with various types of CHD, especially if they have other organ system anomalies, is currently an important part of their medical evaluation (Appendix 1).

The standard metaphase karyotype (450 to 550 bands) is diagnostic for many chromosomal disorders, especially those of chromosome number such as trisomy (trisomy 21) or monosomy (45,X or Turner syndrome). A more sensitive test, high-resolution banding, evaluates chromosomes in prometaphase, which allows for the visualization of a greater number of bands (550 to 850 bands) than the standard karyotype. This technique better defines chromosomal structural abnormalities such as duplications, translocations between chromosomes, and interstitial or terminal deletions. In most centers, 7 to 14 days is required for standard karyotyping and up to 3 weeks for high-resolution banding. More advanced cytogenetic techniques, such as FISH, are required to diagnose more subtle structural abnormalities, such as microdeletions, tiny duplications, and/or subtle translocations. FISH probes (see below) for chromosomes 13, 18, and 21 are currently available for use on interphase (nondividing) cells to diagnose chromosomal trisomies in a more timely fashion, ie, 1 to 2 days, as would be helpful if one of these trisomies were suspected in a neonate.

Chromosomes can be analyzed from a number of sources, including peripheral blood lymphocytes, cord blood, skin fibroblasts, amniotic fluid, chorionic villi, and bone marrow, with peripheral blood most commonly used. Prior blood product transfusions are not likely to interfere with chromosome testing considering the small volume of the transfusion in relation to the total blood volume of the patient, and especially if leukoreduced and/or irradiated blood products have been used.

Amniotic fluid cells are the primary means of prenatal chromosomal diagnosis. Amniocentesis is routinely performed at 15 to 16 weeks’ gestation. Amniotic fluid cells, however, take 1 to 2 weeks to grow and harvest before karyotyping can be done. Chorionic villus sampling involves the biopsy of tissue from the villous area of the chorion transcervically or transabdominally, between 10 and 12 weeks’ gestation. These results are usually available in 10 to 14 days. The major advantage of chorionic villus sampling compared with midtrimester amniocentesis is that chorionic villus sampling allows the results to be available at an earlier stage of the pregnancy, which reduces the period of uncertainty.

In the current era of in vitro fertilization, preimplantation genetic diagnosis for chromosomal abnormalities/aneuploidies and single-gene defects has recently become possible. Preimplantation genetic diagnosis provides chromosomal and mutational analysis of blastocysts that result from in vitro fertilization before implantation. Preimplantation genetic diagnosis is primarily used by patients choosing assisted reproductive services who have concerns regarding risks of specific genetic disorders. The techniques used for prenatal or preimplantation diagnosis have inherent risks and benefits, which should be discussed on an individual basis with the treating physician. For more detail, the reader is referred to recent reviews of prenatal or preimplantation diagnosis.

**FISH Technology**

FISH is a method by which biotinylated test and control DNA probes are hybridized with metaphase chromosomes to determine whether 1 (deletion), 2 (normal), or 3 (duplication) copies of the test region are present. Specific DNA probes can be located by fluorescence microscopy and will identify well-known deletion syndromes such as del 5p (cri-du-chat). Other fluorescent DNA probes are useful in determining microdeletion syndromes that cannot be detected visually. Several disorders, including Williams-Beuren, Alagille, and the 22q11 deletion syndromes, have been associated with a consistent microdeletion that frequently can be detected only by FISH technology. This technology is widely available in almost every cytogenetics laboratory for the syndromes noted.

**Telomere Analysis by Subtelomere FISH**

Tiny deletions, duplications, or subtle translocations involving the most distal ends of each chromosome (telomeres) may be quite difficult to detect by standard or high-resolution karyotype techniques. Newly developed fluorescent DNA probes for many interstitial chromosomal regions now provide the ability to detect abnormalities that involve the subtelomere-telomere regions (subtelomere FISH). The distal segments of the chromosomal telomeres are composed of telomere-associated repeat sequences, and these extend 100 to 300 kb from the terminal repeat sequences. Chromosome-specific unique sequences are present in these terminal regions, and fluorescent DNA probes can be specifically targeted to these areas. The subtelomere regions are thought to contain a very high concentration of genes; thus, rearrangements in these regions may have a significant impact on the phenotype of the individual. Subtelomere FISH probes with fluorescent DNA have been commercially developed for each end of the chromosome arms except for the short arms of the acrocentric (centromere near 1 end) chromosomes. If the karyotype is normal in a patient with dysmorphic facial features, congenital anomalies, developmental delay, and mental retardation, then the clinician should consider ordering subtelomere FISH studies for further genetic evaluation.

Cardiac malformations reported to date in children with subtelomere chromosomal rearrangements include aortic arch anomalies, VSD, atrial septal defect, mitral valve insufficiency, and concomitant pulmonary stenosis with VSD. Most of the published studies of subtelomere abnormalities indicate that a 4% to 9% prevalence of subtle chromosome rearrangements can be detected in children or adults with microcephaly, hydrocephaly, tracheoesophageal fistula, skeletal anomalies, multiple congenital anomalies, polycystic kidney, duodenal atresia, syndactyly, epilepsy, mental retardation, developmental delay, and/or dysmorphic facial features.

The use of subtelomeric FISH analysis has significant utility in individuals with normal karyotypes, especially if there are multiple congenital anomalies that include mental...
retardation or CHD.\textsuperscript{36} By finding a tiny deletion, duplication, or unbalanced translocation, further investigation of other family members can uncover the exact genetic risks faced by the family and the affected individual. As many as 50\% of families can have other individual members with subtelomeric abnormalities.\textsuperscript{37} Because some polymorphic variants and cross-hybridizations of subtelomeric FISH probes are known, families in whom a subtelomeric abnormality is identified should be seen by a medical genetics specialist to provide appropriate evaluation and counseling.

**Methods of Gene Discovery**

Initial strategies of gene discovery were directed toward isolating a protein of interest, sequencing a portion of it, and then cloning the gene that produces that protein. This approach works well for disorders for which the function of the target protein is obvious and facilitates its identification, eg, Pompe disease (acid $\alpha$-glucosidase deficiency). Currently, disease gene discovery can be accomplished by positional cloning, a candidate gene approach, or a combination of these 2 methods.\textsuperscript{38} Positional cloning has been referred to as reverse genetics. In this paradigm, investigators study families with affected individuals to identify a position on a chromosome that must contain the disease gene of interest, utilizing linkage analysis. That disease gene is then identified from among the set of all genes residing in that chromosomal region through cloning techniques. An example of the successful use of this strategy was the identification of the $\textit{NKX2.5}$ gene, for which the locus was defined from linkage analysis of large families.\textsuperscript{3} Some investigators have used this approach to identify a CHD gene in a syndromic disorder that is a single-gene trait. This approach is far less robust for finding disease genes when the disorder arises in a more complex genetic fashion or is heterogeneous, for example, patent ductus arteriosus.\textsuperscript{4} This may be the case for many forms of CHD. Using the candidate gene approach, investigators look for mutations in genes that encode proteins with relevance to the process in question. For CHD, this means that genes that control the formation and development of the heart (also known as cardiogenic genes) are candidates. A combination of these 2 methods, or the positional candidate approach, uses linkage analysis or identification of karyotypic abnormalities to find a region of a chromosome likely to contain the gene of interest. Candidate genes (cardiogenic) in that particular chromosomal region are then evaluated for mutations.

**DNA Mutation Analysis**

The cytogenetic methods described above identify large changes in chromosome number or structure. However, in certain disorders, changes occur at the level of a single gene and must be detected by alternative techniques. Genes are complex structures that include not only regions coding for the protein itself but also other sequences involved in regulation of gene activity. Currently, the coding region for the protein is evaluated for sequence changes for which the biological significance of an altered coding sequence can generally be interpreted. In contrast, the regulatory domains are not usually studied for sequence changes, because the regulatory domains for the gene may not be known, and the biological significance of the altered sequence is difficult to interpret.

Mutation analysis identifies changes in the coding sequence of the gene, including small deletions, insertions, or substitutions of nucleotides that alter the encoded amino acid and consequently protein structure. Most methods employ polymerase chain reaction–based assays. Indirect screening methods, such as denaturing high-performance liquid chromatography\textsuperscript{39} or single-strand conformation polymorphism,\textsuperscript{40} have been used extensively. More expensive exon-by-exon sequencing of genomic DNA has recently emerged. Additionally, newer, more cost-effective direct sequence analysis methods have become available.\textsuperscript{41} Such testing is usually done on DNA obtained from peripheral blood lymphocytes, but other tissues, such as skin, liver, muscle, buccal cells, or saliva, can be used, depending on their availability. DNA testing technology does have some limitations. For example, several types of mutations, including large deletions, other chromosomal structural abnormalities, and some changes that cause splicing errors, are difficult to detect by these approaches.

Once a sequence variation is identified, it is important to consider whether this variation is disease related. The basic criteria used to establish the disease-causing potential of the nucleotide sequence change are that it (1) is predicted to alter the gene coding sense, gene splice site, or regulatory region of the encoded protein; (2) segregates with disease in a kindred; and (3) is not found in unrelated, unaffected control chromosomes. The occurrence of a change in an evolutionarily conserved sequence domain provides additional support that the sequence change is disease causing. Although each of these criteria should be met by any disease-causing mutation, supporting evidence will come from the demonstration that affected individuals from other unrelated families have mutations in the same gene.

Another major problem is the interpretation of the biological importance of mutations. In many instances, little is known of the role of the normal gene product in cardiac development or function, and in some instances, genes were not known to have any role in the heart before mutation identification (eg, in Alagille syndrome). To date, a variety of mutations that cause pediatric cardiovascular disease, including missense and frameshift mutations, have been identified. The extent and heterogeneity of the genes and the mutations identified thus far suggest that they are associated with a variety of pathogenetic mechanisms, including loss of expression, inactivation, or loss of function or gain of function of the mutated allelic products. The challenge of the future is to define the pathogenesis of disease-causing mutations, which in turn will provide opportunities to develop diagnostic and therapeutic strategies as alternatives to those now used.
Loci and Genes Associated With Congenital Heart Defects Identified to Date

Deletion Syndromes Identified by FISH Technology

DiGeorge Syndrome

DiGeorge syndrome was originally considered to be a rare developmental field defect encompassing derivatives of the branchial arch/pharyngeal pouch system. The syndrome is characterized by aplasia or hypoplasia of the thymus, aplasia or hypoplasia of the parathyroid glands, cardiac malformations, and specific facial features. Infants present with CHD, hypocalcemia, immunodeficiency, and facial dysmorphism. Ten to twenty percent of patients with DiGeorge syndrome have visible alterations that result in the loss of the proximal long arm of 1 copy of chromosome 22.44 On FISH, approximately 6% to 28%48 of parents are found to carry the 22q11 deletion.44

Subsequently, it has been shown that patients with the clinical diagnosis of DiGeorge, velocardiofacial (Shprintzen), or conotruncal anomaly face syndromes most often show a common genetic origin, namely, a 22q11 deletion.47 Not all patients with the clinical features of these syndromes have a 22q11 deletion, consistent with heterogeneous causes for the clinical features. For instance, some patients with similar clinical features may have a small deletion of the short arm of chromosome 10, or some of these features may also result from maternal diabetes mellitus or maternal alcohol use.

The clinical features of the 22q11 deletion syndrome are highly variable between affected individuals, even when they are related.48 The most common features include cardiovascular anomalies, palate anomalies, feeding disorders, speech and learning disabilities, renal anomalies, and behavioral disorders. Other abnormalities may include hypocalcemia, immunodeficiency, skeletal abnormalities, and growth hormone deficiency. Typical facial features may also include tubular nose, hypoplastic alae nasi, bulbous tip nose, low-set and/or dysplastic ears, and myopathic facies. A 22q11 deletion is inherited in an autosomal dominant fashion from a parent in approximately 6% to 28% of cases.48 In many familial cases, one of the parents is found to have a 22q11 deletion only after their child with CHD has been diagnosed as affected. All parents affected with 22q11 deletions are then at risk of carrying the deletion-bearing chromosome from an affected parent will be transmitted to the offspring. This is very important information for genetic family counseling.

The most common cardiovascular defects associated with a 22q11 deletion include tetralogy of Fallot, interrupted aortic arch type B, truncus arteriosus, conoventricular VSDs, and aortic arch abnormalities.50–52 Pulmonary stenosis, atrial septal defects, heterotaxy syndrome, and hypoplastic left heart syndrome have also been reported.

### TABLE 1. Estimated 22q11 Deletion Frequency in Congenital Heart Disease

<table>
<thead>
<tr>
<th>Cardiac Defect</th>
<th>Estimated Deletion Frequency, %</th>
<th>Reference(s)</th>
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<tr>
<td>Interrupted aortic arch</td>
<td>50–89</td>
<td>56, 57</td>
</tr>
<tr>
<td>VSDs</td>
<td>10</td>
<td>58</td>
</tr>
<tr>
<td>With normal aortic arch*</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>With aortic arch anomaly†</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Truncus arteriosus</td>
<td>34–41</td>
<td>51, 56, 59, 61, 61, 62, 61, 62</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
<td>8–35</td>
<td>51, 56, 59, 61, 61, 62, 61, 62</td>
</tr>
<tr>
<td>Isolated aortic arch anomalies</td>
<td>24</td>
<td>55</td>
</tr>
<tr>
<td>Double-outlet right ventricle</td>
<td>&lt;5</td>
<td>51, 56, 59</td>
</tr>
<tr>
<td>Transposition of the great arteries</td>
<td>&lt;1</td>
<td>51, 59</td>
</tr>
</tbody>
</table>

*Left-sided aortic arch with normal branching pattern.
†Includes right aortic arch and/or abnormal branching pattern, cervical location, and/or discontinuous branch pulmonary arteries.

Several studies have demonstrated that a 22q11 deletion is commonly found in a subset of patients with specific types of CHD (Table 1). Individuals with both a cardiac defect and an aortic arch anomaly (right aortic arch, cervical location, or abnormal branching pattern) are more likely to have a 22q11 deletion, as are a subset of patients with tetralogy of Fallot associated with absent pulmonary valve syndrome or aortopulmonary collaterals.53–55 Children with double-outlet right ventricle or transposition of the great arteries are rarely found to have a 22q11 deletion (Table 1).51,55–62

It is important to identify the cardiac patient with a 22q11 deletion by FISH testing to evaluate for associated noncardiac features of the syndrome in a timely fashion and to offer accurate genetic counseling. Additionally, a higher operative mortality in some individuals with a 22q11 deletion has been documented,55,64 and the clinician and surgeon should be aware of this when planning surgery and postoperative care, particularly as related to calcium metabolism or immunologic issues.

Discussions have centered around which cardiac patients should be routinely tested for a 22q11 deletion and at what age. It appears reasonable to test all infants with interrupted aortic arch type B or truncus arteriosus for a 22q11 deletion given the high frequency of a 22q11 deletion in those patients (Table 1). Using the same logic, data also support the testing of all infants with tetralogy of Fallot and one of the following associated features: absent pulmonary valve syndrome, aortic arch anomalies (including right aortic arch), pulmonary artery anomalies, or aortopulmonary collaterals (Table 1).53–55 A high frequency of 22q11 deletion also supports testing of patients with both perimembranous VSD and associated aortic arch abnormalities58 or those with isolated aortic arch abnormalities55 (Table 1).

Much debate on testing strategies has focused on infants with tetralogy of Fallot who have a normal aortic arch and branching pattern. This subset comprises a large patient population, of which 6% are estimated to have a 22q11 deletion.51 To clinically detect the deletion-bearing patient, the infant should be evaluated for hypocalcemia, thymic size, typical facial features, palate anatomy, or nasal regurgitation.
with feeding on a routine examination (Table 2). The older child with a suspected 22q11 deletion could be evaluated for speech and learning disabilities, endocrine abnormalities, immune dysfunction, or other recognized syndromic abnormalities (Table 2). However, clinical assessment for syndrome features alone of the at-risk individual may not consistently identify the infant carrying a 22q11 deletion. Therefore, more routine FISH testing of at-risk infants is likely warranted.

In particular, facial features may be the only associated syndromic finding in the newborn and can be difficult to detect in that age group.62 Such patients may be uncommon and would presumably be identified at an older age when other syndromic features and symptoms became more apparent. But these data also argue for a more comprehensive testing strategy to identify all infants with tetralogy of Fallot and a 22q11 deletion. Ultimately, early diagnosis of the patient with a 22q11 deletion allows for appropriate treatment of associated noncardiac anomalies, including appropriate handling of blood products at the time of surgery (leukocyte-depleted and cytomegalovirus-negative blood for the immunocompromised patient). In addition, accurate and timely genetic counseling can be provided to the family, including information on recurrence issues. Other family members can then be tested appropriately. Therefore, early FISH testing in patients with specific types of CHD is currently suggested as outlined in Table 3.

Finally, prenatal testing for a 22q11 deletion should be strongly considered in the fetus with either interrupted aortic arch, truncus arteriosus, tetralogy of Fallot, VSD (perimembranous, conoseptal hypoplasia, or malalignment types only), or aortic arch anomaly.51,55,58 In the fetus, it is much more difficult to diagnose the 22q11 deletion syndrome by clinical appearance alone, because other features, such as facial dysmorphism, will not be sufficiently apparent to exclude the diagnosis. Appropriate genetic and family counseling is of critical importance in this situation.

**Williams-Beuren Syndrome**

Williams-Beuren syndrome (Williams syndrome) is an autosomal dominant disorder characterized by specific cardiovascular defects, infantile hypercalcemia, skeletal and renal anomalies, cognitive deficits, “social personality,” and elfin facies. Most cases arise de novo due to a chromosomal microdeletion. As with other deletion syndromes, Williams syndrome has a broad range of clinical presentations. Typical cardiovascular anomalies include supravalvular aortic stenosis, often in conjunction with supravalvular pulmonary stenosis and peripheral pulmonary stenosis. These arterial abnormalities constitute an elastin arteriopathy or vasculopathy caused by deletion of the elastin gene.65 The degree of cardiovascular involvement and the involvement of the pulmonary or aortic vessels varies widely. The supravalvular aortic stenosis has been shown to progress in many cases, whereas the supravalvular pulmonary stenosis or peripheral pulmonary artery stenosis usually regresses with time.66,67

Approximately 90% of individuals with a clinical diagnosis of Williams syndrome have been found by FISH to have a microdeletion at chromosome 7q11.23,65,68 Molecular analyses comparing clinical phenotype to genotype have demonstrated that this syndrome is a contiguous gene-deletion syndrome, ie, the deletion or alteration of specific genes in the deleted region corresponds with specific clinical features. Deletion of 1 copy of the elastin gene corresponds with the development of vascular manifestations of this disorder. Deletion of different genes in the region accounts for different manifestations of the disorder. Larger deletions, particularly deletions visible cytogenetically, can be associated with more severe clinical phenotypes, including seizures, which

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**TABLE 2. Age-Related Features of the 22q11 Deletion Syndrome**

<table>
<thead>
<tr>
<th>Newborn/infant age group</th>
<th>Findings detailed above</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific types of congenital heart disease (interrupted aortic arch, truncus arteriosus, tetralogy of Fallot, VSD, aortic arch anomaly)</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Aortic arch anomaly or discontinuous branch pulmonary arteries</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Overt or submucous cleft palate, high arched palate, bifid uvula</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Absent, hypoplastic, or abnormally located thymus</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Hypocalcemia</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Nasal regurgitation of feeds</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Feeding disorders/failure to thrive/gastroesophageal reflux</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Facial dysmorphism (especially abnormal ear or nose)</td>
<td>Findings detailed above</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Toddler/school-aged child</th>
<th>Findings detailed above</th>
</tr>
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<tr>
<td>Findings detailed above</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Feeding disorders</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Delayed emergence in speech</td>
<td>Findings detailed above</td>
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<tr>
<td>Hypermastoid speech</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Learning disabilities</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Behavioral disorders, including attention deficit hyperactivity disorder (ADHD)</td>
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</table>

<table>
<thead>
<tr>
<th>Adolescent/adult</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Findings detailed above</td>
<td>Findings detailed above</td>
</tr>
<tr>
<td>Psychiatric disorders, including bipolar disorders and/or schizophrenia</td>
<td>Findings detailed above</td>
</tr>
</tbody>
</table>

**TABLE 3. Suggested Testing Strategy for a 22q11 Deletion in the Congenital Heart Disease Population**

<table>
<thead>
<tr>
<th>All newborns/infants with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
</tr>
<tr>
<td>TOF</td>
</tr>
<tr>
<td>Isolated AAA</td>
</tr>
</tbody>
</table>

Discontinuous branch pulmonary arteries

Any newborn/infant/child with CHD and another feature of the 22q11 deletion syndrome

Any child/adolescent/adult with TOF, TA, IAA, VSD, or AAA not previously tested who has 1 other feature of the 22q11 deletion syndrome (see Table 2)

All fetuses with IAA, TA, TOF, VSD, or AAA (if amniocentesis performed for diagnostic purposes)

Consider all newborns/infants with VSD with normal aortic arch

IAA indicates interrupted aortic arch; TA, truncus arteriosus; TOF, tetralogy of Fallot; and AAA, aortic arch anomaly.

*Perimembranous, conoseptal hypoplasia or malalignment VSD.
are not typically seen in Williams syndrome. Given the clinical variability of Williams syndrome and the fact that many aspects of Williams syndrome are not particularly evident in a young infant or child, especially characteristic facial features, it is appropriate to consider testing all patients with supravalvular aortic or pulmonic stenosis for this specific microdeletion by FISH at the time of diagnosis of the cardiac disease. In addition, if peripheral pulmonary stenosis persists beyond infancy, it is also appropriate to assess these patients with FISH analysis for the Williams syndrome critical region.

Early diagnosis of Williams syndrome is important to initiate treatment for other potential medical problems (Table 4). In particular, hypercalcemia, which often occurs in the first year of life along with hypercalciuria, can be treated with appropriate diet or medication. Because hypercalcemia can be a risk factor for the development of nephrocalcinosis, making this diagnosis is important for prevention of extensive kidney damage, which can lead to renal failure. Screening for thyroid and renal anomalies will uncover anomalies that are unsuspected clinically. Routine follow-up of blood pressure measurements is needed because at least half of adults with Williams syndrome have systemic hypertension, and this can often be detected in childhood or adolescent years. Early identification of Williams syndrome is also essential for planning educational strategies that can enhance learning and development in children with Williams syndrome. The detection of a deletion also adds diagnostic certainty for the family and the responsible clinician. Appropriate testing of other family members and genetic counseling can then occur.

**Single-Gene Disorders**

In the past 15 years, considerable progress has been made toward identifying molecular genetic causes of selected congenital heart defects. As illustrated in the first part of Table 5, a number of selected congenital heart defects have been found to be associated with mutations in a variety of single genes. Some cardiac defects are related to mutations in >1 gene. It is highly likely that additional single-gene abnormalities (mutations) will be defined in the future. DNA testing for most of the genes for isolated congenital heart defects is unavailable except on a research basis at this time; however, testing of some of these genes is transitioning from the research laboratory to clinical availability. The clinician is advised to consult the Gene Tests Web site (http://www.genetests.org), a publicly funded medical genetics information resource, for updates on what testing is currently available.

The identification of causative gene mutations for genetic syndromes is also occurring at a rapid pace. A select group of syndromes in which the underlying single gene has been discovered is also listed in Table 5. For illustration purposes, Alagille syndrome, NS, and Holt-Oram syndrome will be discussed in greater detail. These single-gene disorders reflect the recent identification of genes responsible for congenital heart defects and for multiple other clinical features.

**Alagille Syndrome**

Alagille syndrome, an autosomal dominant disorder, was originally defined as the presence of bile duct paucity on liver biopsy in conjunction with 3 of the 5 following characteristics: cholestasis; cardiovascular, skeletal, or ocular anomalies; or typical facial features. Cardiovascular anomalies occur in >90% of individuals with Alagille syndrome. The most common cardiovascular features include peripheral pulmonary hypoplasia, tetralogy of Fallot, and pulmonary valve stenosis, although left-sided lesions and septal defects are also seen. Liver disease is highly variable from patient to patient and also within affected members of the same family. It is characterized by a paucity of intrahepatic bile ducts and can include chronic cholestasis, minimal liver enzyme elevation, hypercholesterolemia, or liver failure. Additional clinical features of Alagille syndrome are listed in Table 6.

A subset of Alagille patients (3% to 7%) have deletions of chromosome 20p12 detectable by karyotype or FISH analysis. The gene JAG1, which encodes a Notch ligand protein product, has been mapped into the commonly deleted region of 20p12. Mutations of JAG1 have been identified in patients with a broad spectrum of clinical phenotypes of Alagille syndrome, including patients with a predominant cardiac phenotype.
Patients suspected of having Alagille syndrome should undergo a karyotype and FISH analysis to check for a 20p12 rearrangement or deletion. Karyotype and FISH analysis are readily available in most cytogenetics laboratories, and the finding of a deletion or chromosomal rearrangement can be diagnostic for Alagille syndrome. If this diagnosis is confirmed by the cytogenetic testing, the child can be evaluated for other important features of Alagille syndrome, such as liver disease or additional vascular involvement. In addition, the cytogenetic results will most likely have a significant impact on the reproductive decisions some families will make in the future.

More than 90% of individuals with the classic phenotype of Alagille syndrome have a JAG1 mutation when the most sensitive and rigorous methods for mutation detection are used. JAG1 mutation analysis is now clinically available for those patients whose karyotype and FISH analyses are normal. Growing evidence suggests that patients with a strong family history of right-sided defects, such as peripheral pulmonary stenosis, valvar pulmonary stenosis, or tetralogy of Fallot, who do not otherwise fulfill the criteria for Alagille syndrome may also be appropriate for testing in this specific region. The finding of peripheral pulmonary stenosis or hypoplasia of the branch pulmonary arteries in a child, alone or in combination with tetralogy of Fallot, should prompt consideration of testing for Alagille syndrome. All patients with documented JAG1 mutations or suspected Alagille syndrome should have cardiac, hepatic, ophthalmologic (anterior chamber defects, pigmentary retinal anomalies, posterior embryotoxon), orthopedic (butterfly vertebrae), hematologic (bleeding tendency), and renal (structural, cysts, tubular acidosis) evaluations.

### TABLE 5. Genes Associated With Congenital Heart Defects in the Young

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gene(s)</th>
<th>Chromosome Location</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital heart defects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Familial congenital heart disease</td>
<td>NKK2.5 (CSX)</td>
<td>5q34-q35</td>
<td>3, 71–74</td>
</tr>
<tr>
<td>(ASD, atrioventricular block)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-TGA, DORV</td>
<td>CFCL</td>
<td>2p21</td>
<td>75, 76</td>
</tr>
<tr>
<td>D-TGA</td>
<td>PROSIT240</td>
<td>12q24</td>
<td>77</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
<td>ZFP/M/PG2</td>
<td>8q23</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>NKK2.5</td>
<td>5q34-q35</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>JAG1</td>
<td>20p12</td>
<td>79</td>
</tr>
<tr>
<td>Atrioventricular septal defect</td>
<td>CRELD1</td>
<td>3p21</td>
<td>80</td>
</tr>
<tr>
<td>ASD/VSD</td>
<td>GATA4</td>
<td>6p23</td>
<td>81</td>
</tr>
<tr>
<td>Heterotaxy</td>
<td>ZIC3</td>
<td>Xq26</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>CFCL</td>
<td>2p21</td>
<td>75, 76</td>
</tr>
<tr>
<td></td>
<td>ACVR2B</td>
<td>3p21.3-p22</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>LEFTYA</td>
<td>1q42.1</td>
<td>84</td>
</tr>
<tr>
<td>Supravalvar aortic stenosis</td>
<td>ELN</td>
<td>7q11</td>
<td>85, 86</td>
</tr>
<tr>
<td>Syndromes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holt-Oram syndrome</td>
<td>TBX5</td>
<td>12q24</td>
<td>87, 88</td>
</tr>
<tr>
<td>Alagille syndrome (PPS)</td>
<td>JAG1</td>
<td>20p12</td>
<td>89</td>
</tr>
<tr>
<td>Char syndrome (PDA)</td>
<td>TFAP2B</td>
<td>6p12</td>
<td>4</td>
</tr>
<tr>
<td>Noonan syndrome</td>
<td>PTPN11</td>
<td>12q24</td>
<td>90, 91</td>
</tr>
<tr>
<td></td>
<td>KRAS</td>
<td>12p1.21</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>SOS1</td>
<td>2p21</td>
<td>115, 116</td>
</tr>
<tr>
<td>CHARGE association</td>
<td>CHD7</td>
<td>6q12</td>
<td>93, 94</td>
</tr>
<tr>
<td>Ellis-van Creveld</td>
<td>EVC, EVC2</td>
<td>4p16</td>
<td>95, 96</td>
</tr>
<tr>
<td>Marfan syndrome</td>
<td>FBN1</td>
<td>15q21.1</td>
<td>97</td>
</tr>
<tr>
<td>Marfan-like syndrome</td>
<td>TGFB2</td>
<td>3p22</td>
<td>98, 99</td>
</tr>
<tr>
<td>Cardiofaciocutaneous syndrome</td>
<td>KRAS</td>
<td>12p12.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>BRAF</td>
<td>7q34</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>MEK1</td>
<td>15q21</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>MEK2</td>
<td>7q32</td>
<td>101</td>
</tr>
<tr>
<td>Costello syndrome</td>
<td>HRAS</td>
<td>11p15.5</td>
<td>102–104</td>
</tr>
</tbody>
</table>

ASD indicates atrial septal defect; D-TGA, D-transposition of great arteries; DORV, double-outlet right ventricle; PPS, peripheral pulmonary stenosis; PDA, patent ductus arteriosus; and CHARGE, coloboma, heart anomaly, choanal atresia, retardation, and genital and ear anomalies.
has not yet been suspected. This is helpful to make appropriate arrangements for comprehensive evaluation of clinical issues and to provide appropriate genetic counseling to the family regarding recurrence risk.

**Noonan Syndrome**

NS is a genetic multiple malformation disorder that includes short stature, typical facial dysmorphism, webbed neck, chest deformity, and cardiovascular abnormalities.\(^{112}\) The cardiac involvement is observed in 80% to 90% of affected individuals, with valvar pulmonic stenosis and hypertrophic cardiomyopathy being the most common.\(^{112,113}\) Other congenital heart defects observed in NS are secundum atrial septal defect, atrioventricular septal defect, mitral valve abnormalities, aortic coartation, and tetralogy of Fallot. Other non-cardiac features of NS include cryptorchidism, bleeding diathesis, and developmental delay. Additional features are listed in Table 7. Population prevalence has been estimated at

<table>
<thead>
<tr>
<th>TABLE 7. Clinical Features of Noonan Syndrome</th>
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<tbody>
<tr>
<td><strong>Cardiovascular</strong></td>
</tr>
<tr>
<td>Pulmonary artery stenosis or hypoplasia</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
</tr>
<tr>
<td>Valvar pulmonary stenosis</td>
</tr>
<tr>
<td>Atrial septal defect</td>
</tr>
<tr>
<td>Labile systolic hypertension</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
</tr>
<tr>
<td>Persistent cholestasis/jaundice</td>
</tr>
<tr>
<td>Hepatic ductular hypoplasia</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
</tr>
<tr>
<td>Abnormal liver function tests</td>
</tr>
<tr>
<td><strong>Distinctive facies</strong></td>
</tr>
<tr>
<td>Triangular face</td>
</tr>
<tr>
<td>Prominent forehead and chin</td>
</tr>
<tr>
<td>Hypertelorism</td>
</tr>
<tr>
<td><strong>Ophthalmologic</strong></td>
</tr>
<tr>
<td>Posterior embryotoxon</td>
</tr>
<tr>
<td>Axenfeld anomaly</td>
</tr>
<tr>
<td>Ectopic pupils</td>
</tr>
<tr>
<td>Pigmentary retinopathy</td>
</tr>
<tr>
<td><strong>Neurological</strong></td>
</tr>
<tr>
<td>Normal intelligence to moderate mental retardation</td>
</tr>
<tr>
<td>Hoarse voice</td>
</tr>
<tr>
<td><strong>Endocrine</strong></td>
</tr>
<tr>
<td>Delayed puberty</td>
</tr>
<tr>
<td>Growth retardation</td>
</tr>
<tr>
<td>Hypothyroidism</td>
</tr>
<tr>
<td><strong>Renal</strong></td>
</tr>
<tr>
<td>Horseshoe kidney</td>
</tr>
<tr>
<td>Renal compromise</td>
</tr>
<tr>
<td><strong>Other</strong></td>
</tr>
<tr>
<td>Butterfly vertebra</td>
</tr>
<tr>
<td>Conductive hearing loss</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 6. Clinical Features of Alagille Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiovascular</strong></td>
</tr>
<tr>
<td>Pulmonary artery stenosis or hypoplasia</td>
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<tr>
<td>Conductive hearing loss</td>
</tr>
</tbody>
</table>
1 per 1000 to 1 per 2500 live births. The trait is inherited in an autosomal dominant fashion, although a substantial fraction of cases are sporadic.

NS is genetically heterogeneous, which means that there are at least 3 NS disease genes, *PTPN11*, *SOS1*, and *KRAS*. With genetic linkage analysis and then positional candidacy, an NS disease gene on chromosome 12 was identified. It is *PTPN11*, which encodes a protein tyrosine phosphatase called SHP-2. SHP-2 plays an important role in signal transduction for a wide variety of biological processes, including the formation of the semilunar valves. Mutations in the *PTPN11* gene are observed in 40% to 50% of NS patients and are more prevalent among familial cases and among NS patients with pulmonary valve stenosis. NS patients with hypertrophic cardiomyopathy are unlikely to harbor a *PTPN11* mutation. Otherwise, there does not appear to be a strong correlation between the presence or absence of a *PTPN11* mutation and most other aspects of the NS phenotype (eg, mental retardation). Disease penetrance is nearly complete among those with *PTPN11* mutations, although phenotypic variability within families can be substantial.

Clinical mutation testing for *PTPN11*, *SOS1*, and *KRAS* is now available in the United States and elsewhere. These DNA tests can confirm the diagnosis of NS but cannot exclude it due to the genetic heterogeneity (ie, the individual could harbor a mutation in another NS gene that has not been identified as yet). Molecular confirmation is useful in borderline cases, especially in neonates and adults in whom the facial features of NS may not be obvious. Prenatal testing can be done when the fetus is at risk for inheriting a defined *PTPN11* mutation from an affected parent. Similar testing of suspicious prenatal, sporadic cases (eg, a fetus with cystic hygroma and pulmonic stenosis) suffers from the uncertainty that arises from the genetic heterogeneity.

There are 3 NS-related conditions for which *PTPN11* mutations can be found: LEOPARD syndrome, Noonan-like with multiple giant cell lesions, and certain hematopoietic disorders. LEOPARD syndrome is also a multiple malformation disorder; the name is an acronym that designates the cardinal features: multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. A high percentage of affected individuals have *PTPN11* mutations with certain missense defects that appear to be specific for LEOPARD syndrome rather than NS. Noonan-like with multiple giant cell lesions includes all of the features of NS plus the giant cell lesions of bone. The proclivity for involving the maxilla with expansile lesions leads to this disorder being a form of cherubism. Cardiac involvement appears to be highly similar to NS. Unlike LEOPARD syndrome, the *PTPN11* mutations have no specificity in predicting this disorder versus NS.

**Holt-Oram Syndrome**

Holt-Oram syndrome is an autosomal dominant ‘heart-hand’ syndrome that is characterized by congenital heart defects in patients with upper-limb deformities. This syndrome occurs in approximately 1 per 100 000 individuals, and although it can be inherited in a mendelian fashion, a significant portion of cases are sporadic. All patients have preaxial radial ray malformations (eg, triphalangeal, hypoplastic, or absent thumb and/or radial dysplasia), and three fourths of patients have septation (atrial and/or ventricular) defects and/or progressive atrioventricular conduction disease. Human genetic linkage analyses and positional cloning studies of affected families revealed that Holt-Oram syndrome is caused by mutations in the *TBX5* transcription factor gene (chromosome 12q24.1), and the *TBX5* transcription factor has proven to be a key regulator, particularly in combination with other transcription factors such as *NKH2.5* and *GATA-4*, of gene expression during embryogenesis, and loss of its activity markedly impairs development of the heart and limb.

Although there is significant genetic heterogeneity to the broader class of heart-hand syndromes, there is little if any genetic heterogeneity among Holt-Oram patients. Mutational analyses of the *TBX5* gene-coding regions will detect mutations in approximately three fourths of such patients, and the remainder are likely to have mutations in regulatory regions or to have deletions/insertions not detectable by current mutational analysis. Some studies find that fewer than half of Holt-Oram patients have *TBX5* mutations, which suggests genetic heterogeneity. However, these studies have been confounded by aggregation of patients who have other heart-hand syndromes with those who have Holt-Oram. Thus, careful and detailed clinical evaluations of the cardiovascular and other organ systems are essential to distinguish such other clinical syndromes (eg, Rothmund-Thomson syndrome, Okihiro syndrome, thrombocytopenia absent radius syndrome, and VACTERL association [vertebral anomalies, anal atresia, cardiac defect, tracheoesophageal fistula, renal abnormalities, and limb abnormalities]) that share features with Holt-Oram syndrome but are nonetheless clinically and genetically distinct.

Key to the accurate diagnosis of Holt-Oram syndrome is the uniform presence of upper-limb radial ray defects, which may be symmetrical or asymmetrical (even unilateral) regardless of the presence or absence of cardiovascular disease. Such limb deformity, for example, altered structure of a single carpal bone, may be quite subtle and only detectable radiographically, but individuals without such radial ray defects do not have Holt-Oram syndrome. Other limb malformations (eg, syndactyly of digits other than the thumb, polydactyly, or lower-limb defects), craniofacial abnormalities, and/or evidence of noncardiac visceral organ abnormalities (including heterotaxy) make Holt-Oram syndrome unlikely.

Most Holt-Oram structural cardiac defects are either ostium secundum atrial septal defects or muscular VSDs. Complex congenital heart defects have been seen in Holt-Oram syndrome patients with *TBX5* mutations, but they are rare events. Therefore, the demonstration of ostium primum atrial septal defects, membranous VSDs, or congenital valvular disease should at least prompt further detailed clinical evaluations of other organ systems and consideration of other diagnoses.

Among those individuals with Holt-Oram syndrome, most will have *TBX5* mutations that are nonsense or frameshift
mutations that are predicted to produce a 50% reduction in TBX5 gene dosage, that is, haploinsufficiency. Interestingly, there have been several reports of individuals with duplications of chromosome 12q segments encompassing TBX5 (and therefore potentially TBX5 overexpression), and such patients have clinical phenotypes that overlap with Holt-Oram syndrome. A minority of Holt-Oram syndrome is due to missense TBX5 mutations that do not alter the gene’s dosage. Although large family-based studies have suggested that many such missense TBX5 mutations have their greatest impact on either heart or limb development, compared with haploinsufficient TBX5 mutations that markedly deform both organ systems, these genotype-phenotype associations are not necessarily evident in the individual patient with Holt-Oram syndrome and are not clinically useful for predicting the individual patient’s phenotype.

Thus, in the setting of careful clinical evaluations of patients with suspected Holt-Oram syndrome, there is a rather limited role for TBX5 mutational analyses. When diagnostic clarity is not achieved clinically, TBX5 mutational analyses can provide adjunctive information. However, due to technical limitations of genetic assays used, the absence of a detected TBX5 mutation in an individual with a typical clinical presentation does not preclude a diagnosis of Holt-Oram syndrome. Thus, the most valuable setting for TBX5 genetic testing may be in establishing diagnoses for family members of a patient with previously established Holt-Oram syndrome and a known TBX5 mutation. For instance, McDermott et al. used genetic testing to rule out Holt-Oram syndrome in an individual with tetralogy of Fallot whose cousin had well-established Holt-Oram syndrome. TBX5 genetic testing has also been a useful addition to our assisted reproductive armamentarium. When in vitro fertilization is used as a reproductive strategy for an individual affected by Holt-Oram syndrome, blastocysts can be subjected to preimplantation genetic testing in vitro before their transfer back to the mother. If the affected parent’s TBX5 mutation is established before the in vitro fertilization cycle is begun, mutational analyses can occur in a sufficiently rapid and sensitive fashion that they can be the basis for embryo selection to achieve offspring who will not carry the TBX5 mutation and will therefore be unaffected by Holt-Oram syndrome.

Nonsyndromic Single-Gene Disorders

Studies have recently shown that nonsyndromic CHD can result from single-gene defects. Schott et al. identified mutations in NKX2.5 in 4 kindreds with atrial septal defects and atrioventricular conduction delay without other apparent syndromic features. The mutations were found only in affected individuals, were not present in control samples, and were demonstrated to change protein structure or function. Given that some members of these kindreds had either isolated atrioventricular conduction delay or other types of CHD, investigators subsequently studied additional kindred and sporadic cases with isolated atrioventricular conduction delay or CHD for NKX2.5 mutations. These studies identified likely disease-related mutations in a subset of cases with atrioventricular conduction delay and additional sequence alterations in patients with selected types of CHD.

The gene changes in patients with sporadic CHD were not identified in control subjects, and it was difficult to demonstrate their functional significance; thus, their relationship to the disease may not be proved. These studies demonstrate the complexity of the biological interpretation of some alterations and the likely complexity of the genetic contribution to CHD.

Investigators have also identified mutations of GATA4 in 2 kindreds with septal defects and no apparent syndromic features. Once again, the mutations identified were found in affected individuals but not in control samples and were shown to confer changes in protein function. Mutations in additional kindreds and subjects with septal defects have been reported subsequently. It remains to be seen whether mutations of GATA4 will be identified widely in patients with septal defects or in other sporadic cases of CHD: however, these studies highlight the utility of studying large kindreds to identify novel disease genes for CHD, and they demonstrate that single-gene disorders may be found in a subset of CHD. In addition, these studies identify critical molecular pathways involved in cardiovascular development and disease, given that the proteins encoded by NKX2.5, GATA4, and TBX5 are known to interact with one another in experimental systems.

Many cases of nonsyndromic CHD are unlikely to result from single-gene disorders. Instead, many cases of CHD are likely the result of multiple genetic alterations that increase susceptibility to CHD and interact with environmental factors. Already there is evidence of decreased penetrance and marked variability in expressivity of identical genetic alterations. For example, only 40% to 50% of children with trisomy 21 have CHD, and patients with a 22q11 deletion or even a single-gene defect (eg, JAG1) can present with markedly variable features. Such variable expressivity and penetrance is presumably explained by other genetic and environmental factors. These observations and the marked genetic heterogeneity already evident demonstrate the complexity of deciphering the genetic basis of CHD.

Evaluation for Genetic Basis in Children With CHD

Chromosome analysis and FISH testing for specific deletions are now accepted tools for the clinician. If the clinician finds a specific chromosome abnormality, it will provide the family with a clear explanation of the cause, allow the clinician to provide appropriate counseling about recurrence or lack of recurrence, and prompt the physician to investigate other potential medical problems known to be associated with the particular chromosomal anomaly.

Despite the rapidly advancing fund of knowledge, a genetic defect can only be identified through available testing in a minority of patients with CHD. Many of these children have abnormalities of other organ systems that indicate the presence of a known phenotype. In some cases, there may be a single-gene defect for which no testing is clinically available. In other instances, polygenic inheritance with or without an additive environmental component may be implicated. A complete understanding of the interactions between abnormal cardiac physiology and derangements in other organs is important for appropriate management and counseling in such patients. Therefore, it is useful for the physician caring...
for these patients to have an algorithm based on the initial presentation to assess for the presence of noncardiac abnormalities (Appendix 2).

The approach to the newly diagnosed patient with CHD should include routine examination of all relatives for a potential genetic contribution. Identification of some genetic causes of CHD has highlighted the importance of obtaining an accurate medical history of other family members and documenting an extended pedigree. In some forms of cardiovascular disease, for example, hypertrophic cardiomyopathy and Marfan syndrome, the familial nature (autosomal dominant inheritance) is well recognized; however, for other problems, for example, bicuspid aortic valve, family clustering has not been widely appreciated in the past. Recent studies have shown that a familial bicuspid aortic valve is likely to be inherited as an autosomal dominant condition with reduced penetrance. There is a 24% prevalence of bicuspid aortic valve in first-degree relatives of patients with left ventricular outflow tract obstruction. Increasingly, medical practice is evolving toward a recommendation that other family members undergo clinical evaluation, which may include an electrocardiogram and echocardiogram.

Specific assessment for physical features is warranted. In these situations, it is helpful and important to have a geneticist perform a complete examination to help uncover more subtle abnormalities. Other consultants, for example, from neurology, ophthalmology, orthopedic surgery, and otolaryngology, may be needed based on the suspected diagnoses.

Chest radiographs are performed in all newborn inpatients and many older patients who are diagnosed with CHD. Particular attention should be paid to skeletal defects and cardiac aortic arch, pulmonary, liver, and stomach situs. Additional radiographic tests that may also be indicated include abdominal/renal ultrasound, upper gastrointestinal series, liver-spleen scan, head ultrasound, and brain computed tomography or magnetic resonance imaging.

Cytogenetic testing should be considered in the following situations:

1. Any infant or child with the phenotype of a recognizable chromosomal syndrome (eg, trisomy 21 or 18)
2. Because not all chromosomal abnormalities result in a clinically recognizable syndrome, any infant or child with a congenital heart defect combined with (a) dysmorphic features, (b) growth retardation that cannot be explained by the heart defect, (c) developmental delay or mental retardation, or (d) multiple congenital anomalies
3. Infants or children with a family history of multiple miscarriages and/or siblings with birth defects
4. If major cardiac and/or other visceral organ malformations are documented by prenatal ultrasound and/or fetal echocardiogram

Impact on Patients and Families
For individuals with CHD and their families, identification of a genetic cause is very beneficial. This allows confidence in the diagnosis and allows the physician to explain the exact genetic mechanisms to the family. It also alerts the clinician to investigate other organ systems that may be involved in the syndrome and broadens the context of evaluation from the individual to other family members. In instances where a genetic cause such as Alagille syndrome has been identified in a family, genotyping may be very useful for stratifying “asymptomatic” family members into groups who should have cardiac evaluations and those for whom it is not necessary. Genotype-negative individuals have a low risk of developing pediatric cardiovascular disease, and clinical evaluation of such patients is not warranted. On the other hand, serial evaluation of genotype-positive individuals is essential to monitor development of the phenotype.

Ethical Considerations
Predictive genetic testing of children and adolescents has been the subject of numerous recommendations. Although there is no universal agreement about acceptable practices in pediatric genetic testing, consensus exists that pediatric genetic testing should not take place unless there are clinical benefits to be reaped as a direct result of testing before the patient reaches the age of majority. In addition, the struggle to obtain the pediatric analogue of informed consent is particularly important in genetic testing, in part because the long-term social and legal risks of genetic testing for pediatric patients are difficult to predict, and the risks are more difficult for a child to judge. On the other hand, genetic testing may determine a genetic mechanism of disease that provides an important opportunity for genetic counseling that benefits the entire family.
**Summary**

Ongoing research is now demonstrating that variations or alterations in genes contribute to the origin of CHD to a greater degree than previously suspected. This review has summarized the current knowledge of the genetics of CHD and has provided guidelines and algorithms to aid the clinician in making diagnoses and planning care. Many types of genetic testing are currently clinically available; other testing is still in the research phase. Awareness of this rapidly advancing field is important for all clinicians, and a multidisciplinary team approach to the child with CHD is necessary for comprehensive, state-of-the-art care. In addition to physicians and surgeons with expertise in CHD, a geneticist is a highly important member of this team.

Patients with CHD require multidisciplinary care. Their families deserve up-to-date genetic information as it relates to their child’s prognosis and to the kindred’s risk for future inheritance of genetic abnormalities associated with cardiac defects. Obstetricians will have involvement in these issues if prenatal echocardiography demonstrates CHD or if preimplantation genetic diagnosis and in vitro fertilization are requested. Pediatricians require knowledge about these issues in caring for multiple organ systems in children with genetic syndromes that include CHD. Families of these children will need information about recurrence risk. Pediatric cardiologists and pediatric cardiac surgeons are currently well equipped to care for patients with CHD, but they need to constantly update their understanding of the contribution of genetic abnormalities to these birth defects. As children grow into adulthood, internists, obstetricians, cardiologists, and thoracic surgeons will step in to care for CHD as it is superimposed on adult medical issues.

Research discoveries regarding the genetics and inheritance of CHD are rapidly occurring. As in all genetic research, ethical considerations for children with heart disease demand thorough and thoughtful reflection. It is hoped that dissemination of the information in the present report will result in improved diagnoses and care for children and adults with congenital cardiac disease. Through multidisciplinary care and research, the goal to prevent and improve clinical outcomes in CHD will guide future investigations.
### Appendix 1

<table>
<thead>
<tr>
<th>Chromosomal Disorder</th>
<th>Main Features</th>
<th>Percent With CHD</th>
<th>Heart Anomaly</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletion 4p (Wolf-Hirschhorn syndrome)</td>
<td>Pronounced microcephaly, widely spaced eyes, broad nasal bridge (Greek helmet appearance), downturned mouth, micrognathia, preauricular skin tags, elongated trunk and fingers, severe mental retardation and seizures; 1/3 die in infancy</td>
<td>50–65</td>
<td>ASD, VSD, PDA, L SVC, aortic atresia, tetralogy of Fallot, tricuspid atresia</td>
<td>22, 154</td>
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<tr>
<td>Deletion 5p (cri-du-chat)</td>
<td>Catlike cry, prenatal and postnatal growth retardation, round face, widely spaced eyes, epicanthal fold, simian crease, severe mental retardation, long survival</td>
<td>30–60</td>
<td>VSD, ASD, PDA</td>
<td>22, 155, 156</td>
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<td>Deletion 7q11.23 (Williams-Beuren syndrome)</td>
<td>Infantile hypercalcemia, skeletal and renal anomalies, cognitive deficits, “social” personality, elfin facies</td>
<td>53–85</td>
<td>Supravalvar AS and PS, PPS</td>
<td>67, 157, 158</td>
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<tr>
<td>Trisomy 8 mosaicism</td>
<td>Skeletal/vertebral anomalies, widely spaced ears, broad nasal bridge, small jaw, high arched palate, cryptorchidism, renal anomalies (50%), long survival</td>
<td>25</td>
<td>VSD, PDA, CoA, PS, TAPVR, truncus arteriosus</td>
<td>22, 159–162</td>
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<tr>
<td>Deletion 8p syndrome</td>
<td>Microcephaly, growth retardation, mental retardation, deep-set eyes, malformed ears, small chin, genital anomalies in males, long survival</td>
<td>50–75</td>
<td>AVSD, PS, VSD, TOF</td>
<td>163–165</td>
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<tr>
<td>Trisomy 9</td>
<td>Severe prenatal and postnatal growth retardation, marked microcephaly, deep-set eyes, low-set ears, severe mental retardation; 2/3 die in infancy</td>
<td>65–80</td>
<td>PDA, L SVC, VSD, TOF/PA, DORV</td>
<td>22, 166</td>
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<td>Deletion 10p</td>
<td>Frontal bossing, short down-sloping palpebral fissures, small low-set ears, micrognathia, cleft palate, short neck, urinary/genital, upper-limb anomalies</td>
<td>50</td>
<td>BAV, ASD, VSD, PDA, PS, CoA, truncus arteriosus</td>
<td>22, 167, 168</td>
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<td>Deletion 11q (Jacobsen syndrome)</td>
<td>Growth retardation, developmental delay, mental retardation, thrombocytopenia, platelet dysfunction, widely spaced eyes, strabismus, broad nasal bridge, thin upper lip, prominent forehead</td>
<td>56</td>
<td>HLHS, valvar AS, VSD, CoA, Shone’s complex</td>
<td>169</td>
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<tr>
<td>Trisomy 13 (Patau syndrome)</td>
<td>Polydactyly, cleft lip and palate, scalp defects, hypotelorism, microphthalmia or anophthalmia, colobomata of irides, holoprosencephaly, microcephaly, deafness, profound mental retardation, rib abnormalities, omphalocele, renal abnormalities, hypoplasia, cryptorchidism, uterine abnormalities; 80% die in first year</td>
<td>80</td>
<td>ASD, VSD, PDA, HLHS, laterality defects, atrial isomerism</td>
<td>170, 171</td>
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<tr>
<td>Trisomy 18 (Edwards syndrome)</td>
<td>IUGR, polyhydramnios, micrognathia, short sternum, hypertonia, rocker-bottom feet, overlapping fingers and toes, TEF, CDH, omphalocele, renal anomalies, biliary atresia, profound mental retardation; 90% die in first year</td>
<td>90–100</td>
<td>ASD, VSD, PDA, TOF, DORV, D-TGA, CoA, BAV, BPV, polyvalvar nodular dysplasia</td>
<td>22, 172, 173</td>
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<tr>
<td>Deletion 20p12 (Alagille syndrome)</td>
<td>Bile duct paucity, cholestasis, skeletal or ocular anomalies, broad forehead, widely spaced eyes, underdeveloped mandible</td>
<td>85–94</td>
<td>Peripheral PA, hypoplasia, TOF, PS, (left-sided heart lesions and septal defects less common)</td>
<td>79, 174</td>
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<td>Trisomy 21 (Down syndrome)</td>
<td>Hypotonia, hypertensibility, epicanthal fold, simian crease, clinodactyly of fifth finger, brachydactyly, variable mental retardation, premature aging</td>
<td>40–50</td>
<td>AVSD, VSD, ASD, (TOF, D-TGA less common)</td>
<td>22, 175–180</td>
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<td>Deletion 22q11 (DiGeorge, velocardiofacial, and conotruncal anomaly face syndrome)</td>
<td>Hypertelorism, micrognathia, low-set posteriorly rotated ears, “fish mouth,” thymic and parathyroid hypoplasia, hypocalcemia, feeding/speech/learning/behavioral disorders, immunodeficiency, palate/skeletal/renal anomalies</td>
<td>75</td>
<td>IAA-B, truncus arteriosus, isolated aortic arch anomalies, TOF, conoventricular VSD</td>
<td>181, 182</td>
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<td>Monosomy X (Turner syndrome, 45,X)</td>
<td>Lymphedema of hands and feet, widely spaced hypoplastic nipples, webbed neck, primary amenorrhea, short stature, normal intelligence</td>
<td>25–35</td>
<td>CoA, BAV, valvar AS, HLHS, aortic dissection</td>
<td>22, 183–187</td>
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<tr>
<td>Klinefelter syndrome (47,XXY)</td>
<td>Usually normal appearing, tall stature, small testes, delayed puberty, emotional and behavioral problems common, variable mental retardation</td>
<td>50</td>
<td>MVP, venous thromboembolic disease, PDA, ASD</td>
<td>22, 188</td>
</tr>
</tbody>
</table>

CHD indicates congenital heart defects; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; L SVC, persistent left superior vena cava; TOF, tetralogy of Fallot; AS, aortic stenosis; PS, pulmonic stenosis; PPS, peripheral pulmonary stenosis; CoA, coarctation of the aorta; TAPVR, total anomalous pulmonary venous return; AVSD, atrioventricular septal defect; TOF/PA, tetralogy of Fallot with pulmonary atresia; DORV, double-outlet right ventricle; BAV, bicuspid aortic valve; HLHS, hypoplastic left heart syndrome; IUGR, intrauterine growth retardation; TEF, tracheoesophageal fistula; CDH, congenital diaphragmatic hernia; D-TGA, D-transposition of the great arteries; BPV, bicuspid pulmonary valve; PA, pulmonary artery; IAA-B, interrupted aortic arch type B; and MVP, mitral valve prolapse.
## Appendix 2

### Genetic Algorithms for Cardiac Defects

#### I. Pulmonary outflow obstruction

##### A. Pulmonary valve stenosis

1. **Noonan syndrome**
   a) Autosomal dominant
   b) 25% to 70% of cases result from de novo mutation
   c) More likely if pulmonary valve is dysplastic
   d) Also associated with hypertrophic cardiomyopathy (right and/or left ventricle)
   e) Noncardiac phenotype features
      1. Male or female
      2. Short stature
      3. Broad or webbed neck
      4. Unusual chest shape
      5. Characteristic facies
      6. Developmental delay
      7. Cryptorchidism
   f) Genetic testing clinically available
      1. *PTPN11* gene mutation analysis
      2. *KRAS* gene mutation analysis
      3. *SOS1* gene mutation analysis

2. **Alagille syndrome** (see below)

3. **Costello syndrome**
   a) Sporadic occurrence
   b) Also associated with hypertrophic cardiomyopathy
   c) Noncardiac phenotype features
      1. Failure to thrive
      2. Feeding difficulties
      3. Mental retardation
      4. Increased risk of malignancy
      5. Coarse facial features with thick lips
      6. Loose skin
      7. *HRAS* mutations

4. **LEOPARD syndrome**
   a) Autosomal dominant
   b) Noncardiac phenotype features
      1. Hearing loss
      2. Lentigines
      3. Short stature
      4. Similarities with Noonan syndrome
   c) Genetic testing clinically available
      1. *PTPN11* gene mutation analysis

5. **Other chromosomal anomalies**
   a) Deletions of chromosome 1p, 8p, 10p, 22q
   b) Duplications of chromosome 6q, 15q, 19q
   c) Trisomy 8

#### B. Pulmonary artery branch stenosis

1. **Alagille syndrome** (see below)

2. **Williams-Beuren syndrome** (see below)

3. **Other**
   a) Congenital rubella
   b) Ehlers-Danlos syndrome
   c) Noonan syndrome (see above)
   d) LEOPARD syndrome (see above)

#### C. Pulmonary valve atresia (intact ventricular septum)

1. Ring 9 chromosome abnormality

#### II. Aortic outflow obstruction

##### A. Aortic valve stenosis

1. **Chromosome abnormalities**
   a) Deletion of chromosome 11q (Jacobsen syndrome)
   b) Autosomal trisomies (13, 18)
   c) Deletion of 10q
   d) Duplications of 1q, 2p, 2q, 6q, 11q

2. **Noonan syndrome** (see above)

3. **Turner syndrome** (see above)

##### B. Supravalvular aortic stenosis

1. **Williams-Beuren syndrome**
   a) Autosomal dominant
   b) Most cases result from de novo mutation
   c) Noncardiac phenotype features
      1. Characteristic elfin facies
      2. Loquacious personality
      3. Hypercalcemia
      4. Developmental delay/cognitive defects
      5. Connective tissue abnormalities
      6. Renal anomalies
      7. Thyroid disorder
   d) Genetic testing clinically available:
      1. Microdeletion in chromosome 7q11 (elastin gene) detectable by FISH (>95% of cases)
      2. Rare translocations involving 7q11 locus

2. **Isolated supravalvular aortic stenosis, Eisenberg type**
   a) Distinct entity from Williams syndrome
   b) Abnormal facies and mental retardation absent

##### C. Coarctation of the aorta

1. **Turner syndrome**
   a) Noncardiac phenotype features
      1. Female
      2. Unusual chest shape
      3. Widely spaced nipples
      4. Webbed neck
      5. Lymphedema
      6. Short stature
      7. Streak ovaries
Appendix 2. Continued

2. Other chromosomal abnormalities
   a) Deletion of 18p
   b) Duplications of 4p, 4q, 6q, 10p
   c) Autosomal trisomies 8, 9
3. Familial aggregation of left-sided obstructive heart defects
   a) Frequent occurrence in first-degree relatives (9.4%)

D. Aortic atresia/hypoplastic left heart syndrome
1. Chromosomal anomalies
   a) Deletion of 11q (Jacobsen syndrome)
   b) Turner syndrome
   c) Trisomy 13, 18
   d) Deletion of 4p (Wolf-Hirschhorn)
2. Familial aggregation of left-sided obstructive heart defects
   a) Frequent association with bicuspid aortic valve in a parent (5%)
   b) Sibling recurrence risk (2% to 9%)
   c) Proposed inheritance patterns
      (1) Multifactorial
      (2) Autosomal dominant with reduced penetrance
      (3) Autosomal recessive

E. Bicuspid aortic valve
1. Very common cardiac anomaly (incidence 0.9% to 1.36% in population)
2. Association with familial aggregation of left-sided obstructive heart defects
   a) Frequent finding of bicuspid aortic valve in parents of children with other left-sided obstructive anomalies
   b) Frequent association of cardiac anomalies in first-degree relatives (19.3%)
3. Familial bicuspid aortic valve
   a) Autosomal dominant with reduced penetrance
   b) Prevalence 24% in first-degree relatives
4. Turner syndrome (see above)
5. Chromosomal anomalies
   a) Autosomal trisomies 13, 18
   b) Deletion 10p
   c) Duplication 6q

III. Laterality defects (heterotaxy, asplenia/polysplenia)
A. Phenotype
1. Asplenia syndrome (also known as right atrial isomerism)
   a) Cardiac defects
      (1) Right atrial isomerism
      (2) Complex conotruncal defects
      (3) AVSD
      (4) Anomalous location of inferior vena cava (on same side as abdominal aorta)
   b) Pattern of visceral organs
      (1) Asplenia: 99% of patients, more severe than polysplenia
      (2) Bilateral “right-sidedness”
      (3) Symmetrical liver
      (4) Gastrointestinal malrotation
      (5) Right-sided stomach
      (6) Genitourinary, bronchopulmonary, axial skeletal, and central nervous system abnormalities
2. Polysplenia syndrome (also known as left atrial isomerism)
   a) Cardiac defects
      (1) Left atrial isomerism
      (2) Septal defects
      (3) Interrupted inferior vena cava
      (4) Bilateral superior vena cavae
      (5) Partial anomalous pulmonary venous return
   b) Pattern of visceral organs
      (1) Polysplenia: 90% of patients
      (2) Bilateral “left-sidedness”
      (3) Symmetrical or inverted (larger lobe on left) liver
      (4) Gastrointestinal malrotation
      (5) Two or more spleens, can be functionally asplenic
      (6) Extrahaepatic biliary atresia
      (7) Genitourinary, bronchopulmonary, axial skeletal, and central nervous system abnormalities

B. Genotype
1. No well-described genetic syndromes with clinical testing available
2. Reported chromosomal abnormalities
   a) Autosomal
      (1) Chromosome 2 (CFC1 gene encoding CRYPTIC protein)
      (2) Chromosome locus 6q (HTX3 gene)
   b) X-linked: locus Xq26.2 (ZIC3 gene)

IV. Atrial septal abnormalities
A. Secundum ASD
1. Holt-Oram syndrome
   a) Autosomal dominant
   b) Variable expression
   c) Also associated with VSD, variable other defects
   d) Noncardiac phenotype features
      (1) No sex predilection
      (2) Variable preaxial limb defects
      (3) Absent, hypoplastic, or triphalangeal thumbs
   e) Mutations of TBX5 gene on 12q24.1
2. Familial ASD and progressive atrioventricular block
   a) Autosomal dominant
   b) No demographics known
   c) Variable onset of conduction abnormality
   d) Other cardiac anomalies can include VSD, tetralogy of Fallot, and others
   e) No noncardiac features reported
   f) Mutations or haploinsufficiency of NKX2.5 gene on chromosome 5
3. Familial ASD without progressive atrioventricular block
   a) Other cardiac anomalies can include VSD or pulmonary stenosis
      (1) GATA 4 mutations
4. Ellis-van Creveld syndrome
   a) Autosomal recessive
   b) Often single atrium
   c) Noncardiac features
      (1) Male or female
      (2) Polydactyly
      (3) Deformity of upper lip
      (4) Dwarfism with narrow thorax
Appendix 2. Continued

(5) Mutations have been described in Ellis-van Creveld gene at 4p16.1

5. Noonan syndrome (see above)

6. Other chromosomal abnormalities
   a) Deletions of 1, 4, 4p, 5p, 6, 10p, 11, 13, 17, 18, and 22
   b) Trisomy 18, 21
   c) Klinefelter syndrome

7. Other syndromes
   a) Rubinstein-Taybi syndrome
   b) Kabuki syndrome
   c) Williams syndrome
   d) Goldenhar syndrome
   e) Thrombocytopenia–absent radius syndrome
   f) Marfan syndrome (rare)

B. Single atrium (see Ellis-van Creveld syndrome)

C. Ostium primum ASD (see atrioventricular septal abnormalities)

V. Ventricular septal abnormalities

A. VSD
   1. Holt-Oram syndrome (see under ASD)
   2. Familial ASD and progressive atrioventricular block (see ASD)
   3. Familial ASD without progressive atrioventricular block
      a) Other cardiac anomalies can include VSD or pulmonary stenosis
      b) GATA 4 mutation

4. Chromosome abnormalities
   a) Deletions of many chromosomes
   b) Duplications of many chromosomes
   c) Autosomal trisomies 13, 18, and 21

5. Other syndromes
   a) Rubinstein-Taybi syndrome
   b) Goldenhar syndrome
   c) VACTERL association
   d) Smith-Lemli-Opitz syndrome
   e) Ellis-van Creveld syndrome (see above)

VI. Atrioventricular septal abnormalities

A. AVSD, partial and complete
   1. Autosomal trisomies
      a) Down syndrome
         (1) 60% of infants with AVSD have Down syndrome
      b) Occurs also in trisomy 13 and 18
   2. Other chromosome abnormalities
      a) Deletions of 3p25, 8p2, 22q
      b) Duplications of 10q, 11q, 22q
   3. Isolated AVSD
      a) Autosomal dominant AVSD
         (1) Partial and complete
         (2) Gene locus mapped to 1p21p31
   4. Other syndromes
      a) Holt-Oram syndrome (see above)
      b) Noonan syndrome (see above)

B. Single atrium (see Ellis-van Creveld syndrome)

C. Ostium primum ASD (see atrioventricular septal abnormalities)

V. Ventricular septal abnormalities

A. VSD
   1. Holt-Oram syndrome (see under ASD)
   2. Familial ASD and progressive atrioventricular block (see ASD)
   3. Familial ASD without progressive atrioventricular block
      a) Other cardiac anomalies can include VSD or pulmonary stenosis
      b) GATA 4 mutation

4. Chromosome abnormalities
   a) Deletions of many chromosomes
   b) Duplications of many chromosomes
   c) Autosomal trisomies 13, 18, and 21

5. Other syndromes
   a) Rubinstein-Taybi syndrome
   b) Goldenhar syndrome
   c) VACTERL association
   d) Smith-Lemli-Opitz syndrome
   e) Ellis-van Creveld syndrome (see above)

VI. Atrioventricular septal abnormalities

A. AVSD, partial and complete
   1. Autosomal trisomies
      a) Down syndrome
      (1) 60% of infants with AVSD have Down syndrome
      b) Occurs also in trisomy 13 and 18
   2. Other chromosome abnormalities
      a) Deletions of 3p25, 8p2, 22q
      b) Duplications of 10q, 11q, 22q
   3. Isolated AVSD
      a) Autosomal dominant AVSD
         (1) Partial and complete
         (2) Gene locus mapped to 1p21p31
   4. Other syndromes
      a) Holt-Oram syndrome (see above)
      b) Noonan syndrome (see above)
Appendix 2. Continued

(1) Deletions of many chromosomes
(2) Duplications of many chromosomes
B. Truncus arteriosus/interruption of the aortic arch
1. 22q11 deletion syndrome (see above)
2. Trisomy 8
3. Deletion 10p
C. Transposition of the great arteries (D-TGA, L-TGA)
1. Chromosome abnormalities
   a) Trisomy 18, 21
   b) 22q11 deletion syndrome (very rarely)
   c) Many other partial deletions of different chromosomes
D. Double-outlet right ventricle
1. Chromosome abnormalities
   a) Autosomal trisomies 9, 13, 18
   b) Duplication 2p, 12p
   c) 22q11 deletion syndrome (very rarely)
IX. Tricuspid atresia
A. Most cases are sporadic
B. Familial occurrences reported but rare
1. In siblings
2. In association with a conotruncal malformation or annular hypoplasia in family members
C. Chromosome abnormalities reported but rare
1. Deletions: 22q11, 4p (Wolf-Hirschhorn syndrome)
2. Duplications: partial duplication 22 (Cat-eye syndrome)
D. Targeted mutation of gene encoding Fog-2 in mice resulted in tricuspid atresia, thereby suggesting a genetic basis for the disease
X. Ebstein anomaly
A. Most cases are sporadic
B. Familial occurrences reported but rare
1. In siblings and other family members
2. In association with other mitral valve abnormalities in family members
3. In association with familial atrial standstill
C. Chromosome abnormalities reported but rare
1. Trisomy 21
2. Rearrangements of chromosome 11q in association with renal malformation and Pierre Robin sequence
D. Animal studies implicate several possible candidate genes on chromosome 17q
XI. Total anomalous pulmonary venous return
A. Most cases are sporadic
B. Familial occurrences reported
1. In siblings, twins, parents/children, first cousins
2. Chromosome 4p13-q12, autosomal dominant, variable expressivity, reduced penetrance in large Utah-Idaho family
3. Familial scimitar syndrome
C. Trisomy 8

LEOPARD syndrome indicates syndrome consisting of cardinal features of multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness; AVSD, atrioventricular septal defect; ASD, atrial septal defect; OMIM, Online Mendelian Inheritance in Man; and TGA, transposition of the great arteries. Clinical testing is not yet available for many of the syndromes listed in this appendix.
## Disclosures

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<tr>
<th>Writing Group Member</th>
<th>Employment</th>
<th>Research Grant</th>
<th>Other Research Support</th>
<th>Speakers’ Bureau/Honoraria</th>
<th>Ownership Interest</th>
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This table represents the relationships of writing group members that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all members of the writing group are required to complete and submit.
Reviewer Disclosures

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This table represents the relationships of reviewers that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all reviewers are required to complete and submit.

References


Eosinophilic Heart Disease in Acute Myeloproliferative Disorder

Christina S. Reuss, MD; Susan Wilansky, MD

A 34-year-old man with no past medical history presented to our emergency department with facial droop and dysarthria. He had a 1-day history of dyspnea and nonproductive cough, which he attributed to a viral illness. Examination was notable only for the neurological abnormalities in the chief complaint, which resolved during the emergency department evaluation. A 12-lead ECG showed sinus tachycardia, a prolonged corrected QT interval (530 ms), and T-wave inversion suggestive of ischemia (Figure 1). MRI of the brain was negative for acute stroke. Laboratory values were notable for a hemoglobin of 8.8 g/dL, white blood cell count of 75,400 cells/mL with absolute eosinophilia to 22,620 cells/mL, and a platelet count of 31,000/mL. Troponin T was elevated to 0.40 μg/mL, and creatine kinase of 22 U/L. An urgent transthoracic echocardiogram demonstrated a mildly reduced ejection fraction (45%) with apical akinesis. A large echogenic mass with a mobile component consistent with thrombus obliterated the apices of the left and right ventricles (online-only supplementary Movie I; Figure 2). The clinical findings were a transient ischemic attack, leukocytosis with eosinophilia, thrombocytopenia, anemia, and eosinophilic heart disease.

The patient was treated with leukapheresis, allopurinol, hydroxyurea, and intravenous mephalaneisolone sodium succinate for 48 hours to reduce the white blood cell count. Heparin was initiated for the ventricular thrombus, as well as carvedilol for left ventricular dysfunction. Bone marrow biopsy revealed a myeloproliferative disorder (CHIC-2 deletion subtype) with secondary eosinophilia, and imatinib mesylate (Gleevec) was started. Hematologic derangements improved before the patient was discharged from the hospital, and warfarin was initiated for the apical thrombus. At 5 weeks after discharge, there was no evidence of ventricular dysfunction, apical thrombus, or eosinophilic infiltration (supplementary Movie II; Figure 3). There has been interval resolution of his peripheral eosinophilia and myeloproliferative disorder.

Eosinophilic infiltration of the heart was originally described in 1936 by Löffler1 in the postmortem examination of 2 patients seen in a 20-year period with afebrile leukocytosis and eosinophilia, progressive right-sided heart failure with hepatosplenomegaly, and ascites. Cardiac autopsy evaluation showed a layering of fibrosis that obliterated the ventricles but spared the valves.1 Eosinophilic heart disease is now recognized as a manifestation of the hypereosinophilic syndromes, in which up to 50% of patients have evidence of cardiac involvement.2-3 Typical cardiac findings include endocardial fibrosis with extensive mural thrombus occupying the apices of both ventricles (hence the term “obliterative cardiomyopathy”). Thrombus can also extend toward the base to involve the valvular apparatus (typically the posterior leaflet of the mitral valve), with resulting incompetence. Advanced forms include progressive myocardial damage, conduction system disease, and refractory heart failure.

The present case highlights the classic features of eosinophilic heart disease. The echo-dense mass was seen, contiguous with the adjacent myocardium, with a central area of echolucency. This is the echocardiographic appearance of large thrombus burden with central liquefactive necrosis.4 The thrombus overlies an area of normal wall motion, thus differentiating this entity from thrombus formation in association with dilated cardiomyopathy or ischemic ventricular dysfunction. The apical akinesis in our patient may have been a result of focal eosinophilic myocarditis or coronary artery thromboembolism. It is thought that the activated eosinophil that is admixed with thrombus secretes major basic protein and is responsible for the toxic damage to the heart.5 Regression of the intracavitary mass has been reported with treatment of anticoagulation plus interferon3 or with imatinib mesylate (Gleevec) as the sole agent.6 Two-dimensional echocardiography is the primary method for diagnosis of eosinophilic heart disease and should be performed in all patients with peripheral blood eosinophilia.

Disclosures

None.

References


Figure 1. Twelve-lead ECG on admission. Note T-wave inversions in the inferior and precordial leads.
Figure 2. Still-frame echocardiographic image in diastole (A) and systole (B). Hyperechoic mass fills the left ventricular apex, which is consistent with thrombus. The apex is akinetic, yet there is still thrombus in areas of normokinesia.

Figure 3. Still-frame echocardiographic image in diastole (A) and systole (B). There has been interval resolution of the ventricular apical mass and thrombus and normalization of ejection fraction.
Changes in Left Atrial and Pulmonary Venous Anatomy During Respiration
A 4-Dimensional Computed Tomography–Based Assessment and Implications for Atrial Fibrillation Ablation

Joris Ector, MD; Dirk Loeckx, MSc; Walter Coudijzer, MS; Stijn De Buck, MSc; Frederik Maes, MSc, PhD; Steven Dymarkowski, MD, PhD; Jan Bogaert, MD, PhD; Hein Heidbüchel, MD, PhD

The possibility of integrating 3D models of the left atrium (LA) and pulmonary veins (PVs) in 3D mapping systems has recently provided a detailed anatomic reference for electrophysiologists performing atrial fibrillation (AF) ablations. Single-breathhold contrast-enhanced computed tomography (CT) or MRI images are reconstructed to a static 3D surface and registered to the LA geometry acquired by a roving catheter during the procedure. Respiratory changes in LA and PV anatomy, however, can reduce registration accuracy, especially when preprocedural CT images are acquired during inspiration.1

A 46-year-old man was referred for catheter ablation of drug-resistant paroxysmal AF. One day before the procedure, 64-slice cardiac CT was performed during both held inspiration and end-expiration. Images were not gated to the cardiac cycle to reduce patient radiation exposure. Nonrigid image registration2 between inspiratory and expiratory data sets with custom software resulted in a dynamic 3D (ie, 4D) sequence of the combined changes in LA, PV, and pulmonary anatomy during the respiratory cycle. Automatic intensity-based segmentation and surface reconstruction was performed with commercial software (Amira 4.0, TGS Template Graphics Software, Inc, Chelmsford, Mass).

Figure 1 shows the 3D anatomy of the LA and surrounding pulmonary anatomy (upper pane) and the changes in left atrial geometry during respiration (middle and lower panes). The remarkable absolute and relative anatomic changes during respiration are illustrated as a 4D sequence in the supplemental movie file. During inspiration, the inferior PVs show a larger downward movement than the superior PVs, which results in splaying of the ipsilateral PVs.

One day later, lasso-guided electrical isolation of the 4 PVs was performed under general anesthesia with propofol and mechanical ventilation. Merging of the LA 3D surfaces with biplane fluoroscopic imaging was performed as outlined previously3 to assist catheter navigation and ablation. In brief, LA 3D models are shown as an overlay on the fluoroscopic images after registration with a combined angiographic image of the 4 PVs acquired during apnea (ie, expiration; Figure 2). Although the 3D surface acquired during expiration resulted in a near-perfect registration with the angiographic images, the inspiratory 3D surface could not be registered correctly owing to the relative changes in PV and LA geometry, especially in the region of the inferior PVs (yellow dotted circles).

The observed respiratory changes in LA-PV anatomy are concordant with observations made by other authors1 and include splaying of the PVs during inspiration, with larger variability in the position of inferior PVs during respiration. The present 4D analysis for the first time directly relates these changes to the respiratory movements of surrounding pulmonary and diaphragmatic structures.

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Dr Ector is a research assistant of the Fund for Scientific Research, Flanders. Stijn De Buck acknowledges the support of the IWT OZM 080511 research project.

Disclosures
Dr Heidbüchel is a member of the scientific advisory board of Biosense Webster, Inc, and is holder of the AstraZeneca Chair in Cardiac Electrophysiology, University of Leuven. The remaining authors have nothing to disclose.

References
Figure 1. Top, CT-based 3D reconstruction of the LA, PVs (pink, solid surface), and surrounding pulmonary anatomy (yellow, semi-transparent surface) acquired during held inspiration. Middle and Bottom, Changes in LA geometry during respiration. “X-ray images” on the left are calculated from the inspiratory and expiratory CT data and show the actual position of the LA relative to the cardiac silhouette. Cardiac CT was not gated to the cardiac cycle to reduce patient radiation exposure. c indicates carina; d, diaphragmatic pleural surface; RSPV, right superior PV; RIPV, right inferior PV; LSPV, left superior PV; and LIPV, left inferior PV.
Figure 2. Merging of CT-based 3D models of the LA and PVs with biplane fluoroscopic images during AF ablation procedure. Left, Selective angiography of the 4 PVs is performed during apnea (expiration). End-diastolic angiographic images of the 4 PVs are then combined into a single digital subtraction angiographic image that shows the anatomy of the 4 PVs and LA in right anterior oblique (RAO) and left anterior oblique (LAO) views. Middle and Right, Combined angiographic image is used to register the 3D surfaces during the procedure. Although the 3D surface acquired during expiration results in a near-perfect registration with the angiographic images (middle), the inspiratory 3D surface could not be registered correctly because of relative changes in PV and LA geometry, especially in the region of the inferior PVs (yellow dotted circles, right). RSPV indicates right superior PV; RIPV, right inferior PV; LSPV, left superior PV; and LIPV, left inferior PV.
A 54-year-old woman diagnosed with sarcoidosis with lung involvement was admitted because of faintness associated with complete atrioventricular block. Cardiac catheterization showed normal coronary arteries and preserved left ventricular systolic function (online-only Supplemental Movie I), but endomyocardial biopsy revealed cardiac involvement. A pacemaker was implanted uneventfully, but she perceived the operation to be particularly stressful. Three hours after the operation, routine follow-up echocardiography revealed akinesis of the left ventricular apical wall, but no apical thrombi (Figure 1; Supplemental Movies II and III). She had no symptoms, but her ECG showed artificial right ventricular apical pacing of 70 bpm with additional ST-segmental elevation in leads II, III, aVF, and V3 through V6. There were no significant changes in serum markers of cardiac damage (white blood cells, creatine kinase, creatine kinase-MB, and cardiac troponin T), but brain natriuretic peptide was elevated to 680 pg/mL (reference range <40 pg/mL). Coronary angiography performed 2 days after the operation revealed normal coronary arteries, but left ventriculography demonstrated apical and mid–left ventricular ballooning with augmented contraction of the basal segment, indicating that she was suffering from emotional stress–induced Takotsubo cardiomyopathy. Surprisingly, a giant thrombus, which had formed within 2 days, was detected at the apex (Figure 2; Supplemental Movies IV and V). Heparin and warfarin were started concurrently. Fortunately, the thrombus completely disappeared within 7 days, and left ventricular function recovered within 14 days, without any embolic events (Supplemental Movie VI). To the best of our knowledge, this is the first description of Takotsubo cardiomyopathy associated with rapid formation of an apical giant thrombus. Clinicians should note the possibility of rapid thrombus formation and consider the use of anticoagulation therapy in the management of Takotsubo cardiomyopathy.

Disclosures
None.

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The online-only Data Supplement, which contains Movies I through V, can be found at http://circ.ahajournals.org/cgi/content/full/115/23/e620/DC1.

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Figure 1. Transthoracic echocardiograms showing left ventricular apical dysfunction without any thrombus at 3 hours after operation. A, Apical 4-chamber view. B, Two-chamber view. LV indicates left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; and PML, pacemaker lead.

Figure 2. Left ventriculography (right anterior oblique view) showing left ventricular ballooning in the mid and apical segments, with vigorous contraction of the basal segment (arrowheads), and a giant thrombus in the apex of the left ventricle (arrows). LV indicates left ventricle.
Letter by Geier et al Regarding Article, “Hypertrophic Cardiomyopathy Is Predominantly a Disease of Left Ventricular Outflow Tract Obstruction”

To the Editor:

We read with great interest the article by Maron et al reporting hypertrophic cardiomyopathy (HCM) as a predominantly obstructive disease. The authors describe in their carefully conducted study a cohort of 320 patients, 70% of whom present with either resting or provokable obstruction of the left ventricular outflow tract.

Our own data from a cohort of 186 unrelated HCM patients confirm that distribution: 65% of our patients presented with significant left ventricular outflow tract gradient either at rest or on exercise. We assume that the patients examined by Maron et al were also familialy unrelated. However, HCM is an autosomal dominant heritable disorder with a 50% chance of transmission to the offspring. To make a reliable estimation for the prevalence of obstruction in HCM, it would be reasonable to also include other affected family members into this estimation. The wide spectrum of clinical expression is a well-known phenomenon of HCM and is sometimes particularly striking within families. Frequently, clinical signs in other family members who have inherited the disease-causing mutation are hard to detect and require intensive clinical scrutiny. Many affected relatives are asymptomatic, which can obscure the heritable nature of HCM in such families. Thus, the clinical presentation in affected relatives may be confined to ECG-specific signs of hypertrophy or to tissue Doppler abnormalities in the absence of overt hypertrophy. This has been incorporated into a set of modified criteria for diagnosis of HCM in immediate relatives.

In HCM referral centers (like those from the authors), however, there is a strong selection bias for severely affected patients. Therefore, there may be differences between the general HCM population and the HCM cohort of a tertiary referral center. Often patients are referred to specialized centers for further evaluation either when they are symptomatic or when a conspicuous heart murmur has been detected, both of which are predictors of an obstructive form of HCM. It should therefore be considered that the actual prevalence of left ventricular outflow tract obstruction in the general HCM population, as seen in smaller hospitals and outpatient clinics, may be lower than the proportion described by Maron et al.

Disclosures

None.

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Response to Letter Regarding Article, “Hypertrophic Cardiomyopathy Is Predominantly a Disease of Left Ventricular Outflow Tract Obstruction”

We appreciate the comments expressed by Dr Geier and colleagues regarding our recent study on the prevalence and clinical significance of physiologically provocable left ventricular (LV) outflow obstruction in hypertrophic cardiomyopathy (HCM).1 With respect to the influence of patient selection on our data, we agree that the prevalence of outflow obstruction in a community-based cohort of HCM patients may differ from our findings. Therefore, due to the unavoidable patient selection bias present in tertiary referral centers, we were (and are) cautious in extrapolating our data to the general HCM population. In fact, this important point was underscored in the conclusions of this paper, emphasizing that our results were in fact derived from a hospital-based cohort. Nevertheless, whether our reported prevalence of obstruction is somewhat higher (or lower) than in the general HCM population does not lessen the important implications of these data, ie, that a substantial number of HCM patients have LV outflow gradients detectable only with exercise echocardiography and that recognition of such exercise-induced gradients may broaden management options for patients who otherwise may not be identified as potential candidates for surgical septal myectomy (or, alternatively, alcohol ablation).

In our study, virtually all patients were unrelated. In fact, it was the intent of this investigation to prospectively define the prevalence of outflow obstruction in a consecutive cohort of HCM patients who came to medical attention with the fully expressed disease phenotype that is typical of those customarily encountered in clinical practice. We agree that it would also be instructive to characterize the prevalence of LV obstruction within an even broader spectrum of this disease, including family members with HCM. However, that particular assessment was well beyond the scope of our study design. Nevertheless, we did demonstrate that >50% of patients without obstruction at rest who developed an exercise-induced gradient were asymptomatic at that time.1 In this regard, we have no reason to assume that by including asymptomatic family members with HCM, the prevalence of obstruction would necessarily have differed significantly from that reported in our series.

Disclosures

None.

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