Brief Review

Report of the National Heart, Lung, and Blood Institute Working Group on Sex Differences Research in Cardiovascular Disease

Scientific Questions and Challenges

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Although cardiovascular disease (CVD) is a leading cause of death in both women and men,1 accumulating evidence suggests that biological sex is a major determinant for the development and progression of CVD, which adversely affects >1 million people per year in the United States alone.2,3 However, many of the basic mechanisms underlying sex differences in CVD remain unknown. Thus, the National Heart, Lung, and Blood Institute convened a Working Group meeting on September 22, 2014 in Bethesda, MD, to explore the issues relevant to sex differences in CVD, particularly basic research. The goals of the Working Group were to (1) discuss the importance of and challenges in conducting basic research on sex differences in CVD and (2) advise on specific research priorities that will improve our understanding of sex differences in basic cardiovascular biology.

The Working Group consisted of extramural experts involved in sex differences research related to hypertension, myocardial ischemia/reperfusion injury, sex hormones and their receptors, cardiac and vascular cell–based therapy, genetics, and the evolution and function of the sex chromosomes. Representatives from the National Institutes of Health Office of Research on Women’s Health, Office of Extramural Research, Center for Scientific Review, and the Food and Drug Administration were also engaged in the Working Group’s discussions. This article represents the Working Group’s recommendations on major challenges and research gaps associated with sex differences in CVD and the opportunities they create for research moving forward.

Challenges in Conducting Research on Sex Differences in CVD

Assumptions About the Difficulty of Studying Both Sexes

Recent assessments of published literature in biomedical sciences indicate that in preclinical research on animals, males are studied more than females,4 which may obscure key sex differences that could guide clinical studies.5 One common reason for this preference to use male animals is that females are viewed as being inherently more variable than males because of their estrous cycles. However, recent analyses show that gonad-intact females are no more variable than males and that males have their own sources of variability, such as the stress and hormonal changes caused by dominance hierarchies in male mice that are group housed.6,7 Another reason for preferential use of male animals is that inclusion of females would significantly increase the cost of experiments because of animal housing and experimental procedures. However, group size might not have to be doubled in all cases (as discussed in Sample Size and Statistical Analysis section of this article) because the number of animals required depends on the sex effect size, treatment effect, as well as the size of any interactions.

Finding that one sex is protected from disease more than the other raises the question of whether there are sex-biased protective factors that account for the sex difference in incidence or progression of disease. Discovering a novel protective mechanism is, therefore, potentially useful if a therapy can be developed that enhances the protective factor and prevents or ameliorates
Choosing Appropriate Experimental Models to Study Sex Differences

One of the most critical aspects of any experimental design is choosing an appropriate experimental model. This presents a particular challenge in sex differences research because, in addition to the usual consideration of whether the model mimics the human condition/disease, it is also important to consider whether the model mimics the disease in the appropriate sex. Specifically, in most experimental models of CVD, females exhibit lower mortality and less severe forms of the disease compared with their male counterparts. Indeed, many studies suggest that estrogens are cardioprotective in several models of CVD, so including females may elaborate additional protective approaches for both sexes.

Because accumulating evidence suggests that major risk factors for CVD may be sex specific, it is feasible that different animal models need to be used to study different aspects of CVD in the 2 sexes. In other words, sex differences research may not simply be about directly comparing males and females in the same model under the same experimental settings. Furthermore, both coronary and ischemic heart disease are leading causes of death in women as in men, although women develop the disease on average 7 to 10 years later. Thus, direct comparisons between sexes using age-matched animals, as is typically done, may not be appropriate. Because of all these issues, a recent report stressed the importance of scientific validation of preclinical models in studies of sex differences.

Choice of appropriate in vitro models is also critical for studying sex differences in CVD. It is estimated that only 20% to 28% of published articles related to CVD report the sex of the cells used, which may, in part, contribute to the growing concern over reduced reproducibility of research findings in preclinical studies. Most importantly, however, differences in responses of cells derived from females and males may have clinical, interventional, and diagnostic significance. For example, atheroprotective capabilities of bone marrow mononuclear cells have only been observed in cells derived from females. Similarly, T cells are prohypertensive in male but not in female T-cell-deficient Rag-1−/− mice. These observations indicate that the sex of primary cells is an important factor to be considered in experimental design.

Consideration of Age to Optimize Research on Sex Differences in CVD

Despite the knowledge that CVD typically occurs in older adults and differs between the sexes, preclinical studies have generally used young adult males as models of CVD. Females tend to develop more CVD but at a later age than men, thus studying only young males may lead to findings that are misleading and not applicable to either aging females or males. Furthermore, even if young females were used as a model for studying CVD (instead of young males), the data generated might still be inadequate because aging human females also exhibit menopause, and thus the contribution of sex hormones in disease development will be different between young and older females. Thus, it is imperative that both males and females of appropriate age be used in the context of studying CVD.

Sample Size and Statistical Analysis of Sex Differences

Biomedical studies often use animals of only one sex based on the presumption that the added cost of testing both sexes would be prohibitive. This is not necessarily so. When both sexes respond in the same way to a treatment, a 2-sex study with a sample size of n+2 would have the same power as a single-sex study with a sample size of n. Two additional animals are needed to obtain the same residual degrees-of-freedom in the 2-way ANOVA that includes sex as a factor. For any sufficiently powered study, this difference is negligible. However, if we assume that when a response to treatment is present in only 1 sex, the total number of animals required to achieve the same power in a 2-sex study would be approximately double the number required in a single-sex study. In this case, the test for interaction would have the same power as the test for an overall treatment effect and it would be possible to establish that the sexes differ. Of course, instances of graded or intermediate responses will require sample sizes that fall between these 2 extreme cases to achieve similar power. These cases bracket the range of sample sizes required under different models of sex-specific responses. Thus, although it is prudent to use more animals in a 2-sex design, doubling the sample size addresses the worst case. Unless there is strong previous evidence, it is not possible to know which set of assumptions applies before we carry out the study; and in the absence of previous knowledge, a study that includes both sexes presents new information that is not available in a single-sex study. Furthermore, the most reliable means to determine if there is a difference would be to carry out the study with both sexes concurrently. With this in mind, the real cost saving derives from including both sexes concurrently with the first investigation.

The statistical analysis of data from studies that include both sexes is straightforward. Two-way ANOVA provides tests for both main effects (treatment and sex) as well as a test of interaction. However, it is a common practice to split the data from a 2-sex study and evaluate each sex separately. But if a difference is observed in the outcome of the 2 separate statistical tests, this does not necessarily imply a difference between the sexes. Separate analysis can result in a substantial loss of power and consequent failure to detect an effect that is truly present in one or both sexes. Analysis by 2-way ANOVA makes use of all the information available in the data and provides the most powerful test of sex-by-treatment interaction. Collectively, there is an important need to define best practices for statistical evaluation of sex-by-treatment interactions for basic science investigations.

Working Group’s Recommendations on Scientific Questions and Research Gaps in the Area of Sex Differences in CVD

What Are the Causes and Consequences of Sex Differences in Immune Modulation of Blood Pressure?

There is a growing appreciation for the role of the immune system in blood pressure regulation; however, most basic science studies demonstrating that the immune system modulates blood pressure conducted in male animals. Guzik et al showed that
male mice deficient in recombination activating gene (Rag-1−/−) were resistant to the increase in blood pressure induced by angiotensin II infusion. Furthermore, when T cells were transferred back into these B-cell– and T-cell–deficient mice, the angiotensin II–induced increase in blood pressure was similar to levels in wild-type mice. However, when investigators studied the impact of biological sex in this model, they found striking differences. Female Rag-1−/− mice were resistant to hypertension induced by angiotensin II infusion regardless of the sex of the adoptively transferred T-cells Rag-1−/−-M.17 Furthermore, adoptive transfer of female T cells into the male Rag-1−/− host had much lower blood pressure after angiotensin II infusion than the mice that had male T cells adoptively transferred.15 Thus, both the sex of the host and the sex of the T cell are biological determinants of immune modulation of blood pressure. It is unknown whether the cause of this sex difference is because of intrinsic differences between the sex chromosome complement (XX versus XY) or because of differences between the male and female hormonal environment in which T cells mature? We also do not know the clinical consequences of these sex differences in immune modulation of blood pressure. For example, are sex differences in the immune system responsible for the earlier onset of hypertension in men than in women? These and many questions remain to be answered and comparing mechanisms of immune modulation of blood pressure between the sexes can provide powerful clues that could ultimately lead to new therapeutic approaches for treating hypertension in both men and women.

**Why Are Obesity-Related Hypertension and CVD More Prevalent in Females?**

According to the most recent data from the CDC, women have a higher incidence of overweight and obesity than men, and this is especially true for women who are black or Hispanic.18 Although obesity-related hypertension in humans is thought to be mediated by sympathetic activation, it is unclear whether this is true in both males and females.19 Thus, preclinical studies are needed to examine the mechanisms underlying obesity-associated hypertension in both sexes, to examine whether adrenergic blockade has different long-term effects, and whether differences in body fat deposition plays different roles in hypertension in aging males and females.

**What Are the Mechanisms Underlying Sex Differences in Ischemia/Reperfusion Injury?**

Several studies suggest that sex hormones play an important role in ischemia/reperfusion injury, with most evidence pointing to the cardioprotective effects of estrogens.20 It is thought that these effects of estrogens in the ischemia/reperfusion setting may have come from positive influences on cardiac stem cells21 and reduced oxidative stress from diminished reactive oxygen species generated in the mitochondria.22 However, there may be other mechanisms by which estrogens may exert cardioprotection in ischemia/reperfusion, such as the role of mitochondrial steroid receptors that exist in the myocytes and can regulate oxidative stress. The endoplasmic reticulum plays important roles in mitochondrial response to stress (calcium, unfolded protein response, etc.) and how these 2 organelles, that both contain estrogen receptors (ERs), interact has not been determined. These are potential targets for preventing the damage response to ischemia/reperfusion.

**What Are the Mechanisms Underlying Sexual Dichotomy of Predisposition to Cardiac Arrhythmias?**

Abnormal calcium flux has been implicated in the generation of fatal cardiac arrhythmias including in human heart failure.23 Male mice deficient for the 12.6-kDa FK506-binding protein have abnormal sarcoplasmic reticulum calcium regulation in the heart because of the resulting malfunction of the cardiomyocyte ryanodine receptor. The calcium flux that occurs leads to profound cardiac hypertrophy and heart failure in the male mice.24 Interestingly, postnatal female mice with this genetic deletion of 12.6-kDa FK506-binding protein do not develop cardiac hypertrophy unless administered tamoxifen, implicating estrogen protection. Improved understanding of the mechanisms underlying female protection and male susceptibility may lead to development of new therapeutic strategies for heart failure and fatal arrhythmia.

**Why Are Women More Likely to Develop Heart Failure With Preserved Ejection Fraction With Age, Whereas Older Men Develop Heart Failure With Reduced Ejection Fraction?**

Growing evidence suggests that men and women develop different CVDs as they age.25 For example, postmenopausal women tend to develop heart failure with preserved ejection fraction, characterized by diastolic dysfunction, whereas age-matched men develop heart failure with reduced ejection fraction, characterized by systolic dysfunction.26 These differences are especially important because most drugs used to treat heart failure have been developed to treat heart failure with reduced ejection fraction. There are few, if any effective treatment options for heart failure with preserved ejection fraction.27 It is possible that the aging process affect hearts of men and women differently, so that women are predisposed toward problems with myocardial relaxation while men develop pump failure. Still, few preclinical studies have examined the influence of age and sex on the heart, so we know little about male–female differences in myocardial function in aging. Heart failure models using animals of both sexes may improve our understanding of this important disease and key to the development of effective treatments for heart failure with preserved ejection fraction in all patients.

**What Are the Signaling Mechanisms Responsible for Sex Differences in CVD?**

In addition to the well-established action of the ER–estrogen complex to modulate gene transcription, estrogen can also bind to several different ERs located at the plasma membrane and activate membrane delimited signaling.28 The cross-talk between these signaling mechanisms leads to complex downstream signaling. In addition, both nuclear and acute effects of estrogen can individually lead to changes in cell signaling, gene expression, and cell function. There is also cross-talk between the nongenomic and genomic pathways, which work together to modulate cell and organelle function.29 Recently developed experimental models can be used to elucidate some of these complexities associated with ER signaling in CVD. One such model is the mouse lacking the ERα palmitoylation site, in
which signaling is only via nuclear DNA binding and not via membrane signaling. These mice offer insight into the collaboration between non-nuclear and nuclear ERα and ERβ signaling. Other models that may be used for investigating the role of estrogen signaling in CVD is the mouse that expresses the ligand-binding domain of ERα that is targeted exclusively to the plasma membrane. It is important to note that the effects of estrogen and estrogen signaling may only partly be responsible for sex differences in CVD. The contribution of other ovarian hormones as well as androgens should also be considered.

Can Sex-Based Genetic Variation Profiles Be Used to Predict Susceptibility to Developing CVD?

Given the polygenic and multifactorial nature of many CVDs, additional strategies are needed to identify sex-specific loci involved in the mechanisms of CVD. Through genetic mapping studies, it is clear that some cardiovascular trait loci differ between sexes and that these genetic determinants can influence pre- and postmenopausal susceptibility to disease. Future studies that go beyond identification of single-nucleotide variants are needed to investigate the role of genetic variants in promoter response elements for hormone receptors. More complex statistical analyses to evaluate the impact of expression changes on molecular networks and alteration of disease phenotype are also warranted.

How Do Epigenetic Events Contribute to Sex Differences in CVD?

Recent studies have shown that epigenetics may play an important role in determining sex differences in CVD. For example, DNA methylation has been shown to be altered in adult female mice that were exposed to diethylstilbestrol in utero. Others have reported sex- and hormone-dependent differences in DNA Cpg methylation or elevated phospholipase A2, group 7 gene promoter methylation as a sex-specific marker of aging by increasing the risk of coronary heart disease in females. Sex differences in miRNA in the heart have also been reported. On the basis of these reports, considering epigenetics in studies of sex differences in CVD are warranted.

Do Sex Chromosomes Contribute to Sex Differences in CVD?

Although estrogens are implicated as the dominant factor providing cardioprotection in women, emerging evidence suggests that other female-specific factors, such as the presence of 2 X chromosomes in cardiovascular cells, might act to increase, rather than decrease, susceptibility to ischemia/reperfusion injury. In addition, sex chromosome complement has been implicated in animal studies of hypertension. These early studies only hint at possible effects of sex chromosome complement. What is needed is to identify specific X and Y genes that influence CVD in a sex-biased fashion in animal models, and to study how they act within sex-specific hormonal environments.

How Does the Fetal Environment Contribute to Subsequent Disparate Development of CVD in the 2 Sexes?

There are compelling data suggesting that CVD may be programmed in utero and that male and female fetuses may respond differently to the adverse intrauterine environment, resulting in sex differences in CVD later in life. Although several mechanisms coupling the intrauterine environment and future disease susceptibility have been proposed, including epigenetics, mitochondrial dysfunction, and microbiome dysbiosis in other diseases, these have not been studied in great detail in the context of CVD. Another plausible hypothesis is that placental dysfunction may be a key factor leading to fetal programming of CVD. Specifically, studies have shown that changes in placental morphology and dysfunction can predict risks for coronary artery disease, heart failure, and hypertension and that this is sex specific. However, the mechanisms underlying the placental programming of CVD remain unknown.

Altered mitochondrial function and mitochondrial DNA have been shown to be major contributors to the development of many diseases, including CVD. Because mitochondrial DNA is solely derived from the mother, it is conceivable that programming of mitochondrial health can occur in utero, which may determine predisposition to CVD in later life. Thus, understanding the mechanisms of mitochondrial DNA health may be an important biomarker of future CVD.

What Is the Impact of Sex on Stem Cells and Progenitor Cells in the Treatment of CVD?

It is known that increased levels of circulating bone marrow stem cells reduce the risk of death from CVD in both sexes. In addition, sex differences in the regenerative potential of human endothelial progenitor cells have been documented. Animal studies have also shown that female rodent mesenchymal stem cells and skeletal muscle stem cells are more robust in tissue repair than male rodent stem cells because sex differences exist in mesenchymal stem cell growth factor and cytokine production. The stem cell therapy field is shifting its focus from initial feasibility study to optimization of therapeutic efficacy with the goal to achieve a more consistent and sustained clinical potency. During this transition, we face many variables, such as differences in sex, which can complicate aspects of stem cell therapy. However, identification and characterization of the effect of the cell’s sex in stem cell–mediated therapy for CVD would

Table 1. Scientific Challenges in Sex Differences Research on CVD

| Assumptions about the difficulty of studying both sexes |
| Identify the sex-biased protective factors that account for the sex difference in incidence or progression of CVD |
| Choosing appropriate experimental models to study sex differences |
| Determine the conditions under which the male’s pathophysiology does not occur |
| Study females to advance our understanding of disease in males |
| Validate preclinical models used for sex differences research |
| Consideration of age to optimize research on sex differences in CVD |
| Use both males and females to study CVD |
| Improve access to aging animal colonies of both sexes |
| Sample size and statistical analysis of sex differences |
| Analyze experimental data by 2-way ANOVA |
| Determine the best practices for statistical evaluation of sex-by-treatment interactions for basic science investigations |

CVD indicates cardiovascular disease.
sex influences, has the potential to reveal targets that can lead to development of more sophisticated stem cell therapeutic tools and approaches.

### Conclusions

The working group convened by the National Heart, Lung, and Blood Institute identified several scientific challenges (Table 1) and questions (Table 2) for studying sex differences in CVD. However, it is important to note that the recommendations of the working group are limited to the topics discussed at the meeting and that there are many other areas in which sex differences research is still warranted. The overall conclusion of the working group is that understanding of the mechanisms that make the 2 sexes similar, or different, from each other may lead to the development of sex-specific therapies for prevention and treatment CVD.

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### Disclosures

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### Table 2. Scientific Questions for Sex Differences Research on CVD

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<th>Question</th>
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<td>What are the causes and consequences of sex differences in immune modulation of blood pressure?</td>
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<td>• How does sex chromosome complement (XX vs XY) affect the regulation of blood pressure?</td>
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<td>• How does the hormonal environment affect the maturation of T cells and does change how blood pressure is controlled?</td>
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<td>• What are the sex differences in the immune system responsible for the earlier onset of hypertension in men vs women?</td>
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<td>What are the mechanisms underlying sex differences in ischemia/reperfusion injury?</td>
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<td>• What is the role of mitochondrial steroid receptors in myocytes and can they regulate oxidative stress?</td>
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<td>• Does the endoplasmic reticulum interact with the mitochondrion to regulate the response to cell injury?</td>
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<td>• What are the differences in calcium-handling mechanisms that protect females from heart failure and fatal arrhythmia?</td>
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<td>• Will new animal models and the study of both sexes improve our understanding of HFpEF in older females?</td>
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<td>What are the signaling mechanisms responsible for sex differences in CVD?</td>
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<td>• How can animal models improve our understanding of the cross-talk between non-nuclear and nuclear ERα and ERβ signaling?</td>
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<td>Can sex-based genetic variation profiles be used to predict susceptibility to developing CVD?</td>
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<td>• What are the sex-specific loci involved in the mechanisms and pathways of HFpEF?</td>
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<td>How does the fetal environment contribute to subsequent disparate development of CVD in the two sexes?</td>
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<td>• What are the markers of future CVD, in terms of placental programming, microbiome dyshomeostasis in diabetes mellitus, and other diseases, and in mitochondrial DNA health?</td>
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<td>What is the impact of sex on stem cells and progenitor cells in the treatment of CVD?</td>
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<td>• What are the specific targets and trophic factors that affect our understanding of stem cell–mediated therapy and host tissue response?</td>
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CVD indicates cardiovascular disease; and ER, estrogen receptor.


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