HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Hypertension. 2016 May ; 67(5): 802–807. doi:10.1161/HYPERTENSIONAHA.115.06967.

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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Introduction

While 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 cardiovascular disease (CVD), which adversely impacts more than 1 million people per year in the US alone.[2,3] However, many of the basic mechanisms underlying sex differences in CVD remain unknown. Thus, the National Heart, Lung, and Blood Institute (NHLBI) convened a Working Group meeting on September 22, 2014 in Bethesda, Maryland 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

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Disclosures

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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 NIH 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 indicates 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 utilize 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 due to animal housing and experimental procedures. However, group size might not have to be doubled in all cases (as discussed below under "Sample size and statistical analysis") 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 quite useful if a therapy can be developed that enhances the protective factor and prevents or ameliorates disease in both sexes. Thus, the comparison of the two sexes is not just to reverse the under-representation of females in scientific investigations, but has real potential benefit for both sexes.

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.[8, 9]

Indeed, many studies suggest that estrogens are cardioprotective in several models of CVD[9], 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 two 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 7–10 years later.[10] Thus, direct comparisons between sexes using age-matched animals, as is typically done, may not be appropriate. Because of all of these issues, a recent report stressed the importance of scientific validation of preclinical models in studies of sex differences.[11]

Choice of appropriate *in vitro* models is also critical for studying sex differences in CVD. It is estimated that only 20–28% of published articles related to CVD report the sex of the cells used[4], which may, in part, contribute to the growing concern over reduced reproducibility of research findings in preclinical studies.[12] 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.[13] Similarly, T cells are prohypertensive in male but not female T-cell deficient Rag1−/− mice.[14, 15] 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, pre-clinical studies have generally used young adult males as models of CVD. Females tend to develop more CVD but at a later age than men,[16] 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 as 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 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 two-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 two-way ANOVA that includes sex as a factor. For any sufficiently powered study this difference is negligible. On the other hand, if we assume that when a response to treatment is present in only one sex, the total number of animals required to achieve the same power in a two-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 two extreme cases in order to achieve similar power. These cases bracket the range of sample sizes required under different models of sex-specific responses. Thus, while it is prudent to use more animals in a two-sex design, doubling the sample size addresses the worst case. Unless there is strong prior evidence, it is not possible to know which set of assumptions applies before we carry out the study and, in the absence of prior 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 two-sex study and evaluate each sex separately. But if a difference is observed in the outcome of the two 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 two-way ANOVA makes use of the all 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.[14] showed that male mice deficient in recombination activating gene (Rag-$1^{-/-}$) were resistant to the increase in blood pressure induced by angiotensin II (Ang II) infusion. Furthermore, when T cells were transferred back into these B- and T-cell-deficient mice, the Ang 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 Ang 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 Ang II infusion than the mice which 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 due to intrinsic differences between the sex chromosome complement (XX vs XY) or due to 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 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 African American or Hispanic. [18] While 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-cells[21] and/or 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 two organelles, that both contain estrogen receptors, 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 (FKBP12.6) have abnormal sarcoplasmic reticulum calcium regulation in the heart due to 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, post-natal female mice with this genetic deletion of FKBP12.6 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 (HFpEF) with age, while older men develop heart failure with reduced ejection fraction (HFrEF)?

Growing evidence suggests that men and women develop different CVDs as they age.[16] For example, post-menopausal women tend to develop HFpEF, characterized by diastolic dysfunction, while age-matched men develop HFrEF, characterized by systolic dysfunction.[25] These differences are especially important, as most drugs used to treat heart failure have been developed to treat HFrEF. There are few, if any effective treatment options for HFpEF.[26, 27] It is possible that the aging process affect hearts of men and women differently, so that women are predisposed towards problems with myocardial relaxation while men develop pump failure. Still, few pre-clinical studies have examined the influence of age and sex on the heart, so we know very 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 HFpEF in all patients.

What are the signaling mechanisms responsible for sex differences in CVD?

In addition to the well-established action of the estrogen receptor-estrogen complex to modulate gene transcription, estrogen can also bind to several different estrogen receptors (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 non-genomic and genomic pathways which work together to modulate cell and organelle function.[28] 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.[29] 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.[30] 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[31] and that these genetic determinants can influence pre- and post-menopausal susceptibility to disease.[32] 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.[33] More complex statistical analyses to evaluate the impact of expression changes on molecular networks and alteration of disease phenotype are also warranted.[34]

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*.[35] Others have reported sex- and hormone-dependent differences in DNA CpG methylation[36] or elevated phospholipase A2, group 7 gene promoter methylation as a gender-specific marker of aging by increasing the risk of coronary heart disease in females.[37] Sex differences in miRNA in the heart have also been reported.[38] Based on 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 two X chromosomes in cardiovascular cells, might act to increase, rather than decrease, susceptibility to ischemia/reperfusion injury.[39] In addition, sex chromosome complement has been implicated in animal studies of hypertension.[40] 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 two sexes?

There are compelling data suggesting that CVD may be programmed *in utero*[41] and that male and female fetuses may respond differently to the adverse intrauterine environment resulting in sex differences in CVD later in life.[42] While several mechanisms coupling the intrauterine environment and future disease susceptibility have been proposed, including epigenetics, mitochondrial dysfunction and microbiome dyshomeostasis 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.[43] 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.[44, 45] 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.[46] Since mitochondrial DNA is solely derived from the mother,[60] 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 that increased levels of circulating bone marrow stem cells reduce the risk of death from CVD in both sexes.[47] In addition, sex differences in the regenerative potential of human endothelial progenitor cells have been documented.[48] Animal studies have also shown that female rodent mesenchymal stem cells and skeletal muscle stem cells have been found to be more robust in tissue repair than male rodent stem cells because sex differences exist in MSC growth factor and cytokine production.[49, 50] 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 represent a major milestone and opportunity to advance the field. Improvement of our understanding in host tissue response to stem cell-mediated trophic factors, in relation to 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 NHLBI 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 a number of other areas that sex differences research is still warranted. The overall conclusion of the Working group is that understanding of the mechanisms that make the two sexes similar, or different, from each other may lead to the development of sex-specific therapies for prevention and treatment CVD.

Acknowledgments

The authors would like to thank Drs. Leslie Leinwand (University of Colorado at Boulder) and Robert Simari (University of Kansas School of Medicine) for their time and expert scientific opinion shared during the working group meeting. Thank you to Drs. Zorina Galis, Marissa Miller, Denis Buxton, George Mensah and Nakela Cook (NHLBI) for feedback and editing during manuscript preparation.

Sources of Funding

Funding for the Working Group meeting was provided by the NIH/NHLBI, Bethesda, MD.

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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 two-way ANOVA
• Determine the best practices for statistical evaluation of sex-by-treatment interactions for basic science investigations

Table 2

Scientific Questions for Sex Differences Research on CVD

What are the causes and consequences of sex differences in immune modulation of blood pressure?

- How does sex chromosome complement (XX vs XY) affect the regulation of blood pressure?
- How does the hormonal environment affect the maturation of T cells and does change how blood pressure is controlled?
- What are the sex differences in the immune system responsible for the earlier onset of hypertension in men vs women?

What are the mechanisms of postmenopausal hypertension?

- How do obesity, anxiety, and depression contribute to hypertension in aging females?
- How can we adequately control hypertension in aging females that are obese?

What are the mechanisms underlying sex differences in ischemia/reperfusion injury?

- What is the role of mitochondrial steroid receptors in myocytes and can they regulate oxidative stress?
- Does the endoplasmic reticulum interact with the mitochondrion to regulate the response to cell injury?

What are the mechanisms underlying sexual dichotomy of predisposition to cardiac arrhythmias?

- What are the differences in calcium-handling mechanisms that protect females from heart failure and fatal arrhythmia?

Why are women more likely to develop heart failure with preserved ejection fraction (HFpEF) with age, while older men develop heart failure with reduced ejection fraction (HFrEF)?

- Will new animal models and the study of both sexes improve our understanding of heart failure with preserved EF in older females?

What are the signaling mechanisms responsible for sex differences in CVD?

- How can animal models improve our understanding of the cross-talk between non- nuclear and nuclear ERα and ERβ signaling?

Can sex-based genetic variation profiles be used to predict susceptibility to developing CVD?

- What are the sex-specific loci involved in the mechanisms and pathways of CVD?
- When do hormonal promoter response elements control disease phenotype?
- Will more complex statistical analyses help us better evaluate the impact of expression changes on molecular networks and alteration of disease phenotype?

How do epigenetic events contribute to sex differences in CVD?

- Are there specific DNA modifications that are statistically different between males and females?

Do sex chromosomes contribute to sex differences in CVD?

- What are the specific X and Y genes that influence CVD in animal models?
- How do X and Y genes act within sex-specific hormonal environments?

How does the fetal environment contribute to subsequent disparate development of CVD in the two sexes?

- What are the markers of future CVD, in terms of placental programming, microbiome dyshomeostasis in diabetes, and other diseases, and in mitochondrial DNA health?

What is the impact of sex on stem cells and progenitor cells in the treatment of CVD?

- What are the specific targets and trophic factors that affect our understanding of stem- cell mediated therapy and host tissue response?