Special Article

Consideration of Sex Differences in Design and Reporting of Experimental Arterial Pathology Studies—Statement From ATVB Council

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Abstract—There are many differences in arterial diseases between men and women, including prevalence, clinical manifestations, treatments, and prognosis. The new policy of the National Institutes of Health, which requires the inclusion of sex as a biological variable for preclinical studies, aims to foster new mechanistic insights and to enhance our understanding of sex differences in human diseases. The purpose of this statement is to suggest guidelines for designing and reporting sex as a biological variable in animal models of atherosclerosis, thoracic and abdominal aortic aneurysms, and peripheral arterial disease. We briefly review sex differences of these human diseases and their animal models, followed by suggestions on experimental design and reporting of animal studies for these vascular pathologies. (***Arterioscler Thromb Vasc Biol*****. 2018;38:00-00. DOI: 10.1161/ATVBAHA.117.309524.)**

Key Words: atherosclerosis ■ cause of death ■ myocardial infarction ■ peripheral arterial disease ■ prognosis

Cardiovascular diseases are the leading cause of death in the United States, and the number of deaths from cardiovascular diseases in 2013 was approximately the same in both sexes.[1] However, several cardiovascular diseases occur more commonly or at an earlier age in men than women. For example, the prevalence of coronary heart disease (CHD) and myocardial infarction increases with age in both sexes, but is lower in women, with a ≈10-year delay in onset of CHD in women compared with men.[1] Additionally, the symptoms of CHD often present differently in men and women, as do the therapies used.[2] Sex differences in humans and animal models are widespread and observed in virtually every physiological and molecular phenotype. These differences may be because of direct (cis) gene dosage effects on X and Y chromosome gene products, indirect (trans) effects of X and Y chromosome transcripts, or proteins on autosomal genes and gene products, or via sex hormones, the latter of which can be thought of as a trans effect on gonad development and their resulting sex hormones.

In 2014, a working group of the National Heart Lung and Blood Institute at the National Institutes of Health (NIH) explored sex differences in basic science research relevant to cardiovascular diseases and established priorities for understanding these sex differences.[3] This working group enumerated many issues in choosing experimental models and statistical analysis to study sex differences, and also made recommendations on scientific questions related to sex differences.[3] The NIH Office of Research in Woman's Health also held a workshop on sex as a biological variable in preclinical research, which specified the need to use both male and female animals in research, to use sex as a variable in study design, and to report sex-disaggregated data.[4] In 2015, the NIH issued a notice entitled Consideration of Sex as a Biological Variable in NIH-funded Research (NOT-OD-15-102). This notice recognizes that sex is an important biological variable that must be taken into account in the design of research studies in humans and vertebrate animals. The notice states that both sexes must be used in research studies, unless scientifically justified, with the data presented separately for each sex to allow for the identification of sex differences. This issue was then incorporated into the more comprehensive NIH notice entitled Enhancing Reproducibility through Rigor and Transparency (NOT-OD-15-103), which was endorsed by the journals of the American Heart Association. This NIH notice specifically addresses NIH grant proposals and states that planned studies that do not address biological variables such as sex differences must be clearly justified scientifically in the research plan. Study sections have also been instructed to score a grant proposal's recognition of sex as biological variable as a strength or weakness in the study design/approach. A prior policy statement on one aspect of this NIH notice,

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Nonstandard Abbreviations and Acronyms	
AAA	Abdominal aortic aneurysms
Apoe$^{-/-}$	Apolipoprotein E deficient
CHD	Coronary heart disease
HDL-C	High-density lipoprotein cholesterol
HLI	Hindlimb ischemia
IL	Interleukin
LDL-C	Low-density lipoprotein cholesterol
Ldlr$^{-/-}$	LDL receptor-deficient
NIH	National Institutes of Health
PAD	Peripheral arterial disease
TAADs	Thoracic aortic aneurysms and acute aortic dissections
TGF-β	Transforming growth factor-β

establishing mechanisms to report refutations of prior studies, was published by *Arteriosclerosis, Thrombosis, and Vascular Biology* in 2016.[5]

Including both sexes in animal studies may affect the numbers of animals required for experiments, but the resulting findings will likely provide enhanced insight in identifying sex-dependent differences related to cardiovascular disease physiopathology and therapeutic approaches.

The goal of this statement is to frame issues in experimental arterial pathology in the context of sex as a biological variable and to address the following questions:

1. What are the animal models for these arterial pathologies, how well do they model the human diseases, and do the pathologies develop to the same extent in male and female animals?
2. How should studies be designed to account for sex differences in these models?
3. What should be the standard for publication using these models in regard to using both sexes and reporting the data separately by sex? And the related question of whether this standard should be the same for studies focused or not focused on the mechanism of sex effects?

Atherosclerosis

Sex Differences in Human Atherosclerotic Diseases

Atherosclerosis is one of the leading causes of cardiovascular morbidity and mortality. In coronary arteries, atherosclerosis leads to angina pectoris and myocardial infarction. Atherosclerosis progression includes lipid accumulation, intimal thickening, inflammatory cell infiltration, fibrous cap formation, calcification, thrombosis, and rupture.[6]

Many large population-based cohort studies report sex differences in atherosclerotic diseases. The Multi-Ethnic Study of Atherosclerosis measured coronary artery calcium score (derived from ultrafast computed tomography) in men and women of European, African, Asian, and Hispanic descent. For all 4 racial groups, calcification was lower in women than in men at ages 55, 65, and 75, with an approximate 10-year delay of progression in women.[7] Comparably, women lag behind men by ≈10 years in the prevalence of myocardial infarction or CHD.[1] Carotid intimal medial thickness (measured by external ultrasound of the neck) was also greater in men than in age-matched women but the extent of that difference was highly variable depending on the studied population (young healthy individuals versus older populations with or without carotid atherosclerosis).[8–15] Thus, sex plays an important role in human atherosclerosis, with women developing atherosclerosis at a later age than men. However, it is worth noting that the prevalence of atherosclerosis in women >85 years of age is higher than in men of this age.[1]

There is a growing body of evidence that clinical presentations, medical treatment, and outcomes of atherosclerosis exhibit sex differences.[2,16] Acute coronary syndrome is an umbrella term for conditions that cause the sudden onset of disrupted blood flow to the heart, which includes ST-segment–elevation myocardial infarction, non–ST-segment–elevation myocardial infarction, unstable angina, and other atypical presentations. Women first presenting with acute coronary syndrome are more likely to have multiple risk factors than men, including smoking, obesity, depression, hypertension, and diabetes mellitus.[2] Additionally, diabetes mellitus is a stronger risk factor for myocardial infarction in women than in men.[17] Chest pain is the most prevalent symptom of acute coronary syndrome in both sexes, but women are more likely to have atypical presentation than men, such as jaw pain, nausea, and vomiting.[18] Similarly, coronary artery pathology differs between the 2 sexes with women more often presenting with plaque erosion and coronary microvascular dysfunction than men.[2] Women tend to wait longer to seek treatment after their first heart attack, and with their sometimes atypical symptoms, they are less likely to be admitted to coronary or intensive care units.[19] Women admitted to intensive care units had similar outcomes as men, after adjusting for delayed arrival and other risk factors; however, women who were admitted to general medical wards were 89% more likely to die than men after their first heart attack.[19]

What might explain this delayed onset of atherosclerosis in women? LDL-C (low-density lipoprotein cholesterol) levels, a crucial contributor to atherosclerosis, are similar in adult women and men, whereas HDL-C (high-density lipoprotein cholesterol) levels are ≈20% higher in women than men.[1] The inverse association between HDL-C and CHD is irrefutable, but whether high HDL-C is directly protective against CHD, rather than just associated with low CHD, has been justifiably questioned because of several failures of drugs that target HDL-C to decrease CHD, along with a genetic test called Mendelian randomization, which found that common genetic variants associated with HDL-C, and no other lipid traits, were not associated with CHD risk.[20,21] Women have ≈15% lower age-adjusted plasma triglyceride levels than men,[1] which goes along with their higher HDL-C levels, and which may be responsible for the delayed incidence in CHD in women, as a Mendelian randomization and uncommon triglycerides raising genetic variants indicated causality.[22,23] In addition to dyslipidemia, hypertension and risk for thrombosis are associated with atherosclerosis. There is a sex effect on hypertension, but it is age related with women having a lower prevalence than men <45 years of age, a similar prevalence between the ages of 55 to 64, and a higher prevalence after 65 years of age.[1] One risk factor in the opposite direction from CHD incidence

is thrombosis, with women having higher platelet reactivity to specific agonists, and higher fibrinogen levels than men.[16]

The role of female sex hormones in the sex differences observed in atherosclerosis has been extensively studied. Briefly, the longitudinal Nurses' Health Study ongoing from the 1970s to the 1990s and the Women's Health Initiative are major studies that addressed the important question of the role of sex hormone treatment in atherosclerosis. Clinical investigators have deduced that multiple yet to be fully defined factors impact the potential benefits, or contraindications, of hormone replacement therapy for women in this disease, such as the timing of hormone replacement therapy relative to the start of menopause, which might affect the therapeutic window. The readers are referred to a recent review in this area that critically examines the complexity of this issue.[24] In contrast to studies on estrogens, much less attention has been paid to how low testosterone in aging men might affect atherosclerosis.

Overall, the mechanisms by which men and women have different prevalence, clinical manifestations, and outcomes of treatments for atherosclerosis remain to be discovered, which only increases the need for rigorous testing in preclinical models exploring these sex differences.

Animal Models of Atherosclerosis and Evidence of Sex Differences

Ideally, animal models of atherosclerosis should be representative of humans in regard to their lipoprotein metabolism, atherosclerosis pathogenesis, and sites of lesion predilection. Other considerations include the time it takes for lesions to develop, the time and ease of breeding, the cost of acquisition and maintenance, and the ability to perform in vivo imaging and interventions.[25] Although limitations exist,[26,27] *Apoe*$^{-/-}$ (apolipoprotein E deficient) mice and *Ldlr*$^{-/-}$ (LDL receptor-deficient) mice are the most commonly used mouse models to study atherosclerosis.[28–30] A high-fat high-cholesterol Western-type diet, which causes a rapid increase in plasma cholesterol, is frequently fed to these mice to accelerate development of atherosclerosis in these 2 mouse models.[26,31] Atherosclerotic lesions are predominantly present in the aortic root, ascending aorta, aortic arch, and the major branches of the aortic arch (innominate artery, left common artery, and left subclavian artery).[32,33] Mouse atherosclerosis is mostly quantified and characterized in the aortic root region using cross-sections and in the entire aorta using en face staining.[32] Mouse lesions at the fatty streak and fibroproliferative lesion stages have many similarities with human atherosclerosis; and, they provide insights into understanding inflammatory mechanisms of atherosclerosis.[34–36] However, plaques in mice usually do not develop in coronary arteries[26,27] and are less prone to rupture[37–39] compared with human plaques.

Sex differences have been frequently reported in mouse atherosclerosis studies (examples shown in the Table). The presence and extent of these differences is affected by the specific mouse model studied, the genetic background strain, the time point and diet used, and the site of lesion quantification. The most widely reported sex effect on atherosclerosis is that female mice have larger aortic root lesion areas than male mice on several genetic backgrounds and diets.[40–46] This sex effect on aortic root lesion area may be lost or reversed in older C57BL/6 *Apoe*$^{-/-}$ mice.[45,47] However, on some genetic backgrounds, such as Ola129 *Apoe*$^{-/-}$, 129 *Apoe*$^{-/-}$, FVB *Apoe*$^{-/-}$, and FVB/N *Ldlr*$^{-/-}$, lesion size in the aortic root was not significantly different between male and female mice.[40,41,43,46,48] F1 mice bred from C57Bl/6 APOE-Leiden/CETP double transgenics, a dominant model of atherosclerosis, and each of ≈ 100 different strains from the hybrid mouse diversity panel showed overall larger aortic root lesion area in females versus males.[49] There are also reports of sex differences for brachiocephalic artery lesions, but the sex effect is model dependent with larger lesions in females than in males in C57BL/6 *Apoe*$^{-/-}$ mice, but not in FVB/N *Ldlr*$^{-/-}$ mice.[41,42] In addition, sex effects varied between lesions closer to and farther from the brachiocephalic artery bifurcation.[42] There are also conflicting data on sex effects for aortic atherosclerosis measured en face, which use different models, diets, and time points.[50–52]

Sex effects on atherosclerosis have been observed with pharmacological approaches or breeding modifier gene knockouts. For example, the beneficial effect of a PPARγ agonist on atherosclerosis in C57BL/6 *Ldlr*$^{-/-}$ mice was only observed in male mice.[53] Similarly, breeding the interferon γ deficiency (*Ifng*$^{-/-}$) onto the *Apoe*$^{-/-}$ background led to smaller lesions only in male mice.[54]

Mechanistic studies relevant to atherosclerosis are often performed using cells or tissues obtained from mouse models; however, the sex of the donor animal is not routinely disclosed in these studies. Sex effects are prevalent in gene expression studies and in expression quantitative trait locus studies that associate genetic variation near a gene with the expression level of that gene (called a local or *cis*-expression quantitative trait locus). For example, in mice derived from an *Apoe*$^{-/-}$ strain intercross, there were sex-specific associations between atherosclerosis lesion burden and the expression of some cholesterol biosynthesis genes in liver, as well as between the expression of IL-4 (interleukin-4) and oxidative phosphorylation genes in adipose tissue.[55] In a well-powered ex vivo mouse bone marrow macrophage transcriptomic study, 31% of the genes were expressed differentially between the sexes at a conservative *P*-value threshold of 2.3×10^{-6}, although the sex effect on gene expression was small, with most having <20% difference.[56] About 3/4 of the strong *cis*-expression quantitative trait locus identified in this study were shared between male and female macrophages, which probably represents an underestimation because of arbitrary *P*-value threshold effects.[56]

Suggestions for Incorporating Sex into Design and Reporting of Animal Atherosclerosis Studies

General recommendations for animal atherosclerosis studies are described in a recent scientific statement from the American Heart Association.[57] Because sex is a biological variable, with known effects on human atherosclerotic disease and animal models of atherosclerosis, we suggest that all animal studies using atherosclerosis as an end point be performed in both male and female mice, with the data presented separately by sex. The sex of the mice used should be stated in

Table. Selected References Illustrating Variances on Atherosclerosis and Sexual Dimorphism in Mice

Mouse	Diet	Region of Atherosclerosis Measurement	Sexually Dimorphic		
			Yes		No
			Larger in Male	Larger in Female	
Apoe$^{-/-}$	Normal	Aortic root	Older C57Bl/6J[47]	Younger C57Bl/6[45] C57Bl/6[46] C57Bl/6 and DBA/2J[40] C57Bl/6J[43] C57Bl/6J[48]	Older C57Bl/6[45] 129/SvEvTac[46] AKR/J, 129/SV-ter, BALB/cByJ, C3H/HeJ[40] Ola129[43] FVB/NJ[48]
	Modified	En face aorta	C57Bl/6J[50]	C57Bl/6J[51]	
		Aortic root		C57Bl/6J and C3H/HeNHsd[44] C57Bl/6J[42]	
		Brachiocephalic artery	C57Bl/6J (far from bifurcation)[42]	C57Bl/6J (close to bifurcation)[42]	
Ldlr$^{-/-}$	Modified	En face aorta	Older C57Bl/6Jx129 Sv[52]		
		Aortic root		C57Bl/6J[41]	FVB/NJ[41]
		Brachiocephalic artery		C57Bl/6J[41]	FVB/NJ[41]

the results and figure legends, so that one does not have to search through the methods section or supplement to obtain this information. If only 1 sex was studied, strong justification should be provided.

If a statistically significant effect of a drug, diet, or modifier gene is observed only in 1 but not both sexes, this sex effect should not be assumed to be real if the study is not adequately powered. Here, looking at the effect size can be informative; for example, if a treatment decreases lesion area in female mice by 50% meeting $P<0.05$, whereas decreasing lesion area in males by 40% with $P>0.05$, it could be because of chance that the male data were not statistically significant. In this case, we suggest increasing the number of males to achieve significance. In studies that focus on sex differences, it is imperative to be well powered to observe sex-specific effects, and replication in a second experimental cohort may be recommended.

In addition, the sex of mice used for tissue and cell (macrophage, smooth muscle and endothelial cell) analyses should be stated clearly in the methods and results section. Although it might not always be necessary to perform these studies in tissues/cells from both sexes of mice, we would certainly suggest this in studies that focus on the mechanisms for sex differences.

Thoracic Aortic Aneurysms and Acute Aortic Dissections

Sex Differences in Human Thoracic Aortic Aneurysms and Acute Aortic Dissections

The major diseases affecting the thoracic aorta are aortic aneurysms and acute aortic dissections, which are collectively designated as thoracic aortic aneurysms and acute aortic dissections (TAADs).[58] Premature death because of thoracic aortic diseases has ranked as high as the 15th leading cause of death in the United States.[59] The natural history of TAA involving the aortic root and ascending aorta is to asymptomatically enlarge over time until an acute tear in the intimal layer leads to a dissection (termed Stanford type A dissections). With dissection, blood penetrates the aortic wall and separates the aortic layers, causing aortic rupture and other complications. Although medical treatments can slow the enlargement of an aneurysm, the mainstay treatment to prevent premature deaths because of dissections is surgical repair. This is typically recommended when the aneurysm diameter reaches 5.0 to 5.5 cm; however, studies on patients presenting with acute type A dissections indicate that up to 60% present with aortic diameters smaller than 5.5 cm.[60]

TAADs are categorized as syndromic (associated with abnormalities of other organ systems) and nonsyndromic (with manifestations restricted to the aorta). Up to 25% of individuals with TAADs have evidence of a highly penetrant, pathogenic gene variant conferring a high risk for disease and either have a syndrome (eg, Marfan syndrome) or autosomal dominant inheritance of thoracic aortic disease without syndromic features, collectively termed heritable thoracic aortic disease. For individuals with a single-gene variant predisposing to thoracic aortic disease, the vast majority of causative genes are inherited in an autosomal dominant manner. Therefore, men and women are equally likely to inherit the predisposition. However, sex differences in terms of aortic disease presentation, age at onset, and outcomes vary based on the underlying gene. Syndromic TAADs such as Marfan, Loeys–Dietz, and Ehlers–Danlos have been reported to exhibit sex differences, but the size of reported studies has typically not allowed for extensive quantification of sex differences. In a multinational cohort of patients with ascending aortic aneurysm, aortic enlargement developed earlier in males than females.[61] In 113 patients with Marfan syndrome, aortic root dilation was present in 85% of males compared with 73% of females.[62] Recent results from the Genetically Triggered Thoracic Aortic Aneurysms and Cardiovascular Conditions registry demonstrated that adult males were more likely than females to have aortic root dilatation (92% versus 84%), aortic regurgitation (55% versus 36%) and to have undergone prophylactic aortic root replacement (47% versus 24%, respectively).[61,63,64]

These data, describing the largest cohort to date of Marfan patients, demonstrate that even within syndromic TAA associated with single-gene inherited mutations, male sex is associated with more severe and earlier symptoms. The age of onset of aortic events was even more dramatically different based on sex in patients with TGF-β (transforming growth factor-β) receptor type I (*TGFBR1*) mutations, with 90% of men having an event by the age of 60 years compared with only half the women.[65] The proportion of men presenting with aortic dissections was also higher than women. In contrast, there is no such difference based on sex in patients with TGF-β receptor type II (*TGFBR2*) mutations.[65] For smooth muscle α-actin (*ACTA2*) mutations, aortic events are more prevalent in men than women (62% versus 40%) but the age at first aortic event did not differ significantly between men and women.[66]

In addition to autosomal genetic variants, an alteration in a sex chromosome can increase the risk for thoracic aortic disease. Turner syndrome is a disorder that affects only women and results from partial or complete loss of one X chromosome. Women with Turner syndrome are at an increased risk for bicuspid aortic valves, thoracic aortic enlargement, aortic coarctation, and acute aortic dissections.[67]

In individuals with thoracic aortic disease who do not have a clear genetic trigger for disease, men are more likely to be affected than women. Cohorts of patients presenting for surgical repair of either a thoracic aneurysm or an acute dissection are male predominant, with 65% being men.[68] The International Registry of Acute Aortic Dissection for individuals presenting with an acute aortic dissection reported a mean age of presentation of 63 years old and a male predominance of 65%.[69] Women were significantly older and presented later than men after the onset of symptoms. Although women less commonly experience dissections when compared with men for the general population of aortic dissection cases, their outcome is worse, a difference attributed to delayed diagnosis and atypical symptoms at presentation.[70] Women are more likely to die after admission with an acute aortic dissection than men, and inhospital complications of hypotension and cardiac tamponade occur with greater frequency in women than men. Recent data indicate that women are more likely to undergo thoracic endovascular aortic repair than open repair, and have a higher mortality and lower long-term survival after surgery.[71,72]

Animal Models TAADs and Evidence of Sex Differences

Animal models for thoracic aortic disease have primarily been based on engineering mice to disrupt or insert a genetic variant in a gene known to predispose to thoracic aortic disease. The most commonly studied mouse models involve alteration of fibrillin-1 (*Fbn1*), the gene predisposing to Marfan syndrome. These experimental models include: mice that make no fibrillin-1 ($Fbn1^{-/-}$ mice); 20% of normal fibrillin-1 ($Fbn1^{mgR/mgR}$ mice); and, equal amounts of wild-type and mutant fibrillin-1 harboring a disease-causing missense mutation ($Fbn1^{C1041G/+}$ mice, reported previously as $Fbn1^{C1039G/+}$ mice because the human mutation is at location 1039).[73–75] In these mice, severity of disruption of fibrillin-1 production correlates with t severity of the aortic disease. Marfan mice that make no to little fibrillin-1, the $Fbn1^{-/-}$ and $Fbn1^{mgR/mgR}$ mice, die from ruptured thoracic aortas within the first 2 weeks after birth and a few months of age, respectively. In contrast, the $Fbn1^{C1041G/+}$ mice exhibit slowly enlarging aortic root aneurysms but seldom progress to dissection or rupture. Studies using these mouse models did not describe the sex of mice used and have not reported on differences in the aortic phenotype based on sex.[73,76]

Thoracic aortic diseases were also studied in mice with knockin of *Tgfbr1* or *Tgfbr2* mutations, along with mice with fibulin-4 *(Fbln4)* deficiency in smooth muscle cells.[77,78] Unfortunately, these publications did not report whether male, female, or both sexes were studied. For the $Foxe3^{-/-}$ mice and smooth muscle cell-specific knockout of *Tgfbr2*, the aortic phenotype has only been studied in male mice.[79,80] To model hypertension as a driver of thoracic aortic disease, aortic remodeling has been studied after thoracic aortic constriction only in male mice.[81]

Suggestions for Incorporating Sex into Design and Reporting of Animal TAAD Studies

Suggestions for experimental design and reporting to incorporate sex as a biological variable are similar to those that are described for animal atherosclerosis studies. Because sex differences have not been studied extensively in the available genetic TAAD animal models, we suggest that researchers design, perform, and report experiments to include both male and female mice, even if the major purpose of the study is not to determine sex differences. This will help researchers by providing sex-based data that can be incorporated into the design of future experiments and possibly justify the use of only 1 sex.

Abdominal Aortic Aneurysms

Sex Differences in Human Abdominal Aortic Aneurysms

Abdominal aortic aneurysms (AAA) is defined as a focal dilation of the abdominal aorta (50% or more increase of normal aortic diameter) resulting from degeneration and weakening of the aortic wall.[82] The most common location for human AAA is the infrarenal region of the abdominal aorta. AAA is typically asymptomatic unless rupture is impending, which results in severe bleeding with 85% mortality.[83] Between the ages of 50 and 84 years, AAA prevalence is estimated at ≈1.1 million people in the United States, accounting for 1.3% of all deaths and the 10th leading cause of mortality in men over 55 years of age.[84,85] Ultrasound is the routine method to detect the presence of an AAA. According to the US Preventive Services Task Force, screening for an AAA by ultrasound is recommended for men ages 65 to 75 who smoke or have ever smoked, but there is no recommendation for AAA screening in women.[86,87] Even in men, it is unclear whether routine AAA screening is performed according to these recommendations. This is of concern as, aside from endovascular repair once an AAA has reached a size of 5.5 cm, there are no medical therapies that have been demonstrated to attenuate AAA expansion and rupture. Recent studies suggest that short-term survival

benefit decreases over time after endovascular repair, and that repairs are associated with a higher rate of late rupture.[88] Thus, there is an imperative need to develop AAA medical therapeutics for both sexes.

Male sex is the largest nonmodifiable risk factor for AAA, with estimates ranging from 4-fold to 5-fold higher incidence in men than in women, and a 10-fold higher risk to develop an AAA in men compared with age-matched women.[89,90] Hospitalization for ruptured or intact AAA was reported to be 5× more prevalent in men compared with women.[90] Most likely because of these marked sex differences in AAA prevalence, only the Society for Vascular Surgery has recommended screening women (over 65 years of age) who have smoked or have a family history of AAA,[91] as compared with current recommendations for men. Although women have a lower incidence of AAA, they have worse prognosis than men because AAA has been reported to progress faster in women and to rupture at smaller sizes.[92–94] Moreover, women have been reported to experience more complications and to have higher mortality rates than men after open AAA repair.[95] This sex-specific manifestation may relate to an older age of women (estimated at 10 to 15 years) undergoing endovascular AAA repair.[96]

What might explain more progressive growth of AAA in women, despite a well- documented lower AAA prevalence in women compared with men? Vascular anatomy has been suggested to contribute to differences in not only the diagnosis of AAA in men compared with women but also to differences in AAA expansion. For example, if either the ratio of infrarenal to suprarenal diameter of >1.2 or a diameter of >1.5× the normal aortic diameter is used to define an AAA (rather than a 3.0 cm cutoff), AAA prevalence would be estimated at 6.2% to 9.8%, a much higher prevalence than has been reported in women.[91,97] Thus, use of the same diagnostic criteria for men and women may contribute to underdiagnosis of small AAA in women. Other potential mechanistic contributors to more aggressive AAA expansion in women than men include differences in biomechanical and microstructural properties of the aorta or differences in aortic peak wall stress.[98,99] Moreover, less aggressive management of cardiovascular risk factors such as lipid lowering, hypertension, or smoking in women may contribute to more aggressive AAA expansion. These differences suggest that the threshold for repair of AAA may differ between women and men.[96] However, as the same AAA diameter criteria are typically used for both men and women when assessing the need for endovascular repair, women most likely have more advanced disease at the time of surgery.

Animal Models of AAA and Evidence of Sex Differences

There are 3 commonly used AAA mouse models.[100–103] Elastase-induced AAA in mice is performed by perfusion of elastase into the infrarenal aorta. Immediate procedural dilation occurs, followed by a period of ≈7 days of stable diameter, and subsequent slow expansion.[103] Elastase-induced AAA is also used in rat models.[104] Calcium chloride-induced AAA in mice is performed by peri-aortic application of calcium chloride to the infrarenal aorta.[102] Both of these mouse models share the common human AAA location of the infrarenal aorta. A third mouse model involves minor surgery in which a subcutaneously implanted minipump infuses AngII to induce AAA in the suprarenal aorta.[100,101,105]

Sex differences in the calcium chloride-induced AAA mouse model have not been reported. In elastase-induced AAA, male mice have much larger aortic expansion than female mice, and after intra-aortic elastase perfusion the abundance of estrogen receptor alpha mRNA in female aortas was greater than those of males.[106] In female mice with whole-body deficiency of aromatase, the enzyme that converts testosterone to estrogen, protection against elastase-induced AAA was abolished, whereas ovariectomy of wild-type female mice increased elastase-induced aortic dilation.[107] Administration of mesenchymal stem cells from female mice to male recipients before intra-aortic elastase perfusion attenuated AAA growth.[108] These results suggest that estrogen protects females from elastase-induced AAA through estrogen receptor α-mediated suppression of proinflammatory mediators in pivotal cell types. Similar results were observed in a rat model of elastase-induced AAA. In fact, male rats with intra-aortic elastase perfusion have larger and more frequent AAAs than females.[104] Moreover, transplantation of aortas from male rats into males before intra-aortic elastase perfusion resulted in aneurysm formation, whereas transplantation of female aortas into male rat recipients decreased elastase-induced AAAs.[104]

Similar to what has been observed in elastase-induced AAA, AngII infusion into male *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice results in ≈80% to 100% AAA incidence, whereas age-matched females exhibit only ≈20% AAA incidence.[109,110] Sex differences in AAA development and progression in this model have been linked to both sex hormones and sex chromosomes. Early studies demonstrated that ovariectomy of female *Apoe*$^{-/-}$ mice had no effect on AngII-induced AAA, but orchidectomy of males reduced AAA incidence to the level observed in females.[109] These results imply that testosterone, rather than estrogen, contributes to sex differences in AngII-induced AAA. Replacement of testosterone to castrated male *Apoe*$^{-/-}$ mice recapitulated a high incidence of AAA susceptibility, and female *Apoe*$^{-/-}$ mice administered dihydrotestosterone had a high incidence of AngII-induced AAA.[110] Interestingly, a single acute exposure to testosterone during development in 1 day old female *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice resulted in high AAA susceptibility of adult females.[111] This relatively permanent inducement of AAA susceptibility from neonatal testosterone exposures occurred despite a low concentration of serum testosterone in adult females. In contrast, adult male mice require continuous exposure to testosterone to yield increased AAA prevalence compared with females. When female mice were induced to exhibit high AAA susceptibility by neonatal testosterone exposure, the progressive growth of established AAA was decreased by exogenous administration of estrogen.[112] Thus, both estrogen and testosterone may regulate the progressive growth of AngII-induced AAA.

In addition to sex hormones, sex chromosome defects have been suggested to contribute to differences in vascular disease development. Recent studies using the 4-core genotype model to produce XX and XY *Ldlr*$^{-/-}$ mice with female gonads demonstrated that either the presence of the Y chromosome or the absence of a second X chromosome in XY

females, resulted in a profound increase in the incidence and severity of AngII-induced AAA.[113] These effects occurred in XY females exhibiting low serum testosterone concentrations. However, exposure of XY females to exogenous testosterone resulted in striking aneurysm rupture rates (75%), suggesting that both sex hormones (eg, testosterone) and sex chromosomes (XY chromosome complement) influence development and severity of AngII-induced AAA.

Suggestions for Incorporating Sex into Design and Reporting of Animal AAA Studies

Sex differences on the development and progression of AAA are profound, as demonstrated in both human observations and animal studies. Thus for animal models of AAA, it is vital to report on the sex used and to report data separately by sex. The low incidence of experimental AAA in females provides a challenge for studies in which interventions reduce the disease. Based on calculations of statistical power, it is likely that very large numbers of females will be needed to complete the studies. Hence, this provides a rationale for a restriction of studies to only male. However, if the design of a study is to augment AAA formation, inclusion of females may be feasible, albeit with a large enough number of experimental animals/group to achieve significance. We suggest that studies aimed at identifying mechanisms of reduced AAA formation focus on males, as AAA development is much higher in males, whereas studies in both males and females are suggested to identify putatively enhanced mechanisms of AAA.

Additional studies that specifically focus on mechanisms of sex differences in AAA progression are warranted to learn more about mechanisms of AAA formation and progression in females versus males. This may include defining whether AAA therapeutics, as they emerge, exhibit different efficacy against AAA progression in males versus females.

Peripheral Arterial Disease

Sex Differences in Human Peripheral Arterial Disease

Peripheral arterial disease (PAD) technically refers to any vascular disorder (including dissection, vasculitis, aneurysm, thromboembolism, etc) affecting any noncoronary artery (including carotid, subclavian, and renal arteries). In practice, however, the term PAD is generally used to describe atherosclerotic disease affecting the arteries of the lower extremities. PAD is a highly morbid condition associated with reduced quality of life (eg, because of reduced mobility), disease-specific events (eg, ischemic ulcers and amputation), and high rates of adverse cardiovascular outcomes (typically because of concomitant coronary or cerebrovascular disease),[114] therefore it is a rapidly growing public health epidemic estimated to affect over 200 million people worldwide.[115]

PAD is driven by many of the same risk factors as coronary artery disease such as diabetes mellitus, although smoking plays a more prominent role, and there may exist some heritable factors that specifically impact the peripheral vasculature.[116] Historically, PAD was thought to affect men more than women.[117] However, many of these early epidemiological studies were subject to methodological issues that may have skewed their findings.[118] These issues include (1) defining disease presence on the basis of symptoms (which are known to be absent and unreliable in the majority of PAD patients) rather than with imaging or formal vascular laboratory testing; (2) neglecting to consider the presence of subclavian disease or single vessel PAD (this can result in artificially normal ankle-brachial indices, which estimates vessel patency by comparing blood pressure measured at the ankle and the arm[119]); and (3) failing to consider sex-specific differences in normal ankle-brachial indices values because of the higher mean height of men (ankle pressures are higher in taller individuals). For example, leg pain with walking that is relieved by rest (a symptom known as intermittent claudication) was reported more often in men than women in the Framingham Study, the Framingham Offspring Study, and the Rotterdam Study.[118] However, it is now known that women are more likely to have asymptomatic disease or atypical symptoms, which may have led to under-reporting. When quantitative ankle-brachial indices measurements are used, this sex difference is far less apparent, and several studies found essentially equivalent or even higher PAD rates in women (reviewed in[118]). Indeed, a recent study which attempted to model the global prevalence of PAD found that the condition seems to be more common among women worldwide, especially in low and middle income countries despite less smoking in females than their male counterparts in those areas.[115]

Although intermittent claudication is the symptom most often associated with PAD, the majority of patients present with few if any classical complaints. As mentioned above, women experience their disease differently than men, and are significantly more likely to report atypical symptoms, including pain that begins at rest rather than with exertion.[120] This may delay the appropriate diagnosis of PAD and contribute to the observation that PAD is frequently unappreciated in the clinical setting.[121] Women also present with greater functional impairments, including slower walking speeds, reduced quality of life, and higher rates of concomitant depression.[117,120,122,123]

Once diagnosed, women are known to receive less aggressive risk-reducing therapies than men, as shown in the Reduction of Atherothrombosis for Continued Health registry.[124] This occurs even though there are no data to suggest that women respond differently to therapies such as supervised exercise, cilostazol, or other investigational agents.[117,122,125] Women seem to be selected for surgical intervention less frequently than men,[126] but have more postprocedural complications when they do undergo surgery. For example, women have been reported to experience higher rates of vascular access site occlusion and groin hematoma, and may have lower long-term graft patency rates after intervention.[117,122,127] Anatomic differences in vessel size by sex may account for some of these reported events.[122] Data about associations with other long-term outcomes (eg, survival post-intervention) have been inconsistent, due in part to the fact that women remain underrepresented in contemporary PAD-related trials, accounting for only ≈1/3 of study participants.[122]

As discussed above, the impact of estrogen and other sex hormones on the vasculature is complex and may vary by age and duration of exposure. Some observational studies reported a lower incidence of PAD with long-term HRT.[128] However,

these findings were not confirmed in prospective randomized trials such as the Women's Health Initiative of older women well past menopause and the Heart and Estrogen/Progestin Replacement, a secondary prevention trial.[129,130] Conversely, HRT may be associated with need for reintervention and reduced primary patency rates after revascularization.[117,131]

Animal Models of PAD and Evidence of Sex Differences

Although a variety of studies have been performed in rabbits, pigs, dogs, and primates,[132] the most commonly used animal model of PAD is the murine hindlimb ischemia (HLI) model (visually detailed in Niiyama et al[133]). This model can be performed in several ways, but generally includes the permanent ligation and excision of the femoral artery. Typically, this procedure is performed in young, healthy, nonatherosclerotic mice and unfortunately does not accurately model the natural history of human PAD. Instead, many view it as a model of critical limb ischemia, which can be used to study the angiogenic and arteriogenic response to an acute ischemic insult. Changes across genotype or in response to treatment are assessed by measuring tissue perfusion (via Laser Doppler) and vascular anatomy (by microcomputed tomography or histologically[132,134]). Dyslipidemic *Apoe*$^{-/-}$ and diabetic *Lepr*^{db} mice have been proposed as alternative backgrounds for use in HLI studies.[132]

Few studies have evaluated sex differences in the murine HLI model. Instead, most have focused on the remarkable differences across inbred mouse strains, including the observation that C57BL/6 mice have a rather striking collateral reserve and ability to recover after femoral ligation, whereas BALB/c mice frequently develop digital necrosis postoperatively.[132] This phenomenon has been investigated most thoroughly by Dokun et al,[135] who performed linkage analyses on 95 intercrossed mice, where they identified genetic loci that may confer resistance to tissue loss after an ischemic insult. In this study, the authors reported observing no sex-specific differences in tissue necrosis or perfusion ratio. Conversely, a smaller study of 8 male and 8 female C57BL/6 mice reported that female mice displayed modestly lower flow recovery 28 days postoperatively, possibly because of differences in angiogenesis or vasodilator response.[136] One additional study found that androgen exposure could augment the angiogenic properties of male but not female endothelial cells in vitro.[137] These findings were extended in vivo, where androgen treatment was found to rescue defective blood flow recovery after HLI in orchiectomized mice.[137]

Suggestions for Incorporating Sex into Design and Reporting of Animal PAD Studies

Unlike other cardiovascular diseases such as coronary disease and stroke, sex differences are less well described in patients with PAD. This is also the case for animal models of PAD, with relatively few studies formally quantifying differential responses to HLI across the sexes. Accordingly, firm and evidence-based guidance for the design of including both sexes in PAD-related animal studies is not yet possible. Because of the relative dearth of data in human or animal subjects about sex differences in PAD, we suggest that experimental PAD studies such as HLI assays be performed in 1 sex or both sexes, and, for the latter data should be presented separately for each sex. As cost cannot be used as a justification for utilizing only 1 sex in newly NIH-funded studies, we suggest that future studies be planned using both sexes with results reported separately by sex.

Perspectives

Arterial pathologies are complex traits affected by numerous genetic, environmental, and stochastic variables, which should be specified in articles. For example, the genetic background of the mice should be clearly stated, including the extent of backcrossing (N-number) into a specific genetic background. Sex is a critical biological variable to be considered in the design of animal studies to understand universal versus sex-specific mechanisms and test potential therapies. Animal models provide opportunities to understand the complex mechanisms of sex differences in arterial pathologies that cannot be easily studied in humans. Thus, we highlight our general suggestions for sex as a biological variable in arterial pathology experiments in the box text.

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Highlights

- Both sexes should be used in experimental arterial pathology studies unless justified scientifically.
- Studies should be adequately powered to observe sex differences.
- Data should be reported and analyzed separately by sex, and may be combined post hoc with appropriate correction for sex if necessary.
- Sex should be clearly stated in the methods, results, and figures.
- More arterial pathology studies are warranted on sex differences and their mechanisms.
- Sex should be clearly stated for ex vivo cell/tissue studies, but inclusion of both sexes is not required unless sex differences are being studied.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Consideration of Sex Differences in Design and Reporting of Experimental Arterial Pathology Studies—Statement From ATVB Council

Peggy Robinet, Dianna M. Milewicz, Lisa A. Cassis, Nicholas J. Leeper, Hong S. Lu and Jonathan D. Smith

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