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ROLE OF SEX CHROMOSOMES IN SEXUAL DIMORPHISM OF ANGII-INDUCED ABDOMINAL AORTIC ANEURYSMS

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ROLE OF SEX CHROMOSOMES IN SEXUAL DIMORPHISM OF ANGII-INDUCED ABDOMINAL AORTIC ANEURYSMS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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2018

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ABSTRACT OF DISSERTATION

ROLE OF SEX CHROMOSOMES IN SEXUAL DIMORPHISM OF ANGII-INDUCED ABDOMINAL AORTIC ANEURYSMS

Abdominal aortic aneurysms (AAAs), a permanent dilation in the abdominal region of the aorta, is a highly sexually dimorphic disease. AAAs prevalence is ranging from 4-10 fold higher in males than females. Defining the mechanistic basis for reduced (in females) or increased (in males) AAA formation and progression may uncover potential therapeutic targets. The majority of studies examining sexual dimorphism focus on the role of sex hormones. However, genes residing on sex chromosomes, in addition to sex hormones, may contribute to sexual dimorphism of AAAs. For example, the X chromosome contains about 5% of the whole genome, but the role of sex chromosomes genes to sexual dimorphism of cardiovascular diseases such as AAAs is largely unknown. The purpose of this study was to determine the role of sex chromosomes as mediators of sex differences for angiotensin II (AngII)-induced AAAs in hypercholesterolemic mice.

We used the four core genotype murine model, which enables the creation of phenotypically normal male and female mice with an XX versus XY sex chromosome complement, to test the hypothesis that an XY sex chromosome complement promotes AngII-induced AAAs. Transgenic male mice expressing the Sry gene on an autosome, but not on the Y-chromosome, were bred to female low-density lipoprotein receptor deficient mice to create male and female mice with an XX or an XY sex chromosome complement.

In females, an XY sex chromosome complement doubled the incidence and markedly increased the severity of AngII-induced AAAs. To define mechanisms, we examined gene expression patterns in abdominal aortas and demonstrated elevated expression of inflammatory genes that were linked to increased MMP activity and oxidative stress in aortas from XY females. Moreover, administration of testosterone to XY females, to mimic males, resulted in a striking level of aneurysm rupture.

In males, transcriptional profiling of abdominal aortas revealed 450 genes that were influenced by sex chromosomes. Infusion of AngII to XY males resulted in diffuse pathology along the length of the aorta, while XX males developed focal AAAs, with pathology reduced by orchietomy in both genotypes. Thoracic aortas of XY males exhibited adventitial thickening which was not exist in thoracic
aortas from XX males. Following a prolonged period (3 months) of AngII infusions XY males had AAAs with expanded aortic walls, while XX males had thin walled dilated AAAs.

In summary, our findings demonstrate a remarkable effect of sex chromosome complement to regulate aortic vasculature and disease development. Aside from demonstrating mechanisms of sexual dimorphism of aortic diseases, these findings indicate that chronic sex hormone therapy in the aging and transgender population may have cardiovascular ramifications. Moreover, identification of targets influenced by sex chromosomes and/or sex hormones in a manner that predicts disease development may identify sex-specific approaches to cardiovascular therapy.

KEYWORDS: Sexual dimorphism, Sex chromosomes, Testosterone, Abdominal aortic aneurysm, Angiotensin

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03-09-2018
ROLE OF SEX CHROMOSOMES IN SEXUAL DIMORPHISM OF ANGII-INDUCED ABDOMINAL AORTIC ANEURYSMS

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# TABLE OF CONTENTS

Acknowledgements ............................................................................................................................................ iii

List of Tables .......................................................................................................................................................... viii

List of Figures ......................................................................................................................................................... ix

Chapter I. Introduction ........................................................................................................................................ 1

1.1 Abdominal Aortic Aneurysm ......................................................................................................................... 1

1.1.1 Background ................................................................................................................................................ 1

1.1.2 AAAs pathogenesis ................................................................................................................................ 3

1.1.3 Risk factors ................................................................................................................................................ 5

1.2 Sex differences in AAAs ................................................................................................................................. 7

1.3 AAA and The Renin-Angiotensin System ........................................................................................................ 9

1.4 Role of sex hormones and sex chromosomes in AAAs .............................................................................. 14

1.4.1 Sex hormones .......................................................................................................................................... 14

1.4.2 Sex chromosomes ................................................................................................................................... 17

1.5 Four Core Genotype Mouse Model ........................................................................................................... 23

Chapter I A. Statement of the problem ............................................................................................................. 26

Chapter I B. Hypothesis ........................................................................................................................................ 28

Chapter II. Specific Aim 1 ...................................................................................................................................... 34

Summary .............................................................................................................................................................. 34

2.1 Introduction .................................................................................................................................................. 35

2.2 Methods ....................................................................................................................................................... 37

2.2.1 Animals ..................................................................................................................................................... 37

2.2.2 Isolation of bone marrow cells ............................................................................................................... 38

2.2.3 Gonadectomy .......................................................................................................................................... 38

2.2.4 AngII infusion ......................................................................................................................................... 39

2.2.5 Quantification of AAAs ......................................................................................................................... 39

2.2.6 Blood pressure measurements .............................................................................................................. 40

2.2.7 Measurement of plasma and serum components .................................................................................. 40

2.2.8 Quantification of atherosclerosis .......................................................................................................... 40
2.2.9 Abdominal aorta RNA extraction ................................................. 41
2.2.10 DNA microarrays ......................................................................... 41
2.2.11 Zymography ................................................................................. 42
2.2.12 Quantification of oxidative stress in aortic tissue explants 43
2.2.13 Statistical analysis ......................................................................... 43

2.3 Results .................................................................................................. 44
2.3.1 An XY sex chromosome complement promotes expression of inflammatory genes in abdominal aortas .................. 44
2.3.2 An XY sex chromosome complement results in aggressive AAA formation and severity ......................... 46
2.3.3 Aortas from XY females exhibit augmented inflammatory gene expression, matrix metalloproteinase (MMP) activity, and oxidative stress................................................................. 48
2.3.4 Testosterone exposure (as neonates or adults) results in a striking level of aneurysm rupture in XY females ........ 50

2.4 Discussion .......................................................................................... 51

Chapter III. Specific Aim 2 ........................................................................ 79
Summary ........................................................................................................ 79

3.1 Introduction ......................................................................................... 80
3.2 Methods .............................................................................................. 83
3.2.1 Mice .................................................................................................. 83
3.2.2 Orchietomy ....................................................................................... 85
3.2.3 Measurements of plasma and serum components ................. 86
3.2.4 Blood pressure measurements ....................................................... 86
3.2.5 Quantification of AAAs ................................................................. 87
3.2.6 Quantification of AAs and TAAs ..................................................... 87
3.2.7 Quantification of atherosclerosis .................................................. 88
3.2.8 Quantification of aortic stiffness ................................................. 88
3.2.9 Quantification of adventitial and medial diameter in thoracic aorta tissue sections ....................................... 88
3.2.10 RNA extraction and DNA microarrays .................................... 89
3.2.11 Real Time RT-PCR ....................................................................... 90
3.2.12 Statistical analyses ....................................................................... 91

3.3 Results .............................................................................................. 91
3.3.1 Testosterone and/or Sex Chromosome Complement Influenced Abdominal Aortic Gene Expression Patterns and Aortic Stiffness ................................................................. 91
3.3.2 Aortic Aneurysmal Disease was Diffuse in XY Males and Localized in XX Males ......................................................... 93
3.3.3 Aortic Genes Related to AAA Development Exhibited Region-specific and AngII-induced Differences in Abundance ................................................................. 95
3.3.4 Regional Differences in AngII-induced Aortic Vascular Diseases Between XY and XX Males Persisted with Aneurysm Progression ......................................................... 96
3.4 Discussion ........................................................................................................... 97

Chapter IV. General Discussion ........................................................................ 123
4.1 Summary ........................................................................................................... 123
4.2 Importance of studying role of sex chromosomes in disease........... 126
4.3 AAA is a sexually dimorphic disease ............................................................ 127
4.4 Mechanisms of sex chromosome effects on AAA .................................. 129
  4.4.1 Role of sex chromosome complement on aortic gene expression................................. 129
  4.4.2 Role of sex chromosomes on inflammation............................................... 131
  4.4.3 Role of sex chromosomes on the location of aortic pathology........................................ 132
  4.4.4 Role of sex chromosomes on aortic wall thickness............................. 133
4.5 The interaction of sex chromosomes and sex hormones.............. 135
  4.5.1 The interaction of sex chromosomes and sex hormones in experimental animals................................. 135
  4.5.2 The interaction of sex chromosomes and sex hormones in humans......................................................... 138
4.6 Importance of including sex chromosomes in genome wide association studies (GWAS)................................. 139
4.7 Clinical Significance ......................................................................................... 141
4.8 Future directions ................................................................................................. 142
4.9 Limitations of the study ................................................................................... 145
4.10 Concluding remarks ......................................................................................... 146

References .......................................................................................................... 148

Vita ...................................................................................................................... 172
LIST OF TABLES

Table 1.1 Summary of findings of human AAAs studies using RAS inhibitors.....29
Table 1.2 Summary of animal AAA studies modulating the RAS.........................30
Table 1.3 Summary of animal AAA studies modulating the sex hormones.........31
Table 2.1 Biological pathway analysis for genes overexpressed in XY females..58
Table 2.2 Primer sequences.............................................................................. 59
Table 2.3 Complete list of DNA array results...................................................... 60
Table 2.4 Characteristics of female XX and XY mice infused with AngII .......... 62
Table 3.1 Primer sequences for RTPCR ............................................................. 104
Table 3.2 Upregulated genes in XY vs. XX males ............................................. 105
Table 3.3 Biological pathways upregulated in XY and XX males ..................... 106
Table 3.4 Characteristics of XY and XX male Ldlr-/ mice infused with AngII .. 108
| Figure 1.1 | The four core genotype model | 33 |
| Figure 2.1 | Sex chromosome complement influences abdominal aortic gene expression patterns | 63 |
| Figure 2.2 | An XY sex chromosome complement markedly promotes formation and severity of AngII-induced AAAs in female mice | 64 |
| Figure 2.3 | IL1β expression in abdominal aortas | 66 |
| Figure 2.4 | Aortas from XY females exhibited augmented MMP2 activity and dihydroethidium (DHE) fluorescence | 67 |
| Figure 2.5 | Severe AAA rupture in XY females administered dihydrotestosterone (DHT) | 68 |
| Figure 2.6 | Administration of testosterone (400 μg) to 1 day old neonatal female XY mice results in severe AAA rupture when adult XY females are infused with AngII | 69 |
| Figure 2.7 | mRNA abundance of GAPDH in abdominal aortas from XX and XY Ldlr-/- females | 71 |
| Figure 2.8 | Immune/inflammatory signaling is upregulated in the aortas of females with an XY chromosomal complement | 72 |
| Figure 2.9 | Aneurysm type as an index of severity of AAAs and Kaplan Meier Survival Curve from XX and XY Ldlr-/- females infused with AngII | 73 |
| Figure 2.10 | Abdominal aortas from XY females infused with AngII exhibit increased wall volume. Bottom, representative 3-D reconstruction from ex vivo ultrasound analysis of one AAA/genotype | 74 |
| Figure 2.11 | Morphology of abdominal aorta from XX versus XY females prior to AngII infusions | 75 |
| Figure 2.12 | Representative AAA tissue sections from XY females infused with AngII exhibit pronounced CD68 immunostaining in areas exhibiting breaks in medial elastin | 76 |
| Figure 2.13 | Sex chromosome complement has no significant effect on AngII-induced atherosclerosis in thoracic aortas | 77 |
Figure 2.14 Abdominal aortic lumen diameters (A) and survival plots (B) over days of AngII infusions of XX and XY Ldlr/- females administered DHT ........................................................................................................ 78

Figure 3.1 Graphic depiction of experimental design for studies focused on the formation (A) versus the progression (B) of AngII-induced AAAs... 109

Figure 3.2 Sex chromosome complement, sex hormones, and an interaction between these factors influence gene expression patterns in abdominal aortas of XY and XX males ..................................................... 110

Figure 3.3 RT-PCR analysis of mRNA abundance of key genes in abdominal aortas from male XY and XX mice .................................................. 112

Figure 3.4 Pulse Wave Velocity (PWV) from male XY and XX Ldlr/- mice ... 113

Figure 3.5 An XY sex chromosome complement mediates diffuse aortic vascular disease, while an XX sex chromosome complement is associated with discrete aneurysmal disease in abdominal aortas of male AngII-infused mice .......................................................... 114

Figure 3.6 An XX sex chromosome complement results in focal AAA pathology of increased size compared to XY males infused with AngII ....... 116

Figure 3.7 Thoracic aortas from XY males, but not XX males, exhibit adventitial thickening in response to AngII infusions ......................................... 117

Figure 3.8 mRNA abundance of key genes implicated in aneurysm development in thoracic versus abdominal aortas from XY and XX male mice infused with either saline or AngII-infused (1 day) ......................... 118

Figure 3.9 XX males exhibit dilated AAAs while XY males exhibit diffuse aortic disease with prolonged AngII infusions (3 months).................. 120

Figure 3.10 Ex vivo ultrasound analysis of AAAs from XY and XX male mice infused with AngII for 3 months .............................................................. 122

Figure 4.1 Illustration of the role of sex chromosomes on AngII-induced AAA susceptibility in mice ........................................................................ 147
CHAPTER I. INTRODUCTION

1.1 Abdominal Aortic Aneurysms

1.1.1 Background

Abdominal Aortic Aneurysms (AAA) are asymptomatic vascular diseases with a high mortality rate due to aortic rupture that is manifest in older adults. AAAs are permanent dilations of the abdominal region of the aorta, the major blood vessel supplying blood from the heart to all major organs. In the United States, AAAs are defined as an expansion of the infrarenal aorta by more than 50% of the normal internal lumen diameter (Lederle FA, Johnson GR et al. 2000). In Europe, lumen diameter equal or greater than 3 cm is considered aneurysmal (Ashton HA, Buxton MJ et al. 2002), and because of the high probability of rupture, surgical intervention is recommended when the diameter reaches a 5.5 cm limit (Powell and Brady 2004; Jamrozik K et al. 2001). Because no medical treatment has proven effective against AAA progression, intervention by open or endovascular surgical repair is the only available option to reduce the potential for aortic rupture. However, there is still a high mortality rate after repair (Chang DC, Parine RP et al. 2015; Pecoraro F, Gloekler S et al. 2017). The mean 30-day mortality rate for this surgical procedure is 1.1%-7%, which varies among surgical centers and surgeons (Sakalihasan N, Limet R et al 2005).

Men are more susceptible to AAAs than women. The prevalence of AAAs is about 1% in women and 4% in men between 50-79 years old, and
the disease is responsible for 1.3% of all deaths in men aged 65-85 (Lederle FA, Johnson GR et al. 2000; Lederle FA, Johnson GR et al. 2001; Best VA et al. 2003; Sakalihasan N, Limet R et al. 2005). In the last three decades, the incidence of AAAs has increased dramatically (Best VA, Price JF, et al. 2003; Collin J 1988; Fowkes FG, Macintyre CC et al. 1989; Nasim A, Sayers RD et al. 1995). Compared to data in 1981, AAA mortality rate and hospital admissions due to AAAs increased by 2.6 fold and 3 fold, respectively, in 2000, which could be due either to an increase in disease awareness or to an increase in the aged population, or both (Best VA, Price JF, et al. 2003). In 1991, in the United States, there was about 16,696 deaths due to a ruptured aortic aneurysm or surgical complication of aneurysm repair (Gillum 1995). AAAs are estimated to rupture at a rate of 5.6 cases/ 100,000 people/ year (Bengtsson and Bergqvist, 1993), with an increased likelihood of AAA rupture as aneurysm size progresses.

According to Lardaro et al, in the Journal of Emergency Medical Services JEMS, aortic rupture, also they named it as “aortic catastrophe”, is considered one of the five most common causes of sudden unexpected death and urge every emergency medical service provider to take this condition seriously during their early diagnosis of the patient specially if the patient is old and presented with abdominal or back pain (Thomas Lardaro, Jared Mckinney et al. 2015). Unfortunately, the risk of death (85%) is astonishingly high following aneurysmal rupture, and many patients die before ever reaching the hospital.
1.1.2 AAA Pathogenesis

AAA is a complex multifactorial disease, and many destructive factors and cells are involved in damaging the essential components of the aortic wall (elastin and collagen). The pathophysiology behind the cellular and molecular mechanisms that are involved in the development of human AAAs is not well understood. Histologically, AAAs in humans is characterized by medial degeneration, intensive inflammation, thrombus formation, break in the elastic media, and remodeling of wall layers (Busuttil RW et al. 1982; Campa JS, Greenhalgh Rm et al. 1987; Holmes DR, Liao S et al. 1995). AAA tissue sections exhibit infiltration of macrophages, T cells, and B cells (Curci JA, Liao S et al. 1998; Galle C, Schandene L et al. 2005; Xiong W, Zhao Y et al. 2004; Chang TW, Gracon AS et al. 2015) that secrete degradation factors like proteases and elastases which degrade the elastic media (loss of elastin leads to dilation) and collagen (loss of collagen leads to rupture) in the aorta. This combination of degradation will lead to a decrease in the stretching strength of the wall allowing dilation and AAA formation (Saraff K et al. 2003, Barisone C, Charnigo R et al. 2006). Indeed, a frequently used animal model of AAAs targets the elastin in the aortic wall by infusing the aorta with elastase to induce AAA formation. Further, it has been shown that a high proteases enzyme activity in the aorta is positively associated with AAA formation (Alsriraj Y et al. 2017, Halpern VJ et al. 1994). There is a plethora of evidence that matrix metalloproteinases (MMPs) have a
principal role in AAA development. Proteinases are usually enriched in AAA tissue sections (Vine N and Powell JT 1991; Herron GS, Unemori E et al. 1991; Thompson RW, Holmes DR et al. 1995; Freestone T et al. 1995). Additionally, there is a positive correlation between MMP9 (gelatinase) plasma concentration (Lindholt JS et al. 2000), gelatinase mRNA abundance in the abdominal aorta (McMillan WD, Tamarina NA et al. 1997) and AAA size. Moreover, transgenic mice with genetic deletions of MMPs exhibit reduced experimental AAA development (Pyo R, Lee JK et al. 2000, Longo GM et al. 2002). MMP inhibition using the antimicrobial drug, doxycycline, or chemically modified tetracyclines, which also have MMP inhibition properties, have been proven effective in reducing experimental AAAs in most commonly used animal models when given prior to the initiation of the disease (Petrinec D, Liao S et al. 1996; Curci JA, Petrinec D et al. 1998; Bigatel DA, Elmore JR et al. 1999; Manning MW, Daugherty A et al. 2003; Takai S, Jin D et al. 2013). However, MMP inhibition was not effective in blunting AAA progression when administered to mice with an established AAA (Xie X, Daugherty A et al. 2012). In humans, there was no benefit of doxycycline in reducing the growth rate of small AAAs (Meijer CA, Stijnen T et al. 2013; Baxter BT, Pearce WH, et al. 2002). In addition to MMPs, proinflammatory markers are also believed to be involved in AAA formation like interleukin-6, interleukin-1β, interleukin-17, and C-reactive protein (Kokje VBC, Gabel G et al. 2016; Takagi H, Watanabe T et al. 2014; Lindberg S, Zarrouk M et al. 2016;

1.1.3 Risk Factors

There are many risk factors that are positively associated with AAAs which include age, male sex, cigarette smoking, genetics, ethnicity, obesity, hyperlipidemia, low high-density lipoprotein, and atherosclerosis (Derubertis BG, Trocciola SM et al. 2007; Lederle FA, Johnson GR et al. 2000; Kent KC, Zwolak RM et al. 2010). AAA prevalence increases with age in both sexes (Singh K, Bonaa KH et al. 2001). The risk for AAAs increases significantly in people above 60 years old, and the prevalence increases by 2-4% every ten years afterward (Singh K, Bonaa KH et al. 2001).

Smoking is a significant risk factor for AAA formation. Studies found that smokers have 7 fold greater risk for AAA development than non-smokers (Benson RA, Poole R et al. 2016; Norman PE and Curci JA 2013). There is a direct correlation between the number of smoking years and AAA formation and that risk decreases after smoking discontinuation
(Norman PE and Curci JA 2013). Although males and females have a similar association between smoking and AAAs development the decline in the risk after smoking cessation is faster in females than males (Kent KC, Zwolak RM et al. 2010; Lederle FA, Johnson GR et al. 2001; Stackelberg O, Björck M et al. 2014). Smoking also affects the AAA endovascular surgical repair as smokers need more stents and they have a higher risk of stent graft migration than non-smokers (Koole D, Moll FL et al. 2012).

Aside from age, male sex, and smoking, AAAs are at a higher prevalence in Caucasians compared to other races (Benson RA, Poole R et al. 2016; Kent KC, Zwolak RM et al. 2010). Genetic inheritance also increases AAA risk, as AAA prevalence is very high in siblings of AAA patients, especially male siblings they have about 4 fold increased risk for AAAs, and they develop AAAs at an earlier age than people with no family history and it is recommended to start screening the male siblings of patients with AAA at a younger age than males with no family history (Linné A, Lindström D et al. 2012; Salo JA et al. 1999; Linné A, Forsberg J et al. 2016; Linné A, Forsberg J et al. 2017). Obesity has similarly been found to have a positive correlation with AAA formation (Stackelberg O, Björck M et al. 2013; Cronin O, Walker PJ et al. 2013). In contrast, diabetes mellitus has a negative correlation with AAA development (Pafili K, Gouni-Berthold I et al. 2015; Lederle FA, Johnson GR et al. 2000; Le MT, Jamrozik K et al. 2007; Theivacumar NS, Stephenson MA et al. 2014).
Several studies have found no association between the serum concentrations of cholesterol, triglyceride, and low-density lipoprotein and AAA development (Blanchard, Armenian et al, 2000; Golledge J, van Bockxmeer F et al 2010). However, other studies have reported that hyperlipidemia, ischemic heart diseases, and high blood pressure are independently associated with AAAs (Törnwall ME, Virtamo J et al 2001; Derubertis BG, Trocciola SM et al 2007; Kent KC, Zwolak RM et al. 2010).

1.2 Sex Differences in AAAs

AAAs are a highly sexually dimorphic disease, with male sex considered the strongest non-modifiable risk factor (Wanhainen, Bergqvist et al. 2005; Forsdahl SH, Singh K et al. 2009). The AAA incidence in males is 4 times higher than females (Lederle FA, Johnson GR et al. 2001; Katz DJ, Stanley JC, et al. 1997). Despite females protection from AAAs, if females develop an AAA they have higher growth rates, higher rupture rates, rupture at smaller sizes, and worse prognosis after surgical repair compared to males (Noel AA, Gloviczki P et al. 2001; Forbes TL, Lawlor DK et al. 2006; Solberg, Singh et al. 2005; Mofidi R, Goldie VJ et al. 2007). Importantly, female protection against AAAs is diminished after menopause (Bengtsson H, Sonesson B et al. 1996), and the surgical repair for AAA in females is more difficult because AAAs develop at an older age (Bengtsson H, Sonesson B et al. 1996).

Ultrasound screening for AAA is recommended for males aged 65 to 75 years and especially those with a family history of AAAs and
smokers (Hirsch AT, Haskal ZJ et al. 2006). However, because females have a lower incidence, the Society of Vascular Surgery only recommends screening for female smokers and those that have a family history of AAAs (Hannawa K, Eliason et al. 2009; Kent K et al 2004; Derubertis B, Troccoli EJ et al. 2007).

One potential mechanism of sexual dimorphism of AAAs is differences in the vascular anatomy between males and females. Due to size differences, males tend to have larger and stronger aortas than females, and this results in a divergence in their vessel biomechanical properties (Ninomiya OH, Tavares Monteiro JA et al. 2015). The same study also showed that male aortas had a higher failure load and failure tension than female aortas. Another small study showed that the peak wall rupture risk was higher in females (Larsson E, Labruto F et al. 2011). Furthermore, males and females have anatomic differences that result in different hemodynamics between the two sexes. For example, the presence of the uterus in females direct the blood to move away from the iliac and infrarenal aorta and reduce the amount of stress on these vessels (Taylor WR, Iffrig E et al. 2016). This results in divergence in oscillatory stress between males and females. In males, due to the absence of a uterus, there is a high amount of blood circulation that stress the infrarenal aorta and might activate the inflammatory system in the endothelium (Taylor WR, Iffrig E et al. 2016).
While these anatomical differences are known to exist between the sexes, recent studies have examined whether sex hormone replacement therapy also influences AAA development. Clinical studies in females have demonstrated that sex hormone replacement for greater than 5 years decreased the odds ratio of developing an AAA (Lederle FA, Larson JC et al. 2008). Interestingly, it has been shown that females that reach their menopause at a younger age have larger AAAs than females that reach their menopause at an older age (Villard C, Swedenborg J et al. 2011).

1.3 AAAs and The Renin-Angiotensin System

The renin-angiotensin system (RAS) has been implicated in aortic aneurysms as the chronic subcutaneous infusion of AngII to mice led to AAA formation (Daugherty A, Cassis LA et al. 2000, Henriques TA et al. 2004; Henriques TA et al 2008, Zhang X et al 2012; Xie X, Daugherty et al. 2012; Thatcher SE et al. 2014; Thatcher SE et al. 2015; Zhang X et al. 2015; Alsiraj Y et al. 2017). The RAS is a system that consists of multiple components that are produced in different organs and work in harmony to regulate blood volume, blood flow, blood pressure, and electrolyte balance. In this system, angiotensinogen, the main precursor peptide, is produced in the liver and is cleaved to form angiotensin I in the circulation by the enzyme renin secreted from the kidney (Matsusaka T, Niimura F et al. 2012). Angiotensin I is cleaved to form AngII by angiotensin-converting enzyme (ACE) that is primarily distributed in endothelial cells of the
pulmonary circulation. AngII increases the production of reactive oxygen species along with the induction of several other important factors via angiotensin type 1 receptors (AT1Rs) resulting in vasoconstriction, endothelial dysfunction, inflammation, increased level of oxidative stress, as well as migration/proliferation of vascular cells (Rajagopalan S, Kurz S et al. 1996; Daugherty A and Cassis L1999; Daugherty A, Cassis LA et al. 2000; Braun-Dullaeus RC, Mann MJ et al. 1999).

Since 1999, in collaboration with Dr. Alan Daugherty at the University of Kentucky, our laboratory has demonstrated that chronic infusion of AngII to hypercholesterolemic mice results in reproducible AAA formation in the suprarenal abdominal aorta (Daugherty A, Cassis LA et al. 2000, Henriques TA et al. 2004; Henriques TA et al. 2008, Zhang X et al. 2012; Xie X, Daugherty et al. 2012; Thatcher SE et al. 2014; Thatcher SE et al. 2015; Zhang X et al. 2015; Alsiraj Y et al. 2017). The cellular and molecular consequences in AAAs that produced by AngII infusion are medial and adventitial macrophage accumulation, medial dissection, elastin breaks, that lead to lumen expansion, thrombus formation, and adventitial inflammation (Saraff K, Babamusta F et al. 2003). In hypercholesterolemic mice, AngII infusions lead to AAAs in more than 75% of the males and 10%-20% of the females (Henriques TA et al. 2004; Henriques TA et al. 2008; Zhang X et al. 2012). Similarly, in the elastase perfusion model of AAAs, male rats had larger and more frequent AAAs.
than females when aortic dilation was induced by intraluminal elastase perfusion (Ailawadi G, Eliason JL et al. 2004).

Our laboratory demonstrated previously that sex hormones, specifically androgens, are primary contributors to higher AAA susceptibility in male compared to female mice, as ovariectomy had no effect on AAA formation while orchiectomy decreased AAA incidence to the level of females (Henriques TA et al. 2004). Further studies demonstrated that testosterone is a primary mediator of the high prevalence of AAAs in male mice by increasing AT1aR mRNA abundance specifically in the abdominal region of the aorta (Henriques TA et al. 2008; Zhang X et al. 2012).

The RAS, an important contributor to experimental AAAs, display sex differences in the gene expression of a number of its important elements (Wang Y, Pringle KG et al. 2012; Maric-Bilkan C and Manigrasso MB 2012). Interestingly, genes of some of the essential components of this system, including, prorenin receptor, angiotensin I converting enzyme 2 (ACE2), and angiotensin II type 2 receptor (AT2R) are located on the X chromosome. However, previous studies concentrated on gonadal hormones as the primary mediator of sexual dimorphism in the RAS.

The two most important classes of drugs inhibiting the RAS are ACE inhibitors (ACEi) that are used to block the conversion of angiotensin I to AngII, and selective AT1R antagonists (also known as ARBs) that antagonize the binding of AngII to AT1 receptors (Robles NR, Cerezo I et
al. 2014). These drugs suppress the activity of the RAS. Unfortunately, probably due to difficulties in obtaining sufficient sample sizes of AAA patients for large-scale RAS inhibition studies, or because so many AAA patients are already prescribed inhibitors of the RAS, few studies have investigated effects of RAS inhibitors on human AAAs. Studies to date have an inconsistent agreement about the beneficial effect of RAS inhibition in AAA (Table 1.1). In a nationwide cohort study, administration of ACEi reduced all-cause mortality in patients with AAAs who had not undergone endovascular repair (Kristensen KE, Top-Pedersen C et al. 2015). In a population-based case-control study, AAA patients treated with ACEi had less chance of aortic rupture, and this beneficial effect was not seen in patients treated with other antihypertensive drugs, surprisingly including ARBs (although the patient sample size in this group was small) (Hackam DG et al. 2006). In contrast, retrospective analysis over 25 years suggested that ARBs reduced the rate of AAA growth while ACEi was not effective in slowing AAA growth rates (Thompson A, Hafez H 2010). In another retrospective analysis study over a period of 15 years (from 1995 to 2010) using the data files from 3 medical centers in the USA found no effect of ACEi or ARBs on AAA enlargement (Lederle FA, Noorbaloocchi S et al. 2015). Surprisingly, a retrospective study in the United Kingdom reported that ACEi increases AAA expansion (Sweeting MJ, Thompson SG et al. 2010). Most recently, a randomized placebo-controlled trial conducted in 14 hospitals in England found no significant effect of
perindopril, an ACEi, on the growth rates of small AAAs when compared to AAA patients who received placebo or a calcium channel blocker (Bicknell CD, Kiru G et al. 2016).

In standard AAA animal models, modulation of the RAS components pharmacologically or genetically influences AAA formation (table 1.2). Deletion of AT1aR inhibited AAA formation in AngII-infused male mice (Cassis LA, Rateri DL et al. 2007) and in aortic elastase-perfused male mice (Xuan H, Dalman RL et al. 2017). Drug antagonism of AT1R completely inhibited AAA formation in AngII-infused male mice (Daugherty A, Cassis LA et al. 2001) and in aortic elastase-perfused male mice (Iida Y, Xu B, Dalman RL et al. 2012; Xuan H, Dalman RL et al. 2017), while drug antagonism of AT2R increased the severity of AngII-induced AAAs (Daugherty A, Cassis LA et al. 2001). Furthermore, Liao et al. reported that ACEi blunted the development of AAA in elastase-perfused rats (Liao S, Miralles M et al. 2001). Moreover, administration of aliskiren, a direct renin inhibitor, to ApoE-/- mice limited the progression of AngII-induced AAAs (Seto SW, Krishna SM et al. 2014). Additionally, pharmacological activation of ACE2 reduced internal and external aortic diameters as well as decreased the incidence of AngII-induced AAAs in male mice (Thatcher SE, Zhang X et al. 2014) and ACE2 deletion resulted in increased aortic diameter and AAA incidence in AngII-infused male mice (Moran CS, Biros E et al. 2017). These results illustrate that RAS blockade is effective at inhibiting the formation of experimental AAAs, but there is insufficient evidence supporting a role for RAS blockade in human AAAs.
Perhaps these differences arise from the heavy focus on the role of the RAS in AAA formation in experimental studies, versus a need to develop therapies blunting progression of an established AAA in humans.

1.4 Role of Sex Hormones and Sex Chromosomes in AAAs

Both human and rodent males are much more susceptible to AAAs than females. Males are known to differ from females in three ways: sex chromosomes, sex organs, and sex hormones. These three produce multiple phenotypic differences between males and females. Sex chromosomes determine the sex of the fetus. Male and female sex organs produce different sex hormones that generate the main differences between the two sexes.

1.4.1 Sex Hormones

In males, testosterone is the main androgen that is important for many essential physiological functions. Testosterone is responsible for the development and maintenance of testes, sexual drive, secondary sex characteristics, sperm production, sexual behavior, bone density, muscle growth and strength (Murashima A, Kishigami S et al. 2015; Carson JA and Manolagas SC 2015; Windahl SH, Andersson N et al. 2011). Testosterone is synthesized in the Leydig cells of the testes, and its production is regulated by the hypothalamic-pituitary axis, this axis secretes hormones like a gonadotropin-releasing hormone, luteinizing hormone, and follicular stimulating hormones in response to the low level
of testosterone (Tremblay JJ 2015, Potter SJ, Kumar DL et al. 2016). By the action of the enzyme 5α-reductase, small portion of circulating testosterone is converted to the more active form, dihydrotestosterone (DHT). Both testosterone and DHT are binding to androgen receptors (AR). Unlike testosterone, DHT strongly binds to the AR and slowly dissociates from the receptor. That is why DHT has a more augmented action than testosterone (Tóth M and Zakár T 1982; Zakár T, Kaufmann G et al. 1986; Lemus AE, Enríquez J et al. 1997). Testosterone also gets converted to estradiol by aromatization through the action of the enzyme aromatase (Ishikawa T, Glidewell-Kenney C et al. 2006).

In females, the main sex hormones are estrogens, which are a group of steroid hormones that have an important role in female sexual functions. Estradiol is the main estrogen; it is synthesized mainly in the ovary, but estrogens are also produced by the adrenal glands and adipose tissues. Estrogens act by interacting with estrogen receptors (ER alpha or ER beta) (Eyster KM 2016; Fietz D et al. 2014, Walter et al. 1985, Mosselman et al. 1996, Nelsson S and Gustafsson JA 2011).

Many studies on the sexual dimorphism of AAAs have focused on sex hormones (table 1.3). It has been shown that testosterone is a primary mediator for the development of AAAs in both male and female rodents (Henriques T et al. 2004; Zhang X et al. 2015; Zhang X et al. 2012; Davis J, Salmon M et al. 2016; Cho BS, Woodrum DT et al. 2009; Alsiraj Y et al. 2017). Orchiectomy reduced AngII-induced AAA formation (Henriques TA
et al. 2004; Henriques TA et al. 2008, Alsiraj Y et al. 2017) and progression in male mice (Zhang X et al. 2015), and orchiectomy also reduced AAA formation in aortic elastase perfused male rats (Cho BS, Upchurch GR et al. 2009). Furthermore, androgen deletion inhibited AAA formation in both AngII and elastase-induced AAAs of male mice (Huang CK, Chang C et al. 2015; Davis JP, Upchurch GR et al. 2016). Additionally, blockade of androgen receptors reduces AAA formation in elastase perfused male mice (Davis JP, Upchurch GR et al. 2016). In one study, using the AngII-infusion model, it was shown that testosterone increases the expression of AT1aR in abdominal aortas of male mice, and further, this correlated positively with AAA incidence (Henriques T et al. 2008). It has also been shown that exposure of neonatal female mice to a single dose of testosterone increased AT1aR mRNA abundance in abdominal aortas and AAA incidence of adult females (Zhang X et al. 2012; Alsiraj Y et al. 2017). Besides increasing the expression of AT1aR, androgen effects on AAAs have also been attributed to increased oxidative stress (Chignalia A et al. 2012; Alsiraj Y et al. 2017), inflammation (Razmara A et al. 2005; Sinha I et al. 2006), and augmented MMP activity (Woodrum D et al. 2005; Laser A et al. 2014; Alsiraj Y et al. 2017).

In contrast to testosterone, multiple studies have shown that estrogen has a protective effect on AAA development and progression in female and male mice and rats (Martin-McNulty B 2003; Thatcher SE et al. 2015; Laser A et al. 2014; Grigoryants V et al. 2005, Wu XF, Zhang J
et al. 2009). Estrogen decreased inflammatory pathways (Sinha I et al. 2006) and MMP activity in rat aortic explants (Woodrum D, Ford JW et al. 2005). However, our laboratory reported that removal of endogenous estrogen by ovariectomy had no effect on AngII-induced AAAs in female mice (Henriques TA et al. 2004). In contrast, orchiectomy of male mice reduced the formation of AngII-induced AAAs to the level of females (Henriques TA et al. 2004), with AAA incidence restored to normal levels by administration of DHT (Henriques T et al. 2008). Similar to our results, studies in the aortic elastase perfusion model in rats reported a similar conclusion, removal of testosterone from male rats by orchiectomy decreased AAAs while removal of estrogen from females by ovariectomy had no effect on AAAs (Cho BS, Upchurch GR Jr et al. 2009).

1.4.2 Sex Chromosomes

Although testosterone is a primary contributor to sex differences in AngII-induced AAAs, it is unlikely to be the only causative factor. Previous studies in our laboratory demonstrated that hypercholesterolemic female mice administered a single dose of testosterone when they are one day old resulted in a high increase in AT1aR mRNA abundance in the abdominal region of the aorta, and substantially increased the susceptibility to AngII-induced AAA when they are adults (Zhang X et al. 2012). Interestingly, the neonatal testosterone effect was permanent and did not necessitate continued exposure to testosterone. Surprisingly, male and female mice reacted differently to the administration of neonatal
testosterone. The same dose of testosterone administered to one-day old males didn’t change the susceptibility of adult males to AngII-induced AAA development. Moreover, continuous levels of circulating testosterone are required in males to maintain AAA susceptibility (Zhang X et al. 2012), while females were rendered susceptible after a single androgen exposure.

In addition to sex hormones, sex chromosomes have been suggested to contribute to sexual dimorphism of cardiovascular diseases (Winham SJ, de Andade M et al. 2015). Probably the most compelling association between sex chromosomes and vascular disease development is in Turner’s Syndrome, also called monosomy X. Turner’s Syndrome results from the partial or complete absence of the second X chromosome in females, and is associated with significant vascular abnormalities. Specifically, the risk of aortic dissection is increased 100-fold in patients with Turner’s Syndrome (Wong SC et al. 2014; Bondy CA 2008). Moreover, recent studies revealed that sex chromosome complement affects the blood pressure in AngII infused mice (Ji H, Zheng W et al. 2010), which could indicate that other AngII-mediated effects may also be influenced by sex chromosome complement.

Each cell in the human body has a sex, cells carry the genetic information in 23 pairs of chromosomes that are structured of nucleic acids and proteins. One pair of which, because they are related to sex, is called the sex chromosomes while the rest are called autosomes. The autosome pairs are named by numbers from 1 to 22 while the sex
chromosomes are named as X and Y. Male cells have a pair of X and Y while female cells have a pair of two X chromosomes (Buchholz JT 1947; Mirsky AE and Ris H 1951). The male and female reproductive cells, sperm and ovum respectively, are haploid cells and contain only one copy of sex chromosome, the sperm has either the X or the Y chromosome while the ovum has only X chromosome (Munger SC and Capel B, 2012). During reproduction, sperm and ovum combine together to produce the zygote. The sex chromosome of the sperm is the only one that will determine the sex of the fetus; if the sperm that combines with the ovum contains the Y chromosome then the fetus will be a male, but if the sperm contains the X chromosome then the fetus will be a female (Munger SC and Capel B, 2012; Brennan J and Capel B 2004).

There is a small number of genes on the Y chromosome compared to any other chromosome. This is due to the fact that the Y chromosome does not recombine normally as the rest of the genome which result in losing most of its genes during evolution. In fact, other species, like orthoptera, has lost the entire Y chromosome. (Charlesworth B and Charlesworth D 2000; Lahn et al. 2001; Bachtrog et al. 2008; Hughes et al. 2012; Castillo ER, Bidau CJ et al. 2010). The Y chromosome consists of two regions: the pseudoautosomal region and the male-specific region of the Y chromosome (MSY). The pseudoautosomal region is the region at which the Y chromosome exchanges genetic material with the X chromosome during meiosis (Helena Mangs A and Morris BJ, 2007;
Dhanoa JK et al. 2016). The MSY, which represent about 95% of the Y chromosome, consists of the euchromatic and heterochromatic region (Skaletsky H et al. 2003; Jangravi Z et al. 2013). Most of the Y chromosome genes have sex functions (Dhanoa JK et al. 2016). The key gene on the Y chromosome is the Sry (sex determining region on the Y), this gene is responsible for testes formation (Prokop JW and C.F. Deschepper CF, 2015). The Sry has also been implicated in the regulation of some of the RAS genes (Ely D, Boehme S et al. 2011; Araujo FC, Milsted A et al. 2015). In contrast, the X chromosome is morphologically larger and contains more genes than the Y chromosome. In fact, the X chromosome represents about 5% of the cell genome (Johnson N.A. and Lachance J. 2012; Warburton PE et al. 2004).

The presence of two X chromosomes in females leads to a higher gene expression than males, but females inactivate one of the two X chromosomes by the process of X chromosome inactivation (XCI). This process is important to create a balance in gene quantity between XX and XY cells (Lyon MF 1961; Lyon MF 1962; Jeppesen P and Turner BM, 1993; Ines Pinheiro and Edith Heard, 2017). The inactivated X chromosome remains inactive throughout the lifetime of the cell (Barakat TS and Gribnau JX 2012). The mechanism of XCI is complex and highly organized, and the precise mechanism is still unclear. The X chromosome that is going to be inactivated express long non-coding RNA called Xist (Pinheiro I and Heard E 2017; Cerase A, Pintacuda G et al. 2015). The
expressed Xist recruit proteins followed by several epigenetic changes that result in chromatin modifications. Genes close to the X-inactivation center are silenced first and the silencing spread along the length of the chromosome until the whole chromosome is packed and condensed in an inactive form called a Barr body (Barr ML and Bertram EG 1949; Lyon MF 1961; Chaligné R and Heard E, 2014; Huynh KD, Lee JT 2003; Jeppesen P and Turner BM, 1993; Ines Pinheiro and Edith Heard, 2017). There are two types of XCI: random XCI and imprinted XCI. In mice, the X chromosome undergoes imprinted XCI. Here, the inactivation is selective where the X chromosome from the paternal side is selected for inactivation during the early stages of the embryo development (Sado T, Wang Z et al. 2001; Matsui J, Goto Y et al. 2001). However, this paternal X chromosome is later activated in the development cycle and random XCI occurs, that means there is no preference for inactivation, either the maternal or the paternal X chromosome is randomly selected and inactivated (Mak W., Nesterova T.B. et al. 2004; Okamoto I, Arnaud D et al. 2005). In humans and most mammals, the X chromosome is inactivated by the random XCI (Moreira de Mello JC, de Arajo ES et al. 2010; Clerc P, Avner P. 2006; Van den Berg IM, Laven JS et al. 2009). However, some of the genes of the inactive X chromosome are expressed at varying levels (Berletch et al. 2010; Yang F, Babak T et al. 2010; Berletch JB, Yang F et al. 2011; Li N and Carrel L, 2008). In humans, 15% of female X-linked genes escape inactivation while in mice only 3% of the genes from inactive X allele are
expressed (Berletch JB, Yang F et al 2011). Of interest, several genes of the RAS components are located on the X chromosome which includes prorenin receptor, ACE2, and AT2 receptors; the presence of two X chromosomes in females could result in gene dosage effects between males and females. This potential difference is supposedly balanced by X-inactivation that inhibits the transcription of one of the two X chromosomes. However, there are some genes that escape the inactivation. Males have only one X chromosome, and most of the genes on the X chromosome have no equivalent on the Y chromosome. Therefore, genes on the X chromosome, even if recessive in females, will be expressed in males.

An example of an X-linked disease is Turner’s Syndrome, this disease is associated with a 100-fold increase in the risk of aortic dissection (Wong SC et al, 2014; Bondy CA 2008). Another disease linked to the X-chromosome includes cardiac valvular dysplasia (Bernstein JA, Bernstein D et al 2011; Ritelli M, Morlino S, Giacopuzzi E et al. 2017). Cardiac valvular dysplasia are caused by mutations in the FLNA gene on the X chromosome. This disease is characterized by a thickened heart valve that does not function normally (Monteleone PL, Fagan LF 1969; Kyndt F, Gueffet JP, Probst V et al. 2007; Norris RA, Moreno-Rodriguez R, Wessels A et al. 2010). Unlike females, males, because they have only one X chromosome that they received from the mother, manifest this disease even if only one copy of the genes exist. While females need to have two copies of the genes to cause the condition (Bernstein JA,
Bernstein D et al. 201; Ritelli M, Morlino S, Giacopuzzi E et al. 2017). In this way, females that carry the disease can transfer the condition to her offspring. Males, on the other hand, cannot pass X-linked traits to their sons since they inherit the Y chromosome only (Meroni G 1993).

A number of diseases have also been linked to the MSY, such as high incidence of hypertension and heart disease in men (Bloomer LD et al. 2013; Charchar Fj et al. 2012; Voskarides K et al. 2014). Further data have shown that the Y chromosome is linked with inheritance of high blood pressure, the concentrations of total cholesterol and LDL in the blood as well as the coronary artery disease (Shankar RR, Charchar FJ et al. 2007; Charchar FJ, Tomaszewski M, Lacka B, et al. 2004; Charchar FJ, Tomaszewski M, Padmanabhan S, et al. 2002; Ellis JA, Stebbing M, Harrap SB 2000; Charchar Fj et al. 2012). However, mechanisms for this association are unknown. Sadly, the sex chromosomes are not included in the genome-wide association study (GWAS) and the X chromosome despite being assayed on all GWAS studies only 33% of these studies included the X chromosome in the analyses (Wise AL, Manolio et al. TA 2013).

1.5 Four Core Genotype Mouse Model

The four core genotype mouse model (FCG), produces mice with gonadal organs that are independent of their sex chromosome complement and this allows dissection of the relative role of sex chromosome complement versus sex hormones. This model has been
used by multiple investigators and demonstrates that sex chromosome complement contributes to sexual dimorphism of the brain and other tissues in mice (Arnold AP and Chen X 2009; De Vries GJ et al. 2002). Other studies using this model identified that blood pressure responses to infusion of AngII were influenced by both sex hormones and sex chromosomes (Ji H, Zheng W et al. 2010). The FCG was generated from a natural mutation of the gene that is responsible for testes formation, Sry gene, from the Y chromosome (Lovell-Badge R and Robertson E, 1990; Mahadevaiah SK, Odorisio T et al. 1998). This mutation resulted in a mouse that has the XY- (lacking Sry) sex chromosome complement, this mouse does not develop testes but will develop ovaries instead, making it a female that has male sex chromosome complement and female gonads. The insertion of the Sry gene onto an autosome in XY- mouse result in generating a male mouse that expresses Sry (on an autosome but not on the Y chromosome) denoted as XY- (+ Sry). The presence of the Sry gene on an autosome in this mouse lead to the development of testes, allowing the male to breed normally. When XY- (+ Sry) males are mated with normal XX females, 4 genotypes are produced (Figure 1.1):

1. XY- females (abbreviated as XYF, have ovaries)
2. XX females (abbreviated as XXF, have ovaries)
3. XY males (abbreviated as XYM, have testes)
4. XX males (abbreviated as XXM, have testes)
Previous studies using this mouse model indicate that testosterone concentrations are not different between XY and XX males (Gatewood JD, Wills A et al. 2006). If there is a difference in a certain phenotype between XX and XY mice that have the same gonadal phenotype, then the difference is caused by sex chromosomes. That difference could be due to (a) Gene dosage effects from the Y chromosome, because XY mice are affected by Y genes while XX mice are not; (b) Gene dosage effects from the X chromosomes, because XX mice have two X chromosomes, and for genes that escape X-inactivation there could be gene dosage effects; (c) Parental origin of the X chromosome, XY mice receive only maternal X chromosomes while XX mice receive both the paternal and maternal X chromosomes.
CHAPTER I A. STATEMENT OF THE PROBLEM

AAA development is significantly different between sexes. Males are much more susceptible to AAAs than females. Similar to humans, male mice infused with AngII have a fourfold higher prevalence of AAAs compared to females. Paradoxically, while females are less susceptible to AAAs, female AAAs progress more rapidly and rupture at smaller sizes than males. Previous studies focused on gonadal hormones as primary contributors to sexual dimorphism in AngII-induced AAAs. Our laboratory previously demonstrated that sex hormones, specifically testosterone, are primary mediators to marked differences in AngII-induced AAAs between male and female hypercholesterolemic mice.

Our laboratory recently demonstrated that exposing one-day-old hypercholesterolemic female mice to a single dose of testosterone resulted in a high increase in AAA development when adult females were infused with AngII. Interestingly, female mice become susceptible to AAA formation from only a single androgen exposure during neonatal life while males require continued circulating levels of testosterone to stay susceptible to AAA, as gonadectomy decrease AAAs incidence in males and has no effect in females (Henriques TA et al. 2004). Results from these studies indicate that while important, gonadal hormones cannot fully account for the sexual dimorphism of AAA development.

Recent studies indicated that in addition to gonadal hormones, sex chromosome complement influence the blood pressure in AngII-infused mice (Ji H, Zheng W et al. 2010). Moreover, several studies showed that the Sry gene of the Y chromosome regulates the gene expression of some of the RAS
components (Ely D, Boehme S et al. 2011; Araujo FC, Milsted A et al. 2015). In fact, several genes of the RAS are X-linked, genes that escape X inactivation could result in a gene dosage effect. Finally, in human, loss of X chromosome in females, as in Turner’s syndrome, is associated with a high risk of aortic dilations and dissections (Wong SC et al. 2014; Bondy CA 2008). We hypothesize that an XY sex chromosome complement, in addition to sex hormones, promotes AngII-induced AAAs in hypercholesterolemic mice.
CHAPTER IB. Hypothesis

Sex chromosome effects, in addition to sex hormones, contribute to the sexual dimorphism of AngII-induced AAAs.

The following specific aims tested this general working hypothesis:

**Specific Aim 1:** Determine the role of an XY sex chromosome complement with and without exogenous androgen in AngII-induced AAAs in hypercholesterolemic female mice.

**Specific Aim 2:** Examine the role of an XX sex chromosome complement with and without the presence of endogenous androgen in AngII-induced AAAs in hypercholesterolemic male mice.
<table>
<thead>
<tr>
<th>Drug analyzed</th>
<th>Effect on AAA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEI</td>
<td>Reduced risk of ruptured AAA</td>
<td>Hackam DG et al 2006</td>
</tr>
<tr>
<td>ACEI</td>
<td>No effect on AAA growth rate</td>
<td>Thompson A et al 2010</td>
</tr>
<tr>
<td>ARBs</td>
<td>Reduced the AAA growth rate</td>
<td>Thompson A et al 2010</td>
</tr>
<tr>
<td>ACEI</td>
<td>Increased the AAA growth rate</td>
<td>Sweeting MJ et al 2010</td>
</tr>
<tr>
<td>ACEI</td>
<td>Reduced the risk of surgery for AAA</td>
<td>Kristensen KE et al 2015</td>
</tr>
<tr>
<td>ARBs</td>
<td>Reduction in all-cause mortality</td>
<td>Kristensen KE et al 2015</td>
</tr>
<tr>
<td>ACEI</td>
<td>No effect on AAA enlargement</td>
<td>Lederle FA et al 2015</td>
</tr>
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<td>ARBs</td>
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<td>Bicknell CD et al 2016</td>
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<tr>
<td>ACEI</td>
<td>No effect on AAA growth rate</td>
<td>Kortekaas KE et al 2014</td>
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Table 1.2: Summary of animal AAA studies modulating the RAS

<table>
<thead>
<tr>
<th>Application</th>
<th>Mouse model</th>
<th>Effect</th>
<th>References</th>
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<tbody>
<tr>
<td>AT1aR deletion</td>
<td>AngII-induced AAA in mice</td>
<td>decrease</td>
<td>Cassis et al 2007</td>
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<td>AT1aR deletion</td>
<td>elastase-induced AAA in mice</td>
<td>decrease</td>
<td>Xuan H et al 2017</td>
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<tr>
<td>ACE inhibition</td>
<td>elastase-induced AAA in rats</td>
<td>decrease</td>
<td>Liao S et al 2001</td>
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<td>AT1R antagonism</td>
<td>AngII-induced AAA in mice</td>
<td>decrease</td>
<td>Daugherty A et al 2001</td>
</tr>
<tr>
<td>AT1R antagonism</td>
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<td>decrease</td>
<td>Iida Y, Dalman et al 2012</td>
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<td>AT1R antagonism</td>
<td>elastase-induced AAA in rats</td>
<td>decrease</td>
<td>Xuan H et al 2017</td>
</tr>
<tr>
<td>AT2R antagonism</td>
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<td>increase</td>
<td>Daugherty A et al 2001</td>
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<td>Renin inhibition</td>
<td>AngII-induced AAA in mice</td>
<td>decrease</td>
<td>Seto SW et al 2014</td>
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<td>ACE2 deletion</td>
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<td>Moran CS et al 2017</td>
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Table 1.3: Summary of animal AAA studies modulating sex hormones

<table>
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<th>Application</th>
<th>model</th>
<th>findings or effect on AAA</th>
<th>Reference</th>
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<tr>
<td>Estradiol treatment</td>
<td>AngII infused male mice</td>
<td>decrease AAA</td>
<td>Martin-McNulty B, Tham DM et al 2003</td>
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<td>Orchiectomy</td>
<td>AngII infused male mice</td>
<td>decrease AAA</td>
<td>Henriques TA, Cassis LA et al 2004</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>AngII infused female mice</td>
<td>no effect</td>
<td>Henriques TA, Cassis LA et al 2004</td>
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<tr>
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<td>elastase perfusion in</td>
<td>M&gt;F, estradiol decrease AAA</td>
<td>Ailawadi G, Upchurch GR et al 2004</td>
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<tr>
<td></td>
<td>male and female rats</td>
<td></td>
<td></td>
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<td>Tamoxifen</td>
<td>elastase perfusion in</td>
<td>decrease AAA</td>
<td>Grigoryants V, Upchurch GR et al 2005</td>
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<tr>
<td></td>
<td>male rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orchiectomy</td>
<td>AngII infused male mice</td>
<td>decrease AAA</td>
<td>Henriques TA, Cassis LA et al 2008</td>
</tr>
<tr>
<td>DHT administration</td>
<td>AngII infused male and female mice</td>
<td>increase AAA</td>
<td>Henriques TA, Cassis LA et al 2008</td>
</tr>
<tr>
<td></td>
<td>elastase perfusion in</td>
<td>decrease AAA in males</td>
<td>Cho BS Upchurch GR et al 2009</td>
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<tr>
<td></td>
<td>male and female rats</td>
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Figure 1.1. The four core genotype mouse model. X XF is mated to male mice lacking $Sry$ on sex chromosomes but expressing $Sry$ on autosomes to create four core genotypes.
CHAPTER II. SPECIFIC AIM 1

Female Mice with an XY Sex Chromosome Complement Develop Severe Angiotensin II-Induced Abdominal Aortic Aneurysms (Published in the journal of Circulation)

Authors: Yasir Alsiraj, Sean Thatcher, Richard Charnigo, Kuey Chen, Eric Blalock, Alan Daugherty, and Lisa Cassis.

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Summary

Background: Abdominal aortic aneurysms (AAAs) are a deadly pathology with strong sexual dimorphism. Similar to humans, female mice exhibit far lower incidences of angiotensin II (AngII)-induced AAAs than males. In addition to sex hormones, the X and Y sex chromosomes, and their unique complements of genes, may contribute to sexually dimorphic AAA pathology. Here, we defined the effect of female (XX) versus male (XY) sex chromosome complement on AngII-induced AAA formation and rupture in phenotypically female mice.

Methods: Female low density lipoprotein receptor (Ldlr) deficient mice with an XX or XY sex chromosome complement were infused with AngII for 28 days to induce AAAs. Abdominal aortic lumen diameters were quantified by ultrasound, while AAA diameters were quantified at study endpoint. DNA microarrays were performed on abdominal aortas. To mimic males, female mice were administered a single dose of testosterone as neonates or as adults prior to AngII infusions.

Results: Female Ldlr−/− deficient mice with an XX and XY sex chromosome complement had similar sex organ weights and low serum testosterone concentrations. Abdominal aortas from female XY mice selectively expressed Y
chromosome genes, while genes known to escape X-inactivation were higher in XX females. The majority of aortic gene differences in XY versus XX females fell within inflammatory pathways. AAA incidences doubled and aneurysms ruptured in XY females. AAAs from XY females exhibited inflammation, and plasma IL1β concentrations were increased in XY females. Moreover, aortas from XY females had augmented matrix metalloproteinase activity and increased oxidative stress. Finally, testosterone exposure applied chronically, or as a single bolus at postnatal day 1, markedly worsened AAA outcomes in XY compared to XX adult females.

Conclusions: An XY sex chromosome complement in phenotypic females profoundly influenced aortic gene expression profiles and promoted AAA severity. When XY females were exposed to testosterone, aneurysm rupture rates were striking. Mechanisms for augmented AAA severity in XY females include increased inflammation, augmented matrix metalloproteinases and oxidative stress. Our results demonstrate that genes on the sex chromosomes regulate aortic vascular biology and contribute to sexual dimorphism of AAAs. Sex chromosome genes may serve as novel targets for sex-specific AAA therapeutics.

2.1 Introduction

Genes on sex chromosomes have been implicated in inherited forms of cardiovascular diseases (Charchar, Bloomer et al. 2012, Bloomer, Nelson et al. 2013), but much less is known about the influence of sex chromosomes, and their interplay with sex hormones, on development of common complex cardiovascular diseases. Turner’s Syndrome (monosomy X) is associated with significant risk of aortic dissection (Wong, Burgess et al. 2014), suggesting an
important role for sex chromosomes in the regulation of aortic homeostasis. Abdominal aortic aneurysms (AAAs), an insidious vascular disorder without effective drug therapies, are another example of an aortic disease exhibiting genetic inheritance (Clifton 1977; Johansen and Koepsell 1986; Blanchard, Armenian et al. 2000; Rossaak, Hill et al. 2001) and pronounced sexual dimorphism (Morris, Hubbard et al. 1994; Pleumeekers, Hoes et al. 1994; Wilmink, Pleumeekers et al. 1998; Weiss, Rodionov et al. 2014). Understanding the combined influences of sex hormones and chromosomes on aortic vascular biology and disease may identify novel therapeutic targets that are beneficial for treatment of vascular diseases in one sex versus the other. Moreover, as chronic sex hormone therapy is on the rise in aging males and females, as well as in the transgender population, it is important to increase knowledge regarding sex hormone and chromosome effects on the vasculature. We demonstrated previously that AAAs induced in mice by angiotensin II (AngII) infusion exhibit pronounced sex differences. Similar to humans, there is a 4-fold higher AAA prevalence in male compared to female mice infused with AngII (Henriques, Huang et al. 2004). Testosterone was demonstrated as a primary regulator of the formation and severity of AngII-induced AAAs in male mice (Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008). However, not all differences in AAA susceptibility and severity between male and female mice could be explained by chronic presence of testosterone (Zhang, Thatcher et al. 2012). Recent studies demonstrated that blood pressure responses to AngII in mice were influenced by sex chromosomes (Ji, Zheng et al. 2010). Herein, we embarked on an analysis of effects of an XX versus an XY sex chromosome
complement on aortic gene expression profiles and on development and severity of AngII-induced AAAs in phenotypically female mice. Gene expression profiles demonstrated enriched inflammatory genes in abdominal aortas from XY compared to XX females. When XY females were infused with AngII, AAA incidences doubled and aneurysms ruptured.

Exposure of XY females to testosterone, to mimic the male sex hormone environment, led to striking levels of AAA rupture. Our results indicate a previously unrecognized role for sex chromosomes to influence aortic gene expression patterns and profoundly affect AAA severity.

2.2 Methods

2.2.1 Animals

Male mice with an XY-Sry genotype {8-12 weeks of age; 10 times backcrossed on C57BL/6J background, Stock#010905}, were obtained from The Jackson Laboratories and bred to low density lipoprotein receptor deficient (Ldlr−/−) females (Stock# 002207). Genomic DNA was isolated from tail clips and subjected to PCR using a commercial PCR mix (Promega 2X Master Mix, cat#m7123) and specific primers (Table 2.2). DNA fragments were recognized as 350 (mutation) and 200 bp (internal positive control) bands, while XX females displayed only a 200 bp band. Mice were placed on a high fat diet (TD88137, Harlan Teklad, Indianapolis, IN) for the duration of the experiment. In mice administered testosterone as neonates, within 24 hours of birth female mice were
injected subcutaneously with a single dose of testosterone propionate (400 µg/mouse, n=6-12 mice/genotype; Sigma Aldrich, St. Louis, MO). At 8 weeks of age, all females were ovariectomized (OVX) to eliminate any potential confounding influence of female sex hormones (Henriques, Huang et al. 2004). In mice administered DHT as adults, OVX females (8-12 weeks of age) were implanted with a dihydrotestosterone (DHT) pellet (10 mg pellets/60 day sustained release; 0.16 mg/d; n=11-18 mice/genotype) two weeks prior to AngII infusion and continued through study endpoint (Henriques, Zhang et al. 2008) All procedures were in accordance with institutional guidelines and were approved by the animal care and use committee at the University of Kentucky.

2.2.2 Isolation of Bone Marrow Cells

Bone marrow was extracted from the tibias and femurs of XX and XY female mice (2 months of age; n=3 mice/genotype), and bone marrow cells were differentiated into macrophages by supplementing the culture media (RPMI medium, 2% penicillin/ streptomycin, 1% fungizone) with 1 ng/ml of CSF (Colony Stimulating Factor) for 6 days. Total RNA was extracted using the Rneasy fibrous tissue mini kit (Qiagen, cat#74704).

2.2.3 Gonadectomy

Female XX and XY Ldlr⁻/⁻ mice (8-12 weeks of age) were OVX as described previously (Henriques, Huang et al. 2004). Two weeks later (to clear endogenous ovarian- derived hormones), mice were infused with AngII.
2.2.4 AngII Infusions.

Mice were infused with saline or AngII (1,000 ng/kg/min, Bachem, Torrance, CA) by osmotic micropumps (Alzet model 1004 for 28 day infusions, Durect Co., Cupertino, CA). At study endpoint, all mice were euthanized using a ketamine/xylazine mixture (100:10 mg/kg, IP) in sterile saline and blood collection was performed via cardiac puncture.

2.2.5 Quantification of AAAs by ultrasound, ex-vivo AAA measurements, and 3D volume analysis.

Internal suprarenal lumen diameters were quantified on day 0, 7, 14, and 28 of AngII infusions using a 55-MHz probe with a Vevo 2100 high-resolution imaging system (VisualSonics, Inc.). Mice were anesthetized (2-3% isoflurane) and abdominal hair was removed by shaving and applying a depilatory cream. Mice were imaged and allowed to recover on a heating pad. Images were analyzed by two independent observers who were blinded to the study design. Mice with a 50% increase in abdominal aortic lumen diameter from baseline (day 0) were diagnosed with an AAA, and included in statistical analysis for each measure if they completed study protocol. External abdominal aortic diameters were measured at study end point on aortas mounted on a black wax background. Images were taken using a Nikon SMZ800 dissecting microscope and image analysis to obtain maximal AAA diameters was performed using Nikon Elements Version 3.2. AAA incidence was defined as a 50% increase in abdominal aortic lumen diameter on day 28 of AngII infusions and included mice that died from aneurysm rupture. Percent of rupture was calculated as the % of mice within each group that died from a verified rupture of the aorta upon necropsy. Grading of AAA severity was defined previously (Daugherty, Manning
et al. 2001). Three-dimensional volume analysis was performed on abdominal aortas of OVX XY and XX females (n=5 mice/genotype) using the Vevo 2100 system with the 55-MHz probe. Abdominal aortas were scanned at 51μm intervals and a total of 295 images were collected. Each image was analyzed for lumen and wall volume and images were summed to obtain total cubic millimeter volume of abdominal aortas.

2.2.6 Blood pressure measurements.

Systolic blood pressure was measured on conscious mice using a non-invasive computerized tail-cuff system (BP-2000; Visitech Systems, Apex, NC) during week 3 of AngII infusions. Mice were acclimated to the blood pressure system for 2 days and measurements were taken over 5 subsequent days.

2.2.7 Measurements of plasma and serum components.

Total serum cholesterol concentrations were quantified using enzymatic assay kits (Wako Pure Chemical, Richmond, VA, cat#439-17501). Plasma renin concentrations were determined by quantifying angiotensin I concentrations generated in the absence or presence of an excess of exogenous rat angiotensinogen as described previously (Zhang, Thatcher et al. 2012). Angiotensin I was quantified by radioimmunoassay using a commercial kit (DiaSorin, CA-1553, Stillwater, MN). Plasma concentrations of IL1β were quantified using a commercial Quantikine ELISA (R&D Systems, Minneapolis, MN).

2.2.8 Quantification of atherosclerosis.

Aortas were cleaned, opened longitudinally and mounted on a black wax
background to quantify atherosclerosis in the aortic arch and thoracic aorta. Arch and thoracic aorta areas were defined and lesions within this area were summed and normalized to total region area to calculate the percent intimal surface area covered by an atherosclerotic lesion. Quantification was performed using Nikon Elements Version 3.2.

2.2.9 Abdominal aorta RNA extraction

Female (8 weeks of age) XX and XY mice (n=5 mice/surgical group/genotype) underwent sham surgery or OVX, and two weeks later mice were fed the Western diet for 1 week. Aortas were cleaned from extraneous tissues under a dissecting microscope and placed in RNA Later (Ambion, cat#AM7021). Abdominal aortas (diaphragm to the ileac bifurcation) were used for RNA extraction. RNA was extracted using the Rneasy fibrous tissue mini kit (Qiagen, cat#74704), and RNA concentration and quality was quantified by Agilent 2100 bioanalyzer using RNA 6000 Nano labchip kits (Agilent Technologies, Cat# 5067-151). Samples with RIN (RNA Integrity Number) $\geq 8$ were used for either DNA microarray or RT-PCR.

2.2.10 DNA microarrays

Harvested abdominal aortic RNA samples (n=5 mice/surgical group/genotype) did not differ significantly among treatment groups (Agilent Bioanalyzer RNA Integrity Number [RIN]: 9.51 ± 0.07, p= 0.25; [RNA] (ng/ul): 39.5 ± 3.4, p=0.93; 28s:18s ratio: 2.83 ± 0.89, p=0.33- all statistical tests by ANOVA). Extracted RNA was labeled and hybridized to Affymetrix Mouse Transcriptome Assay 1.0 arrays (one array per subject). Signal intensities were
calculated using the RMA algorithm (Bolstad, Irizarry et al. 2003) at the transcript level using Genomics Suite software (Partek, St Louis). Data were transferred to flat files in Excel and associated with vendor-provided annotation data. Pre-statistical filtering retained unique, annotated probe sets with adequate signal intensity (signal intensity ≥ 4.2 on at least 4 arrays in the study). Filtered signal intensities were analyzed by two-way ANOVA to identify significant main effects of genotype (XX versus XY), surgery (sham vs OVX), as well as interaction. The False Discovery Rate (FDR) procedure (Hochberg and Benjamini 1990) as modified by Storey (Storey 2002) was used to control error associated with multiple testing, with an FDR q- value < 0.05 defining significance. The complete list of significant results is provided in table 2.3. Functional categorization for each expression pattern was determined with the prestatistically filtered gene list as a background using DAVID bioinformatic tools (Huang da, Sherman et al. 2009). Currently, DAVID does not support Affymetrix MTA 1.0 IDs, and therefore best match IDs from Affymetrix Mouse 1.0 Exon arrays were used, covering more than 90% of the filtered MTA data set. Raw data are available through the Gene Expression Omnibus (GSE #:81580 www.ncbi.nlm.nih.gov/geo/).

2.2.11 Zymography

Female mice (n=4 mice/genotype) were infused with AngII (1,000 ng/kg/min) for 24 hours, aortic protein (10 μg) was extracted and resolved using SDS-PAGE (7.5%) polymerized in the presence of gelatin (2 mg/ml) to detect MMP activity. Gels were washed with 2.5% Triton X-100 (1 hr) followed by distilled
water (30 min), and incubated overnight (37°C) in Tris buffer containing 10 mM calcium chloride and 0.02% sodium azide. Gels were stained with Coomassie Brilliant Blue for 3 hours and destained using distilled water. Gel images were captured using a Syngene PXi imager; the unstained, translucent digested regions represented areas of MMP2 activity.

2.2.12 Quantification of oxidative stress in aortic tissue explants

Aortas (thoracic and abdominal segments) from XX and XY LdlrΔ/Δ females were cleaned of adherent tissue and placed in culture media (DMEM; 1:1 without phenol red). Aortic explants were incubated with vehicle (saline) or AngII (10 μM) plus testosterone (10 nM) for 24 hrs to simulate conditions of high AAA incidence and severity. Tissues were snap frozen (OCT) in molds for serial tissue sections (10 μm). Aortic sections were incubated with dihydroethidium (DHE, 10 μmol/L) in phosphate buffered saline for 30 min (37°C), and counterstained with DAPI. Images were obtained (100X; 3-5 images/slide) on a Nikon Eclipse microscope (TE2000-U) with a monochrome camera (exposure=300ms). Histogram plots were generated for each image using Nikon Elements software, and areas under the curve for pixel intensities (250-2500) were calculated.

2.2.13 Statistical Analysis

Numerical data are generally illustrated as mean ± SEM and were analyzed using unpaired t-test or Mann-Whitney tests to compare two groups, by two-way repeated measures ANOVA on time for longitudinal data with between group factor of surgery or genotype, or by two-way ANOVA with between group factors of surgery, treatment (e.g., testosterone in some studies), or genotype. Brown-Forsythe tests for homogeneity of variance were performed.
in some cases, which led to the modification of two of the two-way ANOVA’s; these were modified by weighting observations inversely proportionally to their within-group variances. The Kaplan-Meier method was used to estimate survival curves. The incidence of aneurysm formation was analyzed using Fisher’s exact tests. Statistical analysis was performed using SigmaPlot (Systat Software Inc., San Jose CA) or SAS (SAS Institute Inc., Cary NC), and statistical significance was determined at P< 0.05.

2.3 Results

2.3.1 An XY sex chromosome complement promotes expression of inflammatory genes in abdominal aortas.

To define a role of sex chromosomes in regulating aortic gene expression profiles, we bred male mice expressing autosomal Sry (the testes determining gene) to Ldlr deficient females to create XX and XY female offspring (Arnold and Chen 2009). We focused on female XX and XY mice to enable definition of the role of sex chromosomes under experimentally controlled exposures to testosterone, since testosterone is known to promote AngII-induced AAAs in male mice (Henriques, Huang et al. 2004). We first determined effects of sex chromosome complement on gene expression profiles in abdominal aortas from non-infused (AngII-naïve) XX and XY females in the presence (sham-operated) or absence (OVX) of endogenous female sex hormones. Body weights were similar between genotypes prior to surgery (data not shown). Body weights of XX (sham, 22.4 ± 1.0; OVX, 26.8 ± 0.8g; P<0.05), but not of XY females (sham, 22.6 ± 1.5; OVX, 23.1 ± 0.7g; p=0.75) were increased
significantly by OVX compared to sham-operated controls. We performed transcriptome analyses on RNA extracted from abdominal aortas of XX and XY female mice using Affymetrix Mouse Transcriptome Assay 1.0 arrays. A total of 88 genes exhibited highly significant differences (two-way ANOVA, FDR € 0.05, Figure 2.1A, Table 2.3). Remarkably, all 88 genes were significant by the main effect of chromosome only. Volcano plots of the chromosome effect with highly stringent cutoffs (> 2-fold change, P€1x10^{-6}) demonstrated that, as expected, the expression of genes on sex-chromosomes was strongly influenced. Genes (Ddx3y, Uty, Kdm5d, Eif2s3y) within the male-specific region of the Y chromosome (MSY) were significantly increased in female abdominal XY aortas (Figure 2.1B, fold increase in XY). These genes are of interest as the MSY constitutes approximately 95% of the length of the Y chromosome (Skaletsky, Kuroda-Kawaguchi et al. 2003), does not recombine with the X chromosome during meiosis, is inherited from father to son, and has been linked to cardiovascular risk in humans (Skaletsky, Kuroda-Kawaguchi et al. 2003, Bloomer, Nelson et al. 2013). A second group of genes (Kdm5c, Eif2s3x, Kdm6a, Ddx3x) known to escape X- inactivation in mice (Berletch, Yang et al. 2011), were increased in abdominal aortas of XX females (Figure 2.1B, fold increase in XX). A third group of genes (Msl3, Tlr7, Tlr8, Prps2, Arghap6) are X-linked genes, but were increased in abdominal aortas from XY compared to XX females (Figure 2.1B, fold increase in XY). Further, genes across multiple chromosomes were strongly influenced by the presence of the Y chromosome.
Real-time PCR on abdominal aortas validated selected microarray results (Kdm5d, Eif2s3y, Xist) for the main effect of an XY sex chromosome complement (Figure 1C) and verified microarray findings for genes not exhibiting significant differences between genotypes (GAPDH, Figure 2.7). However, mRNA abundance of Uly, a gene on the Y chromosome, was not significantly different between aortas from sham-operated female XX compared to XY mice, and expression was increased by OVX in aortas from XY females, but not XX females (Figure 2.1C). Biological pathway analyses (Table 2.1) revealed prominent upregulation of inflammatory genes in abdominal aortas from XY females (Figure 2.8). These results demonstrate that sex chromosomes influence gene expression profiles of abdominal aortas, with increased expression of inflammatory pathway gene targets in XY compared to XX. Enrichment of genes on the Y chromosome in aortas from XY females, coupled with increased expression of genes known to escape X-inactivation in aortas from XX females, confirm validity of sex chromosome manipulation in phenotypically female mice.

2.3.2 An XY sex chromosome complement results in aggressive AAA formation and severity.

Female (XX) Ldlr−/− mice exhibit far lower incidences (≈ 25-40%) of AngII-induced AAAs compared to age-matched males (≈ 75-90%) (Henriques, Huang et al. 2004, Henriques, Zhang et al. 2008). To define the role of sex chromosome complement on formation and severity of AngII-induced AAAs, sham-operated and OVX female Ldlr−/− mice were infused with AngII for 1 month. Body weights of
AngII-infused OVX XY female mice were decreased significantly compared to OVX XX females (Table 2.4). Plasma renin concentrations were not significantly different between XX and XY AngII-infused females (Table 2.4). Serum testosterone concentrations were modestly, but significantly increased in AngII-infused sham-operated XY compared to XX females, and elevations of serum testosterone concentrations in XY females were eliminated by OVX (Table 2.4). While sex organ weights of XX and XY females were significantly decreased by OVX in XX and XY females, there were no differences between genotypes (Table 2.4). At study endpoint, internal abdominal aortic lumen diameters of XY females with an AAA (as defined by a 50% increase in lumen diameter) were elevated (by 66%) significantly compared to XX females (Figure 2.2A; OVX groups). Moreover, external AAA diameters were increased significantly in XY compared to XX females (Figure 2.2B; OVX groups). AAA incidence (including aneurysm ruptures) increased (by 31%) in XY females (Figure 2.2C) to levels observed previously in XY male mice (Henriques, Huang et al. 2004, Henriques, Zhang et al. 2008). In XX females (sham or OVX), there were no aneurysm ruptures (Figure 2.2D). In contrast, 35% of XY sham-operated females infused with AngII exhibited aneurysm ruptures, with high rupture rates also observed in OVX XY females (29%). Aortas from XY females demonstrated severe aneurysm pathology that frequently extended the entire length of the aorta and was associated with aneurysm rupture (Figure 2.2E; Figure 2.9). As these effects were observed in OVX XY females (Figure 2.2A-E), results suggest that an XY sex chromosome complement augments AAA formation and severity independent of female sex hormones.
To define characteristics of AAAs from XY females, we performed *ex vivo* 3-D ultrasound on AAAs from each genotype (Figure 2.10). Results demonstrate increased aneurysm wall volume in AAAs from XY compared to XX females, and 3-D remodeling from a representative AAA from each genotype illustrates lumen dilation with marked vascular wall remodeling. There were no overt differences in aortic wall morphology of abdominal aortic tissue sections from non-infused XX *versus* XY female mice (Figure 2.11). However, AAA tissue sections from AngII-infused XY females exhibited CD68 macrophage immunostaining in areas exhibiting breaks in medial elastin (Figure 2.12). An XY sex chromosome complement also increased significantly systolic blood pressure responses of female mice infused with AngII (both sham and OVX; Table 2.4). As infusion of AngII augments atherosclerosis in *Ldlr*<sup>−/−</sup> female mice (Daugherty, Manning et al. 2000), we quantified the percent of the intimal aortic surface within the aortic arch and thoracic aorta covered by atherosclerotic lesions. There was not a significant effect of genotype on AngII-induced atherosclerosis in thoracic aortas (Figure 2.13) or aortic arches (data not shown). Moreover, there were no significant differences in serum cholesterol concentrations between genotypes (Table 2.4).

2.3.3 Aortas from XY females exhibit augmented inflammatory gene expression, matrix metalloproteinase (MMP) activity, and oxidative stress.

Gene arrays on abdominal aortas from non-infused XY females
demonstrated increased expression of genes within inflammatory pathways (Table 2.1), and AAA tissue sections of XY females exhibited positive CD68 immunostaining in areas exhibiting breaks in medial elastin (Figure 2.12). We confirmed by RT-PCR increased mRNA abundance of IL1-β, a component of the inflammasome linked to AAA formation, in abdominal aortas from XY \( Ld\ell r^{-/-} \) females (Figure 2.3A). Moreover, mRNA abundance of \( Tlr8 \) was increased significantly in abdominal aortas of XY compared to XX females (O VX groups: XX, \( 1.1 \pm 0.2 \); XY, \( 2.4 \pm 0.4 \Delta Ct \); \( p=0.017 \)). Since immune cell gene pathways were altered in abdominal aortas from XY females, we harvested bone marrow cells from XX and XY \( Ld\ell r^{-/-} \) females and differentiated them to macrophages. Similar to findings from abdominal aortas, \( Tlr8 \) mRNA abundance was increased significantly in bone marrow macrophages (BMM) from XY compared to XX females (XX, \( 1.1 \pm 0.2 \); XY, \( 2.3 \pm 0.2 \Delta Ct \); \( p=0.0001 \)). Moreover, IL1β protein concentrations were increased significantly in media from incubated BMM of \( Ld\ell r^{-/-} \) XY compared to XX females (Figure 2.3B). Finally, plasma concentrations of IL1β were increased significantly in AngII-infused XY compared to XX \( Ld\ell r^{-/-} \) females (Figure 2.3C). As inflammatory genes with increased expression in abdominal aortas from XY females have been linked to activation of MMPs and oxidative stress, two mechanisms implicated in AAA formation and severity (Kadoglou and Liapis 2004, Keeling, Armstrong et al. 2005, Em eto, Mox on et al. 2016), we quantified MMP activity in aortas of female XX and XY mice infused for 1 day with AngII. Aortas from XY females infused with AngII exhibited significant increases in MMP2 activity compared to XX females (Figure 2.4A,
B). Moreover, when aortas were incubated \textit{in vitro} with AngII and testosterone to simulate conditions where AAA incidence and severity are augmented, DHE fluorescence was pronounced in aortas from XY compared to XX females (Figure 2.4C, D).

2.3.4 Testosterone exposure (as neonates or adults) results in a striking level of aneurysm rupture in XY females.

Castration of male mice has been demonstrated to reduce the incidence of AngII- induced AAAs (Henriques, Huang et al. 2004), while administration of DHT augments AAA formation in adult female hypercholesterolemic mice (Henriques, Zhang et al. 2008). To determine if testosterone exerted similar effects to promote AAAs in females with an XX \textit{versus} an XY sex chromosome complement, we used two different paradigms to androgenize females of each genotype by administering an equivalent dose of androgen. We administered the non-aromatizable ligand of the androgen receptor, DHT, to adult female \textit{Ldlr}⁻/⁻ XX and XY mice prior to and throughout AngII infusions. There were no significant differences in body weight (XX, 28 ± 1; XY, 27 ± 1g; P=0.45), sex organ weights (XX, 0.09 ± 0.01; XY, 0.07 ± 0.02 g; P=0.46) and systolic blood pressures (XX, 129 ± 4; XY, 128 ± 7 mmHg; p=0.90) between AngII-infused XX and XY females. Plasma IL1β concentrations were increased significantly in XY compared to XX AngII-infused females (XX, 1.8 ± 0.9; XY, 15.5 ± 4.8; P<0.05). Both genotypes AAA incidences when exposed to DHT (XX, 94%; XY, 100%), and abdominal aortic lumen diameters were similar (Figure 2.14A). However, while 44% of XX females administered DHT died of aneurysm rupture, 73% of XY females
succumbed to rupture (Figure 2.5A; Figure 2.14B). External AAA diameters of the few surviving XY females administered DHT were increased significantly compared to XX females (Figure 2.5B). Due to the extreme incidence of aneurysm rupture and extensive pathology of aortas from XY female mice administered DHT, atherosclerosis could not be quantified. Recent studies demonstrated that transient neonatal exposure of female hypercholesterolemic mice to testosterone resulted in increased adult susceptibility to AngII-induced AAAs (Zhang, Thatcher et al. 2012). Therefore, we administered testosterone to 1 day old XX and XY female Ldlr<sup>-/-</sup> mice and examined effects of AngII infusions on AAA formation and severity in adult OVX females. There were no significant differences in body weight, serum cholesterol concentrations and systolic blood pressures between AngII-infused XX and XY females (data not shown). As reported previously, transient neonatal exposure to testosterone promoted a high incidence of AngII-induced AAAs in adult XX (50%) and XY (58%) females. However, aneurysm ruptures increased significantly in XY (58%) compared to XX (0%) females administered testosterone as neonates (Figure 2.6)

2.4 Discussion

The field of sexual dimorphism in normal vascular physiology and disease is in its infancy as relates to the influence of sex chromosomes. However, the sex chromosomes are one of the most unusual chromosomal differences in mammals, with approximately 1,098 genes (Ross, Grafham et al. 2005) and more than 300 diseases alone mapped to the X chromosome. While there are fewer protein-coding genes on the Y chromosome, genes within the MSY have been linked to
different forms of cardiovascular diseases (Bloomer, Nelson et al. 2013). As an example of sex chromosome influences on the vasculature, females with monosomy X exhibit a 100-fold increased risk of aortic dissection (Wong, Burgess et al. 2014), but mechanisms for these effects are unknown. Our results demonstrate that an XY sex chromosome complement results in increased expression of inflammatory gene pathways in abdominal aortas of female mice. Further, the incidence and severity of AngII-induced AAAs, a vascular disease associated with pronounced inflammation, was increased profoundly in XY females. We demonstrate that elevated expression of inflammatory genes is linked to increased MMP activity and oxidative stress in aortas of XY females, effects known to promote AAA formation and severity. Finally, exposure of XY females to testosterone to mimic the male sex hormone milieu resulted in a striking level of aneurysm rupture. These results advance knowledge by demonstrating a profound effect of sex chromosome complement on aortic biology and vascular disease development. Moreover, results indicate a complex interplay between sex hormones (e.g., testosterone) and sex chromosomes to regulate aortic biology and vascular diseases, which may have cardiovascular implications for chronic sex hormone therapy in the aging and/or trans- gender population. A positive family history of AAA in a first degree relative has been suggested to increase the risk of aortic aneurysm by up to 10-fold (Johansen and Koepsell 1986), indicating genetic contribution to AAA pathogenesis. However, despite the strong predisposition for AAA formation in males, the contribution of sex chromosome effects to AAA inheritance and sexual dimorphism of this vascular disease has not been investigated extensively. Clifton et al, originally described a family of three affected
brothers diagnosed with an AAA (Clifton 1977). Follow-up studies demonstrated that 77% of affected individuals in a cohort of 233 families were male and 79% of disease transmissions were between father and son (Kuivaniemi, Shibamura et al. 2003), suggesting male-lineage transmission of risk. It is unclear from the present study whether increased AAA incidence and severity of XY females resulted from the absence of a second X chromosome, or from presence of genes on the Y chromosome. However, as XY females exhibiting high AAA incidence and severity inherited the Y-chromosome from male fathers, these results suggest that male-lineage may contribute to increased inherited risk of AAAs through influences of the Y chromosome. These results are the first to demonstrate that sex chromosome complement influences expression levels of 88 genes in abdominal aortas of phenotypically normal females. Of note, the high preponderance of changes in expression levels of inflammatory genes indicates potential predisposition to effects of an inflammatory aneurysm stimulus such as AngII. Recent studies identified sex differences in immune cells as contributors to sexual dimorphism of blood pressure responses to AngII (Ji, Zheng et al. 2010; Pollow, Uhrlaub et al. 2014). Our results demonstrate increased mRNA abundance of two inflammatory pathways typically associated with immune cells, namely IL-1β and Tlr8, in abdominal aortas of XY compared to XX aortas, and elevated plasma IL1β concentrations in XY compared to XX AngII-infused females. As elevated Tlr8 expression was also detected in BMM from XY females, these results suggest a contribution of immune cells to baseline differences in abdominal aortic inflammatory gene expression profiles between XX and XY females. Following infusion of AngII, AAAs in XY females had pronounced macrophage composition, elevated MMP-2 activity and aortas from
XY females responded to AngII and testosterone with increased oxidative stress, suggesting a robust inflammatory immune cell response that contributed to increased AAA severity. These results are in agreement with recent findings demonstrating immune cells as mediators of sex differences in response to AngII infusions (Ji, Zheng et al. 2010; Pollow, Uhr laub et al. 2014), and suggest that sex chromosomes may contribute to sexual dimorphism of this immune cell response. Previous findings from our laboratory demonstrated a prominent role for testosterone to promote AngII-induced AAAs (Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008). In this study, an XY sex chromosome complement increased the severity of AngII-induced AAAs in female mice. These results are the first to demonstrate a profound effect of sex chromosome complement, independent of sex hormones, to regulate the severity of AngII-induced AAAs. As testosterone is also a powerful positive regulator of AAA susceptibility in this experimental model, we used two different paradigms of testosterone exposure to examine interactions between sex hormones (e.g., testosterone) and sex chromosomes. Moreover, as serum testosterone concentrations were modestly different between XX and XY females, administration of DHT to adult females to normalize androgen concentrations between genotypes minimized the possibility that modest differences in serum testosterone are a primary mechanism for augmented formation and severity of AngII-induced AAAs in XY females. Results demonstrate a striking level of aneurysm rupture (up to 73%) when XY females were exposed to testosterone as neonates (Zhang, Thatcher et al. 2012) or adults (Henriques, Zhang et al. 2008). The magnitude of the effect of testosterone to promote the aneurysm
rupture, larger than exhibited previously by XY males infused with AngII (Daugherty, Manning et al. 2000; Henriques, Huang et al. 2004; Henriques, Zhang et al. 2008), may have resulted from the dose of androgen. In addition, while females are typically resistant to AAA development (both humans and AngII-induced mice), AAAs in females grow at more rapid rates and rupture at smaller sizes (Grootenboer, Bosch et al. 2009; Sweeting, Thompson et al. 2012; Thompson, Brown et al. 2013; Skibba, Evans et al. 2015). Differences in the physical size, compliance or stiffness of female abdominal aortas (Ninomiya, Tavares Monteiro et al. 2015) may have contributed to more severe effects of androgen to promote aneurysmal rupture of XY female mice. An interesting finding from this study was increased blood pressure responses, as well as modest elevations in thoracic aorta atherosclerotic lesion surface area in AngII-infused XY compared to XX female mice. These results suggest that an XY sex chromosome complement influences distinct cardiovascular responses (AAA, hypertension, atherosclerosis) to AngII. In contrast to these findings, recent studies demonstrated that an XX sex chromosome complement in gonadectomized MF1 mice contributed to a greater blood pressure response to AngII (Ji, Zheng et al. 2010). Differences in genetic background (MF1 versus Ldlr−/−) or diet (standard murine diet versus Western diet) may have contributed to diverging influences of sex chromosome complement on AngII-induced hypertension between studies. Regardless, it is unlikely that the augmented hypertensive responsiveness to AngII of XY females contributed to the robust severity of AAAs, as previous studies suggested that blood pressure was not a major contributor to AngII-induced AAAs (Cassis, Gupte et al. 2009). Future studies will address whether similar or distinct
mechanisms contribute to augmentation of three different cardiovascular effects of AngII in mice with an XY sex chromosome complement. However, as results from DNA arrays do not indicate differences in expression levels of angiotensin receptors in abdominal aortas of XX versus XY females, and plasma renin concentrations were not significantly different between AngII-infused XX and XY females, these results do not support generalized enhanced responsiveness of XY females to AngII. Using computer program scanned electronic medical records, prevalence of confirmed transgender individuals was 4.4 (per 100,000) in 2006, and 38.7 in 2014 (Roblin, Barzilay et al. 2016). Despite the growing prevalence of the transgender population, literature on specific health outcomes for transgender individuals consists of modest cross-sectional studies, some large retrospective studies, small series and case reports. Of these, recent data suggest increased risk of cardiovascular morbidity/mortality in transgender women (Elamin, Garcia et al. 2010). However, the influence of chronic sex hormone therapy on cardiovascular health in transgender women or men has not been examined extensively. In addition to the transgender population, the use of sex hormone therapy, specifically testosterone to improve sexual function in aging males and females, has also increased. Our results suggest that sex chromosome complement influences aortic biology and the development of AAAs, with an XY sex chromosome complement having a profound effect to promote AAA severity. As these effects were augmented in XY females administered testosterone, these results suggest interactions between sex hormones and genes affected by sex chromosomes. These results may have implications related to chronic sex hormone therapy and the cardiovascular system in aging males or females, or in
the growing transgender population.

In summary, we demonstrate that an XY sex chromosome complement promotes expression of inflammatory genes in abdominal aortas of female mice and predisposes to markedly severe AngII-induced AAAs. As 73% of XY female mice exposed to testosterone (to mimic the male sex hormone milieu) exhibited aneurysm rupture, these results demonstrate interactions between sex hormones and genes influenced by sex chromosomes on aortic biology, vascular disease development and severity. Future studies will use this information to develop AAA therapies that have sex-specific efficacy.
Table 2.1. Biological pathway analysis (DAVID) for genes overexpressed in XY females. Gene Ontology categories, the number of significant genes (n) and the probability that a greater or equal number would be found by chance (P Value ≤ 0.01) are listed.

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<th>Upregulated in XY in Comparison With XX</th>
<th>n</th>
<th>P Value</th>
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<td>Immune response–regulating signal transduction</td>
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<td>Tnfrsf1b</td>
<td>tumor necrosis factor receptor superfamily, member B14</td>
</tr>
<tr>
<td>T0120000794.mm.1</td>
<td>Trem4</td>
<td>triggering receptor expressed on myeloid cells-like 4</td>
</tr>
<tr>
<td>T0070002233.mm.1</td>
<td>Uty</td>
<td>ubiquitously transcribed tetratricopeptide repeat gene Y, chY</td>
</tr>
<tr>
<td>T0120000140.mm.1</td>
<td>Wdr1</td>
<td>writ inhibitory factor 1</td>
</tr>
<tr>
<td>T000002775.mm.1</td>
<td>Xist</td>
<td>inactive X specific transcripts</td>
</tr>
<tr>
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<td>Ltb2</td>
<td>latent transforming growth factor beta binding protein 2</td>
</tr>
<tr>
<td>T0400001607.mm.1</td>
<td>Mapk6</td>
<td>mitogen-activated protein kinase kinase kinase 6</td>
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<tr>
<td>T0070002224.mm.1</td>
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<td>monocyte-endothelial adhesion molecule-1 (MENAM1)</td>
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<tr>
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<td>Mpeg1</td>
<td>macrophage expressed gene 1</td>
</tr>
<tr>
<td>T0070004631.mm.1</td>
<td>Lirlb1</td>
<td>leukocyte immunoglobulin-like receptor, subfamily B (with T cell recognition)</td>
</tr>
<tr>
<td>T0120000324.mm.1</td>
<td>Lirrb4</td>
<td>leukocyte immunoglobulin-like receptor, subfamily B, member 4</td>
</tr>
</tbody>
</table>

Table 2.3. Continued.
Table 2.4. Characteristics of female XX and XY mice infused with AngII.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>XX Sham</th>
<th>XX Ovx</th>
<th>XY Sham</th>
<th>XY Ovx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>25.6 ± 0.7</td>
<td>27.5 ± 0.6</td>
<td>25.1 ± 0.7</td>
<td>23.9 ± 0.7**</td>
</tr>
<tr>
<td>Systolic Blood Pressure mmHg</td>
<td>123 ± 4</td>
<td>123 ± 3</td>
<td>131 ± 4**</td>
<td>129 ± 4**</td>
</tr>
<tr>
<td>Serum Testosterone ng/ml</td>
<td>0.11 ± 0.03</td>
<td>0.15 ± 0.03</td>
<td>0.36 ± 0.12**</td>
<td>0.11 ± 0.05*</td>
</tr>
<tr>
<td>Serum Cholesterol mg/dl</td>
<td>1964 ± 335</td>
<td>1934 ± 144</td>
<td>1405 ± 107</td>
<td>1721 ± 246</td>
</tr>
<tr>
<td>Plasma Renin Concentration ng/ml</td>
<td>0.21 ± 0.11</td>
<td>0.26 ± 0.08</td>
<td>0.13 ± 0.06</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>Sex Organ Weights (g)</td>
<td>0.13 ± 0.02</td>
<td>0.04 ± 0.01*</td>
<td>0.11 ± 0.02</td>
<td>0.04 ± 0.01*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n=10-17/group.
* P<0.05 compared to Sham within genotype.
** P<0.05 compared to XX within surgical group.
Figure 2.1 Sex chromosome complement influences abdominal aortic gene expression patterns. (A) Left, total number of probe sets on arrays filtered to retain transcripts with reliable signal intensity (FDR; False Discovery Rate). Right, number of genes whose expression level was significantly altered by main effect of genotype (XX, XY), surgery (ovariectomy, OVX, sham surgery) and interaction between genotype and surgery. (B), Volcano plot illustrating fold change in gene expression (x-axis) and statistical significance (y-axis). Genes labelled in blue exhibited significant increase in XY compared to XX abdominal aortas; genes labelled in red exhibited significant increase in XX aortas. (C), RT-PCR of selected abdominal aortic genes illustrated in (B). A,B: Filtered array data analyzed by 2-way ANOVA. C, 2-way ANOVA; data are mean ± SEM from n = 5 mice/surgery group/genotype. *, P<0.05 compared to XX within surgical group. **, P<0.05 compared to sham within genotype.
Figure 2.2. An XY sex chromosome complement markedly promotes formation and severity of AngII-induced AAAs in female mice. (A), Internal abdominal aortic lumen diameters over time of AngII infusions from mice of each genotype (OVX groups; n = 10-17 mice/genotype at study onset) that exhibited a 50% increase in lumen diameter over baseline, and that survived the 28 day infusion protocol. (B), Maximal external AAA diameters after 28 days of AngII infusion mice with an AAA (from A). Symbols represent individual mice with mean ± SEM as horizontal lines. (C), AAA incidence (which includes aneurysm rupture). The number of mice with an AAA/total number of mice in each group is illustrated above each bar of the histogram. (D), % ruptured AAAs. (E) Aortas from mice of each genotype. Data were analyzed by (A) two way repeated measures ANOVA, (B) unpaired t-test (C) and (D) Fisher's exact test. *, P<0.05 compared to XX within surgical group.
Figure 2.3. IL1β expression in abdominal aortas (A), bone marrow-derived macrophages (BMM, B) and plasma (C) of XX and XY Ldlr<sup>−/−</sup> females. For A, IL1β mRNA abundance was quantified by RT-PCR in abdominal aortas from XX and XY Ldlr<sup>−/−</sup> ovariectomized females. For B, cells were harvested from bone marrow of XX and XY Ldlr<sup>−/−</sup> females and differentiated in vitro to macrophages. IL1β concentrations were quantified in media of BMM cells of each genotype incubated with LPS. For C, plasma IL1β concentrations were quantified from XX and XY Ldlr<sup>−/−</sup> females infused with AngII for 28 days. Data were analyzed by Mann-Whitney test. Circles represent data from individual mice while mean ± SEM are illustrated by horizontal lines. *, P<0.05 compared to XX.
Figure 2.4. Aortas from XY females exhibited augmented MMP2 activity (A, B) and dihydroethidium (DHE) fluorescence (C,D). A, Zymograms of aortic extracts from XX and XY Ldlr<sup>−/−</sup> females infused with AngII for 1 day. B, MMP2 (64 kDa) activity quantified by densitometric scans of zymograms (n = 4/genotype). C, Representative images of DHE stained aortic explants from XX and XY Ldlr<sup>−/−</sup> females incubated with vehicle (V) or AngII+testosterone (10 μM and 10 nM, respectively; T=testosterone). D, Quantification of DHE staining in aortic explant sections from XX and XY females. *, P<0.05 compared to XX (B, or within treatment group in D). **, P<0.05 compared to vehicle.
Figure 2.5. Severe AAA rupture in XY females administered dihydrotestosterone (DHT). Adult female XX (n=18) and XY mice (n=11) were administered DHT (0.16 mg/d) for 2 weeks prior to and throughout AngII infusions. (A) % AAA rupture in XX and XY females. (B) Maximal AAA diameters at study endpoint. Symbols represent individual mice with horizontal lines representing mean ± SEM. Data analyzed by (A) Fisher’s exact test (B) Unpaired t-test. *, P<0.05 compared to XX+DHT.
Figure 2.6. Administration of testosterone (400 μg) to 1 day old neonatal female XY mice results in severe AAA rupture when adult XY females are infused with AngII. Females (n=6 XX, n=12 XY) were administered a single dose of testosterone within 24 hours of birth, and then infused with AngII at 2 months of age. Top, % AAA rupture. Bottom, representative aortas from mice of each group. Data were analyzed using Fisher’s exact test. *, P<0.05 compared to XX.
Figure 2.7. mRNA abundance of GAPDH in abdominal aortas from XX and XY Ldlr<sup>−/−</sup> females. Data were analyzed by 2-way ANOVA. Symbols represent individual mice, while horizontal lines represent mean ± SEM.
Figure 2.8. Immune/inflammatory signaling is upregulated in the aortas of females with an XY chromosomal complement. The standardized gene expression values for each subject in each treatment group are heatmapped (scale bar: -2 to +2 standard deviations) for the top 50 most significant genes by the main effect of chromosome (two-way ANOVA, p ≤ 0.0005). 37/50 genes (left) are expressed more strongly in XY subjects. 86% of these (bolded symbols) have known roles in immune/inflammatory processes (Gene Ontology annotation search; orange- promote signaling; blue- inhibit signaling).
Figure 2.9. Aneurysm type as an index of severity of AAAs (top) and Kaplan Meier Survival Curve (bottom, OVX groups) from XX and XY Ldlr<sup>−/−</sup> females infused with AngII. Type I, dilated lumen in the supra-renal region of the aorta with no thrombus; Type II, remodeled tissue in the supra-renal region that frequently contains thrombus; Type III, a pronounced bulbous form of type II that contains thrombus; Type IV, a form in which there are multiple aneurysms containing thrombus, some overlapping, in the suprarenal area of the aorta; Rupture, aneurysms from mice that died of verified rupture. Data are illustrated as percent of total mice within each group.
Figure 2.10. Abdominal aortas from XY females infused with AngII exhibit increased wall volume (top). Bottom, representative 3-D reconstruction from ex vivo ultrasound analysis of one AAA genotype. Data were analyzed by unpaired t-test; data are mean ± SEM.
Figure 2.11. Morphology of abdominal aorta from XX versus XY females prior to AngII infusions. Representative abdominal aorta tissue sections from non-infused XX and XY females stained with hematoxylin and eosin. Scale bar represents 100 µm.
Figure 2.12. Representative AAA tissue sections from XY females infused with AngII exhibit pronounced CD68 immunostaining in areas exhibiting breaks in medial elastin. Scale bar represents 200 µm.
Figure 2.13. Sex chromosome complement has no significant effect on AngII-induced atherosclerosis in thoracic aortas. Data were analyzed by weighted two-way ANOVA. Symbols represent individual mice with mean ± SEM represented by horizontal lines.
Figure 2.14. Abdominal aortic lumen diameters (A) and survival plots (B) over days of AngII infusions of XX and XY Ldlr<sup>−/−</sup> females administered DHT. A, Data were analyzed by 2-way ANOVA with repeated measures on time. B, Kaplan Meier curve for survival of mice in each treatment group. Data are mean ± SEM from n=11-18 mice/genotype.
CHAPTER III. SPECIFIC AIM 2

Sex chromosome complement defines diffuse versus focal angiotensin II-induced aortic pathology (Published in ATVB)

Authors: Yasir Alsiraj, Sean E. Thatcher, Eric Blalock, Bradley Fleenor, Alan Daugherty, and Lisa A. Cassis.

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Summary

Objective-Aortic pathologies exhibit sexual dimorphism, with aneurysms in both the thoracic and abdominal aorta (AAA) exhibiting higher male prevalence. Women have lower prevalence of aneurysms, but when they occur, aneurysms progress rapidly. To define mechanisms for these sex differences, we determined the role of sex chromosome complement and testosterone on the location and progression of angiotensin II (AngII)-induced aortic pathologies.

Approach and Results-We used transgenic male mice expressing Sry on an autosome to create low density lipoprotein receptor (LdLR) deficient male mice with an XY or XX sex chromosome complement. Transcriptional profiling was performed on abdominal aortas from XY or XX males, demonstrating 1746 genes influenced by sex chromosomes or sex hormones. Males (XY or XX) were either sham-operated or orchiectomized prior to AngII infusions. Diffuse aortic aneurysm pathology developed in XY AngII-infused males, while XX males developed focal AAAs. Castration reduced all AngII-induced aortic pathologies in XY and XX males. Thoracic aortas from AngII-infused XY males exhibited adventitial thickening that was not present in XX males. We infused male XY and XX mice
with either saline or AngII and quantified mRNA abundance of key genes in both thoracic and abdominal aortas. Regional differences in mRNA abundance existed before AngII infusions, which were differentially influenced by AngII between genotypes. Prolonged AngII infusions resulted in aortic wall thickening of AAAs from XY males, while XX males had dilated focal AAAs.

Conclusions-An XY sex chromosome complement mediates diffuse aortic pathology, while an XX sex chromosome complement contributes to focal AngII-induced AAAs.

3.1 Introduction

Aortic vascular diseases are life-threatening conditions that are not typically symptomatic, which is of concern since ruptures result in high morbidity and mortality. Amongst the various risk factors that have been identified for aortic vascular diseases, male sex has emerged as a positive risk factor for aneurysmal disease in the ascending aorta (AA) (Roman et al. 2017), distal thoracic aorta (TAA) (Cheung et al. 2017), and for abdominal aortic aneurysms (AAAs) (Pleumeekers et al. 1995; Zarrouk et al. 2013; Svensjo et al. 2013; Weiss et al. 2014; Vardulaki et al. 1998; Pleumeekers et al. 1994). Recent studies analyzing patients within the Genetically Triggered Thoracic Aortic Aneurysms and Cardiovascular Conditions (GenTAC) registry demonstrated that adult males with Marfan syndrome were more likely than females to have aortic root dilatation (by 8%), aortic regurgitation (by 19%) and the prevalence of previous aortic dissection tended to be higher in males (by 7%) (Singh et al. 2017). Similarly, TAAs are also more prevalent in men (Olsson et al. 2006). Male sex is considered one of the
largest non-modifiable risk factors for AAAs, with estimates ranging from a 2-10 fold greater prevalence in men compared to women (Weiss et al. 2014; Wilmink et al. 1998; Pleumeekers et al. 1994; Morris et al. 1994). A higher prevalence of aortic vascular diseases in men is not restricted to aneurysms, as the incidence of acute aortic dissection is also higher in men than women (Clouse et al. 2004; Wenger et al. 1993; Maitusong et al. 2016). Despite a uniformly lower incidence of various forms of aortic vascular diseases in women compared to men, paradoxically, women exhibit more rapid growth rates of both TAAs and AAAs than men, and aneurysms rupture at smaller sizes (Juvonen et al. 1997; Davies et al. 2002; Nienaber et al. 2004; Cheung et al. 2017; Grootenboer et al. 2009; Skibba, 2015; Sweeting et al. 2012; Thompson et al. 2013). Mechanisms for these sex differences in aortic vascular disease development versus progressive growth have not been defined. Similar to humans, studies from our laboratory demonstrated that AAAs induced by infusion of angiotensin II (AngII) to hypercholesterolemic mice exhibit a high level of sexual dimorphism, with a 4-fold higher AAA prevalence in male compared to female mice (Henriques et al, 2004). Testosterone was identified as a primary contributor to higher AAA prevalence in AngII-infused male mice (Henriques et al. 2004; Henriques et al. 2008; Thatcher et al. 2014; Thatcher et al. 2015). Moreover, administration of testosterone to neonatal female low density lipoprotein receptor (Ldlr) deficient mice infused with AngII augmented development of aneurysms in the ascending and abdominal aorta (Zhang et al. 2012). Adult female mice exposed to testosterone as neonates maintained a high susceptibility to AngII-induced aortic vascular diseases (Zhang
et al. 2012). In contrast, AAA incidence was markedly reduced when adult male mice were castrated (Henriques et al. 2004). These results suggest that while testosterone is a primary mediator of sexual dimorphism of experimental aortic vascular diseases, other differences between males and females may contribute to sexual dimorphism of vascular disease development and severity. In addition to sex hormones, recent studies demonstrated that an XY sex chromosome complement was sufficient to promote a high level of AAA susceptibility in female Ldlr-/- mice infused with AngII (Alsiraj et al. 2017). Moreover, XY females exhibited increased aneurysm rupture, which rose to 73% when females were exposed to dihydrotestosterone to mimic an adult male milieu. These results suggest that sex chromosome complement also has profound effects on the vasculature, which is of interest as women with Turner’s Syndrome (monosomy X) exhibit a 100-fold increased risk of aortic dissection (Wong et al. 2014). The purpose of this study was to define the relative role of sex hormones versus sex chromosome complement on the location and characteristics of aortic vascular diseases following AngII infusions. The four core genotype murine (FCG) model was utilized to assess the relative role of sex hormones versus sex chromosome complement on the formation and progression of AngII-induced AAAs. This model produces mice in which the sex chromosome complement (XX versus XY) is varied independently of gonadal sex (testes versus ovaries). The FCG model was created from a natural mutation of the Sry gene (testis determining gene) on the Y chromosome of mice (XY-), where an Sry transgene was inserted onto an autosome for testes formation and fertility (XY-Sry) (Itoh et al, 2015). Breeding of
XY-Sry male mice to XX females produces FCG: XX and XY males, XX and XY females. Since testosterone promotes AngII-induced AAs and AAAs in male mice (Henriques et al. 2004; Henriques et al. 2008), we performed studies on male Ldlr-/- mice with an XY and an XX sex chromosome complement in the presence or absence of male sex hormones. Since men experience a higher prevalence of AAs, TAAs, and AAAs than women, we hypothesize that testosterone and an XY sex chromosome complement promote vascular disease along the length of the aorta in male mice infused with AngII.

3.2 Methods
3.2.1 Mice.

All studies using mice were approved by the institutional Animal Care and Use Committee at the University of Kentucky and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. Male transgenic mice with deletion of Sry from the Y-chromosome expressing Sry on an autosome (8-12 weeks of age) were backcrossed 10 times on a C57BL/6J background (Stock#010905, The Jackson Laboratories, Bar Harbor MA) and bred to low density lipoprotein receptor deficient (Ldlr-/-) females (Stock# 002207, The Jackson Laboratories, Bar Harbor MA) to generate Ldlr-/- male mice with an XY or an XX sex chromosome complement. DNA was extracted from ear or tail clips and subjected to PCR using a commercial PCR mix (Promega 2X Master Mix, cat#m7123, Fitchburg WI) and specific primers. For transcriptome analysis, male XY and XX Ldlr-/- mice (n = 4-5/group/genotype) were randomly assigned to sham and orchiectomized (ORC) groups. Following surgery, mice were fed a Western
diet (TD88137, Harlan Teklad, Indianapolis, IN) for 2 weeks prior to euthanasia and tissue harvest. For assessment of aortic vascular pathologies (Figure 1A), male XY and XX mice (n = 19-21/group/genotype) bred from transgenic male Sry mice were randomly assigned to sham and ORC groups. In addition, we included XY male Ldlr-/- mice (n = 10) purchased from The Jackson Laboratories (Stock# 002207, Bar Harbor MA) to compare to XY males generated from our transgenic Sry Ldlr-/- colony. As there were no observed differences in data quantified from Ldlr-/- XY males (Sry-bred or from The Jackson Laboratory), data from XY males were pooled between strain sources. Two weeks after surgery, mice were fed the Western diet for 1 week prior to implantation of osmotic minipumps and then through study endpoint. Anesthetized mice were implanted subcutaneously with osmotic minipumps (Alzet model 1004, Durect Co., Cupertino, CA) for infusion of AngII for 28 days (1,000 ng/kg/min, Bachem, Torrance, CA). Ultrasound was performed on anesthetized mice on day 0, 7, 14 and 27 of AngII infusions. In this study, n = 6 sham-operated male XX mice, n = 2 ORC male XX mice, n = 2 sham-operated male XY mice and n = 2 ORC male XY mice died from aneurysm rupture, and thus were not included in some AAA quantification measurements (internal aortic diameters, external AAA diameter). For aortic regional mRNA abundance, male XY and XX Ldlr-/- mice (n = 6-8 mice/sex) were infused with saline or AngII (1,000 ng/kg/min) for 1 day prior to euthanasia and tissue harvest. For prolonged infusion of AngII (Figure 1B), male XY and XX Ldlr-/- mice (n = 19 XY, n = 8 XX) were fed the Western diet for one week prior to implantation of osmotic minipumps containing AngII (1,000ng/kg/min) and the diet continued through study endpoint.
Ultrasound was used on day 27 of AngII infusions to define mice with an AAA (abdominal aortic lumen diameter > 50% increase compared to day 0 of infusions). For each genotype, all mice infused with AngII qualified with an AAA and thus continued in the study. Osmotic minipumps were replaced with fresh AngII solution every 28 days in anesthetized mice. Ultrasound was performed on anesthetized mice on day 0, 14, 27, 42, 56 and 70 of prolonged AngII infusions. In this study, n = 9 male XY mice and n = 3 male XX mice died from AAA rupture and thus were not included in some measures of AAA quantification (e.g., ultrasound or maximal AAA diameters). Thus, illustrated data for these measurements represent mice that survived the 70 days of AngII infusions. At the end of each experiment, mice were euthanatized under anesthesia (ketamine/xylazine, 100:10 mg/kg, i.p.) and blood was collected via cardiac puncture.

3.2.2 Orchiectomy.

Male (8-12 weeks of age) XX and XY Ldlr-/- mice were orchiectomized under isoflurane anesthesia (2-3%) and given pre and post-operative analgesic (24 hr after surgery; flunixin; 2.5 mg/kg). Mice were shaved in the scrotum region and a depilatory cream (Nair, Inc.) applied to the area to remove hair, followed by sterilizing with povidone-iodine/ethanol (three times). A small incision was made in this region. Vas deferens were collapsed using a hemostat and the testes were removed. The vascular supply was ligated by cauterization using a high-temperature fine-tip loop cauterizer and the hemostat was released. The wound site was monitored for bleeding, and the skin was closed by wound clips (Autoclip stapler). The site was then treated with povidone-iodine, and mice were allowed to
recover. The testes were manipulated but left intact in anesthetized sham-operated mice. All mice were allowed to recover from surgery and to clear their endogenous testicular hormones for two weeks before the onset of AngII infusions.

3.2.3 Measurement of plasma and serum components.

Total serum cholesterol and testosterone concentrations were determined using enzymatic assay kits (Wako Pure Chemical, Richmond, VA, cat#439-17501; Alpco, Salem, NH, cat#55-TESMS-E01; respectively). The renin concentration in plasma was measured by quantifying angiotensin I generated in the absence or presence of an excess of exogenous rat angiotensinogen (purified from nephrectomized rat plasma). Plasma was harvested from mice in ice-cold EDTA (0.2 M). Mouse plasma (8 μl) was incubated in buffer (Na2HPO4, 0.1M; EDTA, 0.02 M; maleate buffer, pH 6.5; total volume of 250 μl) containing phenylmethylsulfonyl fluoride (2 μl/250μl reaction volume) for 30 minutes at 37°C in a shaking water bath. The reaction was terminated by placing samples at 100°C for 5 min. Angiotensin I was quantified by radioimmunoassay using a commercial kit (DiaSorin, CA-1553, Stillwater, MN). Plasma renin concentration (ng/ml) is represented as the difference in angiotensin I levels in the absence versus the presence of exogenous angiotensinogen.

3.2.4 Blood pressure measurements.

Blood pressure was quantified using a non-invasive computerized tail-cuff method (BP-2000; Visitech Systems, Apex, NC) at baseline (prior to pump implantation) and during week 3 of AngII infusion. Measurements were recorded at the same time of the day for 5 days (2 days acclimation, 3 days of recording).
3.2.5 Quantification of AAAs.

The abdominal aortic internal lumen diameters were quantified on day 0, 7, 14, and 28 of AngII infusions using a Vevo 2100 high-resolution imaging system (VisualSonics, Inc.). AAA incidence was quantified from the abdominal aortic internal lumen diameter, and defined as a 50% increase in lumen diameter on day 28 of AngII infusions (compared to day 0). AAA incidence included mice that died from aneurysm rupture, which was visually confirmed post-mortem. Other measurements (ultrasound, maximal AAA diameters) included mice that survived the complete study protocol. External abdominal aortic diameters were quantified on excised, cleaned tissue mounted on a black wax background. A Nikon SMZ800 dissecting microscope was used to obtain aorta images and the maximal external diameter of the abdominal aorta was quantified. Using the Vevo 2100 system with a 55-MHz probe, the three-dimensional volume analysis was performed on abdominal aortas of XX and XY males (n = 5 mice/genotype) that were infused with AngII for 3 months. Aortas were scanned at the abdominal region (diaphragm to the ileac bifurcation) using the ultrasound ex vivo at 0.051 mm intervals with an imaging length of 14.986 mm, and a total of 295 images were collected. Each image was analyzed for wall and lumen volume, and images were combined to obtain the total volume of abdominal aortas.

3.2.6 Quantification of AAs and TAAs.

Arch areas were defined by drawing a 3-mm line from the left subclavian artery. Thoracic areas were defined by drawing a 9mm line from the end of the arch area to the diaphragm muscle. Arch and thoracic combined areas were
quantified by drawing a line around the borders and summing the total area of each. Measurements were performed using Nikon Elements Version 3.2.

3.2.7 Quantification of atherosclerosis.

Atherosclerosis was quantified en face in the aortic arch (Daugherty et al. 2000). Lesions were summed and divided by the total arch area to calculate the percent lesion area. Cleaned aortas were cut open longitudinally and mounted on a black wax background using insect pins (Fine Science Tools, cat# 26002-20) to quantify atherosclerosis in the aortic arch and the thoracic aorta.

3.2.8 Quantification of aortic stiffness (pulse wave velocity, PWV).

Aortic stiffness was assessed on anesthetized (2% isoflurane) male XY and XX mice (8 months of age, non-infused, n = 6 mice/genotype) that were placed supine on a heating board with legs secured to ECG electrodes (Du et al. 2015). Doppler probes (Indus Instruments, Webster, TX) were used to noninvasively quantify aortic velocities at the aortic arch and abdominal aorta. PWV was calculated as the distance between the aortic arch and abdominal probes divided by the difference in thoracic and abdominal aortic pre-ejection times. Data are presented as centimeters/second.

3.2.9 Quantification of adventitial and medial diameter in thoracic aorta tissue sections.

Thoracic aortic segments from XY and XX male mice (n = 3 mice/group) infused with AngII for 28 days were formalin fixed, processed through a series of ethanol and xylene baths and embedded in paraffin. Sets of serial 5 micron cross
sections were collected at intervals along each segment and stained with hematoxylin and eosin. Six tissue sections from each mouse were imaged using a Nikon Eclipse 80i microscope. The adventitial layer was defined by encircling a line around the borders of the outer layer of the aortic section. The medial layer was defined by encircling a line around the borders of the middle layer of the aortic section. Analysis was performed using Nikon Elements Version 3.2.

3.2.10 RNA extraction and DNA microarrays.

XY and XX male mice (8 weeks of age; n = 4-5 mice/ genotype) underwent sham surgery or ORC, and after two weeks, mice were placed on Western diet (TD88137, Harlan Teklad, Indianapolis, IN) for one week. RNA from abdominal aortas was extracted using the RNeasy fibrous tissue mini kit (Qiagen, cat # 74704). Harvested abdominal aortic RNA quality and quantity were measured by Agilent 2100 Bioanalyzer using RNA 6000 Nano Labchip kits (Agilent Technologies, Cat # 5067-151). One RNA sample (subject #9) was excluded due to poor quality. The remaining 17 samples had excellent quality and did not differ significantly by treatment group (RNA Integrity Number [RIN]: 9.64 ± 0.03; RNA concentration (ng/ul): 55.7 ± 7.4; 28s:18s ratio: 3.00 ± 0.07; all p-values for main effects of gene and surgery, as well as of interaction terms were n.s. [p > 0.1]). Extracted RNA was labeled and hybridized to Affymetrix Mouse Transcriptome Assay 1.0 arrays (one array per subject; n = 17). Signal intensities were calculated using the RMA algorithm (Bolstad et al, 2003) at the transcript level using Genomics Suite software (Partek, St Louis). Data were transferred to flat files in Excel and associated with vendor-provided annotation data. Pre-statistical filtering
retained unique, annotated probe sets with adequate signal intensity (signal intensity ≥ 4.2 on at least 3 arrays in the study). Filtered signal intensities were analyzed by two-way ANOVA to identify significant main effects of genotype (XY versus XX), surgery (Sham vs ORC), as well as interaction. The False Discovery Rate (FDR) procedure (Hochberg et al. 1990) was used to estimate the error of multiple testing. Functional categorization for each expression pattern was determined with the prestatistically filtered gene list as a background using DAVID bioinformatic tools (Huang da et al. 2009). Currently, DAVID does not support Affymetrix MTA 1.0 IDs, and therefore best match IDs from Affymetrix Mouse 1.0 Exon arrays were used, covering more than 90% of the filtered MTA data set. Raw data are available through the Gene Expression Omnibus (GSE #:81580 www.ncbi.nlm.nih.gov/geo/).

3.2.11 Real Time RT-PCR.

After 1-day of saline or AngII infusion to XY and XX male Ldlr-/− mice (n = 4-6 mice/ genotype/ treatment) RNA was extracted from abdominal and thoracic regions of the aorta using the RNeasy fibrous tissue mini kit (Qiagen, cat # 74704). cDNA was then synthesized using the qScript cDNA Supermix (Quanta Biosciences, cat# 95048-500). Real-time PCR was processed using the SYBER Green FastMix (Quanta Biosciences, cat# 95071-012) on a BioRad quantitative real-time PCR. mRNA abundance was determined using the ΔΔCt method. Gene expression was normalized to the housekeeping gene, beta-2-microglobulin. Primers sequences for the probed genes are listed in Table 1.
3.2.12 Statistical Analyses.

Data are illustrated as mean ± SEM. Data were analyzed using unpaired Student t-tests for two groups, by two-way repeated measures ANOVA on time for longitudinal data with between group factors of surgery or genotype, or by two or three-way ANOVA with between group factors of surgery, region, treatment or genotype. If differences existed between groups, a Holm-Sidak post hoc analysis was performed. Incidence of AAAs and TAAs were analyzed by Fisher’s exact test when two groups were examined. Statistical analysis was performed using SigmaPlot software (Version 12.3) or GraphPad Prism 5. P≤ 0.05 was considered significant.

3.3 Results

3.3.1 Testosterone and/or Sex Chromosome Complement Influenced Abdominal Aortic Gene Expression Patterns and Aortic Stiffness.

We demonstrated recently that an XY sex chromosome complement promoted expression of inflammatory gene pathways in abdominal aortas from female Ldlr−/− mice (Alsiraj et al, 2017). Since previous studies focused on abdominal aortas of XX and XY females, in this study we used RNA extracted from abdominal aortas of XY and XX male mice (non-infused) that were gonadally intact (sham-operated) or orchiectomized (ORC) for Affymetrix Mouse Transcriptome Assay 1.0 analysis. A total of 1,746 genes exhibited highly significant differences (two-way ANOVA, P<0.01, Figure 2A). There was a significant main effect of sex chromosome (450 genes, FDR = 0.32), castration (799 genes, ORC vs Sh, FDR = 0.14) and an interaction between sex chromosome and castration (708 genes, FDR = 0.24;
Figure 2). A frequency histogram of the number of genes (y-axis) found at different p-value cut-offs (x-axis), with chance depicted by the dashed line, illustrates the number of genes influenced by sex chromosome complement, castration, or by an interaction between sex chromosomes and sex hormones at different p-values (Figure 2). Volcano plots of the chromosome effect with highly stringent cutoffs (> 2-fold change, p < 0.01) demonstrated that, as expected, the expression of genes on sex-chromosomes was influenced strongly (Figure 2). Using this level of stringency, five genes (Xist, Arntl, Npas2, Arhgap20, Ighv14-1) were increased significantly in abdominal aortas of sham-operated XX compared to XY males (Figure 2, Table 2). In contrast, 51 genes were increased significantly in abdominal aortas of sham-operated XY compared to XX males (Table 2). RT-PCR on mRNA extracted from abdominal aortas from mice of each genotype and surgical group confirmed microarray results for selected genes enriched in XX (Xist, Npas2, Arntl, Figure 3) or XY aortas (Cyp2e1, Kap, Figure 3). Biological pathway analysis revealed several expression patterns, potentially related to aortic vascular diseases, that were increased in abdominal aortas of XY compared to XX males (immune response, acute inflammatory response), while other pathways were increased in abdominal aortas of XX compared to XY males (e.g., DNA binding, regulation of cell proliferation) (Figure 2, Table 3). To determine if differences in gene expression patterns (e.g., extracellular matrix, Figure 2) between aortas of XY and XX males influenced aortic function, we quantified pulse wave velocity (PWV) as an index of aortic stiffness in non-infused XY and XX Ldlr/-/- males.
Aortas from XY males exhibited significant increases in PWV compared to XX males (Figure 4).

3.3.2 Aortic Aneurysmal Disease was Diffuse in XY Males and Localized in XX Males.

At study endpoint, body weights of castrated (ORC) male mice infused with AngII were decreased significantly compared to sham-operated controls, regardless of genotype (Table 4; P<0.05). Serum testosterone concentrations were similar in sham-operated XY and XX males infused with AngII (Table 4; P>0.05). Castration resulted in significant decreases in serum testosterone concentrations of both genotypes, with no differences between genotypes (Table 4; P<0.05). Systolic blood pressures were not different between genotypes or surgical groups at baseline (XY sham, 111 ± 4; XY ORC, 109 ± 5; XX sham, 110 ± 3; XX ORC, 107 ± 2 mmHg), but increased significantly in AngII-infused XY compared to XX sham-operated males (Table 4; P<0.05), and this difference was eliminated by castration. Plasma renin concentrations were not significantly different between groups (Table 4; P>0.05). Atherosclerotic lesion surface area in the aortic arch was not significantly altered by castration or by sex chromosome complement in AngII-infused male mice (Table 4; P>0.05).

Aortas from XY sham-operated AngII-infused males exhibited diffuse disease that typically extended from the aortic arch to the suprarenal aorta (Figure 5A, top left). In contrast, aortas from XX AngII-infused males exhibited aortic pathology that was generally restricted to the suprarenal portion of the abdominal aorta (Figure 5A, top right). As an index of the extent of aortic pathology, aorta weights were
increased significantly in XY compared to XX males, with aortic weights decreased by castration in both genotypes (Figure 5B; P<0.05). The area of the aortic arch was increased significantly in XY compared to XX AngII-infused sham-operated males (Figure 5C; P<0.05), with reductions in arch areas following castration of XY males. The incidence of aneurysms in the aortic arch or thoracic aorta (TAAs) was markedly higher in XY (44%) compared to XX (8%) AngII-infused males (Figure 5D; P<0.05). This difference was abolished by castration, which resulted in almost complete ablation of AngII-induced TAAs in both genotypes. In contrast to diffuse aortic disease of XY males infused with AngII, XX males exhibited focal aortic disease within the suprarenal portion of the abdominal aorta, with significant increases in external AAA diameters that were abolished by castration (Figure 6A; P<0.05). Similarly, abdominal aortic lumen diameters were increased significantly in XX compared to XY sham-operated males infused with AngII (day 28, Figure 6B; P<0.05). The high incidence of AAAs was not significantly different between XX and XY AngII-infused males (Figure 6C; P>0.05), and castration significantly decreased AAA incidences of both genotypes (Figure 6C; P<0.05). Larger AAAs of XX males were associated with slight, but insignificant increases in aneurysmal rupture (Figure 6D; P>0.05), which were also reduced by castration of XX males. Since XY, but not XX males exhibited considerable aortic pathology in the thoracic aorta, we characterized morphology of tissue sections prepared from thoracic aortas of XY and XX males infused with AngII. Thoracic aorta tissue sections from XY AngII-infused males exhibited pronounced thickening of the adventitia, which was not evident in thoracic aortas of XX males (Figure 7A, B). Adventitial
thickening of thoracic aortas from XY males was abolished by castration (Figure 7A, B). In contrast, medial diameters were not significantly different between XY and XX males, and were not influenced by castration (Figure 7A, C; P>0.05).

3.3.3 Aortic Genes Related to AAA Development Exhibited Region-specific and AngII-induced Differences in Abundance.

Initial studies defined gene expression patterns in abdominal aortas from non-infused male XY and XX mice (Figure 2). To determine if differences in aortic disease development between XY and XX male mice are associated with regional differences in aortic gene expression, and whether AngII regulates aortic genes in a region-specific manner, we contrasted effects of short-term (1 day) infusions of saline versus AngII on abundance of genes within the thoracic versus abdominal aortas of male XY and XX Ldlr/- mice. We chose short-term AngII infusions to limit the extent of overt aneurysm pathology between genotypes. Abundance of angiotensin converting enzyme (ACE), collagen 1a1, and thrombospondin (Thbs1) mRNAs were increased significantly in abdominal compared to thoracic aortas of XX, but not XY saline-infused male mice (Figure 8A-D; P<0.05). Moreover, abdominal aortas of saline-infused XX males exhibited significantly increased mRNA abundance of ACE, collagen 1a1, and Thbs1 compared to abdominal aortas of saline-infused XY males (Figure 8A, C,D; P<0.05). Infusion of AngII resulted in significant elevations in mRNA abundance of matrix metalloproteinase 2 (MMP2), collagen 1a1, and Thbs1 in thoracic, but not abdominal aortas of XY male mice compared to XY saline-infused controls (Figure 8B-D; P<0.05). In contrast, abdominal aortas of XX males did not respond to AngII with increased
expression of MMP2 or Thbs1. However, mRNA abundance of collagen 1a1 was decreased significantly by AngII in abdominal aortas of XX males. As a result, following AngII infusions, marked regional differences in expression levels of MMP2, collagen 1a1, and Thbs1 were evident, with increased expression levels in thoracic compared to abdominal aortas of XY and XX male mice (Figure 8B-D; P<0.05). The magnitude of AngII-induced changes in the regional expression levels of these genes were more pronounced in XY than XX male mice.

3.3.4 Regional Differences in AngII-induced Aortic Vascular Diseases Between XY and XX Males Persisted with Aneurysm Progression.

Despite a lower prevalence of TAA and AAA development in women compared to men, previous studies demonstrated that aneurysm growth rates are more aggressive in women, and aneurysms may rupture at smaller sizes (Juvonen et al. 1997; Davies et al. 2002; Nienaber et al. 2004; Cheung et al. 2017; Grootenboer et al. 2009; Skibba, 2015; Sweeting et al. 2012; Thompson et al. 2013). Therefore, we examined progression of aortic vascular diseases in response to 3 months of prolonged AngII infusions in XY and XX males. Increased abdominal aortic lumen diameters of XX males persisted with prolonged AngII infusions (Figure 9A; P<0.05). Surprisingly, dilated abdominal aortas of XX males were not associated with significantly larger AAA diameters at study endpoint (Figure 9B; P>0.05). These results suggest divergent aneurysm remodeling between genotypes. Similar to 28 day AngII infusions, XY males infused with AngII for 3 months exhibited diffuse aortic disease (Figure 9C), resulting in significant increases in aorta weight (Figure 9D; P<0.05) and incidence of TAAs compared to
XX males (Figure 9E; P<0.05). Since maximal AAA diameters were not different between XY and XX males, but aneurysms of XX males were more dilated, we performed ex vivo ultrasound analysis of AAA 3-D structures following prolonged AngII infusions (Figure 10, representative 3-D images of AAA from each group in C). AAA wall volumes were increased in XY compared to XX males (Figure 10A), while AAA lumen volumes were increased significantly in XX compared to XY males (Figure 10B).

3.4 Discussion

The key novel findings of this study are: (1) Male sex hormones, coupled with an XY sex chromosome complement result in development of diffuse aortic disease associated with adventitial thickening in response to AngII infusions. (2) In contrast, an XX sex chromosome complement in phenotypic male mice results in focal aneurysmal disease within the suprarenal abdominal aorta. (3) These regional differences in aortic vascular disease development are associated with differences in abundance of genes implicated in aneurysmal disease between thoracic versus abdominal aortas of XY and XX male mice. (4) Short-term infusion of AngII superimposes pronounced regional differences in aortic gene expression in both genotypes, with greater differences in XY males. (5) Focal suprarenal AAAs of AngII-infused XX males progress to exhibit pronounced aortic lumen dilation, while progressing AAAs of XY males infused chronically with AngII have thickened vascular wall volumes and aortas exhibit diffuse disease. It is well established that male sex is a significant non-modifiable risk factor for development of AAAs, with men exhibiting a 2-10-fold higher prevalence than women (Weiss et al. 2014;
Wilmink et al. 1998; Pleumeekers et al. 1994; Morris et al. 1994). Recent results, performed on a large population of well-characterized patients with Marfan syndrome, also exhibited a higher prevalence of aortic root dilatation and regurgitation in men compared to women (Roman et al. 2017). Moreover, using data from the Swedish national healthcare registers from 1987 to 2002, the incidence of TAA was 52% in men compared to 28% in women (Olsson et al. 2006). Collectively, these studies indicate that men exhibit a higher prevalence of vascular disease localized to different regions of the aorta compared to women. A variety of mechanisms for these differences have been proposed, ranging from influences of sex hormones and their receptors (Villard et al. 2017), differences in aortic stiffness (Tong et al. 2013), hemodynamic influences, and basic differences in aortic size between sexes (Lindquist et al. 2017; Lo et al. 2014; Sweet et al. 2011; Lederle et al. 1997). Of interest, sex chromosome abnormalities, such as Turners syndrome (monosomy X), are associated with a higher risk of aortic dissection (Wong et al. 2014), another aortic vascular disease that exhibits higher prevalence in men than women (Meszaros et al. 2000). Recent studies from our laboratory demonstrated that an XY sex chromosome complement, when inserted in phenotypic females with low circulating testosterone, was sufficient to increase the incidence and severity of AngII-induced AAAs (Alsiraj et al. 2017). We also noted that exposure of XY females to dihydrotestosterone, to mimic a male-like milieu, resulted in a striking level of aneurysm rupture (Alsiraj et al. 2017). In this study, we used male mice with an XY or XX sex chromosome complement, as males experience testosterone exposures throughout life and testosterone has
been demonstrated to increase AngII-induced AAAs in male or female mice (Henriques et al. 2004; Henriques et al. 2008; Zhange et al. 2012). Our results demonstrate that testosterone, through a sex chromosome-dependent (XY) mechanism, promotes the presence of diffuse aortic vascular disease in male mice infused with AngII. In contrast, aortas from XX males exhibit focal aortic pathology manifest within the suprarenal region of the abdominal aorta, as has been typically described for AngII-induced AAAs that occur in females (Daugherty et al. 2000). These striking differences in regional location of AngII-induced aortic pathology were both sex chromosome and sex hormone-mediated. Several potential mechanisms have been suggested to contribute to sex differences in aortic vascular disease development (Makrygiannis et al. 2014). In this study, gene expression analysis exhibited 1746 genes that were differentially expressed in abdominal aortas of non-infused XY and XX males according to sex hormone (e.g., influenced by castration), sex chromosome complement, or by an interaction between these factors. Pathway analysis of gene array data identified genes related to extracellular matrix, acute inflammatory response, regulation of cell proliferation and the immune response may relate to differences in AAA susceptibility between males and females. Of the aortic genes exhibiting pronounced differences based on sex chromosome complement, Xist, the RNA gene within the X chromosome that is the effector of the X-inactivation process, confirms the validity of sex chromosome manipulation in XX males. Arntl (BMAL1) and Npas2, genes enriched in aortas from XX compared to XY males, are genes expressing proteins that are core components of the circadian clock apparatus. To
our knowledge, there is minimal information related to influences of circadian rhythm on AAA formation and/or rupture. Abdominal aortas from XY males had higher mRNA abundance of Cyp2e1 (a member of the cytochrome p450 enzyme family) and Kap (kidney androgen-regulated protein) compared to XX males. It is unclear if these differences in abdominal aortic gene expression between XY and XX males are related to aneurysm outcomes of the current study. An intriguing difference between XY and XX males infused with AngII was the large adventitial thickening that occurred in the thoracic aorta of XY, but not XX males. Increased adventitial thickening in response to AngII infusions in thoracic aortas may have contributed to the observed augmented aortic stiffening of XY males (Bersi et al. 2016). Since blood pressures were increased significantly in XY compared to XX males infused with AngII, it is possible that hemodynamic differences contributed to these divergent morphologic responses and susceptibility to AngII-induced TAAs between genotypes. However, blood pressure was similar in AngII-infused sham and castrated males, even though TAA incidence was markedly reduced by castration. These results suggest that blood pressure may not be the primary contributor to the development of diffuse aortic vascular pathologies of XY males. Previous investigators demonstrated a large number of genes (1475) that are differentially expressed in the ascending versus descending aortas of female mice (Pfaltzgraff et al. 2014). These differences were ascribed to developmental origins of cells within distinct regions of the aorta. Specifically, smooth muscle cells of the ascending aorta develop from cardiac neural crest and second heart field (Sawada et al. 2017), descending aorta cells develop from somites while those of the
abdominal aorta are derived from mesodermal origin (Majesky et al. 2007). These differences have been suggested to contribute to diverging physiologic and pathophysiologic responses of the aorta, including regulation of the extracellular matrix (Thieszen et al. 1996; Majesky et al. 2007; Thatcher et al. 2015). Notably, previous studies indicate that cells of mesodermal origin that populate the abdominal aorta express androgen receptors (Cunha et al. 1981), and recent studies indicate increased expression of androgen receptors in abdominal aortas of patients with an AAA compared to control aorta (Villard et al. 2017). In this study, with the exception of collagen 1, XY and XX males exhibited the opposite patterns of gene expression in abdominal versus thoracic aortas, with increased thoracic gene expression in XY males, and increased abdominal gene expression in XX males. Notably, infusion of AngII resulted in robust regional differences in expression patterns of several genes with more robust regional differences (e.g., higher in thoracic than abdominal) in aortas from XY males exhibiting diffuse disease in response to the peptide. It is unclear if embryonic origins of vascular wall cells contribute to the observed regional differences in aortic gene expression patterns or the regional responses to AngII. Despite a lower prevalence of aortic vascular disease in women than men, women with AAAs or TAAs appear to exhibit more progressive aneurysmal growth, with a propensity to rupture at smaller aneurysm sizes (Cheung et al. 2017; Lo et al. 2014; Thompson et al. 2013; Sweeting et al. 2012; Larsson, 2011; Mofidi et al. 2007; Solberg et al. 2005). Previous results from our laboratory demonstrated that castration of male mice with established AngII-induced AAAs resulted in aneurysm remodeling, with thin
walled AAAs of castrated males compared to muscularized aneurysms of intact males (Zhang et al. 2015). Results of the current study agree with previous findings, in that AAAs of XY males had large wall volumes as they continued to grow. In addition, the diffuse nature of aortic vascular pathology of XY males persisted with prolonged AngII infusions. In contrast, XX males exhibited dilated thin walled AAAs with prolonged AngII infusions, suggesting an increased propensity of weakened aortas to rupture. These results indicate that sex chromosome-mediated influences on aortic vascular biology may contribute to differences in the progression of aortic vascular disease between sexes. A limitation of the model used in these studies is that it does not define whether the observed phenotype results from genes residing on the Y or second X chromosome (that escape X-inactivation). Moreover, XX males that are derived from this breeding strategy are infertile and have small testes because they lack the Y chromosome genes that are responsible for spermatogenesis. However, as XX mice have serum testosterone concentrations that are not significantly different from male XY mice (Table 4), it is unlikely that these differences influenced experimental findings.

In conclusion, this is the first demonstration that sex chromosome complement, coupled with sex hormones (e.g., testosterone), modulate the region-specific development of aortic pathology in response to AngII. An XY sex chromosome complement in males resulted in diffuse aneurysmal disease along the aorta, while a male XX sex chromosome complement mediated focal abdominal aortic aneurysmal disease. These regional differences in aortic vascular disease...
according to sex chromosome complement were dependent on the presence of male sex hormones. Regional differences in expression levels of genes implicated in aneurysm development between thoracic versus abdominal aortas from XY and XX males may contribute to divergent aneurysm susceptibility and regional location of aortic pathology between sexes in response to AngII. Finally, aneurysm progression and remodeling were differentially regulated by sex chromosome complement, with XX males exhibiting thin walled AAAs and XY males exhibiting thickened AAAs and diffuse aortic disease from prolonged AngII infusions. These studies suggest that sex differences between men and women in development and progression of aortic vascular diseases may arise from the complex interplay between sex hormones and sex chromosome complement in defining regional aortic gene expression, diffuse versus focal aortic pathology, and differential aneurysm remodeling of aortic vascular diseases.
Table 3.1. Primer sequences for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tr>
<td>ACE</td>
<td>Forward: 5'-AGGTTGGGCTACTCCAGGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGTGAGTGGTGTCTGCTTC-3'</td>
</tr>
<tr>
<td>MMP2</td>
<td>Forward: 5'-GGGTTCCATTTTCTTCTTCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCAGCAAGTAGATGCTGCCT-3'</td>
</tr>
<tr>
<td>Collagen1</td>
<td>Forward: 5'-GCTCCTCTTTAGGGGCACT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCACGTCTCACCATTGGGG-3'</td>
</tr>
<tr>
<td>Thbs1</td>
<td>Forward: 5'-CAATTTTCAGGGGTTGCTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCGTTACCAGTTGTTGC-3'</td>
</tr>
<tr>
<td>Xist</td>
<td>Purchased from QIAGEN catalog#330001 PPM68756B</td>
</tr>
<tr>
<td>Kap</td>
<td>Purchased from QIAGEN catalog#QT00113484</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>Purchased from QIAGEN catalog#QT02280278</td>
</tr>
<tr>
<td>Arntl</td>
<td>Purchased from QIAGEN catalog#QT00101647</td>
</tr>
<tr>
<td>Npas2</td>
<td>Purchased from QIAGEN catalog#QT00108647</td>
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Table 3.2. Upregulated genes in XY vs. XX males

<table>
<thead>
<tr>
<th>Genes Upregulated in XY</th>
<th>Genes Upregulated in XX</th>
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</thead>
<tbody>
<tr>
<td>Ddx3y</td>
<td>Xist</td>
</tr>
<tr>
<td>Uty</td>
<td>ArntI</td>
</tr>
<tr>
<td>Kdm5d</td>
<td>Npas2</td>
</tr>
<tr>
<td>Elf2s3y</td>
<td>Arhgap20</td>
</tr>
<tr>
<td>Dbp</td>
<td>Ighv14-1</td>
</tr>
<tr>
<td>Nr1d2</td>
<td></td>
</tr>
<tr>
<td>Nr1d1</td>
<td></td>
</tr>
<tr>
<td>Cyp2e1</td>
<td></td>
</tr>
<tr>
<td>Cidec</td>
<td></td>
</tr>
<tr>
<td>Fam13a</td>
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</tr>
<tr>
<td>Ehhadh</td>
<td></td>
</tr>
<tr>
<td>Sic27a2</td>
<td></td>
</tr>
<tr>
<td>Npy</td>
<td></td>
</tr>
<tr>
<td>Ms4a4a</td>
<td></td>
</tr>
<tr>
<td>Kap</td>
<td></td>
</tr>
<tr>
<td>Dbh</td>
<td></td>
</tr>
<tr>
<td>Snap25</td>
<td></td>
</tr>
<tr>
<td>Pck1</td>
<td></td>
</tr>
<tr>
<td>Atp1b1</td>
<td></td>
</tr>
<tr>
<td>Gnal</td>
<td></td>
</tr>
<tr>
<td>Syt1</td>
<td></td>
</tr>
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<td>Htr3a</td>
<td></td>
</tr>
<tr>
<td>Sic18a2</td>
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<td>Th</td>
<td></td>
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<td>Tubb3</td>
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<tr>
<td>Tubaa4a</td>
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<td>Acne</td>
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<td>Kcna1</td>
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<td>Gatm</td>
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<td>Ddc</td>
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<td>Sic31a1</td>
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<td>Cdh19</td>
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<td>Napb</td>
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<td>Vat1I</td>
<td></td>
</tr>
<tr>
<td>Neff</td>
<td></td>
</tr>
<tr>
<td>Sic6a2</td>
<td></td>
</tr>
<tr>
<td>Syn2</td>
<td></td>
</tr>
<tr>
<td>PIP1</td>
<td></td>
</tr>
<tr>
<td>Klf21a</td>
<td></td>
</tr>
<tr>
<td>Chil1</td>
<td></td>
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<tr>
<td>Pirt</td>
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<td></td>
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<tr>
<td>Cxcl13</td>
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<tr>
<td>Tspan8</td>
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<td>Snord116I2</td>
<td></td>
</tr>
<tr>
<td>Stmn2</td>
<td></td>
</tr>
<tr>
<td>Necab1</td>
<td></td>
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<tr>
<td>Mpz</td>
<td></td>
</tr>
<tr>
<td>Snord116</td>
<td></td>
</tr>
<tr>
<td>Fabp7</td>
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Table 3.3. Biological pathways upregulated in XY and XX males

<table>
<thead>
<tr>
<th>Pathway Description</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated in XY compared to XX</strong></td>
<td></td>
</tr>
<tr>
<td>1. Polyamine biosynthetic process (4 genes):</td>
<td>Amd2, Azin1, Odc1, Srm</td>
</tr>
<tr>
<td>2. Nuclear pore (6 genes):</td>
<td>Ipo7, Kpna1, Kpna2, Nup50, Nup88, Nup93</td>
</tr>
<tr>
<td>3. Purine ribonucleotide binding (31 genes):</td>
<td>Abcf2, Atp2b1, Baz1b, Btaf1, Cars, Cct2, Cct3, Cdk6, Ctps, Ddx21, Ddx3y, Dgkg, Eif2s3y, Eif4a1, Gna1, Gnl3, Hsp90ab1, Hspa4, Iars, Ide, Insr, Map2k3, Map3k6, Mat2a, Psmc5, Rras2, Sar1a, Sgk1, Srp54a, Srp54b, Srp54c</td>
</tr>
<tr>
<td>4. tRNA binding (4 Genes):</td>
<td>Cars, Mettl1, Xpo5, Yars</td>
</tr>
<tr>
<td>5. Nuclear lumen (16 genes)</td>
<td>Ccnl2, Cfl2, Ddx21, Gnl3, Mbd1, Nop2, Nop56, Polr2l, Pop4, Psmc5, Sfq, Srp54a, Srp54b, Srp54c, Srp72, Srrt</td>
</tr>
<tr>
<td>6. Nucleolus (8 genes):</td>
<td>Ddx21, Gnl3, Mbd1, Nop2, Nop56, Pop4, Srp72, Wdr74</td>
</tr>
<tr>
<td>7. Transition metal ion binding (32 genes):</td>
<td>Adamts1, Adamts9, B4galt4, B4galt5, Baz1b, Cars, Dgkg, Dnaja2, Galnt11, Galnt7, Herc2, Iars, Ide, Kdm5d, Klf13, Klf9, Luc7l, Mat2a, Mbd1, Mbnl2, Nr4a3, P4ha1, Polr2l, Prnp, Sec23b, Slc30a1, Spire1, Sqstm1, Ubr2, Uty, Zdhhc14, Zfp597</td>
</tr>
<tr>
<td>8. Protein amino acid N-linked glycosylation (3 genes):</td>
<td>Mgat2, Prkcsh, Stt3b</td>
</tr>
<tr>
<td>9. Extracellular matrix part (7 genes):</td>
<td>Ccdc80, Col1a1, Col1a2, Col4a1, Col5a2, Lamlc1, Sparc</td>
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<tr>
<td>10. Immune response (7 genes):</td>
<td>Ccl6, Ccl9, Cfb, Fcgr2b, Fcgr3, Il31ra, Tlr13</td>
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<td>12. Skin development (3 genes):</td>
<td>Aars, Col1a1, Col5a2</td>
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<tr>
<td>14. Melanosome (3 genes):</td>
<td>Pdia4, Pdia6, Snd1</td>
</tr>
</tbody>
</table>
Table 3.3. (continued)

Upregulated in XX compared to XY

1. Ribosome (8 genes): Mrpl23, Mrpl52, Rpl18a, Rpl36, Rplp1, Rplp2, Rps10, Ubc
2. DNA binding (20 genes): Arap1, Ddx3x, Ercc2, Foxd1, Gli1, Kcnip3, Kdm5c, Msx1, Nr1h2, Nr3c2, Pold2, Polr2e, Polr2f, Relb, Sry, Stra13, Thap3, Zfp395, Zfp593, Zfp687
3. Intracellular non-membrane-bounded organelle (18 genes): Arhgap6, Camk2n1, Cdk5rap2, Eml2, Fgd1, Grm3, Homer3, Iflt1d1, Myl6, Nudt16, Pdlim2, Rpl13, Rpl35a, Rpl37a, Rps16, Sntg2, Ssh3, Ttf1
4. Positive regulation of macromolecule metabolic process (8 genes): C1qtnf2, Jun, Pawr, Pax1, Pias3, Rarg, Six4, Ttf1
5. Postsynaptic density (3 genes): Camk2n1, Grm3, Homer3
6. Structural constituent of ribosome (4 genes): Rpl13, Rpl35a, Rpl37a, Rps16
7. Death (6 genes): Dapk2, Pawr, Pdcd7, Serpinb9b, Spr, Traf2
8. Regulation of cell proliferation (6 genes): Ada, Jun, Pawr, Rarg, Six5, Wnt2
Table 3.4. Characteristics of XY and XX male \( Ldlr^{-/-} \) mice infused with AngII.

<table>
<thead>
<tr>
<th></th>
<th>XY Sham</th>
<th>XY ORC</th>
<th>XX sham</th>
<th>XX ORC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>28.6 ± 1</td>
<td>23.9 ± 0.6 **</td>
<td>31.3 ± 1</td>
<td>28.3 ± 0.8 **</td>
</tr>
<tr>
<td><strong>Serum testosterone (ng/ml)</strong></td>
<td>1.77 ± 0.85</td>
<td>0.07 ± 0.02 **</td>
<td>1.53 ± 0.64</td>
<td>0.14 ± 0.02 **</td>
</tr>
<tr>
<td><strong>Plasma Renin Concentration (ng/ml)</strong></td>
<td>0.37 ± 0.16</td>
<td>0.32 ± 0.09</td>
<td>0.25 ± 0.04</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td><strong>Total serum cholesterol (mg/dl)</strong></td>
<td>1617 ± 242</td>
<td>1450 ± 320</td>
<td>1797 ± 140</td>
<td>1670 ± 115</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>132 ± 3</td>
<td>135 ± 4</td>
<td>119 ± 4 *</td>
<td>135 ± 3 **</td>
</tr>
<tr>
<td><strong>% Atherosclerotic lesion in aortic arch</strong></td>
<td>21 ± 2</td>
<td>20 ± 3</td>
<td>19 ± 3</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from \( n = 9-20 \) group.

*, \( P<0.05 \) compared to XY within surgical treatment

**, \( P<0.05 \) compared to sham within genotype.
A. AAA formation study:
Groups:
1. XY males (sham)
2. XX males (sham)
3. XY males (ORC)
4. XX males (ORC)

B. AAA Progression study:
Groups:
1. XY males (intact)
2. XX males (intact)

Figure 3.1. Graphic depiction of experimental design for studies focused on the formation (A) versus the progression (B) of AngII-induced AAAs.
**A**

2-way ANOVA

- **Total**: 1746
- **Filtered**: 16907
- **2-way ANOVA Significant**: 72688

**XX vs XY (450)**

**Or vs Sh (799)**

- 301
- 78
- 19
- 659
- 43
- 594
- Intrn (708)

**B**

**XX vs XY in Sham**

**C**

**p-value frequency histogram**

- **XX vs XY (FDR = 0.33)**
- **Or vs Sh (FDR = 0.15)**
- **Intrn (FDR = 0.24)**
- **Chance (FDR = 1)**

**D**

**Biological Pathway Analysis (DAVID)**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyamine biosynthetic process</td>
<td>4</td>
<td>3.38E-05</td>
</tr>
<tr>
<td>nuclear pore</td>
<td>6</td>
<td>9.10E-05</td>
</tr>
<tr>
<td>purine ribonucleotide binding</td>
<td>31</td>
<td>9.72E-05</td>
</tr>
<tr>
<td>tRNA binding</td>
<td>4</td>
<td>5.68E-04</td>
</tr>
<tr>
<td>nuclear lumen</td>
<td>16</td>
<td>0.003179</td>
</tr>
<tr>
<td>nucleolus</td>
<td>8</td>
<td>0.010325</td>
</tr>
<tr>
<td>transition metal ion binding</td>
<td>32</td>
<td>0.018911</td>
</tr>
<tr>
<td>protein amino acid N-linked glycosylation</td>
<td>3</td>
<td>0.019381</td>
</tr>
<tr>
<td>extracellular matrix part</td>
<td>7</td>
<td>2.19E-07</td>
</tr>
<tr>
<td>immune response</td>
<td>7</td>
<td>7.07E-04</td>
</tr>
<tr>
<td>acute inflammatory response</td>
<td>4</td>
<td>8.94E-04</td>
</tr>
<tr>
<td>skin development</td>
<td>3</td>
<td>0.002243</td>
</tr>
<tr>
<td>defense response</td>
<td>6</td>
<td>0.003676</td>
</tr>
<tr>
<td>melanosome</td>
<td>3</td>
<td>0.024775</td>
</tr>
</tbody>
</table>

**Upregulated in XY compared to XX**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ribosome</td>
<td>8</td>
<td>5.92E-05</td>
</tr>
<tr>
<td>DNA binding</td>
<td>20</td>
<td>0.005384</td>
</tr>
<tr>
<td>intracellular non-membrane-bounded organelle</td>
<td>18</td>
<td>1.46E-04</td>
</tr>
<tr>
<td>positive regulation of macromolecule metabolic process</td>
<td>8</td>
<td>0.006427</td>
</tr>
<tr>
<td>postsynaptic density</td>
<td>3</td>
<td>0.013833</td>
</tr>
<tr>
<td>structural constituent of ribosome</td>
<td>4</td>
<td>0.023393</td>
</tr>
<tr>
<td>death</td>
<td>6</td>
<td>0.035434</td>
</tr>
<tr>
<td>regulation of cell proliferation</td>
<td>6</td>
<td>0.040395</td>
</tr>
</tbody>
</table>
Figure 3.2. Sex chromosome complement, sex hormones, and an interaction between these factors influence gene expression patterns in abdominal aortas of XY and XX males. A, Total number of probe sets on arrays filtered to retain transcripts with reliable signal intensity. B, Volcano plot illustrating fold change in gene abundance (x-axis) and statistical significance (y-axis). Genes labelled in blue exhibited significant increase in XY compared to XX abdominal aortas; genes labeled in red exhibited significant increase in XX aortas. C, Frequency histogram of the number of genes (y-axis) found at different p-values (x-axis). Chance is illustrated by the dashed line. D, Biological pathway analysis (DAVID) of gene expression comparing pathways upregulated in XY or XX abdominal aortas. Data are mean ± SEM from n = 4-5 mice/genotype.
Figure 3.3. RT-PCR analysis of mRNA abundance of key genes in abdominal aortas from male XY and XX mice (sham, orchiectomized, ORC). A-C, mRNA abundance of genes (A, Xist; B, Arntl; C, Npas2) exhibiting increased expression in gene arrays from abdominal aortas of XY compared to XX male mice. D,E, mRNA abundance of genes (D, Cyp2e1; E, Kap) exhibiting increased expression in gene arrays from abdominal aortas of XY compared to XX male mice. Data are mean ± SEM from n = 4-5 mice/group. *, P<0.05 compared to XY within surgical group as defined by two-way ANOVA with genotype and surgery as between group factors. **, P<0.05 compared to sham-operated within genotype as defined by two-way ANOVA with surgery and genotype as between group factors and Holm-Sidak post hoc analysis.
Figure 3.4. Pulse Wave Velocity (PWV) from male XY and XX Ldlr-/- mice. Data are mean ± SEM from n = 6 mice/group. *, P<0.05 compared to XY as defined by Student’s t-test.
Figure 3.5. An XY sex chromosome complement mediates diffuse aortic vascular disease, while an XX sex chromosome complement is associated with discrete aneurysmal disease in abdominal aortas of male AngII-infused mice. A, Aortas from XY and XX sham-operated males infused with AngII. B, Aorta weight normalized to body weight. C, Area of the aortic arch. D, Incidence of thoracic aortic aneurysms (%) in mice of each genotype and group. Data are mean ± SEM from n = 10-13 mice/genotype/group who survived the 28 day protocol. *, P<0.05 compared to XY within sham-operated determined by two-way ANOVA with sham and genotype as between group factors with Holm-Sidak post hoc analysis (B,C) or by Fisher’s exact test (D). **, P<0.05 compared to sham-operated within genotype determined by two-way ANOVA with sham and genotype as between group factors with Holm-Sidak post hoc analysis.
Figure 3.6. An XX sex chromosome complement results in focal AAA pathology of increased size compared to XY males infused with AngII. A, Maximal AAA external diameters. Symbols represent individual mice with lines representing mean ± SEM. B, Internal diameters of abdominal aortas at selected intervals during infusion of AngII. Data are mean ± SEM of mice surviving the 28 day infusions. C, AAA incidence (percent above each bar). Numbers in boxes under each bar are mice with an AAA/total number of mice per group. D, % Aneurysm rupture (percent above each bar). *, P<0.05 compared to XY within sham-operated determined by two-way ANOVA with Holm-Sidak post hoc analysis (A,B) or by Fisher’s exact test (C,D). **, P<0.05 compared to sham-operated within genotype determined by two-way ANOVA with Holm-Sidak post hoc analysis (A,B) or by Fisher’s exact test (C,D).
Figure 3.7. Thoracic aortas from XY males, but not XX males, exhibit adventitial thickening in response to AngII infusions. A, Representative thoracic aorta tissue sections from XY and XX sham-operated and orchiectomized (ORC) males infused with AngII for 28 days. Boxes are areas illustrated at higher magnification. B, Quantification of the adventitial layer of thoracic aorta tissue sections from mice of each group. C, Quantification of the medial layer of thoracic aorta tissue sections from mice of each group. Data are mean ± from 6 tissue sections from n = 3 mice/group. *, P<0.05 compared to XY within treatment group determined by two-way ANOVA with surgery and genotype as between group factors, with Holm-Sidak post hoc analysis. **, P<0.05 compared to sham within genotype determined by two-way ANOVA with surgery and genotype as between group factors, with Holm-Sidak post hoc analysis. Scale bar represents 100 µm.
Figure 3.8. mRNA abundance of key genes implicated in aneurysm development in thoracic versus abdominal aortas from XY and XX male mice infused with either saline or AngII-infused (1 day). A, Angiotensin converting enzyme (ACE) mRNA abundance. B, Matrix metalloproteinase 2 mRNA abundance. C, Collagen 1α1 mRNA abundance. D, Thrombospondin 1 mRNA abundance. Data are mean ± SEM from n = 5 mice/group/sex. *, P<0.05 compared to thoracic within genotype determined by three-way ANOVA with region, treatment or genotype as between group factors and Holm-Sidak post hoc analysis. **, P<0.05 compared to XY within aortic region determined by three-way ANOVA with region, treatment or genotype as between group factors and Holm-Sidak post hoc analysis. $, compared to saline within region and genotype determined by three-way ANOVA with region, treatment or genotype as between group factors and Holm-Sidak post hoc analysis.
Figure 3.9. XX males exhibit dilated AAAs while XY males exhibit diffuse aortic disease with prolonged AngII infusions (3 months). A, Internal lumen diameters of suprarenal aorta at selected intervals of AngII infusions. B, Maximal AAA external diameters at study endpoint. Symbols represent individual mice with lines mean ± SEM. C, Aortas from mice in each group at study endpoint. D, Aorta weight normalized to body weight. E, Incidence of thoracic aortic aneurysms in mice of each genotype. Data are mean ± SEM from n = 5-10 mice/group. *, P<0.05 compared to XY determined by Student t-test (A-D) or Fisher’s exact test (E).
Figure 3.10. Ex vivo ultrasound analysis of AAAs from XY and XX male mice infused with AngII for 3 months. A, Wall volume. B, Abdominal aortic lumen volume. C, Representative 3-D reconstructions of AAAs of mice from each genotype. Data are mean ± n = 5 mice/group that survived the study protocol. *, P<0.05 compared to XY as defined by Student’s t-test.
CHAPTER IV. GENERAL DISCUSSION

4.1 Summary

The purpose of this study was to examine the role of sex chromosomes versus sex hormones in mediating the sexual dimorphism of AngII-induced abdominal aortic aneurysms (AAAs) in hypercholesterolemic female and male mice.

First, we sought to examine the role of sex chromosomes in the absence of male testosterone on gene expression patterns in abdominal aortas of hypercholesterolemic female (non-infused) mice. We found that 88 genes were expressed differentially in the abdominal aorta of XX versus XY females and the presence of an XY sex chromosome complement in female mice increased the expression of genes within inflammatory pathways in the abdominal region of the aorta. These results suggest that sex chromosome regulation of inflammatory gene expression patterns may play a role in development of AAAs.

Next, we investigated whether sex chromosomes regulate the development of AngII-induced AAAs. Based on previous research demonstrating that male AngII-infused mice have a higher AAA incidence than females (Henriques TA, Huang et al. 2004), we hypothesized that the presence of an XY sex chromosome complement in female mice promotes AngII-induced AAAs. Our data suggest that the presence of an XY sex chromosome complement in female mice does indeed increase the incidence and severity of AngII-induced AAAs.
Previous studies demonstrated that the removal of androgen by castrating male mice decreased the incidence and severity of AngII-induced AAAs and that the administration of dihydrotestosterone (DHT) increased AAA formation in female mice (Henriques TA, Zhang X et al. 2008). These results demonstrate that both sex hormones (e.g., testosterone) and sex chromosome complement (XY) contribute to sexual dimorphism of AngII-induced AAAs. To further examine if androgen exerts similar effects to promote AAAs in XX versus XY females, we androgenized the females of each genotype by giving them the same dose of testosterone. The hypothesis was that administration of androgen would augment the AAA formation both in XX and XY female mice. We administered DHT to adult hypercholesterolemic XX and XY female mice before and throughout AngII infusions. Although both XX and XY females exhibited a high incidence of AAAs, XY females displayed increased mortality due to an astonishingly high rate of aneurysm rupture.

Recent studies from our laboratory showed that a single dose of testosterone to hypercholesterolemic neonatal female mice increased their susceptibility to AngII-induced AAAs once they became adults (Zhang X et al. 2012). However, adult XX females that became susceptible to AAAs from early life testosterone exposures had low circulating testosterone levels, suggesting that testosterone exposures differentially influence AAA development depending on sex chromosome complement. We therefore examined effects of neonatal testosterone administration to XX and XY female mice on adult susceptibility to AngII-induced AAAs. We administered testosterone to one-day old XX and XY
females and infused them with AngII once they became adults to examine their susceptibility to AAAs. Although there was no difference between XX and XY females exposed to testosterone as neonates in AAA incidence, more than half of the XY females died due to aneurysm rupture.

Next, we investigated the role of sex chromosomes in aortic aneurysm pathology in the presence or absence of endogenous male testosterone by using XX and XY hypercholesterolemic male mice. First, we examined the role of sex chromosome complement on gene expression patterns of abdominal aortas from male (non-infused) mice. We found 450 genes that were expressed differentially in abdominal aortas of XY versus XX males. Analysis of gene pathways determined these differential genes were involved in the extracellular matrix, acute inflammatory response, cell proliferation regulation pathways, and the immune response pathway. Further, we found that after 28 days of AngII infusion, an XY sex chromosome complement in male mice induced diffuse pathology along the entire length of the aorta while an XX sex chromosome complement promoted restricted focal aneurysm pathology in the abdominal region of the aorta. Moreover, XY males, but not XX males, infused with AngII manifest thickening in the adventitial layer of the thoracic region of the aortas. Additionally, we infused XY and XX males with saline or AngII for one day and examined mRNA abundance of key genes involved in aortic pathology from the abdominal and thoracic regions of the aorta. There were regional differences in mRNA abundance of aneurysm-related genes between genotypes that were potentiated with AngII. Finally, following prolonged infusion of AngII, males with
an XY sex chromosome complement had diffuse AAAs with thickened aortic walls, while males with an XX sex chromosome complement had discrete focal AAA with a thinned wall aorta.

Overall, these studies support a role for sex chromosome complement in the sexual dimorphism of AngII-induced AAA in hypercholesterolemic mice. An XY sex chromosome complement promotes diffuse and severe aneurysm pathology following AngII infusion while an XX sex chromosome complement contributes to focal aneurysm pathology located to the suprarenal abdominal aorta. Moreover, sex chromosome effects were associated with differences in regional and AngII-induced abundance of mRNAs involved in AAA development and progression.

4.2 Importance of studying the role of sex chromosomes in disease

There are many diseases that are sexually dimorphic. The National Institutes of Health has recently stressed the significance of biological sex in disease prevention and development, urging scientific researchers to include both sexes, males and females, in the experimental design. Most studies examining sex differences focus on the role of gonadal hormones. However, gonadal hormones do not fully vindicate all sex differences.

Sex chromosome complement, in addition to gonadal hormones, may be a primary mediator behind the sexual dimorphism of various chronic diseases. To demonstrate the importance of sex chromosome effects, during the early stages of embryo development, male and female pre-gonadal embryos are different in size; XY embryos are larger and developmentally more advanced (Burgoyne PS,
Thornhill AR et al. 1995; Burgoyne PS 1993). Additionally, even before gonadal differentiation, the genes of male and female blastocysts are expressed differentially (Bermejo-Alvarez P, Rizos D, Rath D et al. 2010). Moreover, the phenomenon of X chromosome inactivation only partially occurs during the preimplantation period (Bermejo-Alvarez P, Rizos D, Rath D et al. 2010), resulting in upregulation of more than 80% of the X-linked genes in female embryos (Bermejo-Alvarez P, Rizos D, Rath D et al. 2010).

In adult females, the presence of two X chromosomes can create gene-dosage differences when compared to males with only one X chromosome. These gene-dosage differences is balanced by the process of X-inactivation, by silencing the transcription of one of two X chromosomes in all cells (Okamoto I et al. 2011; Penny GD et al. 1996; Payer B and Lee JT, 2008). However, some of the genes on the X chromosome could escape the X-inactivation (Carrel L et al. 1999; Yang F et al. 2010). Although the Y chromosome is small and has less genes than other chromosomes, genes within the male specific region of the Y chromosomes have still been associated with several cardiovascular diseases (Bloomer LD, Nelson CP et al. 2013).

4.3 AAA is a sexually dimorphic disease.

Male sex is one of the strongest risk factors for AAAs, with men exhibiting four to ten fold higher prevalence than women (Weiss, Mahlmann et al. 2014; Wilmink, Quick et al. 1998; Peumeekers, Hoes et al. 1994; Morris, Hubbard et al. 1994). Multiple studies from our laboratory and others have demonstrated that male testosterone is a primary contributor to profound differences in susceptibility
to AAAs between adult male versus female mice and rats (Henriques TA, Huang et al. 2004; Henriques TA, Zhang X et al. 2008; Zhang X et al. 2012; Zhang X et al. 2015; Cho BS Upchurch GR et al. 2009; Huang CK, Chang C et al. 2015; Davis JP, Upchurch GR et al. 2016). However, it is unclear if testosterone by itself is sufficient to explain the sexual dimorphism of AAAs. For example, our laboratory has shown that a single dose of testosterone to neonatal female mice was sufficient to make adult females susceptible to AngII-induced AAAs without the need to see the testosterone for the whole life, which is needed in males (Zhang X et al. 2012).

Additionally, multiple studies have shown that sex chromosomes may contribute to sexual dimorphism of certain phenotypes and diseases (Robinson DP, Huber SA et al. 2011; Ji H, Zheng W et al. 2010; Gioiosa L, Chen X et al. 2008; Chen X, Wang L et al. 2015; Li J, Chen X et al. 2014). Recent studies have demonstrated that blood pressure responses to AngII infusion in mice influenced by sex chromosome complement (Ji H, Zheng W et al. 2010). Moreover, Turner’s Syndrome, or monosomy X in females, is associated with a 100-fold increase in risk of aortic dissection and rupture (Wong, Zacharin et al. 2014; Bondy CA 2008). Furthermore, several genes of the renin-angiotensin-system are on the X chromosome, which suggest a possibility of gene dosage effects for genes that escape X-inactivation.
4.4 Mechanisms of sex chromosome effects on AAA

4.4.1 Role of sex chromosome complement on aortic gene expression

Our data indicate that the presence of an XY sex chromosome complement in female mice without AngII infusion enhanced the expression of genes involved in inflammatory pathways in the abdominal aorta, and this was associated with increased oxidative stress and matrix metalloproteinase (MMP) activity when the female mice were infused with AngII. Moreover, XY females infused with AngII exhibited higher AAA incidence and the pathology was diffuse and more severe. Additionally, administration of testosterone to XY females, in order to mimic the male environment, resulted in an immense rate of aneurysmal rupture.

Also as a part of this study, we decided to use intact male mice with an XY or XX sex chromosome complement (meaning phenotypically male mice exposed to testosterone throughout life). Without AngII infusion, there were 450 genes in the abdominal aorta that were influenced by sex chromosome complement between XX and XY males. Analysis of gene array data using DAVID pathway analysis revealed that these genes are involved in pathways that might relate to the sex differences between males and females in susceptibility to AAAs. Similar to our findings, other studies using the four core genotype mouse model have shown that sex chromosomes influence gene expression in other tissues like heart and the central nervous system (Du S, Itoh N et al. 2014; Li J, Chen X et al. 2014). Interestingly, differences in gene expression due to sex chromosome complement in the abdominal aorta were different in males.
compared to females (e.g., not the same genes and/or pathways were found in XX females versus XX males). This may be explained either due to the difference of sex hormones between males and females or due to the presence of the Sry gene in males as previous studies have demonstrated that this gene regulates blood pressure and gene expression of several components of the renin angiotensin system in male rats (Ely D, Boehme S et al. 2011; Araujo FC, Milsted A et al. 2015).

Of interest, from the gene microarray data, two clock genes, Arntl and Npas2, were highly up-regulated in abdominal aortas from XX males compared to XY males. Additionally, these genes were down regulated with castration, a pattern that matches the AAA phenotype. Arntl, also known as Bmal1, is a transcription factor that is part of the clock machinery. Arntl binds to E-box promoters of Npas2, which regulates downstream clock genes like Pers and Cry (Kristin Eckel-Mahan and Paolo Sassone-Corsi 2013). It is unclear if these genes have protective or detrimental effects on AngII-induced AAAs. A recent study suggested that Bmal1 in smooth muscle cells regulates the circadian rhythm of blood pressure (Xie Z, Gong MC et al. 2015). Several studies have shown that aortic smooth muscle cells undergo oscillation in the expression of clock genes (McNamara P, Seo SB et al. 2001; Nonaka H, Emoto N et al. 2001). In our gene expression studies, aortas harvested for the microarray were collected from the mice in the morning between 9:30 am and 11:30 am and it is not known if the expression of the clock genes are also different at other time points between XX and XY mice. Further studies need to be conducted to confirm this. Currently,
there is a dearth of publications about clock genes and aneurysms; however, several studies have reported that aneurysm rupture and dissection demonstrate a circadian pattern (Manfredini R, Portaluppi F et al. 1999; Manfredini R, Boari B et al. 2004; Killeen S, Neary P et al. 2007; Vitale J, Manfredini R et al. 2015). As AAA ruptures mostly peak in the morning, it has been suggested that the morning high sympathetic activity could lead to increases in aortic shear stress and rupture (Manfredini R, Boari B et al. 2004). By coincidence, even the king of Great Britain, George II, who died in 1760 due to the first identified aortic rupture, was reported to have died in the morning around 6 am (Leonard JC 1979).

4.4.2 Role of sex chromosomes on inflammation

AAA is a vascular disease that is characterized with inflamation. In our study we found that XY females infused with AngII have a higher incidence and more severe AAAs than XX females, and this was associated with increased inflammatory responses. Specifically, XY females had increased gene expression of interleukin-1β (IL-1β) and toll like receptor 8 in the abdominal aorta as well as increased plasma concentrations of IL-1β when infused with AngII. Also, abdominal aortas of XY females following AngII infusion had higher MMP-2 activity and oxidative stress. Similarly, XY males infused with AngII had diffuse and more severe aneurysm pathology along the length of the aorta with higher AngII-induced MMP2 mRNA abundance in thoracic aorta than XX males, which may indicate that an XY sex chromosome complement promotes aortic pathology by enhancing inflammatory responses to AngII.
4.4.3 Role of sex chromosomes on the location of aortic pathology

Many studies, human and experimental, illustrated that males have a higher prevalence of vascular diseases localized at different regions of the aorta compared to females (Roman, Weinsaft et al. 2017; Olsson, Granath et al. 2006; Lederle et al. 2001; Weiss et al. 2014; Wilmink et al. 1998; Peumeekers et al. 1994; Morris et al. 1994; Henriques TA et al. 2004; Henriques TA et al. 2008). According to a study of more than 14,000 cases of thoracic aortic diseases in Europe, the incidence of thoracic aortic aneurysm was doubled in men compared to women (Olsson, Granath et al. 2006). Our results indicate that sex chromosome complement has an important role in determining the location of the AngII-induced pathology in the aorta. As mice with an XY sex chromosome complement when infused with AngII developed diffuse pathology along the aorta, while mice with an XX sex chromosome complement developed discrete focal aneurysm only in the abdominal region of the aorta. In males, this phenotype was mediated by both sex hormones and sex chromosome complement as there was no difference between castrated XY and XX males, this is in line with previous results from our laboratory demonstrating that testosterone is needed in normal XY males to maintain high susceptibility to AngII-induced AAAs (Henriques TA et al. 2004; Henriques TA et al. 2008; Zhang X et al. 2012). It is unclear if these results relate to humans as the majority of data from humans are derived from measurements of the intralumenal diameter in the infrarenal region of the aorta. Our data raise the concern and urge the need to perform multiple measurements along the length of the aorta.
4.4.4 Role of sex chromosomes on aortic wall thickness

Interestingly, thoracic aortas from XY males infused with AngII have a thicker adventitial layer than XX males. Also, XY males infused with AngII had higher systolic blood pressures than XX males, which was not different between sham and castrated groups in the XY males. Since the thoracic aortic aneurysm incidence was reduced with castration, this suggests that blood pressure is not the primary cause behind the development of diffuse aortic vascular pathologies in XY males.

Differences in aortic wall thickness between XX and XY males may suggest that males and females have differing levels of aortic components (e.g., muscle versus adventitia) within the aortic wall, and to our knowledge there is no study that has made these types of comparisons. In fact, women with an AAA have a poorer prognosis and AAAs rupture at smaller sizes (Grootenboer et al. 2009; Skibba et al. 2015; Sweeting et al. 2012; Thompson et al. 2013), which could be due to a thin aortic wall because of lower expression of elastin and collagen.

In this study, we found that XY males have higher expression of key genes involved in aneurysm formation in the thoracic aorta while XX males have higher expression in the abdominal aorta. It has been reported that different locations of the aorta have different gene expression patterns (Pfaltzgraff ER, Shelton EL et al. 2014). Furthermore, recently, it has been shown that different regions of the aorta are populated by smooth muscle cells derived from diverse embryonic origins (Sawada H, Daugherty A et al. 2017). This divergence could be the cause
behind variance in the physiological and pathological reactions of the aorta (Thieszen, Rosenquist et al. 1996; Majesky et al. 2007). In our study the patterns of gene expression in abdominal versus thoracic aortas was enhanced with only one day of AngII infusion. These differences in gene expression might contribute to the diffuse versus focal vascular pathology observed between the two genotypes, XX and XY.

Although women have lower risk of aortic aneurysms, paradoxically, aneurysms progress more rapidly and tend to rupture at smaller sizes in women (Cheung et al. 2017; Lo et al. 2014; Thompson et al. 2013; Sweeting et al. 2012; Larsson et al. 2011; Mofidi et al. 2007; Solberg et al. 2005). In this study, we found that XY males developed AAAs with thick aortic walls that continued to expand with prolonged infusion of AngII. On the contrary, XX males developed dilated AAAs that were restricted to the abdominal region of the aorta, and that exhibited profound lumen dilation and thin walls with prolonged AngII infusion. These results are in agreement with previous findings from our laboratory, which showed that normal XY males with existing AngII-induced AAAs remodel to thin walled AAAs after removal of endogenous androgen by castration (Zhang X et al. 2015). This observed difference in wall thickness of AAAs between the two sex chromosome complements, with thin walled AAAs in females may contribute to increased aneurysm rupture (Cheung et al. 2017; Lo et al. 2014; Thompson et al. 2013; Sweeting et al. 2012; Larsson et al. 2011; Mofidi et al. 2007; Solberg et al. 2005).
4.5 The interaction of sex chromosomes and sex hormones

4.5.1 The interaction of sex chromosomes and sex hormones in experimental animals

Our laboratory previously demonstrated that androgen is a primary mediator of AngII-induced AAAs (Henriques TA et al. 2004; Henriques TA et al. 2008; Zhang X et al. 2012; Zhang X et al. 2015). In this study we found that sex chromosome complement has a prominent role to promote the formation and the severity of AngII-induced AAAs as well. In females, unlike males, this role was independent of gonadal hormones. XY females, either sham or ovariectomized, had high AAA incidence, similar to the incidence of normal XY male mice. However, male mice appear to continuously require the sex hormone, testosterone, to maintain susceptibility to AngII-induced AAA, as AAA incidence goes down to the level of females with orchiectomy (Henriques TA et al. 2004; Henriques TA et al. 2008, Alsiraj Y et al. 2017).

The high AAA incidence of XY females could be due to a modest level of testosterone, although this level was at least ten fold lower than that of normal XY males. However, this level of testosterone in females, coupled with the presence of the XY sex chromosome complement, might have a synergistic effect and be detrimental enough to make the “weak” female aorta susceptible to AAA, as it has been reported that female aortas have a lower capacity to absorb force than male aortas (Ninomiya OH, Tavares Monteiro JA et al. 2015). Additionally, our laboratory demonstrated previously that a single dose of
testosterone to normal XX females when they are one-day old was sufficient to permanently promote the formation of AAAs later in life (Zhang X et al. 2012).

Since testosterone is known to promote the susceptibility of AngII-induced AAAs and the testosterone concentration was modestly higher in XY females, we androgenized the two genotypes by exposing XX and XY female mice to the same dose of androgen. This was done either by a single dose of testosterone injected to one-day old neonates or by continuous diffusion of DHT for 6 weeks through an implanted DHT tablet under the skin of adult XX and XY female mice. Our results demonstrate that despite the high incidence of AAAs in both XX and XY females, the administration of testosterone to female mice with an XY sex chromosome complement increased the rate of aneurysm rupture to the highest level that we have ever seen (73%).

This high level of AAA rupture in XY females is even higher than that of XY males infused with AngII (Daugherty A et al. 2000; Henriques TA et al. 2004; Henriques TA et al. 2008). This may be due to the dose of testosterone or the type of androgen used in these experiments, as DHT is the most potent androgen and has an augmented effect compared to testosterone. Alternatively, it may be due to the anatomical nature of the female aorta, as it has been reported that there are sex differences in the size and the vessel biomechanical properties between males and females. Male aortas are reported to be stronger than female aortas, and have a higher failure load and failure tension than female aortas (Ninomiya OH, Tavares Monteiro JA et al. 2015). Also, it has been shown that female aortas have higher peak wall rupture risk than male aortas (Larsson
E et al. 2011). Furthermore, women with AAAs are known to have poor prognosis compared to males as their AAAs grow faster and rupture at a smaller size than male AAAs (Grootenboer et al. 2009; Skibba et al. 2015; Sweeting et al. 2012; Thompson et al. 2013). Thus, we conclude that putting male factors, namely XY sex chromosome complement and testosterone, into females increased AAA susceptibility and severity resulting in the AAAs of females to rupture at a very high rate.

Conversely, in males, the XY sex chromosome complement required testosterone to enhance the susceptibility to AngII-induced AAAs. We found that XY males have diffuse aneurysm pathology in both thoracic and abdominal aortas compared to XX males that have discrete focal abdominal aneurysms. Notably, both XY and XX males lose their susceptibility to AngII-induced pathology when castrated, which is in agreement with previous results (Henriques TA et al. 2004; Henriques TA et al. 2008). The requirement of testosterone in males to induce AngII-induced AAAs could be due to the presence of the Sry gene in males, or due to early surges of testosterone levels during fetal and neonatal life (Motelica-Heino I, Castanier M et al. 1988; Klinga K, Bek E et al. 1978; Tomlinson C, Macintyre H et al. 2004). Early life exposures to testosterone in males may have an organizational influence to imprint the requirement for continuous testosterone exposures to maintain the “activational effect” of testosterone in adults.
4.5.2 The interaction of sex chromosomes and sex hormones in humans

In contrast to experimental AAA studies in animals, in which it is well established that testosterone plays an important role in AAA formation and progression, in humans this association is not so clear, with only one study reporting that low testosterone levels were associated with AAA (Yeap et al. 2010). This group reported that serum total testosterone levels in men with an AAA were 14.5 nmol/ liter compared to 15.5 nmol/ liter in men with no AAA, and concluded that testosterone levels are negatively associated with the development of AAA (Yeap BB, Hyde Z et al. 2010). In this study, there is only a 1 nmol/ liter difference in total testosterone level between non AAA and AAA patients which does not necessarily indicate that low testosterone levels can cause AAAs. In fact, the normal testosterone level in men ranges from 9-38 nmol/ liter (Fischbach FT, Dunning MB III, 2009 Manual of Laboratory and Diagnostic Tests, 8th ed). This means that testosterone levels within this range are sufficient to maintain the features of male sex, including the beneficial and non-beneficial ones, and thus men with an AAA reported by Yeap et al most likely had normal serum testosterone levels. Additionally, the reported testosterone levels in these human studies were measured in men with an AAA. Typically, AAAs are detected by ultrasound screening which is recommended for men at or above the age of 65 (Mussa FF 2015). Thus, it is unclear if serum testosterone levels were influenced before the formation of an AAA. Since the testosterone level before or at the time of AAA formation is unknown, the relationship between testosterone levels and AAA development are not clear.
Possibly, the decline in testosterone levels of aging male AAA patients suggests that sex chromosomes, either alone or in combination with sex hormones, may contribute to the development and progression of AAAs. Previous data from our laboratory show that a single dose of testosterone to female neonates was enough to enhance the AAA susceptibility in adult female mice (Zhang X et al. 2012). In contrast to men, women tend to have an imbalance in their estrogen to testosterone ratio after menopause (Dai W, Li Y et al. 2012; Rohr UD 2002), which could explain, besides aging, why AAA incidences in women increase (e.g., higher testosterone to estrogen ratio) with age with more propensity to aneurysm rupture (Grootenboer et al. 2009; Skibba et al. 2015; Sweeting et al. 2012; Thompson et al. 2013).

4.6 Importance of including sex chromosomes in genome wide association studies (GWAS)

Previous studies have suggested a genetic contribution to AAA development, as people with a family history of AAA have a tenfold increased chance of developing the disease (Johansen K and Koepsell T 1986). In an Australian ultrasound screening study of siblings, AAA patients reported that 43% of their siblings were males and were also diagnosed with having an AAA (Frydman G, Walker PJ et al. 2003). Similarly, a small study in Sweden that screened 108 siblings of AAA patients found that 16 of them with an AAA were brothers (van der Lugt A, Kranendonk SE et al. 1992). Additionally, in a multi-national study looking at familial aggregation of AAAs in families that have at least two individuals with AAAs, 77% of the affected cases were males and most
were brothers (Kuivaniemi H, Shibamura H et al. 2003). The high rate of disease transmission between the male siblings suggests a Y chromosome influence in transmission of the disease. However, our study is the first to provide direct evidence of the role of sex chromosomes in the sexual dimorphism of AAA.

Sex chromosomes are usually neglected and not included in genome wide association studies (GWAS) and these studies tend to focus only on autosomal genes (Wise AL, Gyi L, Manolio TA 2013). This is usually due to technical difficulties including statistical test selection, random X-inactivation, attribution, and the phenomenon of escape from X-inactivation. The omission of sex chromosomes from GWAS could overlook multiple genes on the sex chromosomes that might contribute to the development and progression of several diseases. Results from our studies, which support a role for sex chromosomes in disease development, provide an impetus to include the sex chromosomes in future GWAS analyses.

Taken together, our data indicate that sex chromosome complement regulates aortic gene expression, responses to AngII and disease development, with an XY sex chromosome complement increasing the incidence and the rupture of diffuse aortic aneurysm pathology that progressed with thickened aortic walls while an XX sex chromosome complement was associated with focal AAA development that progressed with profound luminal dilation and thin aortic walls. Effects of an XY sex chromosome complement were exacerbated by testosterone administration, meaning there is a complex interaction between sex
chromosomes and gonadal hormones in regulating vascular diseases and specifically AAAs.

4.7 Clinical Significance

It is important to understand the interplay between gonadal hormones and sex chromosome genes, specifically pertaining to their role in the regulation of the cardiovascular system. Our studies show that genes regulated by the sex chromosome complement may affect vascular biology to enhance inflammation and augment the response to AngII. Searching for the genes that are regulated genes of sex chromosomes and which affect AAA development may uncover sex-specific treatments for this and other AngII-related diseases.

The transgender population is on the rise, but studies on adverse effects from the chronic use of sex hormone therapy on the opposite sex chromosome complement are scarce. Of these, recent data suggest increased risk of cardiovascular diseases in transgender women (Elamin et al. 2010). However, the effect of chronic use of sex hormone therapy on the cardiovascular system in transgender people has not been thoroughly investigated. In addition, the use of sex hormone therapy for other reasons has also increased, specifically androgen use to improve sex drive and to improve the performance ability in elderly and in young males and females.

Our results suggest that sex chromosome complement influences aortic disease development, with an XY sex chromosome complement having a large influence in promoting aneurysm development and severity. These effects were exacerbated when coupled with testosterone. Information from these studies
should help in the future to develop sex specific therapies against aortic aneurysms and other vascular diseases.

4.8 Future directions

Results from these studies demonstrate that the presence of an XY sex chromosome complement in female mice was sufficient to increase the incidence and the rupture rate of diffuse aortic aneurysm pathology, and that AAA formation and severity were augmented by androgen treatment. Further, it was found that XY males develop diffuse aortic disease along the entire length of the aorta while XX males develop discrete focal aneurysm pathology in the abdominal aorta. Moreover, XX and XY male mice exhibited regional differences in aortic gene expression patterns that were also regulated by sex and AngII. However, we did not address whether these effects were due to the presence of the Y chromosome or due to the absence of the second X chromosome. Therefore, a novel study should look to define whether genes on the Y chromosome or gene dosage effects from the second X chromosome influence the development and progression of AngII-induced vascular diseases. I suggest use of the trisomy mouse model (Chen X, Williams-Burris SM et al. 2013), which allows addition of a Y chromosome to an XX female, or an X chromosome to an XY male. The trisomy model can be generated by mating a normal XY male to an XY- female (this female is generated from the 4 core genotype mouse model). According to Chen X et al. 2013, about 30% of the female offspring produced from this breeding scheme would have XXY- sex chromosome complement. XXY- females would then be mated to XY-(Sry+) males (this male is generated
from the 4 core genotype mouse model) to produce offspring that have the following genotypes, XX, XY, XXY, XYY phenotypic females that have ovaries and XX, XY, XXY, XYY phenotypic males that have testes. By comparing XX females to XXY females, or by comparing XY males to XXY males against AAA will enable delineation of whether the presence of the Y chromosome or the absence of the second X chromosome influences development and/or progression of AngII-induced vascular diseases. If results from these studies show that the X chromosome has a protective role against AngII-induced AAAs, these findings then can be confirmed by the use of the XY* mouse model, a model for Turner’s syndrome or monosomy X, a disease associated with high risk of aortic dissection in females with one X chromosome (Wong, Zacharin et al. 2014; Bondy CA 2008). This model produces mice with one X chromosome or two X chromosomes, each in the absence or presence of a Y chromosome, which will result in XX and XO females that have ovaries and XY and XXY males that have testes (Burgoyne PS, Mahadevaiah SK et al. 1998). By studying the AAA in XX versus XO females, this study will confirm the protective role of X chromosome genes in aortic aneurysm, which could possibly open the door for studies that search for the genes influenced by X chromosome genes that make the monosomy X females predisposed to aortic pathologies, which can be tested by looking for genes that are upregulated or downregulated by AngII in the presence or absence of the second X chromosome. The XY* mouse model can be created by mating XY* males to normal XX females. The XY* male is a male mouse has a Y chromosome that has been re-arranged structurally. This Y
chromosome has an aberrant pseudoautosomal region (PAR) that recombines alternately with the PAR of the X chromosome during meiosis (Burgoyne PS, Mahadevaiah SK et al. 1998).

Furthermore, after identifying the sex chromosome (X or Y) that has the protective or the detrimental role in aortic aneurysm, we should move to define the role of sex chromosomes (the detrimental or the protective ones) in aneurysm progression as AAA is usually detected after it has been established and progressed, due to ultrasound screening recommendations for men aged 65 to 75 years with a history of smoking (Hirsch AT, Haskal ZJ, Hertzer NR et al. 2006). The progression studies could be done by using the same mouse models with prolonged AngII infusion and monitor the AAA progression by ultrasound. The ultimate goals for these future studies are finding therapies that blunt the progression of AAAs. Results from our studies indicate that sex chromosomes determine differences in genes within the abdominal aorta that are either located on the X chromosome and are known to escape X-inactivation, or are located on the Y chromosome. These genes are either transcription or translation factors, making them not easy to use as targetable therapy because they control a wide range of genes on the autosomes. However, the autosomal genes that are regulated by genes of the sex chromosomes could be used as potential therapeutic targets. Furthermore, if we can identify genes that are influenced by sex chromosomes, sex hormones, or by AngII in pivotal cell types, this will help in developing a sex-specific therapy that could provide efficacy against the
formation and/or progression of AngII-induced AAAs in male versus female mice which could be applied to humans to blunt AAA progression and/or rupture.

Another interesting future study should look at the role of clock genes, specifically, Arntl and Npas2 in AngII-induced AAA. It would be of interest to look to the expression of these genes in XX versus XY mice in different segments of the aorta that are collected at different times of day, and based on the information from this experiment, studies could be initiated that disrupt the circadian rhythm to see its role in AngII-induced AAAs. Alternatively, we can delete these genes from aortic smooth muscle cells and see their influence on AngII-induced AAAs in males versus females.

4.9 Limitations of the study

First, we were unable to distinguish if the observed phenotype is due to the presence of the Y chromosome or due to the absence of the second X chromosome.

Second, XX males from the four core genotype model are sterile and have small testes due to the absence of the Y chromosome which has genes that are responsible for sperm production. Nonetheless, the levels of serum testosterone were not statistically different between XX and XY males.

Third, XY females are transiently fertile and have a modestly higher level of serum testosterone than XX females, but this level was at least ten fold lower than the testosterone levels of normal males. Additionally, we normalized the
testosterone level between XX and XY females by giving both genotypes the same dose of androgen.

4.10 Concluding remarks

Findings of this dissertation demonstrate that sex chromosome complement influences aortic gene expression as well as the development and the severity of aortic aneurysmal diseases that form in response to AngII infusion in hypercholesterolemic male and female mice. An XY sex chromosome complement in phenotypic females was sufficient to promote the formation of AngII-induced AAAs. Mimicking the male environment, testosterone exposure to XY female mice increased the severity of AAAs by increasing the rupture rate to up to 73%. Male mice with an XY sex chromosome complement developed diffuse aortic aneurysms along the entire length of the aorta while XX males were found to develop discrete focal aneurysm pathology restricted to the suprarenal region of the aorta and both genotypes (XY and XX males) lost the susceptibility to the disease after castration (Figure 4.1). These results demonstrate, for the first time, that the sex chromosome complement has an immense impact on AAA susceptibility, and that these effects are augmented by testosterone.
Figure 4.1 Illustration of the role of sex chromosomes on AngII-induced AAA susceptibility in mice. On the left, the large black arrow means a higher rate of rupture in female XY mice compared to female XX mice. Basal aortic pictures were adapted from Servier Medical Art.

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168


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