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The Relationship Between *FAM5C* SNP (rs10920501) Variability, Metabolic Syndrome, and Inflammation, in Women with Coronary Heart Disease

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by

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ABSTRACT

The leading cause of death among women is coronary heart disease (CHD), a multifactorial disease with polygenic heritability estimated at 50%. Polymorphisms in the family with sequence similarity 5, member C' (*FAM5C)* gene have been associated with myocardial infarction (MI), and one single-nucleotide polymorphism (SNP) has partially accounted for linkage in an acute coronary syndrome subset. The linkage peak on *FAM5C* corresponds directly with a quantitative trait locus for the inflammatory biomarker monocyte chemoattractant protein 1, as well as a linkage peak to metabolic syndrome (MetS). Metabolic syndrome increases the risk of developing CHD, and MI has been positively associated with elevated inflammatory biomarkers. This study was designed as a descriptive pilot gene association study. The purpose was to investigate the variability of the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD. It also examined the association between the variability of the *FAM5C* SNP (rs10920501), MetS, inflammatory markers, and the association with early onset CHD in the presence or absence of MI. A subset of 91 women was derived from an earlier study of women randomized to either a

gender-tailored or traditional cardiac rehabilitation program. The results indicated the T allele of *FAM5C* SNP rs10920501 has a strong protective effect in women with a history of MI. Women with a history of MI and the heterozygous (AT) genotype had a mean age of onset of CHD at 62 years, compared to the homozygous wild type (AA) with a mean age of onset at 55 years, (F (3, 34) = 5.00, $p < .01$). No women in this study with the homozygous variant (TT) had an MI, further demonstrating the protective effective of the T allele. The genotype of *FAM5C* SNP rs10920501 explains approximately seven percent of the variability of age of onset of CHD in women who have had an MI, while holding body mass index (BMI) and smoking history constant. There was no significant relationship between *FAM5C* SNP (rs109320501) and MetS or any inflammatory biomarkers in this sample. In conclusion, *FAM5C* remains a gene of interest in a complex disease process.

Chapter One

Introduction

It is estimated that 16 million American adults have CHD (Lloyd-Jones et al., 2009). In 2004, one in 30 women died from breast cancer. In comparison, in the same year, one in six women died from CHD, making it the leading cause of death for women (Rosamond et al., 2008). Coronary heart disease is a complex disease with numerous risk factors. Many family and twin studies have examined the importance of an environmental and genetic association. Adding to the complexity, the interaction between the risk factors frequently is not additive, but exponential. This is further complicated by the polygenic heritability of CHD, which can exceed 50% (Lusis, Mar, & Pajukanta, 2004; Hardy & Singleton, 2009). Large genome-wide association scans for various risk factors have failed to identify a single locus which accounts for the large population variation, suggesting multi-gene involvement (Lusis, Mar, & Pajukanta).

The chromosomal region 1q25-31 has been identified as a susceptibility region for MI in two independent populations (Connelly et al., 2008; Hauser et al., 2004). The Genomewide Scan for Early-Onset Coronary Artery Disease Study (GENECARD), which included 422 families, identified a 40 megabase region of linkage to CHD on chromosome 1q25-31 (Hauser et al., 2004). *A priori* stratification by sibling pairs with acute coronary syndrome (ACS), revealed a well defined linkage peak (n = 228 families, logarithm base 10 of odds (LOD) =

2.17, at peak microsatellite marker D1S2589/D1S518) (Hauser et al., 2004). This linkage peak correlates exactly with the linkage peak for the inflammatory biomarker monocyte chemoattractant protein 1 (MCP-1) (LOD = 4.27), at peak microsatellite marker D1S1589 identified by the Framingham Heart Study (Dupuis et al., 2005) and with the linkage peak (LOD = 2.59, D1S1589/D1S518) for MetS in the Insulin Resistance Atherosclerosis Study Family Study (Mercuro et al.) (Langefeld et al., 2004). A SNP found within the first intron of the tumor necrosis factor superfamily 4 gene (*TNFSF4*) (rs3850641), has also been found to reside within the chromosome 1q linkage peak and has been strongly associated with MetS and MI in two independent populations (Wang et al., 2005). This genetic overlap gives strong support to a CHD susceptibility gene in this region.

A strong association with MI was identified with SNPs in the 3' end of the FAM5C gene, which was confirmed within an independent CAD case-control dataset (Connelly et al., 2008). One SNP (rs10920501) in this region displayed linkage and association in both samples. Gene expression of rs10920501 was correlated with the *FAM5C* genotype in 83 human aortic tissue samples, of which 58 were unique. Aorta samples were collected from heart transplant donors. The inheritance of the T allele of rs10920501 was significantly associated with the reduction in the expression of *FAM5C* in the aorta. Proliferating aortic smooth muscle cells and human endothelial cells were cultured and reverse transcriptase PCR identified *FAM5C* expression in both cell types. It was shown that *FAM5C* transcript levels decrease with increasing passage of aortic smooth muscle cell,

suggesting that the level of gene expression may play a role in proliferation and senescence of this cell type (Connelly et al.). Higher expression of the *FAM5C* in the cells that make up the artery may be related to an increased risk of MI.

FAM5C is localized to the mitochondria, which has been associated with cell proliferation and apoptosis (Connelly et al., 2008). The location of *FAM5C* within the mitochondria was the basis for the hypothesis that *FAM5C* played a role in regulating cell proliferation and migration, and ultimately the pathophysiology of the vulnerable atherosclerotic plaque (Connelly et al., 2008).

The relationship between *FAM5C*, inflammatory biomarkers, and MetS remains to be clarified and is the basis for the current study (see the Conceptual Model in Figure 1). Understanding the role of *FAM5C* may aid in the development of new biomarkers to assess disease risk and response to treatment, identify susceptibility genes for at-risk individuals, and help guide pharmacogenetic studies to improve interventions to moderate risk. Coronary heart disease is a complex disease caused by a combination of genetic effects and environmental influences. Coronary heart disease is preventable, but will require the elucidation of the genetic predisposition to address all possible targets for risk reduction.

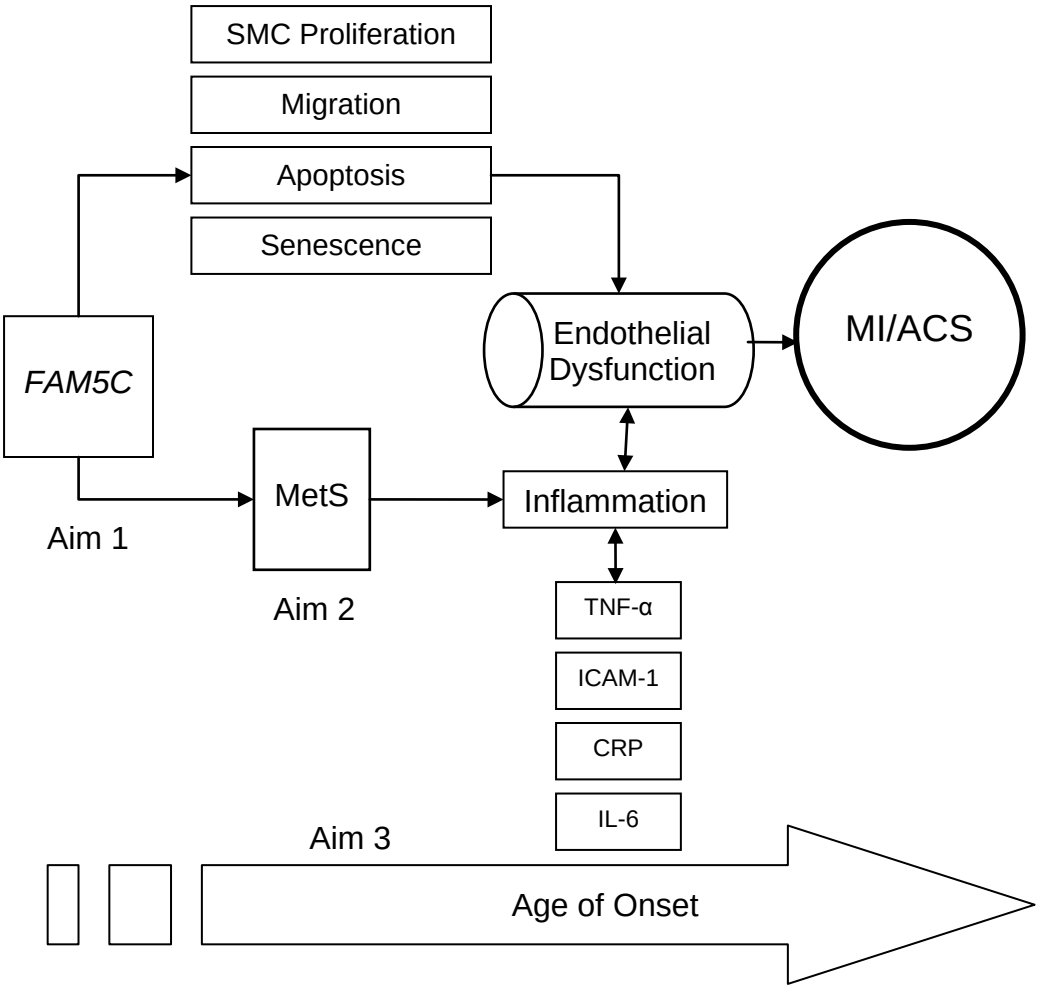

Figure 1. Conceptual Model to Evaluate *FAM5C* SNP rs10920501

Note: Abbreviations; MI: myocardial infarction, ACS: acute coronary syndrome, MetS: Metabolic Syndrome, hs-CRP: high-sensitivity C-reactive protein, ICAM-1: intercellular adhesion molecule-1, IL-6: interleukin-6, TNF-α: tumor necrosis factor-α.

Statement of the Problem

More women than men die from CHD and their outcomes are poorer than men. Alternative targets for risk reduction may improve CHD outcomes in women. Investigating genetic interactions expressed through inflammatory biomarkers will help explain their role in endovascular physiology and provide a target for risk stratification and treatment.

Statement of the Purpose

The purpose of the current study was to explore the variability of the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD, to assess the relationship between *FAM5C* SNP rs10920501, MetS, and inflammatory markers, and to evaluate if *FAM5C* SNP rs10920501 variability was associated with early onset CHD.

Rationale for Single SNP Study

Due to the exploratory nature of the study, a single SNP was selected based on work by Connelly et al. (2008). The *FAM5C* gene has been proposed as a novel MI susceptibility gene by Connelly et al. A single SNP (rs10920501) within the gene has partially accounted for linkage in the GENECARD ACS subset and was associated with MI in CATHGEN sample. Gene expression was also correlated with this SNP in human aortic cells (Connelly et al., 2008). Therefore, the SNP (rs10920501) is considered to be a strong predictor of the *FAM5C* gene and has been selected for the current study.

This SNP has a minor allele frequency of .20 in the European (Utah residents with ancestry from northern and western Europe) population, .32 in the Han Chinese population, .23 in Japanese population, and .172 in the Sub-Saharan African population (International HapMap Project, 2008).

Specific Aims

Aim One

Describe the distribution of the genotype in the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD.

Rationale One. Coronary heart disease is the leading cause of death and disability in women in the United States. Coronary heart disease is a disease with complex gene-environment interactions, in which heritability can exceed 50%. Numerous genome-wide studies have been performed focusing on the most severe phenotype of CHD. The *FAM5C* has been linked by genome-wide and independent case control analysis to MI and ACS (Connelly et al., 2008; Hauser et al., 2004).

In previous trials of *FAM5C*, males were over-represented, comprising 1533 of 2186 subjects or > 70% of the population (Connelly et al., 2008). Very little sex-specific genetic or biomarker data is available for review (Novack, Cutlip, Jotkowitz, Lieberman, & Porath, 2008). Historically, the literature has reported higher mortality rates and poorer outcomes for women with CHD, which warrants further investigation.

Aim Two

Investigate the associations between the variability in the genotype in the *FAM5C* SNP (rs10920501), MetS, and inflammatory biomarkers among women with documented CHD.

Rationale Two. There is overlap of genetic screens in independent data sets, which have validated linkage on chromosome 1 to inflammatory

biomarkers, MetS and MI (Dupuis et al., 2005; Langefeld et al., 2004; Hauser et al., 2004; Wang et al., 2005). The association between *FAM5C* rs10920501, inflammatory biomarkers, and MetS within this region remains to be clarified.

A female cohort is appropriate, in as much as MetS is a contributor to inflammation and is found more frequently in elderly female populations (Feinberg, Schwartz, & Behar, 2008; Zeller et al., 2005; Levantesi et al., 2005, Marroquin et al., 2004). An all female cohort will not permit examination of gender differences, but given the under representation of females in previous studies, the use of an all female sample in the proposed work is seen as an asset.

Aim Three

Evaluate the association between *FAM5C* SNP (rs10920501) and early onset CHD in women.

Rationale Three. The parent study has reported that in a cohort of women enrolled in cardiac rehabilitation (CR), younger women were more likely than older women to have been diagnosed with a MI (Beckie, Fletcher, Beckstead, Schocken, & Evans, 2008). They also had poorer physiologic profiles. Specifically, younger women had higher BMIs, larger waist circumference, increased body fat, and lower high-density lipoprotein cholesterol (HDL-C) levels compared to older women. Younger women were more likely to smoke and express a high degree of psychological distress (Beckie et al., 2008). Additionally, Connelly et al. (2008) has shown that the odds ratio and *p* value improved in when stratifying the sample by age (T allele, $p = 0.009$, $OR = 0.539$).

Innovation

The future of genetics is based on exploring the polygenic basis of complex disease and the gene-environment interaction. The novelty of this study is that it explores a polymorphism of *FAM5C* in a cohort of women, while taking into account MetS and inflammatory biomarkers. The results of this study promote the integration of genetics into the nursing process by providing additional research data on the influence of genetics on complex diseases. This study highlights the importance of gene-gene and gene-gender interactions. Adding to the current knowledge base, this study should facilitate nurses' ability to provide education on the hereditary risk of developing disease, counsel about genetic testing, and manage disease based on genetic testing, all of which ultimately translate into patient care. The study may also impact pharmacogenetics of CHD, as the knowledge of the genetic variants and mechanisms of CHD evolve. As the knowledge base of the genetics of CHD expands, all areas of prevention, detection, diagnosis, and treatment will be positively affected.

Chapter Two

Review of Literature

Introduction

The pathogenesis of CHD has multiple origins. In this chapter, a systematic review of the literature is presented to address the genetic and environmental factors that interact to influence the development and progression of CHD. The chapter begins with a description of the magnitude of CHD in women and the importance of prevention. That is followed by a review of the role of MetS and its link to inflammation. Inflammatory biomarkers, which were included in the parent study, are also explored. A review of landmark genome-wide association studies (GWAS) relative to CHD and an appraisal of the novel *FAM5C* gene are then presented. Lastly, gaps in current knowledge are reviewed.

Coronary Heart Disease and Women

Worldwide, CHD is the single largest cause of death among women (Lloyd-Jones et al., 2009). Nearly half a million women die each year of cardiovascular disease (CVD) (Lloyd-Jones et al., 2009). Women have higher rates of recurrent MI and age-adjusted mortality after the first MI than men (Chandra, 1998; Rogers, 2008). Women 40 years and older have a 43% increased risk of death within the first five years following the first MI, compared with men who have a 33% increased risk (Lloyd-Jones et al., 2009). The human

and economic toll of CHD has reached epic proportions. The estimated cost of CHD in 2010 is approximately $177 billon dollars (Lloyd-Jones et al., 2010). Fortunately, the vast majority of CHD in women is preventable (Mosca et al., 2007).

Differences in lifestyle fail to explain the disparity in the incidence of CHD between genders. Traditional risk factors have been shown to have a high heritability (Lusis, Mar, & Pajukanta, 2004), some of which show gender biases (McCarthy, 2007). For example, there is considerable evidence for sex differences in the heritability of body mass and diabetes. Body mass has been shown to be 50% heritable in women, compared to 35% in men (Pilia et al., 2006). This suggests a possible gender-gene interaction (McCarthy; Pilia, 2006; Schousboe, 2004; Kuusisto, 2008).

The biological etiology of the gender-based differences in the development of CHD may have many sources. The first is the sex-linked chromosomes. Most X-linked traits are more common in men, since a woman would have to inherit mutations in both copies of her X-chromosome. Second, the rate of cumulative risk factors is different between genders. In a study by Leander et al. (2007), smoking was found to interact with family history among women in the development of MI, but not among men. There is growing evidence that genes interact with traditional risk factors in the development of CHD. Third, the effect of sex hormones, including estrogen and testosterone, differ between the genders (McCarthy, 2007). This is seen abruptly at the onset of menopause, when women lose the protective benefits of sex hormones and are at greater risk

for metabolic abnormalities (McCarthy). Menopause leads to a more atherogenic lipid profile (Derby et al., 2009), central adiposity (Toth, Tchernof, Sites, & Poehlman, 2000), and an overall increase in MetS corrected for age (Poehlman, 2002; Levantesi et al., 2005; Zeller et al., 2005). Lastly, there are anatomical and physiological differences that must be considered, including differences in fat distribution and insulin resistance (Mittendorfer, 2005), cardiovascular function (Peterson, 2008), and gene expression (Northoff et al., 2008; Rinn & Snyder, 2005).

In 2007, the American Heart Association published guidelines on CHD prevention in women (Mosca et al., 2007). The guidelines recognized the role of novel risk factors and screening technologies in CHD prevention. The expert panel recommended focused research on different time periods during a woman's life, such as adolescence, pregnancy, and menopause. The guidelines suggested that events unique to a particular life stage might be additive to traditional cardiac risk factors. The panel also recognized that some women are at increased risk for future events, due to known disease or multiple risk factors. The panel suggested that these women should be targeted for aggressive preventative therapy, including control of hypertension, obesity, and stress. The AHA guidelines acknowledged that additional research on the role of genetics in risk factor stratification and the responsiveness to interventions was important (Mosca).

Metabolic Syndrome

Metabolic syndrome has been defined as the presence of three or more of the following risk factors: waist circumference > 88 cm in women and > 102 cm in men, triglycerides ≥ 150 mg/dL, HDL-C < 50 mg/dL in women and < 40 mg/dL in men, blood pressure ≥ 130/85 mmHg, and fasting glucose ≥ 100 mg/dL or the diagnosis of diabetes (Grundy et al., 2006). Estimates suggest that MetS is present in 25% of all adults in the United States and in 40% of those > 60 years old (Adult Treatment Panel III, 2001). Studies have shown that the MetS population is more likely to be female and older (Feinberg, Schwartz, & Behar, 2008; Zeller et al., 2005; Levantesi et al., 2005). An analysis of the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering trial found patients meeting the criteria for MetS had a 19% incidence of death, nonfatal MI, cardiac arrest, or recurrent unstable myocardial ischemia during the 16-week trial, as opposed to 14% in patients without MetS (Schwartz, Olsson, Szarek, & Sasiela, 2005). Metabolic syndrome is associated with dysregulated adipose tissue, which is attributable to inflammation with increased levels of several cytokines (Gustafson, Hammarstedt, Andersson, & Smith, 2007). This increases the risk for cardiovascular atherosclerosis and markedly worsens long-term prognosis (Espinola-Klein et al., 2007).

There is emerging evidence linking obesity to the proinflammatory state of MetS. Adipose tissue is now recognized as an endocrine organ (Kershaw & Flier, 2004). Adipose tissue produces numerous cytokines that contribute to the inflammatory status. Leptin has been associated with total obesity (Friedman &

Halaas, 1998), whereas others such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), plasminogen activator-1 (PAI-1), and adiponectin are more closely linked to abdominal obesity (Friedman, 1998; Fain, 2004; Alessi, 1997; Lihn, 2004; Pitsavos, 2008; Rexrode, 2003). Abdominal adiposity is the strongest predictor of an elevated c-reactive protein (CRP) in older women, whereas total percentage of body fat is the strongest predictor in men (Valentine, Vieira, Woods, & Evans, 2009). In adipose tissue, macrophages are the primary source of TNF-α (Suganami, Nishida, & Ogawa, 2005). Macrophages are the primary mediators of the immune response and represent up to 40% of the infiltrated cells in adipose tissue (Weisberg et al., 2003). Also, TNF-α has been shown to impair insulin signaling in adipocytes and skeletal muscle (Ruan & Lodish, 2004). It has been hypothesized that these cytokines, or “adipokines”, are the link between obesity and other components of MetS (Hivert, 2008; You, 2008).

Metabolic syndrome has been characterized by a state of chronic inflammation. It is accepted that adipose tissue functions as an endocrine organ, which further stimulates cytokine production (Hivert et al., 2008). In a recent study of postmenopausal women with coronary heart disease, 74% of the women met Adult Treatment Panel (ATP) III criteria for MetS (Brown et al., 2008). As obesity and sedentary lifestyles are reaching epic proportions, understanding the interaction of MetS and inflammatory biomarkers has become imperative to improve the early identification, treatment, and prevention of CHD (Hivert et al., 2008).

Inflammatory Biomarkers

Metabolic syndrome has emerged as one of the main contributors to atherosclerosis and has been positively associated with elevated inflammatory markers. Elevated levels of circulating inflammatory markers have been associated with cardiovascular risk factors and all stages of the atherosclerotic process, including endothelial dysfunction, plaque development, plaque instability, and ultimately acute coronary syndromes (Bermudez, Rifai, Buring, Manson, & Ridker, 2002; Athyros, Kakafika, Karagiannis, & Mikhailidis, 2008).

An inflammatory marker that has received considerable attention is CRP. In several prospective studies, CRP has been shown to be an independent risk factor for CHD (Ridker, 2008; Ridker, 2005; Ray, 2005; Lowe, 2006) and MetS (Pischon, 2008; Alizadeh Dehnavi, 2008; Mattsson, 2008). Data has confirmed that CRP is a strong independent predictor of mortality among ACS patients who were treated with early revascularization (Mueller et al., 2002; Zairis et al., 2002). It has been shown that CRP is related to the size of myocardial infarct (De Sutter et al., 2001). In addition, intravascular ultrasound has shown in patients having an acute MI, CPR levels correlate with the presence of plaque rupture (Sano et al., 2003). Also, increased temperature at unstable plaques, measured with an invasive thermogenic catheter, has been related to elevated CRP levels (Stefanadis et al., 2000). In stable post-MI patients, an elevated CRP level is associated with a significantly higher risk for recurrent nonfatal MI or fatal coronary events (75% higher in the highest quartile versus the lowest quartile of high-sensitivity C-reactive protein (hs-CRP)) (Zebrack et al., 2002; Kosuge et al.,

2008). Based on this data, it has been suggested that CRP levels are predictive of short and long term cardiac events.

C-reactive protein is produced primarily in the liver in response to IL-6 secretion. Interleukin-6 is a key regulator of the inflammatory response and is produced in the adipose tissue. C-reactive protein production occurs with a synergistic enhancement of interleukin one (IL-1) and TNF-α (Athyros, Kakafika, Karagiannis, & Mikhailidis, 2008). C-reactive protein can also be found in the endothelium of atherosclerotic plaques, smooth muscle cells, macrophages, and adipocytes (Athyros et al.). C-reactive protein stimulates the expression of adhesion cells, tissue factor, MCP-1, and plasminogen activator one (PAI-1). C-reactive protein activates leukocytes and the complement system, and reduces the bioavailability of nitric oxide. Data suggest that CRP itself potentially attracts monocytes and facilitates the uptake of low-density lipoprotein cholesterol (LDL-C) by macrophages (Zwaka, Hombach, & Torzewski, 2001). In smooth muscle cells, CRP up-regulates the Ang type 1 receptor and stimulates smooth muscle cell proliferation and reactive oxygen species production (Athyros et al.). C-reactive protein and related biomarkers exert a pathogenic role in the atherosclerotic process. This may be reflective of an ongoing cytokine-mediated acute phase response initiated by the innate immune system.

Cytokines are regulatory proteins that have been implicated in the process of inflammation. Among the main cytokines are IL-1, IL-6, interleukin-10, TNF-α, and MCP-1 (Introna, Colotta, Sozzani, Dejana, & Mantovani, 1994; Mantovani, Bussolino, & Dejana, 1992; Mantovani, Bussolino, & Introna, 1997). Interleukin-1

and IL-6 regulate the production of reactant proteins, including CRP. Interleukin-6 may increase plaque instability by driving the expression of matrix metalloproteinases, TNF-α, and MCP-1 (Schieffer et al., 2000).

Elevated IL-6 levels in healthy males correlated with an increased risk of future MI independent of CRP (Ridker, 2000). In the Fragmin and/or Early Revascularization During Instability in Coronary Artery Disease trial, elevated IL-6 was associated with a higher six and twelve month mortality, independent of troponin and CRP levels (Lindmark, Diderholm, Wallentin, & Siegbahn, 2001). In a recent study, IL-6 levels have been shown to be predictive of the onset of an acute ST-segment elevation MI and have prognostic value for future cardiac mortality within two years of the event (Tan, 2008). This data suggests that IL-6 may identify patients with more severe events. However, the application of IL-6 as a routine biomarker for inflammation has been limited by large variation of IL-6 levels within and between individuals and few confirmatory studies (Picciotto et al., 2008; Cava, Gonzalez, Pascual, Navajo, & Gonzalez-Buitrago, 2000).

Tumor necrosis factor-α is a major pro-inflammatory cytokine that has been shown to be significantly elevated in the setting of an acute MI compared to controls (Goswami, Rajappa, Mallika, Shukla, & Kumar, 2009). Tumor necrosis factor-α has also been implicated in myocardial dysfunction and remodeling after coronary events (Nian, Lee, Khaper, & Liu, 2004). In the Cholesterol and Recurrent Events study, recurrent coronary events after MI were associated with higher TNF-α levels compared with controls (Ridker, 2000). In as much as TNF-α has been elevated in acute events independent of other risk factors, it has been

suggested that this inflammatory biomarker should be a target for treatment (Ridker).

Cellular adhesion molecules (CAMs) play a pivotal role in the interactions between leukocytes, platelets, and the vascular endothelium. In cardiovascular disorders, CAMs are involved in atherogenesis and atherosclerotic plaque progression (Haverslag, Pasterkamp, & Hoefer, 2008). Mulvihill, Foley, Murphy, Crean, and Walsh (2000) measured CAMs in patients with ACS at presentation and then after 3, 6, and 12 months. Cellular adhesion molecule levels increased within 10 hours of chest pain onset and remained elevated for 6 months, suggesting the inflammatory stimulus remains sustained (Mulvihill et al.). In patients with unstable angina undergoing coronary stenting, intercellular adhesion molecule (ICAM) and CRP levels were prognostic of those patients at higher risk of having a cardiac event (Doo et al., 2005). However, it was suggested that CRP may play a direct role in promoting the inflammatory component of atherosclerosis by inducing the expression of ICAM-1 (Doo et al., 2005). Recent reports from the Nurses' Health Study and Health Professionals Follow-up Study found, compared to controls, subjects with MetS had a significantly increased relative risk of CHD. After adjustment for MetS, CRP, and ICAM-1 remained an independent predictor of CHD in men (Pischon et al., 2008).

Inflammation has been implicated as a central contributor of the initiation and progression of CHD. Inflammatory biomarkers may have prognostic value in identifying those at risk for future events. Beckie, Beckstead and Groer (2009)

recently published data from the parent study demonstrating that a moderate-intensity exercise program positively affected inflammatory biomarkers and some components of MetS. However, data remains conflicting regarding which biomarker or combination of biomarkers are the most suitable for diagnosis or prognosis of CHD. Through additional research, select markers of inflammation may emerge as targets to direct interventions to minimize CHD risk.

Genetic Susceptibility of Coronary Heart Disease

Advances in Molecular Genetics

Completion of the Human Genome Project, the ability to perform large scale genetic assays, and the study of gene expression have all assisted in advancing the discovery of susceptibility genes for complex diseases. The purpose of the Human Genome Project was to identify all the genes in human DNA and to determine the sequence of the three billion base pairs that make up human DNA (National Institutes of Health (NIH), 2009). Due to rapid technological advancements, the project was completed ahead of schedule in 2003. However, only twenty to twenty-five thousand, or 1.5-2%, of identified genes code for proteins (Human Genome Project Information, 2008). Proteins carry out most of the biochemical activities in a cell and determine how the organism functions. For example, proteins are vital to the immune response, metabolism, and even behavior (Human Genome Project Information, 2008). Little is known about the other 98% of genes (NIH, 2009).

The creation of HapMap in 2005 was a step towards bridging the gap between knowing where the genes are located and understanding the genetic

basis of human health and disease. HapMap is a catalog of human variation or haploytpes in the human genome (International HapMap Project, 2009). HapMap data have accelerated the search for genes associated with disease and been instrumental in finding the genes related to conditions such as obesity and macular degeneration (Hamsten & Eriksson, 2008).

The development of high-throughput genotyping assays can be considered the accelerant for an explosion of genetic studies and gene identification. In the early 1980's, few genes had been identified. Since that time, there has been a steady annual increase, with over 1800 genes identified in 2005 alone (Human Genome Project Information, 2008).

Initially, genetic studies were aimed at establishing a linkage between genes. This was established with microsatellites, which are short tandem repeat segments (Damani & Topol, 2007). Genetic linkage occurs when specific loci or alleles are inherited together. Linkage studies can be challenging to perform because large numbers of families with multiple affected members must be recruited. Every subject's DNA is evaluated with 400 microsatellite markers spaced every 10 cM (or 1 million base pairs) across the genome. The genomes are then evaluated for a "linkage peak" or extensive allele sharing among family members or affected subjects as compared to controls (Damani & Topol, 2007). A significant peak is described as a logarithm of odds ratio score (LOD score) > 3.0, which is a logarithm (base 10) of the likelihood ratio (Hartl & Jones, 2005). A LOD score > 3.0 suggests that the gene is near or within the microsatellite marker (Hartl & Jones, 2005). Identifying genes that cause a specific disease

within a distance of 1 million base pairs has remained difficult (Damani & Topol, 2007). In general, linkage studies will have a low resolution as they are reporting large chromosomal regions with a few million base pairs (Hartl & Jones, 2005). The strongest linkage signals will come from recessive and highly penetrant diseases and it can take years to perform fine-mapping to determine the exact gene that is involved (Hartl & Jones).

Currently, most genotyping is performed with genome-wide association studies. These studies utilize SNPs to establish association with disease (Human Genome Project Information, 2008). Single-nucleotide polymorphisms are differences in a single base of DNA. SNPs account for greater than 90% of human variation, and occur every 100 to 300 bases. Various SNPs can be inherited together, which are called haplotypes. A “tagging SNP” is used to identify a haplotype block or gene region that has a different distribution between the disease group and the control. Each region can then be studied with additional fine mapping, to determine which variants in the gene contribute to the disease (Human Genome Project Information, 2008). A standard microchip array can evaluate 500,000 to 1 million SNPs, which has significantly lowered the cost of gene identification (Hamsten & Eriksson, 2008). The cost of DNA chip technology has dropped by three orders of magnitude over the last 15 years (NIH, 2009). Collectively, these advances have made GWASs for complex diseases possible.

Genome-wide studies are a comprehensive, unbiased approach for identifying genes involved in disease. Early genome-wide studies focused on the

MI phenotype, which is the most severe manifestation of CHD (Topol, Smith, Plow, & Wang, 2006; Connelly et al., 2008). Since that time, many well-powered genome-wide studies have identified loci that are associated with the increased the risk of CHD and MI (Kathiresan, 2009). Several of the most relevant studies are reported here (see Table 1).

Linkage Studies

Eight of the most pivotal genome-wide linkage studies focusing on CHD and MI are reviewed here (Table 1). Each study has implicated a different susceptibility gene for CHD. Only one locus was replicated from one study to the next, each occurring within a population of premature CHD and MI. Linkage was observed on chromosome 2 (2p12) for both premature CHD and MI (LOD 2.70) in the British Heart Foundation Study (Samani et al., 2005), and it corresponded directly with a linkage peak previously identified by Wang et al. (2004) (LOD 3.82), and is very close to the region identified by Pajukanta et al. (2000) (2q21.1-22). This strengthens the evidence that a locus on chromosome 2 is associated with CHD risk (Samani et al., 2005). There has been vast speculation on why there has not been more uniform replication between genome-wide studies. Possibilities include genetic and phenotypic heterogeneity, population stratification, low statistical power and the use of different software programs, differences in the ethnic backgrounds of subjects, and variation of study designs (Heng, 2008).

Table 1

Summary of Landmark Genome-wide Linkage Studies for Coronary Heart Disease

Author	Year	Study population	Number of Affected Subjects	Mean Age	Primary locus	Phenotype
Pajukanata	2000	Finnish	364	< 55M < 65F	2q21.2	Premature CHD & MI
Francke	2001	Mauritian	240	53	16p13	CHD & MI
Broeckel	2002	European	513	52	14	CHD & MI
Harrap	2002	Australian	122	62	2q36	CHD & MI
Helgadottir	2004	Icelandic	713	-	13q12	MI
Wang	2004	American	1,613	44	1p34	Premature CHD & MI
Hauser	2004	American	985	< 51M < 56F	3q13	ACS
Samani	2005	British	4,175 CHD 1,776 MI	53 52	2p11	Premature CHD & MI

Note: Abbreviations; CHD: coronary heart disease, MI: myocardial infarction, ACS: acute coronary syndrome, M: male, F: female.

Helgadottir et al. (2004) carried out a genome-wide study on 296 Icelandic families, including 713 individuals with MI. The study identified two haplotypes spanning the gene arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*) under the linkage peak at 13q12-13 that are associated with MI and leukotriene B4 production (peak LOD 2.86 in females with MI). The first haplotype encodes for 5-lipoxygenase activating protein (*FLAP*). *FLAP* is an important regulator of leukotriene inflammatory mediators. Males had the strongest association with the

at-risk haplotype, with a nearly two-fold increase in risk for MI and stroke (Helgadottir et al., 2004). The same haplotype was later confirmed in a Scottish cohort to be significantly associated with stroke in males (Lohmussaar et al., 2005), and with MI in an unpublished Cleveland study (Topol, Smith, Plow, & Wang, 2006). From the original genome-wide scan, Helgadottir et al. performed additional fine mapping techniques to determine that the five to seven SNP marker haplotype, leukotriene A4 hydrolase (*LTA4H*), accounted for a linkage peak at 12q22 (Helgadottir et al., 2006). Helgadottir et al. also found an ancestry-specific risk of MI, whereas African Americans had three-fold greater risk for MI than subjects with European American ancestry (Helgadottir et al.). This study set three important precedents. First, it demonstrated that two different genes in the same inflammatory pathway could be associated with disease via a single genome-wide scan. Second, it demonstrated that *FLAP* has been implicated in the pathogenesis of atherosclerosis and is an important biomarker for CHD, and lastly ancestry-specific risk for MI was demonstrated for a particular gene.

Wang et al. (2004) conducted genome-wide studies seeking an association with premature CHD. Wang et al. yielded one significant susceptibility loci (*LRP8*) for MI on chromosome 1p34-36 (LOD = 11.68, $p < 10^{-12}$) in 1,613 white pedigrees. A population-based association replication study identified a single SNP (rs5174) with the *LRP8* gene as a significant prognostic indicator of an increased risk of premature CHD and MI ($p = .036$ for CHD and $p = .048$ for MI after Bonferroni correction) (Shen et al., 2007). The precise role *LRP8* plays in the development of an MI remains under investigation. However, it

has been hypothesized that LDL binds to *LRP8*, which activates the phosphorylation of *LRP8*. This increases activation of the p38 mitogen-activated protein kinase, leading to platelet aggregation, thrombosis, and potentially MI (Shen et al.; Hauser et al., 2004).

Of the genome-wide linkage studies that identified susceptibility loci for CHD and MI, three suggested linkage to type two diabetes mellitus (T2DM) (Broeckel et al., 2002; Francke et al., 2001; Harrap et al., 2002). Broeckel et al. performed a GWAS on 513 families to identify regions linked to MI and related risk factors known to be under genetic control. Subjects were identified by screening 93,500 charts at seven cardiac centers throughout Germany. Families were included if at least one sibling had a documented MI, angiographic CHD, or previous coronary revascularization. Genotyping was performed at a spacing of 10 cM, with additional fine mapping at chromosome 14. Linkage with MI was identified at chromosome 14 (LOD = 3.9, $p < .05$). Additional risk factor analysis was carried out to characterize the locus. Serum concentrations of lipoprotein (a) showed linkage at the known apolipoprotein (a) locus on chromosome 6 (LOD = 26.99) and a new locus on chromosome 1 (LOD = 3.8). There was suggestion of linkage for T2DM on chromosome 2 (LOD = 2.96). There was no overlap with the susceptibility locus for MI (Broeckel et al., 2002).

The first evidence of a susceptibility loci for CHD, hypertension (HTN), and T2DM in the context of MetS was presented by Francke et al. in 2001. They conducted a 10cM genome scan with 403 microsatellite markers in 99 families. Families were recruited through a proband with early onset CHD (< 52 years of

age), and criteria for family inclusion included MI or T2DM. In a second step, additional fine mapping was performed on three chromosomal regions where linkage was found. Data suggested a linkage with chromosome 16p13-pter, which partially overlapped a peak for HTN. At the same locus, there was nominal linkage with T2DM in 35 large families of Indian origin. Region 8q23 showed modest linkage between HTN and T2DM (LOD = 2.55, p = .00058), and a previously identified region on 3q27 was replicated revealing linkage between CHD and MI (LOD = 2.13, p = .0009) (Francke et al., 2001).

Harrap et al. (2002) performed a genome-wide study on sibling pairs to identify a susceptibility locus for ACS. Approximately 6000 admissions to cardiac units from 8 Melbourne hospitals were screened for inclusion. Probands were enrolled if they had a documented MI before the age of 70 and had an affected sibling with the same history. Genotyping was completed at a 10 cM resolution. One locus on chromosome 2q36-37.3 showed linkage with ACS (LOD = 2.63, p < .00001), which was replicated with independent markers (LOD = 2.64). This region overlaps with genes encoding for the insulin receptor substrate-1 (IRS1), the HDL binding protein (HDLPB), and the locus *NIDDMI* which has been linked to T2DM. However, only one sibling pair in the present study had T2DM, which did not explain the results (Harrap et al.). This study highlighted the importance of the comparison of pathways and processes implicated in different diseases. It is suggestive that risk factors are genetically related. Further study will reveal new insight into the underlying shared pathogenic mechanisms of complex common disease.

Genome-wide Association Studies

Genome-wide association studies, or GWAS, are a relatively new phenomena based on the rapid progression of technology. In the past three years, GWAS assaying hundreds of thousands of SNPs have identified hundreds of associations of common genetic variants, many of which are associated with CHD (Human Genome Project Information, 2008; Heng, 2008). There are several advantages of GWAS over linkage studies, including the ability to genotype large number of SNPs (Human Genome Project Information, 2008). Studies have progressed from assaying fewer than 100,000 SNPs to more than 1 million, and sample sizes have increased dramatically. An additional advantage is that you do not need to have multiple affected family members for genome-wide association studies. You can use large case-control samples of unrelated subjects for better statistical power. Also, genome-wide association provides a high level of quality control and replication across experiments (Human Genome Project Information, 2008). A summary of landmark GWASs are presented here (see Table 2).

Table 2

Summary of Landmark Genome-wide Association Studies for Coronary Heart Disease

Author	Year	Study Population	N	Mean Age	Primary Loci	Phenotype
Ozaki	2002	Japanese	94	64	6p21	MI
Shiffmann	2008	American	4522	73	*LPA*- 6q26 *VAMP8*- 2p12-p11.2 *TAS2R50*- 12p13.2 *KIF6*- 6p21.2	MI
Chen	2009	American	332	59	9p21	CHD
Large Scale	2009	European	22,755	58	9p21 1p13.3 2q36.3 10q11.21	CHD & MI
Kathiresan	2009	International	25,538	46	21q22 6p24 2q33	Early onset MI
Erdmann	2009	Northern European	19,407	60	3q23.3	Premature CHD
Trégouët	2009	Northern European	8,999	58	6q26-27	CHD

Note: Abbreviations; MI: myocardial infarction, CHD: coronary heart disease.

Ozaki et al. (2002) published one of the first CHD GWAS in 2002. Ninety-four Japanese individuals with MI were initially genotyped through genome-wide association, suggesting the gene *LTA* on chromosome 6p21 was associated with MI. An additional 1,133 subjects were screened for gene variation in the critical region. Two SNPs in the *LTA* region were shown to affect expression level and biologic function of several adhesion-molecules, including ICAM-1, in vascular

smooth muscle in a coronary artery. The genotyping covered less than 100,000 SNPs, which is well below our current standard of at least 250,000 tagging SNPs. However, the study reinforces the pro-inflammatory role in CHD (see Table 2) (Ozaki et al., 2002).

Shiffmann et al. (2008) evaluated 4,522 subjects in the Cardiovascular Health Study for the presence of 74 SNPs. The SNPS were identified in antecedent studies to be associated with MI. Four of the SNPs from genes *LPA, VAMP8, TAS2R50,* and *KIF6* were also associated with MI in this study (Shiffman et al., 2008).

Chromosome 9p21 has been the most widely studied risk variant for CHD and MI, and is the first confirmed independent genetic risk factor for CHD by GWAS (Samani, 2009; Karvanen, 2009; Meng, 2008; Schunkert, 2008; Samani, 2007; Chen, 2009; Anderson, 2008; Paynter, 2009). However, there has been no evidence of gene-environmental interaction in that 9p21 is not associated with traditional risk factors. 9p21 was assessed in one of the largest CHD genetic experiments to date (Large Scale Association Analysis of Novel Genetic Loci for Coronary Artery Disease, 2009). A total of 22,755 participants were recruited from nine European studies. The loci on 9p21 was once again shown to be associated with an increased risk of CHD, as were loci on 1p13.3, 1q41, and 10q-11.21 (Large Scale Association Analysis of Novel Genetic Loci for Coronary Artery Disease).

The Myocardial Genetics Consortium was also a large trial, with a sample size of 19,492 (Kathiresan et al., 2009). They evaluated the association of SNPs

and copy number variants (CNV) with early-onset MI. Copy number variants are differences in DNA structure when compared across genomes (Kathiresan et al., 2009). Copy number variants may account for variation which has not been captured by standard GWAS arrays (Manolio et al., 2009). Variation from CNVs, like SNPs, can arise from a combination of rare or common alleles. Interestingly, highly penetrant CNVs discovered to date have been rather large, effecting many genes (Manolio et al.). Nine SNPs meet genome wide significance. Six had been previously identified: 9p21, 1p13, 10q11, 1q41, 19p13, and 1p32, and three were newly discovered SNPs: 21q22, 6p24, 2q33 (Kathiresan et al.). This study found significant results from genetic mapping of the SNP analysis with a calculated risk of early onset MI of 2.8%; however, it did not find an association with myocardial risk and CNV (Kathiresan et al.).

Erdmann et al. (2009) utilized the Myocardial Genetics Consortium database to evaluate SNPs that had been previously dismissed due to modest effects or low allele frequencies. A three-stage analysis began with genotyping 1,222 German individuals and 1,298 case-controls. The study findings were ultimately replicated in over 25,000 subjects utilizing three databases. Subjects were included if they had premature CHD and a positive family history of CHD. Genotyping was carried out with an Affyemetrix Genome Wide Human SNP Array. The study identified one new CHD risk locus on 3q22.3 ($p = 7.44 \times 10^{-13}$, *OR* = 1.15, 95% *CI* = 1.11-1.19) and a suggestive association with a locus on 12q24.31 ($p = 4.81 \times 10^{-7}$, *OR* = 1.08, 95% *CI* = 1.05 -1.11). The locus on 12q24.31 is clustered near the coding region of *HN1FA* and *C12of43*. These

regions are implicated in T2DM in the young, as well as with increased CRP and LDL levels (Erdmann et al.).

A similar study design, which ultimately included 6,100 cases and 5,600 controls, was utilized to identify gene cluster at the 6q26-q27 locus (Tregouet et al., 2009). This locus encompasses the *SLC22A3-LPAL2-LPA* genes, which have a strong susceptibility locus for CHD. The identified locus partly overlaps the gene which encodes for apolipoprotein(a) (Tregouet).

Genome wide association scans have continued to demonstrate a vast number of susceptibility regions for CHD, most with a low penetrance (Torkamani, Topol, & Schork, 2008). This suggests that CHD is modulated by a large number of low-risk genetic factors. Coronary heart disease has a complex pathogenesis, as demonstrated by the associated risk factors and genetic overlap. *FAM5C* has emerged as a novel gene of interest. *FAM5C* demonstrates the concept of genetic overlap, in as much as it has been associated with MI, the inflammatory biomarker MCP-1 (Dupuis et al., 2005) and MetS (Langefeld et al., 2004).

FAM5C

The GENECARD study, involving six international centers, performed a genome-wide scan on families in which two or more siblings had documented early onset CHD (Hauser et al., 2004). The study included 493 affected sibling pairs, of which 71% were male. Early onset CHD was defined as 51 years of age for men and 56 years old for women. The sample was prospectively stratified into three phenotypic subsets. The first included ACS families, which were defined as

the diagnosis of an MI or unstable angina in at least two affected siblings. The second was the absence of T2DM in all affected siblings, and the third was atherogenic dyslipidemia in any one sibling. Genotypes were analyzed for 395 microsatellite markers. Two regions, 3q13 and 1q25-31, met the criteria for genome-wide significance. A priori stratification by sibling pairs for ACS revealed a well-defined linkage peak on 1q25-31 (n = 228 families, LOD = 2.17, at peak microsatellite marker D1S1589/D1S518) (Hauser et al., 2004).

Three separate studies have identified genetic linkage to the same region on chromosome 1q. The Framingham Heart Study (Dupuis et al., 2005) identified a linkage peak for the inflammatory biomarker monocyte chemoattractant protein 1 (MCP-1) (LOD = 4.27, at peak microsatellite marker D1S1589). This correlated exactly with the linkage peak (LOD = 2.59, D1S1589/D1S518) for MetS in the Insulin Resistance Atherosclerosis Study (Mercuro et al.) Family Study (Langefeld et al., 2004), and the Genetics for Early Onset Cardiovascular Disease Study (GENECARD) identified modest linkage to ACS (LOD = 2.17, D1S1589/D1S518) (Connelly et al., 2008; Hauser, 2004). A SNP found within the first intron of the tumor necrosis factor superfamily 4 gene (*TNFSF4*), (rs3850641) has also been found to reside within the chromosome 1q linkage peak and has been strongly associated with MetS and MI in two independent populations (Wang et al., 2005). However, it was not linked in the GENECARD samples (Connelly et al.; Wang et al.). The precursor for CRP has also been linked to the 1q region (Dupuis et al.; Benjamin et al., 2007). This genetic overlap gives strong support to a CHD susceptibility gene in this region.

The Framingham Heart Study Offspring cohort was used to perform a genome scan of inflammatory biomarkers (Dupuis et al., 2005). A total of 3304 participants with documented CVD were included. Cardiovascular disease was defined by the presence of CHD, stroke, claudication, or congestive heart failure. Four biomarkers of vascular inflammation were measured using enzyme-linked immunosorbent assays. The biomarkers included CRP, IL-6, MCP-1, and ICAM-1. A total of 330 families (1702 individuals) were genotyped using 401 polymorphic markers. An additional 506 individuals without biomarker data were genotyped to include in the linkage analysis to increase the number of shared alleles from common ancestors. After log transforming and adjusting for covariates, the heritability estimates were the highest for MCP-1 at 44%, followed by ICAM-1 at 30.4%, and CRP at 28.2%. IL-6 had the lowest heritability at 13.7%. The maximum LOD score of 4.27 was achieved for MCP-1 on chromosome 1q25.1 at 186 cM marker D1S1589 (nominal p = .000004, genome-wide p = .005). This study found that inflammatory biomarker levels were heritable phenotypes and supports the hypothesis that some chromosome regions may harbor inflammation susceptibility genes (Dupuis et al., 2005).

The IRAS Study also found linkage on at 1q23-q31 at marker D1S1589 (Langefeld et al., 2004). The IRAS study was designed to map genes that predispose to insulin resistance, cardiovascular disease, and obesity. Thirty-five Hispanic pedigrees (216 individuals) were recruited from the San Antonio, Texas (SA) and San Luis Valley, Colorado (SLV). The overall presence of MetS in the sample was 35% as defined by ATP III criteria. A 10 cM genome scan was

conducted on all participants. Nonparametric linkage analysis revealed strong evidence for linkage with MetS to chromosome 1q between markers D1S1589 and D1S1518 in the San Antonio cohort with a LOD score of 2.59 (Langefeld et al.). These results contribute to the growing evidence of an inflammatory locus on 1q. Linkage of T2DM to the 1q21-31 regions has previously been reported in Pima Indians, African Americans, French, European-Caucasian, Hispanic Americans, Utah Caucasians, Amish, and Chinese populations (Hanson et al., 1998; Elbein, Hoffman, Teng, Leppert, & Hasstedt, 1999; Vionnet et al., 2000; Wiltshire et al., 2001; Hsueh et al., 2003; Meigs, Panhuysen, Myers, Wilson, & Cupples, 2002). Within these data sets, there has been significant correspondence between linkage in the 1q region (Wiltshire et al., 2001). The U.K., Utah, French, and Pima populations all fall within a 30 cM interval on 1q (Vionnet et al., 2000; Hanson et al., 1998; Elbein, Hoffman, Teng, Leppert, & Hasstedt, 1999). This is consistent with a common susceptibility locus and coincides with the leakage peak for *FAM5C* on 1q25-31 (LOD 2.17, at peak microsatellite marker D1S1589/D1S518) (Connelly et al., 2008). Replication of linkage results from additional populations will provide vital confirmation of the original findings.

Wang et al. (2005) identified a quantitative trait locus on mouse chromosome 1 that encompasses 11 known genes. This is a homologous region between 1q24.3 and 1q25.1, which includes *TNFSF4* and resides within the FAM5C linkage peak. Polymorphisms in *TNFSF4* are associated with the risk of MI and severity of atherosclerotic disease. Wang et al. demonstrated that mice

with the targeted mutations of *TNFSF4* had significantly ($p < .05$) smaller atherosclerotic lesions than the control mice. Alternatively, mice over-expressing *TNFSF4* had significantly ($p < .05$) larger atherosclerotic lesions than controls. Wang's findings were confirmed in two independent human populations. The first study was the Stockholm Coronary Artery Risk Factor Project, which genotyped a total of 383 subjects with MI before the age of 60 and the Stockholm Epidemiology Heart Program. The Stockholm Epidemiology Heart Program consisted of 2,246 men and women who were treated for a first-time MI and 3,206 controls (Leander et al., 2007). In both populations the less common allele of SNP (rs3850641) in *TNFSF4* was significantly ($p < .05$) more frequent in individuals with MI than in controls. Wang et al. concluded that *TNFSF4* increased the risk of MI in humans.

The GENECARD ACS sibling cohort was correlated with the Catheterization Genetics (CATHGEN) MI case-control dataset (Connelly et al., 2008). CATHGEN participants were recruited through the catheterization laboratories at Duke University Hospital. Controls and cases were chosen on the basis of extent of coronary artery disease (CAD) on the CAD index. The CAD index is a numerical summary of angiographic CAD, which incorporates the extent and distribution of the disease. The sample consisted of 368 MI cases and 289 controls; 83% were male (Connelly et al., 2008).

A peak-wide association screen was performed with 457 SNPs to evaluate for polymorphisms within *FAM5C* and determine if there was an association with MI (Connelly et al., 2008). This correlates to a linkage peak for

ACS on chromosome 1q. One SNP (rs1891586) from the peak-wide screen showed both linkage and association in the ACS sample. Additional fine mapping was performed 15 kilobases upstream and 5 kilobases downstream from the gene, using 67 haplotype tagging SNPs identified by HapMap data. Only 48 SNPs had allele frequencies > 5%. Sixteen of the SNPs showed linkage and/or association, with three SNPs partially accounting for the linkage signal on chromosome 1 by LAMP analysis (Connelly et al., 2008). LAMP analysis uses a maximum likelihood model to provide information on genetic linkage and association from samples of unrelated individuals, sib pairs, trios and larger pedigrees (Li et al., 2006). In the CATHGEN sample, 12 of the 16 SNPs were significantly associated with MI. However, only one of the SNPs in the CATHGEN sample was associated with GENECARD (Connelly et al., 2008). Ultimately, only one SNP rs10920501 (LOD = 2.98, p = 0.018), displayed linkage and association in both samples.

When the CATHGEN MI sample was stratified by age of onset, there was an earlier age of onset (< 55 years) for the subjects with the wild type A allele (J. Connelly, personal communication, May, 9, 2010). This is consistent with identification of the same allele in the GENECARD young affected sample. Gene expression of *FAM5C* SNP rs10920501 was evaluated with 83 aortic tissue samples, of which 58 were unique (Connelly et al., 2008). Human aorta samples were collected from heart transplant donors. Gene expression data showed a decrease in the level of *FAM5C* expression with the presence of the variant T allele, suggesting that higher levels of *FAM5C* may lead to MI. The authors did

not age-stratify the gene expression sample because it was too small (n = 58) (J. Connelly, personal communication, May, 9, 2010).

FAM5C, also known as *BRINP3*, was initially identified in the mouse brain as a gene that is induced by bone morphogenic protein and retinoic acid signaling (Kawano et al., 2004). Bone morphogenic protein and retinoic acid play an important role in the neuronal differentiation of the peripheral nervous system (Kawano et al., 2004). A study by Shorts-Cary et al. (2007) found *FAM5C* was over-expressed in pituitary tumors. Subcellular localization studies showed that *FAM5C* was targeted to the mitochondria. Thus, *FAM5C* is a mitochondrially localized protein and its action can lead to proliferation, migration, and invasion of nontumorogenic pituitary cells (Shorts-Cary et al., 2007). Through multiple signaling cascades, mitochondria play a vital role in the cell cycle, development, and ultimately cell death (McBride, Neuspiel, & Wasiak, 2006). Mitochondrial-controlled apoptosis is considered a crucial event in many disease processes (Rustin, 2002). Localization of *FAM5C* to the mitochondria served as the basis for Connelly et al.'s (2008) hypothesis. They propose that *FAM5C* levels could play a role in the initiation of smooth muscle cell proliferation and migration and/or in the disintegration of smooth muscle cells in the fibrous cap. *FAM5C* expression has been demonstrated in both proliferating aortic smooth muscle cells and human endothelial cells. It was shown that *FAM5C* transcript levels decrease with increasing passage of aortic smooth muscle cell, suggesting that the level of gene expression may play a role in proliferation and senescence of this cell type (Connelly et al.).

Summary

Through overlap of multiple genetic screens in independent data sets, a well-defined linkage peak on chromosome 1 has emerged as a susceptibility region for CHD. A single SNP, rs10920501, can partially account for the linkage peak. *FAM5C is* a localized to the mitochondria and expressed in the human aorta. The relationship between inflammatory biomarkers, MetS, MI, and *FAM5C* remains to be clarified. It is well accepted that inflammation is at the root of these pathologies, but the mechanisms are unclear. Inflammation plays an important role in MetS, and individuals with MetS are at higher risk for CHD. Additionally, vascular smooth muscle pathophysiology is related to inflammation which could explain the MCP-1 QTL linkage (Connelly et al., 2008). Further research is needed to clarify the relationships.

Gaps in Knowledge

Despite recent success in GWAS studies in identifying genes that are associated with CHD, a large proportion of the genetic and metabolic components of CHD remain unattributed. Many of the variants that have been identified are not functional (Bilder et al., 2009). Therefore, the roles of most genetic variants in the pathology of disease have yet to be discovered.

The role of the gene-gender interaction in the etiology of common diseases is becoming more apparent. Most GWAS studies to date have been performed on combined analysis from both genders, using gender as a covariate (Silander et al., 2008). In a case-control Japanese study of MI, results were analyzed separately by gender, revealing that the variants differed for men and

women (Yamada, 2002). A similar result was produced when analyzing MetS (McCarthy, 2007). Further study of the gene-gender interaction and its underlying basis is necessary to clarify inter-individual variation.

Chapter Three

Methods

Introduction

Chapter three presents the methodology of the study. This section includes a description of the study design, the sample, setting, data collection, measures, and the data analysis plan. Limitations are reviewed, as well as the steps taken for the protection of human subjects and plan for dissemination of the study findings.

Design

This study was an exploratory, descriptive pilot gene association study. Data from a subset of patients enrolled in cardiac rehabilitation program tailored for women with CHD was utilized for the study (Beckie et al., 2009). The parent study was funded by the National Institute of Nursing Research (grant number R01NR007678). In the parent study, women were randomized to a traditional cardiac rehabilitation program (CR) or a women's-only behaviorally enhanced, staged-matched intervention. The experimental intervention includes 10 psycho-educational sessions with motivational interviewing, social support, and 36 ECG-monitored exercise sessions over 12 weeks. The institutional review boards of the participating hospital and university approved the parent study. The subjects from the parent study consented for the use of all data, their genetic material, and biological samples in further studies of CHD. Therefore, there was no

additional recruitment or consent required for the study. This was a secondary analysis of the data previously collected.

Sample

Recruitment for the parent study was generated primarily through an automatic hospital discharge protocol, although advertising was also utilized (Beckie et al., 2009). The inclusion criteria for the parent study included women over the age of 21 years, with the diagnosis of a MI, unstable angina, or coronary revascularization within the last year. All participants were oriented to person, place and time, able to read, write, and speak English, and were willing to participate (Beckie, 2006; Beckie, Fletcher, Beckstead, Schocken, & Evans, 2008). Approximately 250 women were enrolled in the parent study. High sensitivity-CRP, IL-6, TNF-α, and ICAM-1 have been measured at three time points (baseline, post-intervention, and at a six month follow-up) in the parent study. Ninety-one subjects from the parent study with stored blood samples were included in the current gene association study (Beckie et al., 2009).

Setting

The study was completed in the University of South Florida, College of Nursing Biobehavioral Laboratory. The clinical data collection and interventions were conducted at the study hospital. DNA purification and genotyping was performed in the Biobehavioral Laboratory at the College of Nursing.

Data Collection

Demographic and clinical information were collected in the parent study by blinded research assistants at three time points including baseline, 13 weeks,

and 37 weeks. Data were stored in a locked cabinet and on password-protected computers in the office of the Principal Investigator. Data used in the study were extracted by the Principal Investigator of the parent study. To assure subject confidentiality, all identifiers were removed from the data. A non-identifying study number was given to link the parent study data and biological samples for the study.

Measures

Clinical Characteristics. Baseline assessments were collected on all subjects in the parent study, which included risk factors for CHD, anthropomorphic measures, and fasting lipid and glucose measurements (Beckie, Fletcher, Beckstead, Schocken, & Evans, 2008). Blood pressure (BP) was measured with a calibrated Accutor plus oscillometric BP monitor (Datascope, Mahwah, New Jersey) according to established guidelines. Body mass index was calculated as weight (kg)/ height (m^2). Twelve hour fasting lipid and glucose measurements were obtained utilizing the Cholestech LDX system (Beckie et al., 2008). Metabolic syndrome was defined by the revised NCEP ATP III criteria (Grundy et al., 2006).

Inflammatory Biomarkers. Blood samples were obtained in all patients prior to completing the baseline assessments for the parent study. The collection was performed between the hours of 0700am and 1200pm, after a 12 hour fast. A total of 5.0 ml of blood was collected via venipucture into a vacutainer tube. The samples were centrifuged at 3500 rpm at 0˚ C; serum samples were then

stored in 1.0 mL eppendorf tubes and immediately placed at -80˚C until further analysis.

High sensitivity-CRP, IL-6, TNF-α, and ICAM-1 were measured using the Luminex 200 IS system. The Luminex system is a laser-based fluorescent analytical system, which is used in conjunction with LINCOplex Bio-assays (LINCO Research St. Charles, Missouri). All assays were analyzed per manufacturers' protocol. Duplicates were run for all samples and concentrations were analyzed using a 5-parameter logistic curve-fitting method. Measures that were obtained with less than the minimum detectable concentration were arbitrarily set equal to half the detection level. Serum hs-CRP was measured in a LINCOplex Human Cardiovascular Disease Panel 2. The sensitivity or minimum detectable concentration for this assay is 6 pg/mL, the intra-assay precision was 8.0%, inter-assay 17.5% (Millpore Technical Publications, 2005b). Interleukin six and TNF-α were measured using the Human Cytokine LINCOplex kit. Sensitivities for these assays are 0.22 and 0.79 pg/mL, respectively. Intra-assay precision was 1.7%, inter-assay precision is < 10% (Millpore Technical Publication, 2006). Intercellular adhesion molecule-1 was measured using the Human Sepsis/Apoptosis LINCOplex kit per manufacturers' protocol. This assay has a sensitivity of 30 pg/mL with intra-assay precision of 5.5%, and inter-assay of 8.7% (Millpore Technical Publication, 2005a).

DNA Specimens. DNA was isolated and purified from the stored samples. The samples were centrifuged for 30 minutes at 4°C to pellet the cells. They were resuspended in 100 uL of phosphate buffered saline for DNA extraction.

The basic steps of DNA isolation are disruption of the cellular structure to create a lysate, separation of the soluble DNA from the cellular debris and other insoluble debris, and purification of the DNA from soluble proteins and other nucleic acids. A QIAmp DNA Mini Kit was selected for this process (Qiagen, Germantown, MD). It has been designed for use with human blood samples through the use of spin columns (Quigen Sample and Assay Technologies, 2003-2009). The cellular structures are enzymatically lysed. DNA binds to the QIAmp silica-gel membrane while debris passes through. Debris is completely removed in two wash steps, leaving pure DNA to be eluted in buffer (Qiaqen).

Extraction and Genotyping. Custom Applied Biosystems TaqMan assays were used to perform polymerase chain reaction (PCR) for the *FAM5C* SNP (rs10920501). The TaqMan assays were used to perform allelic discrimination between the A and T alleles, which have been shown to have a transversion substitution on the (rs10920501) SNP (Connelly et al., 2008). Assays for this SNP were obtained from the manufacturer (Applied Biosystems, Foster City, CA). Different from conventional PCR, TaqMan PCR utilizes a reporter and a quencher that recognizes a specific allele of a SNP. The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye resulting in increased fluorescence by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to the probe and is amplified during PCR. Each sample required the following components: 2.5 uL genomic DNA, 12.5 uL Custom TaqMan SNP genotyping Master Mix (Applied Biosystems, Foster City, CA), 1.25 uL of 20x

TaqMan assay, and 8.75 uL DNAse free water for a total sample volume of 25 uL. Reactions were performed in 96 well plates in duplicate using a Bio-Rad CFX PCR Detection System (Bio-Rad, Hercules, CA). PCR cycling conditions followed the standard TaqMan protocol: Step 1: 95˚C for 10 minutes, Step 2: denaturing at 40 cycles of 92˚C for 15 seconds, Step 3: 60˚C for 1 minute (Applied Biosystems, 2006). Analysis was conducted by BioRad CFX Manager Software version 1.5 (BioRad Laboratories, 2008).

Data Analysis

Data were analyzed using SPSS version 18 for Windows (SPSS Inc., Chicago, Ill.). Initially data were screened for missing data, outliers, and possible confounding factors and to assess whether underlying statistical assumptions for the planned analysis were satisfied. There were no missing data. Descriptive statistics were used to describe study participants. The significance level was set at $\alpha = .05$.

Aim One

Describe the distribution of the genotype in the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD.

Frequency counts and percentages were used to describe the frequency distribution for the variant SNP. Percentages provide the proportion of valid observations found in each of the genotype groups. The SNP has three possible genotypes; the homozygous wild type (AA), heterozygous (AT), and the homozygous variant (TT). Genotype distributions and allele frequencies are reported.

Aim Two

Investigate the associations between the variability in the genotype in the *FAM5C* SNP (rs10920501), MetS, and inflammatory biomarkers among women with documented CHD.

A chi square test was performed to measure the association between genotype and MetS. Separate one-way analyses of variance (ANOVA) were then performed on the log (base-10) transformed values of hs-CRP, ICAM-1, IL-6, and TNF-α from the parent study by genotype. The null hypothesis stated the means of the biomarkers were the same for each genotype group. The results are reported as an *F* test. If the obtained *F* exceeds the critical *F,* the null hypothesis is rejected. The effect size is reported as an eta squared.

Aim Three

Evaluate if the *FAM5C* SNP (rs10920501) is associated with early onset CHD in women.

An ANOVA was used to compare the differences between women with early onset CHD and those who manifested the disease later in life. Early onset CHD for women has been previously defined by Hauser et al. (2004) as occurring at or before the age of 55. Therefore, the age variable was dichotomized to two levels, women age $\leq$ 55 and age > 55. Descriptive statistics were used to describe this subset of study participants. There are three levels of genotyping. The null hypothesis stated that the mean age of CHD onset is equal across all genotypes. The results are reported as an *F* test and the effect size is reported as an eta squared.

A supplemental analysis was performed to determine if there was an association between genotype and age of onset of CHD in women who had an MI. Myocardial infarction was defined as any documented MI in the medical record. Some women had more than one MI prior to study enrollment. This criterion was established to minimize phenotypic heterogeneity. A one-way ANOVA was used to evaluate three levels of genotype and to determine whether there was a significant relationship between genotype and age of onset of CHD in women who had an MI. A linear regression model was used to assess the potential factors that might predict age of onset. The dependent variable was age of onset of disease as first documented in the medical record. The independent variable was SNP rs10920501.

Chapter Four

Results

Introduction

The purpose of this study was to explore the presence of the variant allele in the *FAM5C* gene (rs10920501) in a cohort of women with documented CHD, to assess the relationship between *FAM5C*, MetS, and inflammatory markers, and to evaluate if *FAM5C* variability has predictive value for identifying women with early onset CHD. Chapter four presents the baseline characteristics of the samples and results of the current study. The baseline data included the clinical characteristics, diagnostic criteria for inclusion in the parent study, co-morbidities, and medications that the subjects were on at the time of enrollment to the parent study. The results of the study are presented by aim.

Baseline Characteristics

Ninety-one women from the parent study had inflammatory biomarker data and stored samples available for examination in the current study. Demographic characteristics from the subset of women are presented in Table 3, with comparative data from the GENECARD sample (Hauser et al., 2004). The women in the current study had a mean age of 61.6 years (*SD* = 10.2), with a range of 42 to 82 years. The majority of the women were white (61.5%).

Table 3

Demographic Characteristics of the Sample Compared to GENECARD Sample

Variable	CURRENT n = 91 n (%)	GENECARD* n = 422 n (%)
Age mean (*SD*)	61.6 (10.2)	44.2 (5.8)
Women	91 (100.0)	124 (29.4)
Race/Ethnicity		
Caucasian (Non-Hispanic)	56 (61.5)	392 (92.9)
African American	16 (18.0)	9 (2.1)
Hispanic	19 (22.0)	0

*(Hauser et al., 2004)

The traditional risk factors for CHD are presented in Table 4. Fifty-six percent of the study participants had a history of smoking, with a mean 14.1 $\pm$ 19 pack years. Nearly fifty percent of the women had a self-reported family history of heart disease, and the majority of the women were diagnosed as hypertensive (82.4%), although most were on antihypertensives at the time of enrollment to the parent study. The mean total cholesterol at baseline was 162 $\pm$ 38 mg/dL, the mean HDL-C was 43.4 $\pm$ 12.5 mg/dL, the mean baseline LDL-C was 93 $\pm$ 33.7 mg/dL, with a range from 37-206 mg/dL. Triglycerides also had a wide range from 45- 358 mg/dL, with a mean value of 132 mg/dL. Women in the current study had a mean BMI of 32.4 $\pm$ 7.3 kg/m^2. Thirty-seven percent of the women in the current study were diabetic and 36% met the revised ATP III criteria for MetS (Grundy et al., 2006).

Table 4.

Risk Factors of the Current Study Sample Compared to GENECARD Sample

Variable	CURRENT n = 91 n (%)	GENECARD* n = 422 n (%)
Risk Factors		
Diabetes	34 (37.4)	94 (22.3)
Metabolic syndrome	33 (36.3)	Unreported
History of smoking	51 (56.0)	329 (78.0)
Hypertension	72 (82.4)	240 (56.9)
Familial heart disease	43 (47.3)	422 (100.0)
Total cholesterol, mg/dL	162 (38.0)	239 (64.8)
HDL-C, mg/dL	43.4 (12.5)	38.7 (16.3)
LDL-C, mg/dL	93 (33.7)	146.1 (54.3)
Triglycerides, mg/dL	132 (64.6)	230.1 (138.8)
Body mass index, kg/m²	32.4 (7.3)	29.9 (5.8)
Waist circumference, cm	100.9 (15.2)	Unreported

Note: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Continuous variables are displayed as mean (*SD*) and categorical variables are displayed as n (%). *(Hauser et al., 2004).

Women qualified for inclusion in the parent study based on the diagnosis of an acute MI (29.7%), unstable angina (12.1%), coronary artery bypass graft (CABG) surgery (31.9%), or percutaneous coronary intervention (PCI) (48.4%) within the last year (see Table 5). Medical record review also revealed a previous

history of MI in 17.6% of the women, stable angina in 13.2%, CABG surgery in 7.7%, and previous PCI in 11%. At the time of enrollment, 78% of the women were on beta-blockers, 32% were on angiotensin-converting enzyme inhibitors, 88% were taking aspirin, 67% taking clopidogrel, 93% lipid-lowering agents, 26% were taking nonsteroidal anti-inflammatory agents, and 6.6% were on estrogen replacement.

Table 5

Eligibility Criteria, Co-Morbidities, and Medications of Study Sample

Variable	n = 91 n (%)
Eligibility for the Parent Study	
Acute myocardial infarction	27 (29.7)
Unstable angina	11 (12.1)
Coronary artery bypass graft surgery	29 (31.9)
Percutaneous coronary intervention	44 (48.4)
Co-Morbidities	
Previous myocardial infarction	16 (17.6)
Previous angina	12 (13.2)
Previous coronary bypass graft surgery	7 (7.7)
Previous percutaneous intervention	10 (11.0)
Medications	
Beta-blocker	71 (78.0)
Angiotension converting enzyme inhibitor	29 (31.9)
Aspirin	80 (87.9)
Clopidogrel	61 (67.0)
Lipid lowering agents	85 (93.4)
Nonsteroidal anti-inflammatory agents	24 (26.0)
Estrogen replacement	6 (6.6)

Results by Aim

Aim One- Distribution of the Genotype

The first aim was to describe the distribution of the genotype in the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD. The SNP genotyping distribution included the homozygous wild type (AA), the homozygous variant (TT), and heterozygous (AT). Frequencies and percentages of the genotyping in the current study and those in HapMap distributions are presented in Table 6 (International HapMap Project, 2008).

Table 6

Distribution of the FAM5C SNP (rs10920501) by Genotype

Genotype	Current Sample (n = 91)	HapMap*
	n (%)	Percentage
Homozygous wild (AA)	61 (67.0)	65%
Homozygous variant (TT)	4 (4.4)	5%
Heterozygous (AT)	26 (28.6)	30%

* International HapMap Project, 2008

Important implications of the HWE equilibrium include allele frequencies remain constant across generations, and that for rare alleles, the frequency of the heterozygote far exceeds the frequency of the rare homozygote (Rodriguez, Guant, & Day, 2009). This distribution was tested for deviation from the Hardy-Weinberg equilibrium (HWE). The null hypothesis was that the sample was in HWE. The SNP was found to be in HWE ($\chi^2 = 0.32$, df = 1, $p = .57$); therefore, the null hypothesis was not rejected.

Aim Two- Association Between Genotype, Metabolic Syndrome, and Inflammatory Biomarkers

Aim two investigated the associations between the variability in the genotype in the *FAM5C* SNP (rs10920501), MetS, and inflammatory biomarkers among women with documented CHD. A chi square test was performed to determine if there was a significant relationship between subjects with MetS and without MetS at baseline, and whether this was related to genotype (See Figure 2). Of the total sample, thirty-three subjects (36%) had MetS at baseline. The results indicated that MetS was not related to *FAM5C* SNP rs10920501 (χ^2 = .36, df = 2, *p* =.84). Supplemental analysis also examined the relationship between genotype and diabetes and found no relationship (χ^2 = .28, df = 2, *p* =.87).

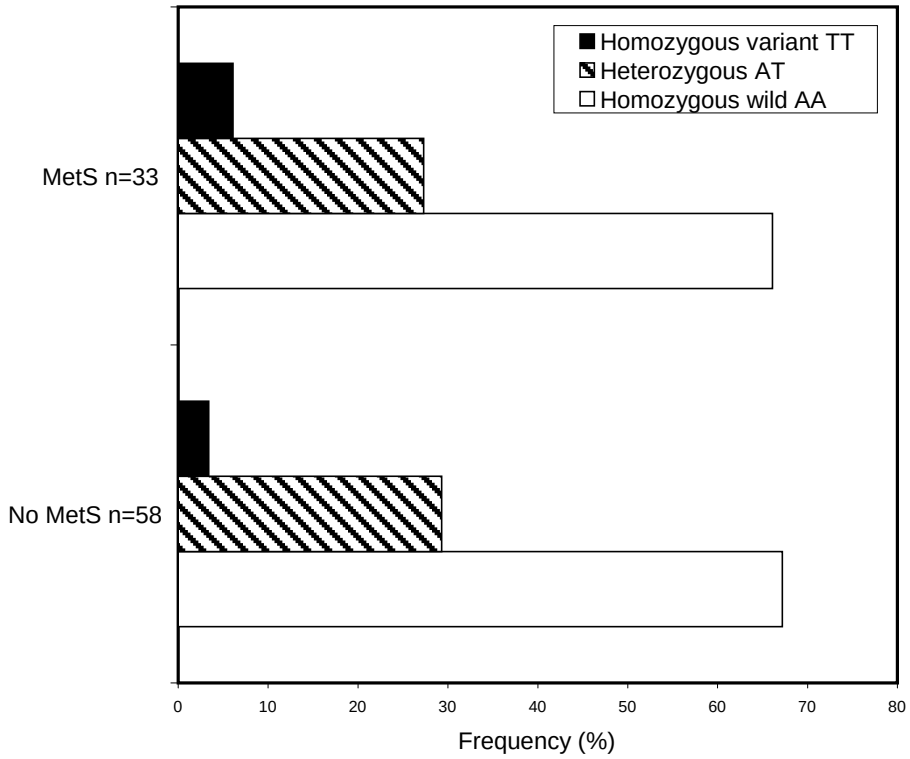

Figure 2. Relationship Between Baseline Metabolic Syndrome and Genotype

Separate one-way analyses of variance (ANOVA) were performed on the log (base-10) transformed means of hs-CRP, ICAM-1, IL-6, and TNF-α from the parent study by genotype (Table 7). The results suggested there was no relationship between genotype of *FAM5C* SNP rs10920501 and any inflammatory markers. The effect sizes (eta²) indicate that there was no systematic relationship between inflammatory biomarkers and *FAM5C* SNP rs10920501 in this study.

Table 7

Relationship Between Inflammatory Biomarkers and FAM5C SNP *rs10920501*

Variable	AA	AT	TT	F	*p*	η^2
	n = 61	n = 26	n = 4	(2,88)		
hs-CRP mg/L	1.32 (.49)	1.37 (.56)	1.31 (.61)	.13	.88	.00
ICAM-1 pg/mL	2.26 (.21)	2.28 (.29)	2.28 (.29)	.04	.93	.00
IL-6 pg/mL	.84 (.45)	1.00 (.60)	.80 (.33)	.94	.40	.02
TNF-α pg/mL	.81 (.33)	.88 (.43)	.88 (.32)	.42	.66	.01

Note: Data are presented as means (*SD*). Inflammatory biomarkers are presented as log transformed data. Abbreviations: AA, homozygous wild AA; AT, heterozygous AT; TT, homozygous variant TT; hs-CRP, high-sensitivity C-reactive protein; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

Aim Three- The Relationship Between FAM5C and Early Onset CHD

Aim three evaluated if the *FAM5C* SNP (rs10920501) was associated with early onset CHD in women. Early onset CHD was dichotomized by a mean age of $\leq$ 55 (n = 35) and a mean age of > 55 (n = 56), which is consistent with the genetics literature. A one-way ANOVA was used to evaluate three levels of

genotype and to determine whether there was a significant relationship with early onset CHD in women. The null hypothesis stated that the mean age of CHD onset is equal across all genotypes. The mean age of women with the onset of CHD at or before the age of 55 was 51.8 years (*SD* 6.6). The mean age of women with onset of CHD after 55 was 67.7 years (*SD* 6.6). When age of onset of CHD was assessed by genotype (Table 8), women with the homozygous variant (TT) genotype on average had a later onset of CHD, with a mean age of 64.5 years (n = 4). However, the ANOVA failed to reach statistical significance (F (2, 88) = .96, p = .39), and the null hypothesis was not rejected.

Table 8

Age of Onset of Coronary Heart Disease by Genotype

Independent Variable	N	Mean (*SD*)	Minimum - Maximum
Homozygous wild (AA)	61	58.5 (11.1)	40 - 82
Heterozygous (AT)	26	61.0 (10.0)	36 - 74
Homozygous variant (TT)	4	64.5 (9.3)	55 -73

Note: Minimum, maximum, and mean presented in years. *SD*, standard deviation.

Clinical characteristics of the sample were examined stratified by age of CHD (≤ 55 years and > 55 years) (see Table 9). Women diagnosed at 55 years or younger were less likely to be diabetic than those diagnosed later in life (χ^2 = 4.23, df = 1, p = .04). The early onset group were less likely to meet the criteria for MetS than the late onset group (χ^2 = 91.00, df = 1, p = <.01), and were more likely to have a previous history of ever smoking (χ^2 = 13.79, df = 1, p = <.01). Women in the early onset group were less likely to be hypertensive than the late

onset group (χ^2 = 43.5, df = 1, p = <.01), and were more likely to have a family history of heart disease than late onset group (χ^2 = 7.69, df = 1, p = <.01). There were no significant differences in the lipid profiles or BMIs between the two groups of women.

Table 9

Risk Factors Stratified by Age of Coronary Heart Disease Onset

Variable	Total Sample n = 91 n (%)	CHD ≤ 55yrs n = 38 n (%)	CHD > 55 yrs n = 53 n (%)	*p*
Risk Factors				
Diabetes	34 (37.4)	11 (28.9)	23 (43.4)	.04
Metabolic syndrome	33 (36.3)	13 (34.2)	20 (37.7)	< .01
Smoking	51 (56)	27 (68.4)	25 (46.2)	< .01
Hypertension	72 (82.4)	29 (76.3)	46 (86.8)	< .01
Familial heart disease	43 (47.3)	22 (57.9)	21 (39.6)	< .01
Total cholesterol, mg/dL	162 (38)	161 (39.9)	162.9 (37.2)	.84
HDL-C, mg/dL	43.4 (12.5)	41.5 (10.2)	44.8 (13.8)	.19
LDL-C, mg/dL	93 (33.7)	91.1 (35.0)	94.2 (32.9)	.68
Triglycerides, mg/dL	132 (64.6)	140.6 (66)	125.9 (63.4)	.94
Body mass index, kg/m^2	32.4 (7.3)	33.8 (8.0)	31.3 (6.8)	.17

Note: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Lipid profile and body mass index results are presented as means (*SD*).

For those women that experienced an MI (n = 38, 41.8%), a one-way ANOVA was conducted to examine the relationship between genotype and age of onset. The null hypothesis stated that for women with an MI, the mean age of

CHD onset is equal across all genotypes. When the women who ever had an MI were assessed by genotype, none had the homozygous variant (TT). This finding is consistent with the strong protective effect of the T allele. Women with the homozygous (AA) genotype had a mean age of onset at 55.1 (*SD* 7.7) years, compared to a mean age of onset of 61.5 (*SD* 10.0) years for the heterozygous (AT) genotype, which was significant (F (1, 36) = 4.61, p = .038). The homozygous wild type (AA) genotype represented 71.1% (n = 27) of the women who experienced an MI (Table 10). Therefore, the null hypothesis was rejected.

Table 10

Age of Onset of Coronary Heart Disease by Genotype in Women with a History of Myocardial Infarction (n = 38)

Independent Variable	n (%)	Mean (*SD*)	Minimum - Maximum
Homozygous wild (AA)	27 (71.1)	55.1 (7.7)	44 - 70
Heterozygous (AT)	11 (28.9)	61.5 (10.0)	46 - 74

Note: Minimum, maximum, and mean presented in years.

A linear regression model was constructed to assess the potential factors that might be predictive of the age of onset of an MI (n = 38). A stepwise method was used to search candidate variables that the literature has suggested are associated with MI. The significant predictors included the homozygous wild type AA genotype, smoking, and BMI. Table 11 displays the predictors, the unstandardized regression coefficients (B), the standard errors associated with the coefficients (SE(B)), the standardized regression coefficients (β), t, and p values. R^2 for regression was significantly different from zero, F (3, 37) = 5.127, p

< .01 (Table 11). The adjusted R^2 of .251 indicates that approximately one quarter of the variability of age of onset of MI is predicted by the homozygous wild type AA genotype, smoking, and BMI. The R^2 change indicates that 6.8% of the variability in CHD age of onset was unique to genotype. The size and direction of the relationships suggest that history of smoking (-.338) is only marginally more predictive of age of onset than homozygous wild type AA genotype (-.293) and BMI (-.303), as indicated by the squared semi-partial correlations. Among women aged 45 to 74 years, women with the homozygous wild type AA genotype had an onset of MI 5.6 years earlier than those with the heterozygous AT genotype.

Table 11

Predictors of the Age of Onset of Coronary Heart Disease in Women with a History of Myocardial Infarction

Predictor Variable	B	SE(B)	β	t	*P*
Constant	77.242	6.947		11.119	.001
Homozygous wild AA	-5.601	2.738	-.293	-2.045	.049
Smoking	-.151	.064	-.338	-2.358	.024
Body mass index	-.414	.194	-.303	-2.130	.041

Note: Adjusted R^2= .183 for lifestyle variables, Adjusted R^2= .251 for the 3 predictor model.

Chapter Five

Discussion

Introduction

This chapter begins with a discussion of the results by aim. Limitations of the current study are reviewed, and the chapter concludes with a summary of conclusions and recommendations for future research.

Discussion of the Results by Aim

The purpose of aim one was to describe the distribution of the genotype in the *FAM5C* SNP (rs10920501) in a cohort of women with documented CHD. Most women were homozygous for the wild type (AA) genotype, with very few homozygous for the protective genotype (TT), as one would expect in a sample of women with established CHD. The genotype frequencies obtained in this study were consistent with those reported by Connelly et al. (2008) and the International HapMap Project (2009). Furthermore, there was not a deviation from the expected proportions in the population under the Hardy Weinberg Equilibrium (Goddard, Ziegler, & Wellek, 2009). The Hardy Weinberg Principle states that genotype frequencies will remain constant, or in equilibrium, across generations unless certain influences are introduced (Hartl & Jones, 2005). Based on this principle, the genotyping results of this study were found to be in equilibrium and free from systematic genotyping errors.

Clinical characteristics of the current sample were compared to the GENECARD study (Hauser et al., 2004). GENECARD was selected for comparison because it identified *FAM5C* as a susceptibility gene for early-onset CHD and found a strong association with *FAM5C* SNP rs10920501 and ACS. Family history of CHD was an inclusion criterion for the linkage analysis in GENECARD. The women in the current study were unrelated, and less than half had a family history of CHD. Overall, the current sample represented an older, heavier group that was significantly more hypertensive than those participating in GENECARD. There were also fewer MIs in the current study group. In GENECARD, more than half of the families had ACS. Diabetes was more prevalent in the current study, although smoking was not. An all female cohort may explain the discrepancy in smoking rates, as historically women have not smoked at the same rates as men (National Health Interview Study, 2008). The women in the current study were more ethnically diverse than the subjects in GENECARD. Nearly all the subjects in GENECARD were Caucasian, compared to slightly over one-half in the current study (Hauser et al.). Lastly, the lipid profiles in the current study at first glance appear to be significantly better than those in GENECARD. However, this must be placed in the context that 92% of the women in the current study were on lipid lowering therapies at the time of enrollment. Unfortunately, these data were not reported from GENECARD. Discrepancy between rates of traditional risk factors in the current study and GENECARD should be put in context with the historical timing of the studies. The GENECARD study was completed in 2002, and the current study was completed

in 2009 (Hauser et al., 2004). During the period between the two studies, risk factor modification evolved considerably (Mosca et al., 2007).

The purpose of aim two was to investigate the association between *FAM5C* SNP rs10920501, MetS, and inflammatory biomarkers among women with documented CHD. The current study did not seek to investigate the numerous genes that are reported to be associated with CRP, ICAM-1, IL-6, and TNF-α. The investigation of the association was based on the known genetic overlap on the same region on chromosome 1q. There have been no previously documented associations in the literature between *FAM5C* SNP rs10920501, MetS, and these inflammatory biomarkers. In the current study, there was not significant evidence to conclude that MetS or diabetes was related to the *FAM5C* SNP (rs10920501). Additionally, no relationship was found between *FAM5C* SNP rs10920501 and the inflammatory markers investigated. Potential explanations for the nonsignificant association between genotypic and phenotypic characteristics of MetS, inflammatory biomarkers, and age of onset include the multi-factorial nature of CHD influenced by the multiple environmental exposures and numerous genetically determined mechanisms underlying disease development and progression. Other potential reasons for the inability to replicate previous study findings include the influences of race, genetic heterogeneity, phenotypic heterogeneity, gene-gene interaction, and low statistical power.

Population stratification occurs when there is marked variation in allele frequencies across subgroups of a population, in the context of baseline

variability in the disease prevalence within the subgroups (Thomas & Witte, 2002). This phenomenon has been recognized as a confounding factor in the replication of genetic studies. The degree of bias hinges on the heterogeneity of allele frequencies and disease risk across and within populations (Thomas & Witte). The IRAS study provides a good example of population stratification within a Hispanic pedigree (Langefeld et al.). The IRAS study provided evidence for linkage on chromosome 1q for MetS, which overlaps with *FAM5C*. The IRAS study was composed of 216 individuals from Hispanic descent from two Hispanic pedigrees, San Antonio and San Luis Valley. When the sample was stratified by geographic region, there was no evidence linking the subjects from the San Luis Valley to chromosome 1q and MetS (Langefeld et al.). This study demonstrates that population stratification may have a significant impact on the ability to replicate genetic findings. According to Liu et al. (2008), it should not be surprising that a significant association identified in one population cannot be found in another. The population in the current study was representative of the minority composition of the Southeastern United States and was more racially diverse than GENECARD (Hauser et al., 2004). Subjects self-identified their ethnic background. It has been suggested that current techniques to control for race and ethnic background are not always effective, in spite of careful sampling. Even when selecting a population based on similar race and shared geography, there can be significant differences that effect allele frequencies and the ability to replicate association studies.

It has been recommended that future studies incorporate genotyping of ancestry informative markers (AIMs) to address population stratification. Ancestry informative markers are a subset of genetic markers that differ in allele frequencies across different populations (Hoggart et al., 2003). It has been suggested that by using AIMs, it will be possible to determine a subject's ethnic background from associations on the marker loci and from associations of the marker with the trait, even without the benefit of demographic data. Ultimately, this should decrease both confounding and selection bias in association studies (Hoggart et al, 2003).

It may be practically impossible to have a sample size large enough to overcome between-study heterogeneity (Liu et al., 2008). Small sample and effect sizes will contribute to the ability to replicate genetic findings. The vast majority of genetic markers discovered in recent association studies have had small effect sizes and explain only a fraction of the genetic contribution to disease. The sample size needed to overcome small effect sizes is based on the heritability of the disease, which includes mean allele frequencies and degree of penetrance (Pawitan, Seng, & Magnusson, 2009). For example, given the weak effect sizes currently reported for T2DM, approximately 800 variants are required to explain a heritability of 40%. It would then take an estimated sample size of 50,000 cases and 50,000 controls to capture 800 common variants with low effect sizes. The sample size to needed to capture rare variants with low effect sizes is staggering. It is estimated that at least 125,000 cases and controls are necessary to discover approximately 1,400 rare variants (Pawitan, Seng, &

Magnusson). The current study had a sample size of 91 women and evaluated a single variant. The effect sizes suggest that regardless of sample size, there was no relationship between *FAM5C* SNP rs10920501 and the inflammatory markers investigated. However, the ability of a single SNP to make a significant contribution to risk prediction of a complex disease remains unlikely. Each SNP increases the probability of CHD by a small margin (Schunkert et al., 2010). Whether a combination of at-risk SNPs will improve genetic risk prediction remains open to debate (Humphries et al., 2004; Janssens, Janicot, Perera, & Bakker, 2004).

Gender-specific risk factors may also have been a confounder in the inability to find an association between *FAM5C* SNP rs10920501, MetS, and inflammatory biomarkers among women with documented CHD. The incidence of CHD varies between genders due to gender-specific genetic risk factors (Opstad, Pettersen, Weiss, Arnesen, & Seljeflot; Mercuro et al.; Tsai et al., 2009). The current study was composed of an all female sample, while GENECARD was a combined gender analysis (Hauser et al., 2004). The GENECARD sample was composed of approximately one-third women (Hauser et al.). The degree to which gender-gene exposures influence the findings can be difficult to assess and quantify (Liu et al., 2008). For example, Silander et al. (2008) evaluated 46 candidate genes for CVD in a Finnish population and found that only half of the reported findings from combined gender analysis are true positives. Silander et al. suggests that genetic risk loci may be more readily detectable in women, while the results for men are confounded by lifestyle risk factors. Sex steroids

and gender-dependent gene expression have also been linked to the differences in the progression and extent of CHD between men and women. Lastly, traditional risk factors, such as hypertension, may also be influenced by gender-specific factors (Mercuro et al., 2010). Ultimately, however, the contribution of the sex-specific differences beyond that of the traditional CHD risk factors remains unknown. Paynter et al. (2010) recently evaluated the contribution of genetic variation from 101 SNPs reported to be associated with CHD in 19,313 healthy white women in the Women's Genome Health Study. They found that genetic risk score was not associated with CVD risk, and that self-reported family history remained the strongest predictor. Similarly, 9p21.3 (rs10757274) has been strongly and significantly associated with cardiovascular events, but knowledge of it has not significantly improved global risk prediction (Humphries, Ridker, & Talmud, 2004). Furthermore, the mechanism for disease manifestation through these genotypes remains largely unknown (Schunkert, Erdmann, & Samani, 2010). The difference in which a particular gene contributes to the same phenotype in each of the sexes is an aspect that should be studied in more depth. A better understanding of the molecular basis of CHD prevalence and clinical manifestations represents a starting point for improved gender-oriented research.

Finally, phenotypic heterogeneity can also be a confounding factor when trying to replicate a genetic association study. An association is considered to be replicated if the same SNP or haplotype is associated with the same phenotype in two or more populations (Peters, 2009). Phenotypic differences of CHD have

been found to be important in other genetic association studies. Girelli et al. (2007) investigated the role of *ALOX5AP* in the development of CHD and MI in patients with or without angiographically documented CHD. Girelli et al. examined 1,431 subjects, of whom 1,047 had severe coronary artery disease. The remaining 384 subjects had normal coronary arteries. The study sought an association with seven *ALOX5AP* SNPs and CHD, allowing for the reconstruction of at-risk haplotypes. Previously identified haplotypes were not replicated in the study. The investigators attributed this discrepancy to phenotypic heterogeneity, which is exacerbated by endemic nature of CHD in western populations.

Previous studies focused on an MI phenotype versus controls from the general population. These controls were not stratified by angiography. Therefore, previous control populations could have had severe atherosclerotic lesions which were not yet clinically evident (Girelli et al.). The MI phenotype is not equal to the presence of CHD (Horne, Carlquist, Muhlestein, Bair, & Anderson, 2008). CHD may eventually progress to ACS and MI, but many patients can live with stable CHD that never progresses to an MI. The GENECARD ACS association subset defined inclusion by MI or ACS, as determined by a sentinel event or diagnostic study (Hauser et al., 2004). By contrast, the CATHGEN MI subset was limited to the documentation of an MI in the medical record (Connelly et al., 2008). This phenotypic heterogeneity in the setting of marginal *p* values calls into question if the *FAM5C* SNP had been previously replicated. The importance of stringent phenotypic criteria cannot be understated if the pathophysiologic mechanisms of CHD and MI are to be understood and eventually translated to clinical practice.

Aim three evaluated the association between *FAM5C* SNP rs10920501 and early onset CHD in women**.** A supplemental analysis evaluated if there was an association between *FAM5C* SNP rs10920501 and age of onset of CHD in women who had ever experienced an MI. When the age of disease onset of the total sample was assessed by genotype, there was a trend toward a protective effect with the T allele, although it did not reach statistical significance. The homozygous variant (TT) genotype had on average the latest onset of CHD, and the homozygous wild type (AA) genotype had the earliest onset of CHD. The heterozygous (AT) was in the middle, creating a stepwise age stratification based on the presence of the T allele. Previous studies of *FAM5C* SNP rs10920501 identified an earlier age of onset (< 55 years) for the subjects with the wild type A allele in the MI and ACS populations. Therefore, given the phenotypic heterogeneity in the current study, it was not surprising that the findings were not replicated utilizing the total sample of women with documented CHD. There was also evidence of different intermediate phenotypes when the current sample was age stratified. The younger women in the current sample with CHD were more likely to have familial heart disease and a previous history of smoking. Additionally, *FAM5C* SNP rs10920501 variant is relatively common. The minor allele frequency (MAF) of *FAM5C* SNP rs10920501 is .20 in the European (Utah residents with ancestry from northern and western Europe) population (International HapMap Project, 2008). The MAF is the frequency of the SNPs less frequent allele in a given population (Hartl & Jones, 2005). However, *FAM5C* SNP rs10920501 represents only one of hundreds or perhaps thousands of

SNPs that have a biological pathway to CHD. The possible gene-gene interaction, which is clearly plausible, can generate a rare composite-genotype from a common variant. For example, two relatively common SNPs, each with a MAF of 0.3, can produce an interacting genotype of 0.3^4 (= 0.0081), assuming independence and interaction among the minor homozygous alleles only (Pawitan, Seng, & Magnusson, 2009). The problem with interacting genotypes becomes more complicated if several SNPs are interacting. If distinct subphenotypes are due to different susceptibility genes, a study that combines them will have diluted effects. Additional variants, like CNV, insertion/deletion, or methylation status will be necessary to add to the prediction power in studies of complex diseases in the future (Pawitan, Seng, & Magnusson, 2009).

In the supplemental analysis, age of disease onset was also assessed in women who had a history of an MI. Nearly two-thirds of women with a history of an MI had the homozygous wild type (AA) genotype, which is consistent with known genotyping frequencies. No women in this study with the homozygous variant (TT) had an MI, which supports the protective effective of the T allele. The major findings of this study are the confirmation of the protective effect of the *FAM5C* SNP (rs10920501) T allele in a population of women with a history of MI. Also, the homozygous wild type (AA) genotype is predictive of the age of CHD onset among women who have had an MI, when holding smoking history and BMI constant.

To demonstrate the clinical relevance, consider two 40 year old women that are destined to have a MI. One woman has the homozygous wild type (AA)

genotype, and the other the heterozygous (AT) genotype. Can we predict which woman will have an MI first? Both women are smokers and have a BMI of 30, which are well established environmental risk factors for MI. The woman with the homozygous wild type (AA) genotype is predicted to have an MI 5.6 years before her counterpart. However, it must be noted that the genetic contribution was fairly small.

To the best of our knowledge, the current study is the first to replicate the protective effective of the *FAM5C* SNP (rs10920501) T allele in a cohort of women. The linear regression suggests that genotype of *FAM5C* SNP rs10920501 exerted its effect on age of onset, independent of life style variables. Acknowledging the relatively small size in the MI subgroup, *FAM5C* SNP rs10920501 should be regarded as a relevant genetic determinant of the age of onset of CHD.

Limitations

Limitations of the current study warrant consideration. All data were collected as part of the parent study. Therefore, any additional demographic variables were limited to those available in the parent study. The findings of this study are limited to a female population with documented CHD and cannot be generalized to the population at large. Since *FAM5C* has been linked to the MI phenotype, a subset was created from the total sample that had a limited sample size. This study was designed as an exploratory pilot, with a convenience sample, utilizing a single SNP. Lastly, CHD has been described as a manifestation of multiple intermediate disease processes, which individually have

genetic and metabolic components (Lanktree & Hegele, 2009), not all of which could be addressed in this study.

Conclusion

FAM5C has shown promise as a novel MI susceptibility gene in the initial GENECARD study. Functional studies have been encouraging regarding the role the gene plays in the atherosclerotic process. Furthermore, *FAM5C* has demonstrated genetic overlap with MCP-1, MetS, and *TNFSF4*. The current study sought to elucidate interactions between *FAM5C* SNP rs10920501 and inflammatory biomarkers, and MetS, and early onset CHD. The current study demonstrated the protective benefit of the T allele in a limited sample size. The homozygous wild type (AA) was significantly associated with an earlier onset of CHD in women who had a history of an MI. The current study found no evidence of an association between FAM5C and hs-CRP, ICAM-1, IL-6, TNF-α, and MetS in an all-female cohort. The differences in study characteristics, outcomes evaluated, and potential gender-gene and gene-gene interactions may have contributed to the nonsignificant results. The current study contributes to the available body of knowledge, and since the inception of the current study there are many more candidate genes under study.

It has been approximately five years since the first genome-wide association studies were published identifying a common risk allele with a large effect size for age-related macular degeneration. Initially, researchers were optimistic about the ability to detect disease with genome-wide association studies. Despite this, only a relatively small amount (< 10%) of the overall genetic

risk of coronary heart disease has been identified (Schunkert et al., 2010). The majority of risk alleles are common and have small effect sizes. Of these, only a small number of the risk alleles are non-synonymous SNPs in exons, which could alter protein structure and function. In fact, most risk alleles reside in either intron, intergenic, of desert gene regions. They may be functional SNPs, but little is known about their biological mechanism. Understanding these mechanisms is required before we can accurately model and measure complex traits.

Phenomics will be of key importance in the post-GWAS era. Phenomics has been functionally defined as the systematic study of multiple phenotypes, across multiple biological scales (Lanktree & Hegele, 2009). It has become increasingly important as high-throughput genotyping drastically increased sample sizes, and new methods have enabled ever-finer mapping of genetic sequences to detect rare variants and CNV not captured on current platforms (Bilder et al., 2009). The application of phenomics requires a transdiscipline approach, which would include expertise in genetics, molecular biology, cell biology, systems biology, and the study of higher level phenotypic expression. While so far it remains unclear to what extent epigenetic factors contribute to phenotypic variance not explained by genomic data, the theoretical potential is vast (Bilder et al.). Phenomics will provide an increased understanding of CHD susceptibility and replication of gene-gene and gene-environment interactions (Lanktree & Hegele).

Currently, traditional risk factors remain the greatest overall predictor of CHD. Even though a genotype may be strongly associated with CHD risk, if its

mechanism is mediated through traditional risk factors, it is unlikely to add significantly to the overall risk prediction (Humphries, Ridker, & Talmud, 2004). Genotypes that have mechanisms outside traditional risk factors and that have a large effect are the most likely to add to risk prediction (Humphries et al.; Paynter et al., 2009). The genetic risk variants require a specific environment to contribute to disease. It is within that environment that there may be modifiable risk factors. A better understanding of these gene-gene, gender-gene, and gene-environment interactions are vital to the understanding of the genetic predisposition to disease, which will aid prediction, prevention, diagnosis, and prognosis, and ultimately guide personalized medicine of CHD.

Recommendations for Future Research

FAM5C SNP rs10920501 has been associated with early onset CHD in women with a previous history of MI. It has been suggested that the mechanism is mediated through the initiation of smooth muscle cell proliferation and migration and/or in the disintegration of smooth muscle cells in the fibrous cap. Inflammation lies at the heart of this pathology and might explain the MCP-1 linkage peak. Future research should explore the relationship between MCP-1 and *FAM5C* more closely. Also, replication of gene expression in a female cohort or gender-specific data to confirm functional significance should be a priority. Ideally, these future gene expression studies would be large enough to allow for age stratification, which has not been possible in previous studies (Connelly et al.). Finally, the genetic pleiotropy of *FAM5C* could be investigated. *FAM5C* has been associated with MI, atrial fibrillation, and left atrial size (Connelly et al.,

2008). These findings are in the setting on the known protective effect of the *FAM5C* SNP (rs10920501) T allele in a young population and evidence that *FAM5C* gene expression may play a role in senescence of smooth muscle cells (Connelly et al.). This begs the question of a possible antagonistic pleiotrophic effect with this SNP. The *FAM5C* gene may provide protection in early life, but have adverse effects later in life. Much more research is needed to address these questions. A better understanding of the genetically triggered pathogenesis of CHD and related interactions will open doors for additional research, risk prediction, and ultimately treatment.

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