ROLE OF THE ARACHIDONATE 5-LIPOXYGENASE ENZYMATIC PATHWAY IN CORONARY HEART DISEASE

By
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To my husband, Joseph Cote Schentrup;
my daughters, Ena Marie and Johanna Cecile Schentrup;
and my son, Maximilian Cote Schentrup

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"I can do all things, through Christ who strengthens me." Philippians 4:13

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Chair: Julie A. Johnson
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Evidence indicates that the arachidonate 5-lipoxyagense enzymatic pathway may play a pivotal role in cardiovascular disease. During this investigation, we achieved three aims. We developed a convenient and accurate pyrosequencing protocol for genotyping a repeat polymorphism in the *ALOX5* gene. We also conducted genetic association studies in populations with both early and established heart disease to determine if genetic polymorphisms in 5LO pathway genes contribute to the variability in both the occurrence and outcomes in cardiovascular disease. Furthermore, we identified a population at risk for heart disease and tested the effect of a leukotriene pathway modifier to affect levels of biomarkers of cardiovascular risk.

We developed a method to genotype an important repeat polymorphism in the promoter region of the *ALOX5* gene, which encodes 5-lipoxygenase. Genotyping this region has previously proven difficult by standard methods which has limited study of this polymorphism. The method presented here has been published and will facilitate future genetic association studies on this polymorphism.

In addition, we assessed whether genetic variation in the genes encoding the enzymes of the 5-lipoxygenase pathway contribute to cardiovascular disease in two populations. The first was in matched subjects from the Women's Ischemia Syndrome Evaluation and the St. James Women Take Heart Project (WISE-WTH) case control study, representing early heart disease. The second were from the INternational VErapamil SR /Trandolapril Study GENEtic case-control Substudy (INVEST-GENES). In the INVEST-GENES population we assessed whether these polymorphisms promote death, myocardial infarction and/or stroke in treated hypertensive patients with coronary artery disease (CAD). We found an effect for *ALOX5* and *LTA4H* polymorphisms to confer race-dependant effects on these outcomes in INVEST-GENES however these same associations were not observed in WISE-WTH.

We further conducted a double-blind placebo-controlled clinical study in subjects at risk for heart disease to determine the effect of pharmacological inhibition of the 5LO pathway leukotriene D_4 receptor by montelukast on cardiovascular biomarkers. In this pilot study, we identified an effect of montelukast to change levels IL1ra, an anti-inflammatory cytokine. Overall, we have generated tools and derived pilot information to help design future studies of the 5LO pathway in similar populations.

CHAPTER 1
BACKGROUND INFORMATION

Introduction

The classical view describing the formation of atherosclerotic plaques in humans, or atherogenesis, involves several phases that begin in adolescence and young adulthood and progress chronically throughout life. Although the specific mechanisms of atherogenesis remain unknown, the most widely accepted theory is that it is a response to endothelial injury in large and medium-sized vessels. Endothelial injury promotes vascular inflammation and negative remodeling, leading to the progression of coronary heart disease. The unfortunate end result of this progression is a cardiovascular event such as myocardial infarction or stroke.[1]

It is now well-accepted that this multi-stage process that leads to heart disease is an inflammatory condition. However, the complexity of inflammation and its signaling has raised many important questions about how heart disease originates and progresses into negative outcomes. Endothelial injury may be attributed to both environmental and genetic factors, such as an overabundance of low-density lipoprotein (LDL) cholesterol in blood or a viral insult. Atherosclerotic lesions begin to form when excess lipoproteins in the blood accumulate and aggregate in the intimal layer of arterial walls at sites of injury. In these lesions, lipoproteins bind to proteoglycan molecules and can then be oxidized. The presence of oxidized lipoproteins enhances the inflammatory response.

Leukocytes adhere to the endothelial wall. Due to the chronic exposure to processes that damage the arterial wall, this inflammatory process occurs over an extended period of time and this activity resembles a chronic inflammatory response involving recruitment of monocytes. These monocytes are recruited and directed to infuse the intima through cytokine signaling. Within the arterial wall, monocytes mature and become macrophages. A hallmark of an

atherosclerotic lesion is the presence of macrophages in the lesion indicative of a chronic inflammatory process. Over time, the plaque lesion becomes enriched with pro-inflammatory mediators including cytokines, chemokines, eicosanoids and lipid mediators. In addition, large amounts of oxidant species are concentrated in plaques.[1]

Macrophages in lesions of endothelial injury ingest oxidized LDL cholesterol that has accumulated, however they are unable to process and eliminate this material. Therefore a continuous accumulation of macrophages occurs in the arterial wall. Macrophages and other inflammatory cells that become laden with oxidized LDL form "foam cells," named for their appearance, which accumulate and form the "fatty streak", which is evident upon microscope analysis of lesions. The chronic inflammatory process also leads to the migration and accumulation of smooth muscle cells over the lesion resulting in the formation of a fibrous cap. The lesion continually increases in size and smooth muscle cells grow over the lesion which begins to obscure the flow of blood through vessels. The vessel compensates by enlarging to increase the lumen size. Further complications of atherosclerotic plaques are endothelial dysfunction, vascular remodeling, obstruction of blood vessels and changes in blood flow and ischemia. Over time, these lesions can either completely obscure the flow of blood through vessel or rupture, leading to severe consequences such as heart attack or stroke[1]. In addition to the factors leading to plaque development, factors that stimulate plaques to rupture are becoming increasingly known. One particular inflammation pathway, the 5-lipoxygenase enzymatic pathway, has received recent interest for its possible role in both of the development and rupture of atherosclerotic lesions.

The Arachidonate 5-Lipoxygenase Pathway

The arachidonate 5-lipoxygenase pathway is just one of several enzymatic pathways that promulgate inflammation. It relies on the activation of fatty acid molecules, known as eicosanoids, derived from arachidonic acid (Figure 1-1).

Arachidonic acid is released from both the nuclear envelope and plasma cell membranes from stored glycerophospholipids by the action of cytosolic phospholipases, particularly phospholipase A_2. It is subsequently metabolized primarily by cyclooxygenases and lipoxygenases to generate a variety of bioactive eicosanoids, including prostaglandins, thromboxanes, and leukotrienes. 5-lipoxygenase is active in bone-marrow derived cells. The action of 5-lipoxygenase (5LO), in coordination with 5-lipoxygenase activating protein (FLAP) (a cofactor), forms the unstable intermediate, hydroperoxeicosatetraenoic acid (HPETE), which degrades into the epoxide leukotriene A_4 (LTA4). LTA4 can be further metabolized by one of two pathways. It can be hydrolyzed to form leukotriene B_4 (LTB4), or alternatively it can be conjugated by glutathione-S-transferase and converted into the cysteinyl leukotrienes, leukotriene C_4 (LTC4), LTD_4 (LTD4) and E_4 (LTE4) (Figure 1-2). Physiologically, leukotriene B_4 (LTB4) activates neutrophil chemotaxis, whereas the cysteinyl leukotrienes (CysLTs) are important mediators of allergic inflammation. Both LTB4 and LTC4 are transported out of leukocytes by specialized proteins. In addition, LTA4 can be delivered to cells lacking all of the upstream synthetic components of the 5LO system through transcellular biosynthesis. Therefore leukotrienes may be formed in epithelial cells, for instance. Neutrophils interact with epithelial cells to deliver LTA4 where it is converted to other leukotrienes. In particular, this interaction has been found to promote CysLT formation, and to contribute to myocardial stiffness and increased coronary resistance in *in vivo* models.[2-3]

Receptors for leukotriene mediators are located on many types of cells. Receptors are G-protein coupled which when stimulated, lead to a cascade of events which further inflammation. LTB4 interacts with the BLT1 receptor and BLT2 receptor. The high-affinity receptor, BLT1, is expressed exclusively in inflammatory cells. In contrast, BLT2 is a low affinity receptor for LTB4 and is expressed in a variety of tissues such as spleen, leukocytes, and ovaries.[4] The physiological functions of BLT2 have not been thoroughly explained, however the results of recent studies appear to suggest that BLT2 plays a role in generating reactive oxygen species, implying a crucial role in the cardiovascular disease process.[5]

Role of 5LO Pathway in Cardiovascular Disease

5LO, FLAP and other downstream components of the 5LO pathway are abundant in atherosclerotic lesions[6]. Evidence indicates that both FLAP and 5LO are overproduced in mononuclear cells in the presence of oxidized low density lipoprotein.[7] In addition, as atherogenesis progresses, the number of cells expressing 5LO in the intima and media are increased.[6] 5LO and its enzymatic products are also significantly more abundant in macrophages of unstable carotid artery lesions when compared to stable lesions.[8] Furthermore, 5LO expression is positively correlated with plaque rupture.[8]

Interestingly, Allen et al.[9] found that the cysteinyl leukotrienes LTC4 and LTD4 induce concentration-dependant contractions in atherosclerotic coronary arteries. In contrast, non-atherosclerotic arteries are not responsive to these leukotrienes. This finding indicates that the 5LO pathway has specific effects on diseased atherosclerotic coronary arteries that may contribute to the worsening of CHD *in vivo*.

Genetic Variation in 5LO genes

The genes that encode the enzymes in the 5LO pathway are polymorphic and many of these polymorphisms have been found to be associated with various phenotypes associated with

inflammation. The following is a brief overview of the current evidence for these phenotypes resulting from genetic variation. The specific variants of interest are listed in Table 1-1.

ALOX5

The *ALOX5* gene encodes arachidonate 5-lipoxygenase, the rate-limiting enzyme in the 5-LO pathway. Genetic variation in the *ALOX5* gene has been proposed to affect the rate and extent of formation of leukotrienes in atherosclerotic lesions. In particular, a common polymorphism has been identified in the transcription factor binding region of the *ALOX5* promoter.[10] Variants in this region cause less efficient transcription of *ALOX5* in *in vitro* studies.[10],[11] and provide the most compelling evidence that polymorphisms in *ALOX5* are important in the development of atherosclerotic plaques. In a 2004 study, carotid intima-media thickness (CIMT) was increased on average by approximately 80 micrometers in subjects with two variant promoter alleles of *ALOX5*. This CIMT is comparable to that found in diabetics, diabetes being the strongest common cardiovascular risk factor for atherosclerotic disease. Also, levels of hs-CRP were increased two-fold among subjects with two variant alleles. Interestingly, increased dietary arachidonic acid significantly increased the atherogenic effect of the variant genotype while dietary intake of n-3 fatty acids reduced the effect. Further studies have also linked variation in the *ALOX5* promoter region to other phenotypes however these have not been universally consistent. In particular to CV disease, Assimes *et al*. were not able to identify an association between SNPs in *ALOX5* with a coronary artery disease (CAD) phenotype although one of these SNPs was correlated with the promoter variant.[12] This SNP (rs12762303), has been found to be linked to the *ALOX5* promoter alleles in a predicable way so as to serve as a surrogate marker for the genotype at this locus in humans.[12] This association is important in Caucasian patients and may also be significant in Hispanic patients, although too few subjects were assessed to draw this conclusion in Hispanics. In African American patients, little

association was identified. Other SNPs in the *ALOX5* gene have been identified as associated with various phenotypes. In particular, the SNP rs2115819[13] was identified as associated with response to montelukast.

ALOX5AP

Helgadottir *et al.*[14] highlighted the implications in cardiovascular disease of genetic variation in the *ALOX5AP* gene, which encodes arachidonate 5-lipoxygenase activating protein (FLAP). In particular, these researchers identified a haplotype, "HapA," that is associated with a nearly 2-fold greater risk of myocardial infarction and stroke in an Icelandic cohort. A further study by Hakonarson *et al.* [15] studied the use of a FLAP inhibitor to treat subjects with previous myocardial infarction who carry HapA to reduce markers of atherosclerotic disease. These researchers found that pharmacological inhibition of FLAP significantly reduced levels of several CV markers as well as LTB4 and urinary LTE4. In addition, the FLAP inhibitor also had a persistent effect to reduce hs-CRP and serum amyloid A, although these changes did not reach statistical significance in the study period. This finding suggests that *ALOX5AP* variation is an important factor in conferring CV risk and that CV markers are useful for measuring this effect. Furthermore, findings of association have been extended to stroke.[16-18]

Since the publication of these studies, others have addressed the relationship between these and other *ALOX5AP* polymorphisms and CV phenotypes. Several have found positive associations, however these results have not been consistent and may depend greatly on the phenotype that is chosen for examination. In particular, a recent meta-analysis examining SNPs and haplotypes associated with HapA and HapB in stroke was not able to identify a definitive link between these variants and a risk for stroke.[19]

LTA4H

LTA4H encodes leukotriene A_4 hydrolase. Recently, Helgadottir *et al.* [20] identified an association between a haplotype in the gene *LTA4H*, called "HapK," and both an increased production of LTB4 and an increased risk for what was defined as a "severe" myocardial infarction (myocardial infarction that occurs in subjects with peripheral vascular disease, stroke or both). The association with HapK was found in four cohorts from Iceland, Philadelphia, Cleveland, and Atlanta, respectively. Further, the association was much more pronounced in African Americans than in European Americans, although the identified haplotype was less prevalent in the African American subset overall. This result indicates that polymorphisms in *LTA4H* may confer differing risk for severe myocardial infarction to subpopulations. SNPs rs1978331 and rs2660845 in *LTA4H* have also been associated with asthma phenotypes.[13, 21]

LTC4S

The *LTC4S* gene encodes leukotriene C_4 synthase (LTC_4 synthase). A recent study in asthmatics highlights the importance of the LTC_4 synthase in the anti-inflammatory response to montelukast.[22] In particular, being a carrier of a variant allele in the 5' region of the *LTC4S* gene (A-444C) reduced the risk of having an asthma exacerbation by 80% compared to the homozygous wild-type. There were significant differences in ethnic groups were seen, where the variant allele was more frequent in whites than African Americans (30% versus 10%). In terms of cardiovascular disease, this gene has become interesting for its role in the CV risk in women. In a study by Iovannisci *et al.*, the A-444C polymorphism was found to be associated with increased carotid intima-media thickness and increased coronary artery calcium in a population of young women. [23]

5-LO Pathway Receptors

The receptors of the leukotriene pathway, while not the focus of this study, may also contain important genetic variation leading to different cardiovascular phenotypes. The receptors, however, have been less studied and will not be addressed here.

Conclusion

These previous findings serve to support a role for the 5LO pathway in CV disease development and/or progression. However, it is not clear whether intervening on this pathway could potentially positively affect the risk for heart disease in a manner that is important for clinical application. In addition, although there is evidence for the role of 5LO pathway genetic polymorphisms in heart disease, it is not clear whether these polymorphisms are important contributors to early CV disease, late CV disease, and whether they confer a higher risk for negative outcomes in patients in whom treatment for CV risk factors, such as hypertension, is already initiated. Therefore, we have two hypotheses:

1) Genetic variation in the 5LO pathway leading to variation in pathway activity will lead to variation in CV disease occurrence, progression and/or outcomes.

2) Since the 5LO pathway is involved in the development and progression of heart disease, inhibiting this pathway pharmacologically will affect cardiovascular biomarkers in such a way that indicates a reduction in the risk for heart disease.

To test our first hypothesis, we will conduct genetic association tests in human subject-derived genetic samples in subjects who are at risk for or currently exhibit heart disease to determine in genetic polymorphisms in 5LO pathway genes may contribute to the variability in both occurrence and outcomes in the spectrum of cardiovascular disease. Our study will be novel since we will explore both the occurrence of heart disease and the incidence of CV outcomes in subjects who have established heart disease.

To test our second hypothesis, we will identify a population at risk for heart disease and test the effect of leukotriene pathway modifiers to affect (presumably reduce) the level of markers of cardiovascular disease. Our study will give clues as to the potential role for the leukotriene pathway in the development of heart disease.

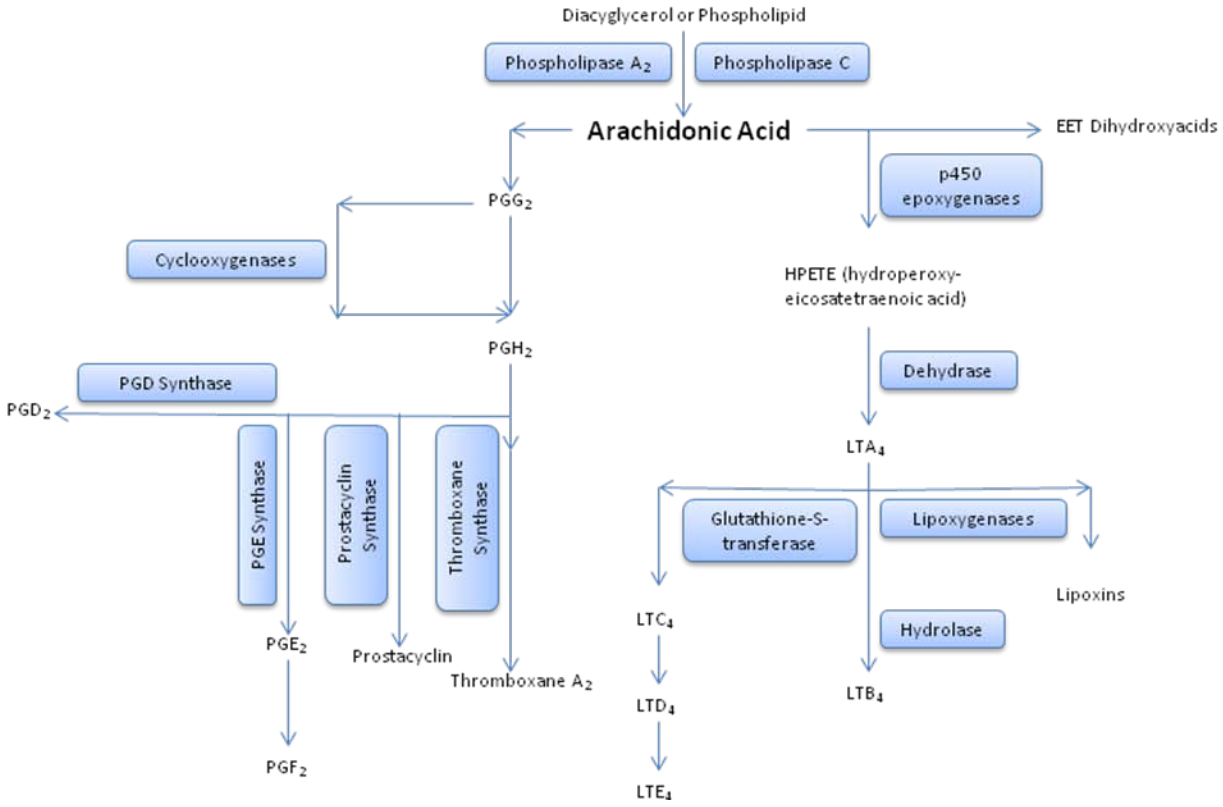


Figure 1-1. The arachidonic acid 5-lipoxygenase pathway of metabolism (PG – Prostaglandin, LT- Leukotrienes)

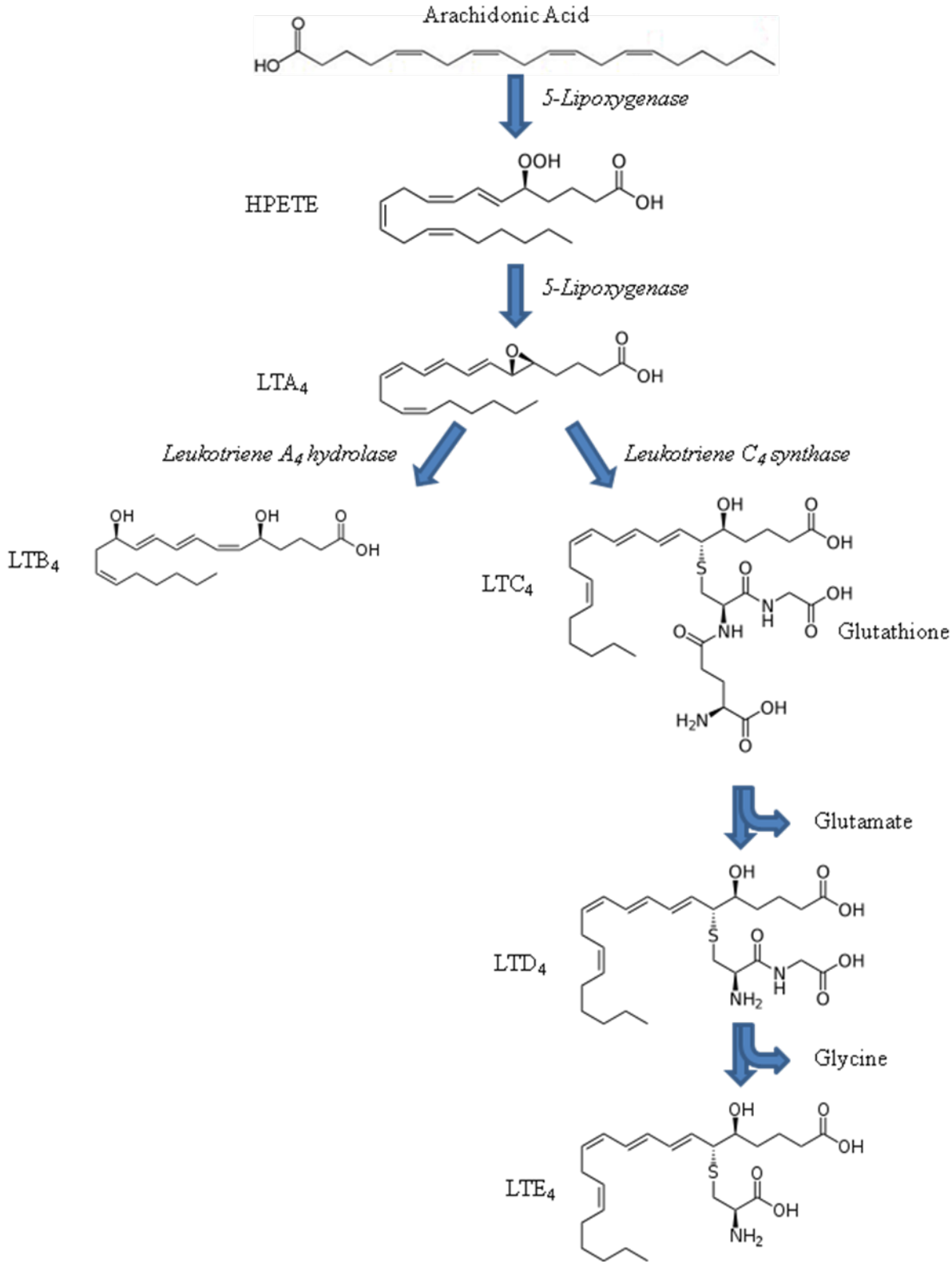


Figure 1-2. Leukotriene synthesis.
HPETE - hydroperoxyeicosatetraenoic acid, LT - leukotriene

Table 1-1. 5-LO pathway variants to be studied

Gene	Chromosome location	Variants	Type of variant
ALOX5	10q11.2	Repeat	promoter
ALOX5AP	13q12	rs4769874	intronic
		rs9551963	intronic
		rs10507391	intronic
		rs17222814	5' upstream region
LTA4H	12q22	rs2660845	5' upstream region
		rs1978331	intron
LTC4S	5q35	rs730012	promoter

CHAPTER 2
ALOX5 PROMOTER POLYMORPHISM ASSAY DEVELOPMENT

Introduction

The *ALOX5* gene encodes arachidonic acid 5-lipoxygenase, the rate-limiting enzyme in the arachidonate 5-lipoxygenase inflammatory pathway. A repeat polymorphism located in the promoter region of this gene is thought to modify the number of binding sites for both Sp1 and Egr-1 zinc-finger transcription factors and may affect transcription of the *ALOX5* gene and thus the amount of 5-lipoxygenase enzyme produced.[24] As such, this polymorphism has been studied in both *in vitro* and clinical studies which implicate it as an important marker for inflammatory diseases, including cardiovascular disease and asthma.[11, 25-27] The role of this polymorphism in these phenotypes is thought to be important across race populations; however previous studies have included limited numbers of subjects from various race or ethnicity groups. This limits the power in these studies to draw decisive conclusions about the role of this polymorphism in diverse populations.[11, 13] We sought to assess the association between this polymorphism and two clinical study populations (described in Chapter 3), however an efficient and effective genotyping method was not readily available. Thus we first developed and validated a novel genotyping method using pyrosequencing technology.

Genotyping the *ALOX5* repeat polymorphism in large clinical studies to further assess its role in disease or in drug response poses particular challenges. The polymorphism is a series of tandem repeats of the nucleotide sequence 5'-GGGCGG-3'. In humans, the variant alleles are formed by varying numbers of these repeats which can range from 2 to 8. The most common allele contains 5 repeat sequences and the three most common variants have a 6-base-pair (1 repeat) deletion, a 12-base-pair (2 repeats) deletion or a 6-base-pair addition, respectively. The GC-rich nature of this genetic region (about 78%), lends itself to considerable secondary

structure in the arrangement of the DNA in this region which presumably accounts for difficulties in genotyping or sequencing this region by traditional methods. In previous studies, this polymorphism has only been genotyped by direct sequencing technology or traditional repeat size fractionation methods, [10, 13, 28] which limits the application to large sample sets.

A SNP in the *ALOX5* promoter region has recently been identified that may be in sufficient linkage disequilibrium in Caucasians to serve as a marker for the wild-type allele of the promoter polymorphism[12]. These researchers found that this SNP does not sufficiently correlate with the repeat polymorphism alleles in African Americans and the correlation between this SNP and the repeat polymorphism in a Hispanic population was assessed in a very small sample size (6 subjects). As such, it is not clear whether this SNP is a suitable marker in highly admixed Hispanic (or other) populations.[12] Therefore, an alternative method to genotype the repeat polymorphism directly is still needed.

Pyrosequencing technology offers a flexible system for genotyping and short sequencing applications. The technology is based on sequencing by synthesis. This method is amenable to determining unknown DNA sequence and also to genotyping multiple allele systems or tandem repeat polymorphisms where simple allelic discrimination is not possible. The read length (up to 250 base pairs depending on the application) makes it particularly relevant in short tandem repeat polymorphism genotyping. However, one shortcoming of this technology is that genotyping "difficult" regions of DNA can result in problems that are similar to those encountered using traditional sequencing methods.[29] These difficulties occur with sequences that include long homopolymers, particularly when this is compounded by a long read-length polymorphism where the pyrosequencing signal drops off as sequencing continues.[30] Furthermore, GC-rich regions are also difficult to genotype by this method and result in very weak signals using

traditional methods.[29] This was evident in our early attempts to genotype the *ALOX5* promoter region using a standard polymerase chain reaction (PCR) amplification protocol and pyrosequencing method. Furthermore, there may also be other factors that are not currently identified that cause this particular polymorphism to be challenging to genotype. Although currently published procedures for amplifying this region of the *ALOX5* promoter are sufficient for various direct sequencing methods, these methods do not allow for sufficient amplification of the region of interest for pyrosequencing based on our experience. Our goal in developing this assay was to overcome the barriers to using pyrosequencing technology to assess the *ALOX5* promoter polymorphism. We hypothesized that an efficient, robust method for amplifying the promoter region by PCR would render the amplicon of sufficient quality to accurately genotype using pyrosequencing methods.

Methods

We developed a method for genotyping the polymorphic sequence in the promoter region of the *ALOX5* gene which effectively addressed the difficulties in genotyping this region. One issue was the potential secondary structure evident in this GC-rich region. Much of this secondary structure can be attributed to syn-anti base pairing of nucleotides (Figure 2-1). We addressed this by utilizing a modified guanine nucleotide, 7-deaza-deoxyguanine, as one of the nucleotides added to our PCR (Figure 2-1). This nucleotide has a modification where atom 7 is changed from nitrogen to carbon. When used in the place of guanine nucleotides, syn- base pairing is eliminated thus reducing the possibility of secondary structure interfering with PCR amplification.

We further optimized the polymerase chain reaction (PCR) protocol for amplification of the genetic polymorphism using a nested PCR approach. This approach was chosen to reduce the occurrence of non-specific bands that were observed by gel-electrophoresis while using

standard PCR conditions to amplify this region. Briefly, a nested PCR method utilizes a set of primers which flanks the target region. These are used in a traditional PCR method. After completion of the first PCR, a second set of primers binding to sequences located closer to the target region are used in a second round of amplification to more specifically amplify the region of interest. A description of nested PCR amplification is given in Figure 2-2.

A gene amplicon surrounding the promoter repeat sequence was prepared using a nested PCR method. DNA samples were utilized at a concentration of 10 ng/µL PCR reactions were conducted using approximately 5 ng of genomic DNA derived from buccal cells. PCR reagents for both nested reactions are given in Table 2-1. Primers were purchased from Operon (Huntsville, AL) and MJ (Waltham, MA) thermal cyclers were used. Primer sequences are listed in Table 2-2. Both nested PCR reactions were conducted using Platinum® Taq polymerase (Invitrogen, Foster City, CA), GeneAmp® 10X Gold Buffer (Applied Biosystems, Foster City, CA) and 7-deaza- deoxyguanine (Roche, Pleasanton, CA). 7-deaza-deoxyguanine was added to deoxyguanosine triphosphate (dGTP) in a ratio of 3:1, and this mixture was added in a 1:1:1:1 ratio with 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine-5'-triphosphate (dCTP) and 2'-deoxythymidine-5'-triphosphate (dTTP) to form the mixture of nucleotides. The second PCR was conducted using biotinylated primers appropriate for pyrosequencing per manufacturer's instructions (Biotage AB, Uppsala, Sweden). Ten microliters of this reaction mix were used for pyrosequencing. Conditions for the nested PCR reactions are given in Table 2-3. In particular, in the second amplification sequence of the nested PCR given in Table 2-3, a touch-down PCR style approach is taken. In this particular approach, the PCR amplification protocol has two phases. In the first phase, the annealing temperature that is used is very close to the melting temperature of the primers. After, in this case, 10 rounds of amplification, the

annealing temperature is dropped to 60°C for 35 rounds of amplification to increase primer binding and improve efficiency of the reaction.

Genotyping by pyrosequencing was conducted using the standard manufacturer protocol (Biotage AB). Briefly, 10 μL of biotinylated PCR product was immobilized on streptavidin-coated Sepharose beads in binding buffer (10 mmol/L Trizma base, 2 mol/L NaCl, 1 mmol/L EDTA, and 0.1% Tween 20, pH 7.6) to a volume of 82 μL at room temperature for 10 minutes. After incubation, beads with bound DNA were isolated by hand-held vacuum probe, and then treated for 5 seconds successively with 70% ethanol, 0.1 M NaOH, and washing buffer (10 mM Tris-acetate). Bound DNA was released into a solution of annealing buffer (20 mmol/L Tris acetate and 5 mmol/L magnesium acetate, pH 7.6) and 10 pmol of sequencing primer of the sequence 5'-CAGGCTCCCGGCTGCCC-3'. This was heated to 80 °C for 3 minutes and then cooled to room temperature.

Pyrosequencing was performed using a Biotage PSQ HS 96 System. The sequence to analyze was CCGCCCCCGCCC[CCGCCC][CCGCCC][CCGCCC][CCGCCC]CCGCA. The pyrosequencing method was capable of detecting 2 to 6 repeats on each allele. During the development of this method, it was observed that the pyrosequencing signal deteriorated some after sequencing 6 repeats (> 40 base pairs). This led to some ambiguity once the number of repeats was greater than 6, therefore it was decided to design the assay to detect up to 6 repeats. Any allele with more than 6 repeats appeared to the observer as the presence of 6 repeats, and therefore particular genotypes with alleles having greater than 6 repeats cannot be determined using this method. The resulting pyrograms were visually assessed and verified by a second observer blinded to the first observer's genotype determinations. The quality of pyrosequencing

calls were assessed by comparing them with 16 DNA samples with known repeat polymorphism genotypes determined by previous methods.

Results and Discussion

The predicted histograms and resulting pyrograms from pyrosequencing are shown in Figure 2-3. For each genotype listed, example pyrograms (top half for each genotype) are given from actual pyrosequencing assays for each *ALOX5* genotype observed. Predicted histograms (bottom half for each genotype) are given for each genotype generated by pyrosequencing software for the *ALOX5* repeat polymorphism genotypes observed in this analysis.

Upon comparing our resulting genotypes with known genotypes that were obtained by traditional sequencing, results were 100% concordant. The *ALOX5* repeat polymorphism genotype frequencies for European Americans, Hispanics and African Americans from the 834 INternational VErapamil SR/Trandolapril Study – GENEtic Substudy (INVEST-GENES)[31] samples and in the 188 St. James Women Take Heart (WTH)[32] samples (European Americans and African Americans) are given in the Figure 2-4 A and B. In addition, genotype frequencies for each common genotype in Hispanic subjects from INVEST-GENES are given in Figure 2-4 C. Frequencies are listed in the Appendix, Table A-1.

The described method for analyzing the *ALOX5* repeat polymorphism has facilitated our analysis of this genetic polymorphism in populations of over 1,000 subjects who are racially and ethnically diverse, and promises to facilitate others. We have successfully implemented this alternative method for genotyping this polymorphism using a nested PCR method and pyrosequencing technology. The relative differences between the genotype frequencies between European American and African American subjects which have been previously identified remained true in this sample, supporting the accuracy of this method for genotyping the *ALOX5* promoter polymorphism. Of note, the frequency of homozygote variants in African American

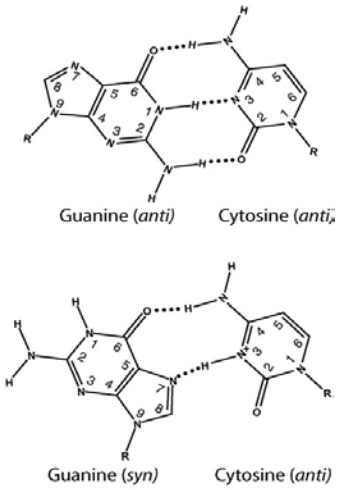
subjects was significantly higher in our samples as compared to previous studies.[13] However, based on both the comparison to samples of known genotypes and also because the DNA of subjects from all race groups were positioned randomly on the 96-well plates, we are confident that the genotypes we obtained are accurate and that differences in frequencies most likely reflects the particular populations we analyzed. Specifically, since the DNA samples used in this study come from patients with or at risk for CAD and the *ALOX5* gene purportedly confers risk for CAD, we would expect the frequency of variant carrier alleles in our population may differ from previous reports in healthy individuals or those without CAD or significant risk factors for CAD. The differences in cardiovascular risk may also account for the differences between the frequency of variant genotypes between the INVEST-GENES African Americans and the WTH African Americans as well (Figure 2-4 A and B).

Several studies have reported the *ALOX5* repeat polymorphism plays a role in several disease phenotypes, including coronary artery disease[11] and asthma.[26-27] Furthermore, it has been found to influence the response to leukotriene modifiers used in the treatment of asthma.[13] Also, *ALOX5* promoter variants appear to have functional consequences in terms of gene expression,[10, 26] although these differences are not consistent across cell-types .[33] Since the allele frequencies of *ALOX5* promoter variants vary considerably between race groups and alternatives to direct genotyping of this polymorphic region are not currently sufficient to characterize this polymorphism in all race groups, the method which has been described is important to further validate the roles of this polymorphism in inflammatory phenotypes.

In conclusion, pyrosequencing offers a convenient and accurate method for genotyping the *ALOX5* promoter repeat polymorphism up to 6 repeats per allele. This method utilizes the reagent 7-deaza-deoxyguanine and also the methods of nested PCR and touch-down style PCR

conditions to optimize this reaction. The method presented has been published and will facilitate both genetic association and pharmacogenomic research on this polymorphism in large samples that are ethnically and/or racially diverse.

A



B

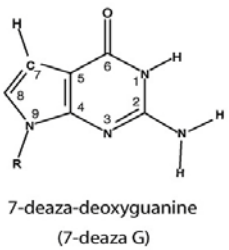


Figure 2-1. Modified nucleotide base-pairing.
A) Anti- and Syn- base-pairing of guanine and cytosine. Syn- base pairing can lead to secondary structure in GC-rich regions. B) 7-deaza-deoxyguanine molecule, has modification at atom 7 (nitrogen to carbon) eliminating the possibility of syn base-pairing

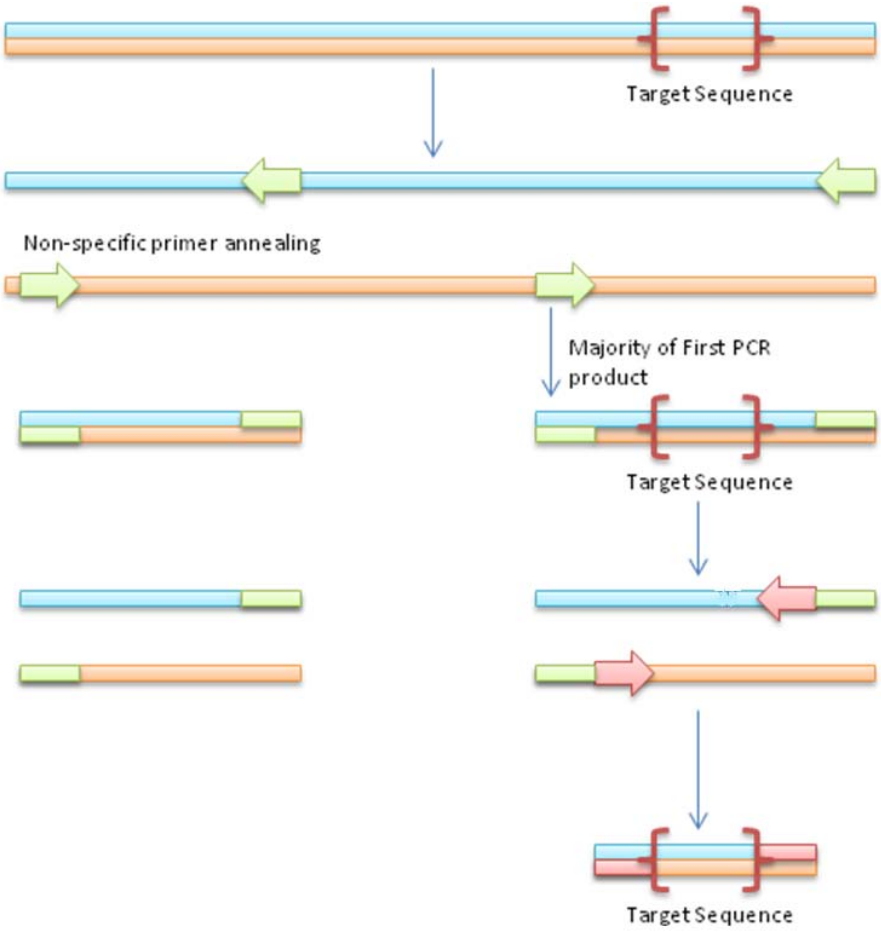


Figure 2-2. Nested PCR scheme.
A target sequence is flanked by primers that also non-specifically amplify another DNA region. A second pair of internally nested primer are used on the first PCR product which amplify around the target sequence but which do not result in non-specific primer amplification.

Table 2-1. PCR reagents and concentrations for both nested reactions

Reagents	Concentration
Platinum Taq polymerase	1.25 U
10X ABI Gold Buffer	1X
Dimethyl sulfoxide	2%
Nucleotide mixture 0.1 mM	0.1 mM
MgCL	0.75 mM
Genomic DNA	5 ng

Table 2-2. Primers (5' to 3')

Primer Sequences	Amount
PCR 1 Forward: GGGTGGCAGCCGAGGT	10 pmol
PCR 1 Reverse: TGTCCAGCAGGTGCTTCTCGC	10 pmol
PCR 2 Forward: Biotin-AGGAACAGACACCTCGCTGAGGAGAGAC	10 pmol
PCR 2 Reverse: GAGCAGCGAGCGCCGGGAGCCTCGGC	10 pmol
Sequencing: CAGGCTCCCGGCTGCCC	10 pmol

Table 2-3. Nested PCR conditions

1st Reaction Step	Temperature/Time	Number of cycles
Initiate	94 / 2 min 1	1
Denature	94 / 30 s	40
Annealing	60 / 30 s	
Extension	72 / 30 s	
Extension	72 / 5 min 1	1
2nd Reaction Step	**Temperature/Time**	**Number of cycles**
Initiate	95 / 12 min 1	1
Annealing	94 / 1 min	10
Extension	68 / 2 min	
Denature	94 / 30 s	25
Annealing	60 / 30 s	
Extension	72 / 45 s	
Extension	72 / 5 min 1	1

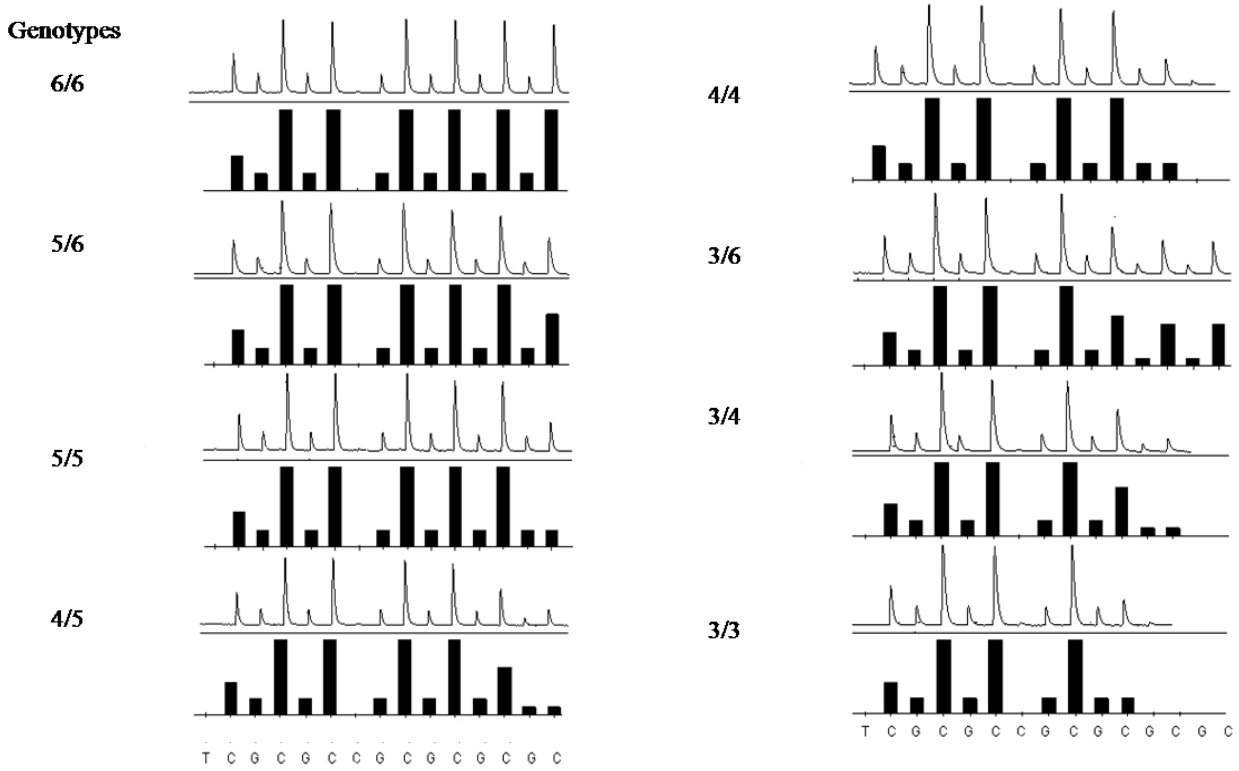


Figure 2-3. Pyrograms and predicted histograms generated through pyrosequencing. For each genotype listed (notation is # *ALOX5* polymorphism repeats in allele1 /# *ALOX5* polymorphim repeats in allele 2), example pyrograms (top half for each genotype) are given from actual pyrosequencing assays for each *ALOX5* genotype observed and confirmed by direct sequencing. Predicted histograms (bottom half for each genotype) are given for each genotype generated by Pyrosequencing software for *ALOX5* polymorphism genotypes observed in the analysis.

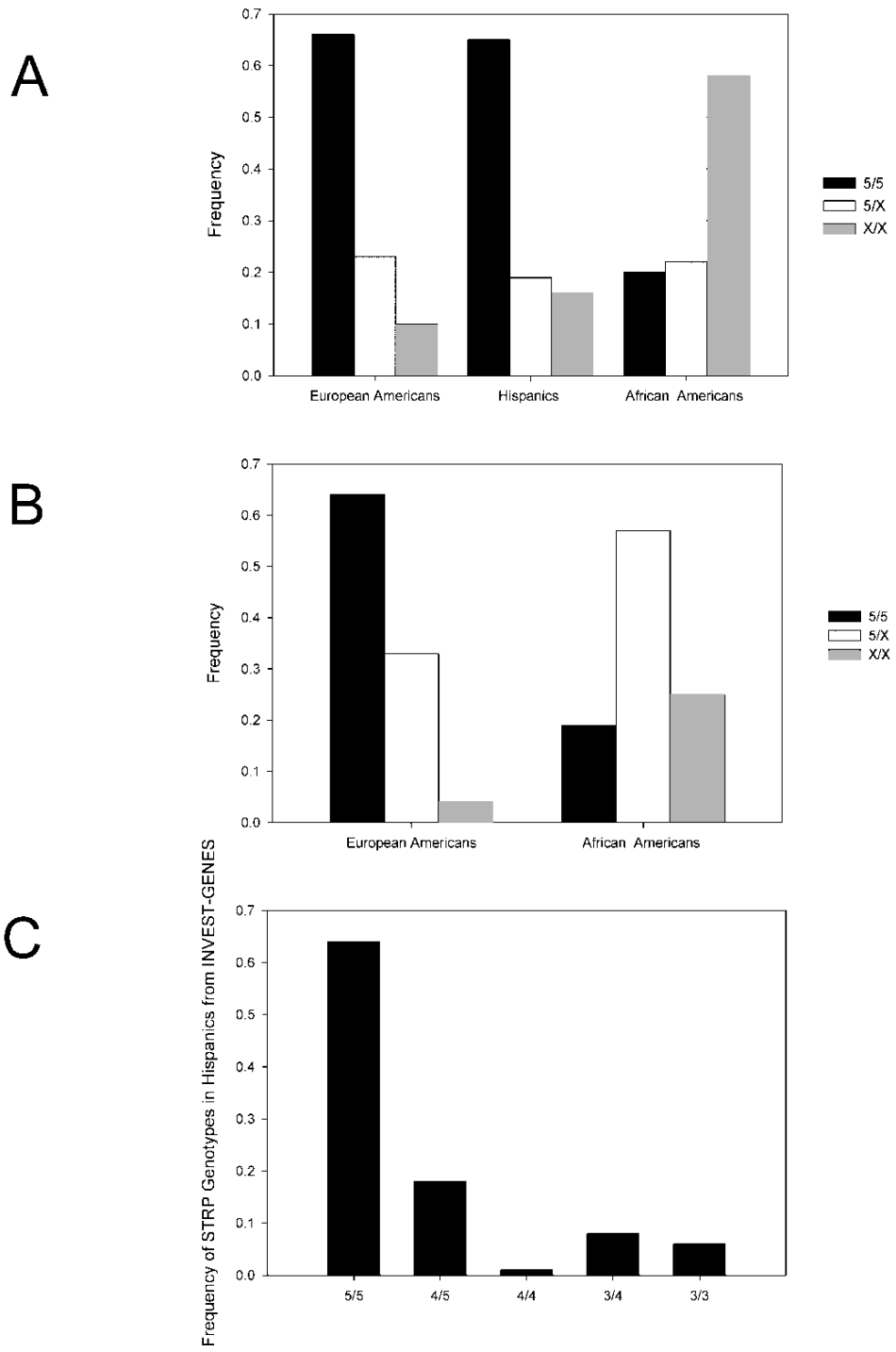


Figure 2-4. Genotype frequencies of the *ALOX5* promoter polymorphism.
A) Frequencies in European Americans, Hispanics and African Americans in INVEST-GENES and B) in European Americans and African Americans from WTH (Hispanic ethnicity is not largely represented in WTH). Genotypes are given as wild-type (5/5), one wild-type allele and one variant allele (5/X) and 2 variant alleles (X/X) in both figures. C) Genotype frequencies for each common genotype in Hispanics INVEST-GENES. Genotypes are given as # *ALOX5* polymorphism repeats in allele1 /# *ALOX5* polymorphism repeats in allele 2.

CHAPTER 3
GENETIC ASSOCIATION STUDIES

Introduction

Based on previous evidence as presented in Chapter 1, the arachidonate 5-lipoxygenase (5LO) enzymatic pathway potentially plays a role in the development and progression of heart disease. Furthermore, these findings indicate that this effect may be modulated at the genetic level, where polymorphisms in 5LO pathway genes may be responsible for conferring some risk for developing CV disease and/or furthering disease progression which leads to CV outcomes such as stroke or myocardial infarction (and ultimately death). However, because of conflicting previous results, it is as yet unclear what the contribution of this pathway is in the spectrum of heart disease, *i.e.* whether this pathway plays a role in the incidence of disease or later on in the progression of disease, or whether its contribution is important all along this continuum. Also, some previous findings indicate that polymorphisms in this pathway may affect males disproportionately to females, and also may confer risk in race populations differently. In order to more fully elucidate the relationship between the 5LO pathway genes and CV endpoints which occur along the continuum of CV disease, we conducted two case-control genetic association studies.

First, in order to address our hypothesis that genetic polymorphisms in 5LO pathway genes confer an increased risk of developing heart disease, we evaluated the occurrence of these polymorphisms in subjects with newly diagnosed coronary artery disease (CAD) as compared to a similar population with risk factors for heart disease but who do not have a diagnosis of CAD. In addition, in order to assess our hypothesis that these same genetic polymorphisms confer an increased risk for the occurrence of negative cardiovascular outcomes in patients with CAD, we evaluated the association of these polymorphisms with the first occurrence of myocardial

infarction, stroke or all-cause death in a clinical trial population of subjects with established coronary artery disease. In addition, we secondarily addressed questions concerning the effect of gender and race on the relationship between this pathway and CVD.

Methods

Case –Control Study #1: Women's Ischemia Syndrome Evaluation (WISE) – St. James Women Take Heart Project (WTH) Case-Control Study

We studied relationships between 5LO pathway genotypes and the incidence of CHD in women in a case-control design. The case-control study group is formed by matching subjects from the Women's Ischemia Syndrome Evaluation (WISE) with subjects from the St. James Women Take Heart Project (WTH).

The WISE study enrolled women from 1996 - 1999 from 4 US locations. It included women who were 18 years of age or older and who were undergoing a clinically-indicated coronary angiogram as part of their regular medical care for chest pain symptoms or suspected myocardial ischemia. Major exclusion criteria for the WISE study included presence of cardiomyopathy, New York Heart Association class IV congestive heart failure, recent myocardial infarction and significant valvular or congenital heart disease.[34] As part of the study, blood samples were collected for analysis of various laboratory parameters and for DNA collection. Coronary angiography was performed on participants and included quantification of epicardial coronary artery size and left ventricular ejection fraction, and also qualitative and quantitative assessment of epicardial coronary artery stenoses. These were designated as levels of coronary artery disease severity as follows: normal/minimal disease = <20% stenosis; mild = 20% to 49% stenosis; significant =≥50% stenosis in any one ma jor epicardial coronary artery. WISE subjects who have < 50% coronary artery obstruction have been adjudicated and determined to have primarily microvascular disease. In contrast, WISE subjects with more than

50% coronary artery obstruction are considered to have primarily macrovascular disease.[35] WISE subjects were contacted at six weeks and then annually to assess various factors, including symptom status, occurrence of cardiovascular events, nonfatal myocardial infarction, coronary angioplasty, coronary bypass surgery, cardiac transplantation and hospitalization for unstable angina, and death. For our case-control study, information at baseline was used to match these subjects (designated "cases") to control subjects selected from the St. James Women Take Heart Project (WTH).

For the St. James Women Take Heart Project (WTH), women from the greater Chicago metropolitan region were recruited to participate in 1992. Subjects were included if they were 35 years or older, had a lack of symptomatic CAD and had the ability to walk on a treadmill at a moderate pace. Women were excluded if they had typical anginal symptoms or myocardial infarction within the previous 3 months, had blood pressures of ≥170/110 mm Hg before initiating the stress test, weighed >325 pounds (because of equipment limitations), or were pregnant. After obtaining informed consent, participants provided data on demographics, lifestyle, behavioral variables, and medical history by self-administered questionnaire, and physical and laboratory assessments were made. Although these subjects had risk factors for CHD, a large majority of these subjects represent a population that did not have active CHD at enrollment. The subjects were followed for 10 years for the primary outcome of all-cause death.[32]

For our case-control analysis, cases are defined as women who were enrolled in the WISE study cohort (N=632) and therefore represent women who have clinical heart disease. Furthermore, WISE samples were separated into 2 groups depending on the degree of stenosis identified in the WISE angiography studies: >50% stenosis and </= 50 %. Control subjects were

selected from the WTH study (N=632). For the purposes of our case-control study, subjects in WTH who had sustained a previous myocardial infarction were excluded. Frequency matching was conducted separately for the two stenosis degree groups in WISE to subjects from WTH based on deciles of age and race, which was self-reported as white or black in both studies based on age and race. Other races were excluded because of low numbers of available samples from which to draw conclusions. Table 3-1 gives a comparison of the WISE and WTH subjects studied as part of the current study.

Case Control Study #2 - International Verapamil SR/Tradolapril Study (INVEST) – GENEtic Substudy

For our second case control study, we assessed relationships between 5LO pathway polymorphisms and the development of adverse outcomes in subjects with existing CHD using a nested case-control study design within the INVEST.[34] The INVEST clinical trial evaluated adverse cardiovascular outcomes occurring with either an atenolol-based or a verapamil SR-based hypertension treatment strategy in 22,576 patients with documented coronary artery disease (CAD) and hypertension. The primary outcome was defined as the first occurrence of death (all cause), nonfatal myocardial infarction (MI) or nonfatal stroke. The design, protocol, and primary outcome have been published in detail elsewhere.[34, 36] Briefly, the protocol assessed patients at baseline, 6, 12, 18, and 24 weeks, and then every 6 months thereafter until 2 years after the last patient was enrolled. At each visit, patients had blood pressure and heart rate measured, clinical assessments performed and additional antihypertensive medications added as needed to meet Joint National Commission VI (JNC VI) blood pressure goals.[37] Subjects were randomized to receive either atenolol 50mg daily or verapamil SR 240 mg daily. If additional blood pressure control was required, atenolol users had hydrochlorothiazide 25 mg daily added and verapamil users had trandolapril 2 mg daily added. These doses were up-titrated over a

course of 12 weeks to attain appropriate blood pressure control. If control was not attained at maximum doses at 12 weeks, atenolol users could then be given additional trandolapril and verapamil users could be given HCTZ. Subjects were followed for occurrence of the primary outcome over an average follow-up time of 3.5 years.

Of the subjects participating in INVEST, genetic samples were collected from 5,979 INVEST subjects residing in mainland United States and Puerto Rico. Genomic DNA was collected using buccal cells from mouthwash samples as previously described.[31] All patients provided written informed consent for participation in the genetic sub-study and the study was approved by the University of Florida Institutional Review Board. Of these 5,979 INVEST-GENES subjects, 258 subjects (as of the last assessment in 2006) had sustained a primary outcome event by the end of the study follow-up time. In order to facilitate genetic association studies in the INVEST-GENES population, a case-control group of samples was assembled previous to the current study. In brief, the 258 subjects who sustained a primary outcome event were designated cases, and were frequency-matched by deciles of age, self-reported race and sex in a ratio of 3:1 to subjects from the INVEST-GENES population who had not sustained a primary outcome event, designated control subjects.[31] Table 3-2 gives a comparison of the case and control subjects in our analysis.

DNA Isolation and Preparation of DNA Plates

Previous to our current study, DNA was extracted from INVEST-GENES samples, WISE samples and most of the WTH samples, and stored appropriately. Approximately 50 WTH samples required DNA extraction using a commercial kit (Qiagen, Valencia, CA) to obtain DNA from whole blood for the purposes of this study. The concentrations of all of the WISE and WTH DNA samples were assessed using a spectrophotometric analyzer (Nanodrop Technologies, Wilmington, DE) using 2 µL of stock DNA/water solution. All samples from

WISE and WTH were normalized using DNAase free water which was added to each sample on 96-well plates to comprise a 10 ng/µL concentration DNA solution in each well. INVEST-GENES samples had been previously normalized.

Genotyping

Polymorphisms in several genes were chosen for genotyping as listed in Table 3-3. In particular, these polymorphisms had been previously described as having a potential effect on the development and/or the progression of cardiovascular disease or other inflammatory disease as described in Chapter 1. Specifically, SNP rs1978331 was only genotyped in INVEST-GENES case control because of the very low number of African Americans in WISE-WTH and because any expected associations were in this group only. Study samples were genotyped using validated genotyping methods as the experimental conditions required. Our primary genotyping methods were the Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Pyrosequencing (Biotage AB, Uppsala, Sweden).

Taqman allelic discrimination assay

The Taqman allelic discrimination assay discriminates between two polymorphic alleles based on the incorporation of one or two fluorescent markers depending on the genotype of the sample at the locus of interest. Based on this genotype, the sample is detected as having one of two types of fluorescent marker (one for homozygotes of one allele, one for homozygotes of the other allele) or both markers (for heterozygotes). To conduct the assay, genotyping reactions were carried out using 2.5 µL Taqman Genotyping Master Mix (Applied Biosystems), 0.0625 µL of probe (with primers), 1.438 µL water, and 10 ng (1 µL) of normalized genomic DNA. The PCR primers and probes were purchased from Applied Biosystems. Primer sequences are proprietary and are purchased from Applied Biosystems in solution conjugated to fluorescent markers. Catalogue numbers for the primers are given in Table 3-3. The samples were exposed

to the following thermocycling conditions: 10 minutes at 95°C, then 15 seconds at 92°C and 1 minute at 60°C repeated 45 times. The Applied Biosystems 7900 HT SNP genotyping platform was used to assess genotypes.

Pyrosequencing assay

Pyrosequencing was used to genotype the *ALOX5* repeat promoter polymorphism, as described in Chapter 1. Pyrosequencing methods for genotyping this polymorphism in the context of this genetic association study have been previously published and are given in chapter 2.[38] Genotyping in this manner was performed on the described samples in both the INVEST-GENES and in the WISE-WTH case-control study.

Statistical Analyses

All statistical analyses were conducted using SAS version 9.13 (Cary, NC) or SPSS version 17 (Chicago, IL). Haplotypes in *ALOX5AP* were derived separately for each racial/ethnic group using PHASE 3.2 (Chicago, IL). Statistical methods for analysis of both case-control studies were similar. Power analysis was conducted using QUANTO ver. 1.2.4 (Los Angeles, CA) to calculate the effect size that we could detect with the power derived from these two fixed-population size studies. For the studied SNPs in both the INVEST-GENES case-control and the WISE-WTH case-control, we planned to do each analysis by race so our power to detect genetic associations is given by race group. For INVEST-GENES, at the 80% power level for minor allele frequencies (MAF) ranging from 0.15 to 0.5, the odds ratios that we can detect are as follows: Caucasians: 1.43-1.59, African Americans: 2.24-2.58 and Hispanics: 1.8-2.04. In WISE-WTH, these values for the same parameters are Caucasians: 1.29-1.4, and for African Americans: 1.82-2.16.

Hardy–Weinberg equilibrium was calculated separately by race/ethnicity using the X^2 test with one degree of freedom. A two-sided p-value of $P<0.05$ was considered significant for all

analyses since analyses were based on previous findings as described in Chapter 1. Unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for occurrence of the primary outcome in each study were calculated using logistic regression. For the WISE-WTH case-control analysis, the covariates included were age, race, BMI, diabetes, degree of coronary artery obstruction (< 50% and ≥ 50%) and Framingham risk score, since these covariates were found to be significant in analyses that did not include genotype. Our primary analysis in WISE-WTH grouped the WISE subjects together irrespective of the percent coronary obstruction. We used degree of coronary obstruction (≥50 or < 50%) as a covariate rather than splitting the sample and analyzing these groups separately. The adjusted logistic regression model for the INVEST-GENES case-control analysis contained covariates from the primary INVEST analysis that were found in the original INVEST analyses to influence the occurrence of CV outcomes. These are age, sex, race/ethnicity, BMI, previous myocardial infarction, history of heart failure, history of diabetes, history of renal insufficiency, and treatment strategy assignment in the original INVEST protocol as previously described.[36] Our primary analyses were conducted using additive statistical models, however all associations were tested as additive, dominant and recessive models so as not to include assumptions about the nature of inheritance for the effects of these polymorphisms and because previous studies are not consistent in the models used. For the INVEST-GENES case-control analysis, we also tested controlling for population stratification using a total panel of 87 AIMs, selected to show large allele frequency differences across three parental populations (West Africans, Indigenous Americans, and Europeans). Maximum likelihood was previously used to estimate each patient's individual ancestry proportions from these three groups. These terms were included in statistical models after first including self-reported race/ethnicity only if models were significantly improved by them.

Results

Genotyping

For our WISE-WTH case control analysis, although 632 of each of WISE and WTH samples were planned for analysis, 586 WISE samples and 628 WTH samples were available in sufficient quantity for genotyping. All 1032 samples for the INVEST-GENES case-control were available. Genotyping success rates are given in Table 3-4. These rates varied markedly between the WISE-WTH study and the INVEST-GENES study, potentially due to several samples with low DNA in the WISE-WTH study. Since these failed genotypes occurred randomly across our assay plates and there were no significant deviations published allele frequencies by race and from Hardy-Weinberg for most of these SNPs except as noted below, we do not believe that these failures accounted for any bias in our genotyping data. For polymorphisms assessed by Taqman assay, the quality of genotyping was assessed by genotyping 5% blinded duplicate samples which had a match rate of > 99%. For the *ALOX5* promoter polymorphism, genotypes were called manually and quality control was conducted on a 5% repeat sample according to the methods described in Chapter 2, which had a match rate of > 99%.

The minor allele frequency for each single genotype was assessed by race and each is given in Table 3-5. Further reported is the frequency of our *ALOX5AP* haplotype of interest, GTA (rs17222814 (G) – rs10507391 (T) – rs17222814 (A)), which was compared with previous reports and found to be similar to previous reports.[39] When compared to HapA, which is the haplotype previously found to be associated with negative cardiovascular outcomes,[14] our haplotype GTA did not incorporate one of the 4 SNPs (rs4769874). This SNP was found to have a very low frequency (5% in Caucasians[40] and 11% in African Americans) and did not provide significant discriminatory power between HapA and the haplotype GTA which we studied.

Furthermore, when we attempted to genotype this SNP using a custom Taqman assay and the genotyping conditions as previously described, the resulting genotypes were not in Hardy-Weinberg Equilibrium for any race group. Therefore we concluded that there were problems genotyping this SNP with this particular assay. Because of the little information gained with this SNP, we did not further pursue genotyping. In addition, there was one additional deviation from Hardy-Weinberg Equilibrium ($p < 0.05$) for SNP rs10507391 in Hispanics only. Since this deviation did not also occur in African Americans and Caucasians and all of these samples occurred randomly on plates together and were genotyped in batches, we believe this to be a random effect of our sample or may be due to ancestral admixture (as we have noted with other SNPs in Hispanics) and not an indication that there were genotyping errors. .

The minor allele frequency for each SNP and for the *ALOX5* promoter polymorphism was assessed by race and compared with previous findings (Table 3-6). These were found to be similar to previous findings for Caucasians, however the variant alleles were more highly represented in our sample of African Americans in INVEST-GENES (little data available on Hispanics).[13] This may be attributed to the composition of our study population which is derived from subjects who all have CAD. Variant *ALOX5* promoter alleles make up the most highly represented alleles in the African American population as compared to the Caucasian and Hispanic populations. Assuming that there is an effect of the variant allele to confer disease or outcomes, in a population of subjects who all have CAD such as in INVEST, there would be a much more noticeable increase relative to a disease-free population in the number of carriers of variant alleles in the African American group than in Caucasian and Hispanic populations where the variant alleles are not highly represented and presumably contribute a much smaller amount to the variation in the occurrence of CAD.

All genetic association study findings were given as dominant models in the text. Additive and recessive models are given in Appendix B. Adjustment for ancestry did not change the statistical significance of any of the models tested and therefore, since ancestral markers were not available for all subjects in equal quantities, these were not included in the results given here.

Genetic Association Studies by Gene: *ALOX5*

ALOX5 alleles were grouped into the number of wild type (5 repeat) alleles (0, 1, or 2). Subjects with more variant *ALOX5* promoter polymorphism alleles were not found to be more highly represented in WISE as compared to WTH in either Caucasians or African Americans (Table 3-7), suggesting a lack of association with CAD. We further assessed the *ALOX5* repeat polymorphism in the INVEST-GENES case control and found that it was significantly associated with an increased risk for the primary outcome, particularly in Caucasians, and was of borderline significance in African Americans (Table 3-7). This association persisted in adjusted analyses.

ALOX5AP

We assessed single *ALOX5AP* SNP associations for the SNPs rs17222814, rs10507391, and rs17222814 in both WISE-WTH and INVEST-GENES. These SNPs were not found in higher frequency among cases in either WISE-WTH or INVEST-GENES. The same was true when we assessed the *ALOX5AP* GTA haplotype in both studies (Table 3-8).

LTA4H

We assessed rs2660845 in the WISE-WTH case control study, where we found it to be significantly associated with the incidence of heart disease (since the variant allele was more highly represented in the WISE subjects as compared to the WTH subjects) in Caucasians in adjusted analysis (Table 3-9). No association was found for rs2660845 in the INVEST-GENES case control study (Table 3-9). We also identified an association between rs1978331 and a reduction in cardiovascular outcomes in African Americans in the INVEST-GENES case-control

study (Table 3-10). This finding remained significant after adjusted analysis. We did not originally plan to genotype this SNP in WISE-WTH because of the very low frequency of African Americans in that sample and the very low power we would have to detect an association in only this race group.

LTC4S

We assessed rs730012 in both WISE-WTH and INVEST-GENES, and found there to be no significant association with our cases (Table 3-11).

Additional Analyses

We conducted several additional analyses, the results of which are included in the Appendix. First, we conducted additional analyses in *ALOX5AP* of the haplotype GTC (rs17222814 (G) – rs10507391 (T) – rs17222814 (C)), which is the most common haplotype found in Caucasians. No association was detected for this haplotype in either WISE-WTH or INVEST-GENES case-control (Appendix Table B-3). In addition, we conducted additional analyses in the WISE-WTH case-control group. WISE subjects with ≥50% coronary obstruction are considered to have primarily vascular disease as compared to those with < 50% obstruction in which microvascular disease is more prominent.[35] Since these two groups of subjects appear to represent two different phenotypes which may have a different genetic basis, we conducted analyses in WISE-WTH by stenosis level (>50% stenosis vs. < 50% stenosis) (Appendix Tables C1-C5). Furthermore, we conducted analyses in only women in the INVEST-GENES case control to more closely compare our two case-control populations (in only Caucasians since this was the largest race group represented and granted us the most power) (Appendix Tables D1-D5). In addition, although we have no pharmacogenomic hypotheses in this study, as an exploratory analysis, we analyzed the effect of the two drug regimens studied in the original

INVEST trial to determine if an effect of drug therapy was evident by genotype in the INVEST-GENES case control study (Appendix Tables E1-E5). None of these results proved significant.

Discussion

We found a few significant genetic associations in our studies. First, we identified that the *LTA4H* polymorphism rs2660845 was more highly represented in cases than controls in the subset of Caucasians in the WISE-WTH case-control study. In addition, we noted an association between the repeat promoter polymorphism in *ALOX5* in Caucasians and between *LTA4H* rs1978331 in African Americans and the primary outcomes in the INVEST-GENES case-control. Overall, from our analyses, our study revealed few significant associations between 5LO pathway gene polymorphism and our outcomes of interest.

Our finding in *LTA4H* would be best replicated by an analysis in a group with a significant number of African American subjects. Previous evidence involving this SNP is primarily in Caucasians and indicates increased risk of the development of asthma and allergies and also an increased risk of myocardial infarction as part of a 5 SNP haplotype (7 SNPs in African Americans) for allele T (whereas in our analysis, we considered the effect of the C allele and found it to be protective). Overall, our study did not provide compelling evidence as to the role of the genetic variation in the *ALOX5* pathway as a whole contributing to heart disease.

Of particular interest is our finding that the repeat promoter polymorphism in *ALOX5* is associated with cardiovascular outcomes in the INVEST-GENES case control study (Table 3-7). Interestingly, in the WISE-WTH analysis, we also found a significant association between this polymorphism and increase risk for the incidence of heart disease in subjects that had >50% coronary obstruction (OR 1.860, 95% CI 1.035-3.342 , p-value 0.038), (Table C-1). Since these subjects have primarily vascular disease rather than microvascular disease, this portion of the WISE-WTH population may be more similar to the INVEST-GENES population. This finding

indicates that the *ALOX5* repeat promoter polymorphism may play a role across the spectrum of heart disease in people with significant vascular disease.

There are several limitations in the design of the WISE-WTH case-control study, one of which includes the complications of comparing two different clinical study populations. There are several important distinctions between these two groups which may have made this particular comparison less appropriate for genetic association studies and may explain our few findings. Both populations are from different geographic areas and therefore may be exposed to different environment factors which may complicate the detection of genetic factors which are associated with cardiovascular disease. Both populations are also temporally different, one primarily recruited in 1992 and the other ending in 1999. We were not able to account for medication use between the two groups since detailed information was not available for both groups. Also, we make the assumption *a priori* that members of the WTH study group did not have CAD. However since these subjects were not evaluated by coronary catheterization they may have had asymptomatic CAD. In addition, there was a significant difference in the rate of having ever smoked between the two groups. Smoking status is a factor that is taken into account in the Framingham Risk Score which we used as a covariate in our logistic regression analyses; however this score only assesses smoking in the past month. Although we analyzed our associations with smoking status as a factor included and found no influence of this factor, it may be that in a sample of this size, the effect of an environmental factor such as smoking, even if not recent, may interfere with the ability to perceive an effect of genotype. Smoking may cause assignment into the WISE group of subjects with symptomatic CAD despite any genetic factor which may confer risk. Our sample size may not have been large enough to be able to statistically adjust for this parameter. Alternatively, it may be that the 5LO pathway does not

contribute substantially to the variation in the risk for early heart disease; however our findings do not clarify this.

In general, the INVEST-GENES case control analysis may be more representative of the effect of 5LO genotype since both arms of the study were randomized with the only major factor of difference being drug therapy. Our strongest evidence was for the *ALOX5* gene. The effects that we saw in this group were comparable to findings from previous studies, indicating that the *5LO* pathway may play an important role in the progression of CAD to CV outcomes.

Table 3-1. Baseline characteristics of WISE and WTH

Characteristic	WISE subjects (n=586)	WTH subjects (n=628)
Age, mean (SD), years	57.7 (11.1)	56.7 (10.7)
Race, #, %		
Caucasian	493 (84.1)	532 (84.7)
African American	93 (15.9)	96 (15.3)
BMI, mean (SD), kg/m^2	29.7 (6.7)	27.2 (5.7)
History of diabetes, #, %	180 (30.8)	27 (4.3)
Framingham Risk Score, mean (SD)	8.5 (6.0)	7.1 (5.2)
$\geq$ 50% stenosis, #, %	204 (34.8)	N/A
Ever smoked, #, %	321 (54.8)	91 (14.5)

Table 3-2. Baseline characteristics in the INVEST-GENES case-control study

Characteristic	Cases (n= 258)	Controls (n= 774)
Age, mean (SD), years	71.6 (9.9)	70.1 (9.3)
Women, #, %	135 (50.2)	389 (51.0)
Race/ethnicity #,%		
Caucasian	163 (60.6)	453 (59.4)
African American	34 (12.6)	97 (12.7)
Hispanic	64 (23.8)	197 (25.8)
Other/multiracial	8 (3.0)	16 (2.1)
BMI, mean (SD), kg/m^2	27.4 (4.8)	29.0 (5.6)
Previous MI, #, %	101 (37.5)	225 (29.5)
History of CHF (class I-III), #, %	30 (11.2)	27 (3.5)
History of Renal Impairment, #, %	16 (5.9)	16 (2.1)
History of Diabetes, #, %	107 (39.8)	219 (28.7)

Table 3-3. SNPs studied as referenced in the National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism (SNP) database

Gene	Polymorphism	Applied Biosystems Catalogue Number
ALOX5	Repeat Promoter Polymorphism	N/A
ALOX5AP	rs10507391	Made to order
	rs9551963	C_30145896_10
	rs17222814	Custom Assay
	rs4769874	C_27929140_10
LTC4S	rs730012	C_644967_10
LTA4H	rs2660845	C_16264985_10
	rs1978331	Made to order

Table 3-4. Genotyping efficiency

Gene	Variant	WISE-WTH Genotyping success rate	INVEST Genotyping success rate
ALOX5	Repeat	91.7	96.6
ALOX5AP	rs10507391	95.0	97.5
	rs9551963	91.6	96.6
	rs17222814	93.8	99.4
LTA4H	1978331	N/A	97.3
	rs2660845	96.5	94.6
LTC4S	rs730012	96.3	96.7

N/A - this SNP was not genotyped in WISE-WTH due to the low number of African Americans in this group

Table 3-5. Minor allele frequencies (MAF) for SNPs and haplotypes studied

		WISE-WTH		INVEST		
Gene	Variant	MAF Caucasians	MAF African Americans	MAF Caucasians	MAF African Americans	MAF Hispanics
ALOX5AP	rs10507391	0.32 (A)	0.19 (T)	0.34 (A)	0.18 (T)	0.50 (A)
	rs9551963	0.49 (A)	0.42 (C)	0.48 (A)	0.46 (C)	0.50 (A)
	rs17222814	0.11 (A)	0.04 (A)	0.10 (A)	0.01 (A)	0.05 (A)
	GTA	0.17	0.10	0.15	0.14	0.14
	GTC	0.43	0.05	0.41	0.02	0.29
LTA4H	rs1978331	NA	NA	0.41 (C)	0.71 (C)	0.42 (C)
	rs2660845	0.27 (G)	0.35 (G)	0.33 (G)	0.39 (G)	0.33 (G)
LTC4S	rs730012	0.29 (C)	0.11 (C)	0.26 (C)	0.09 (C)	0.21 (C)

Table 3-6. Frequencies of *ALOX5* promoter polymorphism variants

	WISE-WTH		INVEST		
ALOX5 variant	Frequency Caucasians	Frequency African Americans	Frequency Caucasians	Frequency African Americans	Frequency Hispanics
5/5	0.66	0.24	0.66	0.20	0.64
5/X	0.25	0.22	0.23	0.24	0.18
X/X	0.08	0.55	0.10	0.56	0.18

5/5 – 2 wild type alleles with 5 repeat elements each
5/X – 1 wild type allele and one variant allele
X/X – 2 variant alleles

Table 3-7. Logistic regression analyses (dominant model) – *ALOX5*

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasians	0.781	1.040	0.790	1.369	0.648	1.073	0.794	1.448
African American	0.285	0.667	0.317	1.401	0.437	0.702	0.287	1.714
			INVEST					
Caucasians	0.005	1.703	1.170	2.477	0.005	1.770	1.193	2.626
African American	0.078	3.157	0.880	11.33	0.054	3.929	0.978	15.78
Hispanics	0.564	1.191	0.658	2.156	0.538	1.221	0.646	2.310

Table 3-8. Logistic regression analyses (dominant model) – *ALOX5AP* GTA (HapA)

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasian	0.760	1.043	0.797	1.365	0.953	0.991	0.740	1.328
African American	0.853	0.910	0.335	2.469	0.812	1.147	0.373	3.528
			INVEST					
Caucasians	0.913	0.977	0.642	1.487	0.878	1.035	0.669	1.600
African Americans	0.742	0.852	0.327	2.217	0.466	0.659	0.215	2.021
Hispanics	0.429	0.751	0.369	1.528	0.419	0.735	0.348	1.551

Table 3-9. Logistic regression analyses (dominant model) – *LTA4H* rs2660845

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasians	0.163	1.195	0.931	1.534	0.030	1.351	1.030	1.772
African Americans	0.454	1.253	0.694	2.260	0.211	1.593	0.768	3.307
			INVEST					
Caucasians	0.429	1.158	0.805	1.665	0.799	1.050	0.721	1.530
African Americans	0.799	1.110	0.497	2.476	0.783	0.876	0.341	2.249
Hispanics	0.730	0.902	0.501	1.624	0.846	1.065	0.562	2.021

Table 3-10. Logistic regression analyses – *LTA4H* rs1978331 – INVEST-GENES ONLY

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			INVEST					
Caucasians	0.567	1.118	0.764	1.636	0.578	1.118	0.754	1.659
African Americans	0.002	0.170	0.055	0.527	0.004	0.129	0.032	0.512
Hispanics	0.697	0.886	0.481	1.631	0.583	0.830	0.427	1.615

Table 3-11. Logistic regression analyses (dominant model) – *LTC4S* rs730012

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasians	0.445	0.907	0.707	1.165	0.700	0.948	0.723	1.243
African Americans	0.480	0.766	0.366	1.605	0.265	0.590	0.233	1.493
			INVEST					
Caucasians	0.837	0.962	0.667	1.389	0.701	1.078	0.735	1.582
African Americans	0.402	0.608	0.190	1.947	0.105	0.321	0.081	1.270
Hispanics	0.171	0.647	0.348	1.206	0.144	0.610	0.314	1.185

CHAPTER 4
LEUKOTRIENES IN CARDIOVASCULAR DISEASE (LICAD) CLINICAL STUDY

Introduction

As described in Chapter 1, the 5LO enzymatic pathway is purportedly involved in the development and progression of CV disease. The precise role of the 5LO pathway in this disease process, although not wholly clear, is to increase inflammatory activity which exacerbates the inflammatory component of this disease. This role has been demonstrated in several studies indicating that leukotrienes are found in increasing levels in progressive stages of atherosclerotic plaques, and appears to be associated with increasing levels of other inflammatory cytokines.[8] Therefore, we hypothesize that by inhibiting the 5LO pathway pharmacologically the risk for CV disease may be ultimately reduced. Furthermore, we believe that this reduction in risk may be reflected in changes in various cardiovascular biomarker levels in plasma.

There are several biomarkers, both validated and experimental, that are indicative of cardiovascular risk. One example is C-reactive protein. C-reactive protein (CRP) is an acute-phase reactant for which levels rise dramatically during inflammatory processes, up to 50,000-fold in acute inflammation, such as infection. It rises above normal limits within 6 hours and peaks at 48 hours in this setting. Its half-life is constant and therefore levels of CRP are primarily driven by the rate of production, which is related to the severity of the precipitating cause.[41] In non-acute settings, levels of CRP detected by a highly sensitive assay (hsCRP) have been found to be related to the degree of CV inflammation and are associated with an increasing risk for CV disease and outcomes.[42-44]

The role of CRP in CVD was the focus of the Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study.[45] This study found that patients with elevated hsCRP levels but LDL levels indicating low or normal

cardiovascular risk had reduced incidence of myocardial infarction and stroke and reduced combined cardiovascular endpoints (myocardial infarction, stroke, arterial revascularization, or hospitalization for unstable angina or confirmed death from cardiovascular causes) after treatment with a potent statin medication. From this study, it is not clear that this effect was because of CRP lowering (37% reduction in CRP was observed) and not due to significant LDL lowering (50% reduction in LDL was observed). However, this finding does indicate that in subjects who would not have had a clear indication for statin therapy based on LDL levels, there is a benefit from statin therapy in subjects with elevated hsCRP. Furthermore, this result implicates elevated hsCRP is a potential independent target of therapy for primary prevention of CV disease and events.

In relation to the 5LO pathway, hsCRP was shown to be elevated in subjects with increased 5LO pathway activity[46], and in addition, a 5-lipoxygenase activating protein (FLAP) inhibitor was shown to reduce hsCRP in subjects with a particular variant FLAP haplotype.[15] Since leukotriene pathway polymorphisms are implicated in the risk for heart disease (see Chapter 1), this further implies that increased activity in this pathway may increase CV risk. This effect on CV risk is supported by previous research.[9] Therefore, it is possible that patients who have increased hsCRP may experience elevated levels of cardiovascular inflammation in part due to increased activity of the leukotriene pathway. One way to assess this hypothesis is by administering a probe drug such as montelukast which reduces the activity of the leukotriene pathway by antagonizing LTD4 receptors (see Chapter 1). If activity of the leukotriene pathway contributes to increased inflammatory activity, particularly in patients with risk for CV disease, then pharmacological antagonism of leukotriene receptors would presumably reduce levels of cardiovascular biomarkers such as hsCRP in this population. Although Allayee *et al*. found a

significant effect on CRP for montelukast in asthmatics,[47] it is unclear whether this affect was primarily seen because the population studied had underlying respiratory disease, a source of inflammation that may have caused CRP to be higher on average due to respiratory disease. We aim to test the hypothesis that administering a 5LO pathway antagonist, montelukast, to subjects with increased risk for CV disease but no significant respiratory disease will reduce levels of hsCRP, indicating a potential for leukotriene pathway antagonism to reduce 5LO pathway activity and therefore CV risk.

In order to modulate the activity of the 5LO pathway in subjects with coronary heart disease risk, we used montelukast. Montelukast is currently marketed for the prophylaxis and chronic treatment of asthma and for the treatment of seasonal allergic rhinitis. As stated, its primary action is as an antagonist specific to the LTD4 receptor, CysLT1. As described in Chapter 1, the action of LTD4 has a pathophysiologic basis for a role in atherosclerotic disease. In addition, recent evidence suggests that montelukast also has a direct inhibitory effect on 5LO *in vitro*.[48] Specific inhibitors of other components of the 5LO pathway such as LTA4H, FLAP and LTC4S are not available commercially, with the exception of a 5LO inhibitor, zileuton. Uncommon but severe hepatotoxicity and four times daily dosing complicates the use of zileuton as a reasonable probe drug to test the influence of the 5LO pathway in CV disease in this pilot study. This, along with the excellent safety profile of montelukast makes it the best option for a probe of the 5LO pathway.

Montelukast is metabolized by the enzymes CYP3A4 and CYP2C9, and has a significant inhibitory effect on CYP2C8. However, since few drugs are primarily metabolized by CYP2C8, there are few potential drug interactions with montelukast. In addition, montelukast has several advantages over another currently marketed LTD4 inhibitor, zafirlukast. Zafirlukast is both a

CYP3A4 and CYP2C9 inhibitor and thus is subject to many common drug interactions. Further, zafirlukast must be taken on an empty stomach, whereas montelukast may be taken without regard to meals. As such, for the purposes of safety and compliance in this clinical study, we used montelukast as our probe drug.

In addition to studying whether montelukast affects hsCRP levels, we evaluated the effect of montelukast treatment on high density lipoprotein (HDL) and triglyceride (TG) levels. These measures have both been found to be influenced by montelukast treatment in previous research.[47] However, again, these prior investigations have focused on populations in which montelukast is indicated for treatment (asthma, allergic rhinitis) and therefore little is known about the effect of montelukast in a population at risk for CV disease where the patients do not have underlying symptomatic respiratory disease.[47] Specifically, Allayee *et al.* identified an effect to reduce both triglyceride and HDL levels in a population of asthmatics.[47] While lowering triglycerides may be a benefit in a population with CV risk, lowering of HDL may actually increase cardiovascular risk. Therefore, in order to derive a more complete picture of montelukast response in populations with cardiovascular risk, we will further explore the effect on lipids in a population without respiratory disease to potentially confirm a novel and perhaps detrimental effect of montelukast on lipid levels.

One caveat with using hsCRP as a biomarker for cardiovascular disease is its variability. Although this variability can be quantified in the setting of large clinical trials such as JUPITER, for the individual patient, variability in hsCRP may confuse the picture of cardiovascular risk. Another way of assessing cardiovascular risk may therefore be to examine downstream markers of inflammation which may vary predictably with cardiovascular risk or which may be affected by other inflammatory pathways that may indicate risk. In particular, increased activity in the

5LO pathway may further influence downstream inflammatory biomarkers that may be measured.[15] Additional biomarkers, when considered along with established biomarkers such as hsCRP, may provide more precise information on which to base treatment decisions. Therefore, in an exploratory analysis, we secondarily assessed the effect of montelukast to change levels of potential cardiovascular biomarkers in blood plasma. We will limit our investigation to a few important potential biomarkers that have been found to be elevated in subjects with hypertension, which is the primary risk factor for heart disease assessed in this study. These include macrophage chemoattractant protein-1 (MCP-1), interleukin-1 receptor antagonist (IL1ra), and epithelial neutrophil activating peptide 78 (ENA-78).

MCP-1 is a chemokine with chemotactic activity for both monocytes and lymphocytes,[49] and which is implicated in the development of inflammatory diseases such as atherosclerosis. In particular, activation of monocytes followed by adhesion to endothelial surfaces and migration of monocytes into the intima of arteries is found in early atherosclerotic lesions and is mediated by chemokines such as MCP-1.[50] Furthermore, enhanced MCP-1 expression in leukocytes has been found in patients with coronary artery disease. Parissis *et al.* identified a significant release of MCP-1 into the circulation of patients with mild to moderate arterial hypertension, as compared to healthy aged-matched controls.[51] This may be associated with endothelial dysfunction and progression of atherosclerosis, which may be triggered by arterial hypertension. It appears this effect is enhanced by the presence of other cardiovascular risk factors such as hyperlipidemia.[51] In addition, Ichiyama *et al.* found that cysteinyl leukotrienes induce MCP-1 in human monocytes and macrophages.[52] In light of this, we propose that antagonizing the activity of cysteinyl leukotrienes may reduce levels of MCP-1 predictably in plasma.

IL1ra antagonizes the binding of the pro-inflammatory cytokine IL-1to its receptors. Therefore, this mediator can antagonize the potential deleterious effects of pro-inflammatory cytokines. In fact, the balance between pro- and anti-inflammatory cytokines is thought to be more important to the disease process than the actual concentrations of pro-inflammatory cytokines alone.[53] IL1ra circulating levels were found to be higher in patients with essential hypertension,[54] and in general it was found that patients with essential hypertension have an altered profile of pro- and anti-inflammatory cytokines, consistent with monocyte activation in the circulation.[53] In relation to the 5LO pathway, reducing the activity of proinflammatory leukotrienes may reduce the circulating levels of IL1ra. It has been shown that IL1ra inhibits LTB4 release by stimulated monocytes.[55] In addition, in certain inflammatory states IL-1 has been found to stimulate LTC4.[56] Therefore, it is plausible that IL1ra may inhibit downstream LTs as well as LTB4. In a state where IL-1β (component of IL-1) is elevated, such as in hypertension,[57] IL1ra may be reflexively affected by inhibiting the activity of LTs. We will test this theory be assessing IL1ra levels in plasma in response to treatment with montelukast.

In addition to monocytes and macrophages, neutrophils play an important role in CVD pathogenesis. Epithelial neutrophil activating peptide 78 (ENA-78) is a chemokine produced by monocytes, neutrophils, platelets, and endothelial cells (among others) and, which functions as a neutrophil activator and attractor.[58] Van de Steen *et al.* found that levels of ENA-78 were modulated by matrix metalloproteinase (MMP) activity.[59] In addition, according to a study by Cippolone *et al.*, leukotriene levels and MMP levels in atherosclerotic plaques are associated,[8] *i.e.* increased levels of leukotrienes appear to stimulate increased production of MMPs. Therefore, it is plausible that modulating leukotriene activity may affect downstream levels of ENA-78 in a predictable way via activity to reduce MMPs.

In order to address our aims, we have conducted a clinical study in which we observe the effect of the leukotriene receptor blocker, montelukast sodium, to change levels of the cardiovascular biomarkers hsCRP, HDL, triglycerides, MCP-1, ENA-78 and IL1ra in plasma.

Methods

Study Protocol

We conducted a double-blind placebo-controlled crossover clinical study (Leukotrienes in Coronary Artery Disease – LICAD) with a planned recruitment of 40 subjects, with 34 completing the protocol. (By the end of the study protocol, enrollment was lower than expected, with 22 participating and 21 with complete data, see discussion section). Subjects were identified and recruited for participation from both previous participants in the Pharmacogenetic Evaluation of Anti-hypertensive Responses (PEAR) study,[60] or by contact through advertisement. All subjects were seen in Gainesville, Florida. Inclusion criteria were designed to capture a population of subjects at risk for heart disease by exhibiting risk factors identified in the Adult Treatment Panel III Guidelines.[61] Inclusion criteria are listed in Table 4-1. In general, targeted subjects were hypertensives, defined for the purposes of this study as subjects with a systolic blood pressure of 140 mmHg or above, or diastolic blood pressure of 90 mmHg or above, a diagnosis of essential hypertension, or treatment for hypertension. Subjects were also eligible if they had triglycerides of 150 mg/dL or above with or without hypertension. Subjects were excluded based on criteria which would either increase patient risk or which would potentially interfere with our ability to assess the effect of montelukast on the biomarkers we were measuring. Exclusion criteria are listed in Table 4-2. In particular, based on findings that imply that montelukast may lower HDL cholesterol,[47] subjects were excluded if their HDL-C level was < 40 mg/dL on screening or, if a previous cholesterol level was available, if it was <40 mg/dL in the previous year. We intended to shed light on the effect of montelukast on HDL in

the context of this study. All subjects' written consent for participation in this study was documented by the investigator and the protocol was approved by the University of Florida Institutional Review Board.

A study scheme diagram is pictured in Figure 4-1. After enrollment, subjects were randomized to either Arm 1 or Arm 2 of the LICAD study. Randomization was blinded to both the investigators and the subjects. Subjects randomized to Arm 1 took placebo once daily. Subjects in Arm 2 took montelukast 10 mg once daily. Subjects continued to take the intervention for 4 weeks and were instructed to avoid taking any anti-inflammatory medications other than montelukast during the study period (a list of common anti-inflammatory agents was provided to each subject). If such a medication was determined by the subject or their healthcare provider to be necessary, the subjects were asked to record this use. After 4 weeks of treatment, the subjects reported to the study site at which time they were crossed-over to the alternate study arm and took either placebo or montelukast for the remaining 4 weeks of the study. No washout period was utilized. Subjects attended one additional study visit after 4 weeks of treatment. At each study visit, subjects had their blood pressure monitored for safety and a 20 mL blood sample collected for the assessment of biomarkers. Adherence was assessed by pill counts.

Multiplex Enzyme-Linked Immunosorbant Assay (ELISA)

Twenty milliliters of whole blood was collected at each study visit using standard venipuncture procedures. Samples (10 mL whole blood) for the collection of serum were allowed to remain at room temperature for at least 30 minutes and then centrifuged for 10 minutes at 3000 RPM at 4C. Serum samples were then remitted to an outside clinical laboratory facility (Quest Diagnostics, Tampa, FL) for evaluation of lipids and hsCRP. The samples (10 mL whole blood) for the collection of plasma were placed on ice immediately after collection

and then processed within 1 hour of collection. Blood samples were centrifuged for 10 minutes at 3000 RPM at 4°C. Plasma was removed and stored in cryotubes at -80°C prior to analysis.

Multiplex ELISA kits were obtained from R & D Systems (Minneapolis, MN). We used Fluorokine MAP Multiplex Human Cytokine Base Kit A and individual analyte kits (Fluorokine MAP Multiplex Human Cytokine Panel A) for MCP-1, IL1ra and ENA-78. We conducted the assay using plasma samples per the manufacturer's recommendation. Briefly, plasma samples were diluted 4X with dilution buffer (R&D Systems). 50 µL of the diluted plasma for each sample were incubated in a 96-well ELISA plate with antibody-containing microbeads (antibodies specific for the analytes of interest) for 3 hours at room temperature. The beads were then washed and rinsed 3X with 100 µL of wash buffer and rinsed using a 96-well plate vacuum manifold. The microbeads were then incubated with 50 µL of a biotinylated antibody cocktail for 1 hour at room temperature. After washing and rinsing 3X with 100 µL buffer, the beads were incubated with a 50 µL solution of strepavidin-containing fluorescent markers for 30 minutes. Again, after washing, the beads were resuspended in 100 µL of wash buffer and analyzed by cytometric fluorescence detection (Luminex 100 IS system, Luminex corp., Austin, TX). All samples were prepared in duplicate in addition to the standard samples and negative control samples. Resulting analyte concentrations were calculated based on a standard curve constructed using Beadview Software (Millipore, Billerica, MA) using standard samples analyzed on the same plate as the experimental samples.

Statistical Analysis

Power was calculated using G*Power (Dusseldorf, Germany). This achievable power and effect size was based on previously observed effects of statin drugs on hsCRP levels,[62] which indicates that 34 subjects are needed to have 80% power to detect an effect size of 0.5 in hsCRP levels. Data from both primary and secondary analytes were converted to percent change as

(montelukast – placebo)/placebo * 100 for each treatment arm. These data were analyzed using UNIVARIATE analysis on the differences between percent change in the placebo arm and the montelukast arm relative to zero, using SAS 9.1 (Cary, IN). An uncorrected two-sided p-value of 0.05 was considered significant, and a Bonferroni correction was planned to account for multiple comparisons after first reviewing the resulting uncorrected values. For non-normally distributed data, a Wilcoxon signed rank test was planned. We assessed an order effect using general linear models to test if there was a significant difference between the two periods of the study. If an order effect was detected, the two treatment periods would be studied separately. If no order effect was detected, the treatment periods would be pooled for analysis. We also assessed a carryover effect by comparing the median values of each biomarker immediately post-placebo by study arm.

Results

Due to difficulties recruiting eligible subjects, we recruited 22 subjects, 21 subjects who had complete data for all visits. Table 4-3 gives baseline demographics of the population studied. A large majority of patients were treated hypertensives (1 subject had SBP > 140 mmHg and DBP > 90 mmHg and a previous diagnosis of hypertension but remained untreated), and all but 1 subject had hypertension as defined by the inclusion criteria (this individual had triglycerides > 150 mg/dL). Subjects were mostly African American and female. The median hsCRP at baseline was 1.7 mg/L (IQR 3.4 mg/L), where 48% were above 2.0 (inclusion requirement for JUPITER trial). This is considered average cardiovascular risk according to the American Heart Association, [63] however the IQR of the hsCRP indicates a large variability of this factor at baseline. Thirty-nine percent had an hsCRP above 3.0 at baseline which is considered high risk.

As seen in Figure 4-2, the distributions for each primary and secondary outcome variable were highly variable at baseline and there were several outliers. No order effect or carryover effect was detected in any of the biomarkers tested (Table 4-4 and 4-5); therefore all results given are for pooled analysis of all treatments. Since resulting concentrations of measured biomarkers were non-normal (Figure 4-3) and several outliers were present, Wilcoxon signed rank tests were used to assess the difference between the percent changes in the placebo and active drug arms. Furthermore, as illustrated in Figure 4-4, the changes in outcome variables associated with montelukast treatment were highly variable as they were for placebo, where changes did not trend mostly upward or downward. The median values for each primary and secondary outcome is given in Table 4-6. The levels of IL1ra were significantly affected by montelukast use (p-value 0.03). Furthermore, as given in Table 4-7, we compared montelukast – baseline to placebo – baseline measurements to assess significant differences between the two arms. Findings for ENA-78 were significant, owing to a large difference between baseline measurements and post-placebo measurements (rather than a difference between baseline and post-montlukast measurements). Therefore when interpreting any significant findings inour primary analysis regarding ENA-78, we would have to take into consideration this placebo effect that was observed. Post-montelukast ENA-78 concentrations were not, however, significantly different than post-placebo ENA-78 concentrations so no primary effect was observed.

We conducted a sub-analysis of the effect of montelukast on subjects with hsCRP > 1.7 (average value found in this study); however this included only 11 subjects and therefore severely limited the power to detect differences in light of the high variability. Treatment with montelukast was not found to affect hsCRP in this group of subjects. Furthermore, none of the clinical factors of age, gender, race, or BMI, were found to affect the results.

Discussion

Inflammation is an important contributor to early atherosclerotic processes. The 5LO enzymatic pathway is a significant contributor to the inflammation process by forming lipid mediators which influence downstream cytokines. Several studies have implicated the 5LO pathway as being involved in CVD pathogenesis. We sought to show that pharmacologic inhibition of the 5LO pathway receptor by montelukast sodium may attenuate the risk for heart disease as indicated by a reduction in cardiovascular biomarkers. Based on our pilot study, we found only an effect of montelukast to change levels of a single secondary outcome biomarker, IL1ra.

As stated, we hypothesized that IL1ra may be affected by inhibition of leukotriene activity. In particular, it was our hypothesis that reduction in leukotriene activity would reduce IL1ra reflexively. However, we observed that IL1ra levels were increased after montelukast use as compared to placebo. In general, this may have occurred because levels of IL1ra are indeed upregulated by leukotriene inhibition, improving the balance of anti-inflammatory to inflammatory cytokines in favor of reducing inflammation. Additional studies directly assessing this effect are needed to further explore this finding.

An important limitation in this study is the lack of power stemming from our low enrollment. Although we had planned an enrollment of 40 subjects of whom 34 subjects would have given us a power of 0.80 to detect an effect size of 0.5, we were only able to recruit 22 in the study period. The primary reasons for this low enrollment were that interested potential subjects were often excluded due to antihyperlipidemia medication use, non-steroidal anti-inflammatory use or anti-allergy medication use. In addition, several had HDL levels less than 40 mg/dL.

Using the same power analysis as we conducted initially, our power to detect an effect size of 0.5 was substantially reduced to 61%. An effect size of 0.62 could be detected with 80% power with the sample size that we were actually able to recruit. Since this is a pilot study, we conducted this analysis of the data both to assess any effect of montelukast on our biomarkers of interest that we could see at this time and also to evaluate the actual variability in our data which will directly affect the effect size we can evaluate. Our original effect size was calculated using information from studies with statins[64] since information on the effect on montelukast to reduce hsCRP in a cardiovascular population was lacking. In addition, in previous studies, observations of hsCRP are highly variable and not normally distributed, which contributes to the difficulties in determining an appropriate sample size needed for out study using montelukast.[64] In fact, we did observe, as expected, considerable variability in our measures of hsCRP and also a lack of normality in the observed measures which both contribute to low power. In addition, the effect of montelukast on our cardiovascular biomarkers of interest is likely much lower than that of statins because of their different mechanisms of action. Using this information we can recalculate our power and determine a more precise number of patients that would need to be recruited if further studies of pharmacological inhibition of this pathway were to be conducted.

Another limitation of this study is that our subject population did not on average exhibit elevated hsCRP although they had other risk factors for CV disease. In studies that previously have shown an effect on hsCRP, such as the JUPITER trial[45], the greatest changes were shown for subjects who had both elevated LDL and elevated hsCRP. In addition, even subjects with low LDL and elevated hsCRP experienced a significant reduction in outcomes with statin treatment. We did not limit our study to subjects with hsCRP levels above a given threshold, and the median hsCRP level was 1.7, indicating an average CV risk for our subjects.[63] In addition,

the total cholesterol for our subjects was not over 200. Therefore, our subject population was even lower risk than the population studied in JUPITER. The low risk profile and lack of consistently elevated hsCRP therefore may contribute to why we did not see changes in hsCRP with montelukast treatment.

A further limitation may be that our choice of montelukast as a leukotriene receptor antagonist did not provide complete blockade of the leukotriene pathway as would a 5LO enzyme antagonist such as zileuton. Zileuton would potently inhibit the enzyme 5LO and all downstream activity, whereas the primary effects of montelukast are known to be as an antagonist of cysteinyl leukotriene receptors. By only antagonizing this one receptor subtype, we do not substantially affect the pathway producing LTB4, a potent neutrophil chemoattractant, and also leave the lipoxin pathway (which are generally anti-inflammatory molecules) intact. Since our choice to use montelukast initially was based primarily on safety and subject compliance, we would consider using of zileuton as our study medication in a similar future study if the safety concerns and compliance issues could be addressed appropriately. Since the time of planning for this study, zileuton was in the process of being formulated to reduce these issues in patients, thereby leaving the possibility of using this new formulation in future studies.

Despite these limitations, it seems unlikely that a study at the planned sample size would have resulted in the observation of significant effects with montelukast for our primary biomarkers of interest. We therefore conclude that montelukast is unlikely to have a future potential role for risk reduction in cardiovascular disease. However, this does not rule out other approaches to better define whether there is a potential role for pharmacological inhibition of the 5LO pathway. Alternatively, as stated above, we should consider studying the effect of a different study medication such as zileuton for future studies. Zileuton has the potential to more

significantly inhibit the 5LO pathway than montelukast and therefore may have a more discernable effect on cardiovascular biomarkers than we saw for montelukast. In addition to using a medication which would have a greater effect on cardiovascular biomarkers, we would increase our sample size to improve our ability to detect an effect for our study medication amid the high variability and non-normally distributed values for our biomarkers of interest. Also, limiting our study population to subjects with elevated hsCRP (> 2.0 mg/L) would provide us with a higher risk population than we studied here and would likely improve our ability to detect meaningful changes in hsCRP elicited by our study medication. Furthermore, other tissues, such as serum or leukocyte cell-culture, may have levels of biomarkers that are affected differently than those in plasma and may be interesting study targets. In addition, we may want to explore biomarkers other than those studies here determine how they are affected by 5LO pathway inhibition.

Table 4-1. Inclusion criteria

Systolic blood pressure > 140 mmHg AND/OR
Diastolic blood pressure > 90 mmHg AND/OR
Previous diagnosis of hypertension AND/OR
Pharmacological treatment for hypertension, AND/OR
Fasting triglycerides > 150 mg/dL in the past year

Table 4-2. Exclusion criteria

Treatment with prescribed anti-dyslipidemia medications
Current treatment with montelukast
Poorly controlled hypertension, where systolic blood pressure is greater than 160 or diastolic blood pressure is greater than 100 at enrollment
History of cardiovascular disease or previous cardiovascular event (including angina pectoris, heart failure, myocardial infarction, stroke)
Diagnosis of diabetes
Active cancer
History of chronic infection currently requiring treatment
History of chronic respiratory disease (asthma, allergic rhinitis, COPD) currently requiring treatment
History of chronic inflammatory disease currently requiring treatment
Chronic use of anti-inflammatory medications such as non-steroidal anti-inflammatory medications or steroid drugs or aspirin at doses > 325 mg daily
Current recreational drug use
HDL-C < 40 mg/dL

Table 4-3. Baseline demographics

Age (SD), years	50.8 (12.5)
Race %	59% African American
Sex %	59% Female
Body Mass Index (SD), kg/m^2	31.1 (7.0)
Treated Hypertension (%)	91%

Table 4-4. Evaluation of order effect

Trait	Median % change by order Active then Placebo Median (IQR)	Median % change by order Placebo then Active Median (IQR)	p-value
hs-CRP	-0.14 (1.52)	0.58 (1.75)	0.38
HDL	0.06 (0.25)	-0.01 (0.28)	0.77
TG	-0.26 (0.74)	-0.08 (0.46)	0.43
MCP-1	-0.07 (0.45)	-0.05 (0.22)	0.80
IL1Ra	-0.08 (0.53)	-0.32 (0.78)	0.28
ENA-78	0.69 (3.26)	0.64 (4.10)	0.89

Table 4-5. Evaluation of carryover effect

Trait	Montelukast then Placebo Median (IQR)	Placebo then Montelukast Median (IQR)	p-value
hs-CRP	3.7 (5.7)	0.90 (4.5)	0.53
HDL	51 (20)	54 (25)	0.86
TG	94 (45)	126 (148)	0.29
MCP-1	83.3 (23)	91.2 (56.2)	0.89
IL1Ra	520 (250)	470 (760)	0.57
ENA-78	594 (1459)	633 (723)	0.62

Table 4-6. Evaluation of montelukast vs. placebo

Trait	Baseline Median (IQR)	Placebo Median (IQR)	Montelukast Median (IQR)	p-value for % change (Montelukast-Placebo)/Placebo
hs-CRP	1.7 (3.4)	1.0 (4.8)	1.0 (7.8)	0.22
HDL	51 (19)	52.0 (22)	50 (20)	0.57
TG	114 (89)	94 (54)	101 (66)	0.33
MCP-1	87.6 (38)	84.4 (33)	94.2 (34)	0.12
IL1ra	501 (323)	518 (428)	587 (811)	0.03
ENA-78	497.5 (607)	631 (749)	527 (885)	0.09

Table 4-7. Evaluation of change from baseline in both montelukast and placebo arms

Trait	Montelukast –Baseline Median (IQR)	Placebo-Baseline Median (IQR)	p-value for comparison
hs-CRP	0.200 (0.82)	0.088 (1.14)	0.50
HDL	-0.020 (0.20)	0.014 (0.16)	0.47
TG	0.078 (0.59)	-0.030 (0.52)	0.49
MCP-1	0.062 (0.35)	-0.027 (0.24)	0.11
IL1ra	0.044 (0.39)	-0.004 (0.40)	0.06
ENA-78	0.210 (2.06)	0.340 (3.20)	0.01

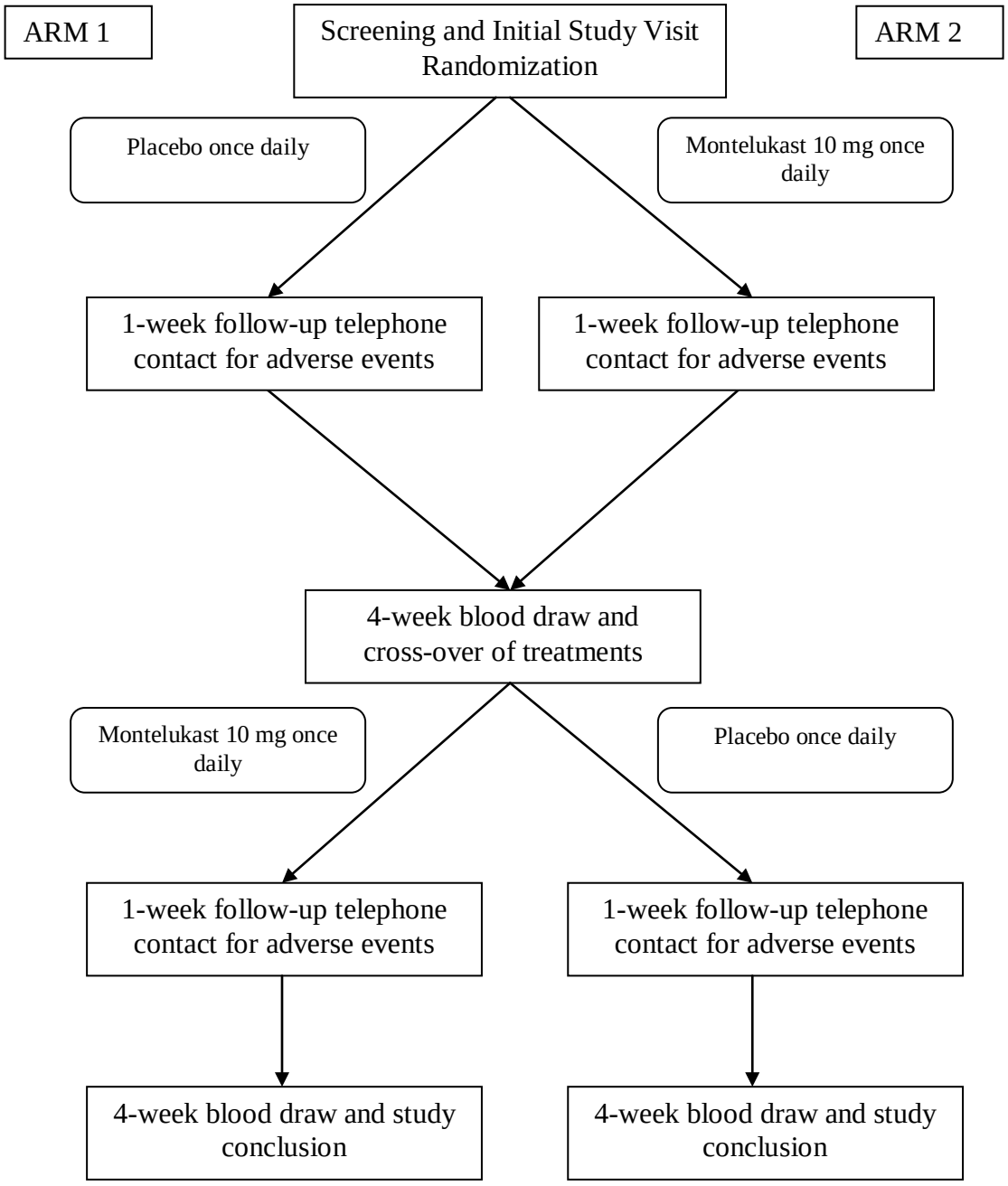


Figure 4-1. Clinical study scheme.

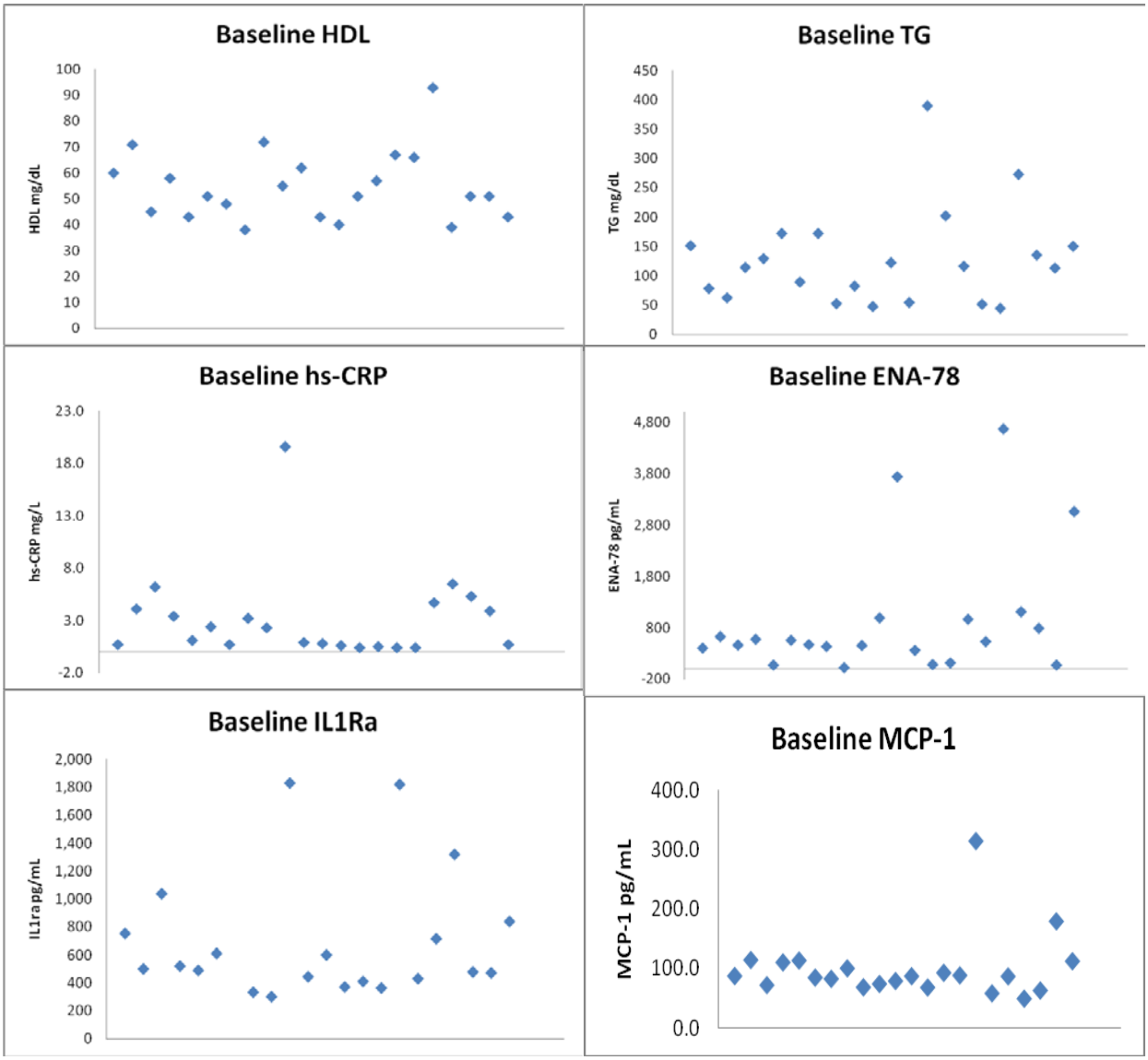


Figure 4-2. Baseline values of HDL, TG, hs-CRP, ENA-78, IL1ra, and MCP-1. This figure illustrates the general variability in the data for these parameters. Each subject in LICAD is represented in each graph by one diamond.

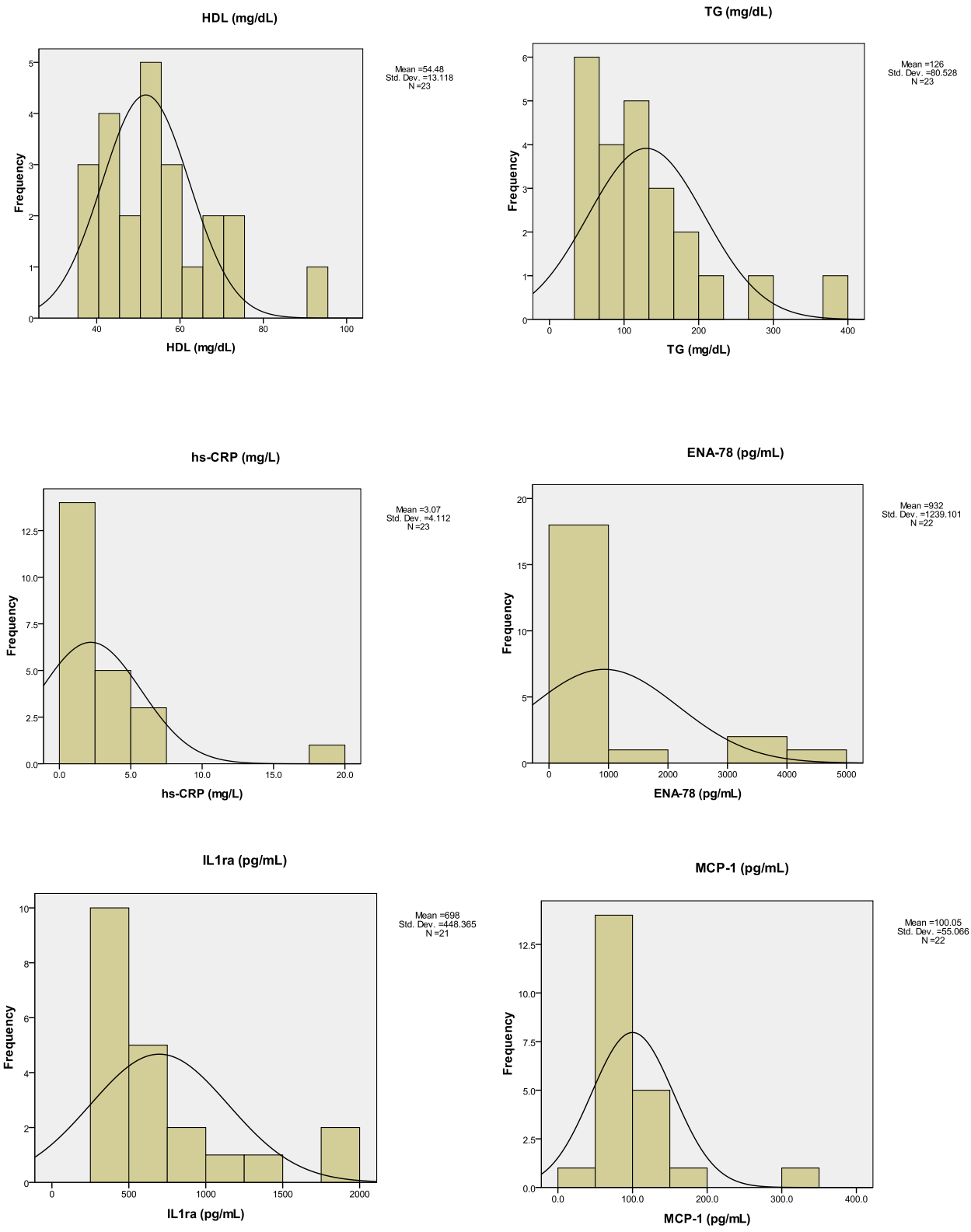


Figure 4-3. Frequency histogram and descriptive information at baseline for each variable.

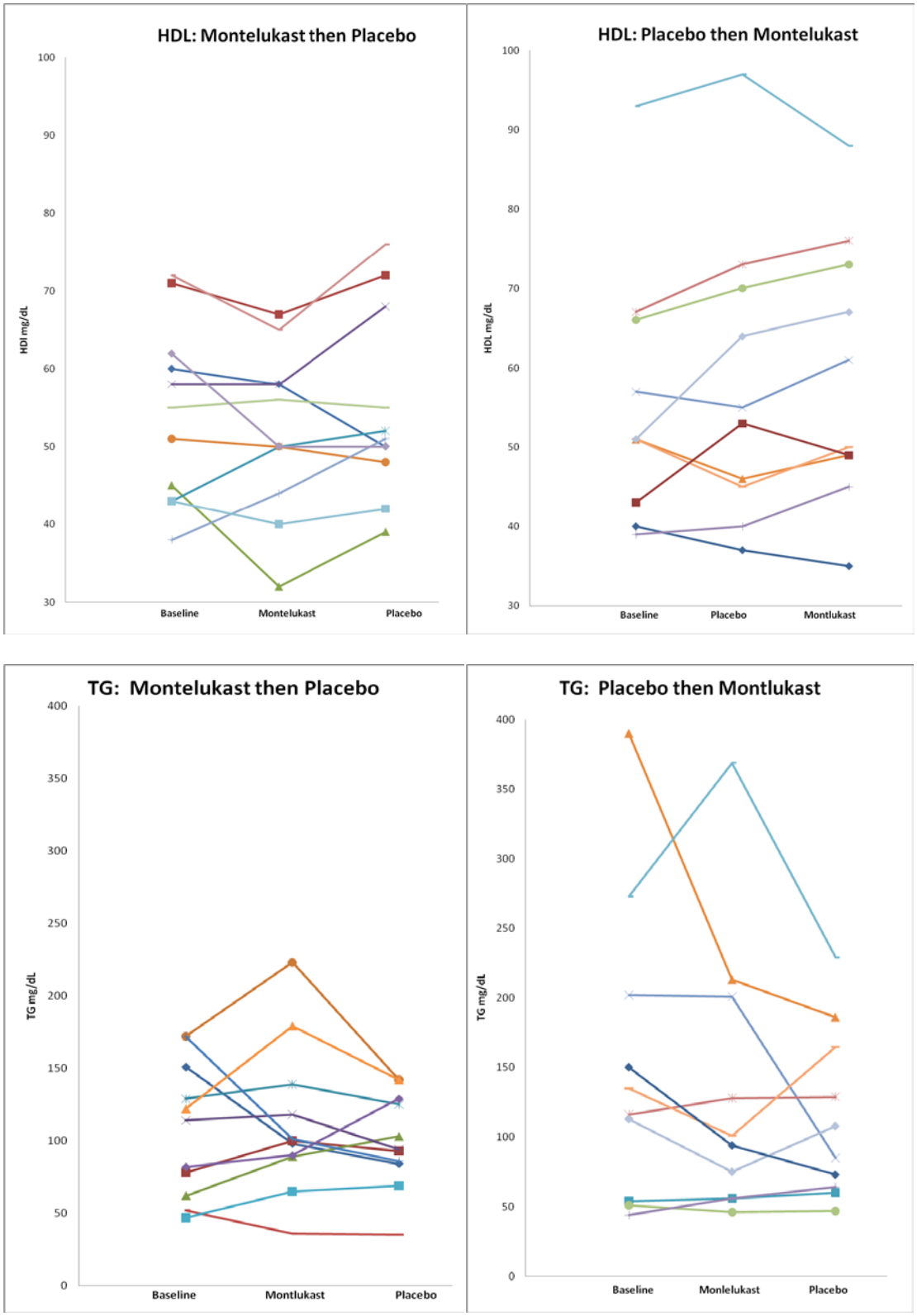


Figure 4-4. Illustrations of the baseline, placebo and montelukast values for the primary measures, HDL, TG and hs-CRP by subject.

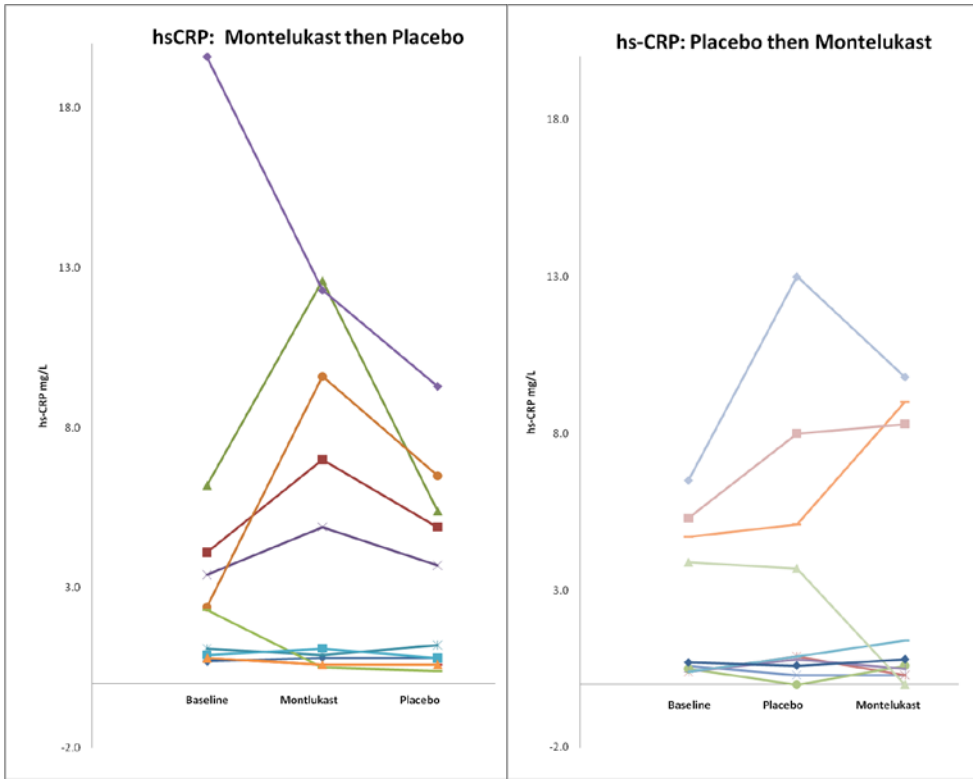


Figure 4-4. Continued

CHAPTER 5
SUMMARY AND CONCLUSION

The arachidonate 5-lipoxygenase enzymatic pathway has been shown in previous studies to play a contributory role in heart disease. In this study, we have identified evidence to support this.

This study is significant to future research for several reasons. First, we describe a protocol to genotype the *ALOX5* promoter polymorphism using pyrosequencing technology, making genotyping this polymorphism more accessible. In addition, we sought to confirm previous genetic association findings in two unique populations representing different spectrums of heart disease. Furthermore, to our knowledge, this is the first clinical study directly addressing the effect of montelukast on cardiovascular biomarkers in a population without primary respiratory disease.

The assay which we developed to genotype the *ALOX5* promoter polymorphism using pyrosequencing technology allows researchers to measure this polymorphism in both small applications and in large populations. The assay relies on reagents easily obtainable from commercial sources and utilizes pyrosequencing technology for detection, although our PCR method may be easily adapted to platforms which allow higher throughput than pyrosequencing. It is our hope that researchers will find this assay useful so that the contribution of the *ALOX5* promoter polymorphism in inflammatory disease, in particular cardiovascular disease, can be more carefully and broadly studied.

In general, we identified two interesting findings in our genetic association analyses. First, we identified a significant association between the *ALOX5* repeat promoter polymorphism and cardiovascular events in Caucasians in the INVEST-GENES case-control study. (OR 1.770, CI: (1.193-2.626 p-value: 0.005). Our finding was statistically significant in Caucasians and there

was a trend for an association in African Americans in dominant models (with the lack of statistical significance likely influenced by our sample size). In particular, this finding is in the setting of CAD (*i.e.* all subjects tested had documented CAD), therefore these data suggest that there is an effect for this polymorphism to confer risk for CV events in the setting of CAD. This expands on previous findings indicating that this polymorphism is associated with markers of CV disease, such as carotid intima-media thickness,[11] but has not been associated with the incidence of CV events when compared to a healthy population.[65-66] It is unclear why this finding was not also identified in our Hispanic population. Previous studies have shown a relationship between dietary fatty acid intake and the strength of association with this polymorphism.[11, 67] Then one possibility may be that in the Hispanics subjects (primarily recruited from Puerto Rico) there may have been environmental differences (*e.g.* dietary patterns) exposures that obscure the association of the *ALOX5* polymorphism to CV events.

Furthermore, we identified in secondary analyses that the *ALOX5* repeat promoter polymorphism is associated in a dominant model with increase risk of cardiovascular disease in Caucasian women in the WISE-WTH case control analysis (OR 1.860 CI: 1.035 – 3.342, p-value: 0.038). In particular, this association is with macrovascular disease, or disease that is characterized by ≥ 50% coronary artery occlusion (no association with microvascular disease was identified in our analysis), which is consistent with the coronary disease present in the INVEST population. This finding further strengthens the conclusion that the *ALOX5* repeat promoter polymorphism is associated with the development of cardiovascular disease, in particular severe CV disease leading to CV events, since in an analysis of the WISE study, women with CAD > 50% went on to have a greater risk of cardiovascular events than those with primarily microvascular disease.[35]

We also identified a significant association between *LTA4H* rs1978331 and the incidence of heart disease in African Americans, noted in the INVEST-GENES case-control analysis (OR: 0.129, CI: 0.032 – 0.512, p-value: 0.004). Although interesting, this finding in only African Americans only lacks support in the previous literature. In addition, the sample of African Americans in which we assessed this polymorphism was limited (132 subjects) as compared to the other populations studied. It is not necessarily surprising to find that the effect of this SNP may be different in the African American population since the MAF in African Americans is much higher than in Caucasians (0.41 in Caucasians vs. 0.71 in African Americans) and the minor allele is protective from CV events according to our data, however it is a finding that should be assessed further in a larger population of African Americans to determine that it is not spurious.

As described in Chapter 3, we had a lack of power within race groups to identify genetic associations with odds ratios that reflect small but important differences cardiovascular risk (30 – 50%) except in our Caucasian subgroups in only those SNPs with MAF close to 0.5. Based on previous findings as described in Chapter 1, it is likely that more of the 5LO pathway gene polymorphisms that we tested are indeed associated with cardiovascular disease and events in the populations that we studied, however the effects of these polymorphisms in our populations of interest may have been smaller than our studies were designed to detect.

In our clinical study, we identified an effect of montelukast to increase levels of IL1ra, one of our secondary biomarkers. In general, this may have occurred because levels of IL1ra may be upregulated by leukotriene inhibition, improving the balance of anti-inflammatory to inflammatory cytokines in favor of reducing inflammation. Since this is a plausible explanation for this finding, this effect should be further explored specifically to determine if it is a spurious

finding, or if it is not, what the nature of this relationship is. The remaining results of this pilot study indicate that the effect of montelukast on most of our biomarkers of interest may have been more subtle than our study was designed to detect (and therefore perhaps not clinically relevant) or, more likely, that montelukast does not significantly affect the biomarkers that we analyzed. For future research, we have determined that we would ideally use the medication zileuton, or another upstream inhibitor of the 5LO pathway, to determine its effect on downstream cardiovascular biomarkers. Based on our study, we hypothesize that a medication such as zileuton that pharmacologically inhibits the 5LO pathway at its rate-limiting step (5-lipoxygenase) is more likely to produce detectable changes in our biomarkers of interest. In addition, we would limit our recruitment to subjects with elevated hsCRP levels to maximize the opportunity to see a reduction in hsCRP by our study medication. Patients with elevated hsCRP are at higher risk for developing cardiovascular disease and therefore may derive more benefit evident in cardiovascular biomarkers from a 5LO inhibitor than other patients with lower risk.

Overall, we designed this study to further characterize the relationship of the 5LO pathway and CVD. We have successfully replicated a previous genetic association finding between the *ALOX5* repeat promoter polymorphism and cardiovascular events. We also determined that this genetic association may be more important in patients with macrovascular disease rather than microvascular disease, which is a novel finding. Our clinical study should be considered exploratory. We assessed the effect of montelukast on cardiovascular biomarkers and determined that in general, montelukast does not significantly affect the biomarkers measured (hsCRP, HDL, triglycerides, MCP-1, IL1ra and ENA-78). This finding alone does not rule out an effect for pharmacological inhibition of the 5LO pathway to reduce the risk of CVD, however it does indicate that montelukast itself has a minimal effect, and that simply inhibiting this

pathway at the LTC4 receptor level may not be sufficient to achieve a meaningful clinical effect to reduce the risk of heart disease. Alternatively, a more upstream pharmacological agent, such as zileuton, may have a more distinct effect and should be studied to further.

APPENDIX A
ALOX5 GENOTYPE FREQUENCIES FOR HISPANICS IN INVEST-GENES

Table A-1. Frequencies of *ALOX5* promoter polymorphism genotypes in Hispanics in INVEST-GENES

Genotype	Frequency
5/5	0.64
4/5	0.18
4/4	0.01
3/4	0.08
3/3	0.06

Genotypes in the form A/B, where A and B are number of repeats in the allele

APPENDIX B
GENETIC ASSOCIATION ANALYSES, ADDITIVE, RECESSIVE AND DOMINANT MODELS

For all of the following tables, CIL refers to the lower limit of the 95% confidence interval, and CIU refers to the upper limit of the 95% confidence interval. The abbreviation OR refers to the odds ratio. All assessments in this Appendix were made using logistic regression procedures according to the methods described in Chapter 3.

Table B-1. *ALOX5* promoter polymorphism

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
WISE-WTH								
Caucasians								
Additive allele 1	0.893	1.022	0.754	1.385	0.847	1.043	0.749	1.451
allele 2	0.738	1.096	0.681	1.761	0.604	1.173	0.692	1.989
Recessive	0.721	1.089	0.682	1.738	0.576	1.160	0.690	1.950
Dominant	0.781	1.040	0.790	1.369	0.648	1.073	0.794	1.448
African Americans								
Additive allele 1	0.914	0.762	0.299	1.941	0.634	0.963	0.326	2.848
allele 2	0.317	0.632	0.291	1.375	0.207	0.604	0.235	1.554
Recessive	0.309	0.721	0.384	1.354	0.208	0.616	0.290	1.310
Dominant	0.285	0.667	0.317	1.401	0.437	0.702	0.287	1.714
INVEST								
Caucasians								
Additive allele 1	0.133	1.561	0.874	2.789	0.076	1.727	0.944	3.162
allele 2	0.008	1.770	1.161	2.698	0.010	1.789	1.149	2.785
Recessive	0.329	1.325	0.753	2.330	0.215	1.449	0.806	2.606
Dominant	0.005	1.703	1.170	2.477	0.005	1.770	1.193	2.626
African Americans								
Additive allele 1	0.063	3.442	0.934	12.681	0.059	3.917	0.952	16.12
allele 2	0.206	2.551	0.598	10.873	0.103	3.972	0.758	20.81
Recessive	0.121	1.920	0.842	4.380	0.207	1.843	0.714	4.758
Dominant	0.078	3.157	0.880	11.329	0.054	3.929	0.978	15.78
Hispanics								
Additive allele 1	0.063	1.949	0.965	3.934	0.075	2.010	0.931	4.339
allele 2	0.278	0.612	0.252	1.485	0.358	0.647	0.256	1.637
Recessive	0.029	2.145	1.081	4.256	0.041	2.191	1.034	4.644
Dominant	0.564	1.191	0.658	2.156	0.538	1.221	0.646	2.310

Table B-2. *ALOX5AP* GTA (HapA)

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
				WISE-WTH				
Caucasians								
Additive allele 1	0.626	1.060	0.801	1.404	0.308	1.039	0.767	1.409
allele 2	0.730	0.911	0.452	1.835	0.281	0.658	0.297	1.459
Recessive	0.758	0.896	0.447	1.799	0.289	0.652	0.295	1.439
Dominant	0.760	1.043	0.797	1.365	0.953	0.991	0.740	1.328
African Americans								
Additive allele 1	0.978	0.682	0.233	2.000	0.975	1.081	0.340	3.437
allele 2	NA	NA	NA	NA	NA	NA	NA	NA
Recessive	NA	NA	NA	NA	NA	NA	NA	NA
Dominant	0.853	0.910	0.335	2.469	0.812	1.147	0.373	3.528
				INVEST				
Caucasians								
Additive allele 1	0.711	0.918	0.585	1.441	0.978	0.978	0.614	1.559
allele 2	0.494	1.379	0.549	3.469	0.473	1.427	0.540	3.772
Recessive	0.466	1.406	0.562	3.517	0.464	1.434	0.546	3.769
Dominant	0.913	0.977	0.642	1.487	0.878	1.035	0.669	1.600
African Americans								
Additive allele 1	0.504	0.710	0.260	1.938	0.384	0.594	0.184	1.919
allele 2	0.778	1.420	0.123	16.35	0.923	1.146	0.071	18.60
Recessive	0.800	0.750	0.081	6.966	0.988	1.020	0.080	13.05
Dominant	0.742	0.852	0.327	2.217	0.466	0.659	0.215	2.021
Hispanics								
Additive allele 1	0.561	0.861	0.428	1.730	0.538	0.793	0.378	1.661
allele 2	0.561	0.530	0.062	4.517	0.630	0.583	0.065	5.231
Recessive	0.998	1.002	0.266	3.766	0.865	0.882	0.207	3.755
Dominant	0.429	0.751	0.369	1.528	0.419	0.735	0.348	1.551

NA – No estimate given because of few or no genotypes in sample.

Table B-3. *ALOX5AP* GTC (rs17222814 (G) – rs10507391 (T) – rs17222814 (C))

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasians								
Additive allele 1	0.764	0.868	0.657	1.417	0.786	0.855	0.632	1.156
allele 2	0.450	0.814	0.550	1.204	0.413	0.789	0.516	1.206
Recessive	0.522	0.893	0.632	1.263	0.483	0.874	0.600	1.273
Dominant	0.258	0.856	0.655	1.120	0.241	0.841	0.629	1.124
African Americans								
Additive	0.971	0.481	0.228	1.014	0.974	0.482	0.201	1.154
	NA	NA	NA	NA	NA	NA	NA	NA
Recessive	NA	NA	NA	NA	NA	NA	NA	NA
Dominant	0.041	0.462	0.220	0.970	0.087	0.469	0.197	1.117
			INVEST					
Caucasians								
Additive allele 1	0.156	1.345	0.893	2.027	0.183	1.335	0.873	2.043
allele 2	0.102	0.578	0.299	1.115	0.091	0.557	0.282	1.099
Recessive	0.016	0.479	0.263	0.874	0.015	0.463	0.249	0.862
Dominant	0.506	1.145	0.769	1.703	0.557	1.132	0.749	1.709
African Americans								
Additive allele 1	0.163	3.241	0.620	16.938	0.410	2.215	0.335	14.66
allele 2	NA	NA	NA	NA	NA	NA	NA	NA
Recessive	NA	NA	NA	NA	NA	NA	NA	NA
Dominant	0.163	3.241	0.620	16.938	0.410	2.215	0.335	14.658
Hispanics								
Additive allele 1	0.280	1.406	0.758	2.608	0.303	1.412	0.732	2.724
allele 2	0.155	2.083	0.758	5.724	0.380	1.670	0.532	5.245
Recessive	0.248	1.760	0.675	4.587	0.532	1.418	0.474	4.248
Dominant	0.175	1.503	0.834	2.712	0.245	1.452	0.774	2.721

NA – No estimate given because of few or no genotypes in sample.

Table B-4. *LTA4H* rs2660845

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			WISE-WTH					
Caucasians								
Additive allele 1	0.772	1.151	0.885	1.496	0.460	1.343	1.011	1.785
allele 2	0.209	1.453	0.894	2.362	0.485	1.394	0.818	2.375
Recessive	0.193	1.369	0.853	2.196	0.435	1.229	0.732	2.061
Dominant	0.163	1.195	0.931	1.534	0.030	1.351	1.030	1.772
African Americans								
Additive allele 1	0.967	1.199	0.642	2.236	0.980	1.477	0.687	3.173
allele 2	0.515	1.474	0.570	3.814	0286	2.139	0.691	6.623
Recessive	0.518	1.344	0.549	3.289	0.309	1.741	0.608	4.811
Dominant	0.454	1.253	0.694	2.260	0.211	1.593	0.768	3.307
			INVEST					
Caucasians								
Additive allele 1	0.293	1.222	0.841	1.777	0.513	1.138	0.772	1.679
allele 2	0.540	0.762	0.320	1.815	0.218	0.562	0.224	1.408
Recessive	0.411	0.700	0.299	1.640	0.170	0.531	0.215	1.312
Dominant	0.429	1.158	0.805	1.665	0.799	1.050	0.721	1.530
African Americans								
Additive allele 1	0.889	0.940	0.394	2.240	0.550	0.732	0.262	2.040
allele 2	0.296	1.958	0.556	6.900	0.533	1.605	0.363	7.101
Recessive	0.252	2.014	0.608	6.672	0.389	1.860	0.453	7.638
Dominant	0.799	1.110	0.497	2.476	0.783	0.876	0.341	2.249
Hispanics								
Additive allele 1	0.728	0.896	0.483	1.662	0.925	1.033	0.529	2.016
allele 2	0.874	0.925	0.355	2.414	0.705	1.222	0.432	3.456
Recessive	0.968	0.982	0.399	2.416	0.712	1.200	0.456	3.162
Dominant	0.730	0.902	0.501	1.624	0.846	1.065	0.562	2.021

Table B-5. *LTA4H* rs1978331 – INVEST-GENES ONLY

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
			INVEST					
Caucasians								
Additive allele 1	0.600	1.115	0.743	1.673	0.676	1.094	0.718	1.666
allele 2	0.657	1.125	0.668	1.895	0.543	1.183	0.689	2.032
Recessive	0.814	1.057	0.664	1.685	0.636	1.124	0.692	1.826
Dominant	0.567	1.118	0.764	1.636	0.578	1.118	0.754	1.659
African Americans								
Additive allele 1	0.002	0.126	0.034	0.468	0.002	0.074	0.014	0.384
allele 2	0.007	0.201	0.062	0.651	0.015	0.168	0.04	0.710
Recessive	0.609	0.811	0.364	1.808	0.942	0.966	0.373	2.501
Dominant	0.002	0.170	0.055	0.527	0.004	0.129	0.032	0.512
Hispanics								
Additive allele 1	0.956	0.982	0.520	1.854	0.789	0.910	0.456	1.817
allele 2	0.304	0.606	0.233	1.575	0.312	0.585	0.207	1.654
Recessive	0.271	0.613	0.256	1.466	0.323	0.620	0.240	1.600
Dominant	0.697	0.886	0.481	1.631	0.583	0.830	0.427	1.615

Table B-6. *LTC4S* rs730012

	Unadjusted				Adjusted			
Gene and variant	p-value	OR estimate	CIL	CIU	p-value	OR estimate	CIL	CIU
				WISE-WTH				
Caucasians								
Additive allele 1	0.266	0.968	0.744	1.258	0.577	0.983	0.739	1.307
allele 2	0.090	0.661	0.412	1.062	0.389	0.803	0.488	1.319
Recessive	0.088	0.671	0.424	1.062	0.387	0.809	0.501	1.307
Dominant	0.445	0.907	0.707	1.165	0.700	0.948	0.723	1.243
African Americans								
Additive allele 1	0.685	0.751	0.352	1.602	0.292	0.668	0.262	1.707
allele 2	0.881	1.072	0.066	17.48	0.124	0.062	0.002	1.661
Recessive	0.930	1.133	0.070	18.40	0.107	0.067	0.003	1.794
Dominant	0.480	0.766	0.366	1.605	0.265	0.590	0.233	1.493
				INVEST				
Caucasians								
Additive allele 1	0.772	0.944	0.642	1.390	0.707	1.081	0.721	1.620
allele 2	0.874	1.059	0.521	2.154	0.867	1.065	0.511	2.219
Recessive	0.820	1.084	0.542	2.167	0.931	1.032	0.505	2.112
Dominant	0.837	0.962	0.667	1.389	0.701	1.078	0.735	1.582
African Americans								
Additive allele 1	0.457	0.642	0.200	2.065	0.113	0.327	0.082	1.304
allele 2	NA	NA	NA	NA	NA	NA	NA	NA
Recessive	NA	NA	NA	NA	NA	NA	NA	NA
Dominant	0.402	0.608	0.190	1.947	0.105	0.321	0.081	1.270
Hispanics								
Additive allele 1	0.217	0.664	0.347	1.271	0.170	0.613	0.305	1.232
allele 2	0.438	0.540	0.114	2.562	0.514	0.588	0.119	2.898
Recessive	0.536	0.614	0.131	2.880	0.631	0.679	0.139	3.304
Dominant	0.171	0.647	0.348	1.206	0.144	0.610	0.314	1.185

NA – No estimate given because of few or no genotypes in sample.

APPENDIX C
WISE-WTH - DEGREE OF CORONARY ARTERY OBSTRUCTION, CAUCASIAN SUBJECTS, ADJUSTED ANALYSIS ONLY

Table C-1. *ALOX5* promoter polymorphism > CAD 50%

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.360	1.894	0.998	3.591
Additive 2 allele	0.670	1.747	0.586	5.210
Recessive	0.464	1.496	0.509	4.401
Dominant	0.038	1.860	1.035	3.342
ALOX5 promoter polymorphism CAD < 50%				
Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.430	0.848	0.576	1.248
Additive 2 allele	0.717	1.029	0.560	1.889
Recessive	0.802	1.079	0.594	1.962
Dominant	0.510	0.889	0.625	1.262

Table C-2. *ALOX5AP* GTA (Hap A) CAD > 50%

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.495	0.986	0.582	1.672
Additive 2 allele	0.440	0.460	0.065	3.263
Recessive	0.438	0.462	0.065	3.256
Dominant	0.846	0.950	0.566	1.596
ALOX5AP GTA (Hap A) CAD < 50%				
Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.327	1.075	0.738	1.567
Additive 2 allele	0.328	0.667	0.276	1.613
Recessive	0.345	0.655	0.273	1.576
Dominant	0.953	1.011	0.707	1.445

Table C-3. *ALOX5AP* GTC CAD > 50%

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.311	0.758	0.436	1.318
Additive 2 allele	0.788	0.948	0.464	1.937
Recessive	0.685	1.136	0.613	2.108
Dominant	0.412	0.802	0.473	1.359
ALOX5AP GTC < 50%				
Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.724	0.907	0.631	1.304
Additive 2 allele	0.271	0.727	0.427	1.239
Recessive	0.296	0.775	0.481	1.249
Dominant	0.436	0.870	0.612	1.236

Table C-4. *LTA4H* rs2660845 CAD > 50%

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.020	1.590	0.954	2.653
Additive 2 allele	0.100	0.468	0.141	1.560
Recessive	0.115	0.388	0.119	1.258
Dominant	0.192	1.388	0.848	2.274
LTA4H rs2660845 CAD < 50%				
Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.660	1.230	0.871	1.738
Additive 2 allele	0.109	1.816	0.977	3.377
Recessive	0.098	1.662	0.910	3.032
Dominant	0.102	1.315	0.947	1.825

Table C-5. *LTC4S* rs730012 CAD > 50%

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.163	1.376	0.619	3.058
Additive 2 allele	0.260	0.777	0.455	1.325
Recessive	0.288	1.519	0.702	3.288
Dominant	0.643	0.891	0.546	1.453
LTC4S rs730012 CAD < 50%				
Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.154	1.024	0.726	1.444
Additive 2 allele	0.786	0.581	0.306	1.101
Recessive	0.079	0.574	0.309	1.066
Dominant	0.693	0.936	0.673	1.301

APPENDIX D
INVEST-GENES - CAUCASIAN WOMEN, ADJUSTED ANALYSIS ONLY

Table D-1. *ALOX5* promoter polymorphism in women

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.586	1.286	0.520	3.179
Additive 2 allele	0.046	1.873	1.012	3.469
Recessive	0.960	1.022	0.428	2.445
Dominant	0.068	1.693	0.962	2.981

Table D-2. *ALOX5AP* GTA in women

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.399	1.333	0.684	2.598
Additive 2 allele	0.224	0.258	0.029	2.296
Recessive	0.204	0.243	0.027	2.152
Dominant	0.741	1.114	0.587	2.118

Table D-3. *ALOX5AP* GTC in women

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.050	1.940	0.999	3.765
Additive 2 allele	0.969	0.982	0.395	2.443
Recessive	0.270	0.640	0.290	1.413
Dominant	0.122	1.654	0.874	3.129

Table D-4 *LTA4H* rs2660845 in women

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.090	0.967	0.540	1.732
Additive 2 allele	0.090	0.337	0.096	1.187
Recessive	0.088	0.342	0.099	1.176
Dominant	0.500	0.825	0.472	1.443

Table D-5. *LTC4S* rs730012 in women

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.468	0.794	0.425	1.482
Additive 2 allele	0.917	1.057	0.371	3.010
Recessive	0.800	1.142	0.409	3.194
Dominant	0.559	0.842	0.473	1.499

APPENDIX E
INVEST-GENES – BY DRUG THERAPY IN CAUCASIANS AS DEFINED IN ORIGINAL INVEST TRIAL, ADJUSTED ANALYSIS ONLY

Table E-1. *ALOX5* atenolol arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.762	1.153	0.460	2.889
Additive 2 allele	0.187	1.589	0.798	3.163
Recessive	0.995	1.003	0.411	2.445
Dominant	0.250	1.429	0.778	2.626

ALOX5 verapamil SR arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.052	2.327	0.992	5.460
Additive 2 allele	0.019	2.088	1.130	3.860
Recessive	0.130	1.902	0.828	4.369
Dominant	0.006	2.157	1.250	3.722

Table E-2. *ALOX5AP* GTA atenolol arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.401	1.348	0.672	2.705
Additive 2 allele	0.268	2.409	0.509	11.41
Recessive	0.303	2.253	0.480	10.57
Dominant	0.266	1.452	0.752	2.804

ALOX5AP GTA verapamil SR arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.340	0.721	0.367	1.413
Additive 2 allele	0.986	1.011	0.283	3.618
Recessive	0.885	1.098	0.310	3.890
Dominant	0.398	0.765	0.410	1.425

Table E-3. *ALOX5AP* GTC atenolol arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.096	1.771	0.903	3.472
Additive 2 allele	0.795	0.874	0.317	2.414
Recessive	0.270	0.602	0.244	1.485
Dominant	0.190	1.548	0.805	2.977

ALOX5AP GTC verapamil SR arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.512	1.224	0.669	2.240
Additive 2 allele	0.077	0.408	0.151	1.102
Recessive	0.028	0.359	0.144	0.894
Dominant	0.990	0.996	0.555	1.788

Table E-4. *LTA4H* rs2660845 atenolol arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.129	1.593	0.873	2.906
Additive 2 allele	0.237	0.389	0.081	1.862
Recessive	0.140	0.313	0.067	1.461
Dominant	0.293	1.362	0.765	2.426

LTA4H rs2660845 verapamil SR arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.398	0.789	0.456	1.367
Additive 2 allele	0.267	0.460	0.117	1.809
Recessive	0.321	0.505	0.131	1.948
Dominant	0.324	0.767	0.453	1.300

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Table E-5. *LTC4S* rs730012 atenolol arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.728	0.891	0.466	1.704
Additive 2 allele	0.529	1.403	0.489	4.021
Recessive	0.458	1.471	0.530	4.079
Dominant	0.935	0.975	0.532	1.788

LTC4S rs730012 verapamil SR arm

Model	p-value	OR estimate	CIL	CIU
Additive 1 allele	0.603	1.161	0.662	2.036
Additive 2 allele	0.698	0.803	0.266	2.427
Recessive	0.621	0.760	0.257	2.249
Dominant	0.743	1.093	0.641	1.865

LIST OF REFERENCES

1. Zipes DP, ed *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine.* 7th ed. Philadelphia, PA: W.B. Saunders; 2005. Zipes DP, Libby P, Bonow RO, Braunwald E, eds.

2. Maclouf J, Sala A, Rossoni G, Berti F, Muller-Peddinghaus R, Folco G. Consequences of transcellular biosynthesis of leukotriene C4 on organ function. *Haemostasis.* Oct 1996;26 Suppl 4:28-36.

3. Sala A, Rossoni G, Berti F, et al. Monoclonal anti-CD18 antibody prevents transcellular biosynthesis of cysteinyl leukotrienes in vitro and in vivo and protects against leukotriene-dependent increase in coronary vascular resistance and myocardial stiffness. *Circulation.* Mar 28 2000;101(12):1436-1440.

4. Subbarao K, Jala VR, Mathis S, et al. Role of Leukotriene B4 Receptors in the Development of Atherosclerosis: Potential Mechanisms. *Arterioscler Thromb Vasc Biol.* February 1, 2004 2004;24(2):369-375.

5. Choi JA, Kim EY, Song H, Kim C, Kim JH. Reactive oxygen species are generated through a BLT2-linked cascade in Ras-transformed cells. *Free Radic Biol Med.* Feb 15 2008;44(4):624-634.

6. Spanbroek R, Grabner R, Lotzer K, et al. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *PNAS.* February 4, 2003 2003;100(3):1238-1243.

7. Fair A., Pritchard K. A. Oxidized Low Density Lipoprotein Increases U937 Cell 5-Lipoxygenase Activity: Induction of 5-Lipoxygenase Activating Protein. *Biochemical and Biophysical Research Communications.* 1994/6/15 1994;201(2):1014-1020.

8. Cipollone F, Mezzetti A, Fazia M, et al. Association Between 5-Lipoxygenase Expression and Plaque Instability in Humans. *Arterioscler Thromb Vasc Biol.* June 2, 2005 2005.

9. Allen S, Dashwood M, Morrison K, Yacoub M. Differential Leukotriene Constrictor Responses in Human Atherosclerotic Coronary Arteries. *Circulation.* June 23, 1998 1998;97(24):2406-2413.

10. In KH, Asano K, Beier D, et al. Naturally Occurring Mutations in the Human 5-Lipoxygenase Gene Promoter That Modify Transcription Factor Binding and Reporter Gene Transcription. *J. Clin. Invest.* March 1, 1997 1997;99(5):1130-1137.

11. Dwyer JH, Allayee H, Dwyer KM, et al. Arachidonate 5-Lipoxygenase Promoter Genotype, Dietary Arachidonic Acid, and Atherosclerosis. *N Engl J Med.* January 1, 2004 2004;350(1):29-37.

12. Assimes TL, Knowles JW, Priest JR, et al. Common polymorphisms of ALOX5 and ALOX5AP and risk of coronary artery disease. *Hum Genet.* May 2008;123(4):399-408.

13. Lima JJ, Zhang S, Grant A, et al. Influence of leukotriene pathway polymorphisms on response to montelukast in asthma. *Am J Respir Crit Care Med.* Feb 15 2006;173(4):379-385.

14. Helgadottir A, Manolescu A, Thorleifsson G, et al. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet.* 2004/03//print 2004;36(3):233-239.

15. Hakonarson H, Thorvaldsson S, Helgadottir A, et al. Effects of a 5-Lipoxygenase-Activating Protein Inhibitor on Biomarkers Associated With Risk of Myocardial Infarction: A Randomized Trial. *JAMA.* May 11, 2005 2005;293(18):2245-2256.

16. Lohmussaar E, Gschwendtner A, Mueller JC, et al. ALOX5AP Gene and the PDE4D Gene in a Central European Population of Stroke Patients. *Stroke.* April 1, 2005 2005;36(4):731-736.

17. Kaushal R, Pal P, Alwell K, et al. Association of ALOX5AP with ischemic stroke: a population-based case-control study. *Hum Genet.* Jun 2007;121(5):601-607.

18. Zhang WL, Yang XM, Shi J, Sun K, Hui RT. Polymorphism of SG13S114T/A in the ALOX5AP gene and the risk for stroke in a large Chinese cohort. *Yi Chuan Xue Bao.* Aug 2006;33(8):678-684.

19. Zintzaras E, Rodopoulou P, Sakellaridis N. Variants of the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene and risk of stroke: a HuGE gene-disease association review and meta-analysis. *Am J Epidemiol.* Mar 1 2009;169(5):523-532.

20. Helgadottir A, Manolescu A, Helgason A, et al. A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. *Nat Genet.* 2005/11/10/online 2005.

21. Holloway JW, Barton SJ, Holgate ST, Rose-Zerilli MJ, Sayers I. The role of LTA4H and ALOX5AP polymorphism in asthma and allergy susceptibility. *Allergy.* Aug 2008;63(8):1046-1053.

22. Lima JJ, Zhang S, Grant A, et al. Influence of Leukotriene Pathway Polymorphisms on Response to Montelukast in Asthma. *Am. J. Respir. Crit. Care Med.* November 17, 2005 2005:200509-201412OC.

23. Iovannisci DM, Lammer EJ, Steiner L, et al. Association between a leukotriene C4 synthase gene promoter polymorphism and coronary artery calcium in young women: the Muscatine Study. *Arterioscler Thromb Vasc Biol.* Feb 2007;27(2):394-399.

24. Silverman ES, Du J, De Sanctis GT, et al. Egr-1 and Sp1 Interact Functionally with the 5-Lipoxygenase Promoter and Its Naturally Occurring Mutants. *Am. J. Respir. Cell Mol. Biol.* August 1, 1998 1998;19(2):316-323.

25. Drazen JM, Yandava CN, Dube L, et al. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat Genet.* Jun 1999;22(2):168-170.

26. Kalayci O, Birben E, Sackesen C, et al. ALOX5 promoter genotype, asthma severity and LTC4 production by eosinophils. *Allergy.* 2006;61(1):97-103.

27. Kim SH, Bae JS, Suh CH, Nahm DH, Holloway JW, Park HS. Polymorphism of tandem repeat in promoter of 5-lipoxygenase in ASA-intolerant asthma: a positive association with airway hyperresponsiveness. *Allergy.* Jun 2005;60(6):760-765.

28. Poole EM, Bigler J, Whitton J, Sibert JG, Potter JD, Ulrich CM. Prostacyclin synthase and arachidonate 5-lipoxygenase polymorphisms and risk of colorectal polyps. *Cancer Epidemiol Biomarkers Prev.* Mar 2006;15(3):502-508.

29. Hube F, Reverdiau P, Iochmann S, Gruel Y. Improved PCR method for amplification of GC-rich DNA sequences. *Mol Biotechnol.* Sep 2005;31(1):81-84.

30. Moore MJ, Dhingra A, Soltis PS, et al. Rapid and accurate pyrosequencing of angiosperm plastid genomes. *BMC Plant Biol.* 2006;6:17.

31. Gerhard T, Gong Y, Beitelshees AL, et al. Alpha-adducin polymorphism associated with increased risk of adverse cardiovascular outcomes: results from GENEtic Substudy of the INternational VErapamil SR-trandolapril STudy (INVEST-GENES). *Am Heart J.* Aug 2008;156(2):397-404.

32. Gulati M, Pandey DK, Arnsdorf MF, et al. Exercise Capacity and the Risk of Death in Women: The St James Women Take Heart Project. *Circulation* September 30, 2003 2003;108(13):1554-1559.

33. Silverman ES, Drazen JM. Genetic variations in the 5-lipoxygenase core promoter. Description and functional implications. *Am J Respir Crit Care Med.* Feb 2000;161(2 Pt 2):S77-80.

34. Pepine CJ, Handberg-Thurmond E, Marks RG, et al. Rationale and design of the International Verapamil SR/Trandolapril Study (INVEST): an Internet-based randomized trial in coronary artery disease patients with hypertension. *J Am Coll Cardiol.* Nov 1998;32(5):1228-1237.

35. Gulati M, Cooper-DeHoff RM, McClure C, et al. Adverse cardiovascular outcomes in women with nonobstructive coronary artery disease: a report from the Women's Ischemia Syndrome Evaluation Study and the St James Women Take Heart Project. *Arch Intern Med.* May 11 2009;169(9):843-850.

36. Pepine CJ, Handberg EM, Cooper-DeHoff RM, et al. A Calcium Antagonist vs a Non-Calcium Antagonist Hypertension Treatment Strategy for Patients With Coronary Artery Disease: The International Verapamil-Trandolapril Study (INVEST): A Randomized Controlled Trial. *JAMA.* December 3, 2003 2003;290(21):2805-2816.

37. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med.* Nov 24 1997;157(21):2413-2446.

38. Schentrup AM, Allayee H, Lima JJ, Johnson JA, Langaee TY. Genotyping the GGGCGG tandem repeat promoter polymorphism in the 5-lipoxygenase enzyme gene (ALOX5) by pyrosequencing assay. *Genet Test Mol Biomarkers.* Jun 2009;13(3):361-365.

39. Girelli D, Martinelli N, Trabetti E, et al. ALOX5AP gene variants and risk of coronary artery disease: an angiography-based study. *Eur J Hum Genet.* Sep 2007;15(9):959-966.

40. Zee RY, Cheng S, Hegener HH, Erlich HA, Ridker PM. Genetic variants of arachidonate 5-lipoxygenase-activating protein, and risk of incident myocardial infarction and ischemic stroke: a nested case-control approach. *Stroke.* Aug 2006;37(8):2007-2011.

41. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest.* Jun 2003;111(12):1805-1812.

42. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-Reactive Protein and Other Markers of Inflammation in the Prediction of Cardiovascular Disease in Women. *N Engl J Med* March 23, 2000 2000;342(12):836-843.

43. Koenig W, Khuseyinova N, Baumert J, Meisinger C. Prospective study of high-sensitivity C-reactive protein as a determinant of mortality: results from the MONICA/KORA Augsburg Cohort Study, 1984-1998. *Clin Chem.* Feb 2008;54(2):335-342.

44. Kuller LH, Tracy RP, Shaten J, Meilahn EN. Relation of C-reactive protein and coronary heart disease in the MRFIT nested case-control study. Multiple Risk Factor Intervention Trial. *Am J Epidemiol.* Sep 15 1996;144(6):537-547.

45. Mora S, Ridker PM. Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER)--can C-reactive protein be used to target statin therapy in primary prevention? *Am J Cardiol.* Jan 16 2006;97(2A):33A-41A.

46. Jeng JR. Plasma C-reactive protein and 5-lipoxygenase-activating protein gene promoter poly-A polymorphism in patients with coronary artery disease. *Cardiology.* 2008;109(1):25-32.

47. Allayee H, Hartiala J, Lee W, et al. The effect of montelukast and low-dose theophylline on cardiovascular disease risk factors in asthmatics. *Chest.* Sep 2007;132(3):868-874.

48. Ramires R, Caiaffa MF, Tursi A, Haeggstrom JZ, Macchia L. Novel inhibitory effect on 5-lipoxygenase activity by the anti-asthma drug montelukast. *Biochemical and Biophysical Research Communications.* 2004/11/12 2004;324(2):815-821.

49. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A.* Apr 26 1994;91(9):3652-3656.

50. Papadopoulou C, Corrigall V, Taylor PR, Poston RN. The role of the chemokines MCP-1, GRO-alpha, IL-8 and their receptors in the adhesion of monocytic cells to human atherosclerotic plaques. *Cytokine.* Aug 2008;43(2):181-186.

51. Parissis JT, Korovesis S, Giazitzoglou E, Kalivas P, Katritsis D. Plasma profiles of peripheral monocyte-related inflammatory markers in patients with arterial hypertension. Correlations with plasma endothelin-1. *Int J Cardiol.* Apr 2002;83(1):13-21.

52. Ichiyama T, Hasegawa M, Ueno Y, Makata H, Matsubara T, Furukawa S. Cysteinyl leukotrienes induce monocyte chemoattractant protein 1 in human monocytes/macrophages. *Clin Exp Allergy.* Sep 2005;35(9):1214-1219.

53. Peeters AC, Netea MG, Janssen MC, Kullberg BJ, Van der Meer JW, Thien T. Pro-inflammatory cytokines in patients with essential hypertension. *Eur J Clin Invest.* Jan 2001;31(1):31-36.

54. Vanhala M, Kautianinen H, Kumpusalo E. Proinflammation and hypertension: a population-based study. *Mediators Inflamm.* 2008;2008:619704.

55. Conti P, Panara MR, Barbacane RC, Bongrazio M, Dempsey RA, Reale M. Human recombinant IL-1 receptor antagonist (IL-1Ra) inhibits leukotriene B4 generation from human monocyte suspensions stimulated by lipopolysaccharide (LPS). *Clin Exp Immunol.* Mar 1993;91(3):526-531.

56. Slotman GJ, Quinn JV, Wry PC, Brathwaite CE, Friedman BM. Unopposed interleukin-1 is necessary for increased plasma cytokine and eicosanoid levels to develop in severe sepsis. *Ann Surg.* Jul 1997;226(1):77-84.

57. Mauno V, Hannu K, Esko K. Proinflammation and hypertension: a population-based study. *Mediators Inflamm.* 2008;2008:619704.

58. Koch AE, Kunkel SL, Harlow LA, et al. Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis. *J Clin Invest.* Sep 1994;94(3):1012-1018.

59. Van Den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem.* Sep 2003;270(18):3739-3749.

60. Johnson JA, Boerwinkle E, Zineh I, et al. Pharmacogenomics of antihypertensive drugs: rationale and design of the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) study. *Am Heart J.* Mar 2009;157(3):442-449.

61. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation.* Dec 17 2002;106(25):3143-3421.

62. Zineh I, Luo X, Welder GJ, et al. Modulatory effects of atorvastatin on endothelial cell-derived chemokines, cytokines, and angiogenic factors. *Pharmacotherapy.* Mar 2006;26(3):333-340.

63. Sabatine MS, Morrow DA, Jablonski KA, et al. Prognostic significance of the Centers for Disease Control/American Heart Association high-sensitivity C-reactive protein cut points for cardiovascular and other outcomes in patients with stable coronary artery disease. *Circulation.* Mar 27 2007;115(12):1528-1536.

64. Zineh I, Arant CB, Wessel TR, Langaee TY, Welder GJ, Schofield RS. Apolipoprotein B/A ration within the normal range is associated with elevated C-reactive protein in apparently healthy women. *Pharmacotherapy.* 2005;25(3):453.

65. Gonzalez P, Reguero JR, Lozano I, Moris C, Coto E. A functional Sp1/Egr1-tandem repeat polymorphism in the 5-lipoxygenase gene is not associated with myocardial infarction. *Int J Immunogenet.* Apr 2007;34(2):127-130.

66. Maznyczka A, Braund P, Mangino M, Samani NJ. Arachidonate 5-lipoxygenase (5-LO) promoter genotype and risk of myocardial infarction: a case-control study. *Atherosclerosis.* Aug 2008;199(2):328-332.

67. Allayee H, Baylin A, Hartiala J, et al. Nutrigenetic association of the 5-lipoxygenase gene with myocardial infarction. *Am J Clin Nutr.* Oct 2008;88(4):934-940.

BIOGRAPHICAL SKETCH

Anzeela Mulaiya Schentrup was born in 1976 in Mississauga, Ontario, Canada, and moved to the United States in 1985. In the United States, she was raised in Hollywood, Florida and then moved to Gainesville, Florida to begin her college studies. She holds a Bachelor of Arts in the history of science, a Master of Science in medicinal chemistry, a Doctor of Pharmacy degree, and a Doctor of Philosophy in Pharmaceutical Sciences all from the University of Florida in Gainesville, Florida. She is a licensed pharmacist in the state of Florida and currently practices at the Gainesville Veteran's Affair Medical Center. In 2004, she started the Clinical Pharmaceutical Sciences graduate program under the guidance of Dr. Julie A. Johnson in the Department of Pharmaceutics at the University of Florida. Dr. Schentrup has earned 3 pre-doctoral fellowships from the American Heart Association, the American Foundation for Pharmaceutical Education and from the American Association of University Women in support of her graduate work. She has authored and co-authored peer-review publications and presented at national conferences. She worked under the supervision of Dr. Johnson in the area of leukotrienes in heart disease. Dr. Schentrup continues to live in Gainesville, Florida with her husband and 3 children.