RT
00604
c.1

Effect of ALOX5AP Gene Variants on Coronary Artery Disease Risk

By

Ahmad A. Alwan

A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

in
Molecular Biology

Lebanese American University

January 2009

Under the supervision of Dr. Pierre Zalloua

LEBANESE AMERICAN UNIVERSITY

Thesis approval Form (Annex III)

Student Name: Ahmad Alwan I.D. #: 200603691

Thesis Title : **"Effect of ALOX5AP Gene Variants on Coronary Artery Disease Risk"**

Program : M.S. in Molecular Biology

Division/Dept : Natural Sciences Department

School : **School of Arts and Sciences**

Approved by:

Thesis Advisor: Dr. Pierre Zalloua [redacted]

Member : Dr. George Baroody [redacted]

Member : Dr. Costantine Daher [redacted]

Date: January 2009

Plagiarism Policy Compliance Statement

I certify that I have read and understood LAU's Plagiarism Policy. I understood that failure to comply with this policy can lead to academic and disciplinary actions against me. This work is substantially my own, and to the extent that my part of this work is not my own I have indicated that by acknowledging its sources.

Ahmad A. Alwan

I grant to the **LEBANESE AMERICAN UNIVERSITY** the right to use this work, irrespective of any copyright, for the University's own purpose without cost to the University or to its students, agents and employees. I further agree that the University may reproduce and provide single copies of the work, in any format other than in or from microfilms, to the public for the cost of reproduction.

Ahmad A. Alwan

ACKNOWLEDGMENTS

My praise be to GOD, most Merciful, who provided me with patience, strength and motivation during this study.

I am very grateful for my advisor, Dr. Pierre Zalloua for the support and encouragement he provided me during my whole study. I have learned from you how to be a hard worker looking always for the best with high self confidence. Your enthusiasm for scientific research is the most important factor for getting this work succeeded.

I would like to thank my committee members, Dr. Costantine Daher and Dr. George Baroody for the efforts they have done to correct my thesis and criticize my work and for their suggestions.

The invaluable help and support that Dr. Mirvat El-Sibai had provided me will be unforgotten. Your help may really change my future. Your name is scratched in my heart and memory. I ask GOD to give you the best because you deserve it.

My big thank goes to Mrs. Sonia Yohanna, for providing me with the needed help to get the required samples for my work and supplying me with the demanded data. I will not forget your kindness.

Special thanks to Mr. Marc Haber for providing me with technical assistance and facilitating my work by supplying me with the materials needed. Your help is highly appreciated.

I would like to thank as well Dr. Daniel Platt for helping me in statistical analysis.

I must acknowledge Mr. Simon Khoury and Ms. May Sanyoura for their help in the thesis work.

I would like to express my gratitude to my teachers, Dr. Fouad Hashwa, Dr. Roy Khalaf, and Dr. Sima Tokijian. I highly appreciate the support and encouragement that Dr. Sima has always provided me in the most difficult periods. I hope that some day I would be able to convey my gratitude.

I must thank Ms. Helena Abu Farah and Ms. Maya Farah for their help in the graduate laboratory.

I would like to thank all my colleagues in the department of molecular biology: Nina, Rana, Rami, Samer, Mazen, Bassem, Dominique, Dina, Pascal, Katia, Tania, Angelique, Jihad, Nizar, Suleiman, Karim, Mirna, Rushdi, Farah, Wissam, Wael and Jalil.

My dearest thanks to my friends: Husein obeid, Mustafa El-Orra, Madi Jarouch, Tarek Jamal, Omar Alshafee, Samer Sammak and Tamer Rammal.

I would like to express my love, gratitude and all the good words may ever found to the individuals who form my world: my parents, brothers, sisters, my wife and my son, Mohammad.

Finally, I would like to thank the Lebanese American University for supporting my work.

ABSTRACT

Effect of ALOX5AP Gene Variants on Coronary Artery Disease Risk

Coronary artery disease (CAD) is a multifactorial disease mediated by genetic and environmental factors. The inflammatory pathway is one of the many pathways which are implicated in CAD pathobiology. Recent studies defined several inflammatory genes as susceptibility genes to CAD. Genetic variations in the *ALOX5AP* gene coding for arachidonate 5-lipoxygenase-activating protein (FLAP) have been shown to predispose to CAD in European populations. The aim of the present study was to validate the role of the genetic variations of *ALOX5AP* gene in CAD in a Lebanese population with angiographically established CAD. Four single nucleotide polymorphisms (SNPs) (the at-risk haplotype B) in the *ALOX5AP* gene were genotyped in 289 young CAD patients (> 50% stenosis, ≤ 52 years) with positive family history of CAD and 227 old subjects proved to be free of CAD (0% stenosis, ≥ 60 years) considered as control group. The four SNPs genotypes were not associated with CAD or myocardial infarction (MI) in our population. No difference in haplotype distributions was detected between CAD cases and controls (P= 0.25). No evidence for association of haplotype B (HapB) with CAD or MI was found (OR= 0.8, P= 0.25; OR= 0.9, P= 0.54, respectively). In conclusion, haplotype B of *ALOX5AP* gene is not associated with increased risk for CAD in the Lebanese population. Our findings dispute the assumption that *ALOX5AP* is a susceptibility gene for CAD and may limit the applicability of FLAP antagonists in prophylaxis of CAD.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

χ^2	Chi-test
AA	Arachidonic acid
aa	Amino acid
AUBMC	American University of Beirut Medical Center
ACS	Acute coronary syndrome
CAD	Coronary artery syndrome
CRP	C-reactive protein
CVD	Cardiovascular system
C-smoking	Cigarette smoking
EDTA	Ethylene diamine tetra acetic acid
FBS	Fasting blood sugar
FLAP	5-lipoxygenase activating protein
FxCAD	Family history of coronary artery disease
Hap	Haplotype
HDL	High density lipoprotein
ICAM-1	Intercellular adhesion molecule-1
LAD	Left anterior descending artery
LCx	Left circumflex artery
LDL	Low density lipoprotein
LT	Leukotriene
LT-R	Leukotriene receptor
LTA	Lymphotoxin alpha
LTA4H	Leukotriene A4 hydrolase
LTB4	Leukotriene B4
LTC4	Leukotriene C4
MI	Myocardial infarction
MPO	Myeloperoxidase
MVD	Multiple vessel disease
Mut	Mutant

OR	Odds Ratio
PCR	Polymerase chain reaction
RCA	Right coronary artery
RHUH	Rafic Hariri University Hospital
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SVD	Single vessel disease
t-chol	Total cholesterol
TG	Triglyceride
VCAM-1	Vascular cell adhesion molecule 1
Wt	Wild type

CHAPTER I

Introduction

1.1. Epidemiology and nature of coronary artery disease

Coronary artery diseases (CAD), including myocardial infarction (MI), continue to be the main cause of fatality in many countries (Mackay *et al.*, 2004). In the US for example cardiovascular disease is responsible for more than 50 % of all deaths and most of these deaths are due to diseases in the coronary arteries (AHA., 2007). Myocardial infarction is expected to be the most common cause of death worldwide by the year 2020 (Murray *et al.*, 1997).

CAD is a multifactorial disease and several risk factors have been well established to increase the risk for CAD. These conventional risk factors include diabetes mellitus, hypercholesterolemia, and hypertension (Mackay *et al.*, 2004). Environmental and behavioral risk factors, such as smoking, high-fat diet and obesity also increase the risk for CAD (Assmann *et al.*, 1999). More recently identified factors that predispose to the disease include hyperhomocystenemia, hyperuricemia, C reactive protein (CRP) (Abchee *et al.*, 2006) and gene polymorphisms in myeloperoxidase gene (MPO) (Düzgünçinar *et al.*, 2008).

1.2. Pathobiology of coronary artery disease

Many pathways implicated in CAD pathogenesis are now well described. An aberration in any of the many steps involved in these pathways creates a pathophysiological condition that leads to the disease. Some of the steps leading to the development of CAD include the loss of the normal function of the arterial endothelium, the accumulation of cholesterol deposits in the sub-endothelial arterial intima, the inflammation of the arterial wall, and finally, the proliferation of smooth muscle cells (Ramos *et al.*, 2007). Inflammatory mediators, such as cytokines, are involved in atheroma formation, rupture of the plaque, and intraluminal thrombosis (Libby., 2002; Hansson., 2005). Here forth we describe the pathobiology of CAD.

The immediate events in the pathogenesis of atherosclerosis take place at the level of the coronary artery endothelium. The endothelium is a very important

structure that maintains the integrity of the vessels. The endothelium controls different processes including thrombosis, leukocyte interaction with the vessel wall, smooth muscle cell proliferation and maintenance of vascular tone via several mediators such as nitric oxide (NO) (Poredos., 2002). Endothelial dysfunction is one of the main pathways through which conventional cardiovascular risk factors, such as smoking, diabetes, and dyslipidemia enhance the development of CAD.

The damaged endothelium results in the reduction of NO release. NO normally acts as vasodilator and as an inhibitor of inflammation and platelet aggregation (Yang *et al.*, 2006). The damaged endothelium allows the entry of Low density lipoprotein (LDL) cholesterol particles through focal lesions formed in it, these particles are then oxidized in presence of reactive oxygen species by macrophages and smooth muscle cells. NO depletion upregulates the synthesis of adhesion molecules on endothelial cell surfaces (VCAM-1, ICAM-1 and E-selectin) (Endemann *et al.*, 2004). These adhesion molecules, along with local cytokines released by local inflammatory cells attract more monocytes to the artery wall. Monocytes then become loaded with the oxidized LDL particles and are transformed to foam cells. These accumulate and aid along with proliferated smooth muscle cells in the formation and growth of the plaque. The plaque is then transformed to a loose plaque after the infiltration of inflammatory cells, smooth muscle cell apoptosis and matrix degradation by matrix metalloproteinases (Figure 1). The plaque thereafter is exposed to rupture which might cause thrombosis and occlusion of the coronary arteries (Lusis., 2000).

1.3. Role of Family history of CAD in coronary artery disease

CAD is considered to be a polygenic disease, with several genes related to the prognosis of the disease. Additionally, the interaction of functional gene polymorphisms with environmental factors plays a significant role in defining the risk of developing CAD (Talmud., 2007). This adds to the difficulty of understanding the disease, however it emphasizes the multifactorial nature it. Most of the known risk factors can indeed be substantially modified through the alteration in health behavior, like dietary choices and the involvement in physical activity. While inherited elements, like genetic makeup and family history of the disease, cannot be

regulated, the detection of those parameters is important for preventative and therapeutic measures.

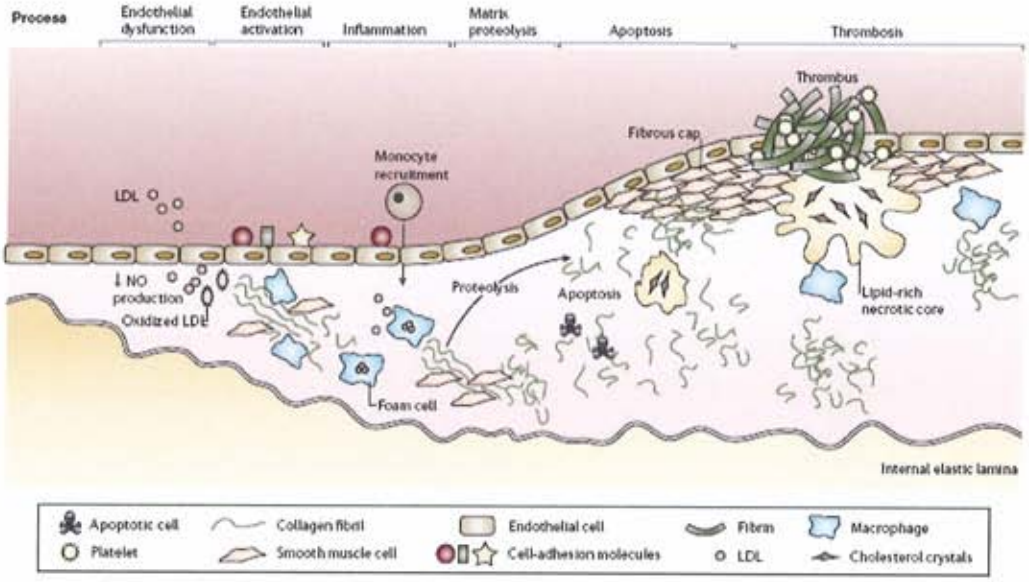

Figure 1. Cellular processes that lead to the development of atherosclerotic plaque. (Watkins *et al.* Genetic susceptibility to coronary artery disease: from promise to progress. *Nature reviews of Genetics*. 2006; 7:163-73)

CAD runs in families, hence family history continues to be one of the most robust independent risk factor for CAD (Lusis *et al.*, 2004; Robin *et al.*, 2007). It was recently shown that a positive family history for CAD is an independent predictor for the disease, confirming a genetic predisposition for the disease (Abchee *et al.*, 2006). Other studies also reported that family history is a strong risk factor for CAD. In the Prospective Cardiovascular Munster (PROCAM) study, the authors reported that family history of MI is an independent risk factor for CAD (Assmann *et al.*, 2002). In the Framingham Heart Study, family history of premature CAD or cerebrovascular disease was found to be a strong risk factor for CAD (Lloyd-Jones *et al.*, 2004). Finally Yusuf *et. al* (Yusuf *et al.*, 2004), showed that family history increased the risk for CAD, even after the adjustment for several conventional risk factors. These data reveal a role of genes that may be distinct from those genes

affecting conventional risk factors, such as dyslipidemia and hypertension. Defining those additional genes and understanding the function of their products is paramount to the understanding of the pathophysiology of the disease.

Family history however, may include common genetic factors as well as shared environmental factors, such as common diet, environmental exposures and similar life style. These factors complicate the nature of CAD development; hence, the detection of the specific genes involved is crucial for proper risk estimation. In addition, in such complex traits, the existence of a risk allele is not by itself sufficient to cause the CAD phenotype and may not systematically be detected to cosegregate with it. CAD is also genetically heterogeneous, and the risk alleles are most likely of variable penetrance and different prevalence in different populations. These factors complicate the identification of CAD susceptibility genes variants (Robin *et al.*, 2007).

1.4. Genomics of coronary artery disease

Despite the complexity of CAD as aforementioned, important progress has been recently made to determine the genomic background of coronary artery disease. This progress is due to the great advancement in the field of genomics, which permits genotyping hundreds of thousands of single-nucleotide polymorphisms (SNPs) in genes which products are implicated in the pathways described above in each subject. These powerful approaches, including genome-wide scans and genome-wide linkage studies, resulted in the identification of new genes that predispose to CAD. These experiments were made possible without depending on previously cited correlation between such gene variants and the disease. Such studies overcame the weaknesses present in the classical association studies that required information about the function of the susceptible genes being investigated (Wang., 2005). Genome-wide studies can also be used in synergy with candidate-gene studies with a focus on genes in the pathophysiological pathways of known risk factors, in order to unravel the complex molecular mechanism of CAD (Damani *et al.*, 2007).

Through genome-wide association studies and genome-wide linkage scans, researchers were able to identify genes, most of which are involved in the

inflammatory pathway of CAD. These include, lymphotoxin- α (*LTA*) (Ozaki *et al.*, 2002), galectin-2 (*LGALS2*) (Osaki *et al.*, 2004), proteasome subunit α type 6 gene (*PSMA6*) (Ozaki *et al.*, 2006), 5-lipoxygenase activating protein (FLAP) (Helgadottir *et al.*, 2004) and leukotriene A4 hydrolase (*LTA4H*) (Helgadottir *et al.*, 2006). Mutations in these genes have been directly correlated with an increased risk of CAD. These genes and their gene products either induce susceptibility to the disease or impair the protection against it.

In a "land mark" study on MI, genetic susceptibilities in the leukotriene pathway that were found to be associated with MI (Helgadottir *et al.*, 2004). Helgadottir *et al.* (2004) showed two genes, the arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*) gene and the Leukotriene A4 hydrolase (*LTA4H*) gene, to be associated with MI (Helgadottir *et al.*, 2004; Helgadottir *et al.*, 2006). In fact, several reports have indicated that the leukotriene pathway plays an important role in the pathogenesis of atherosclerosis, particularly the branch involved in LTB4 biosynthesis.

1.5. Implication of leukotrienes in coronary artery disease

Intracellularly, leukotrienes (LTs), such as LTB4 and cysteinyl LTs (e.g. LTC4) are the products of arachidonic acid oxidation. The release of arachidonic acid from membrane glycophospholipids is stimulated by the phospholipase A2 enzyme. The released arachidonic acid (AA) binds to 5-lipoxygenase-activating protein (FLAP), which is the product of the *ALOX5AP* gene. FLAP transfers the AA to 5-lipoxygenase enzyme, facilitating its conversion to LTA4. LTA4 serves as substrate for LTC4 synthase to generate LTC4/LTD4. LTA4 is then hydrolysed by the enzyme LTA4 hydrolase, which is the product of the *LTA4H* gene, to generate LTB4 (Figure 2).

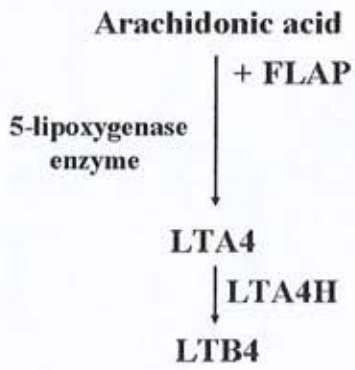

Figure 2. The arachidonic acid oxidation pathway

During inflammatory response leading to CAD, macrophages are the main cells that oxidize AA intracellularly to release LTB4. Intuitively, a gain-of-function mutation in the genes involved in the AA oxidation pathway will lead to an aggravated inflammatory response. LTB4 may act on neighboring cells including, smooth muscle cells (SMCs), and T lymphocytes in a paracrine fashion and on macrophages in an autocrine fashion (Figure 3). LTs perform their physiological actions through binding to and activation of G-protein coupled 7 helix cell surface receptors (Samuelsson *et al.*, 1983). Several cell types such as macrophages, T cells, mast cells, SMCs, and endothelial cells express LT receptors.

LT formation may occur in lamina intima lesions or in the lamina adventitia. In clinically significant coronary artery diseases where atherosclerosis leads to above 50% stenosis of the artery, LT-secreting macrophages form a major part of the plaques (Spanbroek., 2003). The release of LTs in blood vessels leads to edema formation (Dahlen *et al.*, 1981) and coronary artery contraction (Allen *et al.*, 1998). An increase in LT in cultured endothelial cells leads to an upregulation of selectin surface expression, to Von Willebrand factor secretion, as well as to an increase in platelet-activating factor synthesis (Pedersen *et al.*, 1997; Datta *et al.*, 1995). LTB4 may also enhance the inflammatory responses in the arterial walls, by mediating chemotaxis and then transmigration of leukocytes through the vascular endothelium (Lotzer *et al.*, 2005). These leukocytes express LT-Rs and are able to detect and follow the gradient of LTs released by local hematopoietic cells. Moreover, the LTB4-activated leukocytes release lysosomal enzymes which lead to generation of

oxygen reactive species (Samuelsson *et al.*, 1983). This role of LTs in atherosclerosis progression was supported by recent genetic studies on CAD.

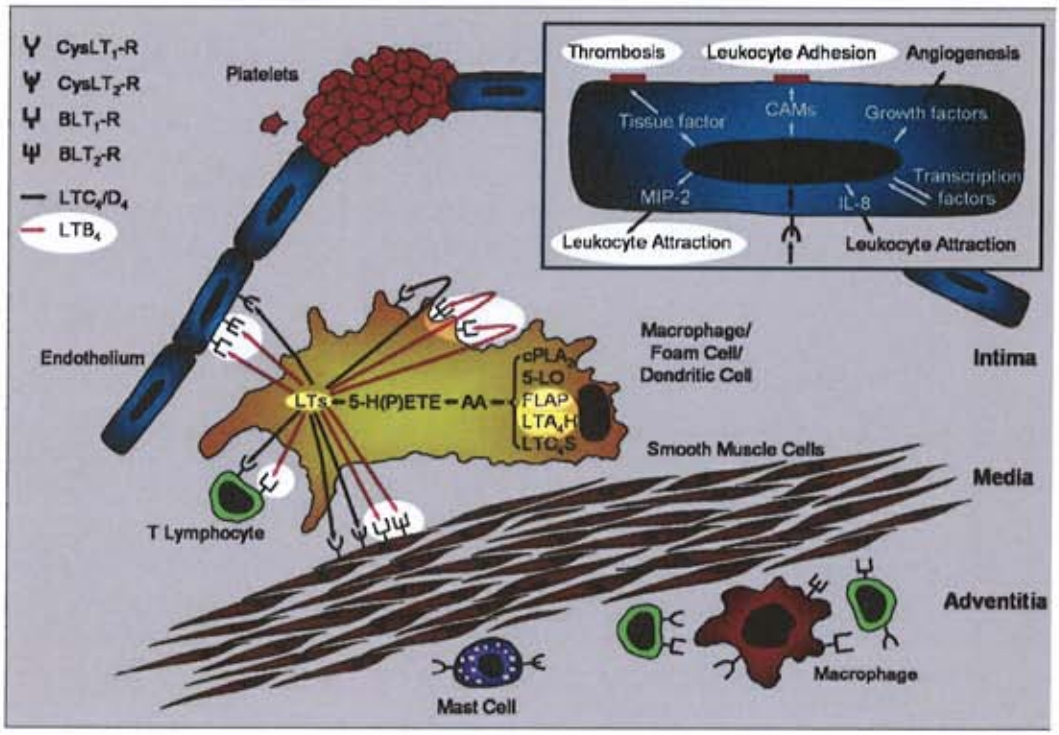

Figure 3. Effects of the inflammatory mediators FLAP and LTB4 on endothelium and smooth muscles of coronary artery are highlighted. (Lotzer K, *et al.* The 5-lipoxygenase pathway in arterial wall biology and atherosclerosis. *Biochimica et Biophysica Acta*. 2005;1736:30-37)

In a recent study involving 713 MI cases, Helgadottir *et al.* (2004) found a linkage between the 13q12-13 chromosomal region, which contains the *ALOX5AP* gene, and a 28.9 kb region that contains 5 exons and encodes a 162 a.a protein. Within that region, the authors identified a haplotype (HapA), consisting of 4 SNPs, which was found to increase the risk of MI by 2-folds. Helgadottir *et al.*(2004) verified their results using a functional assay, where they showed an increased production of LTB4 in vitro in neutrophils that were isolated from HapA carriers compared to control cells (Helgadottir *et al.*, 2004). These results were not supported by another study that could not detect variations in LTB4 levels in the presence of HapA or HapB (Maznyczka *et al.*, 2007).

While Helgadottir *et al.* (2004) established the association between HapA and MI in an Icelandic population, they did not see the same effect in a British cohort (Helgadottir *et al.*, 2004). In the British population they found another haplotype (HapB), which consisted of 4 distinct SNPs, to be more frequent in the cases compared to controls and found HapB to increase the risk for MI (a relative risk of 1.95) (Helgadottir *et al.*, 2004). The association of HapB with CAD was replicated in an Italian population (Girelli *et al.*, 2007) and a German population (Linsel-Nitschke P *et al.*, 2008). Surprisingly, HapA and HapB were not associated with CAD and MI in an American population (Zee R *et al.*, 2006) and a Japanese population (Kajimoto *et al.*, 2005). Finally, in the same genome-wide scan study, Helgadottir *et al.* (2006) used fine mapping to find another haplotype in the LTA4 hydrolase gene (HapK, a 5 to 7 SNP marker haplotype) that correlated with the risk of myocardial infarction and increased production of LTB4 (Helgadottir *et al.*, 2006).

The recent studies aiming to define mutations that correlate to myocardial infarction have added to our understanding of the function of the different genes and proteins involved in the inflammatory pathway that leads to CAD. In a Lebanese cohort, it was shown that family history is an independent risk factor for CAD in patients with > 50 % stenosis (Abchee *et al.*, 2006). These findings may be attributable to the genetic factors that play a significant role in atherogenesis leading to CAD. These results, in addition to the conflicting reports on the association of haplotype B in *ALOX5AP* gene with CAD have led us to investigate the role of *ALOX5AP* gene variants in predisposition to CAD in the Lebanese population. The detection of positive association will serve in the early diagnosis and the identification of high-risk patients and provide another area for therapeutic applications and prophylaxis in our population.

CHAPTER II

Materials and Methods

2.1. Subjects

In this study, we recruited a total of 3000 Lebanese subjects who were referred to the cardiovascular care unit and underwent cardiac catheterization at the American University of Beirut Medical Center (AUBMC) and Rafic Hariri University Hospital (RHUH) in Beirut. Angiograms were performed as a diagnostic procedure for clinical indications and not for the purposes of the study itself; however the results of the angiograms were utilized as our main outcome variable. Patients were mildly sedated prior to cardiac catheterization. Angiography was performed to visualize the left main artery, the left anterior descending artery (LAD), the left circumflex artery (LCx) and the right coronary artery (RCA). All coronary arteries were imaged from different angles. The extent of coronary artery stenosis and the number of diseased arteries were documented for each case. Of these subjects, 289 cases aged $\leq$ 52 years with family history of coronary artery disease had been angiographically confirmed to have a significant coronary artery narrowing >50% stenosis in at least one main coronary artery. Among the cases we identified a subgroup of 55 patients with MI based on medical records showing the echocardiography and enzyme profiles. Moreover, the patients were grouped according to the number of diseased vessels from a total of 4 main vessels examined (left main, left anterior descending, circumflex, and right artery) to single vessel diseased patients (SVD) and multiple vessel diseased patients (MVD). Additionally, 227 control subjects aged 60 years and above, who had normal coronary arteries of 0% stenosis and who were submitted to coronary angiography due to causes other than CAD were considered controls. 127 of these controls had no family history of CAD.

Patients were approached by trained research assistants for discussion on informed consent, prior to the angiographic procedure. Data concerning life style factors, demographic variables, medical history, family history, therapeutic applications and clinical information about subjects were documented. The study protocol was approved by the Institutional Review Board at the Lebanese American University.

2.2. Blood collection and DNA extraction

Venous blood specimens were collected by cardiologists performing coronary angiography; all patients underwent coronary catheterization by Judkins' technique. A blood sample of 20 ml was collected in a sterile syringe from the femoral artery catheterization site of every patient consenting to enroll in the database. EDTA-treated and plain tubes were placed on ice to be delivered to the laboratory department for the measurement of serum lipid profile parameters, CRP, homocysteine, glucose, uric acid and complete blood count. DNA was extracted from a 5 ml peripheral blood samples using the "salting out" method, and stored at -70 °C until genotyping.

2.3. Selection of haplotypes and SNPs

Using the NCBI PubMed Entrez database, we selected the four *ALOX5AP* SNPs constituting haplotypeB (SG13S377, SG13S114, SG13S41 and SG13S35) that were reported to be significantly associated with CAD and MI in a genome-wide association studies and genome-wide linkage scans. The characteristics of each SNP are stated in **Table 1**.

2.4. Genotyping of SNPs

Genotyping of the SNPs mentioned was carried out using the TaqMan Real-time PCR approach. During PCR, the Taq polymerase will partially unwind the perfectly matched probe from the template, cleave the 5' end of the probe with its 5' exonuclease activity, and release the fluorescent reporter into solution. The released fluorescent reporter will no longer be quenched resulting in an increased net fluorescent. Use of two probes, each labeled with a different fluorescent dye, allows detection of both alleles in a single tube.

The concentrations of the DNA samples were determined by ultraviolet spectrophotometry. The DNA was diluted prior to the TaqMan reaction to a concentration of 10ng/μl and the amount used per reaction was 30ng.

PCR: 30ng of genomic DNA was amplified in a 25 µl PCR reaction consisting of 1x master mix (Applied Biosystems, Foster City, CA), 900 nM each of PCR forward and reverse primers, and 250nM each of two TaqMan MGB probes (**Table 2a**; **Table 2b**). PCR was carried out on ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 10 min; 40 cycles of 95 °C for 15sec and 60 °C for 1min (**Table 3**).

Plate read: after PCR amplification, an endpoint plate read was performed on an ABI primer 7900 (Applied Biosystems, Foster City, CA). The fluorescence intensity was measured for each well.

Allele calling: the SDS 2.1 software obtains the fluorescence measurements made during the plate read and generates an allelic discrimination plot with genotype clusters. Sample genotype was called automatically by the software and manually checked.

2.5. Statistical analysis

Statistical analysis was performed with SPSS version 16.0 for Windows (Statistical Package for the Social Science, SPSS Ins., Chicago, IL). Discrete variables were compared by the chi-square test. Continuous variables were expressed as means and quantitative data assessed using student's *t*-test. A value of two-tailed $P<0.05$ will be considered significant. The χ^2 test with one degree of freedom is used to compare the Allele and genotype frequencies among cases and controls. Chi-square test was used to compare the genotype frequency between patient groups with single vessel disease (SVD) and multiple-vessel disease (MVD). Haplotype analysis was carried out using PHASE v2.1. Odds Ratio values were calculated to estimate the association between the haplotypes and the clinical outcome (CAD and MI) and OR>1 was considered as indicator for a positive risk at $P<0.05$.

Table 1: Characteristics of the SNPs genotyped in the *ALOX5AP* gene that define HapB/C haplotypes.

Ref SNP ID	deCODE SNP ID	Gene location	Relative position	SNPs defining **HapB**	SNPs defining **HapC**
rs17216473	SG13S377	Promoter	-5777G>A	A	G
rs10507391	SG13S114	Intron1	2354T>A	A	T
rs9315050	SG13S41	Intron4	26303A>G	A	A
rs17222842	SG13S35	3'-UTR	30375G>A	G	A

Table 2a: The sequence of primers used in target sequence amplification for each polymorphism.

	Primer	
SNP	**Forward**	**Reverse**
SG13S377	gATTgTAggCgTgCACCACTATg	gTggCTCATgCCTATAATCACAAAAC
SG13S114	AAAgATCCAgATgTATgTCCAAgCC	AgggTTTTTgCATATTTTTTTgAACT
SG13S41	TCACCTCACTgAgCATgTCTgTgT	AACATCCCAAAAgAgggTgTTgT
SG13S35	gTgATCAATAATCCTgATTggCCT	ATgCACCCCACAAATACCTACAA

Table 2b: The sequence of the mutant and wild type probes used in genotype detection of each polymorphism.

	Probe	
SNP	**FAM**	**YAK**
SG13S377	AggCCgAggCAggCAgAT	AggCTgAggCAggCAgATCAC
SG13S114	TgCAATTCTAATTAACCTCAATgTTgC	TgCAATTCTTATTTAACCTCAATgTTgC
SG13S41	TgTTgAATCTTTACTCTCAgTTCCTCA	TgAATCTTTACCCTCAgTTCCTCA
SG13S35	AAAAACCgAAAggACCACATCC	AAAAAACTgAAAggACCACATCCC

Table 3: Reagents used in the genotyping of HapB SNPs

Reagent	Concentration per reaction	Volume per reaction
Universal PCR mix	2x	12.5 µl
Primer F	900 nM	1.125 µl
Primer R	900 nM	1.125 µl
Wt probe	250 nM	0.625 µl
Mut probe	250 nM	0.625 µl
DNA	10 ng/µl	3 µl
Water		6 µl

CHAPTER III

Results

Clinical characteristics and demographic data of the study population are shown in **table 4**. The mean age of the control subjects (68 years) was significantly higher than the cases (48 years). Limiting the control subjects to the older individuals ensures that the subject had enough time for the disease to manifest and limiting the cases to young patients ensures that most of the risk factors involved in the early onset disease are genetic and not environmental (which usually happen over a long span of time). The frequency of hypertension was higher in controls than in the cases and this result can be explained by the fact that since these patients were diagnosed early with hypertension they were more likely to take precautionary measures. The two groups were comparable concerning diabetes, obesity, fasting blood sugar (FBS) and uric acid. However, the number of smokers and the levels of total cholesterol, LDL-cholesterol and triglycerides were significantly higher in CAD cases. Males constituted a significant higher percentage among the cases than the controls.

After performing a χ^2 analysis, we found no significant difference in the allele and genotype frequencies between CAD cases and controls (**table 5**). When considering a subgroup of cases who have MI and comparing the allele and genotype frequencies between the MI patients and controls, we still were not able to detect any significant difference (**table 6**). Furthermore, the genotype frequency was similar among single vessel diseased cases and multiple vessel diseased cases (**table 7**).

The distribution of haplotype B and haplotype C between CAD cases and CAD-free controls were similar (P = 0.25, P = 0.43; respectively). The most frequent haplotype was G-T-A-G (**table 8**). However, when comparing the cases with controls with no FxCAD the haplotype B was significantly more frequent in controls (P= 0.034) (data not shown).

The odds ratio for haplotype B and C in estimation to the association with CAD was 0.8 and 0.9, respectively, which indicates no risk for coronary artery disease (**table 9**). The same results appeared in estimation to the association of haplotype B and C with MI where OR was 0.9 and 0.1, respectively (**table 10**).

Table 4: Characteristics of the study subjects

	Cases (n= 289)	Controls (n= 227)	P-values
Age (years)	46.7	68.65	<0.0001#
Sex (male, %)	84.8	46.7	<0.0001*
BMI (kg/m^2)	29.43	29.2	0.62#
C-smoking (%)	58.9	22	<0.0001*
Hypertension (%)	45.5	64.3	<0.0001*
Diabetes (%)	24	19.4	0.213*
Hyperlipidemia (%)	56.6	43.2	0.02*
t-Chol (mg/dl)	209	185	<0.0001#
HDL (mg/dl)	38.67	47.5	<0.0001#
LDL (mg/dl)	133.3	109.4	<0.0001#
TG (mg/dl)	218.4	151.8	<0.0001#
FBS (mg/dl)	120.4	109.3	0.025#
Uric acid (mg/dl)	6	6	0.86#

Means compared by student-t test

* Percentages compared by χ^2 test

Table 5: Genotype and allele frequencies of ALOX5AP HapB SNPs in the study subjects grouped to CAD cases and controls.

ALOX5AP genotype,%	CAD cases (n=289)	Controls (n=227)	P†
SG13S377			
GG	73.4	69.6	0.84
GA	23.5	29.5	
AA	3.1	0.9	
G allele	85.1	84.3	0.875
A allele	14.9	15.7	
SG13S114			
TT	20	17.6	0.779
TA	50.2	52	
AA	29.8	30.4	
T allele	45.1	43.6	0.83
A allele	54.9	56.4	
SG13S41			
AA	74.4	78	0.153
AG	21.4	20.7	
GG	4.2	1.3	
A allele	85.1	88.3	0.5
G allele	14.9	11.7	
SG13S35			
GG	82.3	83.3	0.398
GA	15.9	16.3	
AA	1.7	0.5	
G allele	90.3	91.4	0.787
A allele	9.7	8.6	

† compared by χ^2 test or Fisher's exact test

Table 6: Genotype and allele frequencies of *ALOX5AP* HapB SNPs in the study subjects grouped to MI cases and controls.

ALOX5AP genotype,%	MI cases (n=55)	Controls (n=227)	P†
SG13S377			
GG	69.1	69.6	0.294
GA	27.3	29.5	
AA	3.6	0.9	
G allele	82.8	84.3	0.774
A allele	17.2	15.7	
SG13S114			
TT	21.8	17.6	0.734
TA	47.3	52	
AA	30.9	30.4	
T allele	45.4	43.6	0.797
A allele	54.6	56.4	
SG13S41			
AA	65.5	78	0.053
AG	29	20.7	
GG	5.5	1.3	
A allele	80	88.3	0.1
G allele	20	11.7	
SG13S35			
GG	89.1	83.3	0.531
GA	10.9	16.3	
AA	0	0.4	
G allele	94.5	91.4	0.39
A allele	5.5	8.6	

† compared by χ^2 test or Fisher's exact test

Table 7: Genotype frequencies of *ALOX5AP* HapB SNPs in CAD cases grouped to single vessel diseased cases (SVD) and multiple vessel diseased cases (MVD).

ALOX5AP	CAD cases (n=280)*		
Genotype,%	SVD (n= 42)	MVD (n= 238)	P‡-values
SG13S377			
GG	78.6	72.7	0.72
GA	19	23.9	
AA	2.4	3.4	
SG13S114			
TT	14.3	20.6	0.25
TA	45.2	51.3	
AA	40.5	28.2	
SG13S41			
AA	76.2	73.1	0.87
AG	19	22.7	
GG	4.8	4.2	
SG13S35			
GG	90.5	81.1	0.29
GA	9.5	16.8	
AA	0	2.1	

‡ genotype frequencies compared by χ^2 test

* Data for 9 cases are missing

Table 8: *ALOX5AP* haplotype B/C distribution in the study subjects

ALOX5AP haplotype B/C SNPs (SG13S377, SG13S114, SG13S41, SG13S35)	CAD cases (%)	Controls (%)	P-value (Fisher)
G-T-A-G	53.3	56.8	0.2420
G-T-A-A (Hap C)	11	9.3	0.4396
G-A-A-G	48.8	74	0.0000
G-A-G-G	16.6	11.9	0.9502
A-T-A-G	25.6	29.4	0.1886
A-A-A-G (Hap B)	15.9	18.5	0.2567
A-A-A-A	5.9	5.7	0.6026

Table 9: Odds Ratios for CAD for HapB/C

Hap B/C SNPs	OR for CAD	P-values (Fisher)
G-T-A-A (Hap C)	0.9233	0.4396
A-A-A-G (Hap B)	0.8349	0.2567

(NOTE: most OR p-values are reported as 5% confidence intervals)

Table 10: Odds Ratios for MI for Hap B/C

Hap B/C SNPs	OR for MI	P-values (Fisher)
G-T-A-A (Hap C)	0.1354	0.0132
A-A-A-G (Hap B)	0.9627	0.5476

CHAPTER IV

Discussion

Coronary artery disease is the leading cause of death in the world and particularly in developing countries. Unraveling the genetic basis of this fatal disease in our Lebanese population is crucial for developing the proper prophylactic strategies.

In the present study, we used phenotypically well-characterized subjects to strengthen our study and constitute the proper tool needed to establish a correlation between the studied genotype and the occurrence of coronary artery disease. In this study, we applied a genetic enrichment technique by biasing our recruitment strategy towards young cases ($\leq$ 52 years old). These coronary artery diseased patients will inevitably reflect a lower contribution of lifestyle and environmental factors and are predicted to carry higher genetic significance. Moreover, as mentioned previously, family history of CAD was shown to be a strong predictor of the disease. This has been shown in our population and was confirmed in several studies in the literature. Consequently, we have chosen cases that have a positive family history of CAD aiming to further enrich for the genetic factors predisposing to CAD in the coronary artery diseased group.

In addition our control group included subjects that have undergone angiography and were confirmed to be CAD-free (0% stenosis). In previous studies on genetics factors predisposing to CAD, less stringent control groups were used. These were either population-based (not necessarily confirmed to be CAD-free), or shown by angiography to have non-significant artery stenosis (<50%) (Ozaki *et al.*, 2002; Ozaki *et al.*, 2004; Ozaki *et al.*, 2006; Helgadottir *et al.*, 2004; Helgadottir *et al.*, 2006). However, apparently healthy subjects with minor stenosis may have sub-clinical artery narrowing and may become cases later in life with fully manifested clinical signs. A dramatic example of that was described in a study where a patient reported to have minor stenosis (< 30%) developed significant stenosis (> 70%) in 6 months (Luo *et al.*, 2007). As a result of that, we excluded the controls with non-significant coronary stenosis (< 50% stenosis) from our study. Furthermore, our

controls were much older than the cases (≥ 60 years old) (the difference between the mean ages was 22 years) proposing that such group may indeed have a lower risk of becoming cases later in life and have a relatively low genetic load. The chance for a control with 0% stenosis and older than 60 years to develop CAD is lower than younger individuals. In spite of the age mismatch between the cases and the controls in our study, determining the accurate phenotype leading to proper categorization of the subjects is superseding.

Although we expected young cases to be less exposed to environmental risk factors, smoking was more frequent in cases than controls. The cases showed significantly higher levels of t-cholesterol, LDL-cholesterol, triglycerides and FBS but lower levels of HDL-cholesterol. This might indicate the role of dyslipidemia as a risk factor for CAD in our population.

Our study is the first genetic study on the association of the inflammatory gene *ALOX5AP* with CAD that includes cases and controls with detailed clinical and epidemiological criteria of categorization. This is also the first study on the *ALOX5AP* gene in the Middle Eastern populations.

Inflammation plays an essential role in the pathogenesis of CAD. Several studies reported the contribution of the leukotriene pathway in atherosclerosis and CAD (Dwyer *et al.*, 2004; Funk., 2005; Lotzer *et al.*, 2005). Consequently, we have chosen a candidate gene, which has been previously implicated in the inflammatory pathway, to study. We focused in our study on one cascade, the 5-lipoxygenase (*ALOX5AP*) cascade, in order to validate the association of *ALOX5AP* variants with CAD in the Lebanese population. Surprisingly, our data revealed a lack of association of the at-risk haplotype B forming the *ALOX5AP* variants with CAD (OR=0.83; P=0.25) or MI (OR=0.96; P=0.54) in the Lebanese population. However, when CAD cases were compared to controls with no family history of CAD (n=121), the *SG13S377* genotype showed significant association (P=0.034) and haplotype B frequency was significantly higher in the controls with no FxCAD (P= 0.021). This result strengthens further our previous findings that HapB is not associated with CAD in our population.

Similar results were found in an American population (US male physicians) where the 4-SNP haplotype B was not associated with MI (P=0.08) nor with stroke (P=0.47) respectively (Zee *et al.*, 2006). Another study on an American population (n=811) with European ancestry provided as well no association between HapB (OR=1.12; P=0.31) with acute coronary syndrome (ACS) (Morgan *et al.*, 2007). The same results were seen in German cohort (Lohmussaar *et al.*, 2005) and North American cohort (Meschia *et al.*, 2005) where neither *ALOX5AP* gene variants nor HapB were significantly associated with stroke. On the other hand, the first study characterizing *ALOX5AP* gene as susceptible gene for MI was in Icelandic population (genetic isolate) where HapA was significantly associated with MI (Helgadottir *et al.*, 2005). The same research group found HapB significantly associated with MI in British population (P<0.001). One independent replication study in Japanese cohort provided evidence of an association of *ALOX5AP* gene variants and MI (Kajimoto *et al.*, 2005). In another replication study in Italian population the authors found that, although weak, HapB increase the risk for CAD (OR=1.67; P=0.032) and a new haplotype named haplotype C confers risk to CAD (OR=2.41; P=0.03) (Girelli *et al.*, 2007). In a recent study, HapB was found to increase the risk for MI in German population (P<0.0001) (Nitschke-Linsel *et al.*, 2008). Thus, HapB was found to confer a risk to CAD or MI in European populations but not in North American population and Lebanese population.

The difference in allele, genotype and haplotype B frequencies for the variants under study between these populations, as shown in **table 11**, may be attributed to the differences in ethnic background leading to different SNP linkage disequilibrium and haplotype structures. This difference was clearly observed in the Japanese population where a number of haplotype B frequencies were too low for performing an association analysis and the authors depended on other haplotypes to detect the association of *ALOX5AP* gene with MI (Kajimoto *et al.*, 2005).

Another evidence to the effect of ethnic differences on the genetic susceptibility to CAD is also found in the *LTA4H* gene (encoding for LTA4 hydrolase) which increases the risk for MI depending on the ethnic background of the subjects under study (Helgadottir *et al.*, 2006). Moreover, the previously published reports included

different study subjects with different categorization criteria and different phenotypes (MI, CAD or stroke) which complicates the comparison between different studies.

There was no difference in the genotype frequency of the polymorphisms forming HapB when we compared single vessel diseased cases and multiple vessel diseased cases. This result again provides no evidence of the role of haplotype B in *ALOX5AP* gene in the severity of the disease. In spite of the accumulating evidence on the role of inflammatory mediators in the initiation, progression and severity of the disease (Laxton *et al.*, 2005; Lusis., 2000), the inflammatory mediator FLAP is not involved in the initiation, progression or severity of CAD in our population.

The possibility that our results are false-negative results is unlikely, even with the relatively small sample size, since our selection for the subjects included several inclusion criteria for the purpose of genetic enrichment. Absence of genetic risk factors in our subjects might be unlikely as well, due to the presence of positive family history of CAD in all the cases. Another justification of result disagreement is the existence of false-positive results in the previous studies due to the stratification between cases and controls or the improper categorization of the controls.

It is also worth mentioning that the at-risk-haplotype B is constructed from polymorphisms of no clear effect on the function or the synthesis of the FLAP protein. Moreover, these variants might be associated with disease-causal SNPs in *ALOX5AP* gene, and this association might vary between populations according to the genetic structure of each race. Thus, other haplotypes in *ALOX5AP* gene may be associated with the disease.

Table 11: Frequencies of the alleles forming HapB and haplotype B of the *ALOX5AP* SNPs in different populations (in subjects with CAD or MI)

Population	References	Cases (%)	Controls (%)
Icelandic population	Helgadottir *et al*, 2004		
HapB		*NA*	*NA*
SG13S114 allele A		30.0	34.2
British population	Helgadottir *et al*, 2004		
HapB		7.5	4.0
German population	Linsel-Nitschke *et al*, 2008		
HapB		5.3	*NA*
SG13S377 allele A		14.7	12.9
SG13S114 allele A		33.0	30.9
SG13S41 allele A		92.6	92.7
SG13S35 allele G		91.1	88.9
Italian population	Girelli *et al*, 2007		
HapB		7.5	5.5
SG13S377 allele A		13.8	12.8
SG13S114 allele A		37.0	36.7
SG13S41 allele A		90.6	90.5
SG13S35 allele G		90.5	91.7
Japanese population	Kajimoto *et al*, 2005		
SG13S377 allele A		20.0	18.4
SG13S114 allele A		35.9	35.3
SG13S41 allele A		98.7	99.2
SG13S35 allele G		100	100
North American population	Zee *et al*, 2006		
HapB		6	7
SG13S377 allele A		12.0	13.0
SG13S114 allele A		32.0	32.0
SG13S41 allele A		91.0	91.0
SG13S35 allele G		93.0	91.0
Lebanese population	This study		
HapB		15.9	18.5
SG13S377 allele A		14.9	15.7
SG13S114 allele A		54.9	56.4
SG13S41 allele A		85.1	88.3
SG13S35 allele G		90.3	91.4

CHAPTER V

Conclusion

The genetic contribution of a single gene as the *ALOX5AP* gene to CAD susceptibility might be minute due to the fact that coronary artery disease is a complex multifactorial disease. This necessitates carrying out several validation studies in different populations. In this manner, our study provided significant results showing that haplotype B of the *ALOX5AP* gene is not valuable in predicting atherosclerosis and CAD in the Lebanese population and possibly other populations.

The failure in replicating a positive association of the gene with CAD limits the applicability of FLAP antagonists in routine clinical work. The lack of generalizability of the results due to ethnic differences might impose the individualization of the therapy if present. However, further work is needed to know the causes of variations between the studies. Since family history of CAD has been shown to be an independent predictor of CAD in our population, we suggest the presence of genetic factors increasing the risk to CAD other than *ALOX5AP* gene. This demands further investigation for unraveling the contribution of these risk factors to CAD.

REFERENCES

AHA. (2008). *Heart Disease and Stroke Statistics: Our guide to current statistics and the supplement to our heart and stroke facts*. Retrieved January 18, 2008, from the American Heart Association website: http: //www.americanheart.org/ downloadable/ heart/1200082005246HS_Stats%202008.final.pdf.

Abchee, A., Azar, S.T., Shbaklo, H., Nasrallah, A., Sawaya, F.J., Alam, S., et al. (2006). Predictors of coronary artery disease in the Lebanese population. *Thrombosis Research, 117*, 631-637.

Allen, S., Dashwood, M., Morrison, K., & Yacoub, M. (1998). Differential leukotriene constrictor responses in human atherosclerotic coronary arteries. *Circulation, 97*, 2406-2413.

Assimes, T., Knowles, J., Priest, J., Basu, A., Volcik, K., Southwick, K., et al. (2008). Common polymorphisms of ALOX5 and ALOX5AP and risk of coronary artery disease. *Human Genetics, 123*, 399-408.

Assmann, G., Cullen, P., Jossa, F., Lewis, B., & Mancini, M. (1999). Coronary heart disease: Reducing the risk. *Arteriosclerosis, Thrombosis, and Vascular Biology, 19*, 1819-1824.

Assmann, G., Cullen, P., & Schulte, H. (2002). Simple scoring scheme for calculating the risk of acute coronary events based on the10-year follow-up of the prospective cardiovascular Munster (PROCAM) study. *Circulation, 105*, 310-315.

Choudhury, R. P., Fuster, V., & Fayad, Z. A. (2004). Molecular, cellular, and functional imaging of atherothrombosis. *Nature Reviews. Drug Discovery, 3*, 913-925.

Dahlen, S.E., Bjork, J., Hedqvist, P., Arfors, K., Hammarstrom, S., Lindgren, B., et al. (1981). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: In vivo effects with relevance to the acute inflammatory response. *Proceedings of the National Academy of Sciences of the United states of America, 78*, 3887-3891.

Damani, S. & Topol, E. (2007). Future use of genomics in coronary artery disease. *Journal of the American College of Cardiology, 50*, 1933-1940.

Datta, Y., Romano, M., Jacobson, B., Golan, D., Serhan, C., & Ewenstein, B. (1995). Peptido-leukotrienes are potent agonists of von Willebrand factor secretion and P-selectin surface expression in human umbilical vein endothelial cells. *Circulation, 92*, 3304-3311.

Düzgünçinar, O., Hazirolan, T., Deniz, A., Tokgözoğlu, S.L., Akata, D., & Demirpençe, E. (2008). Plasma myeloperoxidase is related to the severity of coronary artery disease. *Acta Cardiology, 63*, 147-152.

Endemann, D. & Schiffrin, E. (2004). Endothelial dysfunction. *Journal of the American Society of Nephrology, 15*, 1983-1992.

Girelli, D., Martinelli, N., Trabetti, E., Olivieri, O., Cavallari, U., Malerba, G., et al. (2007). ALOX5AP gene variants and risk of coronary artery disease: An angiography-based study. *European Journal of Human Genetics, 15*, 959-966.

Hansson, G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *The New England Journal of Medicine, 352*, 1685-1695.

Helgadottir, A., Manolescu, A., Helgason, A., Thorleifsson, G., Thorsteinsdottir, U., Gudbjartsson, D. F., et al. (2006). A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. *Nature Genetics, 38*, 68-74.

Helgadottir, A., Manolescu, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorsteinsdottir, U., et al. (2004). The gene encoding 5-lipoxygenase activating protein confers risk for myocardial infarction and stroke. *Nature Genetics*, *36* (3), 233-239.

Kajimoto, K., Shioji, K., Ishida, C., Iwanaga, Y., Kokubo, Y., Tomoike, H., et al. (2005). Validation of the association between the gene encoding 5-lipoxygenase-activating protein and myocardial infarction in Japanese population. *Circulation*, *69*, 1029-1034.

Laxton, R., Pearce, E., Kyriakou, T., & Ye S. (2005). Association of the lymphotoxin-alpha gene Thr26Asn polymorphism with severity of coronary atherosclerosis. *Genes* and *Immunity, 6*, 539-541.

Libby, P. (2002). Inflammation in atherosclerosis. *Nature*, *420*, 868-874.

Linsel-Nitschke, P., Gotz, A., Medack, A., konig, I., Bruse, P., Lieb, W., et al. (2008). Genetic variation in the 5-lipoxygenase-activating protein (ALOX5AP) is associated with myocardial infarction in the German population. *Clinical Science*, *115*, 309-315.

Lloyd-Jones, D. M., Nam, B. H., D'Agostino, R. B., Levy, D., Murabito, J. M., Wang, T., et al. (2004). Parental cardiovascular disease as a risk factor for cardiovascular disease in middle-aged adults: A prospective study of parents and offspring. *The Journal of the American Association*, *291*, 2204-2211.

Lohmussaar, E., Gschwenttner, A., Mueller, J. C., Org, T., Wichmann, E., Hamann, G., et al. (2005). ALOX5AP gene and the PDE4D gene in central European population of stroke patients. *Stroke*, *36*, 731-736.

Lotzer, K., Funk, C., & Habenicht, A. (2005). The 5-lipoxygenase pathway in arterial wall biology and atherosclerosis. *Biochimica et Biophysica Acta*, *1736*, 30-37.

Luft, F. C. (1998). Molecular genetics of human hypertension. *Journal of Hypertension, 16*, 1871-1878.

Lusis, A. J., Mar, R., & Pajukanta, P. (2004). Genetics of atherosclerosis. *Annual Review of Genomics and Human Genetics, 5*, 189-218.

Luo, A. K., Jefferson, B. K., Garcia, M. J., Ginsburg, G.S., & Topol, E. J. (2007). Challenges in the phenotypic characterisation of patients in genetic studies of coronary artery disease. *Journal of Medical Genetics, 44*, 161-165.

Mackay, J. H., Powell, S. J., Charman, S. C., & Rozario, C. (2004). Resuscitation after cardiac surgery: Are we agiest? *European Journal of Anaesthesiology, 21*, 66-71.

Maznycaka, A., Mangino, M., Whittaker, A., Braund, P., Palmer, T., Tobin, M., et al. (2007). Leukotriene B_4 production in healthy subjects carrying variants of the arachidonate 5-lipoxygenase-activating protein gene associated with a risk of myocardial infarction. *Clinical Science, 112*, 412-416.

Meschia, J. F., Brott, T. G., Brown, R. D., Crooke, R., Worrall, B. B., Kissela, B., et al. (2005). Phosphodiesterase 4D and 5-lipoxygenase activating protein in ischemic stroke. *Annals of Neurology, 58*, 351-361.

Nathan, L. & Chadhuri, G. (1997). Estrogens and Atherosclerosis. *Annual Review of Pharmacology and Toxicology, 37*, 477-515.

Ozaki, K., Inoue, K., Sato, H., Iida, A., Ohnishi, Y., Sekine, A., et al. (2004). Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-alpha secretion in vitro. *Nature, 429*, 72-75.

Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda T., et al. (2002). Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nature Genetics, 32*, 650-654.

Ozaki, K., Sato, H., Iida, A., Mizuno, H., Nakamura, T., Miyamoto, Y., et al. (2006). A functional SNP in PSMA6 confers risk of myocardial infarction in the Japanese population. *Nature Genetics, 38*, 921-925.

Pedersen, K., Bochner, B., & Undem, B. (1997). Cysteinyl leukotrienes induce P-selectin expression in human endothelial cells via a non-CysLT1 receptor mediated mechanism. The *Journal of Pharmacology and Experimental Therapeutics, 281*, 655-662.

Poredos, P. (2002). Endothelial dysfunction in the pathogenesis of atherosclerosis. *International Angiology, 21*, 109-116.

Robin, N. H., Tabereaux, P., Benza, R., & Korf, B. (2007). Genetic testing in cardiovascular disease. *Journal of the American College of Cardiology, 50* (8), 727-737.

Samuelsson, B. (1983). Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. *Science, 220*, 568-575.

Spanbroek, R., Grabner, R., Lotzer, K., Hildner, M., Urbach, A., Ruhling, K., et al. (2003). Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proceedings of the National Academy of Sciences of the United states of America, 100* (3), 1238-1243.

Talmud, P. (2007). Gene-environment interaction and its impact on coronary heart disease risk. *Nutrition, Metabolism & Cardiovascular Diseases, 17*, 148-152.

Wang, Q. (2005). Molecular genetics of coronary artery disease. *Current Opinion in Cardiology, 20*, 182-188.

Watkins, H. & Farrall M. (2006). Genetic susceptibility to coronary artery disease: From promise to progress. *Nature Reviews: Genetics, 7*, 163-173.

Wung, S. & Aouizerat, B. (2008). Candidate genes of the 5-lipoxygenase pathway in acute coronary syndrome: A pilot study. *Biological Research for Nursing*, *4* (9), 280-292.

Yang, Z. & Ming, X. (2006). Recent advances in understanding endothelial dysfunction in atherosclerosis. *Clinical Medicine & Research*, *4*, 53-65.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., et al. (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet*, *364*, 937-952.

Zee, R., Cheng, S., Hegener, H., Erlich, H., & Ridker, P. (2006). Genetic variants of arachidonate 5-lipoxygenase-activating protein, and risk of incident myocardial infarction and ischemic stroke: A nested case-control approach. *Stroke*, *37*, 2007-2011.