Genetic Susceptibility to Coronary Artery Disease in Type 2 Diabetic Patients

by

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

Master of Science

Molecular Biology

Lebanese American University

2009-2010

Under the supervision of

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LEBANESE AMERICAN UNIVERSITY

Thesis approval Form (Annex III)

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Thesis Title : **Genetic Susceptibility to Coronary Artery Disease in Type 2 Diabetic Patients**

Program : M.S. in Molecular Biology

Division/Dept : Natural Sciences Department

School : **School of Arts and Sciences**

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Date: June 2010

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ABSTRACT

Genetic Susceptibility to Coronary Artery Disease in Type 2 Dibetic Patients

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Type 2 diabetes is a major risk factor for coronary artery disease (CAD). Diabetic patients are three times more likely to develop CAD than non diabetic individuals. Many genes have been studied to test for an association between the different gene variants with CAD in individuals with type 2 diabetes. Of these genes, we selected the most relevant ones including a common allele on chromosome 9p21 (rs2383206), a variant in the uncoupling protein 2 (UCP2) promoter (rs659366), a genetic marker in the potassium voltage gated channel KCNQ1 gene (rs2237892), a proteasome subunit alpha type 6 (PSMA6) gene variant in 5' untranslated region of exon 1 (rs1048990), and the polymorphism in 5' untranslated region of the vascular endothelial growth factor (VEGF) gene (rs2010963). Our purpose is to validate in a Lebanese population the association between these five single nucleotide polymorphisms (SNPs) and CAD in subjects with type 2 diabetes. We selected 752 subjects (352 patients with type 2 diabetes of whom 199 had CAD and 400 subjects without type 2 diabetes of whom 202 had CAD) were genotyped for variants of the 9p21 locus, UCP2 gene, KCNQ1 gene, PSMA6 gene, and VEGF gene. Genotype and allele frequencies between the patients and the control groups were compared using chi-square and logistic regression analyses. In the type 2 diabetic population, allele frequency and Genotype distribution did not differ between CAD patients and controls of the SNPs at the 9p21 region, UCP2 promoter, KCNQ1 gene, PSMA6 gene, or

VEGF gene. When we restricted the analysis to nondiabetic patients (n =400), significant results were obtained in the PSMA6-rs1048990 and the VEGF-rs2010963 polymorphism when comparing subjects with CAD to controls without CAD ($p = 0.027$, $p = 0.036$ respectively). An inverse association between CAD and the GG+GC genotype (OR=0.61) as well as the G allele (OR=0.66) of the PSMA6 polymorphism were observed in the non diabetic population. In addition, the CC genotype of the VEGF-rs2010963 polymorphism associated with CAD in a recessive model in the non diabetic population (OR=1.89). In Conclusion, we could not replicate in a Lebanese population, the associations of the five SNPs studied with CAD in a diabetic population. However, our study suggests that the GG+GC genotypes and the G allele of PSMA6-rs1048990 might be protective against CAD and that the CC genotype of VEGF-rs2010963 could be possible a marker of CAD in a nondiabetic Lebanese population.

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GLOSSARY

AGE: Advanced glycation-end products

AP-1: Activator protein-1

ATP: Adenosine triphosphate

AUBMC: American University of Beirut Medical Center

BMI: Body mass index

CAD: Coronary artery disease

CDKN: Cyclin-dependant kinase inhibitor

CI: Confidence interval

CVD: Cardiovascular disease

ET-1: Endothelin-1

FFA: Free fatty acid

GSIS: Glucose-stimulated insulin secretion

HDL: High-density lipoprotein

HOMA-B: Homeostasis model assessment of beta-cell function

IkB: Inhibitor of kappa B

KCNQ1: Potassium voltage gated channel, KQT-like subfamily member

LAD: Left anterior descending

LCx: Left Circumflex

LDL: Low density lipoproteins

LMCA: Left main coronary artery

MGB: Minor grove binder

MI: Myocardial infarction

mRNA: messenger Ribonucleic acid

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NF-kB: Nuclear factor-kappa B

OR: Odds ratio

PAI-1: Plasminogen activator inhibitor-1

PCR: Polymerase chain reaction

PDR: Proliferative diabetic retinopathy

PKC: Protein kinase C

PSMA6: Proteasome subunit alpha type 6

RCA: Right coronary artery

RHUH: Rafic Hariri University Hospital

ROS: Reactive oxygen species

siRNA: Short interfering RNA

SNP: Single nucleotide polymorphism

TGF-ß: Transforming growth factor-beta

UCP: Uncoupling protein

UPS: Ubiquitin-proteasome system

VEGF: Vascular endothelial growth factor

x^2: chi-test

ACKNOWLEDGEMENTS

I am most grateful to my advisor Dr Pierre Zalloua for his **great research opportunity** and the continuous support throughout my thesis.

I would also like to thank Dr Roy Khalaf and Dr Ralph Abi Habib for accepting to be members of the committee. Dr Khalaf, I especially thank you for recommending this program years ago. I would also like to express my appreciation for Dr. Costantine Daher for always being ready to help me throughout my graduate studies.

Marc Haber for all his help and advice during these 2 year. You were always ready to answer my uncountable questions, thank you for sharing your knowledge M…!

Sonia Youhanna for all her support both as a friend and colleague. I am honored to have worked next to an exceptional person such as you.

Stephanie Saade I doubt that I will ever be able to convey my full appreciation to your support, help, and friendship…I am lacking the words to describe what you offered me during these 2 years.

Exceptional thanks to Ralph Bou Nassif for his endless care and support. Without your continuous encouragement it would have been impossible for me to do it.

A big Thanks for Dr Mirvat Sibai for all the help as well as the fun talks.

All the biology "gang" on the 3rd floor: Helena Abou Farah Awkar, Maya Farah, Valia, Nahla, Jalil, Wissam, Jihad, Wael, Ahmad, Samer, Dana, Sally, Bassem, Rola, Clara, Shunt...and of course Dr Danielle Badro for having to listen to my long stories at lunch...

My sisters Sandy and Sabine-Kim for always being there when I needed them, supporting me until the end. You are all the reason behind my success... A huge gratitude for both the Kokoni and Salloum families.

My friends Jean Carl S, Maya S, David S, Lara H, Hala S , Rami AZ, Nabil N, Marc BN, Youmna M, Yorgui T, Nicolas D, Rim K, Samer T, Rana AM for their continuous encouragements and de-stressing moments.

Amicale des Anciens du College Notre-Dame de Jamhour for the financial help in every step of my education.

To my exceptional parents; my Father Kamal for always looking after me and my mother Lily, my number one supporter who never stopped believing in me, and pushed me to the best. Mom, I dedicate this thesis to you.

Chapter 1

INTRODUCTION

1.1. Epidemiology of Coronary Artery Disease in Type 2 Diabetes

Coronary artery disease (CAD) and type 2 diabetes are both multifactorial chronic diseases whose occurrence is sharply increasing worldwide. The incidence of type 2 diabetes in the Eastern Mediterranean and the Middle East region, including Lebanon, is expected to increase by 81% from 2007 to 2025 (International diabetes federation, 2006). The prevalence of type 2 diabetes in the Lebanese population was recently found to be 15.8% (Hiribli et al., 2005).

Type 2 diabetic patients are four times more prone to cardiovascular disease (CVD) than nondiabetic patients (International Diabetes Federation). The macrovascular and microvascular diseases associated with diabetes include coronary artery disease (CAD), acute myocardial infarction (MI), stroke, heart failure, peripheral arterial disease, retinopathy, nephropathy, and neuropathy (Savage, 1996). Being a major risk factor for the development of any of these complications, diabetes is now being classified as CVD (Grundy et al., 1999).

1.2. Pathophysiology of Vascular Complications Caused by Diabetes

Macrovascular and microvascular diseases due to type 2 diabetes are thought to be the result of the activation of many cell-damaging pathways (illustrated in figure 1) including the activation of protein kinase C (PKC), the formation of advanced glycation-end products (AGEs), and the activation of the hexosamine pathway (Brownlee, 2001; Bartnik et al., 2007).

It was recently shown that hyperglycemia is not the only factor activating these pathways (St. Onge et al., 2009). The main reason for the macrovascular complications in type 2 diabetes is due to insulin resistance leading to an increased free fatty acid (FFA) movement into the arterial endothelial cells (Brownlee, 2005). The increased FFA flux results in an increased reactive oxygen species (ROS) production by ß-oxidation (Brownlee, 2005).

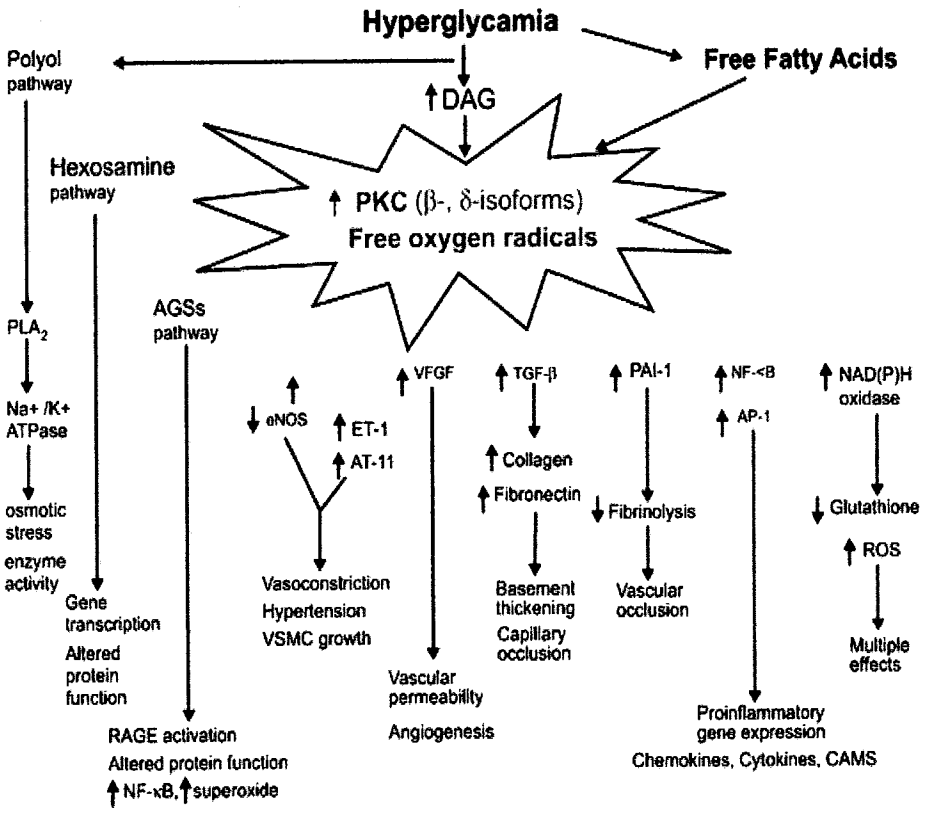

Figure 1. A schematic description of the metabolic consequences of hyperglycemia and free fatty acids overload (Bartnik et al., Hyperglycemia and cardiovascular disease. *Journal of Internal Medicine.* 2007; 262(2):145-56.).

Hyperglycemia and high free fatty acids activate cell damaging pathways including formation of AGEs, activation of PKC, and polyols and hexosamine pathways.

First, the overproduction of ROS activates PKC leading to a series of modifications. These changes include a decrease in nitric oxide (NO) synthesis along with an increase in endothelin-1 (ET-1) leading to vasoconstriction of the endothelium and blood flow abnormalities (Williams et al., 1998; St. Onge et al., 2009). PKC activation also increases the production of vascular endothelial growth factor (VEGF) which affects hyperpermeability of the vascular wall (Brownlee, 2001). Production of transforming growth factor-beta (TGF-ß) is also increased inducing an increase in both collagen and fibronectin amounts which affect basement thickening and capillary occlusion (Koya et al., 1997; Studer et al., 1993). Levels of plasminogen activator inhibitor-1 (PAI-1) are higher following PKC activation thus raising the risk of clot formation due to a decreased fibrinolysis (Feener et al., 1996). An activated PKC increases the activation of nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) which are both transcription factor regulating the expression of inflammatory genes (Brownlee, 2005). A nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase is also activated leading to a further increase in ROS production, thus increasing their multiple damaging effects (Bartnik et al., 2007).

A second pathway is the formation of AGEs. Exposure of proteins and lipids to high glucose concentrations leads to the formation of AGEs that affect the conformation of protein and thus their enzymatic activity (Vlassara, 1997). These AGEs interact with specific surface receptors on cells implicated in atherogenesis (endothelial cells, macrophages, and smooth muscle cell) inducing oxidative stress and thus activating NF-kB which will lead to the expression of a number of genes involved in inflammatory functions (Schmidt et al., 1999).

The third pathway is the hexosamine pathway which is activated by high intracellular glucose levels. Glycolysis then follows except that some of the fructose-6-phosphate goes out of the glycolysis cycle to another set of reactions that ends up by altering transcription factors and gene expression (Sayeski et al., 1996).

Abnormalities in the lipoproteins of diabetic patients are believed to be an additional cause of the increased cardiovascular disease risk in type 2 diabetics (St. Onge et al., 2009). Dyslipidemia characterized by high triglycerides and elevated levels of low density lipoproteins (LDLs) and low levels of high-density lipoprotein (HDLs) are not the only reason for this increased risk since the diabetic LDLs are denser and thus more atherogenic than the non diabetic ones and the diabetic HDLs are less successful in the removal of cholesterol and therefore have impaired antiatherogenic properties (Mazzone et al., 2008).

Microvascular diseases are mostly affected by hyperglycemia. Endothelial cells of the retina, shwan cells and neurons, and mesangial cells in the glomerulus all are incapable of controlling the uptake of glucose during hyperglycemia and thus are at higher risk of damage (Brownlee, 2005). Production of ROS is induced by the high glucose concentration inside the cells leading, by a series of steps, to the activation of the previously discussed pathways including the increase of intracellular AGEs formation, PKC activation, and the activation of both the polyols and the hexosamine pathway (Bownlee, 2005).

1.3. Genomics of CAD in Type 2 Diabetes

Identifying the genes that confer a higher susceptibility to CAD in patients with type 2 diabetes compared to individuals without diabetes is important to further understand the links between both diseases and to improve prevention. The five SNPs chosen for our analysis are among the most importantly studied

polymorphisms for the association of CAD in Diabetic patients. They were replicated in different populations from different ethnic background and displayed contradictory results. These reasons incite us to study these SNPs in our Lebanese Population which was not previously studied and to correlate them with CAD in diabetic patients.

1.3.1. 9p21 Region

Different sequence variants in the 9p21 region were recently identified by genome-wide association studies to be linked with CAD in Caucasians (Helgadottir et al., 2007; Samani et al., 2007). The association was further tested in many other cohorts including the Korean, Japanese and Chinese populations (Hinohara et al., 2008; Shen et al., 2008; Zhou et al., 2008). Other than CAD, the variation on the 9p21 locus was associated with myocardial infarction, type 2 diabetes, and abdominal aortic aneurysm (Saxena et al., 2007; Helgadottir et al., 2008; Matarin et al., 2008).

Doria et al. examined the association of the 9p21 region tagged by single nucleotide polymorphism (SNP) rs2383206 with CAD in individuals with type 2 diabetes. They found that the genetic variants tagged by this SNP constitute major risk factors for CAD in individuals with type 2 diabetes (Doria et al., 2008). Genotypic analysis showed that patients with CAD were more likely to be homozygous for the risk allele G than patients without CAD in the general population, and that this risk was increased in a diabetic population (Del Prato, 2009; Doria et al., 2008).

The SNP rs2383206 is located in a 58-kb linkage disequilibrium region devoid of recognizable protein-coding genes (McPherson et al., 2007). To test the mechanism conferring the risk for CAD, Visel et al. knocked out an orthologous region in mice and compared the messenger RNA (mRNA) expression of

neighboring genes between the wild-type and the double mutants. They found that the expression of CDKN2A and CDKN2B genes in cardiac cells is highly dependant on the presence of the CAD risk interval and thus concluded the existence in this interval of gene regulatory functions acting distantly (Visel et al., 2010).

CDKN2A and CDKN2B genes encode for cyclin-dependent kinase inhibitor proteins that regulate cellular phenotypes such as cell proliferation, cell aging and apoptosis which are functions involved in atheroma formation and CAD (Hannon et al., 1994). CDKN2A regulates pancreatic beta-cells regeneration as well and its overexpression in mice led to diabetes (Krishnamurthy et al., 2006).

1.3.2. UCP2

In 1997, Uncoupling protein 2 (UCP2) was first described to be present in several tissues including pancreatic (Guimeno et al., 1997). UCP2 is present on chromosomal region 11q13 and belongs to the family of uncoupling proteins (UCPs), which are transporters present on the inner mitochondrial membrane (Jia et al., 2009). UCPs are responsible for dissipating the inner mitochondrial membrane proton gradient which is essential for ATP synthesis (Hamada et al., 2008). While UCP1 and UCP3 are tissue specific and expressed in brown adipose tissue and skeletal muscle respectively; UCP2 is ubiquitously expressed and more importantly in the pancreatic islets, the immune system and in adipose tissue (Fleury et al., 1997).

UCP2 has several regulatory roles, two of which will be discussed here. In pancreatic beta-cells, UCP2 down-regulates insulin secretion by lowering ATP synthesis due to the loss of proton gradient (Chan et al., 2004). Higher levels of ATP and of glucose-stimulated insulin secretion (GSIS) were seen in UCP2 deficient mice than in wild-type mice (Zhang et al., 2001). Moreover overexpression of UCP2 through an adenoviral vector inhibited GSIS in insulin

secreting pancreatic insulinoma cells (Hong et al., 2001). In macrophages and in cells of the vascular wall (endothelial and smooth muscle cells), UCP2 plays a role in the modulation of free radical production; it negatively regulates ROS production. In fact, Arsenijevic et al. showed an 80 % increase in ROS production in macrophages following infection in a UCP2 deficient mice compared to wild-type mice thus suggesting a role of UCP2 in the modulation of free radical production (Arsenijevic et al., 2000). Blanc et al provided evidence of larger atherosclerotic lesions in UCP2 gene knock-out mice than in wild type thus proving an antiatherogenic role of UCP2 in the vascular wall (Blanc et al., 2003).

A functional G/A polymorphism at position -866 in the promoter region of UCP2- rs659366 has been extensively studied since the identification of UCP2 as a member of the mitochondrial transporter superfamily (Fleury et al., 1997). Different studies reported varying results about the association of this polymorphism with obesity, fat metabolism and type 2 diabetes in different populations including Caucasian (mainly European), Korean, Japanese, and Chinese populations (Esterbauer et al., 2001; Krempler et al., 2002; Sesti et al., 2003; Wang et al., 2004; Reis et al., 2004).

Bulotta et al. compared the distribution of the -866 G/A SNP between type 2 diabetics and healthy individuals in a Caucasian population from Italy and found the A allele to be associated with a reduced risk of Type 2 diabetes (Bulotta et al., 2005). In a French Caucasian population, Cheurfa et al. further detected an association between the A allele and a reduced risk of CAD in men with type 2 diabetes (Cheurfa et al., 2008).

An increased expression of UCP2 mRNA was associated with the A allele in cultured cells transfected with a UCP2 reporter construct (Krempler et al., 2002). Contradictory results were obtained in human tissues, associating the -866A allele

with an increased or decreased mRNA expression (Krempler et al., 2002; Wang et al., 2004).

1.3.3. KCNQ1

Located on the chromosome 11p15.5, the rs2237892 tags a part of the intron 15 of KCNQ1 (Unoki et al., 2008). KCNQ1 (Potassium voltage gated channel, KQT-like subfamily member 1) gene encodes for the pore-forming subunit of the potassium voltage-gated channel responsible for the repolarization of the cardiac action potential (Barhanin et al., 1996). KCNQ1 gene is mostly expressed in the heart and the pancreas (Unoki et al., 2008). Mutations in the KCNQ1 gene were associated with long QT syndrome (disorder in the electric activity of the heart due to delayed repolarization), abnormalities in the cardiac conduction and congenital deafness due to a loss of function of the channel (Wang et al., 1996; Neyroud et al., 1997). KCNQ1 has also been shown to be expressed in cultured insulin-secreting (INS-1) cells and that a 90% higher insulin secretion results from the selective blockade of KCNQ1 channels (Ullrich et al., 2005).

Recently, genome-wide association study identified SNPs in KCNQ1 gene conferring susceptibility to type 2 diabetes (Unoki et al., 2008). From these SNPs, rs2237892 showed the strongest association with type 2 diabetes (Yasuda et al., 2008). This association was first tested in two Japanese populations then replicated in Chinese, Korean and European populations (Yasuda et al., 2008; Hu et al., 2009). Other than type 2 diabetes, variants in KCNQ1 gene were tested for an association with premature coronary artery disease (Chen et al., 2010).
In the various populations studied, the ancestral allele C of the SNP rs2237892 proved to be the risk allele associated with a higher risk for type 2 diabetes than the minor allele T (Lee et al., 2008; Hu et al., 2009; Qi et al., 2009). On the other hand in the study of the association of KCNQ1 variants with type 2 diabetes and

premature CAD in a Chinese population, Chen et al found that the CC genotype decreases by 90% the risk of developing premature CAD as compared to the TT genotype, thus finding the C allele as the protective one against premature CAD (Chen et al., 2010). They could not however replicate the association of the rs2237892 with type 2 diabetes (Chen et al., 2010).

The mechanisms leading to the susceptibility to diabetes from the risk allele of KCNQ1 still need to be investigated. After associating the C allele of rs2237892 with diabetes, Yasuda et al. searched for an association of this risk allele with levels of insulin resistance by computing the homeostasis model assessment of beta-cell function (HOMA-B) which is a measure of insulin resistance and beta-cell function from basal glucose and insulin concentrations (Mathews et al., 1985). No significant results were obtained for the diabetic individuals; however individuals with CC genotype in the non diabetic group had significantly lower HOMA-B than the other genotypes suggesting a role of the risk allele in the impaired beta-cell functioning (Yasuda et al., 2008). Qi et al replicated these results in the Chinese Han population in which the risk allele C was significantly associated with impaired beta-cell functions (lower HOMA-B values) (Qi et al., 2009).

1.3.4. PSMA6

Gene polymorphisms in pathways involved in the inflammatory process are recently studied for a possible association with CAD or MI ever since the classification of atherosclerosis as an inflammatory disease (Hansson, 2005). Proteasome subunit alpha type 6 gene (PSMA6) gene encodes an alpha subunit of the 20S proteasome which is a core component of the 26S ubiquitin-proteasome system (UPS) (Coux et al., 1996). The UPS is a major regulator of the inflammatory pathway through control of the expression of cytokines (Herrmann

et al., 2010). It has been found to be a key player in the pathogenesis of atherosclerosis including the initiation, progression and complication stages (Herrmann et al., 2010). In the presence of an inflammatory stimulus, the UPS degrades the inhibitor (IkB) of NF-kB, a transcription factor that regulates immune and inflammatory responses (Karin et al., 2000). NF-kB is now free to go into the nucleus and activates inflammatory genes (Beinke et al., 2004).

A common SNP rs1048990 in the 5' untranslated region of exon 1 of PSMA6 confers risk for MI in Japanese population (Ozaki et al., 2006). The association with MI was additionally studied in another Japanese population, as well as in Caucasian and Chinese populations (Takashima et al., 2007; Bennett et al., 2008; Liu et al., 2009). Barbieri et al. further investigated the association of PSMA6 polymorphism with MI in Caucasians with type 2 diabetes and Banerjee et al. tested its association with CAD in a North Indian population (Barbieri et al., 2008; Banerjee et al., 2009).

Ozaki et al. found the minor allele G of rs1048990 to be the risk allele associated with MI and that this polymorphism is a functional one since the G allele enhanced PSMA6 transcription (Ozaki et al., 2006). Different results are obtained in replication studies showing an association of the risk allele with MI in a Chinese population and no association in a Caucasian population (Liu et al., 2009; Benett et al., 2008). Takashima et al. could not replicate the G allele association with MI in a Japanese population; however an association between the risk allele G and the intima media thickness which represents an indicator of coronary atherosclerosis suggests a role of this SNP in the development of CAD (Takashima et al., 2007). In a Caucasian population with type 2 diabetes, a higher G allele and GG genotype frequencies were observed in the MI group compared to those without MI (Barbieri et al., 2008). No association of variants of

rs1048990 was found when comparing CAD patients and controls in the North Indian population (Banerjee et al., 2009).

Ozaki et al used short interfering RNA (siRNA) to test the effect of a higher expression of PSMA6 due to the presence of the risk allele G of rs1048990 on the degradation pathway. They found that in the presence of siRNA specific for PSMA6, IkB remained in the cultured cells for a significantly longer period compared to the degradation of IkB in the absence of siRNA suggesting that a higher PSMA6 expression might be enhancing the degradation of IkB and activating NF-kB thus leading to a more important inflammation (Ozaki et al., 2006).

Marfella et al. further studied the effect of diabetes on ischemic cardiomyocytes and macrophages and found an upregulation of the UPS in human diabetic myocardium (Marfella et al., 2009).

1.3.5. VEGF

The vascular endothelial growth factor (VEGF) gene is located on chromosome 6p21.3. It encodes a growth factor that plays a role in the differentiation and proliferation of vascular endothelial cells thus regulating angiogenesis (Ferrara et al., 1997). VEGF expression is believed to be increased in diabetes due to hyperglycemia and tissue ischemia leading to microvascular complications such as diabetic retinopathy (Aiello et al., 1994). Moreover, a higher VEGF expression was found to be related to vascular complications in diabetics probably through the neovascularization of atherosclerotic plaque (Testa et al., 2008). This neovascularization of the plaque would be bringing additional cellular and soluble lesion components into the vascular wall thus promoting the development of the plaque. (Doyle et al., 2007).

The -634 C/G polymorphism in the 5' untranslated region of VEGF gene was first studied by Watson et al. in 2000 to test the effect of different polymorphisms in the VEGF gene on its expression (Watson et al., 2000). The correlations of the -634 C/G VEGF polymorphism with different diseases have been investigated in various populations. SNP rs2010963 tagging this region was examined for a possible association with diabetic retinopathy in a Japanese population as well as in a Brazilian population of European Ancestry; association of this SNP with MI in Caucasians with type 2 diabetes was also studied (Awata et al., 2002; Errera et al., 2007; Petrovic et al., 2007).

Awata et al. found a significantly higher CC genotype in type 2 diabetic patients with retinopathy compared to diabetics without retinopathy in a Japanese population, but did not find any differences in genotype frequencies when comparing type 2 diabetics to healthy individuals (Awata et al., 2002). On the other hand, Errera et al. reported an association between the CC genotype and the development of the proliferative diabetic retinopathy PDR in a diabetic Brazilian population of European origins (Errera et al., 2007). In a Caucasian population with type 2 diabetes, the CC genotype of the -634 VEGF polymorphism was further associated with MI (Petrovic et al., 2007). The VEGF polymorphism is believed to be a functional one since higher VEGF serum levels were reported in individuals with the CC genotype compared to the CG and GG genotype (Awata et al., 2002; Petrovic et al., 2007).

To better understand the link between type 2 diabetes and CAD, these different association studies searched for polymorphisms that would be associated with CAD in patients with type 2 diabetes in numerous populations including Caucasians, Europeans, Japanese, and Chinese one. Since different results are

reported in various associations studies depending on the population selected, we investigated the association of the 5 different SNPs discussed above with type 2 diabetes and CAD in the Lebanese population. This would help us better understand the molecular genetics of CAD ant type 2 diabetes in the Lebanese population and thus provide us with tools for early and improved detection in the Lebanese population.

Chapter 2

MATERIALS AND METHODS

2.1. Selection of Subjects

The study population of this retrospective case control study consisted of 753 Lebanese subjects selected from the CAD database of the Genomic laboratory of Dr Zalloua. Briefly, this database is made up of 4285 Lebanese subjects who were referred to the cardiovascular care unit and had a coronary angiography performed at the American University of Beirut Medical Center (AUBMC) and Rafic Hariri University Hospital (RHUH) in Beirut. Coronary arteries including the left main (LMCA), the left anterior descending (LAD), the circumflex (LCx), and the right coronary artery (RCA) were imaged from different angles, and the percentage of coronary artery stenosis along with the number of the diseased arteries was documented in each case. Trained researchers collected additional information concerning medical history, family history, and clinical details. Blood samples were collected from each patient consenting to enroll in the study and measurements of lipid profile parameters, C-reactive protein, homocysteine, glucose, and complete blood count were performed. DNA was extracted from a 5 ml peripheral blood samples and stored at 4°C until genotyping.

The Institutional Review Board at the Lebanese American University approved this study.

752 subjects were chosen from our available data based on the following selection criteria: the extent of coronary artery stenosis (0% stenosis in any vessel and >50% in at least one vessel), the diabetic status (diabetic and non-diabetic). The four groups are listed in **Table 1**.

Table 1. Description of the 4 Groups

Description	Count
No Diabetes, No CAD	198
No Diabetes, CAD	202
Diabetes, No CAD	153
Diabetes, CAD	199

2.2. Selection of SNPs

Using the NCBI Pubmed database, we selected five SNPs that were reported to be significantly associated with CAD and type 2 diabetes in different association studies. The characteristics of each SNP and their assay ID (TaqMan® pre-designed SNP genotyping assay, Applied Biosystems) are detailed in **Table 2.**

Table 2. Characteristics of the SNPs Studied.

AB Assay ID	NCBI SNP Reference	Gene Symbol	Location	Mutation
C___1754669_10	rs2383206	CDKN2A-CDKN2B	9p21.3	A/G
C___8760350_10	rs659366	UCP2	11q13.4	G/A
C__16171025_10	rs2237892	KCNQ1	11p15.5	C/T
C__11599359_10	rs1048990	PSMA6	14q13.2	C/G
C___8311614_10	rs2010963	VEGFA	6p21.1	C/G

2.3. Genotyping of SNPs

Genotyping of rs2383206, rs659366, rs2010963, rs1048990, and rs2237892 SNPs was performed with 5' exonuclease (TaqMan®) chemistry on the Applied Biosystems 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). During PCR, the Taq polymerase will partially unwind the perfectly matched probe from the template, cleaving the 5'end of the probe and thus releasing the fluorescent reporter into the reaction solution. The quencher at the

3'end of the probe will no longer have an effect on the reporter, and the fluorescence of the reporter can now be detected. Two probes with two different reporter fluorescing at two different wavelength are used which allows not only the detection of both alleles in the same tube but also ensures that the tube contains DNA (**Figue 2**).

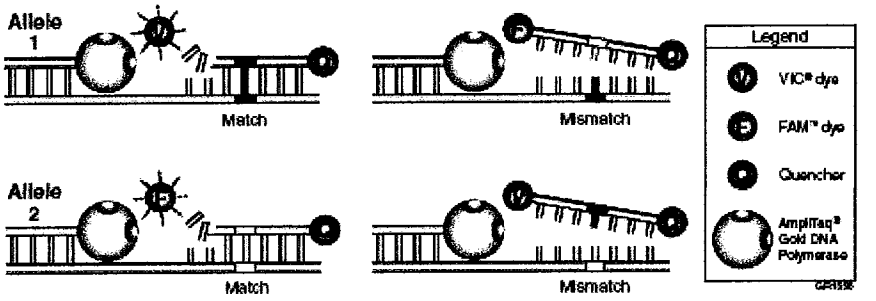

Figure 2. TaqMan® SNP Genotyping Assays: results from matches and mismatches between the target and the probe sequences (Applied Biosystems, 2007, Allelic discrimination assay getting started guide for the 7900HT Fast System). One of the variant probes is labeled with VIC dye and the other with FAM dye. Whenever a match is present the reporter is released by exonuclease activity exhibiting fluorescence.

The DNA was diluted with DNase-free water from the stock DNA samples and 10ng of DNA were used for each reaction. The recommended amount of purified genomic DNA for TaqMan SNP genotyping assay ranges from 1 to 20ng.

In order to perform Real Time PCR, genomic DNA was amplified in a 15ul PCR reaction containing 7.5ul of 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 ul of 20x SNP Ggenotyping assay containing the forward and the reverse primers as well as the TaqMan MGB (minor grove binder) probes (TaqMan pre-designed SNP genotyping assay, Applied Biosystems). The following conditions were used: 95°C for 10min for the activation of the

AmpliTaq Gold enzyme, followed by 45 cycles of 95°C for 15sec for denaturation and 60°C for 1min for annealing and extension.

Following amplification, an endpoint plate-read displays the fluorescent intensity for each well using the 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). A plot of the fluorescence values in each well is generated by the SDS 2.1 software (System Detection Software) indicating the alleles present in each well. Results were also reviewed manually.

2.4. Statistical analysis

Discrete variables were compared by the chi-square test. Continuous variables were expressed as means ± standard deviation and compared by Student's t-test. Allele and genotype frequencies between cases and controls were compared using the chi-square test. A two-tailed p-value of less than 0.05 was considered as significant. Odds ratios (OR) with their 95% confidence interval (CI) were computed for the estimation of the association between the different genotypes or alleles and the clinical outcome. A significant OR of more than 1 was considered as a positive risk. Statistical analyses were performed using the SPSS program for Windows version 17.0 (Statistical Package for the Social Sciences, SPSS Inc., Ill, USA).

Chapter 3

RESULTS

3.1. Clinical Characteristics

The clinical characteristics of the study population are reported in **Table 3**. The diabetic status was a selection criterion since we chose half the CAD group to be diabetic and the other half non diabetic and the same was done for the control group and thus no comparisons were done on the percentage of diabetes between the two groups. The frequencies of hypertension, hyperlipidemia, and family history of CAD were significantly higher in the CAD group than in the controls ($p = 0.001$, 0.001, 0.007; respectively). Glucose, total cholesterol and LDL levels were significantly higher in the CAD group ($p = 0.0078$, 0.0432, 0.0198; respectively). The two groups were comparable concerning the triglycerides and HDL levels ($p>0.05$). There was significantly more males in the CAD group ($p<0.0001$) and the mean age of the CAD subjects was higher than the controls ($p = 0.0009$). Higher body mass index (BMI) were observed in the control group ($p = 0.001$).

Table 3. Clinical characteristics of study population (n=752).

	CAD (n=401)	Controls (n=351)	P-values
Age (years)	60.02 ± 12.25	57.14 ± 11.25	0.0009 #
Male (%)	72.3	49.3	<0.0001 *
Hypertension (%)	67.1	55.6	0.001 *
Hyperlipidemia (%)	53.9	42.2	0.001 *
Family history of CAD (%)	67.7	58.1	0.007 *
Glucose (mg/dl)	130.67 ± 55.79	118.05 ± 46.11	0.0078 #
Total cholesterol (mg/dl)	192.64 ± 57.25	183.36 ± 42.907	0.0432 #
HDL (mg/dl)	41.37 ± 11.31	42.5 ± 13.04	0.3008 #
LDL (mg/dl)	120.32 ± 50.95	110.86 ± 35.13	0.0198 #
Triglycerides (mg/dl)	176.31 ± 98.47	183.65 ± 112.54	0.4356 #
BMI (Kg/m^2)	27.21 ± 4.67	30.18 ± 5.67	0.0001 #

Means compared by student-t test

* Percentages compared by x^2 test

3.2. Genotypic and Allelic Frequencies of CAD vs No CAD Groups

Genotype and allele frequencies did not differ significantly between patients with CAD (having at least one vessel with more than 50% stenosis) and control subjects (no stenosis) in the polymorphisms tagging the 9p21 region, the UCP2 promoter, the KCNQ1 gene, or the PSMA6 gene (**Table 4**). In the rs2010963 variant of the VEGF gene, the genotypic distribution differed significantly between the CAD group and the controls with a higher frequency of the CC genotype in the CAD group (p = 0.049). When comparing the CC genotype to the CG genotype, an association between the CC genotype of the VEGF polymorphism and CAD was seen (OR = 1.57, 95%CI: 1.06-2.31; p = 0.023). No

significant difference was observed in the allelic frequency of this polymorphism between CAD and control groups (p = 0.684).

Table 4. Genotype and allele distribution of the 9p21 region, UCP2, KCNQ1, PSMA6, and VEGF gene polymorphisms in patients with CAD and in control subjects (N=752).

	CAD	No CAD	pvalue
rs2383206			
GG	183	143	
AG	212	207	0.133
G	578	493	
A	212	207	0.241
rs659366			
GG	198	180	
GA	157	140	
AA	42	30	0.645
G	553	500	
A	241	200	0.451
rs2237892			
CC	356	316	
CT+TT	28	29	0.730
C	739	659	
T	29	31	0.492
rs1048990			
GG	13	12	
CG	116	115	
CC	266	217	0.472
G	142	139	
C	648	549	0.276
rs2010963			
CC	87	59	
CG	171	182	
GG	136	110	0.049
C	345	300	
G	443	402	0.684

3.3. Genotypic and Allelic Frequencies of CAD vs No CAD Groups in Non Diabetic Patients

Considering the non diabetic subjects and grouping them according to CAD status, PSMA6-rs1048990 showed significantly different allele and genotype frequencies between CAD patients without diabetes and non diabetic controls in a dominant model where GG and GC were compared to CC. Non diabetic subjects without CAD were found to have higher G allele ($p = 0.025$) and higher GG and GC genotypes than the non diabetics with CAD ($p = 0.027$ in a dominant model; **Table 5**). The computed odds ratio revealed an inverse association between the GG and GC genotype in a dominant model and CAD in non diabetic subjects (OR = 0.61, 95%CI: 0.40-0.94; $p = 0.027$). We observed also an inverse association between the G allele and CAD in non diabetic patients (OR = 0.66, 95%CI: 0.46-0.95). For the VEGF-rs2010963 polymorphism, significant differences in genotype frequencies were observed between subjects with CAD and controls in non diabetic population ($p = 0.036$; **Table 5**). We found an association between the CC genotype and CAD in a recessive model (OR = 1.89, 95%CI: 1.15-3.12; $p = 0.013$).

Table 5. Genotype and allele distribution of the 9p21 region, UCP2, KCNQ1, PSMA6, and VEGF gene polymorphisms in non diabetic patients with CAD and in non diabetic subjects without CAD (N=400).

	No DIABETES		
	CAD	No CAD	pvalue
rs2383206			
GG	88	82	
AG	109	116	0.513
G	285	280	
A	109	116	0.612
rs659366			
GG	107	100	
GA	74	81	
AA	17	16	0.748
G	288	281	
A	108	113	0.659
rs2237892			
CC	175	178	
CT+TT	17	16	0.786
C	366	370	
T	18	18	0.975
rs1048990			
GG+CG	58	78	
CC	138	114	0.027
G	62	85	
C	330	299	0.025
rs2010963			
CC	49	30	
CG	82	101	
GG	64	68	0.036
C	180	161	
G	210	237	0.106

3.4. Genotypic and Allelic Frequencies of Diabetic vs Non Diabetic Patients

When classifying the data according to the diabetic status, no significant differences in genotype and allele frequencies were seen between type 2 diabetic

patients and non diabetic patients in any of the 5 polymorphisms studied (x^2 test and Fischer's test: p>0.05 for all polymorphisms; **Table 6**).

Table 6. Genotype and allele distribution of the 9p21 region, UCP2, KCNQ1, PSMA6, and VEGF gene polymorphisms in patients with type 2 diabetes and in control subjects (N=752).

	Diabetes	No Diabetes	pvalue
rs2383206			
GG	156	170	
AG	194	225	0.674
G	506	565	
A	194	225	0.742
rs659366			
GG	171	207	
GA	142	155	
AA	39	33	0.363
G	484	569	
A	220	221	0.166
rs2237892			
CC	319	353	
CT+TT	24	30	0.239
C	662	736	
T	24	36	0.264
rs1048990			
GG	14	11	
CG	106	125	
CC	231	252	0.611
G	142	147	
C	648	629	0.943
rs2010963			
CC	67	79	
CG	140	183	
GG	114	132	0.860
C	304	341	
G	398	447	0.990

3.5. Genotypic and Allelic Frequencies of CAD vs No CAD Groups in Diabetic Patients

When the analysis considered the diabetic subjects only, further subgrouping according to CAD showed no significant differences in genotype or allele frequencies between CAD patients with diabetes and diabetic controls ($p > 0.05$; **Table 7**).

Table 7. Genotype and allele distribution of the 9p21 region, UCP2, KCNQ1, PSMA6, and VEGF gene polymorphisms in diabetic patients with CAD and in diabetic control subjects (N=352).

	DIABETES		
	CAD	No CAD	pvalue
rs2383206			
GG	95	61	
AG	103	91	0.143
G	293	213	
A	103	91	0.250
rs659366			
GG	91	80	
GA	83	59	
AA	25	14	0.389
G	265	219	
A	133	87	0.157
rs2237892			
CC	181	138	
CT+TT	11	13	0.583
C	373	289	
T	11	13	0.308
rs1048990			
GG	9	5	
CG	62	44	
CC	128	103	0.732
G	80	54	
C	318	250	0.435
rs2010963			
CC	38	29	
CG	89	81	
GG	72	42	0.197
C	165	139	
G	233	165	0.258

3.6. Genotypic and Allelic Frequencies of MI vs No MI Groups

Finally, we grouped our population into a myocardial infarction group (patients who undergone angiography due to MI) and a control group (patients who undergone the angiography for reasons other than MI or unstable angina and who have 0% stenosis) to test the association of the different polymorphisms studied with MI. The genotype and allele frequencies of the 5 SNPs did not significantly differ between the MI group and the controls ($p>0.05$; **Table 8**).

Table 8. Genotype and allele distribution of the 9p21 region, UCP2, KCNQ1, PSMA6, and VEGF gene polymorphisms in patients with MI and in control subjects without MI (N=311).

	MI	No MI	pvalue
rs2383206			
GG	33	99	
AG	41	135	0.729
G	107	333	
A	41	135	0.788
rs659366			
GG	34	116	
GA	31	95	
AA	9	22	0.742
G	99	327	
A	49	139	0.451
rs2237892			
CC	66	213	
CT+TT	4	18	0.558
C	136	442	
T	4	20	0.436
rs1048990			
GG+GC	26	89	
CC	47	141	0.637
G	29	98	
C	117	362	0.709
rs2010963			
CC	11	30	
CG	36	121	
GG	27	83	0.862
C	58	181	
G	90	287	0.911

Chapter 4

DISCUSSION

Gene-gene and gene-environment interactions are crucial for the holistic understanding of complex diseases such as coronary artery disease. This disease has been extensively studied and some factors became well-established as conventional risk factors. These include age, gender, hypertension, hyperlipidemia, family history of CAD. **Table 3** describing our population highlights these well- established factors since CAD group consists of a significantly higher frequency of old, hypertensive, hyperlipidemic males compared to controls group.

In this study, 5 different polymorphisms, rs2383206 in 9p21 region, rs659366 in the UCP2 promoter, rs2237892 in the KCNQ1 gene, rs1048990 in the PSMA6 gene, and rs2010963 in the VEGF gene were analyzed as possible genetic markers of CAD in the Lebanese population and then in a diabetic subgroup of the population. Association of these SNPs with MI was also included. Cases were selected as having at least one of the four coronary arteries with more than 50% stenosis and controls were restricted to subjects with 0% stenosis in all 4 vessels.

The genotype and allele distribution of the rs2383206, rs659366, rs2237892, rs1048990 variants did not reveal any significant association with CAD. In fact, when comparing the CAD group to controls without CAD, only rs2010963 showed significance ($p = 0.049$) in the genotypic distribution. Furthermore, none of these 5 variants shows a significant difference when comparing diabetic to nondiabetic subjects or when comparing diabetic subjects with CAD to diabetics without CAD.

On the other hand, significance can be detected on the level of rs1048990 and rs2010963 when comparing CAD to controls without CAD in a nondiabetic population. Nondiabetic controls had a higher frequency of the G allele of PSMA6-rs1048990 polymorphism than non diabetic cases. In addition, the CC genotype of the VEGF-2010963 polymorphism associates with CAD in the nondiabetic subjects.

Doria et al, found that rs2383206 associates with CAD in presence of poor glycemic control in type 2 diabetes (Doria et al., 2008). However our study notably differs from theirs in the selection of controls. In fact, they selected cases as having more than 50% stenosis based on angiography which is similar to our selection. However their controls were not angiographically selected as our subjects; they ruled out patients with positive stress test from their controls. This raises the issue of subjects who have stenosis but display no clinical symptoms; these subjects might be included in the control group of Doria et al. even though they should be considered as cases depending on their level of stenosis. This possibility is eliminated in our study since all controls are confirmed to have no stenosis in all of the 4 coronary arteries.

Concerning variants in UCP2 and KCNQ1 genes as mentioned earlier, some studies not only failed to replicate association of the polymorphism studied with the disease but also showed contradictory results. For instance, Chen et al. was unable to replicate the association of KCNQ1-rs2237892 with type 2 diabetes in a Chinese population while Yasuda et al. had strongly associated this polymorphism with type 2 diabetes (Chen et al., 2008; Yasuda et al.,2008). In addition, concerning the UCP2 polymorphism, Cheurfa et al. found that the A allele of the rs659366 associated with a reduced risk of CAD whereas Krempler et al. proposed the G allele to be protective against one of the major risk factors

of CAD, diabetes (Cheurfa et al., 2008; Krempler et al.,2002). It is worthy to note that Cheurfa et al. lost this association in women when stratification by gender was performed; the A allele seemed to reduce risk for CAD only in males and not females (Cheurfa et al., 2008).

These different findings and selection criteria can explain the inability to replicate some of the associations in our population.

Concerning the -634 C/G VEGF polymorphism tagged by rs2010963, the CC genotype was found to be associated with CAD in our general population but not in the type 2 diabetic population. Our results also showed that subjects with the CC genotype have 1.57 times more risk of developing CAD than those with the CG or CC genotypes.

The CC genotype has been reported to be associated with MI in a type 2 diabetic population (Petrovic et al., 2007). This polymorphism is suspected to be a functional one with the CC genotype associated with higher VEGF serum levels than the other genotypes in healthy individuals and high VEGF levels are suspected to be involved in acute coronary events (Awata et al., 2002; Petrovic et al., 2007). Our results are in agreement with these functional studies; we reported that individuals with the CC genotype have 57% more risk of developing CAD than the other genotypes and this might be due to the higher VEGF levels in individuals with the CC genotype which are suspected to increase neovascularization of the plaques and thus promoting their growth leading to stenosis. Interestingly the p-value decreases when considering the nondiabetic population. The genotypic distribution between the CAD patients and the controls without CAD barley significant, but when patients were set to be nondiabetic comparison between the CAD and the controls differed significantly

to 0.036 and the risk conferred by the CC genotype compared to other genotypes increased to 1.89.

Furthermore, the non-significant difference between CAD and controls without CAD observed in the PSMA6 polymorphism rs1048990 ($p = 0.472$) became significant ($p = 0.027$) when population was selected as nondiabetic. This trend seen in both rs2010963 and rs1048990 might suggest that diabetes, a major risk factor, might be masking the effect of the variant on the CAD status. Therefore, these polymorphisms will be a risk factor only in a population that is not already a high-risk population.

In addition, the results obtained with PSMA6-rs1048990 are remarkable. There was indeed a significant allelic and genotypic difference between the CAD group and the control group without CAD, yet the risk allele G as suggested by the calculated Odds Ratio (OR=0.66) is a protective one in our population. Such a phenomenon is not uncommon in genetic studies especially in the genetic study of complex diseases such as CAD. It is referred to as the "flip-flop" associations as explained by Lin et al. where two alleles of the same variant are found to be risk alleles in different population (Lin et al., 2006).

The flip-flop phenomenon is implausible if it occurs in populations of same ethnic group; clearly this does not apply in this case since our population has a different ancestry than that of the studied populations of Japanese, Chinese, and European origins. The opposite effect of the same variant allele is explained by the genetic and environmental differences across populations (Lin et al., 2006).
When we analyzed association of the different polymorphisms with MI, none of them showed significance in the allelic distribution. It is important to state that the cases were diagnosed as having MI whereas the controls were subjects

without MI, without unstable angina and having 0% stenosis in all 4 arteries. Even though we selected extreme patients, our study was unable to replicate the association of any SNPs with MI.

Irreproducibility of studies and contradiction in results both have several potential causes identified by meta-analyses of association studies. One of these causes is "inflated positive results" in the original association because the population selected is not representative of the general population. This might also be due to the fact that positive results are favored for publication and researchers are inclined to disregard reporting negative results which lead to biasness (Ferreiros-Vidal et al., 2004). Insufficient sample size also leads to erroneous correlations (Ferreiros-Vidal et al., 2004).
Lack of replication is also due to the collection of data, selection criteria, stratification of population, confounding from population structure, and misclassification of outcome (Cardon et al., 2003; Colhoun et al., 2003). But the most important factor, as we already mentioned, is the genetic heterogeneity across different populations. In fact, a meta-analysis conducted by Ioannidis et al. demonstrated that correlation between the first study and the succeeding studies dealing with the same association is limited (Ioannidis et al., 2001).

The main strength of our study is that our cases and controls were angiographically confirmed. The CAD group was thus verified to have more than 50% stenosis in at least one vessel and the controls were confirmed to have 0% stenosis in any of the vessels thus eliminating the risk of misclassification of controls due to the possible occurrence of asymptomatic obstructive CAD in the control group.

However, our study includes also some limitations. First, small subgroups were used in the analysis of the association between the different genotypes and CAD status in diabetic patients. This might be considered as both limitation and strength because the sample size was considerably reduced only due to our stringent selection criteria. Second, we do not have available information on the age of onset and duration of diabetes in our population therefore selection criteria such as diabetic for more than 10 years as the one used by Petrovic et al. and onset of diabetes at the age of 30 or later as the one used by Bulotta et al. and Doria et al are not possible in our data set (Petrovic et al., 2007; Bulotta et al. 2005; Doria et al., 2008).

Chapter 5

CONCLUSION

Coronary artery disease is a complex disease that engages various pathways such as oxidative stress and inflammatory pathways.

Our study focused on 5 variants thought to be associated with MI, CAD and more specifically in diabetic populations. Our study suggests different results. In fact rs2383206 in 9p21 region, rs659366 in the UCP2 promoter, and rs2237892 in the KCNQ1 gene associated with none of the three mentioned diseases. In contrast, rs1048990 in the PSMA6 gene, and rs2010963 in the VEGF gene associated with CAD in nondiabetic patients. Our study suggests that the GG and GC genotypes along with the G allele of PSMA6-rs1048990 might be protective against CAD and that the CC genotype of VEGF-rs2010963 could be possible a marker of CAD in a nondiabetic Lebanese population.

Further studies with larger sample size are needed to increase the statistical power and be able to further stratify by age, gender, other risk factors such as hypertension and smoking. In the case of multifaceted disease such as coronary artery disease many loci and environmental components interact to cause the disease and thus studies dealing with one locus or with one aspect independently of the others lead to ambiguous results. Thus more comprehensive studies of the mechanisms involved in these polymorphisms and their associations with the disease are needed.

Our study brings to light the complexity yet the importance of genetic association studies in human diseases.

Chapter 6

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