# Genetic Susceptibility to Coronary Artery Disease in Type 2 Diabetic Patients

by

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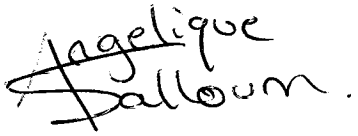

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# ABSTRACT

## Genetic Susceptibility to Coronary Artery Disease in Type 2 Dibetic Patients

by Angelique K. Salloum

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

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*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).

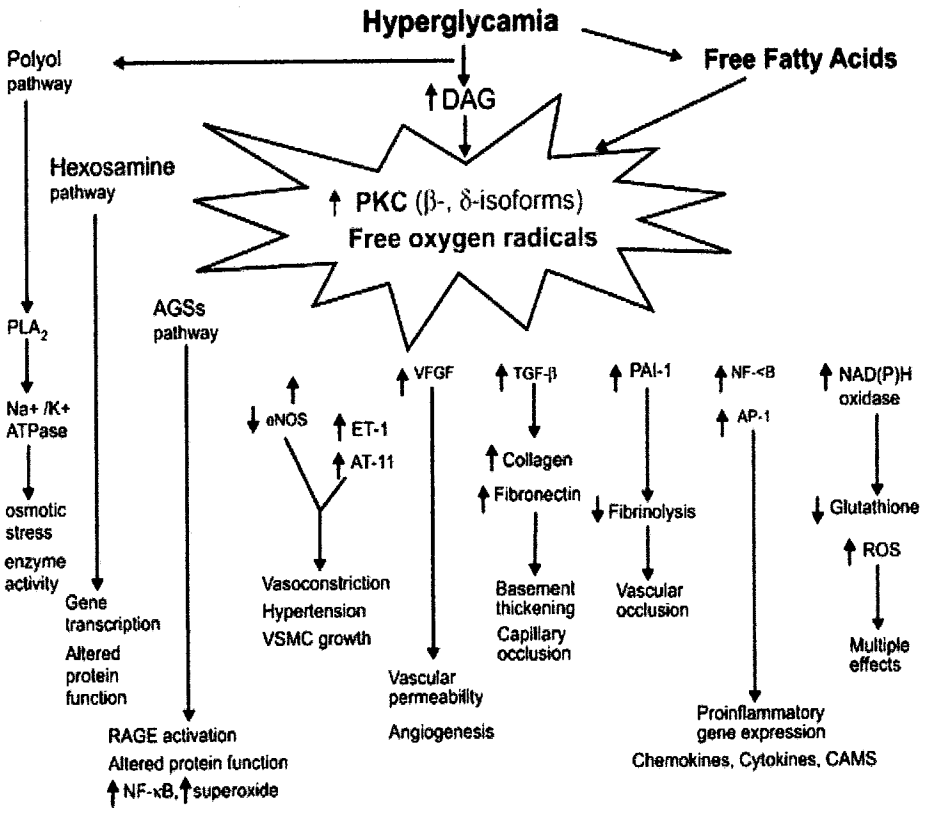


**Figure 1.** A schematic description of the metabolic consequences of hyperglycemia and free fatty acids overload (Bartnik et al., Hyperglycemia and cardiovascular disease. *Journal of Internal Medicine.* 2007; 262(2):145-56.).

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**).

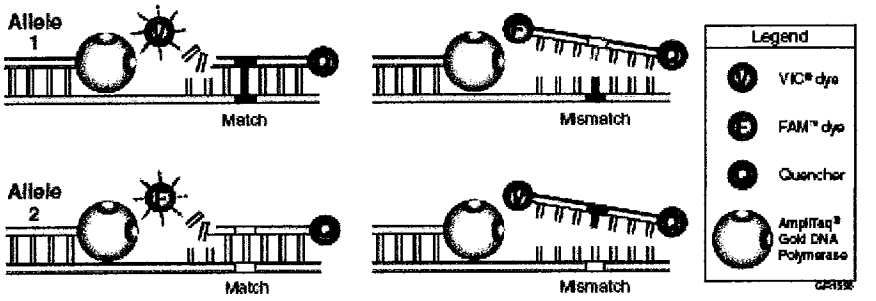


**Figure 2.** TaqMan® SNP Genotyping Assays: results from matches and mismatches between the target and the probe sequences (Applied Biosystems, 2007, Allelic discrimination assay getting started guide for the 7900HT Fast System). One of the variant probes is labeled with VIC dye and the other with FAM 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 |