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Genetic investigation of vascular diseases in the French-Canadian population

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Abstract

Ischemic stroke and ischemic heart disease (IHD) are complex disorders which are influenced by both environmental and genetic factors. To identify genetic risk factors to these disease, we conducted two association studies in a population where a significant founder effect has occurred: the French-Canadians. We studied 10 polymorphisms, in the following 9 genes, which have previously been implicated in the development of IHD and/or ischemic stroke: angiotensinogen (M235T), angiotensin-converting enzyme (287 bp I/D), angiotensin II type I receptor (A1166C), coagulating factor V (Leiden mutation), platelet glycoprotein IIIa (PL^{A1}/PL^{A2} alleles), plasminogen activator inhibitor-1 (4G/5G), apolipoprotein E (E2, E3, and E4 isoforms), methylenetetrahydrofolate reductase (C677T), and stromelysin-1 (5A/6A).

In the first study, we investigated the relationship between the gene variants listed above and 150 IHD patients and 113 controls, who matched on age, gender, and ethnic background. Multiple regression analysis revealed that the only variant associated with IHD was in the platelet glycoprotein IIIa gene. The PL^{A1}/PL^{A1} genotype was found to be associated with IHD ($P = 0.0147$), while the PL^{A1}/PL^{A2} genotype was found to be protective against the development of IHD ($P < 0.05$). In the second study, we investigated the relationship between these same genetic variants and 97 French-Canadians with cerebrovascular disease (59 ischemic strokes, 38 TIAs) and 134 age and gender matched French-Canadian controls. We found that the stromelysin-1 variant was the only one associated with the development of cerebrovascular disease. The 5A/6A genotype was found to be more frequent among cases than controls ($P = 0.0119$), and there was a trend showing that the 6A/6A genotype was more frequent in controls than cases ($P = 0.0504$).

In conclusion, these results provide further support that the development of IHD and cerebrovascular disease, in the French-Canadian population, is in part genetic. New research needs to be initiated in order to identify new genetic variants that can predispose to these diseases. We also need to better understand the interactions of these genetic variants with the established risk factors in the aim of characterizing the etiology of ischemic stroke and IHD; this will in turn provide new avenues for treatment, if not prevention.

Résumé

Les maladies cérébrovasculaires (CV) et cardiaques ischémiques (CI) sont des traîts complexes influencés aussi bien par l'environnement que par des facteurs génétiques. Afin d'identifier des facteurs de risques génétiques pour ces maladies, nous avons éffectué deux études d'associations en utilisant une population où un effet fondateur a été documenté, la population Canadienne Française. Nous avons étudié 10 polymorphismes, retouvés dans 9 gènes, préalablement impliqués dans le dévelopment des maladies CV et CI: angiotensinogène (M235T), 'angiotensin-converting enzyme' (287 bp I/D), récepteur de type I de l'angiotensine II (A1166C), facteur de coagulation V (mutation 'Leiden'), glycoprotéine IIIa des plaquettes (allèles PL^{A1}/PL^{A2}), inhibiteur I de l'activateur du plasminogène (4G/5G), apolipoprotéine E (isoformes E2, E3, et E4), méthylènetétrahydrofolate réductase (C677T), et stromélysine-1 (5A/6A).

Premièrement, nous avons étudié la relation entre les variants génétiques énumérés ci-dessus et une cohorte qui comprenait 150 patients avec maladies CI et 113 contrôles appariés en fonction de l'âge, du sexe, et l'origine éthnique. L'analyse de regressions multiples a démontré que le seul variant génétique associé avec les maladies CI était situé dans le gène de la glycoprotéine IIIa des plaquettes. Nous avons observé que le génotype PL^{A1}/PL^{A1} était associé avec les maladies CI ($P = 0.0147$), alors que le génotype PL^{A1}/PL^{A2} semblait être protecteur contre ces maladies ($P < 0.05$). Deuxièmement, nous avons étudié la relation entre ces mêmes variants génétiques et une cohorte qui comprenait 97 Canadien-Français avec maladies CV (59 accidents cérébrovasculaires ischémiques et 38 attaques ischémiques transitoires) et 134 contrôles Canadien-Français appariés en fonction de l'âge et du sexe. Nous avons trouvé que le variant dans gène de la stromélysine-1 était le seul associé avec le dévelopement de maladies CV. Nous avons observé que le génotype 5A/6A était plus fréquent parmis les patients que les contrôles ($P = 0.0119$), alors que le génotype 6A/6A semblait être plus fréquent chez les contrôles que chez les patients cependant, ce dernier résultat n'était pas statistiquement significatif ($P = 0.0504$).

En conclusion, ces résultats supportent l'évidence qui suggère que le dévelopment des maladies CI et CV, dans la population Canadienne-Française, est influencé par des traîts génétiques. De nouvelles études doivent être entreprises afin

d'identifier de nouveaux variants génétiques qui pourraient prédisposer à ces maladies. Nous devons aussi approfondir nos connaissances sur les interactions entre les variants génétiques et les facteurs de risques déjà établis dans le but de mieux caractériser les maladies CV et CI. Ceci pourra éventuellement aider au dévelopment de meilleurs traîtements et à l'amélioration de l'efficacité de prévention.

Abbreviation list

ACE: angiotensin-converting enzyme

AGT: angiotensinogen

AGT1R: angiotensin 2 type 1 receptor (gene)

Ang. I: angiotensin 1

Ang. II: angiotensin 2

AMI: acute myocardial infarction

APC: activated protein C

apo: apolipoprotein

AT1: angiotensin 2 type 1 receptor (protein)

AT2: angiotensin 2 type 1 receptor (protein)

BMI: body mass index

CAD: coronary artery disease

CVD: cardiovascular disease

D: deletion

DVT: deep vein thrombosis

ECM: extracellular matrix

FDGF: fibroblast derived growth factor

FFA: free fatty acid

IDL: intermediate-density lipoprotein

IHD: ischemic heart disease

IMT: intimal-medial thickening

Hcy: homocysteine

HD: heart disease

HDL: high-density lipoprotein

HTN: hypertension

I: insertion

LDL: low-density lipoprotein

LVH: left ventricular hypertrophy

NO: nitric oxide

MDGF: macrophage derived growth factor

MI: myocardial infarction

MMP: matrix metalloproteinase

MTHFR: methylenetetrahydrofolate reductase

OR: odds ratio

PAI: plasminogen activator inhibitor

PDGF: platelet derived growth factor

PGIb: platelet glycoprotein 1 b

PGIIb: platelet glycoprotein 2 b

PGIIIa: platelet glycoprotein 3 a

rAPC: resistance to activated protein C

RAS: renin-angiotensin system

RR: risk ratio (relative risk)

SMC: smooth muscle cell

TGFβ: transforming growth factor beta

THF: tetrahydrofolate

TIA: transient ischemic attack

TIMP: tissue inhibitor of metalloproteinase

TPA: tissue plasminogen activator

TPL: tissue thromboplastin

VLDL: very low-density lipoprotein

VSMC: vascular smooth muscle cell

vWF: von Willebrand factor

Part I) Introduction

A) Ischemic heart disease (IHD)

1) Demographics

Cardiovascular diseases (CVD) are the leading cause of death in industrialized countries where 51% of deaths are related to IHD (1). IHD is a complex disease which is influenced by both genetic and environmental factors. The incidence of this disease increases with increasing age and is more frequent among males than females. The prevalence of IHD also varies among different ethnic groups and populations (2). Treatment for IHD includes invasive procedures such as angioplasty and bypass surgery, as well as drug therapy to reduce the risk of recurrent manifestations of IHD in survivors of this disease. However, the treatment of IHD with antiplatelets, anticoagulant agents, or other drugs show only modest benefits. The American Heart Association (AHA) reports a 15% reduction in vascular mortality and a 32% reduction in reinfarctions, for survivors of myocardial infarction (MI), with use of these drug treatments (3). It is generally accepted that prevention of IHD remains the most important avenue of treatment.

2) Heart and blood vessel physiology

The human heart is composed of 3 layers. The endocardium is the inner most layer which lines the heart cavities. The epicardium, a delicate membrane, makes up the outer most layer. The myocardium is the middle layer; this is the heart muscle. It is composed of a network of myocytes, single nucleus cells. The myofilaments are the contractile elements of the myocytes and they are arranged in bundles referred to as myofibrils, which are separated by mitochondria and sarcoplasmic reticulum. Blood is supplied to the myocardium by the coronary arteries which originate from the aorta, immediately above the aortic valve. There are 2 main coronary arteries: the right coronary artery and the left coronary artery; the latter bifurcates into the left anterior descending, and the left circumflex coronary artery. In the normal state, blood flow is roughly equal throughout the myocardium, due to autoregulation (4, 5).

The human blood vessels are also composed of three distinct layers. The outer most layer, the adventitia, is composed of connective tissue and elastic fibers (6). The middle layer, the media, comprises vascular smooth muscle cells (VSMC) and elastic fibers. The intima is the inner most layer and can be divided into 2 parts. The

basement membrane, which contains collagens, laminins, and other extra cellular matrix (ECM) proteins, acts to separate the media from the endothelium (7). The endothelium is a layer of cells which are in direct contact with the flowing blood.

3) Definition

IHD is the most common type of heart disease (HD) and is responsible for at least 80% of all deaths from HD (4). It results from the lack of oxygen supply to the myocardium, due to diminished blood flow from the coronary arteries, resulting in ischemia and infarct. IHD is caused by an imbalance between the oxygen demand of the myocardium and the supply of oxygen in the blood from the coronary arteries. Infarction of the tissues, irreversible ischemia, occurs after cell deprivation of oxygen for about 30 minutes (8).

4) Causes

The main cause of IHD is atherosclerosis of the coronary arteries. Initiation of atherosclerosis occurs when the endothelium of the blood vessel, which is stable under normal conditions, sustains a mechanical (e.g. hypertension (HTN)) or chemical injury (9). Infiltration of circulating monocytes into the intima will follow. The monocytes will become activated and produce cytokines and growth factors which will induce ECM growth by VSMC. In response to this, VSMC will migrate from the media to the intima and undergo proliferation. This is the beginning of matrix deposition and plaque growth. The endothelium starts producing various growth factors which contribute to VSMC migration and proliferation. The now unstable endothelium allows infiltration of lipids into the plaque; these lipids are engulfed by macrophages which are called foam cells. This creates a situation where there is an imbalance between matrix deposition and degradation; the accumulation of ECM proteins is favored. At first, there is what is called compensatory enlargement. The atherosclerotic growth occurs outwards to compensate for the increasing intimal volume. Eventually, this mechanism becomes outweighed by the growing mass and stenosis of the vessel is inevitable.

Plaque rupture is a complication of atherosclerosis. The plaque is filled with chronic inflammatory infiltrates and matrix accumulation is not uniform across the plaque. Foam cells, probably derived from monocytes, tend to accumulate in the

shoulder regions of the fibrous plaque (10). This area is less resistant to the mechanical stress imposed by the blood flow and is therefore more likely to rupture. The ulceration of the fibrous plaque cap exposes pro-coagulating substances in the blood vessel wall, and therefore triggers the blood-clotting cascade; thrombus formation is the resulting event. The thrombus can grow and become occlusive, narrowing the bore of the vessel and diminishing the blood flow through the vessel; this leads to increased wall pressure, which further promotes atherosclerosis. A fibrotic organization can also be formed from this thrombus. This involves migration of VSMC into the thrombus where various ECM proteins are deposited, therefore contributing to the progression of atherosclerosis and occlusion of the blood vessel.

5) Manifestations

a) Angina pectoris

Angina pectoris is defined as pain in the chest and it is the most common symptom of IHD. It is not associated with anatomical changes in the myocardium as long as the ischemic episode is not of a duration prolonged enough to cause necrosis. The most common cause of angina pectoris is atherosclerosis of the coronary arteries, which becomes symptomatic when approximately 75% of the lumen is occluded (4). There are two types of angina pectoris. The first, stable angina, is characterized by recurrent episodes of chest pain brought on by a stimulus such as exercise; the pain is of limited duration and is relieved by stopping the activity. The second type, unstable angina, is the most serious form. It is defined as a variety of chest pains which have a less predictable pattern; the pain may occur during rest or sleep. Unstable angina forewarns an imminent danger of MI.

b) Sudden death

Sudden death is defined as the unexpected death form cardiac causes within 1 to 24 hours after the onset of acute symptoms (8). Many forms of HD may cause sudden death, but in 75-90% of cases it is the consequence of IHD. Coronary atherosclerosis underlies most cases of sudden cardiac death, although it can also occur due to acute thrombosis of a coronary artery.

c) Myocardial infarction (MI)

MI is due to coronary atherosclerosis and thrombus formation, causing a reduction in coronary blood flow which results in ischemic necrosis of the myocardium. An infarction is an irreversible injury caused by a prolonged lack of oxygen supply which leads to the death of the specialized cells of the heart muscle. Acute myocardial infarction (AMI) is responsible for the majority of deaths due to IHD (8). It can occur at any age and the incidence increases progressively through life. The event is typically sudden but in 20% of the cases, it is asymptomatic. Clinical diagnosis of AMI is based on the symptoms, the changes in the electrocardiogram (a record of the electric current in the contracting myocardial cells), and the elevation of specific enzymes (lactic dehydrogenase and creatine kinase); these are soluble cytoplasmic enzymes which leak out of the fatally damaged myocardial cells. Many complications can follow an MI; theses include heart failure, arrhythmias, recurrent MI, and stroke.

B) Stroke

1) Demographics

Ischemic stroke accounts for over 80% of all strokes and has a world wide distribution; it is the third leading cause of death and the first leading cause of acquired disability in developed countries (11). Every year, stroke affect 500,000 Americans and 50,000 Canadians, 150,000 and 15,000 of which, respectively, will die from the disease. Therefore, stroke has a substantial economic and social impact with an associated annual cost of approximately 30 billion dollars in the USA and 3.5 billion dollars in Canada. Individuals who are at risk for developing stroke can be treated either by prophylactic means, which confer only a modest decrease in stroke risk, or with invasive procedures such as carotid endarterectomy, the best current treatment. Acute stroke can be treated with thrombolytic agents, but even if a modest reduction in injury can be achieved through these treatments, those affected will still be left with neurological deficits. For these reasons, prevention remains the most beneficial approach.

2) Brain anatomy

A complex vasculature exists that supplies the brain with its needed fuel and oxygen. The main arteries supply blood to the larger parts of the brain while a network of smaller penetrating arteries, which branch off the main ones, act to carry blood to structures deep in the brain. The left common carotid artery (CCA) originates from the brachiocephalic artery, and the right CCA originates from the aorta. Each CCA bifurcates in the neck into the external carotid artery (ECA) and the internal carotid artery (ICA) (12). The ECA runs anteriorly and ordinarily supplies blood to the face. It can serve as collateral circulation if the ICA is occluded. The ICA, which runs more posteriorly, enters the skull, gives off an ophthalmic artery branch, and then bifurcates into the anterior cerebral artery (ACA) and the middle cerebral artery (MCA). By convention, the carotid artery territories are referred to as the anterior circulation (front of brain).

The first branch of the subclavian artery is called the vertebral artery (VA). It runs upwards and backwards, entering the spine between the 6^{th} and 5^{th} cervical vertebra. The intracranial portion of the VA joins with the contralateral VA to form the basilar artery (BA). The BA runs midline, giving off other arteries. The posterior circulation (the back of the brain), which includes the brainstem, is supplied by the VA and the BA. This circulation is constructed differently than the anterior circulation and receives vessels from each side. The circle of Willis, a structure formed by the brain arteries, allows for connections between the anterior circulation on each side, as well as the posterior circulation on each side. Sometimes, if one or more arteries are occluded, the circle of Willis serves as collateral circulation to prevent infarction.

3) Definition

Stroke is a heterogeneous disorder. A stroke is defined as any damage to the brain (or central nervous structures) caused by an abnormality in blood supply (12). It is sudden in onset and causes focal neurological deficit resulting from either occlusion or rupture of cerebral arteries, which lead to either brain ischemia or hemorrhage, respectively. The acquired neurological deficit reflects both the size and location of the infarct.

4) Causes

If we exclude vascular dysplasias, the main causes of stroke are atherosclerosis and HTN (12). Atherosclerosis affects mainly large extracranial and intracranial arteries. However, atheromatous plaques do not develop at random in any location. They tend to form at branchings and curves of the cerebral arteries. This may be due to the change in dynamic of the blood flow at those particular sites. HTN is more likely to damage small penetrating arteries deep in the brain; this process, in conjunction with the concomitant onset of atherosclerosis, is called microatheroma (lipohyalinosis).

5) Manifestations

a) Ischemic stroke

Ischemic stroke accounts for over 80 % of all strokes. It is defined as a lack of blood depriving the brain of its needed fuel and oxygen (12). There are 4 main types of ischemic events. The first, thrombotic stroke, is characterized by the obstruction of the blood flow due to a localized process within a blood vessel (e.g. fibrous plaque and/or thrombus). Embolic stroke is caused by an obstruction of the blood flow due to material which is formed elsewhere in the body. The source of the material is most commonly from the heart. Lacunar stroke, which may be asymptomatic, results from the occlusion of the small penetrating branches of the cerebral arteries; this arises due to the process of microatheroma. Finally, transient ischemic attacks (TIA) can be defined as episodes of focal neurological deficits due to an inadequate blood supply. They can involve deep or superficial arteries, leave no trace of neurological abnormalities upon examination, and always resolve themselves within 24 hours.

b) Hemorrhagic stroke

Hemorrhagic stroke involves blood leakage from one of the cerebral arteries. The bleeding damages the brain by cutting off connecting pathways and causes local or general pressure injury to the brain tissues (12). There are 2 types of hemorrhagic strokes. The first is subarachnoid hemorrhage, where blood leaks out of the vascular bed into the spaces surrounding the brain, via the cerebral spinal fluid pathways. The second is intracranial hemorrhage, where blood leaks directly into the brain substance. Hemorrhagic stroke is thought to have a different etiology than ischemic stroke and for this reason it will not be considered in the remainder of this thesis.

C) Risk factors for ischemic heart disease (IHD) and stroke

Many factors are associated with both IHD and stroke. It is important to recognize that although both genetic and environmental factors play a role in the development of these diseases, nearly all vascular risk factors have a genetic contribution. Diabetes mellitus, HTN, and smoking are three of the most potent independent risk factors involved in the development of these disorders. Other important risk factors include: dyslipidemias (increased total cholesterol, increased low-density lipoprotein (LDL) cholesterol, decreased high-density lipoprotein (HDL) cholesterol, increased triglycerides), left ventricular hypertrophy (LVH), obesity, lack of exercise, stress, male gender, increasing age, positive family history of the disease, personality features, use of oral contraceptives, menopause, hyperhomocysteinemia, and alcohol intake (3, 4, 13, 14). Stroke and MI have also been shown to be risk factors associated with each other (15). Coagulation disorders, vascular malformations, and a variety of genetic disorders (e.g. Marfan syndrome) are also rare causes for increased risk of developing IHD and stroke. These latter causes will be excluded from the scope of this research.

Part II) Genetics of ischemic heart disease (IHD) and stroke

A) Hereditary components of ischemic heart disease (IHD)

1) Family and twin studies

IHD has long been observed to aggregate in families (16). Familial aggregation studies adjusted for known IHD risk factors have shown a high relative risk in first degree relatives. A study by Roncaglioni et al. showed that in first-time MI patients of either sex with a positive family history of MI (first degree relative affected), the adjusted estimate if the RR of MI is 2.0 (95% CI, 1.6-2.5) when compared to cases with a negative family history (17). Their data also show that in both sexes, the risk of MI increases according to the number of relatives affected; RR of 3.0 in cases with two or more relatives affected. Many studies support the hypothesis that a positive family history of IHD is an independent risk factor for IHD. In a 10-year follow-up study of more than 5500 healthy men, multivariate logistic regression analysis identified that family history of MI followed LDL cholesterol as the strongest predictor of MI, at a significance level of $P < 0.01$ (18). In a study by Jousilahti et al., a parental history of premature IHD conferred an adjusted RR for AMI of 1.71 in men and 2.87 in women (AMI < 55 years of age) (19). Even though there is mounting

evidence implicating family history of IHD in the development of IHD, data showing the role of family history as an independent risk factor for IHD are still controversial. Part of the role of family history may lie in the passing on of susceptibility genes, since considerable evidence exists which suggests that part of the basis for developing IHD is genetic (20). This also implicates the passing on of other multifactorial disorders such as diabetes and HTN. Finally, individuals from a same family are exposed to a same environment and therefore tend to share the same risk factors associated with that environment.

The contribution of genetics to IHD can also be observed in a 26 year follow-up study of over 20,000 Swedish twins (21). This study reported that death from IHD at an early age in one's twin is a predictor of the risk of death from IHD, in both men and women. The study found that male monozygotic twins had a 3 fold increased RR of death from IHD than male dizygotic twins. Among females, the RR of death from IHD before the age of 65 years was 14.9 in monozygotic twins and 2.2 in dizygotic twins. This study also concluded that as the age at which one's twin died of IHD increased, the RR of death from IHD decreased in both monozygotic and dizygotic twins, further supporting the role of genetic factors in this disease.

2) Animal studies

To date there is no adequate animal model of IHD. There exist, however, various mouse models of atherosclerosis. Mice are normally highly resistant to atherosclerosis but, through induced mutations, strains can be developed which mimic the disease pathology observed in humans. ApoE-deficient mice, LDL receptor-deficient mice, transgenic mice expressing the human apo B gene, apo E Leiden and apo E R142C mice (mutations for hyperlipoproteinemia III), as well as various inbred strains are some of the lines of mice which are being used to study atherosclerosis (22). These models develop to different stages of atherosclerosis, but none of them develop thrombosis. Their purpose is to provide insight into the pathogenesis of atherogenetic disorders; this should in turn lead to better therapeutic and, possibly, preventive strategies.

B) Hereditary components of stroke

1) Family and twin studies

Although many studies have addressed the influence of family history on the risk of developing stroke, its role in the disorder still remains controversial. Design flaws in these studies with respect to data collection and analysis are in part responsible. However, some studies with larger cohorts and better designs have been able to show an association between family history and the risk of stroke. In the Framingham Offspring Study (23), both paternal and maternal histories of stroke or TIA were associated with an increased risk of stroke (RR = 2.4 and 1.4, respectively). A parental history of IHD was also associated with the development stroke or TIA (RR = 3.33). In a prospective study of over 14,000 middle-aged men and women in Finland, the adjusted RR for developing stroke associated with a positive parental history of stroke was 1.89 (24). In another study from the UK, investigators prospectively assessed the influence of positive family history of IHD or stroke on the RR of developing stroke in over 7000 middle-aged men. The findings suggest that such a family history is associated with an increased risk of stroke, independent of other established risk factors (RR = 1.4) (25).

Twin studies have also shown the contribution of genetics to stroke. In a study involving the National Academy Veteran Twin Registry, analysis of twin pairs by questionnaire revealed an approximate five fold increase in the prevalence of stroke among monozygotic twins when compared to dizygotic twins (26). The concordance rate was 0.177 for monozygotic twins and 0.036 for dizygotic twins, suggesting that genes influence the development of stroke.

2) Animal studies

The spontaneous hypertensive stroke-prone rat (SHRSP) is the most widely studied animal model of cerebrovascular disease since it most resembles the human disease. A genetic study in this rat model clearly showed genetic components to this disease (27). A cross between the SHRSP and the stroke-resistant spontaneously hypertensive rat (SHR) was carried out to remove the hypertension confounding variable and a genome-wide screen was performed on the resulting F2 cohort. Three major quantitative trait loci (*STR1-3*) were identified which could together account for 28% of the phenotypic variance in stroke latency. While the data indicates that *STR-1*

strongly affects stroke latency, *STR-2* and *STR-3* were found to confer a protective effect against stroke.

C) The study of complex traits: association versus linkage

The study of complex traits such as IHD and stroke differs greatly from the straightforward genetics of Mendelian disorders. Genetic linkage studies involve the use of family relatives. This is a way to map unknown susceptibility genes by observing the co-segregation of a disease phenotype with a specific marker allele; markers which are physically linked to a disease-causing gene on a chromosome can be identified. A widely used method of linkage for complex traits is called sib-pair analysis. This involves determining if affected sibling pairs share certain alleles more than 50% of the time. Although the sib-pair approach is widely used for certain genetic traits (28), linkage studies are generally not used for the study of IHD and stroke. The genetic analysis of these diseases is complicated by many factors such as genetic heterogeneity, mode of inheritance, reduced penetrance, epistatic effects, large number of loci (probable lack of a major locus effect), and gene-environment interactions (14, 29). Other problems, such as the variability in phenotypic description and the late-onset of the clinical stage of the disease, make it difficult to recruit families for linkage analysis.

Association studies are a more suitable approach to the study of IHD and stroke. This type of study consists of comparing the prevalence of certain gene polymorphisms between affected and non-affected individuals. The assumption is that the gene polymorphisms which are involved in the causal pathway of the disease will be more frequent in the affected population than the unaffected population. Association studies also have important limitations, such as the applicability of the results to the general population and the interpretation of the results found using small sample sizes. A case-control study design, which can be used for association studies, is based on the previous knowledge of the desired study outcome (IHD and stroke in this case) and investigates about the possible exposures (gene polymorphisms in this case) which might contribute to that outcome. The 'case' group consists only of individuals that have been diagnosed with the disorder. The 'control' group can be of two kinds. The first is family-based controls which require that the parental genotypes be available. For each locus investigated, the parental alleles (mother and father's)

which were not transmitted to the offspring (affected individual) are used as controls. This acts to reduce the genetic complexity associated with multifactorial disorders. Population-based controls is the second and simplest approach. Due to the advanced age of some of the IHD and stroke affected individuals, parents are frequently not available for testing and so unrelated individuals from the general population, who are unaffected by the disorder, can serve as controls. Selection criteria can be introduced to recruit the controls, therefore minimizing the problem of population stratification.

D) The use of founder populations: the French-Canadians

A founder population consists of a limited number of individuals which have broken away from the larger original population and have colonized elsewhere. They establish a growing population that does not mix with neighboring populations. This founder population becomes genetically isolated and some of the genetic variations, present in the parent population, become over- or under-represented. From generation to generation, some alleles may get selected out while other alleles confer an advantage and increase in frequency. Also, alleles may increase or decrease in frequency due to chance alone; this process is called genetic drift. More importantly, the genetic complexity of the population is reduced because of the limited number of founders and subsequent selection and drift.

Founder populations provide distinct advantages for the genetic study of complex traits. There are a relatively small number of ancestral chromosomes suggesting that the genes predisposing to the disease were only introduced a limited number of times into the population, which therefore reduces the genetic heterogeneity. The existence of a founder effect in the French-Canadian population has been confirmed by many studies (30-32).

Part III) Candidate systems and proteins involved in the development of ischemic heart disease (IHD) and stroke

A) The renin-angiotensin system (RAS)

1) General overview

RAS is of renal origin and is an important factor in the regulation of blood pressure, as well as fluid and electrolyte balance. Its regulation has functional significance for many organs and is a central event in vascular pathophysiology. Two

distinct RAS's have to date been identified (33). The first is endocrine RAS, which is plasma-based. The second is local RAS, or tissue RAS, which is extrarenal. Both generally function through the same cascade of two main enzymes. The initiating enzyme in that cascade is renin, an aspartyl protease which is cleaved in many tissues from pro-renin, and which is secreted by the kidneys into the blood stream (34). Active renin's function is to cleave its circulating substrate, angiotensinogen (AGT), into the decapeptide angiotensin I (Ang.I), which in turn is further cleaved by angiotensin-converting enzyme (ACE), the pathway's second main enzyme. The final product is the octapeptide angiotensin II (Ang.II), whose effects are mediated through Ang.II type I and II receptors (AT1 and AT2, respectively).

Ang.II has effects throughout the body including in the kidneys, the heart, and the blood vessel wall (34). Some of its systemic effects, which result in vasoconstriction, include the increase of aldosterone secretion from the adrenal cortex, the increase of vasopressin (a potent vasoconstrictor), and the induction of salt and water reabsorption. Ang.II also has many local effects which are thought to be precursor events to the process of atherosclerosis. Ang.II stimulates various growth factors such as macrophage-derived growth factor (MDGF), fibroblast derived growth factor (FDGF), and platelet derived growth factor (PDGF- a potent mitogen) which contribute to the migration and proliferation of VSMC (33). Ang.II promotes hypertrophy by enhancing vascular myocyte growth and by increasing collagen synthesis, therefore increasing ECM formation. Ang.II plays a role in endothelium dysfunction by oxidizing circulating LDL cholesterol molecules; this process in turn promotes their uptake by endothelial cells (35). These superoxide anions selectively inhibit the relaxation of the endothelium by preventing the release of nitric oxide (NO), a substance normally secreted by the endothelium which acts as a vasodilator, an inhibitor of platelet aggregation and of cellular growth and migration. Plaque instability is also mediated by Ang.II since it acts to generate endothelin and norepinepherin, potent SMC vasoconstrictors. This increases local vasoconstriction, and can result in the fissuring of the soft fibrous plaques. Ang.II also acts to promote thrombosis by stimulating the synthesis of proteins involved in the inhibition of fibrinolysis (thrombus dissolution), such as plasminogen activator inhibitor-1 (PAI-1).

2) Angiotensinogen (AGT)

AGT is the sole known substrate for the enzyme renin and its only proven function is as a precursor to Ang.I in the RAS pathway (36). It is placed in the serine-proteinase inhibitor superfamily even though it has no serine-proteinase inhibitor activity. The major source of synthesis of AGT is the liver, even though hepatocytes don't store it. Although AGT is present in other tissues, it is unlikely that extrahepatic synthesis makes a significant difference in plasma concentration of AGT.

In the endocrine RAS pathway, the conversion of AGT to Ang.I through renin is the rate-limiting step in the production of Ang.II. Altered concentrations of AGT would lead to an excessive or inadequate production of Ang.II. However, circulating renin and AGT are not the only source of Ang.II.; it can be produced locally be enzymes which can cleave Ang.II directly from AGT. AGT is therefore thought to be the rate-limiting factor in the production of Ang.II.

3) Angiotensin-converting enzyme (ACE)

ACE is a dipeptidyl-carboxypeptidase which converts Ang.I to Ang.II. It is mostly located on the endothelial cells of vascular beds (33). ACE is found predominantly in the lung capillaries where almost all of the Ang.I in circulation is converted to Ang.II. ACE activity is detectable in the plasma, and levels of ACE are stable within an individual. In the endocrine RAS pathway, renin is the rate-limiting enzyme involved in Ang.II production, whereas in the local RAS pathway, ACE may have a more important role than renin in influencing levels of Ang.II since there is a relation between local ACE and Ang.II which is independent of renin.

Tissue ACE also acts to inactivate bradykinin. ACE is identical to kinase II, the enzyme which normally inactivates this peptide. Bradykinin is normally released by the endothelium and acts as a potent vasodilator and inhibitor of growth. It works through endothelial cell receptors to cause the release of prostacyclin, NO, and endothelial-derived hypopolarizing factor, all of which are relaxing factors. Bradykinin can also stimulate tissue plasminogen activator (TPA), a fibrinolytic agent. ACE inhibitor drugs have been shown to attenuate the generation of superoxide anions and Ang.II, as well as to stimulate the release of NO and prostacyclin. ACE inhibitors also act as antihypertensive agents and have been shown to cause the regression of

LVH.

4) Angiotensin II type 1 receptor (AT1)

The effects of Ang.II are mediated through two receptors: AT1 and AT2. AT1 mediates most of the functions of Ang.II and is localized in many tissues, including the heart, VSMC, the brain, platelets, monocytes, the kidneys, the adrenal cortex, the placenta, and sperm cells (34, 37). On the other hand, the function of AT2 is less defined and the sites of physiological expression are much less abundant: the brain, the kidneys, the adrenal cortex and medulla, and the uterus. AT2 will not be discussed any further in this thesis.

The stimulation of AT1, a G-coupled receptor, is mediated by phospholipid-derived secondary messengers, activation of protein kinase C, and MAP kinase pathway. This receptor is expressed in low numbers in the body and can be regulated in four different ways: phophorylation, internalization, control of mRNA stability, and at the transcriptional level. AT1 regulation is highly cell- and organ-specific; only a few stimuli will induce the same effect in different organs. The cellular regulation is probably based on the cell-specific activation of intracellular kinases by the particular agonist. In all tissues, the number of AT1 which are functionally available at the cell surface not only determines but also limits the magnitude of the ATR-mediated effects. Therefore, up- and down-regulation of this receptor is important in understanding RAS.

B) The hemostasis system

1) Blood clotting

Blood clotting results from a specific trigger that initiates a cascade of reactions in which inactive enzymes become activated. The system is made up of serine proteases, each of which activates the subsequent enzyme in the series. There are two hemostasis pathways. The first, the intrinsic pathway, is triggered when negatively charged surfaces which underlie the endothelium, such as collagen fibers, become exposed (34, 38). In response to this, clot promoting substances, such as plasma kallikrein, and blood clotting factors, such as factor XII (fac. XII), are pulled from circulation and activated (fac. XIIa). Fac. XIIa then activates factor XI (fac. XIa) which then goes on to activate factor IX (fac. IXa), which in turn will participate in

the activation of factor X (fac. Xa) by forming a complex with activated factor VIII (fac. VIIIa); this latter factor becomes active when it is separated from von Willebrand factor (vWf). The subsequent activation of fac. X also requires the presence of Ca^{2+} as well as phospholipids from aggregated platelets. Fac. Xa will, with the aid of activated factor V (fac. Va), cleave thrombin from prothrombin. The final and fundamental reaction in this cascade, which is catalyzed by thrombin, is the release of fibrin from fibrinogen. Fibrin monomers start to polymerize with each other to form a loose mesh of interlacing strands. This is converted to a tight aggregate in the presence of Ca^{2+} and with the aid of fac. XIIIa, which has been activated by thrombin. This dense aggregate traps blood, serum, and platelets to form a blood clot.

The extrinsic pathway is the second possible pathway in this system. What triggers this pathway in injured tissues is an agent called tissue thromboplastin (TPL). This agent contains clot-promoting properties as it acts to activate factor VII (fac. VIIa). In turn, fac. VIIa will activate fac. X and fac. IX. TPL also provides the phospholipids needed to completely activate fac. X in the extrinsic pathway.

2) Platelets

Platelets are anuclear cells which are pinched off bits of megakaryocyte cytoplasm; the latter are giant cells originating from the bone marrow (6). The platelet membrane contains receptors for many substances including collagen, vWf, fibrinogen, and thrombin. The platelet adhesion mechanism, which does not require platelet metabolic activity, occurs when platelets adhere to the exposed collagens, laminins, and vWf in the blood vessel wall, via integrins. vWf enhances the adhesion by forming a bridge between collagen fibers and a surface receptor, platelet glycoprotein Ib (PGIb). This latter binding leads to the platelet aggregation mechanism which can also be induced by thrombin. Platelets become activated, change shape, discharge their granules, and stick to other platelets. Aggregation takes place only if the surrounding environment contains fibrinogen which adheres to platelet receptors, the glycoprotein IIb/IIIa complex. Activated platelets also release mitogenic and chemotactic substances, such as PDGF and transforming growth factor-beta (TGFβ), which play a role in VSMC proliferation and infiltration of monocytes into the endothelium.

3) Fibrinolysis

The activation of the enzymes of the blood clotting cascade by partial proteolysis is an irreversible process. Once the enzyme is cleaved, it remains active until it is degraded or inhibited by some other means. The fibrinolytic system acts to counter-balance the tendency of clots to form in the blood vessels. With the exception of cerebral microcirculation, all endothelial cells produce thrombomodulin (34, 38). This thrombin-binding protein is expressed on the cell surface. When thrombin binds thrombomodulin, the newly formed complex acts as an anticoagulant by activating protein C (APC). APC, along with its cofactor protein S, inhibit the coagulant properties of fac. VIIIa and fac. Va. It also enhances fibrinolysis by neutralizing PAIs, which normally act to inhibit TPA. This allows TPA to catalyze the release of plasmin from plasminogen. Plasminogen is the active component of the fibrinolytic system since it lyses fibrin and fibrinogen to their degradation products, which will in turn inhibit thrombin.

4) Factor Va (fac. Va)

Thrombin is separated from prothrombin by fac. Xa through reactions which are augmented by fac. Va, Ca^{2+}, and phospholipids. Fac. V, in its native state, has no clot-promoting properties. Fac. Xa alters fac. V to fac. Va, which then attaches itself to the fragment II portion of prothrombin where it acts as an accelerator of fac. Xa. This latter reaction brings about a rapid release of thrombin which, as it is being liberated, alters fac. V to fac. Va by cleavage.

5) Platelet glycoprotein IIIa (PGIIIa)

PGIIIa is part of a fibrinogen receptor on the platelet surface: the PGIIb/PGIIIa complex. This complex is a member of the integrin family of adhesive receptors and exists in an inactive state on the resting platelets (39). Under conditions of high shear stress, such as narrowing of the blood vessel lumen, the interactions between PGIb and vWf will lead to conformational changes in the PGIIb/PGIIIa complex, therefore allowing platelets to bind fibrinogen as well as other platelets. Normal platelet aggregation, which is achieved through this complex, requires an intact fibrinogen receptor.

6) Plasminogen activator inhibitor 1 (PAI-1)

PAI-1 acts to inhibit both TPA and urokinase-type plasminogen activator (UPA) and therefore may be the primary regulator of plasminogen activation in vivo (40). PAI-1 is a glycoprotein synthesized in the blood vessel wall, by both endothelial cells and VSMC, and has a very short circulating half-life. Secretion of PAI-1 can be stimulated by Ang. II and glucose. This protein is therefore subject to dynamic regulation and there exits a fine balance between it and the plasminogen activators. A deficiency in PAI-1 would lead to hemorrhagic consequences. On the other hand, an excess in PAI-1 would cause a prothrombotic state due to the loss of the ability to synthesize plasmin. Plasmin not only acts to degrade fibrin but it also activates matrix metalloproteinases (MMP) as well as TGFβ, which plays a role in the suppression of VSMC proliferation.

C) Fat metabolism

1) Plasma lipids and lipid transport

Important lipids include fatty acids and their derivatives: neutral fats (triglycerides), phospholipids, and sterols (e.g. cholesterol) (34, 38). Major lipids in the plasma do not circulate in free form. Free fatty acids (FFA) are bound to albumin, and triglycerides and cholesterol, the major components of plasma lipids, are transported in the form of lipoproteins. Lipoproteins have a non-polar, hydrophobic core which contains triglycerides and cholesterol esters, and a polar surface which is made up of phospholipids, cholesterol, and apoproteins.

An exogenous source of triglycerides, the major source of dietary fat, is absorbed by the intestine after meals and circulates as very large lipoproteins called chylomicrons. These molecules are rapidly cleared from the plasma by the action of lipoprotein lipase which breaks down the triglycerides to FFA and glycerol. The remaining particles, the chylomicron remnants, are cholesterol-rich lipoproteins which bind liver receptors and are endocytosed. Chylomicrons and their remnants constitute the transport system for exogenous lipids.

The endogenous transport system is made up of very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, and HDL. These are named according to their physical densities: the lowest density contains the least protein and the most triglycerides. The primary endogenous source of triglycerides is the liver. There, triglycerides, along with most of the cholesterol in the liver, are incorporated into VLDL and released into circulation. By the action of lipoprotein lipase, VLDLs become depleted of some of their triglycerides; the molecules that remain are IDL. While about half of IDLs are taken up by the liver, the other half not only picks up cholesterol esters from HDL but also loses more triglycerides to become LDLs. The cholesterol esters in LDL particles are taken up by numerous tissues after interacting with LDL receptors. HDL, which is synthesized in the liver and the intestine, acts to absorb the cholesterol leaving the cell, transfers lipid components between other lipoproteins, and facilitates enzyme activity in the metabolism of lipoproteins.

2) Apoproteins

Apoproteins are the protein constituents of the lipoproteins. The major apoproteins are apo A, C, E, and B; the latter has two forms which differ in their molecular weight: apo B-48 and apo B-100. Chylomicrons contain apo E, C, and B-48 while their remnants contain only apo E, and B-48. VLDLs contain apo E, C, and B-100. One component of apo C, apo C-II, activates lipoprotein lipase. IDL contains both apo B-100 and E; the apo E portion is lost when IDL becomes LDL. Finally, HDL from the liver have both apo E and C while HDL from the intestine only have apo A. LDL receptors, which allow the transport of these particles in and out of the liver as well as extrahepatic tissues, recognize apo E and B-100, but not apo B-48. These proteins permit the lipoproteins to interact with their receptors in order to maintain the proper distribution of fat throughout the body.

3) Apolipoprotein E (apo E)

Apo E is a 33-35 kD polymorphic glycoprotein which is primarily synthesized in the liver but which is also synthesized in the brain, adrenal cortex, and macrophages; it is degraded in the lysosome of the cell. Although apo E's role as a ligand for the apo E/B receptor in the liver has been well characterized, its function in extrahepatic tissue remains poorly understood. Macrophage-derived apo E is thought to play a role in the reversal of cholesterol transport in vivo (41). The synthesis of

macrophage-derived apo E is in large part regulated by the cellular contents of cholesterol, where free cholesterol is a major determinant. The mRNA levels and protein secretion of apo E increase when macrophages take up copper-oxidized LDLs (Cu-oxidized LDL). Although Cu-oxidized LDLs also act to stop the esterfication of cholesterol and cause the inactivation of a lysosomal proteases, which leads to impaired degradation of cholesterol and lysosomal trapping, it has been suggested that the increase in apo E expression caused by Cu-oxidized LDLs may be due to mechanisms other than cholesterol accumulation (41).

D) Homocysteine (Hcy) metabolism

1) General overview

There are two important pathways in the metabolism of Hcy: the methionine pathway, which is present in all mammalial cells, and the transsulfuration pathway, which only exists in the liver, kidney, small intestine, and pancreas (42). Methionine is an essential nutrient which is involved in the synthesis of proteins, S-adenosylmethionine (AdoMet), Hcy, and cysteine. In the cell, about 10% of the AdoMet becomes decarboxylated while the rest goes on to enter the transmethylation pathway, where it acts as a methyl donor and becomes S-adenosylhomocysteine (AdoHcy). This latter molecule must be removed from the system and a way in which this is achieved is by its conversion to Hcy. Although Hcy can bind proteins and be exported out of the cell, it can also be incorporated in one of two pathways. It can enter the transsulfuration pathway where it is converted to cystathionine and then cysteine by the enzymes cystathionine-β-synthase and cystathionase, respectively. This is the only reaction that removes Hcy from the methionine cycle and it is irreversible. Tissues which do not have this pathway require an outside source of cysteine. Hcy can also continue in the transmethylation pathway, where, with the aid of methionine synthase, it gets remethylated. This reaction regenerates both methionine and methyl tetrahydrofolate (THF). In order for this to take place, a methyl group must be obtained from the one carbon pool, which is provided by the derivatives of folic acid. Once folate is absorbed by the intestine, it is methylated either locally or in the liver; it then enters the blood stream (43, 44). 5-methyl THF is the major form of circulating folate in the plasma; it is synthesized from 5,10-methylene THF, by the enzyme methylene THF reductase (MTHFR), this being the only reaction generating 5-methyl THF in the cell. The excessive presence of Hcy in

the cell can be due to an impairment in it's remethylation. This can be caused by either a folate or a MTHFR deficiency. Hcy is thought to have a wide range of effects which are related to the development of CVD. These include increasing procoagulant activity, synthesis of collagen, and proliferation of VSMC (45). In vitro studies have shown that increased Hcy levels can lead to the oxidation of various molecules (e.g. Cu-catalyzed oxidation of hydrogen peroxide) which in turn cause damage to the endothelium (46). Prolonged exposure of cells to Hcy has also been shown to decrease the secretion of NO by the endothelium (47).

E) The extracellular matrix (ECM)

1) General overview

The ECM of vascular tissues is mainly composed of proteoglycans and collagens, which are produced by VSMC and endothelial cells. The ECM also contains elastins, the main component of elastic fibers, fibronectins, and laminins, which are part of the basement membrane (9). The ECM is a dynamic environment where matrix proteins are constantly being synthesized and degraded. In order to maintain matrix integrity there must be a balance between matrix formation and matrix turnover. If this homeostasis is shifted, there is either an excessive production and accumulation of matrix proteins or an enhanced turnover of matrix proteins which causes weakening of the ECM. The remodeling of the ECM is done by proteins of the MMP family which is composed of at least 16 zinc-dependent endopeptidases. There are 4 groups of MMPs: collagenases, stromelysins, gelatinases, and membrane-type MMPs. Most of these are secreted in latent form (zymogen) by SMC, fibroblasts, and inflammatory cells (macrophages). Although each MMP is substrate specific, between them the MMPs can degrade all the components of the ECM. Their proteolytic functions are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (48).

MMPs can be controlled at three different levels: transcription, activation of latent form, and inhibition of TIMP activity. Disruption at any of these levels will cause excess production or degradation of ECM proteins. Atherogenesis involves extensive vascular remodeling. The formation of atherosclerosis is characterized by the accumulation of ECM elements, hence the synthesis of proteins is greater than its degradation. On the other hand, disruption of the fibrous cap is characterized by

weakening of the lesion, therefore the degradation of ECM proteins is greater than their synthesis. The synthesis/turnover ratio of the ECM may vary at different stages and locations of the disease process.

2) Stromelysin-1 (MMP-3)

Of the MMPs, MMP-3 has the widest substrate specificity and can degrade many of the components of the ECM (49). Its principle substrate is proteoglycan, and other substrates include collagens (type II, IV, and IX), laminins, fibronectins, and gelatins. Stromelysin can also activate other members of the MMP family such as collagenases (MMP-1) (50). The regulation of stromelysin occurs mainly at the transcription level. The promoter region of this gene responds to different regulators including PDGF and interleukin-1 (IL-1). The expression of MMP-3 is regulated by agents such as cytokines, growth factors, tumor promoters, and oncogene products. The natural inhibitors of stromelysin-1, TIMPs, are coordinately expressed and regulated by similar agents.

Part IV) Candidate genes for ischemic heart disease IHD and stroke

A) The renin-angiotensin system (RAS)

1) Angiotensinogen (AGT)

The AGT gene is 12 kb long and consist of 5 exons and 4 introns; it is situated on human chromosome 1q42-1q43 (36). The 5'flanking region has regulatory sequences responding to glucocorticoids (increases gene transcription), Ang.II (stabilizes mRNA), cytokines, thyroid hormones, insulin, androgens, and estrogens (51). One variant of this gene is an exon 2 substitution of a T for a C at position 704 of the cDNA. This substitution leads to an amino acid change from methionine (M allele) to threonine (T allele) at residue 235 (refer to Table A) (52).

The M235T variant is present in about 15% of the Western population. It has not only been linked to HTN but it has also been associated with increased concentration of circulating AGT in hypertensives (53). This variant has been reported to confer a two-fold increased risk of IHD, an association which was found to be independent of blood pressure (54). While some data suggest that AGT variants are independent risk factors for the development of coronary atherosclerosis, others have

reported a lack of association between the two (55). In contrast to this, it has been observed that balloon injury in the aorta can activate AGT gene expression in the medial layer, implicating AGT in neovascular tissue proliferation and therefore in CVD (56). In addition, studies have shown that the accuracy of prediction of MI in normotensives, and the RR of cerebral infarction, increased with the increasing number of T alleles among individuals who carry the ACE DD genotype; the clinical relevance of these findings still remains to be determined (57, 58). It is therefore important to initiate further research on the possible implication of this AGT polymorphism in the development of stroke and IHD.

2) Angiotensin-converting enzyme (ACE)

The ACE gene is a 26 exon gene which is 21 kbp long and located on human chromosome 17q23. It had been established that about 50% of the inter-individual variability in plasma ACE concentration is due to a major gene effect (59). It was later shown that this major gene effect was associated with an insertion (I)/ deletion (D) polymorphism in the ACE gene (60). This is a 287 bp *alu* repeat sequence in reverse orientation near the 3'end of intron 16 of the gene.

The ACE gene has merited much attention as a candidate gene for IHD (20). In a study by Rigat et al., the DD genotype was associated with a two-fold increase in levels of circulating ACE when compared to other genotypes (61). This genotype has also been associated with enhanced conversion of Ang.I to Ang.II (62). With respect to MI, a meta-analysis has shown that the D allele confers an increased risk of MI (63). Data from some studies have also shown that homozygosity for the D allele was more frequent in MI cases, which were defined as being of 'low-risk' status, than in controls (64, 65). Other studies have found no association between the ACE I/D polymorphism and MI, coronary atherosclerosis and stenosis (66, 67).

ACE has also been suggested as a candidate gene for stroke (68). There have been many studies which have concluded that ACE levels increase with the increasing number of D alleles and that the DD genotype is in fact associated with stroke (69, 70). Other data suggest that this genotype is more specifically associated with cerebral infarction and lacunar stroke (58, 71). The D allele of the ACE gene has also been associated with a parental and personal history of stroke, and a parental history of

HTN (72, 73). Much controversy still surrounds the I/D polymorphism of the ACE gene. Due to the potentially severe consequences which changes to the RAS may have on the development of stroke and IHD, this variant merits further investigation with respect to its effects on the system and on vascular disease outcome.

3) Angiotensin II type 1 receptor (AGT1R)

The AGT1R gene is situated on human chromosome 3q21-25, with 4 to 5 exons spanning at least 60 kbp. The entire coding sequence of this gene is found within the last exon (37). Many different polymorphisms have been identified in the coding and 3'untranslated region of the AGT1R gene (74). One variant, located in the 5'end of the 3'untranslated region, corresponds with an A (A allele) to a C (C allele) transversion at position 1166 of the mRNA; this change does not alter the amino acid sequence of the AT1 protein.

This AGT1R polymorphism has been studied with respect to CVD. In a cross-sectional study, the C allele was found to be associated with aortic stiffness in over 300 untreated hypertensives (75). Since the association was not present in normotensives, the authors suggest that the presence of HTN may accentuate the effects of Ang.II. Another study has reported a higher prevalence of the C allele among hypertensives than normortensive subjects (76). Alternatively, these authors suggest that changes in the AGT1R gene may act to predispose to HTN.

While some studies have failed to show an association between the A1166C polymorphism and the increased risk of CVD (77), others have data which suggest otherwise. The involvement of the A/C 1166 polymorphism in the development of coronary artery stenosis in the Japanese has been reported (78). In 235 Norwegian females considered to have a 'low-risk' phenotype, the CC genotype was associated with an increased risk of MI (79). Furthermore, a synergistic effect of this polymorphism and the ACE DD genotype has been reported to increase the risk of MI (80). The biological mechanism by which the epistatic effect of these two genes could affect the risk of MI is unknown. Changes in the AGT1R gene may, by themselves or in concordance with the presence of other risk factors, act to reduce or enhance the development of atherosclerosis in diseases such as stroke and IHD, and thus should be further investigated.

B) The hemostasis system

1) Blood clotting: factor V (fac. V)

The fac. V gene is located on human chromosome 1q21-25 (81). A base change from a G to an A at position 1691 of the DNA is located in exon 10 of this gene, 11 nucleotides 5'of the start of intron 10. This substitution results in an amino acid change of arginine to glutamine, at position 506 in the RNA sequence, which is situated in the cleavage site for APC and therefore creates a resistance to APC (rAPC). This mutation is called fac. V Leiden and prevents the inactivation of fac. V by APC, resulting in a procoagulating state.

rAPC is the major cause of inherited deep vein thrombosis (DVT) (82). In more than 90% of cases, fac. V Leiden turns out to be the cause of rAPC (81). This mutation has been found to be present in 3-7 % of two healthy white populations and is to date the most common hereditary blood coagulation disorder. Three percent of the population will be heterozygous for Leiden while 2 in 10,000 live births will be homozygous (83). The risk of thrombosis for heterozygotes increases by 7-10 fold, while the risk for homozygotes increases about 80 fold. It has been suggested that this increase may only be observed if other risk factors are present, since some individuals with rAPC remain asymptomatic. Even though the implication of the Leiden mutation in venous thrombosis has been established, its role in the development of arterial disorders remains controversial.

Although most studies have found no association between the presence of the Leiden mutation and CVD (84-86), some studies present results which suggest that this mutation may be involved in the early development of arterial thrombosis (87). In a case-control study by Rosendaal et al., the Leiden mutation was found to be associated with MI, with a 2.4 fold excess of the mutation when compared to controls, in women aged 18-44 years; this effect was particularly strong in smokers (88).

Many stroke studies have also failed to find an association with the Leiden mutation (84, 89). Some studies have shown the absence of this mutation's influence on early onset of carotid intima-media thickening (IMT), linking its effect to the presence of other CVD risk factors (86). Although the fac. V Leiden mutation has been listed as a risk factor for cerebral venous thrombosis, data implicating it with

cerebral arterial thrombosis remains only a trend (90, 91). Due to this mutation's important involvement in venous thrombosis, it is important to further investigate its potential role in the development of arterial disorders, such as stroke and IHD, which have similar etiologies and potentially more devastating consequences.

2) Platelets: platelet glycoprotein IIIa (PGIIIa)

Two polymorphic variants of the PGIIIa gene are known: PL^{A1} and PL^{A2}. At position 1565, in exon 2 of this gene, the base can be either a T or a C. This leads to a change in the amino acid sequence coding for either a proline (PL^{A1} allele) or a leucine (PL^{A2} allele) at position 33 of the protein sequence (92).

The PL^{A2} allele of the PGIIIa gene has been under investigation for its possible implication in the development of IHD. A study looking at 2252 Caucasians found that the PL^{A2} allele was associated with IHD in 'low-risk' individuals (93). In a study by Weiss et al., data suggest that there is a greater prevalence the PL^{A2} allele among individuals with MI/unstable angina (94). Although the sample size in this study is relatively small, the authors find that there is a statistical difference of more than 7 years in the onset of MI/angina between the cases with at least one PL^{A2} allele, versus cases homozygote for the PL^{A1} allele. In another study looking at 100 patients with coronary syndrome, the adjusted odds ratio (OR) for the PL^{A2} allele was found to be higher in cases younger than 60 years of age: 5.93 versus 2.91 (95). While other studies have also found similar associations between this PGIIIa polymorphism and IHD (96), others have failed to show such an association (97, 98). In a prospective follow-up study of 8.6 years, Ridker et al. observed no association between cases consisting of 374 MI, 209 stroke, 121 DVT, and 704 controls with respect to the PL^{A2} allele of the PGIIIa gene (99).

Very few studies have focused their attention on the possible association between the PL^{A2} allele and stroke, although increased platelet activity has been associated with both stroke and MI (100, 101). In a study of over 600 white Europeans with stroke, the PL^{A2} allele was found to be a significant predictor of stroke in subjects that had never smoked (102). Another study has reported similar findings, where PL^{A2} is associated with stroke risk among white females (103).

Normal platelet function is important in blood clotting. Any change leading to variations in platelet function may alter normal blood coagulating responses, and thus help lead to a disease state. This PGIIIa polymorphism may aid in contributing to changes in the hemostasis system and therefore should be further studied with regard to stroke and IHD, due to the atherosclerotic/thrombotic nature of these diseases.

3) Fibrinolysis: plasminogen activator inhibitor-1 (PAI-1)

The PAI-1 gene is 12.3 kb and consists of 9 exons and 8 introns; it is situated on human chromosome 7q21.3-q22 (104). A common diallelic polymorphism has been described 675 bp upstream from the start of transcription of the PAI-1 gene. One allele has a sequence of five guanines (5G, or I) while the other has only four guanines (4G, or D). It has been demonstrated that both these alleles bind a transcriptional activator, whereas only the 5G allele binds a transcriptional repressor at an overlapping site in the promoter region (105).

The 4G allele of the PAI-1 gene has been implicated in the development of CVD. In a study by Eriksson et al., this polymorphism was shown to influence the basal level of transcription of PAI-1; a two fold increase was detected between 4G and 5G constructs (106). This same study not only found that the increase in the plasma activity of PAI-1 was associated with the increasing number of 4G alleles, but men with a first occurrence of MI before the age of 45 years were also found to have a greater number of 4G alleles. On the other hand, some studies have not found an association between the 4G allele and MI (107).

Although a large amount of the data also suggest that the 4G allele is associated with increased levels of PAI-1 (49, 108), PAI-1 is known to be influenced by many metabolic factors. Many studies have demonstrated that PAI-1 levels are strongly positively correlated with BMI and triglycerides (108-110). This is the link between fibrinolysis and fat metabolism. There is also a link between decreased fibrinolysis and diabetic and obese persons. Insulin resistance influences the synthesis of PAI-1 via its effect on lipid metabolism.

The PAI-1 gene has been found to be a good candidate gene for stroke, although to date a minimal amount of studies have looked at this issue (68). Mean basal level of PAI-1 has been found to be elevated in stroke cases when compared to controls (111). In a study of over 500 ischemic and hemorrhagic stroke patients, the 4G/5G polymorphism was not found to be associated with the disorder (112). Clearly, our understanding of the function of this gene product with respect to other systems, as well as vascular disease, is not yet complete. More studies need to be conducted in order to elucidated the role of this PAI-1 polymorphism in the development of atherosclerotic diseases, such as stroke and IHD.

C) Fat metabolism

1) Apolipoprotein E (apo E)

Apo E is encoded by a gene cluster on human chromosome 19 (113). This gene has 3 codominant alleles called ε2, ε3, and ε4. Of these, ε3 is by far the most frequent in the Caucasian population (77%), followed by ε4 (15%), and finally by ε2 (8%) (114). These alleles code for 3 isoforms of apo E: E2, E3, and E4 respectively. The 3 isoforms differ by an amino acid substitution at one or two sites. E2 has a cysteine residue at both positions 112 and 158, while E4 has an arginine at both these positions. E3 has a cysteine at position 112 and an arginine at position 158. These changes create or eliminate restriction sites for the enzyme *Cfo*I (or *Hha*I).

The apo E gene has been investigated by many studies as a susceptibility gene for IHD. A meta-analysis has revealed that the presence of apo ε4 allele was greater among male cases of IHD than among male controls; a similar trend was observed in women (115). The study failed to find an association between IHD and apo ε2 allele. In another study, over 300 Finns with coronary artery disease (CAD) were assessed for variances in apo E polymorphism with respect to plasma lipids and severity of CAD (116). The data show that plasma total cholesterol and LDL-cholesterol, as did the severity of CAD, increased according to apo E genotype: E2/3 < E3/3 < E3/4 < E4/4; other studies report similar findings (117). Carotid IMT in relation to apo E genotype has also been assessed (118). The data suggest that in both men and women free of symptomatic IHD, carotid IMT increased from E2 to E4 carriers. Although most studies show that the ε4 allele is associated with an increased risk of IHD, some

studies have reported associations between the ε2 allele and IHD (119, 120). A study has found that apo E2/3 genotype is associated with an earlier IHD age of onset (121). Individuals carrying the apo ε2 are thought not to be protected against IHD since this allele is associated with increased levels of plasma triglyceride-rich lipoproteins.

The involvement of apo E in the development of stroke is much more controversial than that of IHD. Many studies have shown data which support the protective effect of the ε3 allele against stroke (122, 123). While some studies report that apo E4 has increased frequency in large vessel cerebrovascular disease, others suggest that apo ε4 is not an important risk factor for stroke in the elderly (124, 125). There also exists a lack of consensus regarding the effect of apo ε2 allele. On the one hand, a study reports that apo ε2 may be a risk factor for stroke (122). On the other hand, a study looking at 150 ischemic stroke cases finds that the presence of the ε2 allele confers protection against stroke before 80 years of age, but that this protective effect seems to be lost beyond this age (126). Although the role of apo E in vascular disease processes still remains to be defined, its involvement in the development of cardio and cerebrovascular disease merits further investigation.

D) Homocysteine (Hcy) metabolism

1) Methylenetetrahydrofolate reductase (MTHFR)

The human MTHFR gene is situated on chromosome 1q36.3. A point mutation that causes the base substitution of a C to a T at position 677 of the DNA has been identified in this gene (127). This change results in an amino acid substitution of alanine to valine at position 222 of the protein sequence. It has been shown that this causes the enzyme to have increased susceptibility to heat inactivation, leaving MTHFR with only 50% residual activity. The three possible genotypes show significant differences with respect to thermolability. Individuals homozygote for the mutation have a distinctly lower range of activity, while heterozygotes have an intermediate range when compared to individuals which do not carry this mutation. There is also strong evidence showing that individuals carrying at least one mutated allele have almost twice the fasting levels of Hcy; homozygotes for this mutation had the highest levels.

Severe MTHFR deficiency, where the enzyme has 0-20 % residual activity, is the most common inborn error of folate metabolism and results in severe hyperhomocysteinemia. The thermolabile variant of MTHFR is thought to result in mild hyperhomocysteinemia, also an accepted risk factor for vascular disease (128). It was first shown that this MTHFR deficiency was involved in vascular disease when a study reported an associated between MTHFR thermolability and CAD (129). Another study reports that this variant is predictive of CAD independently of other risk factors (130). The sequence variant responsible for this sensibility to heat inactivation was then identified at the DNA and protein levels (127). Many studies have since found similar associations. In a study looking at vascular disease in general (cardio, cerebro, and peripheral), it was found that the C677T mutation was a risk factor for atherosclerotic disease (131). These results suggest that plasma folate plays a critical role in Hcy homeostasis, especially in individuals homozygous for this mutation. Although similar data have been shown by other studies (132), some studies have failed to show this association (133, 134). Even though the role of Hcy in the development of atherosclerotic disease is not yet clear, the evidence suggest some form of implication and therefore systems which may directly or indirectly influence the plasma levels of Hcy should be investigated with respect to the risk of diseases such as stroke and IHD. The C677T mutation in the MTHFR gene, which leads to alterations in the remethylation of Hcy, should be closely looked at in the vascular disease process.

E) The extracellular matrix (ECM)

1) Stromelysin-1 (MMP-3)

Stromelysin-1 is located on the long arm of human chromosome 11. The gene has 10 exons and 9 introns and spans 8-12 kbp of DNA (49). A polymorphism in the promoter region of this gene has been identified 1171 bp upstream from the start transcription site. The I/D of an A creates a run of either 5 or 6 adenosines (5A vs. 6A allele). It has been found that this polymorphism plays a functional role in the expression of MMP-3. The 6A allele has been shown to have preferential binding to a nucleoprotein, possibly a transcriptional repressor, which in turn leads to a lower level of gene expression; the 5A allele shows levels of expression two folds greater than the 6A allele (135).

Disruption of the ECM homeostasis has been studied with respect to the pathology of vascular disease processes. An imbalance in connective tissue remodeling, as well as an increase in MMP expression, is observed in many studies (136-138). Other studies have found that stromelysin expression is present in atherosclerotic plaque and that the mRNA transcripts localize to SMC and foam cells of the fibrous cap (50, 139). This data has lead to the hypothesis that MMP-3 may be involved in the vascular tissue remodeling associated with the pathology of atherosclerosis development and plaque rupture. It has been suggested that changes in the stromelysin-1 promoter sequence might lead to over/under expression of the gene, in turn leading to local alterations in the normal turnover process of ECM proteins. The 5A/6A polymorphism has been studied with respect to coronary atherosclerosis. In a sample of 72 men with CAD, data show that the 6A/6A genotype is associated with significantly greater progression of the disease when the baseline stenosis is smaller than 20% (107). Therefore the 6A allele, which has a lower stromelysin-1 expression, would favor matrix deposition and growth of atherosclerosis; 5A/5A individuals may be protected from this at baseline. The 5A allele has been suggested as a recessive protective allele in a study which observed that drug prevention of narrowing of the vessel diameter after by-pass surgery was more effective in 5A/5A individuals when compared to other genotypes (140). Although the role of this polymorphism with respect to advanced lesions and plaque rupture remains to be elucidated, stromelysin-1 is a good candidate gene for stroke and IHD due to its possible implication in the progression of atherosclerosis.

F) Hypothesis of the study

The following is a genetic investigation of IHD and ischemic stroke in the French-Canadian population. There is thus far sufficient evidence to support that these diseases are genetically determined, at least in part, and so the scope of this association study will comprise the nine genes, and respective polymorphisms, discussed above (for summary see Table A). The prevalence of genotypes will be analyzed and discussed with respect to disease status as well as disease risk factors.

Table A Candidate genes and variants for stroke and ischemic heart disease				
Gene	System	Role	Polymorphism	Region of change
AGT	**RAS**	**Precursor to Ang. I**	**Base substitution T704C, Met235Thr**	**Exon 2**
ACE	RAS	Converts Ang. I to to Ang. II	I/D 287bp	3' end of intron 16
AGT1R	**RAS**	**Mediates effects of Ang. II**	**Base transversion A1166C**	**5' end of 3' un-translated region**
Fac. V	Hemostasis	Helps fac. Xa to release thrombin	11 nuc. from intron 10 G1691A, Arg506Glu	Exon 10
PGIIIa	**Hemostasis**	**Part of the platelet fibrinogen receptor**	**Base substitution T1565C, Pro33Leu**	**Exon 2**
PAI-1	Hemostasis	Inhibits fibrinolysis	I/D, 5G/4G	Promoter - 675 bp
Apo E	**Fat metabolism**	**Receptor ligand for various lipoproteins**	**E2, E3, E4 isoforms**	**Residues 112 and 158**
MTHFR	Hcy metabolism	Remethylation of Hcy	Base subtitution C677T, Ala222Val	Exonic
MMP-3	**ECM**	**Degrades ECM**	**I/D, 6A/5A**	**Promoter - 1171 bp**

Part V) Materials and Methods

A) Subjects

1) Ischemic stroke study

Ninety-seven (67 men and 30 women) successive French-Canadian patients with ischemic stroke, who were identified from the McGill Cerebrovascular Clinic (Montreal General Hospital (MGH)) and the St-Luc Hospital, were enrolled into the study. The *inclusion criteria* were: a documented TIA or ischemic stroke (presumed atherosclerotic) in an 80 year old or younger patient of French-Canadian ancestry, defined as having 4 French-Canadian grand-parents. Standard definitions of TIA and ischemic stroke were applied (141). Given the demographics of the French-Canadian population, requiring that the proband's 4 grand-parents be French-Canadian avoided the introduction of non-French-Canadian genes from immigrants who came to Quebec after the first World War. The cut-off age of 80 years was introduced to prevent focusing on young patients with stroke, which would bias the study towards finding more penetrant genes that may not be as important as a cause for the more common atherosclerotic variety of strokes. The purpose of this study was to identify genes that predispose to the more common forms of stoke and therefore patients who are at greater risk to develop these types of stroke were included in the study. Due to the difficulty in obtaining aged-matched controls, patients older that 80 years of age were

excluded from the study. No lower age limit for exclusion was established since the non-atherosclerotic types of stroke that are more common in young individuals were eliminated by the exclusion criteria. The *exclusion criteria* were: presence of a traumatic dissection of an artery, embolic stroke of cardiac origin, cerebral hemorrhage, vasculitis, aneurysms, other vascular malformations as well as other non-atherosclerotic causes of stroke (hypercoagulability, antiphospholipid syndrome, etc.). Informed consent was obtained from each patient according to a protocol approved by the Research Ethics Committee of both McGill University and the MGH Research Institute. A detailed clinical questionnaire (see 'questionnaire information' section below) and a pedigree were completed for each patient, and verified by a Clinical Research Nurse. Data validation was done using the patient's hospital records and confirmation of stroke type was done using the patient's CT scan/MRI; ambiguous diagnoses were reconfirmed by a neurologist. For each patient, blood samples were obtained for biochemical analysis (total cholesterol, LDL, HDL, glucose, and fibrinogen), DNA extraction, and to start lymphoblastoid cell lines in order to provide an ample supply of DNA as well as RNA and protein that may be needed for certain parts of the study at a later time.

2) Ischemic heart disease (IHD) study

One hundred and seventy four successive patients with IHD were collected at the Montreal Cardiology Institute. Their *inclusion criteria* were: documented presence of IHD (angina, MI non-Q, MI) in individuals of at least 18 years of age. Their *exclusion criteria* were: CVD caused by obvious provoking factor (i.e. due to non-atherosclerotic reasons) such as arrhythmia, hypotension, severe trauma, major surgery, active present bleeding disorders, etc. The *exclusion criteria* which we imposed were: non-French-Canadian ancestry. Informed consent was obtained from each patient according to a protocol approved by the Research Ethics Committee of the Montreal Cardiology Institute. A detailed questionnaire was also obtained for each patient (see 'questionnaire information' section below) by a Clinical Research Nurse; data was validated using the patient's hospital records. Blood samples were obtained from each patient for the purpose of DNA extraction. Twenty four patients were excluded from our study to non-French-Canadian ancestry; one hundred and fifty patients (111 men and 39 women) were available for genotyping.

3) Controls

Controls used in theses studies were free of both IHD and stroke. Since the controls obtained from the participating Research Institutes mentioned above were subject to the same inclusion/exclusion criteria, they were pooled for a total of 176 controls (77 men and 99 women); the respective Research Ethics Committee's approval was obtained to allow the use of these controls in derived studies. This allowed to increase the number of controls that could be matched by ethnic background, age, and gender, as well as to increased statistical power to both studies. A total of 134 controls (77 men and 57 women) were used for the ischemic stroke study and a total of 113 controls (74 men and 39 women) were used for the IHD study; the rest were excluded due to the age-matching criteria.

4) Questionnaire information

The following lists and explains how the clinical data was recorded for both the stroke and the IHD cohorts, unless specified otherwise. *Family history of CVD* (IHD study only): at least one first-degree relative affected with any cardiac disorder. *Family history of ischemic stroke* (stroke study only): at least one first-degree relative affected with ischemic stroke. The *index event* is the event which brought the patient into the study: either stroke, IHD, or control. *Index event for IHD* cases: most severe IHD event by gradient: MI being the most severe, followed by MI non-Q, unstable angina, and stable angina being the least severe. *Index event for stroke* cases: most recent stroke. Since a TIA is not a stroke, we only recorded TIAs which had occurred before the event of a stroke or if only TIA has occurred (i.e. no stroke). *Multiple events* are recorded as such: for IHD cases: listing of the presence of other IHD events (according to severity gradient) which are less severe than the index event, if applicable. Only multiple MIs are recorded while any other IHD manifestations occurring more than once are only recorded one time. For stroke patients: chronological presence of other strokes and/or TIA occurring before the index event, if applicable. TIAs between strokes are not recorded, and multiple TIAs before a stroke are recorded only once, as a single event. *Study age*: age at which the individuals were entered into the study. *Age of onset*: for IHD cases: age at first IHD event; for stroke cases: age at first stroke or TIA. *Circulation* (stroke cases only): location of cerebral circulation affected by the event : anterior, posterior, or unknown. *First vascular event*: the patient's first vascular event of any kind. *Age of first*

vascular event: age at which the first vascular event of any kind occurred. *Other vascular diseases*: presence of vascular diseases other than the type of vascular disease in the index event (i.e. stroke or IHD). *Gender*: male or female. *Menopausal status*: pre-, post-, or menopausal. *Height* and *weight* for body mass index (*BMI*) measurements (stroke study only). *Obesity status* (IHD study only): must have been diagnosed as being obese by a doctor. *Diabetes status*: presence or absence of diabetes, diagnosed by a doctor. *Glucose level* (stroke study only): measured at time of entry into the study (non-fasting); normal range 3.3-6.4 mmol/L. *Dyslipidemia status*: presence of any dyslipidemia, including elevated cholesterol and/or triglycerides, diagnosed by a doctor or from the biochemical measurements (non-fasting) when entered into the study (stroke study only); desirable total cholesterol < 5.19 mmol/L, LDL-C < 3.37 mmol/L, and HDL-C > 1.1 mmol/L. *Fibrinogen levels* (stroke study only): measured at time of entry into the study; normal range: 1.84-4.15 g/L. *Smoking status*: smokers must be presently smoking or have stopped less than 3 months ago, non-smokers must have never smoked, and ex-smokers must have stopped more than 3 months ago. *HTN status*: presence or absence of HTN diagnosed by a doctor or by blood pressure measurement recorded at time of entry into the study; blood pressure over 140/90 is the definition of HTN.

B) Genetic analysis

Leukocyte DNA was extracted from 15 ml of venous blood, using a method described elsewhere (142), and stored at 4°C. All polymerase chain reactions (PCRs) used to detect polymorphisms were carried out with 100 ng of genomic DNA as a template, using a Perkin Elmer (9700) Thermal Cycler.

Angiotensin-converting enzyme (ACE)

Determination of the ACE I/D polymorphism was achieved in a total mix of 15μL containing 0.87X *Taq* buffer by Perkin Elmer (containing 15 mM $MgCl_2$), 130 μM deoxynucleotide triphosphates (dNTP), 0.23 μg/μL bovine serum albumin (BSA), 0.043 U/μL *Taq* DNA polymerase (Perkin Elmer), and 1.73 ng/μL of each primer; the sense primer was 5' CTGGAGACCACTCCCATCCTTTCT 3' and the antisense primer was 5' GATGTGGCCATCACATTCGTCAGAT 3'. Samples were amplified at 95°C for 1 minute (hot start), followed by 35 cycles at 95°C for 30 seconds, 57°C for 45 seconds, and 72°C for 1 minute; final extension at 72°C for 5 minutes. The

PCR products (490-bp I and 190-bp D) were run on a 2% agarose gel (Gibco) and visualized with ethidium bromide (EtBr) and ultra violet (UV) light.

Angiotensinogen (AGT)

Determination of the AGT M235T polymorphism was achieved in a total mix of 13.0 μL containing 1X *Taq* buffer, 200μM dNTP, 0.23 μg/μL of BSA, 0.07 U/μL *Taq* DNA polymerase, and 8 ng/μL of each primer; the sense primer was 5' CAGGGTGCTGTCCACACTGGACCCC 3' and the antisense primer was 5' CCGTTTGTGCAGGGCCTGGCTCTCT 3'. Cycling and digestion conditions have been previously described (143). Digested PCR products were separated on a 2.5% agarose gel and visualized by EtBr and UV light. Amplification yielded a product of 165 bp; the substitution of a T for a C at position 704 of the DNA creates a half site for the enzyme *Tth 111* I and therefore yielded fragments of 141 and 24 bp.

Angiotensin II type I receptor (AGT1R)

Determination of the AGT1R A1166C polymorphism was achieved using the same PCR mix as the one listed for the AGT polymorphism. The sense primer was 5' ATAATGTAAGCTCATCCACC 3' and the antisense primer was 5' GAGATTGCATTTCTGTCAGT 3'. PCR cycling conditions were: 35 cycles at 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 30 seconds. PCR products were digested overnight at 37°C with 0.24 U/μL of *Dde*I enzyme with 1X buffer #3 from New England Biolabs (NEB). Products were separated on a 2.0% agarose gel and visualized by EtBr and UV light. Amplification yielded a product of 350 bp; the polymorphism creates a restriction site for *Dde*I and digestion yielded fragments of 200 and 150 bp.

Factor V (fac. V)

Determination of the fac. V Leiden mutation was achieved using the same PCR mix as the one listed for the AGT polymorphism. Cycling and digestion conditions, as well as primer sequences, have been previously described (87). Products were separated on a 2.0% agarose gel and visualized by EtBr and UV light. Amplification yielded a product of 220 bp. Digestion of this product with the enzyme *Mnl*I yielded products of 37, 67, and 116 bp-fragments. The presence of the Leiden mutation causes the aberration of a cleavage site for the enzyme *Mnl*I and therefore yielded fragments of 67 and 153 bp.

Platelet glycoprotein IIIa (PGIIIa)

Determination of the PGIIIa PL^{A1} and PL^{A2} alleles was achieved using the same PCR mix as the one listed for the AGT polymorphism. The sense primer was 5' TTCTGATTGCTGGACTTCTCTT 3' and the antisense primer was 5' TCTCTCCCCATGGCAAAGAGT 3'. PCR was carried out at 94°C for 1 minute, followed by 35 cycles at 94°C for 45 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Amplification yielded a 266 bp-fragment. PCR products were digested overnight at 37°C with 0.5 U/μL of *Nci*I enzyme and 1X buffer #4 (NEB). The digested fragments were run out on a 2% agarose gel and visualized with EtBr and UV light. The PL^{A2} allele causes the addition of an extra restriction site for *Nci*I, resulting in fragments of 216 and 50 bp.

Plasminogen activator inhibitor-1 (PAI-1)

Identification of the PAI-1 4G/5G polymorphism was done in a total mix of 13.0 μL containing 1X *Taq* buffer, 192 μM dNTP-A, 154 μM [^{35}S-P]dATP, 25 μM dATP, 0.23 μg/μL of BSA, 0.07 U/μL *Taq* DNA polymerase, and 8 ng/μL of each primer; the sense primer was 5' CACCACCCCACCCAGCACACC 3' and the antisense primer was 5' GGCCGCCTCCGATGATACACG 3'. PCR was carried out at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 59°C for 40 seconds, and 72°C for 1 minute; final extension at 72°C for 10 minutes. Amplification yielded products of 122 bp (4G allele) and 123 bp (5G allele). These products were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

Apolipoprotein E (apo E)

Determination of the apo E alleles (ε2, ε3, and ε4) was done in a total mix of 25 uL containing 1X *Taq* buffer, 0.1 μg/μL of BSA, 0.07 U/μL *Taq* DNA polymerase, 200μM dNTP, 0.1% dimethyl sulfoxide (DMSO), and 6 ng/μL of each primer; the sense primer was 5' AGACGCGGGCACGGCTGTCCA 3' and the antisense primer was 5' CCCGCACGCGGCCCTGTTCC 3'. PCR was carried out at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute and annealing at 74°C for 45 seconds; final extension at 72°C for 5 minutes. PCR products were digested overnight at 37°C with 1 U/μL of *Cfo*I enzyme and 6X buffer REact 1 (Gibco). The resulting fragments were separated on a non-denaturing 12% polyacrylamide gel and visualized by EtBr staining and UV light. The ε2, ε3, and ε4 alleles yielded the following band patterns: 91 and 83 bp, 91 and 48 bp, 72 and 48 bp,

respectively.

Methylenetetrahydrofolate reductase (MTHFR)

Identification of the MTHFR thermolabile variant was achieved using the same PCR mix as the one listed for the AGT polymorphism. The sense primer was 5' TGAAGGAGAAGGTGTCTGCGGGA 3' and the antisense primer was 5' AGGACGGTGCGGTGAGAGTG 3'. PCR was carried out at 94°C for 3 minutes, followed by 30 cycles at 94°C for 15 seconds, 61°C for 15 seconds, 72°C for 30 seconds; final extension at 72°C for 5 minutes. Amplification yielded a 198 bp-fragment. PCR products were digested overnight at 37°C with 0.6 U/μL of *Hinf*I enzyme and 2X buffer #2 (NEB). The presence of the thermolabile variant (C to T change at the DNA level) creates a restriction site for the enzyme *Hinf*I and therefore generates fragments of 175 and 23 bp.

Stromelysin-1 (MMP-3)

Identification of the stromelysin-1 5A/6A polymorphism was achieved in a total mix of 25 μL which contained 0.8X *Taq* buffer, 160 μM dNTP-A, 160 μM [^{35}S-P]dATP, 20 μM dATP, 0.08 μg/μL of BSA, 0.03% DMSO, 0.08% Triton X-100, 0.1 U/μL *Taq* DNA polymerase, and 6.4 ng/μL of each primer; the sense primer was 5' CTCCTGCCTCAACCTCTCAAAG 3' and the antisense primer was 5' ATCACTGCCACCACTCTGTTCTC 3'. PCR was carried out at 94°C for 5 minutes, followed by 30 cycles at 94°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute; final extension at 72°C for 10 minutes. Amplification yielded products of 200 bp (5A allele) and 201 bp (6A allele). These products were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

C) Statistical Analysis

Data for the age variable are represented by means +/- the standard deviation (SD). The calculation of mean differences was done using an unpaired *t*-test. The frequencies for all other variables were estimated using the Pearson chi-square test; the Yates corrected chi-square test was used when the cell's expected values were low, therefore correcting for data which didn't follow the chi-square distribution. A P-value smaller than 0.05 was considered to be statistically significant. Analyses were also carried out by means of stepwise logistic regression in order to estimate the influence of the interaction of several variables in predicting the outcomes; stroke and

IHD were used as the dependent variables in these models. Correction for multiple testing was not performed in any of these analyses..

Part VI) Results

A) Demographics

1) Stroke study

Table # 1 gives the distribution of case and control patients with respect to selected variables. The case group was comprised of 97 stroke/TIA patients (67 men and 30 women) which were on average 63.2 years of age (+/- 9.6 years), while the control group was made up of 134 individuals (77 men and 57 women) which were on average 62.1 years of age (+/- 9.4 years); the average age of the cases was not significantly different from that of the controls ($P > 0.25$). The average age of the male cases did not significantly differ from that of the male controls ($P > 0.25$), nor did the average age of the female cases with respect to that of the female controls ($P > 0.05$). Although there is a greater proportion of males in the case group (69%), as opposed to the control group (57.5%), the two groups do not significantly differ from each other with respect to male gender ($P = 0.097$). As expected, most of the other variables which have been previously associated with the development of cerebrovascular disease, were more frequent in cases than in controls. This applies to smoking, HTN, diabetes, and dyslipidemia. This did not apply to family history of stroke where the presence of a positive family history of stroke did not differ between cases and controls ($p = 0.09$); only 97 of the 134 controls had information on family history of stroke.

Table # 1	Stroke study demographic table					
	cases (n)	cases years +/- SD	controls (n)	controls years +/- SD	t-test	P-value
Average age:						
All	97	63.2 +/- 9.6	134	62.1 +/- 9.4	0.683	> 0.25
Males	67	62.5 +/- 10	77	61.9 +/- 10.8	0.346	> 0.25
Females	30	67.7 +/- 8.4	57	64.9 +/- 7.8	1.569	> 0.05
Other variables:	cases (n)	cases (%)	controls (n)	controls (%)	$\chi 2$	P-value
Male gender	97	69	134	57.5	2.755	0.097
Family history of stroke	97	29.9	97	24.7	0.415	0.5193
Smoking	97	81.4	133	46.6	27.23	< 0.001
Hypertension	97	66	134	25.4	36.339	< 0.001
Diabetes	97	14.4	134	2	10.549	0.0012
Dyslipidemia	97	59.8	134	26.1	25.148	< 0.001

The prevalence of stroke types according to the index event is described in table # 2. In this cohort, thromboembolic stroke was the most frequent type of event (49.5%), followed by TIA only (39.1%), lacunar stroke (6.2%), and unknown type of stroke (5.2%). Within each stroke type (Type %), males had a greater frequency of that respective event than did females. Table # 3 describes the stroke cases irrespective of the index event. Individuals which have had multiple strokes of different types are represented here; this means that some cases have been counted more than once to depict the population in this manner. The most frequent type of stroke remains thromboembolic stroke (n = 51), followed by TIA only (n = 38); lacunar and unknown types of stroke have the same frequency (n = 7). Among cases, 11 individuals have had a TIA before a stroke of any kind. The incidence of a single cerebrovascular event was 68%, while that of 2 or 3 events was 27.8% and 4.2%, respectively; none of the cases had more than 3 cerebrovascular events.

Table # 2	Stroke cases			
Stroke type	Gender	Total (n)	**Total %**	**Type %**
Thrombo-	**M**	32	33	66.7
embolic	**F**	16	16.5	33.3
	Total	48	49.5	100
Lacunar	**M**	4	4.1	66.7
	F	2	2.1	33.3
	Total	6	6.2	100
TIA only	**M**	26	26.8	68.4
	F	12	12.3	31.6
	Total	38	39.1	100
Unknown	**M**	5	5.2	100
	F	0	0	0
	Total	5	5.2	100
Grand total		97	100	100

Table # 3	Stroke cases			
Stroke type	Males (n)	Females (n)	Total (n)	%
Thrombo-embolic	34	17	51	
Lacunar	5	2	7	
Unknown	6	1	7	
TIA only	26	12	38	
TIA before stroke	10	1	11	
1 event	43	23	66	68
2 events	22	5	27	27.8
3 events	2	2	4	4.2
4 events	0	0	0	0

2) Ischemic heart disease (IHD) study

The IHD cohort is represented in table # 4, with respect to selected variables. The cases (n = 150) were on average 64.97 years of age (+/- 8.2 years) and the controls (n = 113) were on average 64.1 years of age (+/- 8.7 years); these age groups did not significantly differ from each other ($P > 0.2$). The average age of the male cases (n = 111) was 64.3 years while that of the male controls (n = 74) was 62.95 years, and the average age of the female cases (n =39) was 66.9 years while that of the female controls (n =39) was 66.3 years. In both sexes, the average age did not significantly differ between cases and controls ($P > 0.15$ and $P > 0.25$, respectively). Moreover, the case and control groups did not differ from each other with respect to male gender ($P = 0.1738$). With regard to the other variables, cases had a greater

frequency of smoking, HTN, diabetes, and dyslipidemia, as would be expected. Positive family history of CVD and obesity did not differ between the case and control groups (P = 0.0978 and P = 0.3709, respectively); however, information on obesity was only available in 23 of the 113 controls.

Table # 4	Ischemic heart disease study: demographic table					
	cases (n)	cases years +/- SD	controls (n)	controls years +/- SD	*t*-test	P-value
Average age:						
All	150	64.97 +/- 8.2	113	64.1 +/- 8.7	0.65	> 0.2
Males	111	64.3 +/- 8.1	74	62.95 +/- 9.7	0.99	> 0.15
Females	39	66.9 +/- 8	39	66.3 +/- 6.1	0.155	> 0.25
Other variables:	cases (n)	cases (%)	controls (n)	Controls (%)	$\chi 2$	P-value
Male gender	150	74	113	65.5	1.85	0.1738
Family history of CVD	150	55.3	113	44.2	2.74	0.0978
Smoking	148	67.6	112	48.2	9.103	0.0026
Hypertension	150	54.7	113	24.8	22.449	< 0.001
Diabetes	150	21.3	113	2.7	17.904	< 0.001
Dyslipidemia	150	80	113	26.5	72.975	< 0.001
Obesity	148	7	23	0	0.801	0.3709

Table # 5 describes the IHD cases with respect to the index event. MI was the most frequent type of IHD which was observed (42%), followed by unstable angina (36%), MI non-Q (17.3%), and stable angina (4.7%). Within each IHD type (Type %), males had a greater frequency of that respective event than did females. Table # 6 describes the IHD cases, taking into account the occurrence of multiple MIs. Of these cases, 82.5% have had only 1 MI, while only 17.5% have had 2 or more MI events. Of the males, 80.4% (n = 41) have had only 1 MI, and of the females, 91.7% (n = 11) have had a single MI when compared to cases which have had 2 or more MI events.

Table # 5	Ischemic heart disease (IHD) cases			
IHD type	Gender	Total (n)	**Total %**	**Type %**
MI	M	51	34	81
	F	12	8	19
	Total	63	42	100
MI non-Q	M	18	12	69.2
	F	8	5.3	30.8
	Total	26	17.3	100
Unstable	M	35	23.3	64.5
angina	F	19	12.7	35.5
	Total	54	36	100
Stable	M	7	4.7	100
angina	F	0	0	0
	Total	7	4.7	100
Grand total		150	100	100

Table # 6	Ischemic heart disease (IHD) cases			
IHD type	Males (n)	Females (n)	Total (n)	%
Only 1 MI	41	11	52	82.5
Multiple MIs	10	1	11	17.5

B) Genes

In the following section, the distribution of genotype frequencies between cases and controls, for both the stroke and IHD studies, are shown with respect to each gene (and respective polymorphism) investigated. Allele frequencies were not calculated for comparison of distributions due to the issue of multiple testing; many tests need to be performed with these complex traits, due to a large number of variables implicated, and genotypes provide the appropriate insight into the biological relevance of the polymorphisms with respect to the disease outcome. Odds ratio were also not calculated, due to the multiple testing issues mentioned above; chi-square values gives a good estimate of the differences in genotype distribution between cases and controls with respect to the disease outcome. This section also includes the results of a more stringent analysis of these genotype distributions with respect to selected variables. These variables are: family history of IHD/stroke, smoking (IHD study only), obesity (IHD study only), gender, HTN, diabetes (IHD study only), and dyslipidemia.

1) Angiotensinogen (AGT)

Table # 7 and # 8 give the distribution of AGT M235T genotypes frequencies in cases and controls for the stroke and IHD study, respectively. In both studies, there was no significant difference in genotype frequencies between cases and controls. Further analysis by stratification for selected variables did not reveal any differences in genotype frequencies (data not shown).

Table # 7 **Stroke study**						
Angiotensinogen (M235T variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
MM	37	38.1	50	37.3		
MT	51	52.6	62	46.3		
TT	9	9.3	22	16.4		
Total	97	100	134	100	2.605	0.2718

Table # 8 **Ischemic heart disease study**						
Angiotensinogen (M235T variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
MM	56	37.3	40	35.4		
MT	61	40.7	53	46.9		
TT	33	22	20	17.7		
Total	150	100	113	100	1.236	0.5391

2) Angiotensin-converting enzyme (ACE)

The genotype frequencies of the ACE I/D polymorphism, in cases and controls of the stroke and IHD studies, are represented in tables # 9 and 10, respectively. No significant difference was observed between the case and control groups, in either study, with respect to genotype distribution. Analysis by stratification for selected variables did not reveal any differences in genotype frequencies (data not shown).

Table # 9 **Stroke study**						
Angiotensin-converting enzyme (287 bp I/D)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	20	20.8	21	15.7		
ID	45	46.9	63	47		
DD	31	32.3	50	37.3		
Total	96	100	134	100	1.237	0.5388

Table # 10 **Ischemic heart disease study**						
Angiotensin-converting enzyme (287 bp I/D)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	18	12	16	14.2		
ID	71	47.3	55	48.7		
DD	61	40.7	42	37.1		
Total	150	100	113	100	0.458	0.7953

3) Angiotensin II type I receptor (AGT1R)

The frequency of the genotype distribution of the AGT1R A1166C variant between cases and controls of the stroke and IHD studies are represented in tables # 11 and 12, respectively. No significant difference was observed between these two groups, in either study. Analysis controlling for the selected variables mentioned above did not identify any differences in genotype frequencies (data not shown).

Table # 11 **Stroke study**						
Angiotensin II type I receptor (A1166C variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
AA	41	42.3	58	43.3		
AC	48	49.5	61	45.5		
CC	8	8.2	15	11.2		
Total	97	100	134	100	0.691	0.7077

Table # 12 **Ischemic heart disease study**						
Angiotensin II type I receptor (A1166C variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
AA	62	41.3	48	42.5		
AC	69	46	54	47.8		
CC	19	12.7	11	9.7		
Total	150	100	113	100	0.55	0.7596

4) Factor V (fac. V)

Frequencies of the coagulating fac. V Leiden mutation, in cases and controls of the stroke and IHD studies, are shown in tables # 13 and 14, respectively. The genotype distribution between the case and control groups, in both studies, was nearly identical (P = 1). Data stratification, according to the selected variables mentioned above, failed to identify any differences in genotype frequencies between the case and control groups, in either studies (data not shown).

Table # 13 **Stroke study**						
Factor V (G1691A variant: Leiden mutation)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
GG	93	96.9	129	96.3		
GA	3	3.1	5	3.7		
AA	0	0	0	0		
Total	96	100	134	100	0	1

Table # 14 **Ischemic heart disease study**						
Factor V (G1691A variant: Leiden mutation)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
GG	145	96.7	109	96.5		
GA	5	3.3	4	3.5		
AA	0	0	0	0		
Total	150	100	113	100	0	1

5) Platelet glycoprotein IIIa (PGIIIa)

Tables # 15 and 16 show the frequency of genotype distribution of the PGIIIa PL^{A1}/PL^{A2} polymorphism between cases and controls, for both the stroke and IHD studies respectively. There was no significant difference observed between the case and control groups for the stroke study (P = 0.5955). However, there was a trend showing a difference in the genotype frequency distribution for this gene polymorphism, between cases and controls of the IHD study, although it was not statistically significant (P = 0.0507). The PL^{A1}/PL^{A1} genotype was found to be more frequent among the case group of the IHD study (P = 0.0147), while the PL^{A1}/PL^{A2} genotype was found to be more frequent among control group (P < 0.05). Upon further investigation, controlling for the selected IHD risk factors mentioned previously, the genotype distribution of the PGIIIa PL^{A1}/PL^{A2} polymorphism was found to be significantly different between the case and control groups with respect to male gender, HTN, and dyslipidemia (data not shown). Among males, 111 cases and 74 controls, the frequency of the PGIIIa PL^{A1}/PL^{A2} variant was significant at the P = 0.0081 level. We find that the PL^{A1}/PL^{A1} genotype in more frequent among male cases (P = 0.0033), while the PL^{A1}/PL^{A2} genotype is more frequent among male controls (P = 0.0079); no association was found with the PL^{A2}/PL^{A2} genotype according to gender. Among 82 cases and 28 controls with hypertension, the genotype frequency of the PGIIIa PL^{A1}/PL^{A2} variant was observed to be significantly different (P = 0.0037). We observed a trend showing that the PL^{A1}/PL^{A1} genotype was more

frequent among hypertensive cases than controls, although this was not significant (P = 0.0502). We also observer that the PL^{A2}/PL^{A2} genotype was more frequent among hypertensive controls than cases (P = 0.0196). Among non-hypertensives, the PL^{A1}/PL^{A2} genotype was found to be in greater frequency in controls than in cases (P = 0.0424). The genotype frequency was also found to be significantly different between 120 cases and 30 controls which have dyslipidemia (P = 0.0164). We found that the PL^{A1}/PL^{A1} genotype to be in greater frequency among dyslipidemic cases than controls (P = 0.0351), while that the PL^{A1}/PL^{A2} genotype is more frequent in dyslipidemic controls than cases (P = 0.0111).

Table # 15 **Stroke study**						
Platelet glycoprotein IIIa (T1565C variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
A1A1	69	7.9	88	65.6		
A1A2	23	24	40	29.9		
A2A2	4	4.1	6	4.5		
Total	96	100	134	100	1.037	0.5955

Table # 16 **Ischemic heart disease study**						
Platelet glycoprotein IIIa (T1565C variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
A1A1	115	76.7	71	62.8	5.95	0.0147
A1A2	31	20.7	37	32.7	4.92	< 0.05
A2A2	4	2.6	5	4.5		
Total	150	100	113	100	5.962	0.0507

6) Plasminogen activator inhibitor-1 (PAI-1)

Tables # 17 and 18 show the frequency of the genotype distribution of the PAI-1 I/D polymorphism in cases and controls of the stroke and IHD studies, respectively. No significant difference was found between the case and control groups in either study. No other significant differences were found upon stratification according to the selected variables previously listed (data not shown).

Table # 17 **Stroke study**						
Plasminogen activator inhibitor-1 (I/D at -675 bp)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	20	22.6	32	23.9		
ID	50	48.7	62	46.2		
DD	26	28.7	40	29.9		
Total	96	100	134	100	0.767	0.6814

Table # 18 **Ischemic heart disease study**						
Plasminogen activator inhibitor-1 (I/D at -675 bp)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	34	22.7	27	23.9		
ID	72	48	52	46		
DD	44	29.3	34	30.1		
Total	150	100	113	100	0.108	0.9475

7) Apolipoprotein E (apo E)

The frequency distribution of the apo E isoforms between cases and controls for the stroke and IHD studies are shown in table # 19 and 20. respectively. No significant difference in genotype frequencies was observed between the case and control groups of these two studies. Further investigation found that, in the IHD study, the apo E isoforms differed in genotype frequencies between diabetic cases (n = 32) and controls (n = 3) (P = 0.0307). No other differences in frequency were observed (data not shown).

Table # 19 **Stroke study**						
Apolipoprotein E (E2, E3, and E4 isoforms)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
E2E2	1	1	2	1.5		
E2E3	10	10.3	19	14.3		
E2E4	2	2.1	1	0.8		
E3E3	68	70.1	88	66.2		
E3E4	15	15.5	23	17.2		
E4E4	1	1	0	0		
Total	97	100	133	100	3.15	0.6768

Table # 20 **Ischemic heart disease study**						
Apolipoprotein E (E2, E3, and E4 isoforms)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
E2E2	2	1.3	2	1.8		
E2E3	14	9.4	12	10.7		
E2E4	2	1.3	1	0.9		
E3E3	93	62	78	69.6		
E3E4	37	24.7	19	17		
E4E4	2	1.3	0	0		
Total	150	100	112	100	5.492	0.4824

8) Methylenetetrahydrofolate reductase (MTHFR)

Tables # 21 and 22 represent the frequency of genotype distribution for the MTHFR C677T variant between cases and controls of the stroke and IHD study, respectively. Differences in genotype distribution between cases and controls, in either studies, were not observed. Upon stratification of the data, according to the selected variable listed above, the only difference in genotype frequencies which was observed was between diabetic cases (n = 32) and controls (n = 3) of the IHD study (P = 0.01) (data not shown).

Table # 21 **Stroke study**						
Methylenetetrahydrofolate reductase (C677T variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
TT	16	16.6	18	13.5		
TC	45	46.9	65	48.9		
CC	35	36.5	50	37.6		
Total	96	100	133	100	0.434	0.8048

Table # 22 **Ischemic heart disease study**						
Methylenetetrahydrofolate reductase (C677T variant)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
TT	21	14	18	16.1		
TC	70	46.7	57	50.9		
CC	59	39.3	37	33		
Total	150	100	113	100	2.446	0.4851

9) Stromelysin-1

The frequency of the stromelysin-1 I/D polymorphism genotype distribution between cases and controls of the stroke and IHD studies are represented in tables # 23 and 24, respectively. We observed a difference in genotype distribution between the case and control groups of the stroke study (P = 0.0236). We find that the I/D genotype is significantly more frequent in stroke cases than controls (P = 0.0119). We also observe a trend showing that the I/I genotype is more frequent among controls than stroke cases, although this was not significant (P = 0.0504). After stratification for the selected variables listed previously, it was determined that, in the stroke study, there was a significant difference in genotype frequency between 67 cases and 72 controls with no family history of stroke (P = 0.0288) (data not shown). Data also revealed that, in the IHD study, the stromelysin-1 genotypes differed between the case (n = 32) and control (n = 3) groups which were found to have diabetes (P = 0.0022). No other differences were observed (data not shown).

Table # 23 **Stroke study**						
Stromelysin-1 (I/D at -1171 bp)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	19	19.8	43	32.3	3.828	0.0504
ID	56	58.3	54	40.6	6.331	0.0119
DD	21	21.9	36	27.1	0.55	0.4582
Total	96	100	133	100	7.491	0.0236

Table # 24 **Ischemic heart disease study**						
Stromelysin-1 (I/D at -1171 bp)						
Genotypes	Cases (n)	Cases (%)	Controls (n)	Controls (%)	$\chi 2$	P-value
II	41	27.3	37	33		
ID	68	45.4	46	41.1		
DD	41	27.3	29	25.9		
Total	150	100	112	100	2.349	0.5032

C) Logistic regression analyses

The following section lists the results of the logistic regression analyses which were done for both the stroke and IHD studies. Only patients for which all data was available for all variables were used in this part of the analysis. These variables were taken into account for the models in both studies (unless specified otherwise): family history of IHD/stroke, smoking, diabetes, gender, age, HTN, dyslipidemia, obesity (IHD only), as well as all the genotypes for the polymorphisms (listed previously) in the genes of AGT, ACE, AGT1R, fac. V, PGIIIa, PAI-1, apo E, MTHFR, and stromelysin-1. For each variable which is retained in the final model, there is a corresponding coefficient which is the multiplicative factor of that variable in the overall equation used to predict the outcome (stroke or IHD). Negative coefficients indicate that the presence of that variable decreases the chance of being a success event in this model; the opposite is true for positive coefficients. The Goodness of Fit chi-square is used as a measure of how well the data fits the given model; therefore, the larger the P-value, the better the data fits the model.

1) Stroke study

Ninety-five stroke cases and 95 controls were used in this part of the analysis. Table # 25 describes the final results of the logistic regression analysis for the stroke study. In this study, the model used the 'control' status as the success event. This means that estimated values for the variables entered into the models are calculated are in terms of increasing/decreasing the chances of being a control. The variables

which remained predictive of stroke in the final model, and their coefficients, are: HTN (-1.523), dyslipidemia (-1.135), age (2.86), and smoking (-0.8659). In this model, the presence of HTN, dyslipidemia, and smoking decrease the chances of being a control. The age variable has been stratified into 'presently young' (< 53 years) and 'presently old' (≥ 53 years); 53 years is the means age of the stroke cohort used for this analysis, minus 1 SD. The coefficient of the age variable indicates that being 'presently old' increases the chances of being a control. The Goodness of Fit chi-square for this model is P = 0.225, indicating that the data is a good fit for this model.

Table # 25	Stroke study- Logistic regression		
Cases (n)	**Controls (n)**		
95	95		
Variables	**Coefficient**	**Standard error**	**Goodness of fit P-value**
Smoking	-0.8659	0.378	
Age	2.86	1.11	
HTN	-1.523	0.347	
Dyslipidemia	-1.135	0.35	
Constant	-0.8974	1.13	0.225

2) Ischemic heart disease (IHD) study

Table # 26 describes the results of the logistic regression analysis for the IHD study. One hundred and forty-eight IHD cases and 112 controls were used in this part of the analysis. In this study, the model used the 'case' status as the success event. This means that estimated values for the variables entered into the models are calculated are in terms of increasing/decreasing the chances of being a case. The variables which remain predictive of IHD, and their corresponding coefficients, are: smoking (0.7503), PGIIIa (0.8989), HTN, (0.8143), diabetes (2.016), and dyslipidemia (2.318). In this model, the genotype PL^{A1}/PL^{A1} is found to increase the chances of being a case. The Goodness of Fit chi-square for this model is P = 0.3, indicating that the data is a good fit for this model.

Table # 26	Ischemic heart disease study- Logistic regression		
Cases (n) 148	Controls (n) 112		
Variables	Coefficient	Standard error	Goodness of fit P-value
Smoking	0.7503	0.326	
PGIIIa PL^{A1}/PL^{A1}	0.8989	0.336	
HTN	0.8143	0.338	
Diabetes	2.016	0.802	
Dyslipidemia	2.318	0.328	
Constant	-2.538	0.448	0.3

Part VII) Discussion

These association studies were conducted in order to identify genetic risk factors predisposing to stroke and/or IHD, using a founder population: the French-Canadians. We studied known polymorphisms, which have previously been implicated in the development of stroke and/or IHD, in the following genes: AGT (M235T variant), ACE (287 bp I/D), AGT1R (A1166C variant), fac. V (Leiden mutation), PGIIIa (PL^{A1}/PL^{A2} alleles), PAI-1 (4G/5G variant), apo E (E2, E3, and E4 isoforms), MTHFR (C677T variant), and stromelysin-1 (5A/6A variant). We also assessed the role of the following risk factors for stroke and/or IHD in both the stroke and IHD cohorts (unless specified otherwise): age, gender, diabetes, dyslipidemia, HTN, smoking, family history of stroke/IHD, and obesity (IHD study only).

The stroke study was comprised of 97 French-Canadian stroke/TIA cases and 134 age and sex matched French-Canadian controls, who were free of stroke and IHD. The average age of the cases (63.2 years) was not significantly different from that of the controls (62.1 years) ($P > 0.25$), and the presence of male gender was not significantly different between the two groups ($P = 0.097$). The control group was not matched for any other stroke risk factors since imposing a too stringent definition of the control group would have greatly limited the number of controls available for enrollement into the study; this would in turn have reduced the statistical power to detect any differences between the case and control groups. We instead chose to record the presence/absence of other vascular risk factors (see 'Materials and

methods' for details), and control for these in the analysis. As expected, the initial analysis revealed that the presence of smoking, HTN, diabetes, and dyslipidemia, established risk factors for stroke, were more frequent in cases than controls. From the logistic regression analysis, smoking, HTN, age ('presently old'), and dyslipidemia were retained in the model, indicating that their effect on the outcome of stroke remained significant in the presence of other variables. The fact that diabetes was not retained in this model suggests that its effect on the development of stroke is less important in this population. From this analysis, we observe that being 'presently old' increases the chances of being a control (coefficient = 2.86). This is simply an artifact of the way the controls were recruited into the study. We targeted controls which were older than our cases to assure that they would be past the age of disease onset of the cases. These analyses reconfirmed our assumptions that the case group which we had collected was in fact representative of the 'at risk' population for stroke; the risk factors for stroke are expected to be higher in individuals which have the disease than those who don't (the control group). Vice versa, this also served to reconfirm that our control population was representative of individuals who are free of cerebrovascular disease.

We also found that the presence of a positive family history of stroke was not significantly different between cases and controls. Although some studies have found an association between parental history of stroke and presence of stroke in the offspring (23, 25), the role of family history in the development of stroke remains ambiguous. The development of stroke implicates the interaction of many genetic and environmental factors; possibly, the required combination of these factors for stroke may be difficult to observe from generation to generation. This could explain the lack of a positive association between family history of stroke and stroke in our study. It is also noteworthy that information on family history of stroke was only available in 97 of the 134 controls; this might have, by chance, biased the results of the analysis by excluding controls which had a negative family history of stroke. It has also been suggested that study design may be responsible for the reported inconsistencies on the role of familial aggregation of stroke (144). One problem lies in the different definition given to positive family history of stroke. Another problem is the heterogeneous nature of stroke, and the fact that the definition of stroke may differ between studies; different stroke types are thought to have different pathophysiologies

and therefore different pathogenesis. Although our case group consists of individuals who have cerebrovascular disease, not all have experienced the same type of stroke. Irrespective of the index event, our cases consists of 51 thromboembolic strokes, 7 lacunar strokes, 7 strokes of unknown type, and 38 individuals which have only experienced TIAs. This mixture of stroke types may very well contribute to the lack of association between stroke and family history. A larger case group may be needed in order to explore the role of family history with respect to stroke type.

In the IHD study, the case group consisted of 150 French-Canadian IHD patients while the control group consisted of 113 French-Canadians, who were free of IHD and stroke. The controls were matched for ethnic background, age, and gender; they were not matched for any other vascular risk factors for the same reasons mentioned above. The average age of the case group (64.97 years) did not differ from that of the control group (64.1 years) ($P > 0.2$), and the presence of male gender was not significantly different between the two groups ($P = 0.1738$). In the initial analysis, we found that the presence of smoking, HTN, diabetes, and dyslipidemia were more frequent in cases than in controls; this was expected since these are considered to be 'traditional' risk factors for IHD. From the logistic regression analysis, we observe that all these variables still have a significant effect on the development of IHD in the presence of each other. This helped to establish that our case group was representative of the 'at risk' population for IHD. It was expected that these risk factors be less frequent in the control group, which represents individuals who are free of IHD. We found that the presence of a positive family history of CVD was not significantly different between the two groups ($P = 0.0978$). Although the role of family history in the development of IHD has been listed as risk factor for IHD in many studies (18, 19), this issue still remains controversial (145). The basis of familial aggregation of IHD is the aggregation of IHD risk factors, both genetic and environmental. As with the development of stroke, the complex nature of IHD may make family history a poor independent risk factor. The lack of association between family history of CVD and our IHD cases may, on the one hand, lie in the definition of family history. We looked at family history of CVD and not IHD, which might have biased the analysis. The control group may be comprised of a large number of individuals which have a family history of heart disease which is not ischemic in nature, therefore masking a possible association. Moreover, the case group is not homogeneous with respect to

IHD sub-type. Out of the 150 IHD cases, 42% have had an MI, while 4.7% have only experience episodes of stable angina. This variability might also bias the identification of an association of family history with the case group in this study.

Genes

Renin-angiotensin system (RAS): angiotensinogen (AGT), angiotensin-converting enzyme (ACE), and angiotensin II type I receptor (AGT1R)

RAS plays an essential role in the regulation of blood pressure. AGT is the precursor to Ang. I in the RAS pathway. A known polymorphism in the AGT gene, the M235T variant, results due to a base substitution (T704C) in exon 2 of this gene (52). This variant has been linked to HTN and associated with increased circulation of AGT in hypertensives (53). It has also been associated, in some studies, with increased risk of IHD and cerebral infarction (54, 57). In our studies, there was no significant difference in genotype distribution between cases and controls, with respect to this variant. Even among hypertensives, no relationship was observed between the M235T variant of this gene and the development of stroke and/or IHD. The effect of this variant on the RAS and in the development of atherosclerotic diseases, if any, remains to be elucidated. This polymorphism is not in the renin cleavage site and may only serve as a marker for another functional polymorphism, which would affect the local availability of AGT and therefore alter the local production of Ang. II. This in turn could effect the vasculature and lead to a diseased state.

ACE is the enzyme which converts Ang. I to Ang. II in the RAS pathway. Ang. II is a potent vasoconstrictor and proliferator of VSMC and ECM, changes which are involved in the development of atherosclerosis. Inhibition of NO, an endothelium vasorelaxant, and increased production of Ang. II have both been associated with increases in plasma ACE (62). The 287 bp I/D polymorphism in the ACE gene, situated at the 3'end of intron 16, has been associated with variations in ACE plasma levels. Although it is thought that this polymorphism is probably not responsible for the changes in circulating levels of ACE, but instead linked to another functional variant, it has merited great attention as a potential marker of vascular disease. The DD genotype of this gene has been implicated in different aspects of vascular disease, including the development of MI and stroke (65, 70). In our studies,

no association was found between the ACE genotypes and the development of stroke and/or IHD. While some studies have reported associations in cases which were defined as 'low-risk' (64), we report no association with this ACE polymorphism and patients which can be considered as 'low-risk': non-diabetics, non-dyslipidemics, non-smokers, and non-hypertensives. It is possible that ACE may not be involved in the development of early atherosclerosis, but may only be involved in thrombus formation. This hypothesis is supported by a study which reports that in healthy 'low-risk' individuals, especially women, the ACE DD genotype is associated with significantly higher levels of PAI-1 (146). In vitro and in vivo studies have also demonstrated that Ang. II acts to increase PAI-1 levels and plasma concentration (147, 148). We have not measured the plasma levels of PAI-1 in the present cohorts, but are considering doing so in a new collection of patients. We have also not tested for the possible interactions between this ACE variant and the I/D polymorphism of the PAI-1 gene. This may help to understand the link between the RAS and the hemostasis system in the French-Canadian population.

In the RAS pathway, Ang. II mediates most of its effects through the AT1, which is coded by the AGT1R gene. The A1166C variant is a known polymorphism of this gene, and is situated at the 5'end of the 3'untranslated region. In some studies, this polymorphism has been associated with arterial stenosis and increased risk of MI (78, 79). In both our stroke and IHD studies, we observe no associations between this polymorphism and the development of stroke and/or IHD. The influence of the AGT1R A1166C polymorphism on vascular structural alterations, as well as its role in the pathology of vascular diseases, have not yet been confirmed. It may be that this polymorphism is not functional in the development of vascular diseases, but is in linkage disequilibrium with a yet unidentified functional variant of this gene.

In these studies, as in others, it is not yet clear if and how the above polymorphisms affect the RAS and, consequently, the development of stroke and/or IHD. Although some studies find an association between these individual polymorphisms and the development of atherosclerotic diseases, many studies, such as ours, do not (55, 66, 77). Though the results of our studies suggest that these polymorphisms are not involved in the development of stroke and IHD in French-Canadians, other explanations can justify these findings. Do to small sample sizes, we

were unable to stratify our cohorts according to specific disease sub-type. Most associations to these polymorphisms are reported to exist with regard to a specific manifestation of stroke and/or IHD. Moreover, we did not test for interactions between these variants in relation to disease outcome. It has been reported in some studies that a synergistic effect exists between the ACE DD genotype and either the AGT M235T variant or the AGT1R A1166C variant, influencing the development of atherosclerosis (57, 80). It may be that many functional variants from the RAS are needed in order to detect an effect on the development of stroke, IHD, or other vascular diseases. It may also be that these polymorphisms manifest themselves only at a specific stage in the development of stroke and IHD, a disease stage which might not be represented by our cohorts. It has been suggested that changes in the RAS contribute to increase the susceptibility of thrombus formation and are not involved in the early development of atherosclerosis. Segregation of the stroke and IHD cases according to thrombotic outcomes may prove fruitful in elucidating the effect of RAS, and these variants, in the etiology of these disorders.

The hemostasis system: factor V (fac. V), platelet glycoprotein IIIa (PGIIIa), and plasminogen activator inhibitor-1 (PAI-1)

Fac. Va plays a crucial role in the blood coagulating cascade by helping fac. Xa in activation of thrombin, the second to last step in the formation of a blood clot. A common mutation in the fac V gene, the Leiden mutation, results in the alteration of the binding site for APC, its natural inhibitor; this resistance to APC has been found to be the major cause of DVT (82). Even though small and large vessel disease are thought to be different, there have been many studies looking at the potential influence of this mutation on the development of arterial diseases. Some studies report that the Leiden mutation may be involved in the development of large vessel disease, but most have not found any associations (84-87). Our studies confirms previous reports of a lack of association between the Leiden mutation of the fac. V gene and the development of stroke and IHD. Although it could be concluded that this mutation is not a risk factor for stroke and/or IHD in French-Canadians, this might be an overstatement. As suggested by other studies, this mutation may play an important role in the development of large vessel disease in the young (149), and patients which experience advanced atherosclerosis may therefore not survive to old age. Since our stroke and IHD cohorts are on average 63.2 and 64.97 years of age, respectively, this

would explain the nearly identical distribution of this mutation among the case and control group, as well as the absence of any individuals which are homozygote for this mutation. This theory is reinforce by a study which reports that the Leiden mutation is not a risk factor for large vessel disease in a cohort of elderly patients which includes many angina and TIA cases (150). These sub-types of IHD and stroke, respectively, have less severe manifestations of atherosclerosis and thus patients may survive to older age. The fact that no homozygotes for Leiden were observed in the study's cohort also supports this hypothesis. Another theory is that since some individuals with the Leiden mutation remain free of DVT, other risk factors may be needed in order to observe the effects of this mutation on DVT; this may also be true for IHD and stroke. The recruitment of larger cohorts will permit a more segregated analysis with respect to IHD and stroke risk factors.

PGIIIa is part of the fibrinogen receptor on the platelet surface. The binding to fibrinogen is an indispensable step in the platelet aggregation mechanism and therefore in the formation of a blood clot. The PL^{A1} and PL^{A2} alleles of the PGIIIa gene code for different protein structures, and have been investigated for their possible involvement in vascular diseases. The PL^{A2} allele has been associated with an increased risk of IHD and stroke (94, 102). In our stroke study, we find no association between this PGIIIa polymorphism and stroke. Although these results suggest that this variant may not be involved in the development of stroke in French-Canadians, we have not yet conducted an analysis stratified by stroke type due to small sample size. Since this protein is involved in the formation of a blood clot, the influence of this variant on stroke may be more evident in outcomes which result due to thrombotic consequences, such as thromboembolic stroke.

We find that the PL^{A1}/PL^{A1} genotype is associated with increased risk of IHD ($P = 0.0147$), while the PL^{A1}/PL^{A2} genotype is protective against IHD ($P < 0.05$). From the logistic regression analysis, the PL^{A1}/ PL^{A1} genotype was entered into the model and remained significant. Therefore, when controlling for other variables, PGIIIa still had a significant effect on the development of IHD (coefficient = 0.8989, SE = 0.366). These results are contrary to what has been previously reported in the literature. While some studies report an association between the development of atherosclerotic diseases and the PL^{A2} allele, some report that this polymorphism does

not confer any increased risk for these disease (84, 95). To date, there have been no reports implicating the PL^{A1} allele in IHD. Although this polymorphism causes a change at the amino acid level, which leads to a different tertiary structure of the glycoprotein, the two variants are antigenically distinguishable and no functional variance has yet been attributed to this polymorphism (151). A possible explanation for the contrasting result between our study and that of others is that the PL^{A1} and PL^{A2} alleles may each be in linkage disequilibrium with another yet unidentified functional variant present in the French-Canadian population. Differences in ethnic background have previously been discussed as a reason for the discrepancies between studies. Differences in gene frequencies, with respect to different ethnic backgrounds, have been reported for PL^{A2} (152, 153). Another plausible explanation for these results is that the function of this polymorphism is affected by another interacting gene product. A functional variant in one of the many genes involved in blood clotting, such as PGIIb (the other part of the fibrinogen receptor) or the fibrinogen molecule itself, could counteract the effects of this PGIIIa polymorphism, in the French-Canadian population. Finally, these results could be insignificant and have occurred due to small sample size. Under- and over-sampling of the PL^{A2} and PL^{A1} alleles in the control and case groups, respectively, could have biased the results. Although we will attempt to replicate our results in a larger cohort, an in depth investigation on the possible function of this PGIIIa polymorphism will be required to better understand the role of this gene in vascular disease in many different populations. An investigation of the potentially different binding efficiencies of PGIIIa product variants, with respect to different environments, might help us to better understand platelet function in blood coagulation and thrombus formation.

Many different associations were observed between cases and controls in our IHD study. We observed a susceptibility effect of the PL^{A1}/PL^{A1} genotype with respect to the presence of male gender, HTN, and dyslipidemia. We also find a protective effect of the PL^{A1}/PL^{A2} genotype with respect to male gender and dyslipidemia, while the presence of both PL^{A2} alleles was found to have a protective effect in hypertensives. Due to the small sizes of these sub-groups, and the complex nature of these associations, we will try to reproduce these results in a larger cohort before evaluating the biological significance of these findings.

PAI-1 is an inhibitor of the fibrinolytic system. An I/D polymorphism in the PAI-1 gene results in a run of either 5 or 4 guanines; allele 5G and allele 4G, respectively. The 4G allele causes the abolition of a transcriptional repressor binding site, and has been associated with increases in basal levels of PAI-1; it has also been associated with the development of CVD (106). In both our stroke and IHD studies, we report no association between this PAI-1 polymorphism and the development of these diseases. Since this protein functions to inhibit blood clot dissolution, this variant could play a role in the development of thrombosis and not early atherosclerosis. As one study suggests, the problem with this association may lie in the definition of specific IHD manifestations, such as MI (154). These authors suggest that the 4G allele seems to be associated with thrombosis, and not with the development of atherosclerosis, therefore different types of MI should be considered. Since our cohorts were not stratified according to different manifestations of stroke or IHD, it is possible that these cohorts represent a mix of both early and late stages of atherosclerosis, therefore potentially masking an association. Another plausible explanation for this lack of association is that this polymorphism acts to influence plasma levels of PAI-1, but is either not a genetic risk factor for IHD and stroke, or requires the interactions of other genetic or environmental risk factors to significantly contribute to the disease outcome. Although increased levels of PAI-1 are observed in CVD patients, these findings must be interpreted with caution due to the acute phase nature of the PAI-1 protein (155). With respect to stroke, the authors of one study also suggest that this lack of association may partly be attributed to the undefined influences of PAI-1 at the time of acute stroke (112). More in depth knowledge on the normal response of this protein in vascular disease state is required in order to better understand the role of this polymorphism in the etiology of stroke and IHD.

Fat metabolism: apolipoprotein E (apo E)

Apo E serves as receptor ligand for various lipoprotein particles. The apo E gene codes for 3 isoforms, E2, E3, and E4. While the apo E4 isoform has been shown to increase susceptibility to IHD in some studies (115), a consensus on the role of these three isoforms in the development of stroke has yet to be reached. Although we do not observe an association between these apo E isoforms and the case and control groups of either studies when looking at these groups as a whole, we do find a significant difference in apo E genotype distribution between diabetic cases and

controls of the IHD study (P = 0.0307). However, these results should be interpreted with caution since the case group is relatively small (n = 32), and the control group is comprised of only 3 individuals. An attempt to replicate this result in a larger cohort is in progress.

The involvement of these apo E isoforms in the development of stroke and IHD may, on the one hand, lie in the efficiency of cholesterol clearance from circulation. The apo ε2 allele is associated with lower levels of LDL than apo ε3, due to its low receptor affinity which leads to up-regulation of LDL receptors (113). The apo ε4 allele is associated with higher levels of LDL than apo ε3, due to its high receptor affinity which leads to receptor down-regulation; this results in an accumulation of plasma IDL, and thus an increased conversion of IDL to LDL which accumulates in the plasma. On the other hand, macrophage-derived apo E and its role in the reversal of cholesterol transport may also play a role in the disease process. In a study by Cullen et al., data has shown that in normal macrophage the secretion of apo E differs between genotypes: E3/3 secretes 77% and 30% more than E2/2 and E4/4 respectively (156). This variance in secretion has no effect on cholesterol homeostasis. When the cells become exposed to acetylated LDLs, not only is the secretion of apo E altered (most increase in E4/E4 and least in E2/E2) but the effectiveness in cholesterol efflux also varies. E2/2 macrophage are shown to be more efficient at disposing of cholesterol; this may be due in part to apo E2 resistance to reuptake due to its low affinity for its receptor. In E3/3 macrophage, the accumulation of cholesterol is less, due to a high basal expression of apo E. Lastly, E4/4 macrophage secrete the most apo E but, due to enhanced binding and reuptake of cholesterol, have the least effective net efflux of cholesterol. The increase in macrophage derived apo E which occurs in response to altered LDL particles may represent a mechanism to facilitate cholesterol removal during atherogenesis; some variants of apo E may prove to be more effective in this process. It may be that the ε2 and ε4 alleles of apo E play atherogenic roles through different mechanisms, at different periods of the development of the disease. In order to observe this effect, larger cohorts with a more segregated analysis with respect to stroke and IHD manifestations and risk factors will be required.

Homocysteine metabolism: methylenetetrahydrofolate reductase (MTHF R)

The enzyme MTHFR plays an important role in the remethylation of Hcy in the cell. A common mutation in the MTHF R gene, the C677T variant, has been associated with a reduction in enzyme specific activity, due to an increase in enzyme thermolability. This variant has been associated with increased risk of developing atherosclerotic diseases (131). In the present stroke and IHD studies, we have found no difference in genotype distribution between the cases and controls when looking at these groups as a whole. However, we have observed a difference in genotype distribution between the diabetic cases (n = 32) and controls (n = 3) of the IHD study (P = 0.01). Although this difference is significant, the small number of individuals in each group, especially the control group, forces us to question the value of this association. We will attempt to reproduce this result in a larger cohort before placing too much weight on this finding. The lack of association which we report can be explained in many ways. It is hypothesized by Frosst et al. that the C677T mutation lies in the region of MTHFR which could be involved in folate binding; this normally would act to stabilize the enzyme. Not only has the influence of folate on Hcy levels been reported by many studies (157, 158), but hyperhomocysteinemia has also been established as a risk factor for vascular diseases (159, 160). It has been suggested that a possible gene-environment interaction could explain this lack of association between this MTHFR variant and atherosclerotic diseases (161, 162). Therefore, in populations where the folate intake is high, the mutation in MTHFR may not result in mild hyperhomocysteinemia and so may not be a marker for vascular disease. This situation could apply to our French-Canadian cohorts. In the present cohorts, we have not measured the levels of folate nor Hcy. We are doing so in the second set of cohorts which are presently being collected, since these indices might help to elucidate the function of this MTHFR variant in the development of stroke and IHD. We have also not investigated the effects of this mutation in concordance with other gene polymorphisms, such as the apo E isoforms. The TT genotype of the MTHFR leads to an increase in total Hcy, which is associated with an increase in the oxidation of LDL. In turn, this could lead to an increase in macrophage derived apo E production, as discussed previously. In the presence of certain apo E genotypes, the net cholesterol efflux from the cell could be reduced, therefore contributing to atherogenesis. The possible interactions between Hcy metabolism and fat metabolism may result in increased susceptibility to stroke and/or IHD and merit further investigation.

The extracellular matrix (ECM): stromelysin-1 (MMP-3)

MMP-3 is part of the MMP family which acts to degrade ECM. The I/D polymorphism, situated in the promoter region of this gene, results in the presence of either 6 or 5 adenosines (6A allele and 5A allele, respectively). The 6A allele has been associated with the early development of coronary atherosclerosis, while it has been suggested that the 5A allele may confer protection against these early stages of the disease (107, 140). In our IHD study, we only find a significant difference in stromelysin-1 genotype distribution between diabetic cases (n = 32) and controls (n = 3) (P = 0.0022). As discussed previously, the small number of subjects in the control group makes these results invalid. An attempt will be made to reproduce these findings in a larger cohort.

In our stroke study, we find a significant difference in genotype distribution between the case and control groups as a whole (P = 0.0236). We report the same finding in stroke cases and controls which do not have a family history of stroke (P = 0.0288). This is in accordance with our previous findings that family history of stroke is not associated with the development of the disease in this cohort. We would also expect to see a significance difference in stromelysin-1 genotype distribution between cases and controls which do have a family history of stroke, albeit this is not what we observe. This lack of association is probably due to a small sample size, and therefore we will replicate these results in a larger cohort. Specifically, we report that the heterozygous genotype, 5A/6A, is significantly more frequent in stroke cases than controls (P = 0.0119). We also find a trend indicating that the 6A/6A genotype is more frequent in controls than in stroke cases, although this did not reach significance (P = 0.0504). Although these results contradict what we would have predicted from findings in the literature on IHD studies, where the 6A allele is associated with IHD, our results can be explained in many ways. The 6A allele may be a risk factor to early atherogenesis, due to its high affinity for its transcriptional repressor, which results in an decrease in the basal levels of MMP-3. This will cause a decrease in ECM degradation and remodeling, and thus an increase in matrix deposition. The 5A allele, on the other hand, may also act to increase stroke susceptibility, but at the late stages of the disease. This allele is characterized by a high basal level of MMP-3 production due to its low affinity for its transcriptional repressor. This results in increased ECM degradation, plaque instability and rupture, which can lead to either progressive

growth of the atheroma or thrombosis; these are mechanisms of disease which may be more important in stroke than in IHD. While individuals which have the 5A/5A genotype may be protected, in part, at early stages of atherosclerosis, and individuals which have the 6A/6A genotype may be protected, in part, from thrombus formation, individuals which have both these alleles, the 5A/6A genotype, may have increased susceptibility at many stages of the disease, therefore increasing the risk of disease outcome. Our stroke cases may be representative of those individuals, but since we did not analyze these cases with respect to stroke type, this can not be confirmed. Although our controls tend to have a higher frequency of the 6A/6A genotype and may not be protected against the early stage of the disease process, the fact that they have not experienced a stroke may be explained in part by the absence of other genetic factors, such as the simultaneous presence of the 5A allele. Another important fact to consider is that this polymorphism was not found to have a significant effect on stroke outcome in the regression analysis. Although this could be due in part to small sample size, it may also be explained by the potentially small effect of this gene on the development of stroke, in the presence of other stroke risk factors such as HTN, smoking, and dyslipidemia.

Strengths and weaknesses of the stroke and ischemic heart disease studies

There are many strong points to mention about these two association studies. First and foremost is the use of a founder population, the French-Canadians. This serves to decrease the genetic heterogeneity, an important factor to keep in mind when studying the genetics of complex diseases such as stroke and IHD. An important aspect of association studies is the use of appropriate controls. Our control group not only shared the same ancestral background as our case group, but they were also selected to match for age and gender, important non-modifiable risk factors for both diseases. These controls did not have any clinical manifestations of either stroke or IHD, making them appropriate controls for both studies. Atherosclerotic based diseases, such as stroke and IHD, should, on some levels, be looked at together since they are risk factors for one another. Individuals which are free of one disease but have clinical manifestations of the other should not be used as controls for studies on either stroke or IHD. For all the above reasons, our selected control group is thought to provide a reliable estimate of both genetic and environmental stroke and/or IHD risk factors in the population from which the cases were recruited. Our studies also

showed that the selected case groups were representative of the 'at risk' individuals for stroke and IHD in the base population, due to the increased frequency of established risk factors in these groups. A unique and important feature of these studies is the nature of the genetic investigation. We studied a total of 10 polymorphisms, which are either functional or thought to be in linkage disequilibrium with a functional variant, in 9 different stroke/IHD candidate genes, which belong to 5 major systems involved in the normal functioning of the vascular system. Our investigation allowed us to observe if one or many of these polymorphisms, in concert with established stroke/IHD risk factors, played a role in the development of stroke and/or IHD in the French-Canadian population.

Some of the weaknesses of these studies revolve around the small number of case subjects in each cohort. In the study of these complex disease, having a low number of cases creates a problem for stratification by disease sub-type in the analysis, leaving each resulting group with a small number of patients, and therefore decreasing the statistical power. One can still conduct an analysis on the group as a whole, which is what we have done, but for heterogeneous diseases such stroke and IHD certain associations may only be revealed when the group is stratified according to disease sub-type. This type of stratification will help to elucidate the etiologies of these diseases; we are presently continuing the recruitment of cases and controls, for both studies, and hope to be able to perform such analyses in the near future. Another aspect which can be considered as a weakness of these studies is the omission of certain risk factors in the studies or in the analyses. Data on circulation (stroke study), age of onset, first vascular event, age of first vascular event, and presence of other vascular disorders was ascertained, but was not used in the analysis. This data will be used for comparison between disease sub-types (i.e. cases with thromboembolic stroke vs. TIA only) and is therefore not addressed in the scope of this thesis. Other data which was recorded but not analyzed, due to too much missing information, include measurements of fibrinogen levels and BMI; these variables will be looked at in future analyses. Other stroke and/or IHD risk factors which were not recorded include alcohol intake and the use of oral contraceptive; these are presently being recorded for the new patients being entered into this study. Menopausal status was recorded but was not used for stratification due to the small number of females which were not post-menopausal. Data on physical exercise, stress, and personality features was not

recorded due to the bias and qualitative nature of these factors; obtaining a standardized quantitative response from the candidates would have been too difficult. Lastly is the issue of correcting for multiple testing, which was not done in these two studies. Since there are a large number of possible interactions between all the variables for which we have to test, associations which we have found might have, in some instances, lost statistical significance as a result of correction for multiple testing. Instead, we prefer to replicate each studies' associations in another independent sample which is presently being collected. We are aware of the importance of correction for multiple testing and intend to apply this to the next cohorts, which will be larger in size than the present ones, and therefore more resistant to multiple testing.

Part VIII) Conclusion

In conclusion, the present studies demonstrated the importance of several factors in the development of IHD and stroke among the French-Canadians. Firstly, we reconfirm the importance of 'traditional' stroke/IHD risk factors such as dyslipidemia, HTN, smoking, and diabetes in the development of these disorders. Secondly, we provide further evidence to support the genetic basis of stroke and IHD by identifying genetic variant, such as PGIIIa PL^{A1}/PL^{A2} and stromelysin-1 5A/6A, which seem to contribute to the outcome of IHD and stroke, respectively.

Clearly, advances in the field of vascular disease research need to include the simultaneous investigation of environmental and genetic risk factors. Populations which are 'at risk' for diseases such as stroke and IHD need to be better targeted in order to elucidate the etiologies of these diseases. To do so, we must impose a more stringent definition of the case and control groups, not only to eliminate result discrepancies between studies, but to better understand the disease process. This will in turn lead to more specialized treatment for those affected with the disease, and better prevention programs for those at risk of developing stroke and/or IHD.

Part IX) References

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