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UMI
Pharmacogenomics and Genetic Risk Factors of
Coronary Artery Disease

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ABSTRACT

Coronary artery disease (CAD) is the most prevalent disorder and the leading cause of death worldwide. There are a number of CAD medications, which are effective and safe in most patients, but have been associated with adverse reactions such as angioedema induced by angiotensin I-converting enzyme inhibitors (AE-ACEi). In this study, we identified aminopeptidase P (APP) activity as an endophenotype for AE-ACEi, which is a heritable quantitative trait (heritability = 0.336 ± 0.251 SD) and is significantly reduced in a majority of our cases. Although initial mutation screening did not reveal any coding variants in XPNPEP2, which encodes membrane-bound APP, subsequent linkage analysis of APP activity in eight families provided a maximum LOD score (3.75) for this locus. Sequencing of additional cases identified a splice variant (314_431del) and a non-coding polymorphism (rs3788853) in this locus, which cosegregate with low plasma APP activity. The latter accounts for the linkage signal and is associated with AE-ACEi (P = 0.036). In addition, we identified other potential loci for APP activity and demonstrated that certain ACEi (Captopril and Enalapril) non-specifically inhibit APP activity. Furthermore, we detected polymorphisms associated with reduced APP and ACE activities among females with estrogen-dependent inherited angioedema.

We also conducted a genetic investigation of depression among CAD patients to identify common susceptibility loci which might explain the correlation between these diseases. Our candidate gene association study identified a polymorphism (rs216873) in the von Willebrand factor gene that was significantly associated (P = 7.4 x 10^-5) with elevated depressive symptoms in our CAD cohort. These results suggest that risk factors for atherosclerosis also underlie susceptibility to depression among CAD patients.
This dissertation contributes to the field of genetics and pharmacogenomics of CAD. A better understanding of the toxic effects of CAD drugs will assist in the development of safer and more effective treatments. In addition, our results may facilitate clinical assays to identify individuals who are susceptible to angioedema prior to ACEi or estrogen therapy. Finally, our genetic investigation of depression in CAD patients reveals a novel drug target (VWF) for treatment of depression in cardiac cases.
RÉSUMÉ

La maladie coronarienne athérosclérotique (MCA) est la cause de mortalité la plus enregistrée à l'échelle mondiale. Bien qu'un grand nombre de médicaments soient prescrits pour traiter la plupart des individus en souffrant, certains de ceux-ci peuvent entraîner des effets secondaires comme l'angioedème lors de la prise d'inhibiteurs de l'enzyme de conversion de l'angiotensine (AE-iECA). Nous avons établi que l'activité de l'aminopeptidase P (APP) est un endophénotype de l'AE-iECA et qu'elle représente un trait quantitatif et génétiquement transmissible (héritabilité = 0.336 ± 0.251 DS) car son activité est réduite dans la majorité des individus affectés. Malgré un criblage génétique antérieur n'ayant pas révélé de variations génétiques dans la séquence codantes du gène XPNPEP2, une analyse par liaison génétique de l'activité de cet enzyme dans huit familles a maintenant permis d'établir une valeur LOD (logarithm of odds) maximale de 3.75 à ce locus. Le séquençage de cas additionnel a permis l'identification d'une variation d'épissage (314_431del) et d'un polymorphisme non-codant (rs3788853; P = 0.036) à ce locus et ceux-ci sont observés en parallèle avec la faible activité plasmatique de l'APP. Nous avons identifié d'autres loci possiblement liés à l'activité de l'APP et démontré que certains iECA (Captopril et Enalapril) inhibent cette activité de façon non-spécifique. Nous avons aussi identifié des polymorphismes associés avec une faible activité APP et ECA chez des femmes souffrant d'angioedème estrogène dépendant de forme héréditaire. Cette thèse contient également une étude génétique de la dépression chez des patients avec MCA. L'étude de gènes candidats a révélé un polymorphisme (rs216873; P = 7.4 x 10^{-5}) à l'intérieur du gène codant pour le facteur von Willebrand (vWF) qui est associé avec un risque accru de dépression chez ces patients.
Cette dissertation contribue à la génétique et la pharmacogénomique des MCA. Une meilleure compréhension des MCA permettra l'élaboration de meilleures médications. Il est possible que ces résultats aident à l'amélioration des essais cliniques sur la MCA en facilitant l'identification d'individus plus à risque de présenter de l'angioedème. Enfin, l'étude de la dépression des individus avec MCA permettra peut-être d'établir une nouvelle cible (VWF) pour traiter la dépression chez ceux-ci.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Guy Rouleau, who encouraged and supported me throughout my PhD. I would also like to thank Dr. Patrick Dion for his mentorship, guidance and friendship over the years. In addition, I appreciated the helpful advice of my supervisory committee, Drs. Erwin Schurr, Eric Shoubridge and Jean-Lucien Rouleau. I would like to acknowledge all the participants involved in our genetic studies. My projects were possible through numerous collaborations with many clinicians and scientists who contributed to sample collection, biochemical characterisation of proteins and statistical analyses. I would especially like to thank Drs. Albert Adam, Marie-Pierre Dube, Nancy J. Brown and Karen Binkley for all their efforts. Moreover, there were several colleagues who deserve recognition for their technical assistance including Dr. Inge Meijer, Mr. Daniel Rochefort, Mses Judith St-Onge, Annie Levert and Sandra Laurent. I would also like to acknowledge all the friends who supported and encouraged me including Dominique Verlaan, Lan Xiong, Jean-Baptiste Rivere, Paul Valdmanis, Ariela Abecassis, Claudia Gaspar, Adele Cantegrel, Faith Au-Yeung, Christiane Messaed and Shawn Stochmanski. Finally, I thank my husband, Nader Ghasemlou, my parents and my brother for their unwavering support. Nader, you give me the strength to pursue my ambitions and the courage to confront any challenges that arise along the way. In addition, I am grateful for the support of the National Institute of Health and the National Heart, Lung and Blood Institute which funded my projects as well as the Heart and Stroke Foundation of Canada, Funds de la Recherché du Santé du Quebec (FRSQ), McGill University, and the Canadian Institute of Health Research (CIHR) for supporting me throughout my graduate training.
CONTRIBUTION OF AUTHORS

Chapter 2.2


Duan: Manuscript writing, study design, gene sequencing and SNP genotyping, RNA isolation, reverse-transcriptase polymerase chain reactions (PCR), file (genotype, phenotype, pedigree marker) preparation for linkage and statistical analyses

Nikpoor: Sample collection and linkage analysis

Dube, Flury, Foroud: Linkage analysis

Molinaro, Adam: Characterization of aminopeptidase P activity in plasma

Meijer, Dion: Assisted gene screening experiments and RNA work

Rochefort, Saint-Onge: Assisted SNP and microsatellite genotyping experiments

Brown, Gainer, Potier, JL Rouleau, Agostoni, Cugno, Simon, Clavel, Potier, Wehbe, Benarbia, Marc-Aurele, Chanard: Sample collection and clinical evaluation

GA Rouleau: Supervision and manuscript revision
Chapter 3.2


Molinaro and Adam: Manuscript writing, study design, characterization of Bradykinin, des-Arg⁹-BK and aminopeptidase P

Duan: Manuscript writing, study design, allele-specific PCR, file (genotype, phenotype, pedigree and marker) preparation for heritability estimate and association analysis

Chagnon and Moreau: Assisted characterization of proteins

Simon, Clavel, Lavaud, Boileau, Lepage: Sample collection and clinical evaluation

Chanard and Rouleau: Supervision and manuscript revision

Chapter 4.2

Qing Ling Duan, Karen Binkley, Guy A. Rouleau. Genetic analysis of factor XII and bradykinin catabolic enzymes in a family with estrogen-dependent inherited angioedema.

*Accepted in the J. Allergy Clin Immunol.*

Duan: Manuscript writing, project design, gene sequencing and genotyping assays

Binkley: Sample collection, clinical evaluation, manuscript writing

Rouleau: Supervision and manuscript revision
Chapter 5.2


McCaffery: Manuscript writing, project design, candidate gene selection, supervision

Duan: Manuscript writing, project design, SNP selection for Illumina custom design genotyping assay, DNA preparation and quantification

Frasure-Smith, Lespérance, Théroux: Sample collection, clinical evaluation, candidate gene selection

Rouleau: Supervision and manuscript revision

Barhdadi, Dubé: Association analysis

Chapter 6.2


Duan: Manuscript writing, project design, DNA preparation and quantification, SNP and microsatellite genotyping experiments

Dube, Barhdadi: Association analysis
Frasure-Smith, Lespérance, Théroux: Sample collection and clinical evaluation

McCaffery, Rouleau: Supervision and manuscript revision
CLAIMS FOR ORIGINALITY

1. The measurement of plasma APP activity demonstrated that this quantitative trait is significantly reduced in a majority of our AE-ACEi cases and is partially determined by genetic factors. Linkage analysis of this endophenotype in eight large families identified a QTL on chromosome X, which contained a candidate gene for APP activity (*XPNPEP2*).

2. Mutation screening of this QTL identified a genomic deletion resulting in erroneous splicing of the mRNA transcript, which encodes a truncated protein, in one AE-ACEi case with zero APP activity. The same mutation co-segregates with low APP activity in other members of his family. This deletion demonstrated the role of *XPNPEP2* in susceptibility to AE-ACEi. In addition, a non-coding polymorphism was identified at this locus in eight affected cases, which accounts for the linkage signal and is significantly associated with AE-ACEi.

3. Measurement of APP activity in human plasma samples treated with several commonly used ACEi drugs demonstrated non-specific inhibition of APP by two ACEi drugs: Captopril and Enalapril.

4. This pharmacogenomic investigation of AE-ACEi is the first to identify a susceptibility locus for this potentially fatal adverse reaction of ACEi. In addition, we determined that this adverse reaction is a complex trait that may be determined by more than one genetic locus as linkage signals for APP activity were also detected on
chromosomes 6, 8 and 9. Moreover, mutation screening of \textit{XPNPEP2} identified more than one genetic variation regulating APP activity suggesting allelic heterogeneity. Finally, factors other than APP activity may increase risk of AE-ACEi, which may or may not be genetic.

5. Polymorphisms in \textit{ACE} and \textit{XPNPEP2}, associated with decreased expression/activity of ACE and APP, were found in an Italian family with multiple cases of estrogen-dependent inherited angioedema. This suggests that impaired BK metabolism also plays an important role in susceptibility to this familial form of angioedema.

6. Genotyping of polymorphisms in the \textit{TCF7L2} gene resulted in significant association with type 2 diabetes in a French Canadian cohort of CAD patients. This cohort has a higher prevalence of diabetes than the general population as high blood glucose is a known risk factor for CAD. Furthermore, we demonstrated that obesity increases risk for diabetes independently and additively with variants in this gene.

7. A candidate gene association study of depression in CAD patients identified a novel locus (\textit{VWF}) and mechanism for depression (platelet aggregation) among individuals who also have heart disease. Genetic variants in this gene may increase susceptibility to both diseases and account for their correlation.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5-HTTLPR</td>
<td>Serotonin Transporter</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin I-Converting Enzyme Inhibitor</td>
</tr>
<tr>
<td>ADR</td>
<td>Adverse Drug Reaction</td>
</tr>
<tr>
<td>AE</td>
<td>Angioedema</td>
</tr>
<tr>
<td>AN69</td>
<td>Negatively Charged Membrane for Hemodialysis</td>
</tr>
<tr>
<td>ANGII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>APP</td>
<td>Aminopeptidase P</td>
</tr>
<tr>
<td>AR</td>
<td>Anaphylactoid Reaction</td>
</tr>
<tr>
<td>B1R</td>
<td>B1 Receptor</td>
</tr>
<tr>
<td>B2R</td>
<td>B2 Receptor</td>
</tr>
<tr>
<td>BDI-II</td>
<td>Beck Depression Inventory II</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass index</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>calcium channel, L type, alpha 1C subunit</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CHRM2</td>
<td>cholinergic muscarinic receptor 2</td>
</tr>
<tr>
<td>CIINH</td>
<td>First component Inhibitor</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
</tr>
<tr>
<td>CNR1</td>
<td>cannabinoid receptor</td>
</tr>
<tr>
<td>CPN</td>
<td>Carboxypeptidase N/Kininase I</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>Des-Arg⁹-BK</td>
<td>Des-Arginine9-Bradykinin</td>
</tr>
<tr>
<td>DPPIV</td>
<td>Dipeptidyl Peptidase IV</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>EDIA</td>
<td>Estrogen-Dependent Inherited Angioedema</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen Response Element</td>
</tr>
<tr>
<td>F12</td>
<td>Coagulation Factor XII</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol anchor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
</tr>
<tr>
<td>h²</td>
<td>heritability</td>
</tr>
<tr>
<td>HAE</td>
<td>Hereditary Angioedema</td>
</tr>
<tr>
<td>HBP</td>
<td>High Blood Pressure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>hcAPP</td>
<td>Human Cytosolic Aminopeptidase P</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HGP</td>
<td>Human Genome Project</td>
</tr>
<tr>
<td>hmAPP</td>
<td>Human Membrane-Bound Aminopeptidase P</td>
</tr>
<tr>
<td>HMWK</td>
<td>High Molecular Weight Kininogen</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary Adrenal Cortical Axis</td>
</tr>
<tr>
<td>HSR</td>
<td>Hypersensitivity Reaction</td>
</tr>
<tr>
<td>HTR2A</td>
<td>Serotonin Receptor</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg equilibrium</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of Odds</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of Mercury</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral Endopeptidase</td>
</tr>
<tr>
<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative Trait Locus</td>
</tr>
<tr>
<td>$r^2$</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SERPING1</td>
<td>First component Inhibitor gene</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SOLAR</td>
<td>Sequential Oligogenic Linkage Analysis Routines</td>
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<tr>
<td>SSCP</td>
<td>Single Strand Conformation Polymorphism</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half-Life</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Transcription Factor 7-like 2 gene</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular cellular adhesion molecule 1</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cells</td>
</tr>
<tr>
<td>XPNPEP2</td>
<td>Membrane-Bound Aminopeptidase P gene</td>
</tr>
<tr>
<td>XPNPEPL</td>
<td>Cytosolic Aminopeptidase P gene</td>
</tr>
<tr>
<td>$\lambda_R$</td>
<td>Relative Risk</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION

1.1 CORONARY ARTERY DISEASE

Cardiovascular disease (CVD) is the number one cause of death worldwide, with an estimated 17.5 million deaths in 2005, representing 30% of all global deaths (World Health Organization). CVD is also a leading cause of disability and the largest health care burden in developed countries (Public Health Agency of Canada 1998; Liu et al. 2002; National Heart, Lung and Blood Institute 2008). The projected economic cost of CVD this year in the United States is $448 billion (National Heart, Lung and Blood Institute 2008). CVD is the general term for a group of disorders affecting the heart and/or blood vessels including coronary artery disease (CAD; also known as coronary heart disease (CHD) or atherosclerotic heart disease), stroke, hypertension, viral infections and other diseases affecting the heart muscles. CAD represents more than 50% of all CVD cases and is the leading cause of death (Public Health Agency of Canada 1998; National Heart, Lung and Blood Institute 2008). The risk of developing CAD by age forty is 49% for men and 32% for women (Lloyd-Jones et al. 1999).

CAD is a chronic condition caused by plaque deposits in the arteries which reduce the supply of oxygenated blood to the heart. These plaque deposits form during atherosclerosis, an inflammatory response initiated by damage to the inner lining (endothelium) of the arteries, which may be triggered by various factors such as smoking, high blood pressure, diabetes, and inflammation (Winifred 1995). Endothelial damage provokes white blood cells (WBC) to congregate at the lesion site and initiate an inflammatory immune response (e.g. the release of cytokines such as interleukin 6 (IL-6)). As the endothelium is gradually weakened by inflammation, more WBCs accumulate and...
large low density lipoproteins (LDL) are able to penetrate the arterial wall. The WBC and LDL combine to form plaque deposits inside the artery, which calcify over time to reduce the artery's ability to contract and expand. This "hardening of the arteries" reduces the supply of oxygen to the heart, causing the myocardial cells of the heart muscles to die, which may manifest as chest pain (angina) and fatigue in some individuals. However, 50% of men and 63% of women do not report any clinical symptoms prior to a heart attack (myocardial infarction (MI)) (Thom et al. 2006). MI results from the rupture of the plaque deposits, leading to the subsequent formation of blood clots at the rupture site or loosened pieces to move through the arteries to other sites and potentially obstruct blood flow to the heart (Ashley 2004).

CAD may be diagnosed using a number of tests and procedures (Ashley 2004). For example, a blood test may be used to measure the level of C-Reactive Protein (CRP), which is an inflammatory marker that is released during atherosclerosis and may be used to predict heart disease risk (Pearson et al. 2003). Another non-invasive test is an electrocardiogram, which measures the rate and rhythm of heart beats via electrodes attached to the chest, arms and legs. This test is usually carried out while the patient exercises on a treadmill or stationary bicycle to generate stress onto the heart. In addition, an echocardiogram is a test that uses sound-waves (ultrasound) to provide information about the size, shape and movement of the heart valves and the ventricular myocardium. This identifies parts of the heart muscle that contract poorly, which represent areas of the heart that is receiving decreased amounts of oxygenated blood or parts that may have been damaged from poor blood supply. Alternatively, CAD may be determined using a coronary angiogram, which releases a dye into the coronary arteries
followed by an X-ray to show an outline of the arteries and allows the identification of any obstructions in the path of blood flow.

Although the clinical manifestations of CAD commonly begin between middle and late life, recent studies show that the majority of atherosclerosis begins in childhood (Ford 2003). The early onset of atherosclerosis and clustering of CAD within families suggest that genetic factors contribute to the pathogenesis of this disease. To date, numerous genetic loci have been associated with CAD (Online Mendalian Inheritance in Man (OMIM) 608901) and some of its risk factors including hypertension (OMIM 145500), type 2 diabetes (OMIM 125853), and depression (OMIM 608516) and hypercholesterolemia (OMIM 143890). In addition, other factors increase the risk for CAD such as high blood pressure (HBP), high fat diet, high alcohol consumption, high ratio of low to high density lipoproteins (LDL:HDL), diabetes, low physical activity, obesity, smoking, stress and depression (Ashley 2004). Thus, CAD is a multifactorial condition determined by various genetic and non-genetic elements.

Despite the fact that CAD remains the number one killer worldwide, the mortality rate has significantly declined throughout the past four decades (National Heart, Lung and Blood Institute 2008). This is due to substantial improvements in the prevention, diagnosis and treatment of CAD. The risk of CAD may be reduced significantly through life style choices such as maintaining a low fat or cholesterol diet, low alcohol consumption, regular exercise, reducing stress and quitting smoking (Kromhout et al. 2002; The National Cholesterol Education Program2002; De Backer et al. 2003). Surgical procedures are also used to prevent MI such as balloon angioplasty and coronary artery bypass graft. Finally, CAD may also be treated using various medications such as
antihypertensives (e.g. angiotensin I-converting enzyme inhibitors (ACEi)), statins to reduce cholesterol and antiplatelets (e.g. aspirin) to inhibit formation of blood clots (Ashley 2004). As with most other drugs, there is considerable interindividual variability in response (efficacy and toxicity) to these medications used to treat CAD and other cardiovascular diseases (Torpet et al. 2004).

1.2 OVERVIEW OF OBJECTIVES

This dissertation includes pharmacogenomic studies of two potentially fatal adverse drug reactions (ADRs) associated with ACEi, a large class of drugs used to treat CAD. We hypothesize that genetic factors predispose some individuals to develop these ADRs during ACEi therapy. The primary aim of both studies is to identify genes that underlie these ADRs. A better understanding of the genetic risk factors will facilitate the development of genetic assays to predict susceptible individuals prior to drug administration. Such tests would prevent unnecessary suffering and deaths from these acute ADRs. Furthermore, revelations about the biochemical mechanisms underlying these ADRs will assist in the rational development of safer CAD drugs as well as efficient treatment for these ADRs.

Chapters 5 and 6 of this dissertation describe the genetic investigations of two major risk factors for CAD, depression and type 2 diabetes. Numerous studies have confirmed the association between depression and recurrent cardiac events as well as higher mortality among depressed CAD patients (McCaffery et al. 2006). Type 2 diabetes, characterized by elevated blood glucose levels which damage the blood vessels (Henry et al. 2003) and accelerate atherosclerosis (Beckman et al. 2002), has also been
associated with higher CAD risk and mortality. The aim of these studies is to identify genetic loci which increase susceptibility to these common CAD risk factors. A better understanding of depression and type 2 diabetes would assist in the prevention and treatment of these conditions as well as CAD.

This general introduction provides a literature review of the ADRs associated with ACEi drugs as well as depression and type 2 diabetes. In addition, this chapter outlines the aims of this dissertation and the mapping approaches used to identify genetic loci for these multifactorial traits.

1.3 PHARMACOGENOMICS OF ANGIOTENSIN I-CONVERTING ENZYME INHIBITORS

1.3.1 Pharmacogenetics and Pharmacogenomics

Medications which treat or prevent the development of most illnesses show variable drug efficacy across a population and ADRs in certain individuals (Evans et al. 2001). This limits the therapeutic benefits and results in morbid and sometimes fatal toxicity. ADRs are estimated to be directly responsible for up to 7% of all hospitalizations, are a leading cause of death and represent $75 billion in healthcare costs each year in the United States (Lazarou et al. 1998).

A number of factors can influence an individual’s response to a drug such as age, gender, diet, smoking, alcohol consumption, disease status, or interactions with other medications (Coleman 2005). In addition, it has been observed that differences in drug response are often greater among unrelated individuals of a population than among
family members or in the same individual (or monozygotic (MZ) twins) at various times, which indicates that genetic factors play a role (Vesell 1989). Genetics may account for 20-95% of variability in drug response (Kalow et al. 1998).

The earliest association between heritable variation and differential drug metabolism was documented in the 1950s by Kalow and colleagues who found that variants in the gene encoding butyrylcholinesterase led to impaired hydrolysis of the muscle relaxant succinylcholine, manifesting as muscle paralysis (Kalow 1956; Kalow et al. 1959). Subsequent studies identified common genetic variants in the gene encoding N-acetyl transferase among individuals who were unable to metabolize Isoniazid for treatment of tuberculosis (Evans et al. 1960), Hydralazine for lowering blood pressure (Timbrell et al. 1980) and Procainamide for cardiac arrhythmias (Reidenberg et al. 1975). Individuals with impaired acetylation were more likely to experience a number of adverse reactions to these drugs (Drayer et al. 1977). These earlier studies gave rise to the field of pharmacogenetics and subsequently, pharmacogenomics. Although these terms are often used interchangeably in reference to the study of genetic factors underlying differential drug response, the latter more specifically refers to the use of a genome-wide approach. To date, numerous variants in genes encoding drug metabolizing enzymes, transporters and targets (e.g. receptors) have been described to affect drug response (Eichelbaum et al. 2006).

Pharmacogenetics or pharmacogenomics have the potential to personalize medicine by establishing the accurate drug dosage required for an individual based on the presence of one or more DNA variations. For example, single nucleotide polymorphisms (SNPs) in the CYP2C9 (cytochrome P450 oxidation enzyme) (Margaglione et al. 2000)
and VKORC1 (Vitamin K epoxide reductase protein) (D'Andrea et al. 2005; Rieder et al. 2005) genes determine the metabolism of a class of anticoagulants collectively known as warfarin. Testing of these polymorphisms improves predictions of the therapeutic warfarin dosage (Gage et al. 2008). In addition, genetic screening prior to drug administration could identify those who are susceptible to develop potentially fatal ADRs. For example, SNPs in the gene encoding thiopurine S-methyltransferase increase risk of a potentially fatal adverse reaction to chemotherapeutic agents (thiopurine drugs), a condition known as myelosuppression (decrease of bone marrow activity, resulting in fewer red blood cells, WBC and platelets) (Yates et al. 1997). Genetic testing to predict ADRs and dosage would replace the current trial and error method for drug therapy, which frequently results in non-response among susceptible individuals, inefficient drug doses resulting in negative effects or even death as result of acute toxicity.

1.3.2 Hypertension

Blood pressure (BP) is a measurement of the force of blood flow against the walls of the arteries when the heart beats (systolic BP) and when it is at rest (diastolic BP). Hypertension is a prevalent disease affecting one out of three Americans (systolic BP > 140 mmHg and diastolic BP > 90 mmHg) while another 28% of Americans have pre-hypertension (systolic BP between 120-139 mmHg and diastolic BP between 80-89 mmHg) (Chaturvedi 2004). Before age 45, hypertension is more common in men than in women, but BP increases with age among women so that this condition is more common among post-menopausal women than men of the same age (Lawes et al. 2006). It is also more prevalent in populations originating from Africa, Middle East, Southeast Asia, and
among Native Americans (Lawes et al. 2006). Other factors increasing risk for hypertension include family history (suggesting genetic risk), older age, high sodium diet, alcohol consumption, inactivity, obesity, high ratio of LDL: HDL and diabetes (Ashley 2004). Thus, hypertension is also a multifactorial disease that can be managed through lifestyle changes such as regular exercise and a healthy diet.

Hypertension is a common risk factor for CAD, stroke, kidney disease and other CVDs (Chaturvedi 2004). HBP can damage the walls of the arteries to predispose to atherosclerotic heart disease. In addition, hypertension can damage the heart by causing the muscles to thicken (hypertrophy) and function abnormally (dilate and contract with less force). HBP affecting smaller arteries, which supply blood to other organs such as the brain, eyes, and kidneys, can also predispose to other diseases such as stroke, retinopathy, and renal failure, respectively.

Many drugs are available to lower blood pressure such as diuretics (eliminate excess water and salts from the body), beta-blockers (reduce heart rate), ACEi, angiotensin receptor blockers (ARBs) or calcium channel blockers (cause blood vessels to relax and dilate). Because hypertension is a risk factor for other CVDs, these medications also effectively prevent heart attacks and strokes as well as treat kidney failures and diabetes. Usually, these medications are prescribed in combinations to effectively lower BP. For example, individuals with diabetes and kidney disease are usually prescribed a cocktail of ACEi, ARB and diuretics (The American Society of Hypertension). However, it has been observed that some people respond better to certain drugs than to others as all above mentioned drugs have been associated with ADRs in some individuals.
1.3.3 Angiotensin I-Converting Enzyme Inhibitors

The first ACEi (Captopril) was developed in 1975 for the treatment of hypertension. Since then, a number of other ACEi medications with different brand names (e.g. Benazepril, Enalapril, Fosinopril, Lisinopril and Quinapril) have become available and are increasingly prescribed for the treatment of other CVDs (Yusuf et al. 2000). While ACEi effectively lower BP (Waeber et al. 1982), it also decreases complications and mortality in patients with CAD, diabetes, left ventricular dysfunction, post-MI and stroke, as well as other CVDs (Yusuf et al. 1992; Lewis et al. 1993; Chobanian et al. 2003). Thus, ACEi is increasingly prescribed to treat or prevent a number of disorders affecting the heart or blood vessels, making it one of the most widely used medications worldwide, with more than 40 million patients (Agostoni et al. 2001).

The therapeutic effects of these drugs (Figure 1) result from inhibition of ACE (Kininase II), which acts on the renin-angiotensin system to convert angiotensin I to angiotensin II (Ang II), a potent endogenous vasoconstrictor. ACE also acts on the kallikrein-kinin pathway by metabolizing bradykinin (BK), an important vasodilator, to its inactive products (Figure 1). Thus, ACE inhibition prevents the formation of Ang II and the breakdown of BK, resulting in dilated blood vessels and lower BP. ACE inhibition may also decrease the risk of other CVDs through suppression of the renin-angiotensin pathway (Lonn et al. 1994). For example, Ang II increases vascular permeability through contraction of endothelial cells, which accelerates atherosclerosis (Greenwald et al. 1994). Therefore, decreased Ang II in the presence of ACEi could slow atherosclerosis and decrease the risk of CAD. Moreover, increased BK levels have been
shown to improve coronary blood flow (Groves et al. 1995), increase glucose uptake by myocytes (Rosen et al. 1983), and decrease infarct size in animal models (Martorana et al. 1992; Wall et al. 1994), which helps to treat CAD, diabetes and MI, respectively. A common deletion polymorphism in the ACE gene, resulting in higher gene expression (Rigat et al. 1990), has been associated with increased risk for CVDs (Schunkert et al. 1994; Yoshida et al. 1995).

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<th>Renin-angiotensin system</th>
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Figure 1. The role of angiotensin I converting enzyme inhibitors on the renin-angiotensin and kallikrein-kinin systems. Modified from (Komajda et al. 2000).

1.3.4 Adverse Reactions Associated with ACE Inhibitors

Despite the therapeutic benefits, ACEi have been associated with a number of ADRs and an estimated death rate of 0.2% (Coats 2002). Considering the widespread use of these drugs, the impact of a potentially fatal reaction to these drugs is substantial. The most common and least threatening adverse effect of ACEi is a chronic, dry cough that affects
up to 20% of patients (Dykewicz 2004). More serious side effects are hyperkalemia (high potassium levels), complications during pregnancy, angioedema (AE-ACEi), and hypersensitivity reactions (HSR-ACEi; also known as anaphylactoid reactions (AR-ACEi)) during hemodialysis (Israel et al. 1992). Thus, patients taking ACEi have to be monitored closely and are taken off these drugs when serious ADRs develop.

Angioedema (AE) is a common clinical syndrome that is associated with various pathogenic conditions (Greaves et al. 1991). It is characterized by localized swelling of subcutaneous or mucosal tissue layers, which affects the face (e.g. lips, tongue), upper airways, gastrointestinal tract (GI), genitalia, more rarely, the hands or feet (Israel et al. 1992; Agostoni et al. 2004). Most AE episodes are allergic reactions that may be treated with antihistamines and other anti-inflammatory drugs. However, a fraction of these do not represent allergic reactions, referred to as non-histaminergic AE, and do not respond to such drugs. Untreated AE affecting the respiratory tract may require emergency surgical procedures to prevent death by asphyxiation (Agostoni et al. 2001). Acute AE of the GI tract may cause violent abdominal pain, vomiting and/or diarrhea (Agostoni et al. 2004).

Most non-histaminergic AE cases have a inherited impairment of the protein that inhibits the first component of the complement pathway known as the C1 esterase inhibitor (C1INH; OMIM 106100)) (Davis 1988; Agostoni et al. 1992). To date, more than 150 mutations have been identified in the SERPING1 gene, which encode C1INH, resulting in decreased concentration (hereditary angioedema (HAE) type I) or decreased activity of CIINH (HAE type II). A deficiency in C1INH activates the complement...
pathway and increases the production of BK through activation of coagulation Factor XII and kallikrein (see Figure 2 below) (Schapira et al. 1983).

A fraction of AE cases occur in individuals with normal CIINH concentration and function. For instance, AE is a well known adverse reaction of ACEi (Wood et al. 1987; Slater et al. 1988; Vleeming et al. 1998) and familial AE has also been associated with increased estrogen levels (Warin et al. 1986; Binkley et al. 2000; Bork et al. 2000). In addition, an idiopathic HAE has been described in some families (Kranke et al. 2000; Gupta et al. 2004). While the pathophysiology of AE cases with normal C1INH levels are poorly understood, previous studies suggest that, similar to HAE, BK is an important mediator (Israelii et al. 1992; Davis 2003).

AE-ACEi occurs in 0.1 to 0.7% of Caucasian patients (Slater et al. 1988), but is estimated to be 4.5-fold more prevalent in black Americans (Brown et al. 1996; Coats 2002). Although this racial difference may be the result of both genetic and non-genetic influences, many variants known to regulate drug response differ in allele frequency among different ethnic groups (Tate et al. 2004), suggesting that genetics may also contribute to AE-ACEi risk. However, environmental factors are also important as smokers taking these drugs have a 3-fold greater AE risk (Coats 2002). The incidence AE-ACEi is likely underestimated since clinical symptoms may develop months or even years after starting ACEi therapy, thus obscuring its relationship with the drug (Agostoni et al. 2004). There is currently no method to predict individuals who have higher risk of developing AE-ACEi.

As mentioned above, another potentially fatal adverse reaction associated with ACEi therapy is AR or HSR that occurs during haemodialysis with a negatively charged
membrane (Verresen et al. 1990; Parnes et al. 1991). Hemodialysis filters the blood to remove waste (e.g. potassium and sodium) as well as extra fluids that build up as a result of kidney failure, which stem from diabetes or other conditions. HSR-ACEi manifest as a combination of AE symptoms (e.g. swelling of the face and/or tongue) with severe hypotension (fall of systolic BP below 100 mmHg with faintness and nausea), flushing, and dyspnoea within 20 minutes of starting haemodialysis with a negatively charged membrane (Verresen et al. 1990). One study reported HSR in 57% of ACEi patients within minutes of starting haemodialysis (Verresen et al. 1990). Since some ACEi patients never develop HSR even after repeated sessions of haemodialysis, this indicates there is an individual susceptibility to ACEi-associated HSR. Some studies suggest that AE and HSR induced by ACEi share a common mechanism. HSR patients develop acute AE during hemodialysis characterized by swelling of the face and respiratory tract (Bright et al. 1999).

1.3.5 The Bradykinin pathway

Previous reports have suggested that BK plays an important role in the pathophysiology of both ACEi-induced AE and HSR (Israel et al. 1992; Schaefer et al. 1993; Verresen et al. 1994; Nussberger et al. 1998). BK is released from high-molecular-weight kininogen (HMWK), when the coagulation factor XII is activated to hydrolyze plasma kallikrein (Figure 2). This hydrolysis occurs in response to tissue injury or when plasma comes in contact with a negatively charged surface such as during hemodialysis (Bhoola et al. 1992). BK binds to the transmembrane receptor, B₂R, which is constitutively expressed, to cause vasodilation and increased permeability of the blood vessels (Brown 2001).
Figure 2. Bradykinin (BK) formation and degradation. BK is a vasodilatory peptide that is normally shortlived, degraded into inactive products by angiotensin converting enzyme, aminopetidase P, Neutral Endopeptidase and Dipeptidyl Peptidase IV. BK is also converted to the potent vasodilator, Des-Arginine⁹-BK, by Carboxypeptidase N, which is in turn degraded by angiotensin converting enzyme and aminopetidase P.

ACE is the primary enzyme that cleaves BK into its inactive metabolites (Sheikh et al. 1986; Murphey et al. 2000). The metallopeptidase X-prolyl aminopeptidase (aminopeptidase P (APP)) represents the secondary pathway while other metabolizing proteins include carboxypeptidase N (CPN; kininase I), neutral endopeptidase (NEP) and dipeptidyl peptidase IV (DPPIV). In the absence of ACE inhibition, CPN transforms approximately 4% of BK into its active metabolite des-arginine⁹-bradykinin (des-Arg⁹-
BK) (Blais et al. 1999; Blais et al. 2000). This carboxy-truncated metabolite, des-Arg$^9$-BK binds to the B$_1$ receptor (B$_1$R), the expression of which is induced by inflammation (e.g. up-regulated by cytokines) and is involved in chronic inflammation (Marceau et al. 1998). Des-Arg$^9$-BK, in turn, is broken down primarily by APP, while ACE serves as the secondary catabolic enzyme (Cyr et al. 2001).

During episodes of AE and HSR induced by ACEi, high levels of BK have been measured in the plasma of affected patients (Verresen et al. 1994; Krieter et al. 1998; Nussberger et al. 1998). However, there was no increase in cleaved HMWK, the precursor of BK, which differs from HAE cases caused by a deficiency in C1INH (Agostoni et al. 1999). This indicates that reduced BK degradation, rather than an increased production, plays an important role in ACEi-induced AE. Thus, anomalies in one or more of the proteins which metabolize BK, such as APP, CPN, NEP and DPPIV may increase an individual’s risk of developing AE-ACEi.

Studies have reported reduced plasma APP activity among AE-ACEi cases (Adam et al. 2002), which is correlated with significantly reduced des-Arg$^9$-BK degradation (Blais et al. 1999; Molinaro et al. 2002). In the presence of ACE inhibition, CPN activity is significantly increased to transform a greater fraction (28% vs. 4%) of BK into des-Arg$^9$-BK (Blais et al. 1999). This active metabolite accumulates among individuals with lower APP activity as this enzyme is the only enzyme degrading des-Arg$^9$-BK when ACE is inhibited (Cyr et al. 2001). One study reported that ACEi patients treated with an APP inhibitor (Apstatin) developed an inflammatory response (Kim et al. 2000). Accumulated levels of des-Arg$^9$-BK have been shown to cause proinflammatory effects in vivo (Blais et al. 1997; Blais et al. 1999). In addition, a study demonstrated
elevated expression of the B₁R in rats and mice which were treated with ACEi (Marin-Castano et al. 2002). Thus, previous data indicates that lower plasma APP activity, resulting in increased des-Arg⁹-BK levels, may predispose individuals to AE-ACEi.

A decrease in DPPIV activity and concentration were measured in a cohort of black Americans who developed AE-ACEi (Lefebvre et al. 2002). In addition to degrading BK, DPPIV is the primary catabolic enzyme of substance P when ACE is inhibited (Ahmad et al. 1992). Moreover, increased BK levels have been shown to activate the release of substance P from nerve fibers (Kopp et al. 1997). Mice models have demonstrated that elevated BK and substance P cause AE-ACEi through increased vascular permeability (Emanueli et al. 1998; Campos et al. 2000). Thus, reduced DPPIV, contributing to elevated BK and substance P, may predispose to AE-ACEi in black populations who also have an increased risk.

Smoking may increase risk for AE-ACEi also through the activation of the BK and/or substance P pathways. One study had measured higher BK concentrations among smokers compared to non-smokers (Pretorius et al. 2004). It has been proposed that inflammation as result of smoking activates the kinin pathway to increase BK, des-Arg⁹-BK as well as induce the expression of the B₁R which binds the latter peptide (Van 1999).

We hypothesize that genetic factors which regulate the activities of APP, DPPIV and other enzymes in the BK and/or substance P pathways determine risk of AE and HSR associated with ACEi drugs. Identification of these genes may facilitate genetic assays to predict individuals who are susceptible to these potentially fatal ADRs and may lead to the development of effective treatments for AE-ACEi.
1.4 DEPRESSION AND CORONARY ARTERY DISEASE

Depression is a mood disorder that affects approximately 7% of Americans (Kessler et al. 2003). Clinical symptoms include prolonged (two weeks or more) feelings of sadness, hopelessness, decreased interest in usual activities or hobbies, lack of energy, change in appetite or weight, thoughts of suicide or death. It is estimated that up to 15% of depressed individuals eventually commit suicide (Davies et al. 2001). Risk factors for depression include family history, environmental factors including stress and trauma, biological causes such as imbalances in neurotransmitters, and may result from other diseases such as stroke and CAD. A meta-analysis of twin and family studies indicates that major depression is more common among close relatives of depressed probands (Sullivan et al. 2000). Across five twin studies, the mean heritability (familial aggregation due to additive genetic factors) of major depression was 37%. Multiple genetic loci have been associated with major depression (OMIM 608516).

The diagnosis of depression is based on criteria listed in the "Diagnostic and Statistical Manual of Mental Disorders, fourth edition" (DSM-IV), which was published by the American Psychiatric Association in 1994. One of the most widely used assessment tools to evaluate the severity of depression is the Beck Depression Inventory II (BDI-II), which is a survey consisting of 21 questions (Beck et al. 1996), modified from an earlier version (Beck et al. 1961). Each item of this questionnnaire corresponds to symptoms of depression listed in the DSM-IV and is given a score based on the patient’s answer ranging between 0 and 3. A total BDI-II score of 0-13 suggests no depression, 14-19 is considered to reflect mild depression, 20-28 is reflective of moderate
depression while 29-63 is compatible with severe depression. This scale of depression severity is recommended by the National Heart Lung and Blood Institute for all depression studies in relation to CVD as it has been highly reliable across different populations (Davidson et al. 2006).

Numerous studies have reported higher incidence of depression (15-20%) among cardiac patients (Lett et al. 2004). The association between depression and CAD was first observed by Carney and colleagues, who reported that 78% of cardiac patients with major depression experienced another cardiac event compared to only 35% of non-depressed cardiac controls (Carney et al. 1988). Since this initial report, more than twenty studies have since documented significant associations between depressive symptoms and recurrent cardiac events (reviewed in (Lett et al. 2004)). Several of these studies also reported that depression is associated with higher prevalence of CAD among individuals with no known history of heart disease (Anda et al. 1993; Pratt et al. 1996; Penninx et al. 2001; Rudisch et al. 2003). Furthermore, CAD patients with depression suffer higher rates of morbidity and mortality (Frasure-Smith et al. 1995; Frasure-Smith et al. 1999).

Several mechanisms have been proposed to account for the association between depression and CAD (Musselman et al. 1998; Carney et al. 2002; Joynt et al. 2003; Lett et al. 2004). For example, it has been argued that links between depression and CAD may be due to an association of depression with other cardiovascular risk factors (e.g., smoking, reduced physical activity, hypertension, diabetes), poor CAD prognosis, cardiac-related disability, and toxicity caused by antidepressants. However, common genetic effects may also underlie the vulnerability to both diseases. In a study of 2,731
male twin pairs from Vietnam, it was reported that common genetic factors account for 20% of the correlation between depressive symptoms and CAD (Scherrer 2003). Taken together with the previous reports of heritability of depression and CAD individually, this study strongly suggests common genetic influences for depression and CAD. The identification of genes that predispose to depression among cardiac patients will help to better treat CAD and reduce mortality.

We hypothesized that genetic variations contributing to risk of depression and CAD may be identified in candidate genes that are involved in biological pathways thought to contribute to both (see review (McCaffery et al. 2006)). For example, inflammation is involved in all stages of atherosclerosis, ranging from leukocyte recruitment to the injured endothelium to the rupture of plaque deposits (Libby et al. 1999; Libby et al. 2002). Chronic inflammation has been shown to predict the development of cardiovascular events among persons with no known CAD. Inflammatory markers such as cytokines (e.g. IL-6, TNF-α (Harris et al. 1999; Ridker et al. 2000)), cellular adhesion molecules (e.g. ICAM-1 (Hwang et al. 1997; Malik et al. 2001)) and acute phase reactants (e.g. CRP (Kuller et al. 1996)) have each been shown to increase risk for CAD among individuals with no previous history of cardiac disease. Studies have also reported that several of these inflammatory markers are associated with depression. For example, an elevation of IL-6 has been associated with depression (Maes et al. 1995; Dentino et al. 1999; Lutgendorf et al. 1999; Kiecolt-Glaser et al. 2002; Miller et al. 2002; Moreno et al. 2002). As IL-6 stimulates release of CRP, it is not surprising that elevations in CRP are also seen among depressed individuals (Miller et al. 2002; Danner et al. 2003). Since inflammation may contribute to the development of CAD and
depression (Kiecolt-Glaser et al. 2002), it is possible that genetic variation related to inflammation could increase risk to both diseases.

The platelet activation pathway has also been associated with both depression and CAD. For example, serotonin (5-HT) contributes to CAD by inducing platelet aggregation and vasoconstriction through binding to its receptor (5-HTR2A) prior to reuptake by the serotonin transporter (5-HTTLPR) (De Clerck 1991). Several investigations have shown increased expression of 5-HTR2A among depressed patients (Biegon et al. 1987; Arora et al. 1989; Biegon et al. 1990). In addition, decreased 5-HTTLPR expression has also been reported among depressed patients (Briley et al. 1980; Langer et al. 1981; Paul et al. 1981; Nemeroff et al. 1988; Nemeroff et al. 1994). This suggests that depressed patients may be particularly vulnerable to platelet aggregation and vasoconstriction associated with CAD (Musselman et al. 1998).

Other pathways which may also underlie the association between CAD and depression include the sympathetic nervous system (Li et al. 2001; McCaffery et al. 2002; Finley et al. 2004), hypothalamic-pituitary adrenal (HPA) cortical axis (Wust et al. 2004; Wust et al. 2004) the parasympathetic nervous system (Neumann et al. 2005) as well as the omega 3/6 metabolic pathway (Covault et al. 2004). For example, polymorphisms in FKBP5, encoding a glucocorticoid receptor-regulating co-chaperone, have been associated with HPA axis response in depression (Binder et al. 2004). In addition, the long-chain fatty acid co-A ligase 4 (FACL4), a key enzyme required for the incorporation of omega-3 and -6 fatty acids into phospholipid membranes, has also been associated with major depression (Covault et al. 2004).
1.5 TYPE 2 DIABETES AND CORONARY ARTERY DISEASE

Diabetes is the sixth most common cause of death in the United States and is associated with life threatening and disabling consequences including heart disease, stroke, hypertension, kidney disease, nervous system disease, blindness and amputations (American Diabetes Association, 2005). This condition is characterized by impaired metabolism of glucose, resulting in abnormally high levels of blood sugar (hyperglycemia) (Tierney 2002). Elevated glucose levels result from insufficient production of insulin by the beta cells of the pancreas (Rother 2007). In Type 1 diabetes (OMIM 222100), this is due to an autoimmune destruction of the pancreatic beta cells. Type 2 diabetes (OMIM 125853) results mainly from insulin resistance in target tissues. Clinical symptoms of hyperglycemia include excessive production of urine (polyuria), excess thirst, blurred vision, weight loss, and lethargy (Tierney 2002).

In 2002, 9.3% (~19,300,000) of U.S. adults over the age of 20 were diabetic, and an additional 26.0% had a impaired fasting glucose (Cowie et al. 2006). Over 90% of diabetes cases are estimated to be type 2 diabetes. Obese adults (Colditz et al. 1990; Harris et al. 1998) and individuals who gain weight over the course of adulthood (Chan et al. 1994; Colditz et al. 1995) present a significantly greater risk for type 2 diabetes than non-obese individuals. In addition, a family history of diabetes also increases an individual’s risk of developing this disease, suggesting a genetic susceptibility to type 2 diabetes (Barroso 2005).

Diabetes increases risk of atherosclerosis and other CVDs due to excess levels of glucose, which can damage the arteries by making the walls thicker and less elastic (Henry et al. 2003). In addition, high levels of glucose increase the viscosity of the blood
(Cinar et al. 2001), making it more difficult to pass through the arteries. Individuals with type 2 diabetes have greater risk of atherosclerosis in the large arteries (e.g. that supply blood to the heart) and develop atherosclerosis at an earlier age (Beckman et al. 2002). Diabetes also increases mortality among patients with CAD (Huxley et al. 2006).

Identification of genes that underlie this disease will improve our understanding of its pathogenesis and assist in the better treatment and prevention of CAD as well.

**1.6 GENETIC MAPPING OF HUMAN COMPLEX TRAITS**

**1.6.1 Genetics of Complex Traits**

Most common diseases (e.g. CAD, hypertension, depression, type 2 diabetes) and quantitative traits (QTs; e.g. BP, body mass index, cholesterol) are referred to as multifactorial or complex traits because these are influenced by multiple genetic and non-genetic factors. One measure of the effect of genetics on susceptibility to common diseases is the increased risk to relatives ($\lambda_R$). For example, among 21,000 Swedish twins followed for 26 years, the relative risk of fatal CAD when one twin died of CAD before the age of 55 years was 8.1 among male MZ twins compared to 3.8 among male dizygotic (DZ) twins (Marenberg 1994). Among women, this risk was 15.0 for MZ twins relative to 2.6 for DZ twins. Another measure of genetic susceptibility is heritability ($h^2$), which estimates the fraction of the phenotypic difference among individuals that can be explained by genetic factors. The $h^2$ of CAD estimated in the above mentioned twin cohort after 36 years of follow-up were 57% and 38% among males and females, respectively (Zdravkovic 2002). In fact, the $h^2$ of most common diseases is estimated to range between 30-50% (Levy et al. 2000; Florez et al. 2003).
The majority of known disease genes underlie Mendelian (single-gene) disorders with fewer genes identified for complex traits (Hugot et al. 2001; Ogura et al. 2001; Rioux et al. 2001; Guo et al. 2004). However, as most human diseases do not follow Mendelian inheritance patterns, it is of great medical interest to identify the genetic basis of complex traits. Unlike Mendelian disorders which have a major effect gene at a single genetic locus, complex traits result from the interactions of multiple gene products with each other and/or with the environment (e.g. diet, smoking, medications) (Strachan 1999). Thus, variations in several genes, each with only modest effects on the phenotype, may determine a complex trait. This makes gene mapping for complex traits more difficult using traditional approaches such as genome wide linkage analysis (Altmuller et al. 2001). Recent technological advances in human genetics have accelerated the identification of genes contributing to complex traits by facilitating large-scale association studies. This section will review the methods for mapping genes underlying human complex traits, recent technological advancements which facilitate human genetic mapping, and current limitations.

1.6.2 History of Human Gene Mapping

One of the earliest associations between heritable variation and human disease susceptibility was reported in 1956 by Clarke et al., who observed that patients with duodenal ulcer often have type O blood (Clarke et al. 1956). By the 1970s, many proteins containing common amino acid variants were found in patients with various diseases, which led to the hypothesis that frequent genetic variations altering protein structure or function could influence risk for common diseases (Harris 1970). Discovery of more abundant DNA polymorphisms in the early 1980s known as restriction fragment
length polymorphisms (RFLPs) marked the beginning of human genetic mapping to identify disease-causing genes (Botstein et al. 1980). These DNA polymorphisms did not directly cause disease, but were used as genetic markers to locate disease-causing genes based on the close physical proximity of the marker and the putative disease gene. This is because recombination during meiosis rarely separates two loci that are very close on a chromosome, which forms the basis of linkage analysis (Morton 1955). Positional cloning by linkage analysis identifies a disease locus based on its proximity to a polymorphic marker, without prior knowledge of gene function. This offers an unbiased approach to identify candidate loci underlying a phenotype.

The first comprehensive map of genetic markers consisted of approximately 400 RFLPs (Donis-Keller et al. 1987). These were not very informative as each marker was bi-allelic and difficult to genotype using southern blots. The second genetic map was based on microsatellite markers (mostly (CA)n repeats), which are more abundant, highly informative (multi-allelic) and easier to type (Weissenbach et al. 1992). The development of dense genetic maps, combined with the wide-spread use of PCR, led to the identification of 50 disease genes by 1995 (Collins 1995). The human genome project (HGP), initially released in 2001 and completed in 2003, identified more than 10,000 highly polymorphic markers (Collins et al. 1996; Broman et al. 1998; Lander et al. 2001; Venter et al. 2001).

Revolutionary advances in human genetics during the past decade have dramatically improved genetic mapping of human traits. Currently, the molecular basis of 2352 human phenotypes has been identified, although most are Mendelian traits (OMIM). In addition, more than 11 million single nucleotide polymorphisms (SNPs)
have been identified in the human genome, of which more than 5 million have been validated by multiple groups (National Center for Biotechnology Information (NCBI); International Hapmap project). Although SNPs have limited informativeness (biallelic) compared to multiallelic microsatellites, this is compensated by their high abundancy in the human genome and the capacity to generate high-throughput genotype data. New technology developed over the past few years allows for large numbers of SNPs to be typed rapidly and at low cost in a large sample size. Such large genetic studies are also facilitated by new computational tools for management and analysis of large datasets.

1.6.3 Phenotype

Phenotypes of interest for genetic investigations are usually disease status or risk factors for disease. Disease status is often a dichotomous trait (affected or unaffected) but many diseases have quantitative precursors. For example, CAD is a disease with multiple quantitative risk factors such as BP, cholesterol levels, body mass index, blood glucose, and depressive symptoms. Although it is of great medical interest to investigate the genes underlying a disease, it is sometimes more advantageous to study a quantitative trait that confers disease risk (Newton-Cheh et al. 2005). This is especially important when the disease is difficult to diagnose so that several different diseases are often grouped together. For example, stroke is a group of heterogeneous disorders that are difficult to distinguish clinically and are often misdiagnosed as the same disease. In addition, quantitative traits are often easier to measure, thereby reducing error and increasing heritability of the phenotype. BP is an example of a trait that can be measured with high accuracy and $h^2$ estimates of BP indicate that there is a strong genetic contribution (Hottenga et al. 2005).
There are several other strategies for narrowing the definition of a phenotype for genetic investigations. For example, an early age of onset for complex traits such as Alzheimer's disease, breast cancer, and CAD indicates greater heritability and genetic homogeneity. The mode of inheritance for early onset Alzheimer's disease resembles an autosomal dominant Mendelian disease (OMIM 104310). Genetic heterogeneity may also be reduced by including only individuals with a family history of the disease or to focus on specific ethnic groups. For example, founder populations such as the Ashkenazi Jews, French Canadians and Amish populations in the US are ideal for genetic investigations as fewer disease alleles are represented in each population due to the fact they descended from a small number of ancestors. In addition, one may also choose to include only individuals whose phenotypic measurements fall at extreme ends of the trait distribution (i.e. BP).

Once an unambiguous phenotype is diagnosed or measured, it is critical to determine the genetic basis for this trait. It is possible for a trait to run in families because of shared environment (e.g. diet, physical activity, education) rather than a genetic contribution. The importance of shared environment among family members is illustrated by a study which reported a recessive gene for attending medical school as result of ignoring common environmental factors within families (McGuffin et al. 1990). As mentioned above, the genetic contribution to a trait may be determined by measuring the risk to a relative of an affected proband ($\lambda_R$) or the heritability of the trait ($h^2$). In addition, twin studies, adoption studies, and segregation analysis within families determine the genetic contribution to a trait (Strachan 1999).
1.6.4 Linkage Analysis

Positional cloning by linkage analysis requires the ascertainment of families with one or more affected individuals. These pedigrees are genotyped for a set of a few hundred/thousand polymorphic markers evenly spaced across the genome at intervals of several megabases (Marshfield Clinic Resource). Large families containing multiple affected members provide more power to detect the location of a disease gene as it is assumed that all affected individuals within one family share the same mutation. However, smaller families are often easier to collect. Linkage studies involving more than one smaller family have higher risk of genetic heterogeneity (the same phenotype may be caused by mutations in different loci).

Linkage analysis calculates the likelihood that a marker or set of markers are linked (recombination fraction $0 < 0.5$) or not to a disease locus (recombination fraction $0 > 0.5$) (Morton 1955; Terwilliger et al. 1994). The ratio of these likelihoods is converted into a LOD (logarithm of the odds) score. A LOD less than -2 indicates that the marker is not linked to the disease whereas a LOD of 3 or greater indicates linkage. That is, a LOD of 3 suggests that the odd of the marker being linked to the disease gene is 1000:1 with a 5% chance of error.

Whole genome linkage analysis is a comprehensive search of the entire genome for loci that contribute to a phenotype. The first successful gene identification using this approach was published in 1986 for chronic granulomatous disease (Royer-Pokora et al. 1986). Since then, positional cloning has successfully mapped genes for many other Mendelian disorders such as duchenne muscular dystrophy, cystic fibrosis, Huntington disease, adult polycystic kidney disease, breast cancer. This approach was less successful
for mapping complex traits that often result from multiple loci of modest effects. There are few examples of genes identified through linkage analysis for complex traits such as Crohn’s disease, type 1 diabetes, stroke and type 2 diabetes (Altmuller et al. 2001; Hugot et al. 2001; Gretarsdottir et al. 2003; Guo et al. 2004; Grant et al. 2006).

Linkage analysis for complex traits differs from Mendalian diseases as the mode of inheritance (i.e. autosomal dominant, autosomal recessive, X-linked) is unknown. Softwares developed for linkage analysis of Mendalian traits (e.g. LINKAGE or GENEHUNTER) require specification of the mode of inheritance. Linkage analysis of complex traits uses a non-parametric or model-free method that does not require this information. Examples of such programs include the Sequential Oligogenic Linkage Analysis Routines (SOLAR) (Almasy et al. 1998) and MERLIN (Abecasis et al. 2002). These programs identify haplotypes that are shared in excess among affected relatives, which are identical by descent (IBD), as these are expected to contain the disease causing gene.

1.6.5 Genetic Association Studies

Association calculates the rate of co-occurrence between a certain allele and phenotype within a population. That is, allele 1 is associated with disease D if people who have D also have allele 1 more (or less) often than expected based on the frequencies of the disease and allele in the population. A genetic marker may be associated with a trait for a number of reasons (Cordell et al. 2005): 1) the polymorphism increases the risk for the trait; 2) the polymorphism does not affect risk but is highly correlated with a nearby risk allele (linkage disequilibrium (LD)); 3) the association is due to some underlying population stratification or admixture (e.g. a common trait in one ethnic group may be
positively correlated with any allele that also happens to be common in that population); 4) natural selection (e.g. individuals with the polymorphism or another strongly correlated polymorphism nearby are more likely to survive and reproduce).

Traditionally, association studies represent an important step in the fine-mapping of genetic loci initially detected by linkage. This is because the candidate regions identified by linkage analysis usually span a large chromosomal region (e.g. several megabases) containing several genes. Association analysis has the potential to locate a susceptibility factor within a large chromosomal region by using the strong correlations among markers located close together (LD) as a mapping tool. That is, the alleles at two nearby loci co-occur more often than expected by chance. The gene for cystic fibrosis was identified using a combination of linkage and association analyses (Kerem et al. 1989).

In recent years, technological advances coupled with a better understanding of LD patterns in the human genome have made genome-wide association studies (GWAS) involving hundreds of thousands of markers more affordable, resulting in the identification of more than 50 disease loci (McCarthy et al. 2008). Since most of the genome falls into blocks of strong LD, within which most genetic polymorphisms are correlated with one another, it is possible to select a single ‘tag’ polymorphism (Johnson et al. 2001) for genotyping that will capture most variants in that haplotype. This tag marker provides information about the nearby variants which are not genotyped, thereby reducing the number of markers needed for genotyping, but does not decrease the power of a study (Zhang et al. 2005). In addition to identifying new polymorphisms, the primary goal of
the HapMap project is to identify tag SNPs across the entire genome in order to facilitate GWAS.

The threshold for significance is critical for association studies of complex diseases as the risk of false-positive results is higher than that for linkage analysis. This is due to the fact that a true association may not exist between any of the genotyped markers and the phenotype of interest. Furthermore, each association test carries an independent risk of false positive results. To reduce risk of an erroneous association, the significance threshold is modified to control for the number of independent tests such as the Bonferroni correction factor, which divides the p value threshold (0.05) by the number of tests or markers. Because of the high risk for false-positives, it is important that an association can be replicated in different populations. In a review of the literature, only 6 of 166 associations were replicated by at least 75% of subsequent studies (Hirschhorn et al. 2002). The failure to replicate the association may be explained by small sample sizes, the modest effects of the causal variants on disease risk or genetic heterogeneity.

1.6.6 Bioinformatics

Once a chromosomal region has been isolated by linkage and/or association analysis, candidate genes in that region may be identified using a number of bioinformatics tools. The HGP has made physical mapping of a candidate region obsolete as the complete genomic sequence is available on public databases such as National Center for Biotechnology Information (NCBI) and University of California at Santa Cruz (UCSC) genome browser. This sequence data may be used to design primer pairs for mutation screening experiments. In addition, these websites contain sequence data for numerous
other species such as the mouse, chimpanzee and dog, which allows comparisons to be made with human data to predict gene structure and potential regulatory sequences. Other bioinformatics tools include programs that predict gene structure (e.g. promoter, transcription factor binding sites, splice sites), expression data (to determine if the candidate gene is expressed in biologically relevant tissues), 3D modeling (e.g. for validation of mutation found), and sequence alignment programs (Fox et al. 2006). Finally, bioinformatics tools allow genes to be prioritized for mutation screening based on known or suggested function.

1.6.7 Mutation Screening

In principle, genetic variation contributing to a common disease may be rare (frequency < 1% in the population) or common (frequency > 1% in the population). Most of the genetic variations in humans are common with allele frequencies ≥ 5% (Sachidanandam et al. 2001; Gabriel et al. 2002), which suggests that the majority of random genetic variations are evolutionarily neutral (Pritchard 2001; Reich et al. 2001). In addition, genetic variations underlying common traits may be coding or non-coding. Coding changes may be missense, nonsense, splicing, deletion or insertion, which represent 90% of all known mutations listed in the Human Gene Mutation Database of the Institute of Medical Genetics of Cardiff (Table 1). The remaining 10% of mutations are small regulatory lesions or gross deletions and insertions, rearrangements and repeat variations. The unbalanced ratio of coding to non-coding mutations may be explained by the fact that most gene-screening efforts to date have mostly focused on the coding sequences of genes as non-coding DNA is poorly annotated.
<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Number of Entries</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>micro-lesions</strong></td>
<td></td>
</tr>
<tr>
<td>missense/nonsense</td>
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</tr>
<tr>
<td>splicing</td>
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<td>776</td>
</tr>
<tr>
<td>small deletion</td>
<td>9526</td>
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<tr>
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<td>gross insertion</td>
<td>513</td>
</tr>
<tr>
<td>complex rearrangements</td>
<td>419</td>
</tr>
<tr>
<td>repeats</td>
<td>147</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>58257</strong></td>
</tr>
</tbody>
</table>

Table 1. Number of entries in the Human Genetic Mutations Database by mutation type as of July 2008.

Non-coding variants only recently became associated with complex traits (Hirschhorn et al. 2002). Several reports predict that most complex traits are mediated by non-coding variations (Mackay 2001; Korstanje et al. 2002; Birney et al. 2007). However, it remains difficult to locate potentially regulatory regions in non-coding DNA. Genome wide linkage and association studies suggest that 1-3% of genes have common cis-acting variants, which are located close to the gene it regulates (Cheung et al. 2005; Stranger et al. 2005). For example, of 144 functionally significant polymorphisms tested by reporter assays, one quarter were located more than one kb upstream or 3’ to the gene (Rockman et al. 2002). However, polymorphisms known as trans-acting elements may also regulate the expression of another gene located elsewhere in the genome. Yeast and
mice studies have shown that trans-acting loci accounts for the majority of variability in gene expression (Brem et al. 2002; Schadt et al. 2003; Yvert et al. 2003). However, testing the potential function of non-coding variants is more difficult and remains a challenge in human genetics.

1.6.8 Validation

The identification of a genetic variant in a candidate gene may be followed by several validation steps. For linkage studies, segregation of the mutation with the disease haplotype is confirmed within the linked families. Next, the allele frequency is determined in a control population. If absent or less frequent among unaffected controls, this gene may be screened in other affected cases or families with the same phenotype to identify new mutations in the same gene. In the case of association studies, the mutation is genotyped in a large cohort of cases and controls to determine association with the phenotype of interest. If suggestive or significant association is shown, the association is replicated in other populations with the same disease.

Several experimental approaches can be used to determine the biological significance of the identified genetic variations. For instance, cultured cell lines (e.g. lymphoblasts or fibroblasts) of affected and control individuals may be used to compare the expression of the gene of interest using methods such as quantitative real-time PCR and Western blots. If the variation is located in regulatory regions, reporter gene assays (e.g. Luciferase) can be used to compare the gene expression level correlated with each allele. In addition, cellular and animal models can be developed to study the effect of overexpression or down regulation of the gene. This can be achieved through the use of
transient expression vectors or small interfering RNA (siRNA) in cells and the development of transgenic or knockout animal models. Currently, the choice of animal models is not only limited to mouse as various organisms (e.g. drosophila, nematode, zebrafish) have been developed to evaluate the disease causative nature of a gene of interest.

1.6.9 Limitations

It is often impossible to find a genetic marker that cosegregates perfectly with a complex trait (Lander et al. 1994). For example, some individuals who have the susceptibility allele may not manifest the disease (incomplete penetrance) or certain individuals without the susceptibility allele develop the disease as result of non-genetic causes (phenocopies). Thus, the susceptibility allele may increase risk for a disease without causing it. Another explanation for imperfect cosegregation of a marker and trait is locus heterogeneity, whereby mutations in one of several genes may result in the same disease or trait. Therefore, a chromosomal region may segregate with a disease in some individuals or families but not others. It is also possible that some traits require the simultaneous presence of several mutations in multiple genes (polygenic inheritance). Genetic mapping studies of polygenic traits are complicated in that a single locus is neither necessary nor sufficient to produce the trait. Another complication of genetic mapping for complex traits is the high frequency of disease-risk alleles. It is often difficult to distinguish a common variant causing a prevalent disease from a benign polymorphism that is frequent in a population. Moreover, epigenetic factors (non-DNA variations such as methylation patterns) and genetic imprinting (differential activity of the paternal and maternal alleles) may alter gene expression to cause disease. Common mutation
screening experiments do not detect such epigenetic variations. Finally, mitochondrial
inheritance may also affect phenotype and lead to imperfect segregation with a nuclear
 genetic marker.

1.6.10 Benefits

Genetic investigations of human traits provide insight into the molecular mechanisms
underlying human diseases. Developments in human genetics during recent decades have
allowed the identification of genes for numerous Mendelian disorders (OMIM). Gene
identification has led to the development of treatments for many of these diseases
(Brinkman et al. 2006). These include novel drug targets, gene transfer, antisense or
RNAi technology for dominant disease, and embryonic stem (ES) cell therapy (O'Connor
et al. 2006). In addition, gene identification for Mendelian disorders have also led to
better prevention of diseases such as prenatal screening for Phenylketonuria (PKU)
(Scriver 2007). The advent of high-throughput technology (genotyping and
computational tools) during the past few years have made large-scale, genome-wide
association studies a feasible approach for gene identification, which has proven to be
more successful for the identification of genes for complex traits as these often have
modest effects on phenotype. Since most human traits and diseases are complex in
nature, this increased ability to locate genes for complex diseases has enormous medical
implications.

In recent years, many pharmaceutical companies have invested in genomics
research to identify novel drug targets as well as to predict negative response to drugs.
For example, most drugs for common diseases such as CAD have been associated with
one or more ADRs. As each drug developed costs hundreds of millions to billions of
dollars and years of investment for a pharmaceutical company, the potential loss is substantial if a drug is removed from the market or prohibited from sale due an ADR in certain individuals. Numerous drugs have been withdrawn from the market because of safety concerns (Need et al. 2005). In addition, there has been a steady decline in the annual number of drugs approved by the Food and Drug Administration (FDA) in the past decade. For example, a high incidence of angioedema associated with vasopeptidase inhibitors (Coats 2002), which inhibit ACE and NEP in the BK pathway, led to a FDA rejection which resulted in devastating loss for the pharmaceutical company Bristol Myers Squibb. If pharmacogenomics can identify individuals who are at risk of ADRs or those who will respond positively, this can improve the rate of FDA approval and reduce costs of clinical trials. Furthermore, genetic tests for predicting ADR risk would reduce the rate of ADR-related mortality.
CHAPTER 2: ANGIOEDEMA ASSOCIATED WITH ANGIOTENSIN I-CONVERTING ENZYME INHIBITORS

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Reference:

2.1 RATIONALE

Our pharmacogenomic investigation of ACEi drugs began in 2000 with the collection of AE and HSR cases. As outlined in the previous chapter, a number of studies had suggested that the BK pathway plays an important role in the pathogenesis of both ADRs. In particular, two reports identified impaired degradation of des-Arg$^9$-BK, the active metabolite of BK, among cases who had developed AE and HSR during ACEi therapy (Blais et al. 1999; Blais et al. 1999). Given that APP is the main metabolizing enzyme of BK and des-Arg$^9$-BK in the presence of ACE inhibition (Blais et al. 2000, we hypothesized that genes regulating this enzyme may determine risk of AE and HSR associated with ACEi. We measured APP activity using a previously described assay in the plasma samples of our cases, which were isolated from whole blood (Simmons et al. 1992). Although our collaborators had developed several antibodies for quantification of APP levels, these did not successfully detect APP in human plasma samples (A. Adam, personal communication).

There are two known forms of APP in humans: cytosolic and membrane-bound (hcAPP, hmAPP), encoded by two different genes: XPNPEPL, XPNPEP2. These genes were cloned prior to the completion of the human genome project (Sprinkle et al. 1998; Sprinkle et al. 2000), which enabled our initial mutation screening experiments. Although a previous study had suggested that hmAPP is the main contributor to plasma APP activity as this isoform is located on the extracellular membrane of endothelial cells (Ryan et al. 1996), we included both APP genes in our genetics study. In addition, we hypothesized that other modifier genes may also regulate APP activity. Thus, we
performed a genome wide scan using the families of our affected cases to identify new candidates.

Several major developments in human genetics occurred in the early years of this project which facilitated our pharmacogenomic study. For example, the human genome sequence, initially released in 2001 (Lander et al. 2001; Venter et al. 2001), made available dense microsatellite maps (Marshfield Clinic Resource) for genome wide scans and publically accessible human genomic sequences for mutation screening experiments. In addition, the release of the mouse genomic sequence in December 2002 and alignment with the human genome allowed for comparative genomics to identify potentially functional regions of non-coding DNA. Finally, the availability of variance component linkage analysis programs such as the Sequential Oligogenic Analysis Routines (SOLAR) (Almasy et al. 1998) enabled us to use large pedigrees for linkage analysis of complex quantitative traits.
2.2 A VARIANT IN \textit{XPNPEP2} IS ASSOCIATED WITH
ANGIOEDEMA INDUCED BY ANGIOTENSIN I-CONVERTING
ENZYME INHIBITORS

2.2.1 Abstract

Angiotensin I-converting enzyme inhibitors (ACEi), which are used to treat common cardiovascular diseases, are associated with a potentially life-threatening adverse reaction known as angioedema (AE-ACEi). We have previously documented a significant association between AE-ACEi and low plasma aminopeptidase P (APP) activity. With 8 large pedigrees, we hereby demonstrate that this quantitative trait is partially regulated by genetic factors. We tested APP activity using a variance component quantitative trait linkage (QTL) analysis of a 10-cM genomewide microsatellite scan enriched with 7 markers over 2 candidate regions. We found significant linkage (LOD = 3.75) to a locus that includes the \textit{XPNPEP2} candidate gene encoding membrane-bound APP. Mutation screening of this QTL identified a large coding deletion segregating in one pedigree and an upstream single nucleotide polymorphism (C-2399A SNP), which segregates in the remaining 7 pedigrees. Measured genotype analysis strongly suggests that the linkage signal for APP activity at this locus is accounted for predominantly by the SNP association. In a separate case-control study (20 cases and 60 controls), we found significant association of this SNP to ACEi-induced AE \((P = 0.0364)\). In conclusion, our findings provide supporting evidence that the C-2399A variant in \textit{XPNPEP2} is associated with reduced APP activity and a higher incidence of AE-ACEi.
2.2.2 Introduction

Angiotensin I-converting enzyme inhibitors (ACEi) are a class of drugs used by over 40 million patients worldwide for the treatment of cardiovascular diseases such as hypertension, congestive heart failure and diabetes (Unger et al. 1994; Brown et al. 1998). Angioedema (AE) associated with ACEi therapy (AE-ACEi) is a potentially fatal adverse event that affects 0.1-0.7% of Caucasian patients (Israili et al. 1992; Vleeming et al. 1998) and is 4-5-folds more prevalent among African Americans (Brown et al. 1996). This racial difference suggests that genetic factors modulate AE risk but environmental factors are also important since smokers taking ACEi have an increased susceptibility to AE (Coats 2002; Kostis et al. 2004). The incidence of AE-ACEi is likely underestimated as the clinical symptoms could develop years after starting ACEi therapy, thus obscuring its relationship with the drug and often leads to misdiagnosis (Agostoni et al. 2004).

A majority of AE-ACEi cases do not respond to anti-histamines or corticosteroids, indicating that these are not allergic reactions (Agostoni et al. 2001). Currently, there is no effective treatment for AE-ACEi and no method for identifying individuals with increased susceptibility to this adverse reaction. Better understanding of the pathogenetic mechanism underlying this type of AE may also have significant implications for AE associated with other vasopeptidase inhibitors (i.e. Omapatrilat), which block the activities of both ACE and neutral endopeptidase (NEP). The AE risk associated with these drugs is even higher than with ACEi (Coats 2002) and has curtailed the regulatory approval for usage of these vasopeptidase inhibitors to treat cardiovascular diseases.

Previous reports have suggested that bradykinin (BK), a potent vasodilatory and pro-inflammatory nonapeptide, plays a central role in the pathophysiology of AE-ACEi
BK is rapidly degraded in the plasma of healthy individuals by angiotensin I-converting enzyme (ACE) and aminopeptidase P (APP) (Bhoola et al. 1992). Kininase I enzymes normally transform a minute fraction (3.5%) of BK into its active metabolite des-arginine$^9$-bradykinin (des-Arg$^9$-BK) (Blais et al. 2000). This carboxy-truncated metabolite is in turn broken down by APP and ACE (Cyr et al. 2001). In the presence of ACE inhibition, however, Kininase I activity is increased (transforms 28% of BK into des-Arg$^9$-BK) and APP acts as the major metabolizing enzyme of both BK and des-Arg$^9$-BK (Blais et al. 2000).

An increase of BK has been measured in plasma of patients during episodes AE-ACEi, but unlike hereditary forms of AE (OMIM 106100), there is no increase in cleavage of the BK precursor, high molecular weight kininogen (HK) (Nussberger et al. 1998; Agostoni et al. 1999; Cugno et al. 2003). This suggests that impaired BK metabolism, rather than an increased production, plays an important role in AE-ACEi. We previously reported significantly lower plasma APP activities in patients with a history of AE-ACEi, which is strongly correlated with a significant decrease in des-Arg$^9$-BK degradation in vitro (Blais et al. 1999; Adam et al. 2002; Molinaro et al. 2002). Blais et al. demonstrated that half of AE-ACEi cases has an enzyme defect involved in des-Arg$^9$-BK metabolism (Blais et al. 1999). Accumulated levels of des-Arg$^9$-BK has been shown to cause proinflammatory effects in vivo (Blais et al. 1997; Blais et al. 1999). One study reported that ACEi patients who received subcutaneous injections of the APP inhibitor, Apstatin, developed local inflammations (Kim et al. 2000). Thus, previous data indicate that reduced plasma APP activity may predict increased risk for AE associated with ACEi therapy.
The aim of this study is to determine whether plasma APP activity is regulated by genetic factors and to identify the quantitative trait locus (loci) which confer susceptibility to AE-ACEi. There are 2 known APP enzymes in humans, membrane-bound (mAPP) and cytosolic APP (cAPP). The gene encoding the former is XPNPEP2 (OMIM 300145), localized to chromosome Xq26.1 (Sprinkle et al. 1998) and the latter is the product of XPNPEPL (OMIM 602443) on chromosome 10q25.1 (Sprinkle et al. 2000). While these represent good candidate genes for the inter-individual variability in plasma APP activity, other genetic loci may serve as important regulators. Plasma APP activity have been shown to form a continuous distribution in the general population (Cyr et al. 2001), suggesting that plasma APP activity is a complex quantitative trait likely influenced by multiple genetic loci and nongenetic factors (e.g. smoking, hormone replacement (Gallagher et al. 1999)). Identification of the genetic factors underlying reduced plasma APP activity would provide a better understanding of the pathogenesis of AE-ACEi and may facilitate the development of a clinical assay to detect those individuals with greater AE risk.

2.2.3 Subjects and Methods

Blood and Plasma Samples

The ethics committees from Centre Hospitalier de l'Université de Montreal, Institut de Cardiologie de Montreal, and McGill University reviewed and approved all protocols involving human subjects. Informed consent was obtained from all participants. DNA extraction from blood employed a standardized protocol (Gentra Systems).
Participants

Cohort 1 (Figure 1a), comprised of 5 Caucasian pedigrees (88 DNA samples), was collected for a genomewide microsatellite scan followed by a variance component linkage analysis for APP activity. Cohort 2 (Figure 1b), consisting of 3 Caucasian pedigrees (35 DNA samples), was subsequently ascertained for genotyping of 11 microsatellite markers, which cover 4 genomic regions that provided LOD scores > 1 in the linkage analysis of cohort 1 and the locus including the XPNPEPL candidate gene. Plasma samples were also collected from all 123 participants for characterization of APP activity. The kindreds were ascertained from Canada (Montreal) and France based on the presence of one individual in each family with a history of AE and/or another acute adverse affect related to ACEi therapy, anaphylactoid reactions (AR-ACEi) (Bright et al. 1999). The clinical diagnosis was not considered for linkage analysis due to lack of power. Instead plasma APP activity measurements were used as the quantitative trait in a variance component linkage analysis.

DNA from 20 independent AE-ACEi cases (supplemental Table 1) was extracted for mutation screening experiments. All cases were Caucasians with a history of AE-ACEi originating from Canada (n = 7), Belgium (n = 3) and USA (n = 10). Plasma samples were available for all AE cases except two (USA). The onset of clinical symptoms ranged from a few hours up to 8 years after starting ACEi therapy. In addition, we collected 3 unrelated Caucasian controls to match each AE patient for country of origin and gender (n = 60). Controls from Belgium and Canada had no history of ACEi therapy whereas the medical histories were unknown for USA controls.
Controls were not matched for age as we did not find age effects in our samples, which is in agreement with previous findings (Cyr et al. 2001).

Characterization of Plasma APP Activity

APP activity was measured in plasma using a modification of an assay previously described (Simmons et al. 1992). Our assay used the synthetic substrate Arginine-Proline-Proline. Plasma APP activity is expressed as nanomoles of Arginine released per minute per millilitre of plasma sample (1 unit = 1 nmol/min/ml).

Genome Scan and Microsatellite Genotyping

A 10 cM genomewide microsatellite scan (marker panel SS4 on the ABI-3700 DNA analyzer at the Genome Quebec Innovation Centre, Montreal, Canada), enriched for 7 markers flanking the two APP candidate genes on chromosomes 10 and X (D10S534, D10S1741, D10S562, DXS1212, DXS8057, XPNPEP2_CA, DXS1047), was performed on cohort 1 (total of 397 microsatellite markers). Primers for the XPNPEP2_CA repeat marker located upstream of XPNPEP2 (approximately 4 kb) were designed for this study: sense 5'-GCTCTTTCCCCCTGCTGTGT-3’, antisense 5’-GGTGCTGTGGGGTGCCCTCATC-3’.

Genotyping of the 11 genome-scanned markers in cohort 2 were conducted in our laboratory. Genomic DNA was amplified by radiolabeled (α-35S-dATP) PCR. The products were separated by electrophoresis on 6% denaturing polyacrylamide gels. The alleles were sized by comparison to the M13mp18 sequence ladder, and each individual
was assigned a genotype. Marker-allele sizes and frequencies were obtained from the Fondation Jean Dausset CEPH database.

Statistical Analyses

The SOLAR (Sequential Oligogenic Linkage Analysis Routines, version 2.1.4 Official) (Almasy et al. 1998) program was used to estimate heritability of plasma APP activity and to perform a variance component linkage analysis. Age and sex were considered as covariates, but were not significant ($P > 0.05$). APP activity was transformed for normality to avoid convergence failure of SOLAR by using the Box-Cox transformation (Box et al. 1964) such that $T(\text{APP}) = \left( \left( \text{APP} + 1 \right) \times 0.40 - 1 \right) / 0.40 \times 4$. The two-point (all chromosomes) and multipoint (autosomes only) variance component linkage analyses were conducted for Box Cox transformed plasma APP activity in cohorts 1 and 2. Empirical LOD adjustment based on 10,000 simulations was used for all linkage analyses. QTL results of the genomewide scan for the 5 pedigrees in cohort 1 were adjusted with an empirical LOD factor of 0.98220 (results shown in Figure 2). QTL results of cohorts 1 + 2 were corrected with an empirical LOD adjustment factor of 0.74595 (Table 1).

A measured genotype approach using variance component analysis (Soria et al. 2000) in SOLAR was used to test for the C-2399A SNP genotype-specific differences in plasma APP activity means at the XPNPEP2_CA marker. This approach accounts for the different relationships among family members in performing association on the measured genotypes and quantitative trait (Box Cox transformed plasma APP activity). The three genotypes of the C-2399A SNP, CC, CA and AA were coded as -1, 0, and 1 in females.
and the C and A hemizygous genotypes in males as -1 and 1, respectively. The measured genotype test was conducted on all families except pedigree VI, which segregates the coding deletion accounting for the APP variation in this family. Other statistical analyses were conducted in SAS v9.1.3. All tests were two-sided.

Mutation detection

Oligonucleotides for polymerase chain reaction (PCR) were designed from the genomic sequences for human XPNPEP2 (accession AL023653) and XPNPEPL (accession AL354951), obtained from the National Centre for Biotechnology Information (NCBI) database. The exons, 5' and 3' untranslated regions of both candidate genes were amplified using genomic DNA and radiolabeled deoxyadenosine triphosphate for single-stranded conformational polymorphism (SSCP) analysis. Electrophoresis of PCR products was performed on 9.5% polyacrylamide (5% glycerol) and on 50% mutation detection enhancement gels (Biowhittaker Molecular Applications, Rockland, Me), followed by autoradiography. Samples showing altered migration patterns were selected for sequencing. The coding regions of XPNPEP2 were sequenced in all AE-ACEi cases with APP activity < 10 units. All sequencing was performed at Genome Quebec Innovation Centre, Montreal, Canada. Variations that altered a restriction enzyme digestion site were genotyped in additional individuals using restriction fragment length polymorphism (RFLP) assays. The silent variant in exon 6 of XPNPEP2 (SNP rs3747343) was genotyped by EcoNI digestion.

The C-2399A SNP was genotyped using a modification of the allele-specific PCR assay previously described (Germer et al. 1999). This required 3 PCR primers for each
polymorphic site (sequences available upon request): one common and two allele-specific oligonucleotides. The allele-specific primers differed only at the position of the most 3’ nucleotide. Two standard PCRs were performed using the common primer and either one of the allele-specific primers. Products were visualized on 1.5% agarose gels by electrophoresis.

*mRNA Analysis*

Extraction of total RNA from suspended lymphoblast cells employed a standardized protocol (Qiagen). Synthesis of cDNA required 2 steps: 1) annealing oligoDT primers and random hexamers to the RNA template in an initial reaction mix (3 µg of total RNA, 1 µg each of oligoDT primers and random hexamers, and HPLC-grade water), incubated at 70°C for 3 minutes; 2) extension with the addition of a second reaction [1 µl of Moloney murine leukemia virus reverse transcriptase (100U), 10 µl of 5x First Strand Buffer, 5 µl of 0.1 M DTT, 1µl of RNase Inhibitor and 2 µl of dNTP (25mM)], incubated at 37°C for 1 hour. Primer pairs for PCR amplification of cDNA were designed from the transcript sequence of the human *XPNPEP2* gene (accession U90724), obtained from the NCBI database. We applied a nested PCR protocol for amplification of the cDNA to compensate for the low gene expression of *XPNPEP2* in lymphoblasts (Venema et al. 1997). Products from the first PCR were diluted 30 fold and then used as template DNA in the second PCR. Amplified products were visualized on 1.5% agarose gels and then sequenced.
2.2.4 Results

Quantification of Plasma APP Activity

The mean plasma APP activity among our AE-ACEi cases was 13±3 units. There was a non-significant gender difference (t-test, p=0.2549) with mean APP values of 15±4 units for women and 9±6 units for men. These mean values for AE-ACEi patients differ from those previously reported in healthy Caucasians (24 ± 9 and 19 ± 7 units in women and men, respectively) (Cyr et al. 2001). Moreover, the majority (67%) of our AE-ACEi cases have APP values < 10 units, which is comparatively more than 10% previously reported for the general population (Cyr et al. 2001). However, a third of our affected patients have normal or high plasma APP activity (> 20 units), suggesting that additional factors are likely involved in determining risk to AE-ACEi.

Mutation Screening of Candidate Genes

DNA from 10 unrelated individuals with plasma APP activities < 10 units and a history of AE-ACEi were screened for mutations in XPNPEP2 and XPNPEPL by SSCP. A band variant was detected in exon 6 of XPNPEP2 in 4 individuals. Sequencing revealed a T to C substitution (SNP rs3747343), which does not result in an amino acid change. Genotyping by RFLP in the family members of these cases and 20 healthy individuals showed no correlation between this variant and plasma APP activity. No other sequence variants in the candidate genes were detected in these subjects.

Given the absence of a causative mutation, we concluded that if a functional variant conferring risk to AE-ACEi exists within these candidate loci, it might be located in regulatory regions affecting transcription, splicing, or message stability. Alternatively,
APP activity might be regulated by other genetic loci. For example, variants in genes coding for transcription factors might modulate expression of the APP coding gene(s). Genome-wide studies in yeast have shown that 75% of transcripts are regulated by trans-acting elements (Yvert et al. 2003). Previous studies have also shown that expression differences in humans can be explained by genetic variations located elsewhere in the genome [e.g. Wilm’s Tumor (Discenza et al. 2004)]. Thus, we conducted a genome-wide scan and variance component linkage analyses in addition to further mutation screening experiments involving additional AE-AEi cases in order to identify other potential genetic loci regulating APP activity.

Variance Component Linkage Analysis

Cohort 1 (Figure 1a), included in the genome scan and characterized for plasma APP activities, was tested for variance component QTL analysis using SOLAR. The mean of the transformed trait in the 5 pedigrees was 16.8 units with standard deviation of 6.3 units, skewness of -0.17 and kurtosis of -0.14. Heritability of APP activity was estimated at 37.5% ± 26.5 (P = 0.0336), indicating that the observed phenotypic variability within these families partly results from genotypic differences. The genome scan performed on cohort 1 resulted in the identification of 3 loci with LOD scores > 1 in multipoint linkage analysis (Figure 2a) on chromosomes 6, 8, and 9, and one region on chromosome X that included the XPNPEP2 candidate gene with a two-point LOD = 2.15 (Figure 2b).

We proceeded to genotype cohort 2 (Figure 1b) for 11 genome scanned microsatellite markers covering the 4 regions above and the XPNPEPL candidate gene (Table 1). Heritability of plasma APP activity for all 8 pedigrees combined was
estimated at 33.6% ± 25.1% (S.E), with a p-value = 0.0452. Analyses of the combined genotype data from cohorts 1 and 2 produced a maximum two-point LOD score of 3.75 for the marker XPNPEP2-CA (Table 1). Review of the linkage data strongly suggested that the XPNPEP2 locus is a major genetic factor controlling APP activity in our families despite no mutation found in the initial mutation screening experiments. This locus, however, is not the only regulator of APP activity as multipoint linkage analysis in cohort 1 (Figure 2a) provided strong linkage to chromosome 6 (LOD score = 3.47), but the linkage signal was greatly reduced in combined analyses of both cohorts (LOD score = 1.43). Our linkage analysis also provided positive linkage to loci on chromosomes 8 and 9. Further investigation of these loci is necessary to either confirm or reject linkage.

Mutation Screening of the XPNPEP2 Locus

Given evidence of strong linkage to the XPNPEP2 locus, we sequenced the coding regions of this candidate gene in all available AE-ACEi cases with APP activities < 10 units. A coding deletion in XPNPEP2 was detected in the proband of pedigree VI (Figure 1b) (not included in the initial mutation screening experiments) who first suffered an anaphylactoid reaction (AR) associated with ACEi during haemodialysis and subsequently suffered an AE-ACEi episode. The 175 bp genomic deletion (g.2953-3127del) includes 16 bp at the 3’ end of exon 2, the donor splice site and a fragment of the downstream intron (Figure 3a). The deleted allele is also present in 3 relatives of this AR/AE patient, who all have negligible or reduced plasma APP activities (Figure 3b). The 2 men with relatively high plasma APP activities in this family each have a normal
copy of this gene. This deletion was not found in other AE-ACEi cases or controls included in this study.

Total RNA from cultured lymphoblastoid cells of the AE/AR-ACEi male was used to determine the effects of this deletion on the mRNA. Sequencing of his cDNA revealed an erroneously spliced transcript missing exon 2 and a segment of exon 3 (314_431del). This mutant transcript encodes a premature stop codon, which presumably translates into a truncated protein of 38 amino acids that is significantly shorter than the wild-type mAPP of 674 amino acids (Figure 3c). The first 21 amino acids of the protein constitute the signal peptide for the human mAPP as previously predicted by the SignalP program and is consequently cleaved off in the mature protein (Nielsen et al. 1997). The mutant protein lacks the predicted active site residues involved in metal ion coordination and proton shuttling as well as a glycosylphosphatidylinositol (GPI) anchor (Molinaro et al. 2004).

Next, we sequenced upstream regions of XPNPEP2 that are highly conserved in the mouse (UCSC; April 2002 freeze) to identify potential regulatory variants. We identified one SNP (C-2399A; rs3788853) that segregates in 7 of our 8 linkage families (genotypes shown in Figure 1). The A allele is absent only in the pedigree segregating the coding deletion described above. The two probands in pedigrees I and V also lack the A allele, which segregates in their families. Interestingly, only pedigree I contributes to the linkage signal at the XPNPEP2_CA marker (LOD = 0.99). We further sequenced 3 kb upstream, 1 kb downstream and the coding region of XPNPEP2 in all AE-ACEi patients with the A allele but did not detect any other sequence variations that might be in linkage disequilibrium with the C-2399A SNP.
The MatInspector program (Genomatix Suite) predicts that this substitution potentially affects the binding sites of 2 trans-acting elements: hepatic nuclear factor 4 (HNF-4) and peroxisome proliferator activated receptors/retinoid X receptor (PPAR/RXR) heterodimer. The biological significance of this SNP remains to be determined. We have conducted quantitative RT-PCR and northern blots without success due to low expression of XPNPEP2 in human lymphoblastoid cells (Venema et al. 1997). We have yet to ascertain more relevant tissues (i.e. kidney, lung) for functional analysis.

Measured genotype analysis of the C-2399A SNP

To evaluate the extent to which the C-2399A SNP accounts for the linkage observed at the XPNPEP2 locus, we repeated linkage analysis at the XPNPEP2_CA microsatellite marker after regressing out the effect of the C-2399A SNP association by including it as a covariate in the linkage model (Almasy et al. 2004). The trait mean calculated on the 7 pedigrees segregating the SNP (Figure 1) was 17.25 units. The standard deviation in the model with the SNP as covariate was 5.55, compared to 6.70 in the model without the SNP as a covariate, and the proportion of variance explained by the covariate SNP was 0.3149. The beta term for the SNP genotypes in the model was -4.67. The SNP upstream of XPNPEP2 was significant as a covariate in the model ($P < 0.0001$). Using all pedigrees except family VI, which segregates the XPNPEP2 coding deletion, the linkage signal diminished substantially in two-point linkage at the microsatellite marker XPNPEP2_CA from LOD = 3.24 to LOD = 0.54. This marked diminution in LOD score indicates that the linkage signal for plasma APP activity at this locus is mostly accounted for by the C-2399A SNP association.
Association of the C-2399A SNP with AE-ACEi

We genotyped 20 independent AE-ACEi cases and 60 unrelated, matching controls for the C-2399A SNP. The A allele was present in 8 out of the 20 AE patients. By allele counting, the A allele was found at a frequency of 11.1% in our controls and 27.3% in our AE-ACEi cases. By genotype analysis in cases versus controls, where the male hemizygote is considered to be equivalent to the female homozygote, yields a $P = 0.0364$ (Armitage trend test) when comparing AE cases to population controls.

2.2.5 Discussion

This report provides evidence that variable plasma APP activity is partially regulated by genetic factors. We estimated that 34% of the phenotypic variation in our linkage families results from genotypic differences. Linkage analysis significantly identified a quantitative trait locus (LOD = 3.75) near XPNPEP2, a candidate gene encoding mAPP. Despite the absence of mutations detected in the initial gene-screening experiments, further investigation of this locus identified 2 sequence variations. The genomic deletion segregating in pedigree VI results in an erroneously spliced transcript that is predicted to translate into a severely truncated protein. The C-2399A SNP genotype significantly accounts for the linkage signal at this QTL and is significantly associated with AE-ACEi ($p = 0.0364$). No other variants that may be in linkage disequilibrium with the A allele were found at this locus in additional sequencing experiments, further suggesting that this SNP may be responsible for the observed reduction in plasma APP activity.

The above variants were found in 9 of 20 AE-ACEi cases (supplemental Table 1). This suggests that the low plasma APP activity observed in additional AE-ACEi patients
may result from other genetic loci or environmental factors. Our multipoint variance component linkage analysis of cohort 1 provided evidence of significant linkage to a locus on chromosome 6 (LOD score = 3.47) and positive linkage to loci on chromosomes 8 and 9. Multipoint linkage analysis including cohort 2, however, reduced linkage to the chromosome 6 locus (LOD score = 1.43) as well as the LOD scores for the other 2 autosomal loci. Genotyping of additional markers at these loci in more families are necessary to either confirm or reject linkage.

Given that a third of our AE patients have normal plasma APP activity (> 20 units), this quantitative trait clearly explains a fraction of the AE risk associated with ACEi therapy. Individuals who are not genetically predisposed to have low APP activity may develop AE-ACEi due to non-specific inhibition of APP by certain kinds of ACEi drugs (Hooper et al. 1992) or other unidentified causes. Other potential risk factors include elevated levels of the sensory neuropeptide substance P, which correlate with reduced amounts of dipeptidyl peptidase IV activity (Ferreira et al. 2000; Lefebvre et al. 2002). In addition, clinical trials for new vasopeptidase inhibitors (inhibits NEP and ACE) result in higher AE incidence than with ACE inhibition alone, suggesting that NEP activity may play a role in AE risk. Furthermore, other non-genetic factors (i.e. smoking and estrogen replacement) may contribute to AE-ACEi risk.

Our study confirms previously published data showing that reduced plasma APP activity is a relatively common phenotype in the general population (Lefebvre et al. 2002). Other than predisposition to AE-ACEi, no clinical phenotype has been associated with low APP activity to date. This suggests that its physiological role may be non-essential or there is functional redundancy. Furthermore, certain ACEi patients with low
APP activity do not develop AE during therapy. This suggests that factors (i.e. environmental triggers) acting in pathways other than des-Arg⁹-BK degradation may contribute to AE risk.

Finally, low APP activity may determine risk to other ACEi-associated adverse effects. We previously reported an association between reduced plasma APP activity and anaphylactoid reactions during hemodialysis induced by ACEi therapy (Blais et al. 1999a). Further genetic investigations are necessary to determine if variants in the XPNPEP2 locus, such as those described here, play a role in AR-ACEi risk. Other adverse reactions associated with ACEi therapy include chronic cough that affects approximately 10-15% of ACEi patients. It would be interesting to further characterize plasma APP activity in these patients and screen XPNPEP2 as candidate susceptibility gene for these side effects of ACEi.

In conclusion, this report demonstrates that genetic variants at the XPNPEP2 locus are partially responsible for reduced plasma APP activity. Furthermore, we show a significant association between the C-2399A polymorphism and AE-ACEi. Thus, we have successfully mapped a quantitative trait locus, which may in part predict susceptibility to a potentially fatal adverse reaction associated with one of the most commonly used drugs worldwide. Our study represents a potential application of pharmacogenomics in health care, a field destined to transform medical care but where there have been few successes to date. Our findings in addition to further studies may facilitate the rational design of safer drugs for treating cardiovascular diseases as well as clinical assays for predicting individuals who are genetically more liable to develop AE associated with ACE or other vasopeptidase inhibitors.
2.2.6 Acknowledgements

We would like to thank all the participants in this study; Dr. André Toulouse for molecular expertise; Ms. Dominique Verlaan for careful review of this manuscript; Ms. Nicole Gervais and Ms. Kateri Brisebois for technical assistance; the logistic assistance of Hospal France. This work was funded by the National Institute of Health. GAR and GM are supported by the Canadian Institutes of Health Research. AA receives funding from Fonds de Recherche en Santé du Québec.
2.2.7 Tables

Table 1

Variance Component Two-Point QTLs for Markers Genotyped in Cohorts 1 and 2.

<table>
<thead>
<tr>
<th>Panel Markers</th>
<th>cM pos.</th>
<th>Chrom. band</th>
<th>Cohorts 1 and 2 (8 pedigrees)</th>
<th>Cohort 1 only</th>
</tr>
</thead>
<tbody>
<tr>
<td>D6S1017</td>
<td>63.3</td>
<td>6p21.1</td>
<td>0.97</td>
<td>2.62</td>
</tr>
<tr>
<td>D6S1280</td>
<td>73.1</td>
<td>6p12.3</td>
<td>0.18</td>
<td>1.09</td>
</tr>
<tr>
<td>D6S1056</td>
<td>102.8</td>
<td>6q16.1</td>
<td>1.32</td>
<td>1.68</td>
</tr>
<tr>
<td>D8S277</td>
<td>8</td>
<td>8p23.1</td>
<td>0.22</td>
<td>0.69</td>
</tr>
<tr>
<td>D8S373</td>
<td>164.5</td>
<td>8q24.3</td>
<td>1.72</td>
<td>1.76</td>
</tr>
<tr>
<td>D9S925</td>
<td>32.2</td>
<td>9p22.2</td>
<td>0.16</td>
<td>1.71</td>
</tr>
<tr>
<td>D9S1122</td>
<td>75.9</td>
<td>9q21.2</td>
<td>0.13</td>
<td>0.76</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers Flanking Candidates</th>
<th>cM pos.</th>
<th>Chrom. band</th>
<th>Lod 1</th>
<th>Lod 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10S1220</td>
<td>70.2</td>
<td>10q11.23</td>
<td>0.24</td>
<td>0.74</td>
</tr>
<tr>
<td>DXS8057</td>
<td>136.4</td>
<td>Xq25</td>
<td>2.1</td>
<td>1.82</td>
</tr>
<tr>
<td>DXS1047</td>
<td>143.2</td>
<td>Xq25</td>
<td>2.04</td>
<td>0.28</td>
</tr>
<tr>
<td>XPNPEP2_CA</td>
<td>146</td>
<td>Xq25</td>
<td>3.75</td>
<td>2.15</td>
</tr>
</tbody>
</table>

NOTE--Eleven microsatellite markers that were already part of the analysis in cohort 1 were genotyped in cohort 2 to cover the 4 loci that provided LOD scores > 1 in cohort 1 and the region proximal to the XPNPEPL candidate gene (D10S1220).
Table 2

C-2399A SNP Genotypes in ACEi-Associated AE Cases and Matched Controls.

<table>
<thead>
<tr>
<th>C-2399A SNP Genotype</th>
<th>AE-ACEi Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td># Males</td>
<td># Females</td>
<td>Mean APP (units)</td>
</tr>
<tr>
<td>CC or C</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>CA</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>AA or A</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total # Samples</strong></td>
<td><strong>20</strong></td>
<td><strong>13±3</strong></td>
</tr>
</tbody>
</table>

NOTE-- Mean APP activity is represented as units of Arginine released per minute per mL of plasma sample.
Supplemental Table 1

XPNPEP2 Genotypes in 20 AE-ACEi Patients.

<table>
<thead>
<tr>
<th>AE-ACEi Patient No.</th>
<th>APP (units)</th>
<th>Gender</th>
<th>Origin</th>
<th>C-2399A SNP</th>
<th>Other Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>M</td>
<td>MTL</td>
<td>C</td>
<td>g.3217del3391</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>M</td>
<td>BELGIUM</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>M</td>
<td>USA</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>unknown</td>
<td>M</td>
<td>USA</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>F</td>
<td>MTL</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>F</td>
<td>MTL</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>24</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>48</td>
<td>F</td>
<td>USA</td>
<td>C/C</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>F</td>
<td>MTL</td>
<td>C/A</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>F</td>
<td>MTL</td>
<td>C/A</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>F</td>
<td>BELGIUM</td>
<td>C/A</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>unknown</td>
<td>F</td>
<td>USA</td>
<td>C/A</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>M</td>
<td>USA</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>M</td>
<td>MTL</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>M</td>
<td>MTL</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>F</td>
<td>BELGIUM</td>
<td>A/A</td>
<td></td>
</tr>
</tbody>
</table>

Note.— APP activity is represented as units of arginine released per minute per milliliter of plasma sample. The C-2399A SNP genotype is shown as C/C, C/A, or A/A for females (F), and C or A for males (M). The coding deletion resulting in an erroneously spliced transcript is represented as g.3217del3391.
Cohorts included in variance component linkage analysis. One member of each kindred, depicted in black, developed AE and/or AR associated with ACEi therapy. All available members were quantified for plasma APP activity, shown as numbers, which represent units of Arginine released per minute per mL of plasma. The C-2399A SNP genotypes are shown as C/C, C/A or A/A for females and C or A for males. a, DNA samples from cohort 1 were included in a genomewide microsatellite scan, which was analyzed for linkage using a variance component method (SOLAR program). b, Eleven genome scanned markers were genotyped in cohort 2 to further evaluate linkage. A large coding deletion (DEL) segregates in pedigree VI.
Figure 2

Results of the variance component QTL analyses in the 5 pedigrees of cohort 1. All results were corrected empirically for $\alpha \leq 0.05$. a, String diagram of autosomal multipoint results, LODs > 1 are shown next to a LOD scale. b, Two-point linkage analysis results including chromosome X.
Figure 3  

a. The deleted genomic region of 175 bp consists of 16 bp of exon 2 (in blue), the 3' donor splice site (in green) and intronic sequences. 

b. The deleted allele (DEL) is present in 4 members of this pedigree who have negligible or reduced (in one female heterozygote) plasma APP activity. The two men with normal alleles (wt) both have high APP measurements. 

c. The deletion results in an abnormally spliced mRNA transcript, coding for a premature stop codon that is predicted to translate into a severely truncated protein of 38 amino acids. This short peptide would not be membrane-bound as it lacks a glycosylphosphatidylinsitol (GPI) anchor attached to the alanine (A) at position 650.
CHAPTER 3: HYPERSENSITIVITY REACTIONS ASSOCIATED WITH ANGIOTENSIN I-CONVERTING ENZYME INHIBITORS

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Reference:

3.1 RATIONALE

Linkage analysis of plasma APP activity and mutation screening of the XPNPEP2 gene, as described in the previous chapter, identified genetic variants associated with both the quantitative trait and AE-ACEi. Given previous evidence that impaired des-Arg⁹-BK degradation may also play an important role in the pathogenesis of HSR-ACEi (Blais et al. 1999), we hypothesized that variants in the XPNPEP2 locus may also increase risk for this ADR. In addition, two of our AE-ACEi cases from the previous chapter (proband of pedigrees IV and VI) also developed HSR-ACEi during hemodialysis, which further supports our hypothesis for a common underlying mechanism. Moreover, HSR-ACEi patients present AE symptoms during hemodialysis such as swelling of the face and respiratory tract causing dyspnoea (Bright et al. 1999).

All of our HSR cases were treated with an ACEi and had developed this ADR during hemodialysis with a negatively charged membrane. Contact with a negatively charged surface is known to activate the BK pathway. In addition, these cases were simultaneously taking ACEi. Given the fact that some individuals treated with ACEi do not develop HSR (even after repeated hemodialysis sessions) indicates an individual susceptibility to this ADR that is independent of dialysis with a negatively charged membrane.

All HSR-ACEi cases included in this study were collected in the early 1990s. Due to the high incidence of this ADR associated with ACEi, the Food and Drug Administration (FDA) issued a warning to physicians not to combine these drugs with dialysis using negatively charged membranes (Alert 1992). As a result, the frequency of this ADR declined, making collection of new cases more difficult.
3.2 KININ-DEPENDENT HYPERSENSITIVITY REACTIONS IN HEMODIALYSIS: METABOLIC AND GENETIC FACTORS.

3.2.1 Abstract

Although the association of angiotensin I-converting enzyme inhibitors (ACEis) with a negatively charged membrane is thought to be responsible for hypersensitivity reactions (HSRs) during hemodialysis, we hypothesize that these complications are due to changes in plasma aminopeptidase P (APP) activity and genotype. To test this hypothesis, we measured plasma APP activity in 14 patients who suffered HSR (HSR+) while dialyzed with an AN69 membrane and simultaneously treated with an ACEi. APP activity was also studied in a control group (n = 39) dialyzed under the same conditions, but who did not suffer any side effect (HSR-). We found significantly decreased plasma APP activity (P = 0.013) in HSR+ subjects as well as altered degradation of endogenous des-Arginine⁹-bradykinin, with a significantly lower β value (P < 0.001). The same analytical approach was taken in 171 relatives of HSR+ patients. Variance component analysis suggested that genetic differences may explain 61% of the phenotypic variability of plasma APP activity (P < 0.001) and the kinetic parameters that characterized kinin degradation. We also showed that the C-2399A single-nucleotide polymorphism at the XPNPEP2 locus was a significant predictor of APP activity in the 39 HSR- controls (P = 0.029). Furthermore, a recessive genetic model for the A allele disclosed a significant difference in mean APP activity by genotype (P < 0.001). Finally, our study defined the nonspecific inhibition of recombinant APP by some ACEis. In conclusion, this paper highlights the complexity of HSR in hemodialysis, suggesting, as with angioedema, that
these rare, but life-threatening adverse events are governed by several metabolic and genetic factors.
3.2.2 Introduction

Although beneficial in the treatment of various cardiovascular disorders and the progression of renal failure in diabetic and non-diabetic patients (Lewis et al. 1993; Brown et al. 1998), angiotensin I-converting enzyme inhibitors (ACEis) are associated with several side effects, for which symptoms largely depend on the clinical context. Hypersensitivity reactions (HSRs) or anaphylactoid reactions, which mimic symptoms of immediate hypersensitivity, are observed in patients chronically hemodialyzed with synthetic, highly permeable, electronegative membranes while taking ACEi (Schaefer et al. 1994). These reactions typically occur in the first minutes of the dialysis session, and their intensity is usually mild to moderate, but may be life-threatening. Numerous factors have been hypothesized to cause HSR (Revillard 1990; Jaber et al. 1997; Sundaram et al. 1997; Horl 2002). However, reported increases in plasma bradykinin (BK) during episodes of HSR (Schulman et al. 1993; Fink et al. 1994; Verresen et al. 1994) in hemodialyzed patients suggest a role of kinins in the genesis of these adverse reactions.

The nonapeptide BK is released from high molecular weight kininogen by plasma kallikrein during activation of the contact system. It is subsequently metabolized, mainly by three zinc metallopeptidases (Bhoola et al. 1992; Cyr et al. 2001). ACE and X-Pro-aminopeptidase (aminopeptidase P (APP)) are the first and second inactivating metallopeptidases of BK, respectively. Carboxypeptidase \( N \) transforms BK into its carboxytruncated active metabolite, des-Arginine\(^9\)-BK (des-Arg\(^9\)-BK), which constitutes minor metabolic pathway unless ACE is inhibited. Des-Arg\(^9\)-BK is also degraded by ACE and APP. In this case, however, APP represents the main peptidase activity whereas ACE plays a secondary role.
BK exerts its pharmacological activities through its constitutively expressed kinin B₂ receptor (Leeb-Lundberg et al. 2005), although des-Arg⁹-BK binds to the B₁ receptor, the synthesis of which is increased by different cytokines (Marceau et al. 1998). Both B₁ and B₂ receptor agonists have several physiological activities (Leeb-Lundberg et al. 2005) including the ability to dilate the peripheral vasculature, directly and indirectly (resulting in hypotension), constrict the pulmonary airways (evoking bronchospasms), and induce the release of histamine from mast cells, which are hallmarks of HSR.

Human APP, a hydrolase that cleaves N-terminal imido bonds normally protected from attack by other known aminopeptidases, exists in two known forms: a glycosylphosphatidylinositol-anchored membrane form (hmAPP) and a cytosolic form (hcAPP). mRNA of both APP forms have been found in different tissues (Ersahin et al. 2005). We previously reported significantly lower plasma APP activity in patients who suffered ACEi-linked acute side effects (Blais et al. 1999; Blais et al. 1999; Adam et al. 2002; Molinaro et al. 2002; Molinaro et al. 2002). When ACE was inhibited, the low APP activity was associated with an increased half-life of exogenously added (Blais et al. 1999) or endogenously generated des-Arg⁹-BK (Molinaro et al. 2002; Molinaro et al. 2002).

We reported recently (Duan et al. 2005) that genetic factors partially regulated the activity of APP in eight families, each with one member who developed ACEi-associated angioedema (AE). Heritability of plasma APP activity was estimated to be 34% (± 25%). Sequencing the upstream region of the XPNPEP2 gene coding for hmAPP, we found a single-nucleotide polymorphism (SNP) C-2399A, for which the A allele was linked with
low plasma APP activity. In addition, we observed a deletion of 175 bp in one case of AE. This deletion resulted in a truncated protein of 38 amino acids (Duan et al. 2005).

In the present study, we tested the hypothesis that HSR may occur not only from the association of a negatively charged membrane (physicochemical factor) and an ACEi (pharmacological agent), but is also dependent on the ability (metabolic aspect) of the dialyzed patient to degrade kinins, generated from activation of the contact system of plasma. We measured APP activity, and defined the metabolism of the endogenous kinins BK and des-Arg⁹-BK in the plasma of 14 patients who suffered HSR (HSR+) while dialyzed with an AN69 membrane and simultaneously treated with an ACEi. These same parameters were also measured in 171 relatives of these HSR+ patients and a control group (n = 39) dialyzed under the same conditions, but who did not suffer any side effect (HSR-). Furthermore, we calculated the importance of heritability on APP activity, and investigated kinetic parameters among the families of HSR+ patients. Finally, we tested the nonspecific inhibition of different ACEi on a recombinant wild-type form of hmAPP (Molinaro et al. 2005).

3.2.3 Materials and methods

Participants

The study protocol was conducted according to French regulations and was approved by Ethics Committees from the Centre hospitalier de l'Université de Montréal (CHUM), the Institut de cardiologie de Montréal, McGill University, and the Université de Rennes (France). Informed consent was obtained from all participants. Patients were from five different nephrology-hemodialysis centers in France (Hôpital Universitaire and ARPDD,
Reims, Centre Hospitalier La Beauchée, Saint-Brieuc, Centre Hospitalier Louis-Pasteur, Cherbourg, Centre Hospitalier Intercommunal de Cornouaille, Quimper) and one in Canada (Hôtel-Dieu de Saint Jérôme, Québec). Sampling took place at these sites between July 2001 and December 2003. Fifty-three patients on chronic hemodialysis, 39 HSR- and 14 HSR+, were enrolled in this study. Their main clinical characteristics are shown in Table 1. In addition, 171 relatives of the 14 HSR+ patients participated in a medical interview and donated blood samples for genetic linkage research (Figure 1).

Hemodialysis

All patients were chronically hemodialyzed three times per week, for average session duration of 240 min. In all instances, the dialyzer, either flat sheet or hollow fiber, was equipped with the AN69 polyacrylonitrile membrane (Gambro-Hospal, Meyzieux, Lyon, France). The blood flow rate comprised between 250 and 350 ml/min. Unfractionated heparin was used as anticoagulant. The dialysate contained bicarbonate and was of the highest bacterial and endotoxin quality. Adequacy of dialysis was assessed by $Kt/V$ for urea higher than 1.2. Dialyzers were not reused. In those patients who developed HRS, ACEi was withdrawn, and they were shifted to another type of membrane, that is, a neutral membrane.

Hypersensitivity reaction

The presence of HSR was defined on the basis of clinical criteria proposed recently by Bright et al. (Bright et al. 1999) in a survey of HSR as an incident involving two or more of the following symptoms occurring within 5–20 min of starting dialysis: abdominal
cramps, nausea, vomiting or diarrhea; shortness of breath, chest tightness, wheezing, or bronchospasms; facial, labial and/or lingual swelling, AE or laryngeal edema; hypotension (> 20 mmHg drop in systolic blood pressure); flushing, or warmth; numbness or tingling of the fingers, toes, lips, or tongue. All the symptoms disappeared in 2–6 h after the end of dialysis, whether the session was completed or interrupted.

Dialyzed patients who presented HSR (HSR+ group) and their pedigree are represented in Figure 1. The etiology of renal failure was diabetes mellitus ($n = 1$, propositus of family II); glomerular disease ($n = 7$, propositus of family I, III, IV, V, VI, VIII, XIV); chronic interstitial nephritis ($n = 2$, propositus of family VII, X); nephroangiosclerosis ($n = 2$, propositus of family XI, XIII); polycystic renal disease ($n = 1$, propositus of family IX); and unknown ($n = 1$, propositus of family XII).

At the time of the HSR episode, eight patients were treated with captopril (propositus of family I, II, V, VI, IX, X, XI and XIV), four with enalapril (propositus of family VII, VIII, XII, XIII), one with benazepril (propositus of family V), and one with lisinopril (propositus of family III).

The group of patients who never presented any side effect while dialyzed in the same conditions as HSR+ served as the control group (HSR- patients). At the time of blood sampling, these patients were treated with the following ACEi: ramipril ($n = 24$), enalapril ($n = 5$), perindopril ($n = 7$), and fozinopril ($n = 3$).

Laboratory investigations

Blood and plasma samples: Venous blood was sampled at the start of dialysis and before heparin administration. Blood was collected in BD Vacutainers either on Na-citrate (1/10
v/v) for metabolic investigations or on K₂-ethylenediaminetetraacetic acid for DNA analysis. Citrated blood was centrifuged at 2000 g, plasma was collected and stored in sealed tubes at -80°C before shipping to the reference center. K₂-ethylenediaminetetraacetic acid samples were stored at room temperature until DNA extraction.

Reagents: The ACEi enalapril was from Merck Frosst Canada (Kirkland, Québec, Canada); the ACEi ramipril was a generous gift from Dr W Linz (Sanofi Synthelabo-Aventis GmbH, Germany); BK and des-Arg⁹-BK were acquired from Peninsula Laboratories (Belmont, CA, USA); the internally quenched fluorescent substrate K(Dnp)PPGK(Abz) (Molinaro et al. 2005) was synthetized by Professor A Carmona (Department of Biophysics, Escola Paulista de Medicina, UNIFESP, São Paulo, Brazil). All other fine chemicals and inhibitors were from Sigma (Montreal, Québec, Canada).

Metabolic investigations

Measurement of APP enzymatic activity in plasma: APP activity was assessed kinetically using the K(Dnp)PPGK(Abz) quenched fluorescent substrate. This original method was developed and validated recently in our lab (Molinaro et al. 2005). The substrate allows sensitive kinetic measurement of plasma APP activity with intra- and inter-assay coefficient of variation (CV)% equal to 6 and 10%, respectively (Molinaro et al. 2005). One unit of APP activity corresponds to 1 pmol of K(Dnp)PPGK(Abz) hydrolyzed per min/ml.

This analytical approach has the advantages of kinetic measurement of enzyme activity, and similar affinity of the enzyme for the new substrate (K_m: 20 ± 5 μM) when
compared to its natural substrate, des-Arg\(^9\)-BK (\(K_m: 56\pm13 \, \mu\text{M}\)) (Molinaro et al. 2005); it contrasts with values for the synthetic tripeptide Arg-Pro-Pro (\(K_m: 837 \pm 75 \, \mu\text{M}\)) previously employed to assess APP activity.

**Metabolism of endogenous BK and des-Arg\(^9\)-BK in plasma**

*Contact system activation:* Plasma was activated as described earlier for a group of normal healthy people (Cyr et al. 2001) and AE patients (Molinaro et al. 2002). Briefly, 1 ml of plasma was preincubated with enalapril for 20 min at 37\(^\circ\)C in polypropylene tubes at a concentration (130 nM) which totally inhibited ACE activity. The contact system was then activated by incubation of the plasma with glass beads (37\(^\circ\)C, with agitation). The reaction was stopped after various incubation periods (0–60 min) by adding cold anhydrous ethanol at a final concentration of 80\% vol/vol. The samples were then incubated at 4\(^\circ\)C for 1 h and centrifuged (4\(^\circ\)C, 15 min, 3000 g) for the complete precipitation of protein. The supernatant was decanted and evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY, USA) before quantification of the immunoreactive peptides BK and des-Arg\(^9\)-BK.
Measurement of immunoreactive BK and des-Arg⁹-BK: The residues of evaporated ethanolic extracts were resuspended in 50 mM Tris/HCl buffer (pH 7.4) containing 100 mM NaCl and 0.05% Tween-20. After resuspension, residual BK and formed des-Arg⁹-BK were quantified by two specific competitive enzyme immunoassays (Decarie et al. 1994; Raymond et al. 1995).

Briefly, both immunoassays use purified polyclonal immunoglobulin G raised against the C-terminal portion of the peptides, responsible for the B₁ or the B₂ pharmacological activities. Antibodies were raised in rabbits against the C-terminal part of BK covalently linked to bovine serum albumin using glutaraldehyde. The tracer used was BK labeled with digoxigenin-3-O-methylcarboxyl-ɛ-aminocaproic acid-N-succinimide ester (DIG-O-Su, Roche diagnostic, Laval, Québec, Canada) purified by high-pressure liquid chromatography and characterized by mass spectrometry and radioimmunoassay. Kallidin (Lys-BK) and BK are 100% crossreactive with these antibodies. None of the BK metabolites (des-Arg⁹-BK or Lys-des-Arg⁹-BK) present a significant crossreactivity (< 1%) with the anti-BK antibodies. The calibration curve was characterized by an ED₅₀ of 7.37 pg/well (50 μl). The CV for each value of the calibration curve is less than 5%. Intra- and inter-assay CV of BK measurements were lower than 4.0 and 7.4%.

For the quantification of des-Arg⁹-BK, polyclonal antibodies were generated in rabbit against Cys-Lys-Aca-Lys-des-Arg⁹-BK coupled with maleimide-activated keyhole Limpet Hemocyanin and purified by gel filtration (PD-10). The tracer was obtained by coupling des-Arg⁹-BK to DIG-O-Su. After purification by hydrophobic chromatography, the tracer was also characterized by mass spectrometry and radioimmunoassay. Lys-des-
Arg⁹-BK and des-Arg⁹-BK exhibit identical 100% immunoreactivity for the C-terminal peptide-specific antibodies. Neither BK nor Lys-BK crossreact in the assay and different metabolites of kinins present less than 0.1% cross reactivity. The calibration curve is characterized by an ED₅₀ of 6.96 pg/well (50 μl). The CV for each value of the calibration curve is less than 6%. The intra-assay CV calculated at three concentration levels varies between 5 and 8%, and the inter-assay CV is lower than 10%. The bound DIG-BK/DIG-des-Arg⁹-BK is reacted with anti-DIG Fab labeled with horseradish peroxidase. The reaction was revealed by adding o-phenylenediamine and read by absorbance at 530 nm.

**Mathematical treatment of the kinetic profiles of BK and des-Arg⁹-BK:** The following mathematical model \( y = k \ t^\alpha \ e^{\beta t}, \ t > 0 \), was fitted to the concentrations of endogenous BK and des-Arg⁹-BK measured at different times (t) for each plasma sample. This 3-parameter (k, \( \alpha \) and \( \beta \); \( k > 0, \ \alpha \) and \( \beta > 0 \)) model corresponds to a form similar to gamma distribution (Rice 1995) and has been described and validated earlier (Cyr et al. 2001). The \( \alpha \) and \( \beta \) parameters are respectively related to the shape of the first and the second part of the curve corresponding to the formation and the degradation of each peptide. These \( \alpha \) and \( \beta \) parameters allow the calculation of other kinetic parameters: time of the maximum: the value of t for which the maximum of the curve was obtained: \( t = \alpha / \beta \); maximum: the value of the maximum of the curve which corresponds to the value of the curve for \( t = \alpha / \beta \); AUC: the area under the curve which is mathematically given by \( k \Gamma(\alpha + 1)/\beta^{\alpha+1} \), where \( \Gamma(\alpha + 1) \) is the gamma function; half-life of formation (\( t_f \)): the value \( t_f \) in the interval 0 to \( \alpha / \beta \) for which \( t^\alpha \ e^{\beta t} = (0.5) (\alpha / \beta)^\alpha \ e^{-\alpha} \); half-life of degradation (\( t_d \)): the
value \( t_d \) in the interval \( \alpha/\beta \) to \( \infty \) for which \( t^\alpha e^{\beta t} = 0.5 \) \( (\alpha/\beta)^\alpha e^{\alpha} \); \textit{slope of the half-life of formation}: the value of the slope of the curve at half-life formation = \( k e^{\beta t} t^\alpha (\alpha - \beta t_d) \); and \textit{slope of the half-life of degradation}: the value of the slope of the curve at half-life degradation: \( k e^{\beta d} t_d^\alpha (\alpha - \beta t_d) \).

Genetic investigations

\textit{Heritability}: To test the hypothesis that genetic factors could influence the variability of APP activity and of kinetic parameter values for the metabolism of BK and des-Arg\(^9\)-BK, heritability was estimated among the combined families of the 14 HSR+ patients. For this analysis, probands were not excluded on the basis that the pedigrees were not selected for APP activity levels and covariates were not included in the analysis.

\textit{Genotyping}: A commercially available protocol (Gentra, Minneapolis, MN, USA) was applied for DNA extraction. Genotyping of C-2399A SNP, described elsewhere (Duan et al. 2005), required the modification of an allele-specific polymerase chain reaction assay (Germer et al. 1999).

The SNP genotypes served two purposes: (1) to compare allele and genotype frequencies between HSR+ and HSR- patients; (2) to evaluate associations between SNP genotypes and phenotypes (APP activity or different kinetic parameters of BK and des-Arg\(^9\)-BK) in both groups (HSR- and HSR+).
Pharmacological investigations

Production and purification of human recombinant soluble membrane APP: A secreted form of wild-type human APP was engineered and fully characterized (Molinaro et al. 2005).

Inhibition studies of recombinant APP with metallopeptidase inhibitors: Kinetics parameters were measured with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The data were plotted to fit the one-site competition equation by nonlinear regression, with imposed constraints (-5.0 to 110% activities).

All enzyme activity assays were performed at 37°C. Assay mixtures minus recombinant APP served as controls. Stock solutions of different inhibitors (ramipril, apstatin, enalapril, captopril, and lisinopril) were successively diluted in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid 0.1 M (pH 7.4). The final concentrations tested ranged from 0 to 1 or 10 mM. Reactions were in triplicate and incubated at 37°C for 90 min, with or without a metallopeptidase inhibitor. Hydrolysis of the fluorogenic substrate K(Dnp)PPGK(Abz) was performed as follows: reactions were in 150 μl volume in opti 96-well plates containing 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.4), 10 μM substrate, and purified recombinant enzymes. Fluorescence was measured kinetically (every 5 min) with a FL600 microplate fluorescence plate reader (BioTek, Winooski, VT, USA) with excitation of 340 nm and an emission of 420 nm.
Statistical analysis.

For kinetic parameters and enzyme activities, the means of the parameters for the two groups (HSR+ and HSR-) were compared by t-test with the Satterthwaite-Welch approach, taking into account the possible heterogeneity of variances (Neter J 1996) using SPSS, version 13.0. P < 0.05 values were considered statistically significant.

The heritability coefficients of APP and the different kinetic parameters of kinins were calculated on z-scored values by SOLAR (Sequential Oligogenic Linkage Analysis Routines, version 2.1.4 Official) (Almasy et al. 1998) with robust estimation of means and variance according to t-distribution. P < 0.05 values were considered statistically significant.

The APP means were compared between the genotype groups by one-way analysis of variance, followed by linear contrasts; a t-test was used for the recessive genetic model (Neter J 1996). SNP association results were statistically significant at P<0.001.

3.2.4 Results

Metabolic investigations

APP activity in plasma: APP values of the HSR+ patients and their relatives appear in Figure 1. Figure 2 presents the distribution of APP activity in both groups of dialyzed patients compared to that of the reference population (Cyr et al. 2001; Molinaro et al. 2005). Mean activity (82 ± 107 U) in HSR+ plasma was significantly lower than in HSR- (193 ± 199 U, P = 0.013) and the reference samples (317 ± 248 U, P = 0.000). Mean plasma APP activity in the dialyzed patients corresponded respectively to the 11th
(HSR+) and 38th percentile (HSR-) of the reference population. In the HSR- group, there was no significant difference in mean APP values in diabetic patients compared to other kidney diseases ($P = 0.516$). Similar results were obtained between diabetes and glomerular diseases ($P = 0.689$), diabetes, and interstitial nephritis ($P = 0.074$) (Table 1).

**Metabolism of endogenous BK and des-Arg$^9$-BK in plasma:** Figure 3 illustrates the mean metabolic profiles of BK and des-Arg$^9$-BK during the activation of HSR+ and HSR- plasma in the presence of an ACEi. No discrepancy in BK was detected for the different kinetic parameters (Table 2), except for a lower $\alpha$ among HSR+ patients ($P = 0.043$), which characterized BK generation from high molecular weight kininogen. The main metabolic difference between HSR+ and HSR- patients occurred in des-Arg$^9$-BK degradation. In the HSR+ group, a lower $\beta$ value ($P < 0.001$) reflected the altered degradation of this B$_1$ receptor agonist by APP in the presence of an ACEi. The des-Arg$^9$-BK degradation anomaly was responsible for a higher maximal concentration of the peptide ($P = 0.048$) during the activation period. When Benjamini’s correction (Benjamini Y 1995) for multiple comparison was applied to these kinetic parameters, two independent of APP activity became nonsignificant ($\alpha$ for BK, $P = 0.371$; $t_{1/2}$ slope of formation ($P = 0.093$) of des-Arg$^9$-BK).

**Genetic investigations**

*Heritability estimates:* In members of the 14 families of HSR+ patients, heritability of APP was estimated at 0.61 (± 0.18), indicating that genetic factors may explain 61% of the phenotypic variability in plasma APP activity ($P < 0.001$). This significant heritability estimate involved the kinetic parameters, which characterized the degradation
of BK and des-Arg⁹-BK, and depended mainly on APP activity when ACE was inhibited (Table 3). In such cases, the P-values obtained by Benjamini's correction did not affect the heritability estimates.

Genotyping: The genotyping results of C-2399A SNP of the XPNPEP2 gene in HSR+ and HSR- patients are presented in Table 4. The SNP was found to be a significant predictor of APP activity in the 39 HSR- controls (A and AA: 0 ± 0, CA: 251 ± 167, C and CC: 222 ± 206 pmol K(Dnp)PPGK(Abz) hydrolyzed/min/ml; analysis of variance: P = 0.029). Linear contrast on the genotyped groups was significant (P = 0.012). Furthermore, a recessive genetic model for the A allele disclosed a significant difference in mean APP activity by genotype (A and AA: 0 ± 0; CA and C and CC: 228 ± 197 pmol K(Dnp)PPGK(Abz) hydrolyzed/min/ml; t-test: P < 0.001). However, the SNP was not found to be predictive of HSR status as the association between groups (HSR+ and HSR-) and genotypes was not significant (Fisher's exact test: P = 0.4289).

Pharmacological investigations

Inhibition studies of recombinant APP with metallopeptidase inhibitors: The effect of various inhibitors on the hydrolysis of K(Dnp)PPGK(Abz)NH₂ by a recombinant soluble hmAPP is illustrated in Figure 4. The selective APP inhibitor apstatin and the sulfhydryl group-containing captopril were the most potent inhibitors (IC₅₀ of 0.6 ± 1.1 and 17 ± 1.1 μM, respectively). The other ACEi tested only inhibited at higher concentrations (mM ranges for enalapril) or not at all (lisinopril and ramipril).
3.2.5 Discussion

HSR were rare in the 1980s, but this incidence increased significantly during the 1990s owing to the widespread use of ACEi in patients dialyzed with a negatively charged membrane (Verresen et al. 1990). This association prompted a warning from American federal agencies (Alert 1992).

As other rare acute side effects of ACEi, HSR could happen when at least three different factors are present (Figure 5). The first one is the presence of a drug that inhibits specifically ACE but could also nonspecifically inhibit another metallopeptidase as APP involved in the metabolism of kinins. The second factor may be the negatively charged membranes which is the physicochemical trigger for the release of kinins. The third factor is a metabolic factor which is at least in part genetically regulated. It characterizes the capacity of the dialysed patient to metabolize kinins once ACE is inhibited. In this paper, we have explored the metabolic and the genetic aspect of the AR. We have also evidenced a nonspecific inhibition of APP by some ACEi.

Despite the recent reduction of HSR incidence owing to decreased membrane surface electronegativity, there is still a persistent risk of this severe adverse reaction during hemodialysis, especially among patients taking an ACEi. As the triggering factor (Z potential of the membrane) contributing to HSR is already known, the metabolic factors predisposing to it have yet to be fully understood. In this report, we address questions concerning the factors that control the metabolism of kinins in the presence of an ACEi. In this case, the metabolism of kinins (mainly of the $\beta_1$ receptor agonist) depends on APP activity.
Although the nature of the isoform (hmAPP, hcAPP, or both) responsible for plasma APP activity remains unknown, we recently published the analysis of a genome-wide microsatellite scan, which yielded highly significant linkage between plasma APP activity and a marker flanking the XPNPEP2 gene encoding hmAPP. Linkage was not obtained for hcAPP (Duan et al. 2005). This suggests that APP activity in plasma is mainly or uniquely the result of hmAPP. hmAPP is mainly localized on the external site of the plasma membrane of endothelial cells, on the brush border membrane of epithelial cells in the intestine, and in renal proximal tubules (Ersahin et al. 2005). hmAPP has also been found in human heart preparations where it contributes to BK metabolism (Blais et al. 2000). It has been hypothesized that hmAPP on endothelial cells inactivates BK and locally produced des-Arg<sup>9</sup>-BK. Suppression of hmAPP activity by apstatin, a commercially-available inhibitor, has highlighted the pathophysiological role of this metallopeptidase. However, the pharmacological effect of its blockade becomes evident mainly in the presence of an ACEi. In fact, inhibition of APP in rats synergizes the hypotensive action of injected BK, but this hypotension-potentiating influence is mostly seen in the presence of lisinopril (Kitamura et al. 1999). In humans, apstatin has no effect on the inflammatory reaction induced by local subcutaneous BK injection in the forearm. However, the APP inhibitor acts synergistically with quinapril to augment the wheal response to exogenous BK (Kim et al. 2000).

In this study, we measured APP activity in the plasma of HSR-, HSR+, and their relatives. For this purpose, we used the newly described, internally quenched fluorescent substrate K(Dnp)PPGK(Abz). This substrate, which mimics the NH<sub>2</sub>-terminal sequence
of the physiological substrate BK/des-Arg9-BK, is highly specific for APP activity (Molinaro et al. 2005).

With plasma samples obtained from the reference population (Cyr et al. 2001), we demonstrated that APP activity measured according this new analytical approach, was not only correlated with that published previously with the Arg-Pro-Pro (RPP) substrate \( r = 0.921, P < 0.001 \), but also exhibited a higher correlation with the degradation rate of des-Arg9-BK in the presence of an ACEi \( r = 0.822, P < 0.001 \) (Molinaro et al. 2005). With this new substrate, we found significantly lower APP activity in HSR+ compared to both HSR- dialyzed patients and the reference population.

As in our previous reports involving AE patients, the metabolic profiles of endogenous BK and des-Arg9-BK in HSR+ plasma were obtained by activating the contact system with glass beads in the presence of an ACEi. This mimics what happens in vivo when the plasma of an ACEi-treated patient comes in contact with a negatively charged membrane at the beginning of hemodialysis. Under our experimental conditions, we did not obtain evidence of any anomaly in the metabolism of the nonapeptide BK. Abnormal breakdown of des-Arg9-BK, assessed by significant differences in the \( \beta \) parameter that depended only on APP activity, led to a significant increase of maximal peptide concentration during the in vitro incubation period.

Similar metabolic anomalies among ACEi patients who developed HSR+ or AE indicate that one or several common genetic predisposition(s) may partially explain the pathophysiology of both adverse reactions. In the 14 families of HSR+ patients, we obtained heritability estimates for APP activity that were even higher than those calculated previously for AE (Duan et al. 2005). This may be explained by different
factors: the number of families (14 vs 8), the new analytical approach, but also the difference in the clinical context of side effects. We also found a significant association between low levels of APP activity (and the capacity to degrade des-Arg⁹-BK) and the C-2399A SNP polymorphism in the XPNPEP2 gene identified previously (Duan et al. 2005), lending further credence to concepts that genetics, most commonly SNPs as candidate gene factors, determine interindividual variability in risk for the disease (Rao et al. 2005). The C-2399A polymorphism was found to be predictive of low APP activity, but not sufficient for phenotype characterization, as is usually the case for quantitative traits.

As early as 1992, Hooper et al. (Hooper et al. 1992) reported that several ACEi inhibit APP purified from the pig kidney. Given this, we could not exclude that an ACEi nonspecifically inhibited APP activity among HSR+ patients. This was particularly true in dialyzed patients with impaired renal excretion of most ACEi. We showed that, in addition to the selective APP inhibitor apstatin, the sulfhydryl-containing ACEi captopril could also inhibit APP at μM concentrations. Captopril was the first ACEi to be used clinically. It is characterized by a relatively weak inhibition constant (\(K_i=1700\) vs 200 and 7 pM for enalapril and ramipril, respectively) and short half-life, necessitating multiple daily takes, at higher dosages, to achieve clinical efficacy. The concentration of captopril and other ACEi has not been documented in the plasma of dialyzed patients at the time of HSR episodes. Nevertheless, it is conceivable that, under these conditions, the high plasma concentrations needed to therapeutically inhibit ACE, coupled with poor clearance in dialyzed patients, could lead to the nonspecific inhibition of other metallopeptidases, such as APP, resulting in the accumulation of the B₁ agonist.
This nonspecific inhibition of APP by captopril and the important proportion of patients treated with the ACEi in the HSR+ group (8/14 vs 0/39 in HSR-) could plead for its role in the pathophysiology of some HSRs. Among the eight HSR+ patients treated by this ACEi at the time of the HSR+ episode, six had low or undetectable APP activity, but two (propositus of family X and XI) had activity (268 and 337 U, respectively) equal to or higher than the 50th percentile of the reference population. We could then hypothesize that APP activity could have been inhibited \textit{in vivo} by captopril at the time of the HSR episode.

This study highlights the complexity of HSR, as is the case with AE and other side effects of metallopeptidase inhibitors (ACEis and vasopeptidase inhibitors). These observations also raise important questions about the regulation of APP enzymatic activity in human plasma, and, ultimately, about the pharmacological activity of B₁ agonists in humans, and particularly in dialyzed patients. The factors that regulate plasma APP activity are not known and must be identified. Plasma APP activity is regulated at least two different levels. First, at the level of synthesis in cells by yet unidentified transcriptional and possibly post-transcriptional mechanisms responsible for the quantity of APP bound at the cell surface by a glycosylphosphatidylinositol anchor. Studies are now ongoing in our laboratory, using cells expressing APP, such as endothelial cells and human embryonic kidney cells (HEK 293 cells), to better characterize these mechanisms. The second level at which plasma APP activity is controlled is at cleavage of the glycosylphosphatidylinositol anchor and release of the protein in plasma. Phospholipase C has been shown to be a candidate for such release. More interesting, however, is the recent report that ACE has
glycosylphosphatidylinositolase activity (Kondoh et al. 2005). As both APP and ACE are often expressed in the same cells, it will be interesting to see whether ACE levels affect membrane-bound APP.

Our study also raises a question about the pathophysiological meaning of our in vitro observations: the consequences in vivo of B₁ agonist accumulation during activation of the contact system of plasma in the presence of an ACEi. In a rat model, we have previously demonstrated a pro-inflammatory effect of des-Arg⁹-BK when its corresponding B₁ receptor is expressed (Moreau et al. 2005). More recently, we have also observed in the pig that chronic enalapril treatment induces B₁ receptors in the kidneys (Moreau et al. 2005). However and contrarily to BK (Schaefer et al. 1993; Verresen et al. 1994), no des-Arg⁹-BK concentrations have been reported until now in human and particularly in plasma of dialyzed patients. The present results could be used as a basis for a clinical study in which plasma BK and des-Arg⁹-BK concentrations will be documented in HSR+ and HSR- patients. The association of these circulating levels of both B₁ and B₂ agonists with the clinical symptoms and the inflammatory status of these patients could elucidate the relative role of B₁ and B₂ receptors in these rare but potentially serious side effects. These results could also help us to understand the regulation of B₁ receptor expression in dialyzed patients and could objectivate the usefulness of non-peptidic B₁ antagonists, now in clinical development, for the treatment of metallopeptidase inhibitor side effects (Moreau et al. 2005; Moreau et al. 2005).
3.2.6 Acknowledgements

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3.2.7 Tables

Table 1 | Main clinical characteristics of patients who presented HSRs during hemodialysis (HSR+) and those without (HSR−), while treated with ACEi

<table>
<thead>
<tr>
<th>Variable</th>
<th>HSR+</th>
<th>HSR−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>14</td>
<td>39</td>
</tr>
<tr>
<td>M/F</td>
<td>5/9</td>
<td>21/18</td>
</tr>
<tr>
<td>Age (extremes)</td>
<td>50 (29–71)</td>
<td>53 (24–78)</td>
</tr>
<tr>
<td>Time on dialysis (months)</td>
<td>38.4 ± 16.8</td>
<td>54.0 ± 21.6</td>
</tr>
<tr>
<td>Diabetes type 2</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Nephroangiosclerosis</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Glomerular disease</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Chronic interstitial disease</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Polycystic disease</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

ACEi, angiotensin I-converting enzyme inhibitor; F, female; HSR, hypersensitivity reaction; M, male.
Values are mean ± s.d.
All dialyzers were equipped with the AN69 membrane and ACEi was prescribed for more than 1 month.
Table 2 | Parameters characterizing the γ model fitted to kinetic parameters of endogenous kinins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BK HSR−</th>
<th>HSR+</th>
<th>HSR−</th>
<th>HSR+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha (α)</td>
<td>0.81 ± 0.20</td>
<td>0.69 ± 0.18</td>
<td>0.87 ± 0.30</td>
<td>0.70 ± 0.16</td>
</tr>
<tr>
<td>P=0.043</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formation t₀ (min)</td>
<td>1.30 ± 0.39</td>
<td>1.15 ± 0.52</td>
<td>6.54 ± 5.88</td>
<td>7.79 ± 6.83</td>
</tr>
<tr>
<td>P=0.247</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formation t₀ slope</td>
<td>14 349 ± 4163</td>
<td>18 747 ± 11 041</td>
<td>451 ± 202</td>
<td>737 ± 413</td>
</tr>
<tr>
<td>P=0.168</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum (nm)</td>
<td>523 ± 137</td>
<td>559 ± 101</td>
<td>79 ± 46</td>
<td>239 ± 275</td>
</tr>
<tr>
<td>P=0.375</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of maximum (min)</td>
<td>7.1 ± 1.4</td>
<td>7.3 ± 2.1</td>
<td>39 ± 47</td>
<td>112 ± 239</td>
</tr>
<tr>
<td>P=0.780</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta (β)</td>
<td>0.12 ± 0.04</td>
<td>0.10 ± 0.04</td>
<td>0.04 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>P=0.168</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degradation t₀ (min)</td>
<td>21.3 ± 5.2</td>
<td>23.5 ± 6.5</td>
<td>107 ± 106</td>
<td>160 ± 124</td>
</tr>
<tr>
<td>P=0.218</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degradation t₀ slope</td>
<td>-12169 ± 841</td>
<td>-2107 ± 1101</td>
<td>-74 ± 43</td>
<td>-80 ± 58</td>
</tr>
<tr>
<td>P=0.829</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (pmol/ml min)</td>
<td>11.8E3 ± 4.5E3</td>
<td>14.0E3 ± 4.70E3</td>
<td>16.5E3 ± 40.5E3</td>
<td>31.6E4 ± 1.04E6</td>
</tr>
<tr>
<td>P=0.135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AUC, area under the curve; BK, bradykinin; des-Arg⁹-BK, des-Arginine⁹-BK; HSR, hypersensitivity reaction.
Values are means ± s.d.
Table 3 | Heritability analysis of kinetic parameters for the kinins BK and des-Arg⁹-BK with SOLAR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BK</th>
<th></th>
<th></th>
<th>des-Arg⁹-BK</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heritability</td>
<td>Error</td>
<td>Probability</td>
<td>Heritability</td>
<td>Error</td>
<td>Probability</td>
</tr>
<tr>
<td>Alpha (α)</td>
<td>0.08</td>
<td>0.112</td>
<td>0.220</td>
<td>0.10</td>
<td>0.099</td>
<td>0.113</td>
</tr>
<tr>
<td>Formation t₀ (min)</td>
<td>0.19</td>
<td>0.136</td>
<td>0.043</td>
<td>0.29</td>
<td>0.132</td>
<td>0.004</td>
</tr>
<tr>
<td>Formation t₀ slope</td>
<td>0.21</td>
<td>0.108</td>
<td>0.009</td>
<td>0.37</td>
<td>0.145</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Maximum (nm)</td>
<td>0.33</td>
<td>0.127</td>
<td>&lt; 0.001</td>
<td>0.51</td>
<td>0.150</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Time of maximum (min)</td>
<td>0.50</td>
<td>0.156</td>
<td>&lt; 0.001</td>
<td>0.24</td>
<td>0.155</td>
<td>0.035</td>
</tr>
<tr>
<td>Beta (β)</td>
<td>0.35</td>
<td>0.138</td>
<td>0.001</td>
<td>0.36</td>
<td>0.131</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Degradation t₀/₂ (min)</td>
<td>0.56</td>
<td>0.147</td>
<td>&lt; 0.001</td>
<td>0.22</td>
<td>0.153</td>
<td>0.051</td>
</tr>
<tr>
<td>Degradation t₀/₂ slope</td>
<td>0.34</td>
<td>0.126</td>
<td>&lt; 0.001</td>
<td>0.39</td>
<td>0.151</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AUC (pmol/ml.min)</td>
<td>0.30</td>
<td>0.149</td>
<td>0.008</td>
<td>0.34</td>
<td>0.159</td>
<td>0.005</td>
</tr>
</tbody>
</table>

AUC, area under the curve; BK, bradykinin; des-Arg⁹-BK, des-Arg⁹-in⁹-BK; SOLAR, Sequential Oligogenic Linkage Analysis Routines.
Table 4 | Genotype distribution of the *XPNPEP2* C-2399A polymorphism in HSR+ and HSR− patients

<table>
<thead>
<tr>
<th></th>
<th>A and AA</th>
<th>CA</th>
<th>C and CC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSR+</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>HSR−</td>
<td>6</td>
<td>7</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
<td><strong>9</strong></td>
<td><strong>33</strong></td>
<td><strong>52</strong></td>
</tr>
</tbody>
</table>

HSR, hypersensitivity reaction.
Figure 1 | Cohorts ascertained for linkage analysis. One member of each kindred (the propositus), depicted in black, developed HSR associated with ACEI therapy. Willing participants are depicted in white. Non-participants are shaded. The SNP genotype and the APP activity are indicated for each member of the different families. SNP genotypes were not available for family XIII.
Figure 2 | **Distribution of APP activities.** Distribution of APP activities in HSR+ and HSR− patients compared with the reference population.  
*P = 0.013 HSR+ vs HSR−; †P = 0.000 HSR+ vs reference.
Figure 3 | Metabolism of endogenous BK and des-Arg⁹-BK. Mean kinetic model-fitted profiles of formation and degradation of (a) BK and (b) des-Arg⁹-BK for HSR+ (straight line) and HSR− (dashed line) patients after activation of the contact system with glass beads in the presence of enalapril.
Figure 4 | Specific and nonspecific inhibition of human recombinant soluble membrane APP. Effect of increasing concentrations of various inhibitors on recombinant soluble hmAPP-catalyzed hydrolysis of K(Dnp)PPGK(Abz)NH₂ as described in Materials and Methods. The data were plotted to fit the one-site competition equation by nonlinear regression with GraphPad software and imposed constraints (-5.0 to 110% activities). IC₅₀ obtained were 0.6 ± 1.1 μM for apstatin (APS), 17 ± 1.1 μM for captopril (CAP), and over 1 mM for enalapril (ENA). Curves for lisinopril (LIS) and ramipril (RAM) did not converge (no inhibition).
Figure 5 | Hypothesis for a multifactorial nature of the HSR in hemodialysis. HSR results from the meeting of at least three different factors: a physicochemical factor triggers the contact system of plasma and liberates BK, a pharmacological agent inhibits ACE activity, and a metabolic aspect which characterizes the capacity of the patient to metabolize kinins.
CHAPTER 4: ESTROGEN-DEPENDENT INHERITED ANGIOEDEMA

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Reference:

Qing Ling Duan, Karen Binkley, Guy A. Rouleau. Genetic analysis of factor XII and bradykinin catabolic enzymes in a family with estrogen-dependent inherited angioedema. Accepted by the *Journal of Allergy and Clinical Immunology*.
4.1 RATIONALE

The first report of an estrogen-dependent inherited form of angioedema (EDIA) identified an Italian family with multiple affected cases over three generations (Binkley et al. 2000). Three of the affected sisters from this family, their spouses and children, reside in Canada and were collected for our genetic study. Initially, we set out to genotype this family for rs3788853 in the XPNPEP2 gene, which is associated with reduced APP activity as described in the previous two chapters. Although Binkley and Davis had excluded the role of C1INH as the cause of angioedema symptoms in this family, no previous study had studied the role of BK degradation in EDIA. Thus, the aim of this study was to determine if variants (e.g. rs3788853 and I/D in ACE) associated with reduced BK degradation, could account for EDIA cases as well. However, prior to beginning our project, two independent studies reported a gain of function mutation in exon 9 of coagulation factor XII gene (F12), which activates the production of BK, in four estrogen-associated angioedema families (Cichon et al. 2006; Dewald et al. 2006). This new gene does not exclude the possibility that impaired BK degradation may also increase risk for EDIA.

The clinical symptoms experienced by all three females were identical to HAE types I and II, which result from decreased C1INH function or expression. An important distinction was that these females developed AE symptoms only during pregnancy and hormone replacement therapy. Two additional family members who did not participate in this study developed AE symptoms within days of starting oral contraceptives. AE symptoms were considered as the first sign of pregnancy in this family. One case reported that she knew she was pregnant when her period was only two days late because
one side of her face swelled up, which also happened to her mother and sisters when they were pregnant. For all three cases, AE symptoms began within two to three weeks after conception and persisted throughout each pregnancy. Although there was no fatality in this family to date, the clinical similarities to HAE and AE-ACEi indicated that this risk should be considered as fatality from laryngeal edema in HAE is approximately 25% (Binkley et al. 2000). Also, there is currently no treatment of this form of AE since traditional therapies for HAE are not effective in these EDIA cases. For instance, administration of C1INH concentrate is not useful and androgen therapy is prohibited during pregnancy.

The members of this family were very eager to participate in our genetic study in hopes that we could identify asymptomatic members and male carriers whose daughters should be screened prior to pregnancy and to determine who should avoid estrogen therapy (e.g. contraceptive pills and hormone replacement therapy).
4.2 Genetic analysis of factor XII and bradykinin catabolic enzymes in a family with estrogen-dependent inherited angioedema

4.2.1 Abstract

Background: Recent studies reported a gain of function mutation in the gene encoding coagulation factor XII among five German and French families with estrogen-associated angioedema, who share a common ancestor. The role of this factor and/or additional pathways that might contribute to elevated bradykinin (BK) levels remains to be determined in other families with estrogen-dependent or associated inherited angioedema.

Objective: The purpose of this study was to determine if mutations in the factor XII gene and/or polymorphisms in the genes for aminopeptidase P (APP) and angiotensin I-converting enzyme (ACE), which have been associated with increased BK levels, contribute to estrogen-dependent inherited angioedema in a large family of Italian origin.

Methods: We screened the coding regions of the F12 and XPNPEP2 genes, encoding factor XII and membrane-bound APP, respectively, for genetic variants in the affected females. In addition, we genotyped this family for the insertion/deletion (I/D) polymorphism in the ACE gene, which accounts for variable ACE levels.

Results: The three affected females all have the threonine to lysine (Thre328Lys) mutation, associated with higher factor XII activity. In addition, they have at least one A allele of rs3788853 at the XPNPEP2 locus, associated with lower APP activity, and at least one I allele in ACE, associated with lower ACE activity.
Conclusion: A missense mutation in $F12$ is present in three affected females of this family with estrogen-dependent inherited angioedema. In addition, these three affected females have polymorphisms associated with lower levels of both APP and ACE, the major enzymes responsible for BK degradation. Thus, our study suggests that multiple genes may contribute to estrogen-dependent or associated inherited angioedema, and explain some of the observed heterogeneity.
4.2.2 Introduction

Classic forms of hereditary angioedema (HAE types I and II) are autosomal dominant disorders resulting from decreased levels or function of the C1 esterase inhibitor (C1 INH) (Cyr et al. 2001; Davis 2003). These manifest as localized swelling of parts of the face, the upper respiratory tract, the gastrointestinal tract, genitalia, the hands and/or feet (Online Mendelian Inheritance of Man (OMIM) 106100) (Agostoni et al. 2004). Inadequate C1 INH fails to restrict the activity of coagulation factor XII (Hageman factor) and kallikrein, leading to increased production of bradykinin (BK), a potent vasodilator and an important mediator of angioedema (Davis 1988; Davis 2003). Additional forms of hereditary and/or sporadic angioedema, which are clinically indistinguishable from classic HAE, have also been reported among individuals with normal C1 INH concentration and function. These include cases of unknown etiology (idiopathic angioedema) (Champion et al. 1969; Cicardi et al. 1999), drug-induced (e.g. angiotensin I-converting enzyme inhibitors (ACEi) (Vleeming et al. 1998) and plasminogen activators (rt-PA) (Rafii et al. 2005)), or angioedema that affect only women (Binkley et al. 2000; Bork et al. 2000). The latter forms are known as estrogen-dependent (or associated) inherited angioedema (EDIA or HAE type III; OMIM 610618)) as they depend on or are worsened by elevated levels of endogenous (e.g. pregnancy) or exogenous estrogen (e.g. oral contraceptives or hormone replacement therapy).

Recent studies identified a gain of function mutation in the gene encoding factor XII (F12) among females with estrogen-associated angioedema originating from Germany and France who shared a common ancestor (Cichon et al. 2006; Dewald et al. 2006). This Thr328Lys substitution in exon 9 of F12 was associated with increased
factor XII activity but not protein levels (Cichon et al. 2006). Earlier studies reported that an estrogen response element (ERE) in the promoter region of the gene contributes to increased gene expression during elevated estrogen states (Klein-Hitpass et al. 1989; Farsetti et al. 1995). Taken together, women with increased estrogen levels and a gain of function mutation in \( F12 \) have elevated levels of a highly active factor XII. It is hypothesized that increased activity and expression of factor XII contributes to higher BK production (Figure 1), which increases vascular permeability and risk of angioedema (Proud et al. 1988; Nussberger et al. 2002). Thus, similar to classic forms of HAE, estrogen-associated inherited angioedema may result from increased levels of BK.

In this study, we investigated the \( F12 \) locus in a family of Italian origin with a previously well characterized, strictly estrogen-dependent angioedema phenotype (Binkley et al. 2000). Binkley and Davis had confirmed the absence of mutations in the coding and 5' untranslated region (UTR) of C1-INH gene (\( SERPING1 \)) as well as the 5'UTR of \( F12 \) among the affected females of this family (Binkley et al. 2000). In addition, we extend our investigation to examine the potential contribution of BK degradation pathways to the EDIA phenotype in this family. As shown in Figure 1, elevated BK levels may result from increased production (e.g. higher factor XII activity) or decreased degradation due to deficiencies of several catabolic enzymes. For example, ACE (angiotensin I-converting enzyme) and APP (aminopeptidase P) are the primary and secondary enzymes responsible for the degradation of BK (Bhoola et al. 1992), with additional enzymes representing minor pathways (Figure 1). Furthermore, ACE and APP also represent the major pathways for the degradation of the active metabolite of BK known as des-Arginine\(^9\)-BK (des-Arg\(^9\)-BK) (Blais et al. 1999; Blais et al. 1999). Genetic
variants in XPNPEP2, resulting in reduced APP activity (Duan et al. 2005) and higher BK and des-Arg⁰-BK levels (Molinaro et al. 2006), have been associated with angioedema induced by ACE inhibitors (Duan et al. 2005). Also, this study investigates the potential role of an insertion/deletion (I/D) polymorphism in the ACE gene, which accounts for 50% of the variability in human serum ACE levels (Rigat et al. 1990). Previous studies have demonstrated that the I allele is associated with lower expression of the ACE mRNA (Suehiro et al. 2004) and decreased degradation of BK (Murphey et al. 2000).

### 4.2.3 Materials and Methods

DNA was extracted from blood samples of 29 members of a family of Italian origin with multiple EDIA cases, which was previously described by Binkley and Davis (Binkley et al. 2000). Figure 2 shows a modified pedigree comparable to the earlier report with several additional members who did not participate in the previous study (e.g. spouses and children). Blood samples for the current investigation were collected in October 2007 from participants who now reside in Canada and represent a subset of the original pedigree. For example, only three affected females (II:2, II:7, II:8) were available for our study whereas three of their affected relatives (II:4, III:4, III:9) reside in Italy and were not available to participate in this study. The phenotypic details for this family may be found in the earlier report by Binkley and Davis (Binkley et al. 2000). In brief, all affected females of this family experienced angioedema symptoms (without urticaria) throughout all their pregnancies (with multiple episodes) as well as during estrogen replacement therapy but not any time in between. Their angioedema symptoms included
swelling of the face and extremities, airway obstruction, as well as abdominal pain and vomiting, which are similar to HAE types I and II. All participants provided informed consent and all protocols were approved by the Ethics Committees at the Centre Hospitalier de l'Université de Montreal and Sunnybrook Health Sciences Centre, Toronto, Canada.

The UTRs and exons of F12 and XPNPEP2 were sequenced for three affected females (II:2, II:7, II:8) and two unrelated male controls (spouses of II:7 and II:8). These coding regions were amplified using intronic primers in standard polymerase chain reactions (PCR) and sequenced at the Genome Québec Innovation Centre in Montreal, Canada. Sequence traces were viewed and analyzed by the program Seqman (DNASTAR package) to identify genetic variants. Furthermore, all family members (n = 29) were screened for exons 9 of F12, genotyped for rs3788853 in XPNPEP2 as well as the ACE/ID polymorphism. Exon 9 of the F12 gene was amplified using primers: 5'-ACACCAAGGCAAGCTGCTAT -3' and 5'- GCTGGCCGGAATCTAGCTC-3', with an optimal annealing temperature of 55°C. The rs3788853 polymorphism located 5' of XPNPEP2 was genotyped using allele specific PCR with one common forward primer: 5'-AACCCTCCCCACGTTGAATCA-3' and a choice of two allele specific reverse primers: 5'-GCACTGCTGAAATAGCAGTTGTTAG-3' and 5'-GCACTGCTGAAATAGCAGTTGTTAT-3' in parallel PCRs with a annealing temperatures of 63°C, followed by gel electrophoresis using 1.5% agarose gel for genotype calling (Duan et al. 2005). Duan et al. had previously determined the high efficacy and reproducibility of this allele specific genotyping method by comparisons with sequencing results in more than 300 DNA samples. The I/D in ACE was genotyped
using primers: 5'- CTGGAGACCACCTCCCATCCTTTCT-3' and 5'-GATGTGGCCATACATCCGTACAGAT-3', with an optimal annealing temperature of 58°C. Genotypes for this I/D polymorphism was determined using agarose gel electrophorsis following a standard PCR as the D allele was 287 bp shorter than the I allele (490 bp).

4.2.4 Results

Figure 2 shows that all three affected females with a history of EDIA (II:2, II:7, II:8) are heterozygous for Thr328Lys (c. 1032 C>A), a gain of function mutation in exon 9 of the \( F12 \) gene, which has been previously described (Cichon et al. 2006; Dewald et al. 2006). In addition, this family has five male carriers (III:5, III:19, IV:3, IV:5, IV:7) and one female heterozygote (IV:6) with undetermined phenotype. At age 20, this female carrier has not demonstrated symptoms of EDIA, in keeping with the fact she has never been pregnant or received exogenous estrogen (oral contraceptives or estrogen replacement).

In addition to the gain of function mutation in \( F12 \), all three affected females have at least one copy of the A allele at the SNP rs3788853, located 5' of \( XPNPEP2 \), which codes for membrane-bound APP. This allele has been associated with decreased APP activity, decreased BK and des-Arg9-BK degradation as well as angioedema induced by ACE inhibitors (Duan et al. 2005; Molinaro et al. 2006). Ten other blood relatives within this family also carry this polymorphism but are unaffected, including the asymptomatic female (IV:6) with the Thr328Lys mutation in \( F12 \). Screening of the coding regions of \( XPNPEP2 \) yielded no mutations in the affected members of this family.
Finally, the three affected females have at least one copy of the inserted (I) allele in intron 16 of the \textit{ACE} gene (Figure 2), which is associated with lower levels of ACE (Rigat et al. 1990; Suchiro et al. 2004). Based on previous studies, one can infer that the individuals with the I/I or I/D genotypes have significantly lower ACE levels compared to individuals with the D/D genotype. Three male relatives of these affected females also have the I/D genotype and eight relatives have the I/I genotype (five male and three female). The female carrier with undetermined phenotype (IV:6) with the F12 mutation and C/A genotype at rs3788853 in the \textit{XPNPEP2} locus also has the I/I genotype in \textit{ACE}, associated with low levels of ACE.

4.2.5 Discussion

In this report, we identify a gain of function mutation in coagulation factor XII gene among three affected EDIA cases in a previously well characterized family (Binkley et al. 2000). High estrogen levels, acting via an ERE in the promoter region of the \textit{F12} gene, increase expression of the mutant factor XII with a gain of function, which is thought to contribute to increased BK levels and angioedema. While this mutation has been documented in families from Germany and France (Cichon et al. 2006; Dewald et al. 2006), we are the first to report this mutation in a family of Italian origin, demonstrating that \textit{F12} determines risk for EDIA in other European populations. It remains to be determined if this Italian family shares a common ancestor with the German and French families previously described. Our study demonstrates that genetic screening of \textit{F12} in additional families with EDIA is warranted, regardless of their country of origin.
Additional studies are necessary to determine the role of this gene in determining EDIA risk in non-European populations.

This study is the first investigation to examine the role of BK catabolic pathways in EDIA. ACE and APP represent the primary and secondary degradation pathways of BK, respectively, with reversed importance in the degradation of des-Arg⁹-BK, an active metabolite of BK (Figure 1). Reduced levels or activities of ACE and/or APP would result in reduced degradation and increased levels of BK and des-Arg⁹-BK, and therefore, greater risk of angioedema (Blais et al. 1999). All three affected females studied in this family have at least one copy of the A allele of rs3788853 at the XPNPEP2 locus, which has been associated with low APP activity and increased risk for angioedema during ACEi therapy (Duan et al. 2005). This minor allele (frequency of 27% in the Caucasian population (CEU) of the Hapmap project) is also present in ten unaffected relatives and two unaffected individuals married into the family. This supports the previous hypothesis that individuals with reduced APP activity are asymptomatic unless a biologically relevant molecule, such as ACE, is also deficient or inhibited (Duan et al. 2005). Genotyping of the ACE I/D polymorphism revealed that all three affected females studied also had at least one copy of the I allele, which is associated with reduced ACE expression. This allele is also common in the general population with reported genotype frequencies of 25.6% (I/I), 48.3% (I/D) and 26.1% (D/D) (Scheer et al. 2005). This polymorphism has been associated with reduced gene expression (Suehiro et al. 2004) and higher BK levels (Murphey et al. 2000).
The effect of estrogen on the levels and/or activities of BK catabolic enzymes may have particular importance in generation of the estrogen-dependent nature of the phenotype in this family. Previous studies have demonstrated that estrogen suppresses ACE expression (Stevenson et al. 2004) and increases BK concentrations (Sumino et al. 1999; Nogawa et al. 2001), independent of the ACE I/D genotype (Sumino et al. 2003). While the effect of estrogen on APP activity is unknown, a recent report suggests that androgens increase APP levels (Drouet et al. 2008). As androgens and estrogens often have antagonistic effects, it is not unreasonable to speculate that estrogen may reduce APP activity. In normal individuals, high estrogen states would facilitate high BK levels through both increased production (increased factor XII) and reduced degradation (reduced ACE and possibly APP levels). In affected members of this family, the normal effects of estrogen on BK levels would be accentuated by the presence of the mutant factor XII with a gain of function, and the polymorphisms associated with pre-existing low activities of ACE and APP.

Although specific measurements for factor XII, APP and ACE levels were not performed, this study suggests that genetic variants in multiple loci may contribute to angioedema, possibly through their effects on BK levels. The presence or absence of genetic variants at these additional loci may contribute to the observed heterogeneity of clinical phenotypes (ie, estrogen-dependent vs. estrogen-associated angioedema) (Binkley et al. 2003), and even heterogeneity within the classic forms of HAE. Further studies of BK catabolic enzymes in other families with EDIA would help delineate these issues.
This report also demonstrates the application of predictive testing to identify presymptomatic carriers of risk alleles, which will aid in the management of estrogen-dependent inherited angioedema. As the affected members of this family have reported multiple episodes of near-fatal laryngeal edema, the prevention of clinical symptoms in at-risk individuals may be life saving. For example, a female of undetermined phenotype (IV:6) carries one risk allele of the Thr328Lys mutation in F12 as well as alleles associated with lower APP and ACE. This genetic information will allow this individual to avoid symptoms by selecting contraceptive strategies other than estrogen-containing agents, and later in life, alternative strategies to estrogen-containing hormone replacement regimes to manage menopausal symptoms. In addition, we have also identified several male family members who carry the mutation, but have never been affected due to the strict estrogen dependence of the phenotype in this family. Identification of such male carriers will allow targeted screening of their offspring so that symptoms can be avoided in subsequent generations.
4.2.6 Acknowledgements

We would like to thank the family for their participation in this study, Ms. Olga Likhodi for sample collection, and Dr. Patrick Dion for careful review of this manuscript. We acknowledge support for this study from the Canadian Institute of Health Research (GAR), and the Allergy, Asthma and Immunology Society of Ontario (KB). QLD was supported by the Heart and Stroke Foundation of Canada.
Figure 1. Proposed mechanisms for BK production and degradation in estrogen-dependent inherited angioedema. The Thr328Lys mutation in exon 9 of F12 increases activity of the factor XII protein. High estrogen levels, acting via the estrogen response element (ERE) in the promoter region (located between nucleotide positions 44 to 31) increases expression of the over-active factor XII, leading to increased Bradykinin (BK) production from high molecular weight kininogen via activated kallikrein. This nonapeptide (R₁-P²-P³-G⁴-F⁵-S⁶-P⁷-F⁸-R⁹ (Arginine¹-Proline²-Proline³-Glycine⁴-Phenylalanine⁵-Serine⁶-Proline⁷-Phenylalanine⁸-Arginine⁹)) is an important mediator of angioedema but is normally short-lived as it is cleaved by several enzymes. ACE and APP represent the primary and secondary degradation pathways of BK with reversed importance in the degradation of Des-Arginine⁹-BK (R₁-P²-P³-G⁴-F⁵-S⁶-P⁷-F⁸), an active metabolite of BK, which may also contribute to angioedema. Other known enzymes involved in the degradation of BK include CPN, DPPIV and NEP, resulting in inactive metabolites. The relative contribution of each degradation pathway is represented by the width of arrows.
LEGEND

- Female, male: unaffected
- Female: affected
- Male: obligate carrier
- Female, male: phenotype unknown
- Deceased
Figure 2. Segregation of the Thr328Lys mutation in F12, rs3788853 in XPNPEP2 (abbreviated XP2), and ACE I/D polymorphism. Affected females (blackened circles) are heterozygous for Thr328Lys, carry at least one copy of the A allele of rs3788853 as well as at least one copy of the I allele in ACE. Individual IV:6 is a young female who is heterozygous for Thr328Lys, rs3788853 and homozygous for the I allele in ACE, however, her phenotype is unknown since she has not experienced high estrogen states. There are five male carriers of the Thr328Lys mutation, eleven individuals with at least one copy of the A allele of rs3788853, and eleven relatives with one or more copies of the I allele in ACE. Note that rs3788853 is located on the X chromosome, thus males are hemizygotes.
CHAPTER 5: DEPRESSION IN CORONARY ARTERY DISEASE PATIENTS

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Reference:

5.1 RATIONALE

As outlined in chapter 1, the association between depression and CAD has been well established by more than twenty studies. Nevertheless, the mechanism for this association remains unclear. We hypothesize that genetic variation(s) that increase risk for depression and CAD could account for the co-occurrence of these two diseases. Moreover, we identified a list of candidate genes which contribute to six biological pathways that might determine susceptibility to both CAD and depression. Many of these genes have been previously associated with depression and/or CAD but some were selected based on their biological relevance (e.g. code of key protein in the relevant pathway).

We began our candidate gene association study of depression in CAD patients prior to the completion of phase I of the International Hapmap project (October 2005), when genome wide association studies (GWAS) were not yet routine as genotyping cost remained relatively high. To minimize expenses, we selected tag SNPs across 59 candidate genes for genotyping using the Illumina platform. Tag SNPs identified using Haploview software was based on linkage disequilibrium (LD) or high correlation between neighbouring SNPs, defined by a coefficient of determination \( r^2 \geq 0.8 \). This permitted us to reduce the number of SNPs for genotyping across a gene without loss of power (Zhang et al. 2005). Our SNP selection was based on available genotype data generated prior to April 2005 in the CEPH population, under the assumption that this Caucasian population was representative or similar to our French Canadian population (Service et al. 2007)
5.2 GENETIC PREDICTORS OF DEPRESSIVE SYMPTOMS IN CARDIAC PATIENTS.

5.2.1 Abstract

Objective: To conduct a candidate gene study focusing on key elements of the inflammation, platelet aggregation, endothelial function and omega-3 and -6 fatty acid metabolism pathways to identify genetic predictors of depressive symptoms in cardiac patients.

Background: Numerous studies suggest that the prevalence of depression is greater among cardiac patients than in the general population. Although several biological mechanisms have been proposed to account for this effect, little attention has been paid to the possibility of genetic contributions to depressive symptoms in cardiac patients.

Methods: Over 700 single nucleotide polymorphisms were successfully genotyped on 17 different chromosomes in 59 genes among 977 cardiac patients of French-Canadian descent, all of whom had completed the Beck Depression Inventory – II (BDI-II).

Results: One SNP, rs216873, within the von Willebrand factor gene (VWF) was significantly associated with BDI – II scores following statistical correction for multiple comparisons. Several additional SNPs related to endothelial dysfunction, platelet aggregation, inflammation and/or previously associated with depression in the literature were identified as suggestive of association (P values < 0.01).

Conclusions: These results suggest that genetic variation related to endothelial dysfunction is predictive of depressive symptoms in cardiac patients and that endothelial dysfunction may be a novel mechanism contributing to depressive symptoms in this patient population.
5.2.2 Introduction

Numerous studies have documented a disproportionately high prevalence of depression among cardiac patients. For example, the prevalence of depression for the U.S. population at large is approximately 7% (Kessler et al. 2003), whereas the prevalence of depression ranges from 15-20% in cardiac patients, with the highest rates often seen among those who recently experienced a cardiac event (Lett et al. 2004). In a recent community-based survey, 12 month prevalence of depression among cardiac patients was approximately twice the prevalence among people not reporting any chronic illness (Egede 2007). This heightened level of depressive symptoms has consistently been associated with greater risk for cardiac mortality (Lett et al. 2004). Several mechanisms have been suggested to account for the greater prevalence of depression among cardiac patients, including the stress of a poor prognosis, altered neuroendocrine function, heightened platelet activity, systemic inflammation, or the neurological effects of disease processes (Carney et al. 2002; Lett et al. 2004). However, little attention has been paid to the potential for a genetic contribution to depressive symptoms with CAD.

To identify specific genes that may influence depressive symptoms in CAD, we conducted a candidate gene study for depressive symptoms in cardiac patients. We included nearly 700 SNPs within 59 genes, focusing on key elements of three biological pathways associated with depressive symptoms in CAD, inflammation, platelet aggregation and omega-3/6 fatty acid metabolism (Carney et al. 2002; Frasure-Smith et al. 2004; Lett et al. 2004). We also included additional genes previously associated with depression (e.g. CREB1) and select genes from other biological pathways thought to be associated with depression and CAD (e.g., hypothalamic-pituitary adrenal cortical,
sympathetic nervous system and parasympathetic axes). We limited our study to cardiac patients of French-Canadian descent to limit allelic heterogeneity and the possibility that our results might be confounded by the effects of population stratification or genetic admixture (Davignon et al. 1993; Heyer et al. 1995).

5.2.3 Methods

Participants

The study included French Canadian patients with CAD from two cohorts: the POLYMORPHISME project and the Epidemiological Study of Acute Coronary Syndromes and the Pathophysiology of Emotions (ESCAPE) (Lesperance et al. 2004). All patients had angiographic evidence of greater than 50% blockage in at least one major coronary artery or a documented myocardial infarction (MI). Participants were recruited from the Montreal Heart Institute and Hôpital Sacré-Cœur in Montreal between November 19, 1998 and April 4, 2002. Projects received ethical approval from the ethics boards of the participating hospitals before beginning recruitment and each participant provided written informed consent. While the POLYMORPHISME project was limited to French Canadians, 25% of the participants in ESCAPE had other backgrounds, and only those with four French Canadian grandparents were eligible for the current genetic analyses. Blood samples were available for 482 POLYMORPHISME participants and 602 subjects from ESCAPE.

To test the feasibility of combining the two cohorts for analysis and the validity of the genetic data, we tested the successfully genotyped samples (459 from POLYMORPHISME and 568 from ESCAPE). First, we computed the average identity
by state between each pair of individuals based on the present genotyping to identify related individuals or those who may have participated in both studies. We identified 47 duplicated individuals (≥ 98% identity) and 3 related individuals (86-97% identity) across the two cohorts and removed one from each pair for subsequent analysis. This resulted in 977 participants available for study, 416 from POLYMORPHISME and 561 from ESCAPE. To test for population stratification, we estimated the genomic control variance inflation factor (VIF) (Devlin et al. 1999; Bacanu et al. 2000) using 50 uncorrelated SNPs as "null" SNPs. To do so, we randomly chose one SNP from each gene, we removed 6 SNPs because the genotype test, the allele test or the trend test showed significant association with depressive symptoms and removed another 3 SNPs because they were correlated. We kept 50 SNPs, among which no pair was correlated with $r^2 > 0.2$. The VIF coefficients were computed using the CASECONTROL procedure in SAS. VIFs for ESCAPE (VIF $\lambda = 0.88$), POLYMORPHISME (VIF $\lambda = 0.70$) and the combined cohort (VIF $\lambda = 0.40$) were all less than 1.0, in agreement with an absence of population stratification effect in this cohort.

We also constructed quantile-quantile (Q-Q) plots of the trend statistics with the 50 selected SNPs. We showed that the observed statistics were consistent with the expected values given the assumption that they have been sampled from a chi square distribution with one degree of freedom. This was true for the ESCAPE cohort alone, for the POLYMORPHISME cohort alone and for the complete data set (data not shown). Next, we compared the allele frequencies between the two cohorts. The trend test for differences between SNP allele frequencies in the two cohorts and the associated quantile plot showed good agreement between the two cohorts (data not shown).
Measures.

Cardiovascular disease: Data on cardiac history (left ventricular ejection fraction, MI, coronary bypass surgery, angioplasty, stroke) were abstracted from medical records. Fasting lipid profiles, height, weight, blood pressure, marital status, education, physical activity, smoking and current medications were assessed at the time of the blood sampling.

Depressive symptoms: Depressive symptoms were measured using the 21-item Beck Depression Inventory (BDI-II (Beck et al. 1996)), a self-report questionnaire commonly used to assess depressive symptoms, and recently recommended for inclusion in studies of CAD and depression by a National Heart, Lung and Blood Institute working group (Davidson et al. 2006). In the current analyses the BDI-II demonstrated high internal consistency (α = 0.91).

Genotyping

Candidate genes were selected based on their relevance to a biological pathway of interest or prior association with depression in the literature (McCaffery et al. 2006). As a first step in identifying candidate genes that might be associated with depressive symptoms in cardiac disease, we focused on genes related to inflammation, platelet aggregation, endothelial function and omega-3/6 fatty acid metabolism. Genes coding for key elements of other relevant biological pathways (e.g. hypothalamic-pituitary-
adrenal cortical axis) were included to the extent possible. A list of the 59 candidate genes targeted as part of this study is presented in Supplemental Table 1.

SNPs were selected based on public information available in April 2005 (dbSNP [genome build 35]). We initially chose 768 SNPs in and around our candidate genes, which met the following criteria: (1) minor allele frequency > 0.05, (2) absence of other flanking variants within 60 nucleotides, (3) score from Illumina ≥ 0.6, (4) submission to dbSNP by more than one source, and (5) validation status. Priority was given to non-synonymous changes located in coding regions and SNPs showing prior association with depression and/or heart disease in the literature. We also used genotype data available in April 2005 from the International HapMap project (www.hapmap.org) to identify tag SNPs, which represent different haplotype blocks within our candidate genes.

Using an Illumina platform, 733 SNPs were successfully genotyped on 17 different chromosomes in 59 genes. Of these SNPs, 33 were not polymorphic, 28 SNPs had minor allele frequencies that were less than 0.01 and were removed from analysis, and one heterozygous SNP on the X chromosome was removed, leaving 671 SNPs available for analysis. Six heterozygous genotypes of males at SNPs on the X chromosome were replaced by missing values.

Statistical Analysis

We used a general linear model (GLM) to test for association of SNPs with depressive symptoms in the sample (McCaffery et al. 2007). In all analyses, a log transform was used for the BDI-II scores to correct the positive skew of the distribution. In order to define the best regression model, we considered the following covariates: age, sex,
obesity, physical activity, smoking status, total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, left ventricular ejection fraction and MI, coronary artery bypass surgery, coronary angioplasty and stroke prior to interview. We did a univariate analysis for each in relation to BDI-II scores using a GLM procedure in SAS. Sex was the only covariate significantly related to the BDI-II ($P < 0.0001$).

**SNP association test:** We used a genotype trend model where the additive allelic effect is captured with the model: $y = \mu + \beta_1 \text{allele} + \beta_2 \text{sex}$, where $y$ denotes the log transformed Beck score, $\mu$ is the mean term, $\beta_1$ models the additive allelic effect, and $\beta_2$ models sex. The genotypes were coded as -1, 0, +1 for the three genotypes, where -1 represents the homozygote state for the common allele, 0 the heterozygous state, and +1 the homozygous state for the minor allele. This model was built using the GLM procedure in SAS, which gives a Fisher analysis of variance test and a student test for regression coefficients. For the genetic association, we considered the type III SS p-value of the allele effect term, which was adjusted for sex effects. Association tests with X-chromosome data are valid under this model. To control for multiple testing, we calculated the effective number of independent markers ($M_{\text{eff}}$) from the computation of eigenvalues of the correlation matrix of SNPs as described by Li and Ji (Li et al. 2005). The $M_{\text{eff}}$ method is more accurate than Bonferroni correction when SNPs are moderately correlated. We estimated the $M_{\text{eff}}$ to 239 effective independent tests from 671 SNPs, which corresponds to a significance threshold of 0.000209.
We conducted haplotype association testing using each individual’s probability of having a particular haplotype according to haplotype estimation by expectation maximization in GLM and logistic regression according to previously described methods (Schaid et al. 2002; Zaykin et al. 2002). The linear and the logistic regressions were performed for haplotype of SNP pairs with adjustment for gender. Haplotype odds ratios were estimated by comparing the frequency equivalent of haplotype probabilities of the most significant haplotype with a positive estimate of $B_j$ to the frequency equivalent of the combined probabilities of the other haplotypes.

Given the established association of sex with depressive symptoms, we further investigated possible statistical interaction effects between sex and SNPs for each SNP in exploratory analyses. Using the GLM procedure in SAS, we tested the interaction term of the model $y = \mu + \beta_1$allele + $\beta_2$.sex + $\beta_3$.(allelexsex), where $y$ denotes the log transformed BDI-II score, $\mu$ is the mean term, $\beta_1$ models the additive allelic effect, $\beta_2$ models sex and $\beta_3$ the interaction between SNP’s additive effect and sex.

5.2.4 Results

Participants

Demographic information and descriptive statistics of the study participants are presented in Table 1. Participants were on average 59 years of age; 21% female, 49% currently married with a mean education level of approximately 11 years. The vast majority of participants (95%) experienced a prior cardiac event, as defined as a previous MI, coronary artery bypass surgery or angioplasty. The percentage with left ventricular ejection fraction < 45% was 19%. The most common cardiac medications were beta-
blockers (75%), statins (73%) and angiotensin-converting enzyme inhibitors (45%),
while only 12% were treated with anti-depressant medications. The mean BDI-II score
was 10.2, with 26% scoring 14 or greater, suggestive of elevated depressive symptoms
(Beck et al. 1996).

Hardy-Weinberg Equilibrium

Following adjustment for multiple comparisons using the methods of Li and Ji (Li et al.
2005), all but six SNPs were consistent with Hardy-Weinberg Equilibrium using the 977
individuals. The six SNPs not in Hardy-Weinberg Equilibrium were rs10489181,
rs2024131, rs2109118, rs3750752, rs6454676 and rs7396243 (P's < 5.2 X 10^{-15}). These
SNPs were included in analyses.

Genetic association

P values for the genetic associations with log transformed BDI-II scores including
adjustment for sex are presented in Figure 1. Following correction for multiple testing,
one SNP, rs216873 in intron 38 of the vonWillebrand factor (VWF) gene was found to be
significantly associated with depressive symptoms (P = 7.4 x 10^{-5}). The 665 cardiac
patients homozygous for the CC allele had a mean score of 9.39 (median: 7.0,
interquartile range: 9.00); the 283 CT heterozygotes had a mean score of 11.70 (median:
9.0; interquartile range: 11.00); and the 29 TT homozygous patients had a mean score of
13.40 (median: 10.0, interquartile range: 11.00). The minor allele (T) was found to have a
frequency of 0.1728 in the full population. When dichotomizing the depression
phenotype into cases with Beck scores ≥ 14 and controls with Beck scores < 14 we
calculated an allele odds ratio of 1.51 95% CI (1.17, 1.94) for the T allele of rs216873, with an allelic population attributable fraction of 0.0821.

*VWF* is a large gene covering 175,797 bp of genomic DNA with 52 exons and 32 SNPs of the gene were included in the test-set. Overall, we found only limited linkage disequilibrium between VWF SNPs, and in particular SNP rs216873 was located in a small LD block and showed LD to a neighboring SNP, rs216856, 9 kb downstream with $D' = 0.98$ and $r^2 = 0.39$ and SNP rs216805 16 kb upstream with $D' = 0.96$ and $r^2 = 0.09$. The T-T haplotype of SNPs rs216856 and rs216873 was strongly associated with the log transformed BDI-II score ($P = 1.86 \times 10^{-5}$) in GLM regression, and logistic regression with the dichotomized BDI-II score ($P = 7.9 \times 10^{-4}$) provided an OR estimate of 1.54 (1.08, 2.20) for the T-T haplotype versus other haplotypes. The T-A haplotype of SNPs rs216873 and rs216805 was also strongly associated with the log transformed BDI-II score ($P = 2.95 \times 10^{-5}$) in GLM regression, and logistic regression with the dichotomized BDI-II score ($P = 8.89 \times 10^{-4}$) provided an OR estimate of 1.54 (1.08, 2.20) for the T-A haplotype versus other haplotypes.

As can be seen from Figure 1, in addition to the one SNP passing the multiple comparison threshold, several SNPs provided suggestive association (uncorrected $P < 0.01$) (Fallin et al. 2005) with depressive symptoms. The P values, associated alleles and minor allele frequencies are listed in Table 2. Several of the genes are related to endothelial function or platelet aggregation, namely *VWF* and the genes coding for vascular cellular adhesion molecule 1 (*VCAM1*), calcium channel, L type, alpha 1C subunit (*CACNA1C*) and the serotonin2a receptor (*HTR2A*). Additional suggestive
associations were identified within cannabinoid receptor \( (CNRI) \) and the cholinergic muscarinic receptor 2 \( (CHRM2) \).

In exploratory analyses, interactions of genotype with sex in predicting depressive symptoms were examined. Thirty-nine interactions were significant at the 0.05 level but were not adjusted for multiple comparisons. Of note, among the nominally significant interactions, 10 SNPs were in \( VWF \), including rs216856 \( (P = 0.02) \), identified as highly suggestive in the primary analyses. The majority of SNPs within \( VWF \) showed stronger association with depressive symptoms in women. These results suggest that genetic associations with depressive symptoms may differ by sex, in particular associations of \( VWF \) with depressive symptoms.

In a second set of exploratory analyses, we examined the one SNP showing statistical significance in the full sample, rs216873, for association with depressive symptoms within the two cohorts to determine if a consistent pattern of effects is seen in both cohorts. In the POLYMORPHISME sample, the 277 cardiac patients homozygous for the CC allele had a mean BDI-II score of 9.35 (median: 7.0; interquartile range: 9.00); the 127 CT heterozygotes had a mean score of 10.91 (median: 8.0; interquartile range: 10.00); and the 11 TT homozygous patients had a mean score of 15.33 (median: 14.0; interquartile range: 11.00) \( (P = 0.009) \). In ESCAPE, the 388 cardiac patients homozygous for the CC allele had a mean score of 9.42 (median: 8.0; interquartile range: 9.50); the 156 CT heterozygotes had a mean score of 12.34 (median: 10.0; interquartile range: 12.00); and the 18 TT homozygous patients had a mean score of 12.22 (median: 9.0; interquartile range: 12.00) \( (P = 0.003) \). These results suggest a similar pattern of association of rs216873 with depressive symptoms within the two cohorts.
5.2.5 Discussion

This is the first candidate gene study to focus on key elements of several biological pathways implicated in the association between depression and CAD as genetic predictors of depressive symptoms in cardiac patients. Our results suggest that genetic variation relevant to endothelial dysfunction and platelet aggregation contributes to the expression of depressive symptoms in cardiac patients. Specifically, we identified one intronic SNP marker, rs216873, within the vonWillebrand factor (VWF) gene that was significantly associated with depressive symptoms after conservative correction for multiple comparisons. The von Willebrand factor is involved in recruiting platelets to the injured endothelium from the earliest stages of atherosclerotic lesion and, when elevated in concentration, is a strong predictor of endothelial dysfunction and risk factor for atherosclerosis (Jager et al. 1999; Rumley et al. 1999; Whincup et al. 2002; Yarnell et al. 2005). In addition to rs216873, there were several other SNPs relevant to endothelial dysfunction and platelet aggregation that showed highly suggestive associations with depressive symptoms, including additional markers within VWF and markers within VCAM1, CACNA1C and HTR2A. Additional signals were identified in CHRM2 and CNR1. Overall, these results indicate that genetic variation relevant to endothelial dysfunction and platelet aggregation may predict depressive symptoms in cardiac patients and that endothelial dysfunction may represent a novel pathway contributing to depressive symptoms in these patients.

It is important to note that these associations did not appear to be attributable to associations with CAD severity (de Jonge et al. 2006), as left ventricular ejection fraction, previous cardiac history, and current cardiac medications were not related to
depressive symptoms in this sample. We hypothesize that the observed association between markers within \textit{VWF} and depressive symptoms reflects cerebrovascular endothelial function and emerging cerebrovascular disease.

\textbf{vWF, endothelial function and neurocognitive function}

It is quite plausible that vWF is associated with depressive symptoms because it impacts cerebrovascular disease and neurocognitive function. The vWF is a strong predictor of endothelial dysfunction. Damage to the endothelium in brain vasculature can result in changes in permeability of the blood-brain barrier, changes in vascular autoregulation and a cerebral prothrombotic state (Wardlaw et al. 2003). For example, it has been suggested that blood-brain barrier impairment with leakage of serum components from small vessels may be a critical component in the development of vascular dementia (Hanon et al. 2003; Ueno et al. 2004; Ueno et al. 2004). Consistent with this hypothesis, plasma vWF appears to be elevated among patients with vascular dementia and stroke (Kario et al. 1996; Stott et al. 2001). Variation in intron 2 of the \textit{VWF} gene has also been associated with acute ischemic stroke (Dai et al. 2001).

Cerebrovascular lesions along the striato-pallido-thalamo-cortical circuits are associated with frontal syndrome (executive deficit, low insight, psychomotor retardation), as well as with depression, and the concept of vascular or atherosclerotic depression has been introduced to describe late onset depression associated with cerebrovascular disease (Krishnan et al. 1995; Alexopoulos et al. 1997). Indeed, patients with late onset depression show more evidence of subcortical disease and impairment on
executive and verbal and nonverbal memory tasks, relative to those with depression with an earlier onset (Salloway et al. 1996).

The gene coding for vonWillebrand factor (VWF) is located on chromosome 12 and encompasses over 175 kb and 52 exons. The associated SNP in this study, rs216873, is located in intron 38. The nearby SNP rs216856 is located in intron 42. If functional, these intronic SNPs may alter a splice site to affect the mRNA transcript or a regulatory element that affects gene expression. However, it is likely that these polymorphisms are in linkage disequilibrium with a functional mutation that is yet to be determined in this gene. Exons spanning this LD block make up the D4, B1-B3 and C1-C2 domains of the vWF, which are involved in the binding of platelet integrin (αIIbβ3, aka glycoprotein IIb/IIIa). The C-terminal knot domain is also proximal, which is involved in the dimerisation of the mature vWF protein.

Other highly suggestive associations

Although not statistically significant in this study, it is notable that several of the genes identified as mildly associated with depressive symptoms in cardiac patients are relevant to endothelial dysfunction and cerebrovascular disease. VCAM1 is involved in the recruitment and adhesion of inflammatory cells to the injured endothelium and is a strong predictor of endothelial dysfunction and atherosclerosis in patients suffering from pre-existing disease (Blankenberg et al. 2001; Blankenberg et al. 2003). As such, it is plausible that that VCAM1 contributes to the exacerbation of cerebral endothelial dysfunction and associated neurocognitive compromise through the recruitment and adhesion of inflammatory cells to lesion sites. It is also notable that a gene coding for
the L-type calcium channel $\alpha_{1c}$ subunit ($\text{CACNA1C}$) was suggestive of association with depressive symptoms as it has been hypothesized that calcium leakage from the cerebrovasculature may directly confer some of the neuronal damage associated with vascular depression (Alexopoulos et al. 1997). Augmentation of antidepressant treatment with nimodipine, a calcium channel blocker, presumably treating some of the vascular disease underlying vascular depression, has been shown to improve depressive symptoms among patients with vascular depression (Taragano et al. 2001; Taragano et al. 2005).

The gene coding for the serotonin$_{2A}$ receptor ($\text{HTR2A}$) may be related to depression through effects on central nervous system serotonin. However, the serotonin$_{2A}$ receptor also mediates the effects of serotonin on platelet aggregation and, among those with existing endothelial dysfunction, vasoconstriction (De Clerck 1991), suggesting a vascular mechanism through which HTR2A may be associated with depressive symptoms.

The final two genes identified as suggestive of association have been associated with depression in earlier reports. The muscarinic receptor$_2$ ($\text{CHRM2}$) gene plays a key role in acetylcholine neurotransmission and vagal tone and has previously been associated with depression (Comings et al. 2002; Wang et al. 2004). The gene coding for the cannabinoid receptor, $\text{CNR1}$, has, in at least one study, been associated with depression in Parkinson’s Disease (Barrero et al. 2005).

A recent paper found a polymorphic region upstream from the serotonin transporter gene (5-HTTLPR) to predict diagnosis of depression among Caucasian cardiac patients (Otte et al. 2007). In the present analyses, none of the SNPs in the region of the serotonin transporter gene were associated with depressive symptoms, as measured
by the BDI-II at the level considered to be highly suggestive in this study. Further, 5-HTTLPR was also not significantly associated with depressive symptoms in this study before \((P = 0.63)\) or after adjustment for sex \((P = 0.52)\).

Limitations

It is important to note several limitations to our study. First, it is well known that many initial reports of genetic association are not replicated in subsequent studies and we recognize the importance of replication of these initial results. Within our study, we explored the association of rs216873 with depressive symptoms in the two cohorts: POLYMORPHISME and ESCAPE. The SNP was nominally associated with depressive symptoms in both cohorts, suggesting a consistent pattern of effect in the two cohorts. Nonetheless, it should be noted that replication across cohorts was not an a priori hypothesis of this study and the p values are uncorrected for multiple comparisons of the large number of markers tested in this study within the two cohorts. Future research should attempt to replicate association of this SNP or neighboring SNPs in LD both in homogeneous populations, such as the French-Canadians, as well as in more diverse populations to confirm the association and to examine the relevance of this finding for other population groups.

By design, we limited our sample to cardiac patients to examine genetic predictors of depressive symptoms in the context of cardiac disease. Although we implicitly hypothesize that different mechanisms may underlie depressive symptoms in the context of cardiac disease, we did not formally test this hypothesis in this study. It is quite possible that some of the markers identified may confer risk for depressive
symptoms in the general population (e.g., HTR2A). In addition, a number of cardiac medications directly impact pathways thought to be associated with depression (e.g., statins can reduce levels of pro-inflammatory cytokines) and may have obscured some genetic associations with depressive symptoms in this patient population.

Our results suggest that the association of VWF with depressive symptoms in cardiac disease may be stronger among women than men. However, we did not have sufficient statistical power to examine gender differences. In each of the cohorts, men outnumbered women by approximately a 3:1 ratio.

Lastly, we examined in detail common SNP variation in 59 genes to determine whether these genes, largely related to biological pathways previously hypothesized to underlie the association between depression and CAD, predict depressive symptoms in the context of cardiac disease. Although sufficient to query specific genes of biological interest, the candidate gene approach is necessarily limited by the current knowledge of the biology of depression in cardiac patients. Thus, the candidate gene approach should be complemented with genome-wide association to further the identification of novel genes and pathways that contribute to the expression of depressive symptoms in cardiac patients.
Table 1. Demographic characteristics, cardiac history and depression symptoms in 977 CAD patients from the ESCAPE and POLYMORPHISME cohorts.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean or percent</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic variables</strong></td>
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<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>59.29</td>
<td>10.38</td>
</tr>
<tr>
<td>Education (y)</td>
<td>11.41</td>
<td>4.26</td>
</tr>
<tr>
<td>Female (%)</td>
<td>21.29%</td>
<td></td>
</tr>
<tr>
<td>Married (%)</td>
<td>48.52%</td>
<td></td>
</tr>
<tr>
<td><strong>Risk factors and cardiac history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedentary (%)</td>
<td>43.49%</td>
<td></td>
</tr>
<tr>
<td>Current daily smoker (%)</td>
<td>19.75%</td>
<td></td>
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<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>132.23</td>
<td>22.28</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>74.87</td>
<td>11.07</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.26</td>
<td>4.59</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.69</td>
<td>1.04</td>
</tr>
<tr>
<td>High density lipoprotein cholesterol (mmol/L)</td>
<td>1.149</td>
<td>0.29</td>
</tr>
<tr>
<td>Previous MI</td>
<td>79.22%</td>
<td></td>
</tr>
<tr>
<td>Coronary Bypass</td>
<td>27.23%</td>
<td></td>
</tr>
<tr>
<td>Angioplasty</td>
<td>70.62%</td>
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</tr>
<tr>
<td>Previous MI, Coronary Bypass or angioplasty</td>
<td>95.09%</td>
<td></td>
</tr>
<tr>
<td><strong>Left ventricular ejection fraction &lt; 45%</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricular ejection fraction</td>
<td>18.70%</td>
<td></td>
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<td><strong>Medications at Baseline Interview</strong></td>
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<tr>
<td>Beta-blockers (%)</td>
<td>75.23%</td>
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<tr>
<td>Angiotensin-converting Enzyme Inhibitors (%)</td>
<td>45.14%</td>
<td></td>
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<tr>
<td>Hypoglycemics (%)</td>
<td>16.48%</td>
<td></td>
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<tr>
<td>Calcium-channel blockers (%)</td>
<td>27.94%</td>
<td></td>
</tr>
<tr>
<td>Statins (%)</td>
<td>73.29%</td>
<td></td>
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<tr>
<td>Long-acting Nitrates (%)</td>
<td>19.18%</td>
<td></td>
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<tr>
<td>Antidepressants (%)</td>
<td>12.18%</td>
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<tr>
<td><strong>Depressive symptoms</strong></td>
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<tr>
<td>Beck Depressive Inventory-II Score</td>
<td>10.18</td>
<td>8.59</td>
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<tr>
<td>Beck Depressive Inventory-II &gt; 14</td>
<td>26.41%</td>
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aData is missing for 132 participants.
### Supplemental table 1. Candidate genes for depressive symptoms in CAD by pathway

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<th>Genes</th>
<th>Short name</th>
<th>Location</th>
<th>Genes</th>
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<tr>
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<td></td>
<td></td>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
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<tr>
<td>Tryptophan hydroxylase 2</td>
<td>TPH2</td>
<td>12q15</td>
<td>Myeloperoxidase precursor</td>
<td>MPO</td>
<td>17q23.1</td>
</tr>
<tr>
<td>5-HTT</td>
<td>SLC6A4</td>
<td>17q11.1-q12</td>
<td>Interleukin 6</td>
<td>IL6</td>
<td>7q21</td>
</tr>
<tr>
<td>5-HT2</td>
<td>HTR2A</td>
<td>13q14-q21</td>
<td>Interleukin1b</td>
<td>IL1B</td>
<td>2q14</td>
</tr>
<tr>
<td>Thrombolodulin</td>
<td>THBD</td>
<td>20p11.2</td>
<td>Intercellular adhesion molecule</td>
<td>ICAM1</td>
<td>19p13.3-13.2</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>F2</td>
<td>11p11</td>
<td>Vascular cell adhesion molecule</td>
<td>VCAM1</td>
<td>1p32-p31</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>VWF</td>
<td>12p13.3</td>
<td>Monocyte chemoattractant protein 1</td>
<td>SCYA2</td>
<td>17q11.2-q12</td>
</tr>
<tr>
<td>Thromboxane A synthase 1</td>
<td>TBXAS1</td>
<td>7q34</td>
<td>E-selectin</td>
<td>SELE</td>
<td>1q22-q25</td>
</tr>
<tr>
<td>Thromboxane A2 receptor</td>
<td>TBXAS2</td>
<td>7p33.3</td>
<td>P-selectin</td>
<td>SELP</td>
<td>1q22-q25</td>
</tr>
<tr>
<td>Parinergic receptor P2Y1</td>
<td>P2RY1</td>
<td>3q25</td>
<td>Tumor necrosis factor - alpha</td>
<td>TNF</td>
<td>6p21.3</td>
</tr>
<tr>
<td>Parinergic receptor P2Y12</td>
<td>P2RY12</td>
<td>17p13.3, 3q24-q25</td>
<td>Plasminogen activator-inhibitor-1</td>
<td>SERPINE1</td>
<td>7q21.3-q22</td>
</tr>
<tr>
<td>Cyclooxygenase 1</td>
<td>PTGS1</td>
<td>9q32.2-q33</td>
<td>Cyclooxygenase-2</td>
<td>CRP</td>
<td>1q21-q23</td>
</tr>
<tr>
<td>Cyclooxygenase 2</td>
<td>PTGS2</td>
<td>1q25.2-q</td>
<td>Calcium channel, L-type</td>
<td>CACNA1C</td>
<td>12p13.31</td>
</tr>
<tr>
<td>Gprotein beta 3 subunit</td>
<td>GNB3</td>
<td>12p13.31</td>
<td>Cytokine receptor</td>
<td>CCR2</td>
<td>3p21</td>
</tr>
<tr>
<td>cAMP responsive element binding protein</td>
<td>CREB1</td>
<td>2q33.3</td>
<td>Tyrosine hydroxylase</td>
<td>TH</td>
<td>11p15.5</td>
</tr>
<tr>
<td>Platelet glycoprotein Ib</td>
<td>GP1BA</td>
<td>17p13.2</td>
<td>Aromatic l-aminodecarboxylase</td>
<td>DDC</td>
<td>7p11</td>
</tr>
<tr>
<td>Platelet glycoprotein IIb</td>
<td>ITGA2B</td>
<td>17q21.1-q21.3</td>
<td>Dopamine beta hydroxylase</td>
<td>DBH</td>
<td>9q34</td>
</tr>
<tr>
<td>Platelet glycoprotein IIIa</td>
<td>ITGB3</td>
<td>17q21.32</td>
<td>Phenylalanine-N-methyltransferase</td>
<td>PNMT</td>
<td>1q22-q22</td>
</tr>
<tr>
<td>Omega 3/6 metabolism</td>
<td></td>
<td></td>
<td>Adrenoreceptor - beta1</td>
<td>ADRB1</td>
<td>10q24-q26</td>
</tr>
<tr>
<td>delta5 desaturase</td>
<td>FADS1</td>
<td>11q12.2</td>
<td>Adrenoreceptor - beta2</td>
<td>ADRB2</td>
<td>5q32-q34</td>
</tr>
<tr>
<td>delta6 desaturase</td>
<td>FADS2</td>
<td>11q12.2</td>
<td>Adrenoreceptor - alpha 1a</td>
<td>ADRA1A</td>
<td>8q21</td>
</tr>
<tr>
<td>delta9 desaturase</td>
<td>FADS3</td>
<td>11q12.2</td>
<td>Adrenoreceptor - alpha 1b</td>
<td>ADRA1B</td>
<td>5q33</td>
</tr>
<tr>
<td>HLOL/ELOVL5</td>
<td>ELOVL5</td>
<td>6p12.1</td>
<td>Adrenoreceptor - alpha 2 a</td>
<td>ADRA2A</td>
<td>10q24-q26</td>
</tr>
<tr>
<td>Long chain fatty acid Co-A ligase Type 4</td>
<td>FACL4</td>
<td>Xq23</td>
<td>Monoamine oxidase A</td>
<td>MAOA</td>
<td>Xp11.23</td>
</tr>
<tr>
<td>HPA</td>
<td>NR3C1</td>
<td>5q31.3</td>
<td>Monoamine oxidase B</td>
<td>MAOB</td>
<td>Xp11.23</td>
</tr>
<tr>
<td>Glaucocorticoid receptor</td>
<td></td>
<td></td>
<td>Adrenochrome oxidase</td>
<td>ACR1</td>
<td>7q22</td>
</tr>
<tr>
<td>11beta-HSD1</td>
<td>HSD1B1</td>
<td>1q32.2</td>
<td>Acetylcholine transferase</td>
<td>CHAT</td>
<td>10q11.2</td>
</tr>
<tr>
<td>11beta-HSD2</td>
<td>HSD1B2</td>
<td>16q22.1</td>
<td>Acetylcholine musclecarino 2 receptor</td>
<td>CHRM2</td>
<td>7q33</td>
</tr>
<tr>
<td>Angiotensin converting enzyme</td>
<td>ACE</td>
<td>17q23</td>
<td>Neuronal nitric oxide synthase</td>
<td>NOS1</td>
<td>12q24</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>AVP</td>
<td>20p13</td>
<td>Endothelial nitric oxide synthase</td>
<td>NOS3</td>
<td>7q36</td>
</tr>
<tr>
<td>Vasopressin receptor Ib</td>
<td>A2PR1B</td>
<td>1q32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MISC</td>
<td>CNR1</td>
<td>6q14-q15</td>
<td></td>
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</tr>
</tbody>
</table>

**Location**: Chromosome location(s) for each gene.
5.2.7 Figures

Figure 1. Genetic association with Beck Depression Inventory – II scores among 977 cardiac patients, statistically adjusted for sex. VCAM1 – vascular cellular adhesion molecule 1 gene; CNR1 – cannabinoid receptor gene; CHRM2 – muscarinic$_{2A}$ receptor gene; CACNA1C – calcium channel, L-type, $\alpha_{1c}$ subunit gene; VWF – von Willebrand factor gene; HTR2A – serotonin$_{2A}$ receptor gene.
CHAPTER 6: TYPE 2 DIABETES IN CARDIAC PATIENTS

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Reference:

6.1 RATIONALE

Diabetes is a known risk factor for CAD as elevated levels of glucose in the blood can damage the endothelial cells of the blood vessels, contributing to atherosclerosis (see chapter 1). It was not surprising then that 21.7% of our combined CAD cohort, described in the previous chapter, also has diabetes. This is significantly higher than the incidence of diabetes estimated in the general population (e.g. 9.3% of U.S. adults over the age of 20, 3% of Canadians between the ages of 35 and 64, 10% of Canadians over the age of 65). We hypothesized that genetic factors which increase risk of type II diabetes in the general population may also account for the high incidence of this disorder among patients with a history of cardiac disease.

This chapter consists of an association study of polymorphisms in the transcription factor 7-like 2 gene (TCF7L2) with diabetes in our CAD cohort. These polymorphisms had been previously associated with type II diabetes in other Caucasian populations without CAD (Grant et al. 2006). Our study is the first to test the association of this gene with diabetes among cardiac patients. In addition, we were the first to test the interaction of these SNPs with obesity, which is another risk factor for type II diabetes. This test determines if the polymorphisms in this TCF7L2 increase risk of diabetes independent of obesity or directly influence obesity.
6.2 OBESITY AND TCF7L2 VARIANTS INCREASE RISK FOR TYPE 2 DIABETES AMONG CARDIAC PATIENTS

6.2.1 Introduction

A microsatellite marker, DG10S478, in the transcription factor 7-like 2 gene (TCF7L2) was previously associated with type 2 diabetes in three Caucasian populations (Grant et al. 2006). This association followed earlier reports by the same group (Reynisdottir et al. 2003), and a separate team (Duggirala et al. 1999), which showed suggestive linkage to chromosomal 10q. Grant and colleagues demonstrated that allele X (composite of all but the shortest allele) of DG10S478 conferred an increased risk for type 2 diabetes of 45% and 141% among heterozygotes and homozygotes, respectively. Since this report, numerous groups have replicated the association in various populations and extended it to include two intronic SNPs (rs12255372 and rs7903146) (Yi et al. 2005; Cauchi et al. 2006; Damcott et al. 2006; Florez et al. 2006; Groves et al. 2006; Humphries et al. 2006; Saxena et al. 2006; Scott et al. 2006; Weedon et al. 2006; Zhang et al. 2006; Chandak et al. 2007; Field et al. 2007; Helgason et al. 2007; Horikoshi et al. 2007; Marzi et al. 2007; Mayans et al. 2007; van Vliet-Ostaptchouk et al. 2007). In this study, we investigated the combined effect of obesity and genotype at DG10S478 and rs12255372 in predicting risk for type 2 diabetes in a sample of French Canadian cardiac patients.

6.2.2 Research Design and Methods

Population. Patients of French Canadian descent with established coronary artery disease (CAD) recruited in two earlier studies, POLYMORPHISME (n = 484) and ESCAPE (n =
were included. All participants were identified between November 1998 and April 2002 at the Montreal Heart Institute and Hôpital Sacré-Coeur and gave written informed consent. Protocols were approved by the ethics committees at both institutions.

Measures. Type 2 diabetes was defined as use of diabetes medications or fasting blood glucose > 126 mg/dL (7.0 mmol/L). Body mass index (BMI) was calculated as weight (kg)/height (m²). Obesity was defined as BMI ≥ 30 kg/m².

Genotyping. DNA extraction from blood used a standard protocol (Gentra Systems). DG10S478 was genotyped by radiolabeled (α-35S-dATP) polymerase chain reactions (PCR) using a standard protocol and primers designed from the genomic sequence of human TCF7L2 (NM_030756; http://www.ncbi.nlm.nih.gov/). Products were separated by electrophoresis on 6% denaturing polyacrylamide gels. Allele sizes and frequencies were obtained from the CEPH database (http://www.cephb.fr). Taqman assays designed by Applied Biosystems (AB) were used to genotype rs12255372. Products were analyzed with a spectrophotometer (AB) equipped with sequence detector software (SDS 2.2.2.).

Statistical Analyses. Markers were tested for Hardy-Weinberg equilibrium (HWE) by using the exact test (Guo et al. 1992). Three genetic models were used for case-control association testing: the chi-square genotype test; the chi-square allele test; and the Cochran-Armitage trend test on genotypes, which tests for additive allele effects on
disease risk. 100,000 Monte Carlo permutations were performed to obtain exact p-value estimates. Mantel-Haenszel statistics were used for stratified analyses by obesity. Two-sided p values are reported. Genotype by obesity interaction was evaluated by testing the significance of the interaction term in a full logistic regression model with additive genetic effects modeled as -1 (G/G), 0 (G/T), 1 (T/T) for the three genotype categories. The association between rs12255372 and BMI was measured using a genotype trend genetic model for an additive allelic effect captured by a regression model for BMI. The model was built using the GLM procedure in SAS, which gives a Fisher and T test for each component. All data were analyzed using SAS 9.1.3 and SAS/Genetics (SAS Institute Inc., Cary, NC, USA).

6.2.3 Results

Population. In contrast to non-diabetic patients, those with diabetes were significantly older, had fewer years of education, more obese with significantly higher BMI, had greater systolic blood pressure and were more likely to have undergone coronary artery by-pass surgery CABG ($P < 0.05$).

Genetic association tests. Of the total population, 1037 (96.2%) were successfully genotyped for DG10S478 and 1004 (93.1%) for rs12255372. Both markers were in HWE ($P = 0.9731$ and $P = 0.8557$) and in strong linkage disequilibrium ($D^* = 0.977$ and $r^2 = 0.970$). Using the nomenclature of Grant and colleagues (Grant et al. 2006), allele 0 of DG10S478 was the smallest and most frequent (68.2%) and is strongly associated with
allele G of rs12255372. All other alleles (X) of DG10S478 (31.8%) were strongly correlated with allele T of rs12255372.

The rate of diabetes increased with an increasing dose of allele X of DG10S478 or allele T of rs12255372, and among obese compared to non-obese participants (Table 1). The association was consistent for both groups, but stronger in non-obese CAD patients. Also, rs12255372 was more closely associated with type 2 diabetes, which may simply reflect the relative instability of the microsatellite.

Logistic regression modeling showed that the interaction between obesity and genotype did not approach statistical significance ($P > 0.34$). Table 1 depicts the joint effects of these risk factors for type 2 diabetes using the non-obese group without a risk allele as the reference sample. The highest risk was observed among obese individuals who carried at least one risk allele. Within each genotype group, obesity was strongly associated with increased risk of diabetes ($P < 0.001$). The regression model also showed an association between rs12255372 and BMI ($P = 0.0481$) with a decrease of BMI towards the T/T genotype. This association was not detected using a dominant genetic model.

### 6.2.4 Conclusions

Concordant with previous reports, our study confirms that polymorphisms in TCF7L2 ($P < 0.0001$) and obesity ($P < 0.001$) are both associated with increased risk for type 2 diabetes in the French Canadian founder population. We observed that the genetic association was stronger in non-obese CAD patients with lower BMI and confirmed previous reports that allele T of rs12255372 is associated with reduced BMI ($P = 0.0481$).
Furthermore, we did not detect an interaction between obesity and genotype, suggesting that these are independent risk factors for type 2 diabetes. A recent paper by deCODE proposed that a second SNP, rs7903146, correlated with DG10S478 and rs12255372, may be the risk variant or its closest known correlate (Helgason et al. 2007). However, previous studies show that none of these associated polymorphisms explain the linkage of type 2 diabetes to this chromosome 10q region (Cauchi et al. 2006; Grant et al. 2006), suggesting that another variant(s) nearby accounts for this linkage and may be the functional variant(s). Screening the mRNA transcripts in future studies may reveal splice variants or alternative exons undetected by screening the genomic DNA.
6.2.5 Acknowledgements

We would like to thank all the participants and Dr. Patrick A. Dion for careful review of this manuscript. This work was funded by the National Institute of Health (HL077442; JMM). QLD is funded by the Heart and Stroke Foundation of Canada. GAR is supported by the Canadian Institutes of Health Research. Collection of data for the ESCAPE study was supported by the Medical Research Council of Canada and an unrestricted grant from GlaxoSmithKline (POP-37744), the Charles A. Dana Foundation, the Montreal Heart Institute Research Fund, the Pierre David Fund, and the Fondation du Centre Hospitalier de l'Université de Montréal.
6.2.6 Tables

Table 1. Rate of type 2 diabetes and odds ratios for each genotype, stratified by obesity, with corresponding genetic association test results.

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<thead>
<tr>
<th>DG10S478</th>
<th>Allele test</th>
<th>Genotype test</th>
<th>Trend test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(p-value*)</td>
<td>(p-value*)</td>
<td>(p-value*)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00057</td>
<td>0.00233</td>
<td>0.00057</td>
</tr>
<tr>
<td>Non-obese</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>86 (17.6%)</td>
<td>110 (25.1%)</td>
<td>33 (30.0%)</td>
</tr>
<tr>
<td>No diabetes (%)</td>
<td>402 (82.4%)</td>
<td>329 (74.9%)</td>
<td>77 (70.0%)</td>
</tr>
<tr>
<td>Obese</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>43 (13.5%)</td>
<td>61 (19.6%)</td>
<td>24 (28.6%)</td>
</tr>
<tr>
<td>No diabetes (%)</td>
<td>276 (86.5%)</td>
<td>250 (80.4%)</td>
<td>60 (71.4%)</td>
</tr>
<tr>
<td>Mantel-Haenszel&lt;sup&gt;†&lt;/sup&gt;</td>
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<table>
<thead>
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<th>Allele test</th>
<th>Genotype test</th>
<th>Trend test</th>
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</thead>
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<td>Non-obese</td>
<td>1</td>
<td>1.57 (1.02, 2.40)</td>
<td>2.57 (1.45, 4.55)</td>
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<tr>
<td>Obese</td>
<td>2.19 (1.37, 3.15)</td>
<td>3.98 (2.46, 6.43)</td>
<td>3.39 (1.42, 8.10)</td>
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<table>
<thead>
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<th>Genotype test</th>
<th>Trend test</th>
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<td>(p-value*)</td>
<td>(p-value*)</td>
<td>(p-value*)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>80 (17.2%)</td>
<td>104 (23.9%)</td>
<td>35 (34.0%)</td>
</tr>
<tr>
<td>No diabetes (%)</td>
<td>386 (82.8%)</td>
<td>331 (76.1%)</td>
<td>68 (66.0%)</td>
</tr>
<tr>
<td>Non-obese</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>38 (12.8%)</td>
<td>58 (18.8%)</td>
<td>24 (31.2%)</td>
</tr>
</tbody>
</table>
CHAPTER 7: DISCUSSION AND CONCLUSION

This chapter summarizes the results of this dissertation and the implications for disease pathogenesis. It also addresses the limitations of our genetic investigations and the efficacy of linkage and association analyses in the study of human complex traits. Furthermore, recent developments in AE-ACEi, depression and type 2 diabetes research are discussed along with the molecular mechanisms underlying these particular diseases. Finally, future research avenues are outlined to follow-up our genetic investigations and to better understand susceptibility of ADRs associated with ACEi as well as depression and Type 2 diabetes associated with higher CAD risk and mortality.
Angioedema Associated with ACE Inhibitors (AE-ACEi)

In this pharmacogenomic study, we mapped a gene (*XPNPEP2*) underlying a human quantitative trait (APP activity) by linkage analysis. This represents one of the first successful QTL mapping experiments which identified a gene underlying a human complex trait by positional cloning. Furthermore, we identified two mutations in this QTL that co-segregate with the quantitative phenotype and are present in nine of our twenty AE-ACEi cases. A coding deletion (g.2953-3127del) resulting in alternative splicing of the mRNA transcript (314_431del) was found in one AE-ACEi case, which also co-segregates with low APP throughout his family. A non-coding polymorphism (rs3788853 or C-2399A), present in eight affected cases, predicts the linkage signal at this QTL and is significantly more prevalent among our AE-ACEi cases compared to controls ($P = 0.0364$).

Our linkage study suggests that other QTLs may also regulate APP activity including loci on chromosomes 6, 8 and 9. These loci may contain modifier genes that regulate the expression of *XPNPEP2* (e.g. transcription factors) or encode proteins that interact with the APP enzyme to modify its activity (e.g. antagonists). In this report, we determined that pedigree V of cohort 1 is not linked to the QTL on chromosome X. Subsequent analysis of this pedigree confirmed that it contributes to most of the linkage signal observed on chromosome 6 (unpublished data). Further investigation of chromosomes 8 and 9 will determine whether these represent spurious or true linkage signals. However, it is possible that non-genetic factors also regulate plasma APP
activity as genetic factors do not account for all of phenotypic variability observed (estimated \( h^2 = 37.5\% \pm 26.5 \text{ SD, } P = 0.0336 \)). In addition, the bimodal distribution of APP activity among our cases (six have normal APP activity) indicates that factors other than a deficiency in APP may predispose individuals to AE-ACEi.

It is notable that we did not detect linkage on chromosome 10q25.1, which contains the gene encoding cytosolic APP (XPNPEPL). This supports a previous study suggesting that plasma APP activity is a measurement of hmAPP as this isoform is localized on the extracellular membrane of vascular endothelial cells, facing the lumen of blood vessels (Ryan et al. 1996). In contrast, hcAPP is ubiquitously expressed within the cytoplasm of cells (Vanhoof et al. 1997), which is less likely to contribute to plasma measurements. The contribution of these two APP isoforms to plasma APP activity may be tested by knocking out the expression of either genes in mice.

This pharmacogenomic study confirmed previous reports that decreased APP activity may be an important mediator of AE-ACEi (Adam et al. 2002; Molinaro et al. 2002). We demonstrated that this quantitative trait is heritable, regulated by genetic factors and may be used as a biomarker for this acute ADR of ACEi. The results of our study may facilitate the development of clinical assays to predict individuals who have higher risk of AE prior to starting ACEi therapy. This would decrease the incidence of such a potentially fatal drug complication. However, further studies are necessary to identify other risk factors, genetic and non-genetic, for this multifactorial disorder.

**Hypersensitivity Reactions Associated with ACEi (HSR-ACEi)**

In this study, we identified APP activity as an endophenotype of HSR-ACEi. We determined that plasma APP activity was significantly reduced \( (P = 0.013) \) among our
fourteen HSR cases compared to thirty-nine ACEi-treated controls who did not develop HSR during hemodialysis. Using the families of these cases, we confirmed that a significant proportion of the phenotypic variability results from genetic factors \( (h^2 = 0.61 \pm 0.18 \text{ SD}, P < 0.001) \). In addition, we reported increased production \( (P = 0.011) \) and decreased degradation \( (P < 0.001) \) of des-Arg\(^9\)-BK, the proinflammatory metabolite of BK, among our affected individuals. Although increased BK \( (P = 0.043) \) was measured among our affected cases, there was no significant decrease in BK degradation \( (P = 0.168) \). These results suggest that HSR is not a direct consequence of hemodialysis using a negatively charged membrane, which is known to activate the BK pathway. Instead, our study suggests that HSR-ACEi results from a metabolic anomaly (reduced APP activity and/or decreased des-Arg\(^9\)-BK degradation) that is partially regulated by genetic factors (e.g. \( XPNPEP2 \)) and may also be a direct consequence of ACEi therapy.

The non-coding polymorphism (rs3788853) in \( XPNPEP2 \) was present in six of our fourteen HSR-ACEi cases, which correlated with reduced APP activity. However, this variant was not significantly more prevalent among our cases compared to controls \( (P = 0.4289) \). This suggests that additional variants at this QTL as well as other loci regulate APP activity in the majority of our HSR-ACEi cases with reduced APP. In addition, factors other than an inherited deficiency in APP activity may increase risk of HSR in some individuals. In this study, we demonstrated that two commonly used ACEi drugs (Captopril and Enalapril) also inhibit of APP activity at therapeutic doses in human plasma samples, which confirms the results of an earlier study performed in pigs (Hooper et al. 1992). This non-specific inhibition of APP may account for HSR-ACEi cases who are not genetically predisposed to low APP activity. In addition, APP inhibition by ACEi
may further decrease the activity of APP among individuals who already have reduced APP activity. It would be interesting to determine if there is a higher incidence of AE or HSR among individuals taking Captopril and Enalapril.

**Estrogen-dependent inherited angioedema (EDIA)**

In this publication, we replicated two previous studies (Cichon et al. 2006; Dewald et al. 2006) by identifying a coding missense mutation (Thr328Lys) in the *F12* gene among three female relatives with a history of EDIA. We were the first to identify this mutation in an Italian family with EDIA, thereby demonstrating that this rare mutation is present in European populations other than German and French. This mutation has been associated with increased activity of the coagulation factor XII, which activates the production of BK. In addition, our study was the first to investigate the role of other enzymes in the BK pathway among EDIA cases. We reported that in addition to the gain of function mutation in *F12*, all three female EDIA cases have either one or two copies of the inserted (I) allele in the *ACE* gene as well as the risk (A) allele at rs3788853 SNP in *XPNPEP2*. The I allele in *ACE* has been associated with reduced ACE activity (Rigat et al. 1990) and the A allele of rs3788853 has been associated with reduced APP activity (described in chapters 2 and 3 of this dissertation). The presence of these common polymorphisms in the affected females suggests that decreased degradation in addition to increased production of BK and des-Arg⁹-BK contribute to increased risk of EDIA in this family.
Depression in Coronary Artery Disease Patients

The incidence of depression in two French Canadian cohorts with a history of CAD was 26%, which is greater than the estimated 7% in the general population (Kessler et al. 2003). This supports previous studies that reported an association between depression and CAD (Carney et al. 2003). In this study, we identified a common, intronic polymorphism (rs216873) that is significantly associated with elevated depressive symptoms (higher BDI-II score) in a cohort of French Canadian CAD patients. This SNP surpassed significance threshold after adjustment for multiple comparisons using both the $M_{eff}$ (Li et al. 2005) and Bonferroni methods (unpublished data). The T allele of this SNP in the $VWF$ gene is associated with greater risk of depression (BDI-II score $> 14$) as determined by an odds ratio of 1.51 (CI 1.17, 1.94) in our CAD cohort. This genetic association was stronger among female cardiac patients compared to their male counterparts. Although not included in this publication, we determined that the T allele was also associated with elevated plasma concentrations of vWF among females of the ESCAPE cohort ($P = 0.011$) but not males ($P = 0.3$) (unpublished data). Measurements for vWF levels were not available for the POLYMORPHISME cohort. However, it is important to note that the number of female participants was too low to draw any conclusions about gender differences as the ratio of males to females was 3:1. Replication of our association results in larger CAD populations is necessary to determine if this gender difference is true.

In unpublished, exploratory analysis involving 409 individuals without CAD (73 with depression, 336 without depression), collected as part of the POLYMORPHISME
project, rs216873 in VWF was not associated with depression (unpublished data). It is also interesting to note that this SNP was not found to be associated with CAD (unpublished data). Again, replication studies involving larger cohorts are needed to confirm these preliminary data. If replicated, this would suggest that the genetic variants in the VWF gene, leading to increased protein concentrations, predispose to depression only in patients who have a history of CAD.

**Type 2 Diabetes and Obesity**

This report replicates the association found between genetic variants in the TCF7L2 gene and type 2 diabetes in a French Canadian cohort of CAD patients. We determined that this association is stronger among non-obese CAD patients compared to obese type 2 diabetes patients. In addition, we reported no interaction between genotype and obesity. Together, this suggests that obesity and genotype at these variants are independent risk factors for type 2 diabetes. This suggests that life style changes leading to weight loss might be beneficial in reducing the risk of type 2 diabetes regardless of genotype risk.

Subsequent mutation screening of the coding regions of this gene in 7 individuals with low BMI as well as the risk genotypes at DG10S478 (X/X) and rs12255372 (T/T) did not reveal any genetic variants (unpublished data). To date, no other study has reported a potentially functional variant at this genetic locus that could account for the linkage signal as well as the association with type 2 diabetes. Nevertheless, numerous studies have replicated the association between these variants in TCF7L2 with type 2 diabetes in multiple populations.
7.2 LIMITATIONS OF STUDIES

As AE and HSR associated with ACEi therapy are infrequent ADRs, it was a challenge to collect large cohorts for genetic association studies. Consequently, rs3788853 in \textit{XPNPEP2} was tested for association with AE-ACEi and HSR-ACEi using small numbers of affected cases and unaffected controls. In addition, we limited our genetic studies to Caucasians given previous reports that susceptibility to AE-ACEi differ between white and black populations (Brown et al. 1996; Lefebvre et al. 2002). The collection of additional AE-ACEi patients, ACEi treated controls as well as black American AE-ACEi cases and controls is underway for replication studies. The ascertainment of new HSR-ACEi cases is more difficult as result of a warning issued by the FDA in 1992, which recommended replacement of the negatively charged dialysis membranes to a more neutral membrane (Alert 1992). Thus, although HSR-ACEi was a frequent occurrence during hemodialysis during the 1990s (Verresen et al. 1990), this ADR is currently rare.

Although we demonstrated the effect of the coding deletion in \textit{XPNPEP2} on mRNA splicing, we did not determine the biological significance of the non-coding SNP (rs3788853) at this locus. As all of our AE-ACEi and HSR-ACEi cases gave informed consent to participate in our study, their ADR episodes were not fatal, although fatal cases have been reported (Byrne et al. 2000). The use of living human participants limited our access to tissues for functional analysis of genetic variants. In addition to DNA and plasma collection, we isolated lymphoblast cells from the majority of our participants. Despite the fact that these cell lines are a continuous and reliable source of RNA and protein, gene expression in lymphoblasts is not necessarily representative of what occurs in other tissues which may be more relevant to the condition being
investigated here. For example, we demonstrated that expression of XPNPEP2 is too low for quantification by reverse-transcriptase and real-time PCR as well as Northern Blotting. Sequencing of cDNA to characterize the deletion mutation in this gene required a nested PCR protocol, which used diluted PCR products as DNA template for a second round of PCR in order to obtain sufficient product for sequencing. An earlier study reported the highest expression of this gene in kidney and liver cells (Venema et al. 1997) but such tissues are not available from our patients. Thus, we were unable to characterize the function of this polymorphism in the available cell lines of our AE-ACEi patients.

Another option to determine the biological significance of rs3788853 in XPNPEP2 would be the use of an in vitro model (e.g. cell transfection assays). However, genotype data from the recently completed HapMap project (The International HapMap Consortium2005) indicates that this SNP is strongly correlated with numerous other polymorphisms across a 35 kb haplotype block. As there are more than 80 SNPs within this region, it would be difficult to determine which polymorphism in this LD block is functional. In addition, genome sequencing of additional species have shown that this SNP is not conserved across species although it is located in close proximity to a conserved domain. Finally, the fact that two of our AE-ACEi probands did not have this polymorphism while the SNP segregates with plasma APP activity in their families (chapter 2) further suggests that this SNP is not functional but is in LD with the functional SNP. Thus, we opted to not pursue further investigations to characterize this associated SNP in order to focus instead on the identification of new candidate genes for AE-ACEi.
A limitation of our association study of depression was that only CAD patients were included, which may not be reflective of the general French Canadian population. For example, 26% of our CAD cohort had depression (BDI-II score > 14), which is much more prevalent than in the general population. We chose to include CAD patients only in our study due to concerns about the disease status of our control cohort. This small number of controls (n = 409) had been admitted into the cardiac ward of the Montreal Heart Institute with self-reported chest pains. However, based on angiographic evidence, none had experienced a MI or had blocked arteries. Thus, none of these individuals were diagnosed with CAD. Nevertheless, they were recruited at a center for cardiac disease and follow-up on these controls were not done to determine if they later developed CVD. Also, their admittance into the hospital may have been related to a mood disorder such as elevated anxiety. The rate of depression among this control group is 17%, which is higher than that estimated in the general population. Thus, it remains to be determined if the associated genes identified in this study increase risk for elevated depressive symptoms in the general population or if they are specific to CAD patients.

Another limitation of our association study of depression was that the majority (78.8%) of our CAD participants were male. Although we detected a stronger association between rs216873 in VWF and depression among female CAD cases, the small number of female participants did not allow us to detect a significant gender difference. Finally, our genetic investigation of depression in CAD patients was restricted to a set of candidate genes. When the project began in 2004, a GWAS was not feasible due to the high cost of SNP genotyping. A GWAS of depression in CAD
patients might reveal new candidate genes that may account for the association between these two disorders.

7.3 Efficacy of Linkage Analysis and Association Studies

The application of variance component linkage analysis to identify a locus for a human quantitative trait was successful in this study. As the disease of interest is an infrequent ADR induced by ACEi therapy, families were collected through a single affected member, which did not provide sufficient power to detect linkage using affected status. Instead, we successfully identified an endophenotype (APP activity) of AE-ACEi for use in linkage analysis. An additional advantage of using this quantitative trait was that APP activity can be accurately measured using thoroughly tested assays, which minimized phenotyping error. Due to the fact that our families were collected through an affected individual with low APP activity, the phenotypic distribution across our families was negatively skewed and had to be transformed to normality because variance component linkage analysis assumes that a continuous trait is normally distributed (Box and Cox 1964). Linkage analysis of APP activity provided significant linkage to a QTL that contains a biologically relevant candidate gene. Subsequent mutation screening experiments identified two genetic variants that co-segregate with low APP activity and are associated with AE-ACEi. Thus, we were able to clone a gene by linkage analysis, which underlies a human quantitative trait and predisposes to a multifactorial disease.

Prior to our investigation, only a handful of genes had been reported for human traits by positional cloning such as for ovarian and breast cancers (BRCA1 and BRCA2) (Miki et al. 1994; Wooster et al. 1995), Alzheimers disease (PSEN1) (Theuns et al. 2005).
2000), and Crohn’s disease (NOD2) (Hugot et al. 2001; Ogura et al. 2001). The majority of genes within QTLs had previously been identified in animal models such as mice and rats (Korstanje et al. 2002). Although more genes have since been mapped in other human QTLs in recent years, positional cloning projects for human complex traits have been less successful than for human Mendelian diseases (Altmuller et al. 2001) and QTLs in other species such as the mouse due to breeding strategies (Flint et al. 2005). This may be explained in part by the fact that linkage analysis assumes that one gene has major effects on the phenotype of interest. While this is true for some complex traits and diseases (e.g. certain forms of breast cancer, Alzheimer’s disease, type 1 diabetes), many human traits are affected by multiple genes that individually contribute only a small fraction of the overall phenotype (e.g. schizophrenia, asthma, hypertension). In addition, environmental factors (e.g. diet, smoking, drugs) can alter the phenotypic outcome. The success rate of positional cloning by linkage analysis has improved in recent years with the use of larger numbers of families (Cox et al. 2001), extended pedigrees and denser genome maps (Kong et al. 2002) (Stefansson H and Sigurdsson E, 2002) (Middleton, FA and Pato MT, 2004). Nevertheless, the popularity of association studies for complex traits has increased in recent years with the advent of cheaper, high throughput genotyping technologies (McCarthy et al. 2008).

This PhD dissertation also included a candidate gene association study, which successfully identified a susceptibility gene, VWF, for depression among CAD patients. Although association studies are accredited with a greater power to detect genes that have modest effects on a phenotype, such studies require a larger set of markers and a larger cohort. For example, whereas our linkage study included less than 400 markers across
the entire genome, we genotyped 768 SNPs across 59 candidate genes in our association study. This is because linkage analysis tests for co-segregation of a marker and disease gene within a family over one or two generations, while association depends on the co-inheritance of a marker and disease allele within a population, which descended from common ancestors over many generations. In addition, our association cohort included nearly 1100 CAD patients and 400 CAD controls whereas our linkage study included only 8 families (n = 123). To further increase our power to detect association, we identified tag polymorphic markers based on Hapmap data instead of genotyping randomly selected SNPs across our candidate genes (Zhang et al. 2005). The associated SNP found in this study surpassed even the most conservative correction factor for multiple testing known as the Bonferroni Factor. However, several additional SNPs in this gene and other genes did not survive this stringent correction factor but yielded highly suggestive association results. The fact that several of these genes contribute to a common biological pathway (endothelial function and platelet aggregation) relevant to depression among CAD patients further supported our association results. Although replication is key to any association study, our association study suggests a novel mechanism for the correlation between depression and CAD.

7.4 MOLECULAR MECHANISMS UNDERLYING DISEASE

Adverse Reactions Associated with ACEi

As outlined in Chapter 1, the kinin (BK and des-Arg⁹-BK) pathway is thought to underlie the pathogenesis of AE as well as HSR associated with ACEi. The kinin pathway may be activated as a result of tissue damage or during hemodialysis via contact with a negatively
charged membrane to produce BK and its active metabolite, des-Arg⁹-BK (Bhoola et al. 1992). Normally, these kinins are rapidly degraded but are known to accumulate in the presence of an ACE inhibitor (Cyr et al. 2001), which results in vasodilation and lower BP (Brown 2001). Kinins are also known to increase vascular permeability and inflammation, which predispose to angioedema (Dendorfer et al. 1999; Brown 2001). These physiological effects of BK and des-Arg⁹-BK may be increased by impaired metabolism due to decreased activity of one or more of the kininases which degrade these peptides. Previous studies have reported significantly decreased BK and des-Arg⁹-BK degradation among individuals who developed AE-ACEi and HSR-ACEi (Blais et al. 1999; Blais et al. 1999; Molinaro et al. 2002). It has also been demonstrated that when ACE is inhibited, APP acts as the primary enzyme for the degradation of both BK and its active metabolite, des-Arg⁹-BK (Cyr et al. 2001). Furthermore, in the presence of an ACEi, the activity of another metabolic enzyme known as CPN is increased to transform a higher fraction of BK into des-Arg⁹-BK (Blais et al. 1999). Thus, a deficiency of APP would lead to accumulated levels of both kinins. However, as other kininases (e.g. NEP, CPN, DPPIV) also degrade BK, higher levels of des-Arg⁹-BK accumulate as result of increased formation (increased CPN activity) and decreased degradation (reduced ACE and APP activity). Our pharmacogenomic investigations identified DNA variations in the gene encoding membrane-bound APP, which are correlated with reduced protein activity in some of our AE/HSR-ACEi patients. Thus, a deficiency in APP activity is regulated by genetic factors and predispose to ADRs associated with ACEi drugs.

The pathogenesis of AE-ACEi may be different between black and white populations. The incidence of angioedema is nearly 5 folds higher among black
Americans compared to white Americans (Brown et al. 1996; Coats 2002). It has been proposed that decreased DPPIV concentration and activity confers risk to AE-ACEi in this population (Lefebvre et al. 2002). In a small sample of black Americans who developed AE-ACEi, we identified several polymorphisms in the DPPIV gene that are significantly associated with this ADR \( (P < 0.01) \) (unpublished data). When ACE is inhibited, DPPIV is the primary pathway for the degradation of substance P, a neurotransmitter that increases vascular permeability in mouse models (Emanueli et al. 1998; Campos et al. 2000). The release of substance P from nerve cells is activated by increased BK (Kopp et al. 1997). Thus, in the presence of an ACEi and a genetic predisposition to lower DPPIV, both BK and substance P levels accumulate to increase risk of AE-ACEi.

ACEi drugs may directly increase risk for AE or HSR associated with these drugs by non-specific inhibition of APP activity. Simultaneous inhibition of both ACE and APP would impair BK and des-Arg^9-BK degradation, leading to increased risk of angioedema. This may explain some of the AE-ACEi cases with normal APP activity when not treated with ACEi.

**Estrogen-Dependent Inherited Angioedema**

Both increased production and decreased degradation of kinins may be involved in the pathogenesis of EDIA. A missense mutation in exon 9 of the *F12* gene leads to increased protein activity and activation of the BK pathway. In women with elevated estrogen levels (e.g. during pregnancy, administration of contraceptive pills, and hormone replacement therapy), this hormone further increases the expression of *F12*. This
combination of increased expression and activity of Factor XII leads to the higher production of BK, which results in increased vascular permeability and greater risk for angioedema. However, as with AE-ACEi, impaired BK degradation resulting from decreased ACE and APP activity may contribute to higher levels of BK and its active metabolite, des-Arg⁹-BK.

**Depression in CAD patients**

The vWF is a membrane-bound protein that is synthesized by the endothelial cells during early stages of atherosclerosis when the endothelial walls become damaged and acts by recruiting platelets to the injury site. Thus, vWF elevation is a marker for CAD but it is unknown how it could increase risk of depression. Previous studies have reported that increased vWF levels is associated with vascular diseases such as stroke and vascular dementia (Stott et al. 2001). Vascular diseases, in turn, have been associated with late-onset depression (Salloway et al. 1996). We hypothesize that endothelial damage as result of increased vWF in circulation may alter permeability of the endothelial wall of the blood vessels. In the brain, it would impair the Blood Brain Barrier (BBB), causing leakage of pro-inflammatory cytokines from the blood vessels into the brain and subcortical vascular disease. Thus, increased vWF may predispose to depressive symptoms. Our hypothesis is also supported by earlier reports that a compromised BBB, leading to neuronal and glial damage, is critical to the development of vascular dementia (Hanon et al. 2003; Ueno et al. 2004; Ueno et al. 2004).
**Type 2 Diabetes**

Since the publication of our paper replicating the association between polymorphisms in the *TCF7L2* gene and type 2 diabetes in two French Canadian CAD cohorts, numerous other studies have replicated this association in additional cohorts. One of these publications identified another SNP, rs7903146, which was more closely associated with type 2 diabetes in West African and Danish populations (Helgason et al. 2007). Homozygotes for the less common allele (T/T) have a five fold higher expression of *TCF7L2* in human islet cells, which was correlated with reduced insulin secretion among type 2 diabetic patients (Lyssenko et al. 2007).

The *TCF7L2* gene encodes a transcription factor that regulates the expression of multiple genes in the Wnt signaling pathway. For example, it regulates the expression of the *proglucagon* (*glu*) gene, which codes for the hormone glucagon-like peptide-1 (GLP-1) (Yi et al. 2005). This product reduces blood sugar levels by activating insulin and inhibiting glucagon release from the pancreas, as well as increasing insulin sensitivity and reducing food intake by inducing a feeling of fullness (satiety) (Turton et al. 1996; Kieffer et al. 1999; Meeran et al. 1999; Peters et al. 2001). Grant *et al.* suggested that genetic variants in the *TCF7L2* gene leading to reduced gene expression and consequently, elevated blood sugar levels, might predispose individuals to type 2 diabetes. This was supported by the research of Florez *et al.*, which found a decrease in insulin secretion among carriers of the risk-conferring genotype, who also had higher risk for diabetes (Florez et al. 2006).
7.5 POTENTIAL RESEARCH AVENUES

The association of variants identified in \textit{XPNPEP2} need to be replicated in larger cohorts of AE-ACEi cases and ACEi treated controls. Functional assays (e.g. luciferase reporter assays using transfected cell lines) may be applied to test the effect of non-coding SNPs such as the one identified in the \textit{XPNPEP2} locus (rs3788853) or other proximal SNPs which are in LD with it. Another method to validate the role of \textit{XPNPEP2} in pathogenesis of AE-ACEi is to generate an animal model which is deficient in this protein. A rat model showed that a deficiency in \textit{DPPIV} predisposed to AE when treated with ACEi drugs (Byrd et al. 2007). An animal model deficient in APP may be generated by knocking out/knocking down the \textit{XPNPEP2} gene or by means a commercially-available inhibitor (Apstatin). These animals may be used to assess risk of AE by administration of ACEi drugs.

We have identified several polymorphisms in the \textit{DPPIV} gene, which are significantly associated with reduced DPPIV levels and activity as well as increased risk for AE-ACEi among a small sample of black Americans (15 cases: 66 controls) (unpublished data). As we did not identify a coding variant in this gene among our affected cases, future mutation screening experiments will focus on the non-coding regions of this gene. Bioinformatics tools may be used to prioritize genomic regions for mutation screening. For example, Dragon ERE (estrogen response elements) identified several ERE motifs within the introns of \textit{DPPIV}, in proximity to our associated SNPs. These are considered good candidate regions for further mutation screening as increased estrogen levels have been shown to decrease DPPIV concentrations (Durinx et al. 2001). The same study measured lower levels of DPPIV among women compared to men.
Furthermore, the importance of this gene may increase as a new class of drugs are emerging which inhibit DPPIV, used for the treatment of type 2 diabetes and may be combined with ACEi drugs.

Other genes involved in the kinin and substance P pathways may also be good candidate genes for AE-ACEi. For example, neutral endopeptidase (NEP) is another protein that degrades BK and substance P. Drugs that inhibit both NEP and ACE were associated with a 3-fold higher incidence of AE compared to drugs that inhibit only ACE (Coats 2002). The F12 gene is also be a good candidate for AE-ACEi. Higher production of BK as result of increased Factor XII activity or concentration may also contribute to elevated kinin levels measured in these angioedema cases. This may also help to explain for the higher incidence of AE-ACEi among females compared to males taking ACEi drugs (Kostis et al. 2005). Additional candidate genes include: prekallikrein, CPN, B1R and B2R. We propose a candidate gene association study using tag SNPs across all the genes which contribute to the BK and substance P pathways. This may reveal new candidates as well as identify genes that have modest effect on degradation but may increase susceptibility to AE-ACEi nonetheless.

The effect of estrogen on the expression of other genes contributing to the kinin pathway may be further investigated. In addition to the up-regulation of F12 and inhibition of DPPIV, estrogen has been known to regulate the expression of numerous other genes via the ERE. For example, this hormone is known to decrease the expression of ACE (Sumino et al. 1999; Nogawa et al. 2001; Proudler et al. 2003; Sumino et al. 2003; Stevenson et al. 2004), which results in increased levels of BK (Sumino et al. 1999; Nogawa et al. 2001). Moreover, a recent study demonstrated that androgens increase the
expression of APP (Drouet et al. 2008), which further explains for the lower incidence of AE-ACEi among male ACEi patients. As estrogens often have antagonistic effects to androgens, it is possible that the female hormone reduces APP activity.

Efforts to replicate the genetic association between polymorphisms in the VWF gene and other suggestive associations are underway in other populations. The aim of these replication studies is to test the association of these SNPs with depression in the general population as well as to test their association with CAD itself. In addition, a five year follow-up of our French Canadian CAD cohorts will be used to determine if these SNPs as well as elevated depressive symptoms are associated with recurrent CAD episodes in our French Canadian cohort. Finally, a genome wide association study of depression in CAD patients may be useful to identify new candidate genes, which were not included in our study.
7.6 CONCLUSION

Our pharmacogenomic study identified a susceptibility locus for a potentially fatal ADR of ACEi. A deleterious mutation in the *XPNPEP2* gene, encoding membrane-bound APP, results in an erroneously spliced transcript in one AE-ACEi, which also segregates with APP activity in his family. A second variant in this locus co-segregates with reduced activity, accounts for the linkage signal at this QTL and are associated with AE-ACEi. However, other genetic loci and/or non-genetic factors may also regulate APP activity. For example, suggestive linkage to chromosome 6 has been observed in one family that was not linked to the *XPNPEP2* locus. In addition, we demonstrated the non-specific inhibition of plasma APP activity by two ACEi drugs (Captopril and Enalapril). Moreover, it is possible that factors other than reduced APP activity contribute to increased risk of AE-ACEi such as reduced DPPIV activity/concentration. Finally, our study suggests that impaired degradation, as well as increased formation, of kinins (BK and des-Arg⁹-BK) may contribute to an estrogen-dependent form of inherited angioedema.

Our genetic investigations of depression and type 2 diabetes among CAD patients identified susceptibility loci that might explain the higher CAD risk associated with both diseases. We identified genes (*VWF, VCAM1*) contributing to atherosclerosis (e.g. endothelial dysfunction and platelet aggregation) which were associated with depression among patients with a history of heart disease. Thus, our results suggest that a novel mechanism may account for depression among cardiac patients. In addition, we reported that a known locus regulating blood glucose homeostasis is associated with type 2 diabetes in our cohort of CAD patients who have a higher rate of diabetes.
In conclusion, this PhD dissertation contributed to the field of pharmacogenomics, complex trait genetics and CAD. The identification of susceptibility loci for ADRs associated with ACEi may facilitate the development of genetic testing assays to predict at risk individuals prior to drug administration. In addition, a better understanding of the pathogenic mechanisms underlying these ADRs will assist in the development of safer drugs for CAD and effective treatments for these ADRs. Thus, my PhD project represents a potential application of pharmacogenomics in health care, a field destined to transform medicine but where there have been few successes to date. Furthermore, we identified genetic loci that might explain for increased risk of CAD among depressed and diabetic patients. Insight into the factors underlying the association between these diseases will aid in the management and care of patients to reduce CAD risk and mortality. Finally, this dissertation demonstrated the application of both linkage and association analysis for genetic mapping of complex human traits. Since most human diseases are complex/multifactorial in nature, there is enormous potential for these genetic approaches in medicine. Recent revolutionary advances in human genetics have facilitated complex trait genetics. As genotyping technologies and computational tools for extracting biologically relevant information from large datasets continue to evolve at a fast pace, this is the most exciting time for complex genetics!
ELECTRONIC RESOURCES

Center for Medical Genetics, Marshfield Medical Research Foundation,
http://research.marshfieldclinic.org/genetics/

Centre d'Etude du Polymorphisme Humain, http://www.cephb.fr/

HUGO gene nomenclature Committee, http://www.gene.ucl.ac.uk/nomenclature/

Human Gene Mutation Database of the Institute of Medical Genetics of Cardiff
(www.hgmd.cf.ac.uk/ac/index.php)

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APPENDIX

Ethical approval

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