

**SINGLE NUCLEOTIDE POLYMORPHISM OF B-TYPE NATRIURETIC
PEPTIDE AND CAROTID ULTRASOUND IMAGING AS BIOMARKERS
TO DETERMINE THE SEVERITY OF ATHEROSCLEROSIS**

by

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Abstract

Cardiovascular disease (CVD) has been the number one cause of death in the world for several decades and will continue to worsen as a global epidemic. The underlying cause of CVD is atherosclerosis: most myocardial infarctions are caused by atherosclerotic plaque build-up in the coronary arteries, also known as coronary artery disease (CAD). Even though the exact mechanism is unknown, hemodynamic disturbance is a potential precursor for atherosclerotic development. The natriuretic peptide system, especially B-type natriuretic peptide (BNP), plays an important role in hemodynamic regulation. Genetic variants, such as single nucleotide polymorphisms (SNPs), of the BNP gene affect its expression and correlate with increased circulating BNP levels. The goal of this thesis research is to determine if SNPs of the natriuretic peptide system contributes to atherosclerotic development, and if these genetic variants can help predict CVD.

We recruited 513 patients from Kingston General Hospital, who were undergoing coronary angiography, the clinical standard for detecting CAD. Blood sample was collected from each patient for genetic analysis. BNP gene SNP, rs198389, was significantly associated with severe CAD in women but not in men. It suggests that the mechanism of atherosclerosis development may differ between the sexes.

In the second portion of the thesis, we investigated the role of genetic mutation in atherosclerosis in the carotid arteries, major arteries in the neck. Ultrasound was used to reveal and quantify carotid plaques, which correlated with the severity of plaques in coronary arteries. BNP genetic variant, rs198389, was associated with the extend of atherosclerosis in the carotid artery.

In the final part of the thesis, we evaluated major adverse cardiovascular events such as heart attack, stroke and cardiovascular death. The SNP rs198389 was found to be a significant predictor for early cardiovascular events, as well as recurrent events.

In summary, this thesis demonstrates that the BNP genetic variant rs198389 may contribute to the development of atherosclerosis in different arterial beds across the body, and it has the potential to be an important tool to predict adverse cardiovascular events.

Co-Authorship

All work presented in this thesis is original. Terry Y. Li completed all experimental work, analysis of data and manuscript preparation unless specified otherwise. Contribution to the experimental design, interpretation of data and manuscript preparation were Marie-France Héту, M. Yat Tse, Amer M. Johri and Stephen C. Pang.

Cathy S. McLellan was the lead interventional cardiologist who carried out coronary angiography and analysis of images. Marie-France Héту carried out ultrasound image analysis and contributed to the evaluation of major adverse events. Will D. King contributed to statistical analysis.

All co-authors contributed revisions to the published manuscripts and consented to publication. The primary principle investigators in these studies are Amer M. Johri and Stephen C. Pang, who provided funding, laboratory space, equipment and reagents.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
BMI	Body mass index
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CCA	Common carotid artery
CIMT	Carotid intima media thickness
CVD	Cardiovascular disease
CT	Computed tomography
Cx	Circumflex (coronary artery)
eGFR	Estimated glomerular filtration rate
GWAS	Genome-wide association study
HDL	High density lipoprotein
HF	Heart failure
HR	Hazard ratio
ICA	Internal carotid artery
IHD	Ischemic heart disease
IL	Interleukin
IMT	Intima media thickness
LAD	Left anterior descending (coronary artery)
LAM	Leukocyte adhesion molecule
LDL	Low-density lipoprotein
LM	Left main (coronary artery)
MACE	Major adverse cardiovascular event

MI	Myocardial infarction
MMP	Metalloproteinase
MPH	Maximal plaque height
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NP	Natriuretic peptide
ANP	Atrial natriuretic peptide
BNP	B-type natriuretic peptide
CNP	C-type natriuretic peptide
NT-proBNP	N-terminal prohormone BNP
RAAS	Renin-angiotensin-aldosterone system
RCA	Right coronary artery
ROS	Reactive oxidative species
RR	Relative risk
PCI	Percutaneous coronary intervention
PDA	Posterior descending artery
SNP	Single nucleotide polymorphism
TNF	Tumor necrosis factor
TPA	Total plaque area
US	Ultrasound
TPA	Total plaque area

Chapter 1

General Introduction and Literature Review

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality in the world.¹ In particular, ischemic heart disease (IHD) and stroke account for a combined 15.2 million deaths worldwide in 2016, more than one quarter of total deaths. These diseases have remained the leading causes of death globally in the last 15 years.¹ In Canada, 51,396 deaths (20% of totally deaths) and 13,551 deaths (5.3% of totally deaths) were due to heart diseases and cerebrovascular disease, respectively in 2016.² According to the most recent data from 2012/13, about 2.4 million (8.5%) Canadian adults aged 20 years and older lived with diagnosed IHD, with an addition of about 158,700 new diagnoses each year.³ The same 2012/13 dataset showed that there were 741,800 stroke survivors in Canada.⁴ CVD also represents an enormous financial burden on the Canadian economy, costing more than 20.9 billion Canadian dollars every year.⁵

The situation in the United States is even more dire. In 2015, 41.5% of the US population had at least one CVD condition, while every one in three deaths was due to CVD. The current annual medical cost of CVD is approximately 318 billion US dollars with an additional 237 billion US dollars in indirect cost. The total cost of CVD (medical and indirect) is projected to reach 1.1 trillion US dollars in year 2035.⁶

In developing countries, heart diseases, along with cancer, are rapidly replacing communicable disease as leading causes of mortality and morbidity.⁷ In recent decades, IHD has risen to the number one cause of deaths in most lower-middle- to upper-middle-income countries and the third cause of deaths in low-income countries.¹ CVD has an even greater impact in developing countries due to their massive populations. In 2014, 45% of total deaths in China was due to CVD, mounting to over 4.4 million deaths and remaining on the rise.⁸ Consequently, CVD

will continue to worsen as a global epidemic, which demands a tremendous amount of effort in understanding its pathophysiology and discovering novel predictive and therapeutic strategies.

Ischemic heart disease, the deadliest type of CVD, is characterized by myocardial ischemia, an imbalance between myocardial oxygen supply and demand, resulting in myocardial hypoxia and accumulation of waste metabolites. Clinical manifestations of myocardial ischemia range from various degrees of angina pectoris, uncomfortable sensation in the chest, to myocardial infarction, where necrosis of cardiomyocytes occurs. IHD is caused by atherosclerotic disease of the coronary arteries, also known as coronary artery disease (CAD).⁹

The coronary arteries supply the heart muscle with oxygen and nutrients. The large components of the coronary arterial vasculature lie within the epicardial fat, are therefore termed epicardial arteries (Figure 1.1). Two main arteries arise from the ascending aorta just above the left and right cusps of the aortic semilunar valve. Left main (LM) coronary artery passes between the left atrium and the pulmonary trunk and branches into the left anterior descending (LAD) artery and the circumflex (Cx) artery. The LAD artery runs within the anterior interventricular sulcus toward the apex of the heart and gives off branches that supply the anterior walls of ventricles and the interventricular septum. The circumflex artery travels within the left atrioventricular sulcus and passes around the left border of the heart to reach the posterior aspect of the heart. It supplies the lateral and posterior wall of the left ventricle. The right coronary artery (RCA) courses through the right atrioventricular sulcus, passing posteriorly between the right atrium and ventricle. It supplies blood to the right ventricle. The posterior descending artery (PDA) travels within the posterior interventricular sulcus and supplies the posterior walls of ventricles and the interventricular septum. The PDA is a branch of the RCA in 85% of the population and such coronary circulation is termed right dominant. In approximately 8% of the population, the PDA arises from the circumflex artery, resulting in a left dominant circulation. In the remaining population, both the RCA and the circumflex contribute to the heart's posterior

blood supply, forming a codominant circulation. In coronary artery disease, atherosclerotic plaque build-up or rupture in these epicardial coronary arteries or their major branches impede blood flow, causing various degrees of myocardial ischemia.

The second deadliest killer in the world, stroke, is an interruption of blood flow to the brain, causing paralysis, slurred speech and/or altered brain functions. About nine of every 10 strokes are ischemic strokes, caused by atherosclerotic blockage in the cerebral arteries.

Hemorrhagic strokes caused by blood vessel bursting are much rarer.⁶ Even though IHD and stroke are often seen as separate diseases in the public eye, they are focal manifestations of the same systemic disease – atherosclerosis.

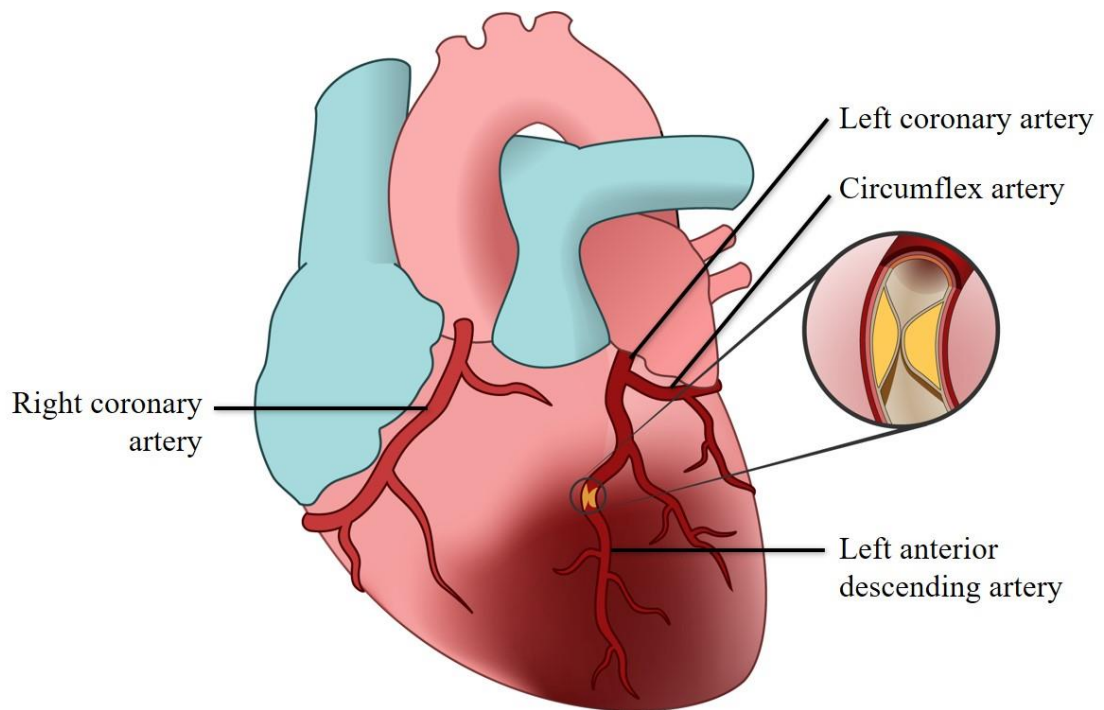


Figure 1.1. Schematic diagram of main epicardial arteries.

Magnified area demonstrates a luminal stenosis that causes myocardial infarction distal to the stenosis (shown in darker colour). Adopted from Wernham and Herr (2017).¹⁰

1.1 History of atherosclerosis

1.1.1 Atherosclerosis is an ancient disease

Due to the current alarming epidemic of CVD, atherosclerosis is often thought as a modern disease, caused by dietary excess, physical inactivity, and tobacco smoking.⁷ However, it is in fact a very ancient disease. In 1852, pathologist Johann Czermak¹¹ was the first to report atherosclerosis in ancient people. During the autopsy of the mummy of an elderly Egyptian woman, Czermak found “multiple considerably large and calcified plaques” in her descending aorta. In 1909, Samuel Shattock¹² found atherosclerosis in the aorta of the Pharaoh Menephtah, believed to have been the pharaoh referred to in Exodus. In 1911, Sir Marc Armond Ruffer¹³ published histologic evidence of atherosclerosis in multiple 3,000-year-old Egyptian mummies. He believed that atherosclerotic lesions were as frequent 3,000 years ago as they were in the early 20th century. Because of the destructive nature of the process, relatively few autopsies have been performed on ancient human remains.⁷ Advances in medical imaging technology, however, allow noninvasive, nondestructive “autopsies” in living humans as well as long-dead mummies. X-ray computed tomography (CT) is capable of detecting calcified deposits within atherosclerotic plaque with high sensitivity.⁷ The presence and degree of calcification of the coronary arteries as measured by CT has been shown in large contemporary, multiethnic population studies to be closely correlated with future coronary events.¹⁴ With the help of CT scanning, Murphy and colleagues¹⁵ demonstrated bilateral carotid calcific atherosclerosis in the Iceman, a 5,300-year-old mummy discovered in the Italian Alps. Their discovery represents the earliest documentation of atherosclerosis in humans. A century following Ruffer’s work, the Horus study team (named after the ancient Egyptian deity) used CT scanning to find atherosclerosis in 20 of 44 Egyptian mummies who lived between 1981 BCE and 364 CE (common era).^{16,17} One of the mummies imaged, Princess Ahmose-Meryet-Amon, had atherosclerosis in all her major vascular beds, including her right coronary and left anterior descending coronary arteries. She is thus far the

earliest human in history with CAD.¹⁸ However, ancient Egyptian culture and lifestyles might have had unique attributes relative to atherogenesis. Moreover, mummification in Egypt during this time was primarily performed on elite Egyptians of high socioeconomic status.¹⁹ To address these issues, the Horus team subsequently obtained whole body CT scans of 137 mummies from four different geographical populations spanning more than 4,000 years, including ancient Egypt, ancient Peru, the Ancestral Puebloans, and southwest America and the Unangan of the Aleutian Islands. They found evidence of atherosclerotic lesions in 47 mummies across all four preindustrial populations, including a preagricultural hunter-gatherer population. The Horus team argued that atherosclerosis was an inherent component of human aging and not characteristic of any specific diet or lifestyle.¹⁹

1.1.2 Early pathological discoveries

The word “atherosclerosis” derived from the Greek roots *athere-*, meaning “gruel,” and *-skleros*, meaning “hardness,” which literally describes the hardened buildup in arteries.²⁰ The first reports of “hardening of the arteries” were recorded as early as the 16th century.²¹ In the mid- to late 18th century, William Heberden was among the first of a group of English physicians to describe “a disorder of the breast” (angina pectoris).²² He and colleagues Edward Jenner and Caleb Hillier Parry also described the process of calcification of the coronary arteries.²³ Lobstein introduced the term “arteriosclerosis” in the early 19th century on the basis of autopsy observation of gross pathologic anatomy. His observations were expanded by the renowned German pathologist Rudolph Virchow, who used similar detailed microscopic analysis of postmortem specimens.⁷ In 1858, Virchow²⁴ recognized the inflammatory nature of arteriosclerotic plaques: “In some, particularly violent cases, the softening manifests itself even in the arteries not as the consequence of a really fatty process, but as a direct product of inflammation.” Virchow also understood atherosclerosis as an active process of tissue reaction rather than a mere encrustation

of thrombus or deposition of fatty material, stating that “the frequency with which cells in a state of fatty degeneration are found in inflamed parts, affords sufficient proof, that in the course of inflammatory processes, which it is impossible we should ever regard as simply passive processes, such transformations must take place.” Virchow’s concept of atherogenesis, elements of which appear strikingly modern, unfortunately yielded to the view of atherosclerosis as a primarily passive cholesterol storage disease for more than a century.²⁵

1.1.3 Modern pathological discoveries

In the latter half of the 20th century, considerable advances in our understanding of atherogenesis have come from the work by Russell Ross, who published the “response to injury” theory in a series of articles throughout the 1970s.²⁶⁻²⁹ He postulated that atherogenesis was initiated by endothelial injury and outlined events following the injury with the focus on smooth muscle cell proliferation.²⁹ The development of monoclonal antibodies enabled rigorous cell identification in the atheroma, confirming that most lipid-laden cells, known as foam cells, arise from mononuclear phagocytes, although smooth muscle cells and endothelial cells can also become engorged with lipids.^{30,31} The role of the mononuclear phagocyte as an effector of atherogenesis emerged with the characterization of macrophage-derived mediators, such as cytokines.³²

In the 1990s, supported by the American Heart Association’s committee on vascular lesions, Stary et al.³³ expanded on Ross’s theory and outlined six distinct morphologic stages of atheroma formation based on histologic composition and structure. These stages delineate the continuous development of atherosclerosis consisting of a multitude of pathogenic processes,³⁴ which will be described in details in the next sections. Over the last couple of decades, the concept that inflammation plays a primordial role in atherogenesis has re-gained the spotlight.²⁵

Numerous studies have shown that most atherogenic processes were facilitated by a complex network of cytokines and proinflammatory mediators.^{25,35,36}

1.2 Normal arterial structure and function

The arterial wall consists of three layers, as shown in Figure 1.2. The most inner tunica intima is a single layer of endothelial cells that lines the lumen of the vessel and comes in direct contact with the circulating blood. The simple squamous endothelium resides on a connective tissue basal lamina, that composes the subendothelial layer consisting of reticular and/or collagen fibres. The tunica intima is separated from the middle layer, tunica media, by an internal elastic lamina. The media consists of smooth muscle cells and extracellular matrix, and serves the contractile and elastic functions of the vessel. The elastic component, more prominent in large arteries (e.g. the aorta), stores the pulsatile kinetic energy from the cardiac ventricles and helps propel the continuous laminar flow of the blood. The muscular component, more prominent in smaller arteries (e.g. distal branches of coronary arteries), constricts or relaxes to alter vessel resistance and therefore blood flow. An external elastic lamina separates the media from the outer-most layer, tunica adventitia. Depending on the size of vessels, the adventitia may contain nerves, lymphatics, and blood vessels called vasa vasorum that nourish the smooth muscle cells near the periphery of the arterial wall.

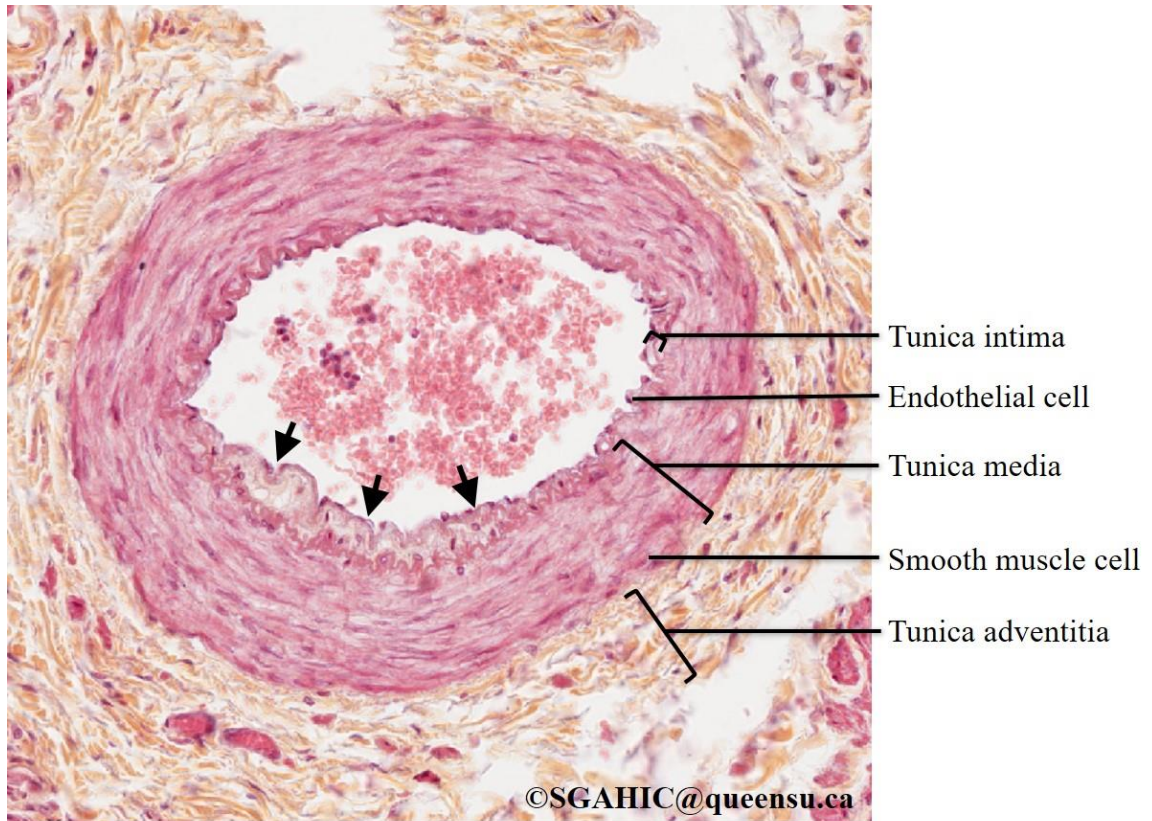


Figure 1.2. Micrograph of a small muscular artery. Arrows show an area of intimal thickening. The subendothelial space enlarges potentially due to endothelial dysfunction and development of foam cells in the space. Adopted from the Scalable Gross Anatomy and Histology Image Catalogue (2000).³⁷

1.2.1 Vascular endothelial cells

In a healthy artery, the endothelium performs structural, metabolic, and signaling functions that maintain homeostasis of the vessel wall. The tightly adjoined endothelial cells form a barrier that controls the passage of large molecules and blood cells from the circulation into the subendothelial space.

Normal endothelial cells produce antithrombotic molecules that either prevent blood from clotting or promote fibrinolysis. Some of these molecules reside on the endothelial surface (e.g. heparan sulfate, thrombomodulin, and plasminogen activators), while other antithrombotic molecules enter the circulation (e.g. prostacyclin and nitric oxide [NO]).²⁰

Endothelial cells also modulate contraction of smooth muscle cells in the tunica media by paracrine secretions that include vasodilators (e.g. NO and prostacyclin) and vasoconstrictors (e.g. endothelins). These substances alter the vascular resistance and therefore luminal blood flow.²⁰

Furthermore, the endothelium also modulates the immune response of the vessel wall. Healthy endothelial cells resist leukocyte adhesion and thereby oppose local inflammation. In summary, without pathologic stimulation, the normal endothelium is selectively permeable, non-thrombogenic, vasodilatory and anti-inflammatory.

1.2.2 Vascular smooth muscle cells

Smooth muscles cells within the tunica media have both contractile and secretory capabilities. Various vasoactive substances modulate the contractile function, resulting in vasoconstriction or vasodilation. Smooth muscle cells also synthesize and secrete the collagen, elastin and proteoglycan that form the bulk of the vascular extracellular matrix. Additionally, smooth muscles cells produce vasoactive and inflammatory mediators, including interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α).²⁰

In normal arteries, most smooth muscles cells reside in the tunica media. However, under certain conditions such as atherogenesis, medial smooth muscle cells can migrate into the subendothelial space of the intima, proliferate and augment production of extracellular matrix molecules.

1.3 Pathogenesis and progression of atherosclerosis

The development of atherosclerosis is complex and does not necessarily follow a sequential path.²⁰ Although the process can be generalized into the following steps, the cells of atherosclerotic lesions continuously interact and modify each other's behavior, shaping the plaque into one of many possible phenotypes. The process of atherogenesis and plaque progression is summarized in Figure 3.1. An atherosclerotic lesion begins with endothelial activation and dysfunction, which allow entry and modification of lipids within the subendothelial layer.³⁴ Modified lipids serve as proinflammatory mediators that initiate leukocyte recruitment and foam cell formation. Foam cell secretions contribute to the migration of smooth muscle cells into the intima layer. Smooth muscle cells proliferate and elaborate extracellular matrix, which traps lipoproteins, forming the typical atherosclerotic plaque with a thrombogenic lipid core covered by a fibrous cap. Plaque growth can protrude into the arterial lumen and impede blood flow. Such flow-limiting plaque can result in tissue ischemia, causing symptoms such as angina pectoris or intermittent claudication of the extremities. Many acute coronary syndromes are the result of plaque disruption, where the fibrous cap ruptures, exposing prothrombotic molecules within the lipid core and precipitating an acute thrombus that suddenly occludes the arterial lumen.²⁰

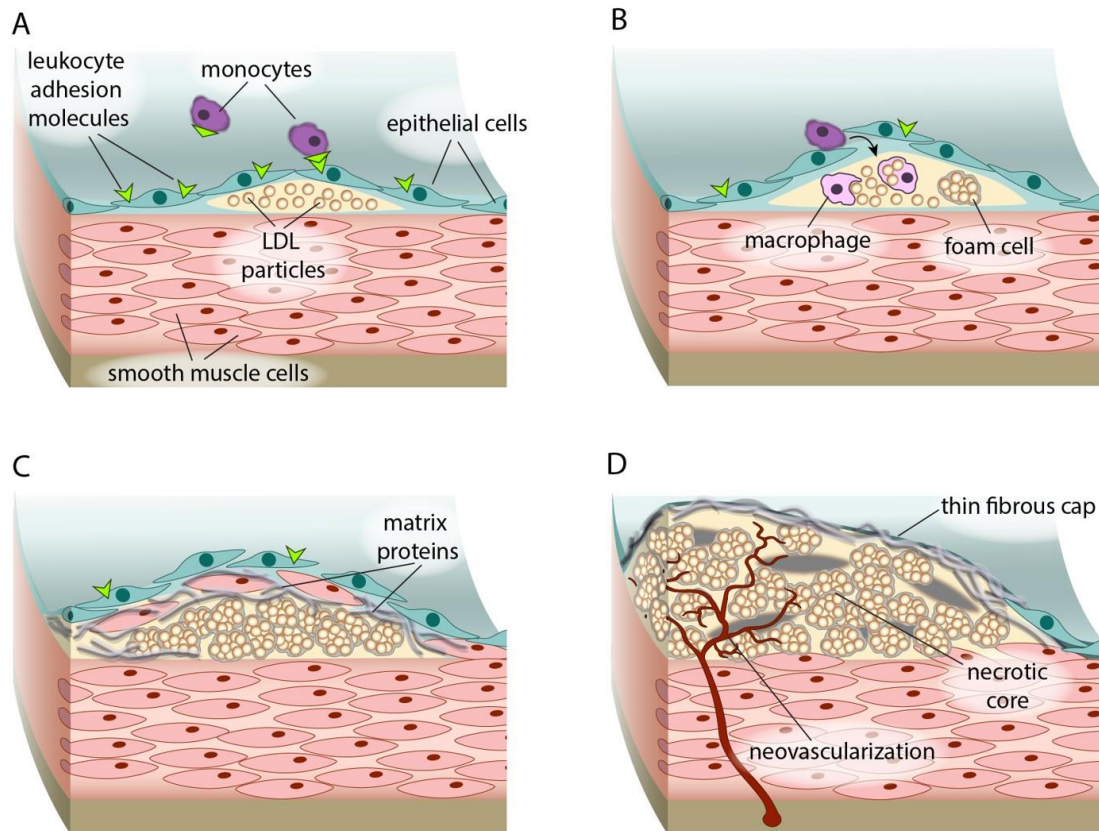


Figure 1.3. Schematic diagram of atherogenesis and plaque progression.

A) Accumulation of lipoprotein particles in the intima induces local cytokine elaboration. These cytokines promote increased expression of adhesion molecules that direct leukocyte migration into the intima. B) After entering the artery wall, monocytes differentiate into macrophage and imbibe modified LDL to form foam cells. C) Smooth muscle cells migrate into the intima layer and secrete extracellular matrix that contribute to the fibrous cap of the plaque. D) Foam cell apoptosis forms necrotic core of the plaque which induces neovascularization. Dying foam cells produce MMPs that degrade extracellular matrix macromolecules, thinning the fibrous cap.

Adopted from Johri et al. (2017).³⁸

1.3.1 Endothelial dysfunction

The quiescent state of endothelial cell mentioned previously can be disturbed by a variety of noxious stimuli. Cardiovascular risk factors, including dyslipidemia, hypertension, diabetes and tobacco smoking, are all associated with over-production of reactive oxygen species – notably, superoxide anion – or increased oxidative stress, both of which reduce endothelial NO bioavailability and promote cellular damage. Endothelial cells under such stress promote local inflammation and become “activated”. Endothelial activation is manifested by impairment of endothelium’s role as a permeability barrier, the release of inflammatory cytokines, increased production of cell surface adhesion molecules that recruit leukocytes, altered release of vasoactive substances, and interference with normal antithrombotic properties.

1.3.2 Hemodynamic shear stress

The predisposition of certain regions of arteries to develop atherosclerosis supports the role of hemodynamic shear stress. Pathology studies reported a clustering of lesions in the left anterior descending artery immediately after and at the first diagonal branch origin.³⁹⁻⁴¹ In the circumflex artery, atherosclerotic involvement was more frequent around the origin of the first marginal branch.³⁹⁻⁴¹ At bifurcation level, regions opposite to flow divider experiences lower values of wall shear stress.⁴² In the carotid artery, due to the asymmetrical nature of the bifurcation, patterns of disturbed flow are usually located on the outer wall of the sinus, where they generate low and oscillating levels of wall shear stress.^{43,44} Cross-sectional studies in patients indicated that the areas of low and/or oscillating wall shear stress correspond to areas of plaque formation, with the outer wall of the proximal internal carotid artery being primarily affected.⁴³ On the contrary, the apex of the bifurcation and the inner wall of the external carotid artery are sites of high wall shear stress, which act protectively against atherogenesis.⁴⁴

Regions of the arterial tree with uniform geometry are exposed to an undisturbed, unidirectional flow, which exerts a physiologic shear stress, whereas arches and branches are exposed to a disturbed, oscillatory flow, which exerts low shear.⁴⁵ Atherosclerotic lesions occur predominantly at sites of low shear, whereas regions of the vasculature exposed to a physiologic shear are protected.⁴⁶ A physiologic shear protects against atherogenesis via a tri-molecular mechanosensory complex (platelet and endothelial cell adhesion molecule 1 [PECAM-1], vascular endothelial cell cadherin and vascular endothelial growth factor receptor 2 [VEGFR2]) expressed on endothelial cells.⁴⁷ These mechanosensory receptors convert mechanical forces into numerous biochemical signals by influencing lipid permeability, inhibition of the cell cycle, suppression of pro-thrombotic tissue factor activity⁴⁸ as well as anti-inflammatory activation of endothelial cells.⁴⁹⁻⁵² This latter effect is mediated by inhibition of pro-inflammatory mitogen-activated protein (MAP) kinase signaling^{49,52} and alteration of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activity and function.^{50,51} Low and oscillatory shear stress as well as disrupted and turbulent flows are able to reduce nitric oxide production and to induce pro-inflammatory mediator synthesis through processes such as NF- κ B-dependent transcription, circulating monocytes recruitment, impaired flow regulated vasorelaxation and reversal of the anti-apoptotic, anti-proliferative and anti-oxidative functions of the endothelium.⁴⁸

Lumen narrowing can result in increased flow velocity at the “throat” of the plaque, low shear stress in the upstream region, and disturbed flow in the form of directionally oscillatory shear stress in the downstream “shoulder” of the plaque.⁴⁵ These local shear stress conditions promote the formation of a rupture-prone plaque phenotype upstream of the lesion and additional growth downstream of the plaque.⁵³

1.3.3 Lipoprotein entry and modification

The dysfunction endothelium no longer serves as an effective barrier between blood and the arterial wall. Increased endothelial permeability allows the entry of low-density lipoprotein (LDL) into the intima. LDL accumulates in the subendothelial space by bind to proteoglycans in the extracellular matrix. “Trapped” LDL particles have increased residence time within the vessel wall and are more likely to undergo chemical modifications that can promote the development of atherosclerotic lesions.²⁰

Oxidation of LDL can result from the local action of reactive oxygen species and prooxidant enzymes derived from activated endothelial cells, smooth muscle cells or macrophages that penetrate the vessel wall. In diabetic patients with sustained hyperglycemia, glycation of LDL can occur. These biochemical alterations render LDL antigenic and proinflammatory. They also contribute to the inflammatory mechanisms initiated by endothelial dysfunction, and continue to promote inflammation throughout the life span of the atherosclerotic plaque.²⁰

1.3.4 Inflammation and atherosclerosis

Inflammatory mechanisms play a crucial role in the formation of atherosclerotic plaques. Leukocyte recruitment and proinflammatory cytokines participate pivotally in the early phase of atherogenesis.⁵⁴ The recruitment of leukocytes depends on the expression of leukocyte adhesion molecules (LAMs) on the endothelial luminal surface and on chemoattractant signals (e.g. monocyte chemoattractant protein-1 [MCP-1]). These molecules direct diapedesis (passage of cells through the intact endothelial layer) into the subintimal space. Two major subsets of LAM persist in the inflamed atherosclerotic plaque: the immunoglobulin gene superfamily (especially vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecule-1 [ICAM-1]) and the selectins (particularly, E- and P-selectin).²⁰ These LAMs and chemoattractant signals direct

mainly monocytes to the forming lesion. After monocytes adhere to and penetrate the intima, they differentiate into macrophages, which imbibe lipoprotein particles to form foam cells. The foam cells secrete inflammatory cytokines, reactive oxygen species, and other mediators, which further promotes the recruitment of more leukocytes.⁵⁴ Ingestion of modified LDL causes foam cell apoptosis, forming the necrotic core of the plaque. Actions of the macrophages amplify the local inflammatory response, for example by producing matrix metalloproteinases (MMPs) that degrade extracellular matrix macromolecules that lend to the strength of the plaque's fibrous cap. Fracture of a weakened fibrous cap permits blood to contact thrombogenic constituents of the plaque's necrotic core.⁵⁴ Other innate immune mechanisms can drive superficial erosion, another modus of thrombotic events. Therefore, inflammation drives atherosclerosis and ultimately triggers thrombotic plaque complications, which commonly cause myocardial infarction (MI), stroke, and cardiovascular death.⁵⁴

1.4 Cardiovascular risk factors

In the early 20th century, most investigators in the field viewed atherosclerosis as an inevitable process of aging. But in 1948, the landmark Framingham Heart Study began to examine the relationship between specific attributes and CVD, establishing the concept of atherosclerotic risk factors. Among later studies, the Multiple Risk Factor Intervention Trial (MRFIT) screened more than 325,000 men, offering an opportunity to correlate risk factors with subsequent CVD and mortality. Of the major risk factors, those that are not correctable include advanced age, male sex, and heredity – that is, a history of CAD among first-degree relatives at a young age (before age 55 for a male relative or before age 65 for a female relative). Risk factors for atherosclerosis amenable to modification include undesirable concentrations and composition of circulating lipids (dyslipidemia), tobacco smoking, hypertension, diabetes mellitus, and lack of physical activity and obesity.²⁰ According to epidemiologic data, nine modifiable risk factors

account for more than 90% of the proportion of risk of a first myocardial infarction.⁵⁵ However, other studies argued for a much lower contribution. Chockalingam and colleagues suggest that classic risk factors account for 50% of the attributable risk of atherosclerosis.⁵⁶

In the presence of cardiovascular risk factors, endothelial dysfunction can be detected before there is any angiographic evidence of disease or increased intima-media ratio on ultrasound examination. Many of the cardiovascular risk factors, including hyperlipidemia, hypertension, diabetes and smoking, are associated with overproduction of reactive oxygen species and/or increased oxidative stress, both of which reduce vascular NO bioavailability and promote cellular damage.⁵⁷ Hence, increased oxidative stress is considered to be a major mechanism involved in the pathogenesis of endothelial cell dysfunction and may serve as a common pathogenic mechanism of the effect of risk factors on the endothelium.⁵⁷

1.4.1 Dyslipidemia

A large and consistent body of evidence establishes abnormal circulating lipid levels as a major risk factor for atherosclerosis. Observational studies have shown that societies with high consumption of saturated fat and prevalent hypercholesterolemia have greater mortality from coronary disease than countries with traditionally low saturated fat intake and low serum cholesterol levels. Data from the Framingham Heart Study and other cohorts have shown that the risk of ischemic heart disease increases with higher total serum cholesterol levels. In particular, elevated levels of circulating LDL correlate with an increased incidence of atherosclerosis and coronary artery disease. Conversely, elevated high density lipoprotein (HDL) particles associate with protection against atherosclerosis, often attributed to HDL's ability to transport cholesterol away from the peripheral tissues back to the liver for disposal.

Hypercholesterolemia promotes atherogenesis by more than just increasing the availability of LDL particles for entering the subendothelial space – it also exacerbates

endothelial dysfunction. An elevated level of circulating cholesterol produces an alteration of the arterial vasodilatation dependent of endothelial cells. Nitric oxide production in hypercholesterolemia appears to be increased, whereas its bioactivity is decreased. In hypercholesterolemia, there is an excess endothelial superoxide generation, which is not only capable of inactivating NO but may also increase LDL oxidation processes. As part of a vicious circle, it further promotes endothelial cell damage through generation of other reactive oxygen species.⁵⁸ Studies showed that cholesterol levels even in the normal range may be inversely related to endothelium-dependent vasodilation. This suggests that lowering cholesterol levels even when it is within the normal range may improve the production and release of endothelium-dependent NO and hence improve endothelial function.⁵⁹

In addition to dietary intake, several genic causes of elevated LDL exist. Familial hypercholesterolemia is a group of genetic defects including mutations of the LDL receptor, of apolipoprotein B and of proprotein convertase subtilisin/kexin type 9 (PCSK9), a protease involved in regulation of the LDL receptor. Heterozygotes with familial hypercholesterolemia display high plasma LDL levels and have about 20-fold risk of developing premature CAD when left untreated. Homozygotes who completely lack functional LDL receptors may experience vascular events, such as acute myocardial infarction, as early as the first decade of life.⁶⁰ On the other hand, loss of function mutations in the gene that encodes the enzyme PCSK9 augment LDL receptor levels on cell surfaces, boosting LDL clearance and yielding lower LDL concentrations in blood. Individuals with this favorable mutation thus are exposed to lifelong low levels of LDL, and have been shown to experience 80% fewer cardiac events than do those with the typical genotype.⁶¹

1.4.2 Diabetes mellitus

Death and disability due to atherosclerotic CVD occurs earlier, with greater severity and with more diffuse distribution among patients with diabetes mellitus (both type I and type II), compared to non-diabetic individuals.^{62,63} About two-thirds of deaths in people with diabetes are due to CVD: of these, approximately 40% are from IHD, 15% from other forms of heart disease, principally congestive heart failure, and about 10% from stroke.⁶⁴ The increased risk for atherosclerotic CVD in diabetes could not be fully accounted for by associated traditional cardiovascular risk factors, and the presence of diabetes in conjunction with other risk factors appeared to cause a synergistic rather than additive additional risk.⁶⁵

Diabetes is a heterogeneous disorder with hyperglycemia required for its diagnosis. *In vitro* and *in vivo* studies where hyperglycemia was induced in the absence of elevated lipids demonstrated the direct effect of hyperglycemia on endothelial dysfunction,^{66,67} atherosclerotic lesion severity and complexity,⁶⁸ and plaque burden.⁶⁹ Even short exposure to high glucose concentrations is sufficient to reduce NO bioavailability⁷⁰ and endothelial-dependent vasodilation,⁶⁶ while increasing endothelial cell leukocyte adhesion.⁶⁷ In the setting of hyperglycemia, vascular smooth muscle cells undergo phenotypic switching from a quiescent, contractile state to an activated, proliferative, migratory, and dedifferentiated state.⁷¹ High glucose concentrations also lead to increased production of reactive oxidative species (ROS),⁷² macrophage inflammation and enhancement of response to inflammation.⁷³

Insulin stimulates endothelial nitric oxide synthase (eNOS)-induced production of nitric oxide (NO) by endothelial cells via the PI3-kinase/Akt pathway. Defects along the insulin signaling pathway seen in insulin resistance and diabetes result in decreased eNOS activity and decreased NO production, promoting endothelial dysfunction.⁷⁴ Productions of the vasoconstrictors, endothelin-1 and angiotensin II, are increased in the presence of compensatory hyperinsulinemia and contribute further to endothelial dysfunction and hypertension.⁷⁵ Insulin

resistance may also activate multiple inflammatory signaling pathways that stimulate atherosclerotic processes.⁶⁵

Diabetes and dyslipidemia commonly occur together, with lipid abnormalities affecting 60-70% of type 2 diabetes.⁷⁶ LDL-cholesterol particles undergo glycation in diabetes and become more atherogenic even in the absence of overt increased LDL concentration.⁷⁷ Diabetic dyslipidemia is also characterized by elevated triglycerides, low HDL-cholesterol, and higher concentrations of apolipoprotein B-containing particles. This is a consequence of elevated free fatty acid level and decreased degradation of apolipoprotein-B in the insulin resistant state.⁶⁵

Patients with diabetes are at increased risk for recurrent atherothrombosis.⁷⁸ Experimentally-induced hyperinsulinemia and hyperglycemia results in elevated circulating tissue factor procoagulant activity and other prothrombotic proteins.⁷⁹ Individuals with diabetes are also more likely to have calcified atherosclerotic lesions.⁸⁰ Hyperglycemia increases post-translational protein modification and promotes glycation end-product development, which potentiates and accelerates vascular calcification, respectively.^{81,82} Thrombosis and calcification increases the complexity of atherosclerotic lesions and lead to higher rates of major cardiovascular events.⁶⁵

1.4.3 Hypertension

Elevated blood pressure (either systolic or diastolic) augments the risk of developing atherosclerosis. The association of blood pressure with CVD does not appear to have a specific threshold. Rather, risk increases continuously with progressively higher pressure values. Systolic pressure predicts adverse outcomes more reliably than does diastolic pressure, particularly in older persons.²⁰

Hypertension may accelerate atherosclerosis in several ways. Animal studies have shown that elevated blood pressure injures vascular endothelium and may increase the permeability of

the vessel wall to lipoproteins. Endothelial dysfunction has been documented in both the forearm and coronary vascular beds of patients with essential hypertension.⁵⁷

Hypertension may also promote atherogenesis by contributing to a prooxidant and inflammatory state. Angiotensin II, a mediator of hypertension, acts not only as a vasoconstrictor but also as a stimulator of oxidative stress and as a proinflammatory cytokine. Oxidative stress in the vascular wall might decrease NO bioavailability. NO induces vasodilation, inhibits adhesion of platelets and leukocytes to the endothelium, prevents platelet aggregation, and inhibits proliferation of smooth muscle cells.⁵⁷

Cyclic circumferential strain, as it is increased in hypertensive arteries, can enhance smooth muscle cell production of proteoglycans that bind and retain LDL particles, promoting their accumulation in the intima, and facilitating their oxidative modification.²⁰

1.4.4 Tobacco smoking

Numerous studies have shown that tobacco smoking predisposes to atherosclerosis and IHD. Even low level smoking leads to adverse outcomes, but the heaviest smokers have the greatest risk of cardiovascular events.²⁰

Tobacco smoke contains a variety of substances, such as nicotine and free radicals, which can promote atherosclerosis in several ways. Several studies have reported that smoking is associated with endothelial dysfunction. Evidence exists to show that smoking causes oxidative stress which may inactivate NO and enhance oxidative modification of LDL. Studies in the human forearm vascular bed have demonstrated decreased flow-dependent dilation in chronic smokers due to inappropriate stimulation of the sympathetic nervous system by nicotine and a reduced vasodilator response to acetylcholine, that was related to increased oxidative stress.⁵⁷ Additionally, tobacco smoking decreases circulating HDL levels and increases platelet

adhesiveness. Extrapolation from animal experiments suggests that smoking not only accelerates atherogenesis but also increases the propensity for thrombosis.²⁰

1.5 Atherogenesis involves vasoactive systems

Atherosclerosis is a multi-factorial disease that involves many physiological systems. Vasoactive systems regulate arterial blood pressure and circulating volume. Elevated blood pressure may contribute to the initiation and development of atherosclerosis. Vasoactive systems include the NO system, the renin-angiotensin-aldosterone system, the endothelin system and the natriuretic peptide system (see Figure 1.4).

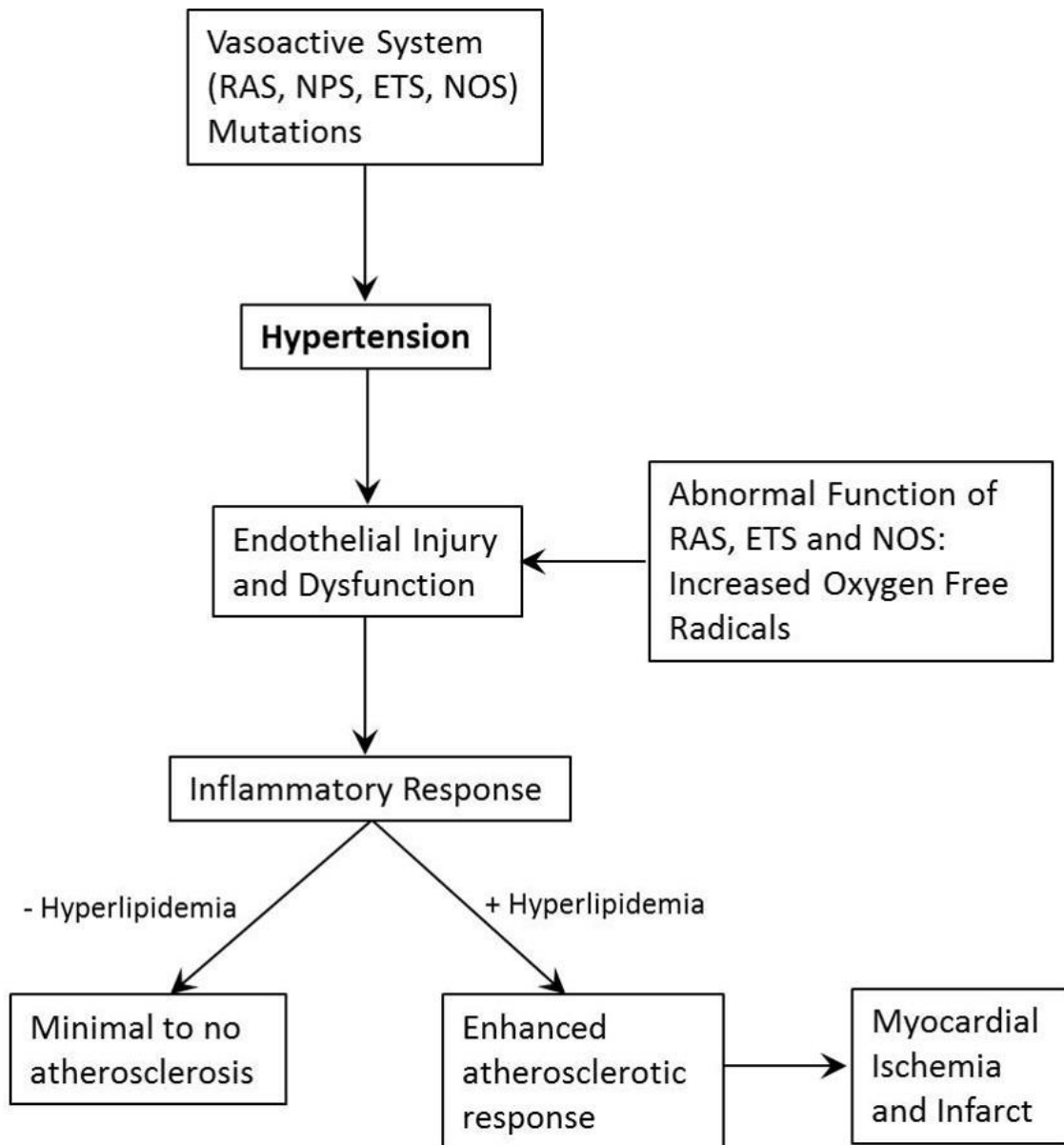


Figure 1.4. Flow diagram showing the proposed involvement of vasoactive systems in atherogenesis. RAS, renin-angiotensin system; NPS, natriuretic peptide system; ETS, endothelin system; NOS, nitric oxide system.

1.5.1 The nitric oxide system

Nitric oxide (NO) is an endothelium-derived relaxing factor produced in the vascular endothelial cell wherein nitric oxide synthase (NOS) catalyzed the oxidation of L-arginine and oxygen to produce NO.⁸³ NO plays an important role in regulating vascular tone, lowering lipid levels, and inhibiting the expressions of adhesion molecules, platelet aggregation as well as vascular smooth muscle cell proliferation.⁸⁴ There are three NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), Under physiological state, nNOS and eNOS is more inclined to express, but under pathological state iNOS is more likely to express.⁸⁵

NO is a multifunctional signaling molecule involved in the maintenance of metabolic and cardiovascular homeostasis. NO is also a potent endogenous vasodilator and involved in key processes that suppress the development of atherosclerosis. NO bioavailability indicates the production and utilization of endothelial NO. Its decrease is related to oxidative stress, lipid infiltration, the expressions of some inflammatory factors, and the alteration of vascular tone, which plays an important role in endothelial dysfunction.⁸³

1.5.2 The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a hormone system that acts on multiple physiologic pathways primarily by regulating blood pressure and fluid balance, but also by local autocrine and paracrine actions. Renin is produced by juxtaglomerular cells of the kidney. Angiotensinogen is synthesized and secreted by the liver. Its cleavage by renin leads to the production of angiotensin I. Angiotensin I is then converted into angiotensin II, catalyzed by angiotensin converting enzyme (ACE) in the lung. ACE also presents in endothelial cells and produces angiotensin II locally in blood vessels. Angiotensin II increases blood volume and pressure by binding to specific angiotensin receptors, primarily AT1 and AT2.⁸⁶

Under pathophysiologic conditions, RAAS also contributes to the development of atherosclerosis and its various manifestations, both directly and indirectly through the actions on other systems. RAAS mainly acts as a promoter of atherosclerosis by its action on blood vessels, and by promoting the development of hypertension, insulin resistance and diabetes, obesity, vascular and systemic inflammation. As RAAS plays a key role in the pathogenesis of CVD, RAAS genes have been extensively studied as candidate genes for atherosclerosis and coronary artery disease. Several polymorphisms of its genes have been found to be in relationship with atherosclerosis and cardiovascular diseases.⁸⁶

1.5.3 The endothelin system

Endothelins (ETs) comprise a family of three peptides, ET-1, ET-2 and ET-3 derived from three distinct genes.⁸⁷ ET-1 is the predominant isoform in the cardiovascular system produced by vascular endothelial cells, vascular smooth muscle cells, cardiomyocytes, fibroblasts and epithelial cells.⁸⁸ The human EDN1 gene codes for a biologically inactive 212 amino acid preproendothelin-1 (PPET1). The promoter of the EDN1 gene contains numerous regulatory elements and its transcription is regulated by many hormonal and environmental stimuli.⁸⁹ Specific furin-like proteases cleave PPET1 into the big endothelin-1, from which the 21 amino acid ET-1 is produced through the proteolytic action of endothelin converting enzymes.⁹⁰ ET-1 exhibits low plasma concentration because of the quasi irreversible feature of its receptor binding and the clearing after binding,⁹¹ but ET-1 concentrations are 100-fold higher within the vascular wall, suggesting a paracrine mode of action.⁹²

The actions of ET-1 are mediated via two G-protein-coupled receptors, endothelin receptor A (ET_AR) and endothelin receptor B (ET_BR). The actions of ET-1 include arterial and venous vasoconstriction, direct positive inotropic effects on cardiomyocytes, growth promoting effects on vascular smooth muscle cells and proliferative actions on fibroblasts.⁹³ Most of these

effects are mediated by the ET_AR, while ET_BR stimulation can result in the release of vasodilators and ET-1 clearance, actions that antagonize the ET_AR.⁹⁴

ET-1 is the most potent endogenous vasoconstrictor known to date, hence its role in hypertension has been under thorough investigation, while its significance in atherosclerosis is newly emerging.⁹³ The vasoconstrictor effects of ET-1 are even more potent in the coronary arteries compared with peripheral arteries, due to unique characteristics of coronary vascular physiology.⁹⁵ For example, the coronary endothelium expresses fivefold lower eNOS and threefold higher ET-1 levels than the aortic endothelium.⁹⁶ ET-1 was found to contribute to more vasoconstriction in arteries with atherosclerotic lesions and significant stenosis than in normal arteries. Thus the functional response to ET-1 may depend on the presence of CAD and its severity.⁹⁷

ET-1 induces endothelial dysfunction in the coronary circulation through several mechanisms. Endothelial dysfunction may be associated with an imbalance between NO and ET-1, in favor of the latter.⁹⁸ ET-1 can inhibit the activity of eNOS, thereby decreasing NO bioavailability.⁹⁹ ET-1 enhances the formation of reactive oxygen species (ROS) and is associated with local oxidative stress and inflammation.⁹³ ET-1 also stimulates the mitogen-activated protein kinase pathway, which is involved in cell growth and proliferation, and may accelerate atherosclerotic progression.¹⁰⁰ Finally, ET-1 may interfere with glucose and lipid metabolism, further exacerbating atherosclerosis.⁹³

1.5.4 The natriuretic peptide system

The natriuretic peptide system consists of three members, atrial natriuretic peptide (ANP), B- type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). The human ANP gene is located on the short arm of chromosome 1 and is expressed in atrial myocytes to produce the 151 amino-acid pre-pro ANP.¹⁰¹ Pre-pro ANP is processed to a pro ANP of 126 amino acids,

which is released into the circulation. The secreted pro ANP further undergoes cleavage and post-translational modification by the enzyme corin yielding the 28 amino-acid biologically active form of ANP.¹⁰¹

The human BNP gene is also located on chromosome 1 and consists of three exons and two introns.¹⁰² BNP is synthesized by ventricular myocytes first as a pre-pro hormone of 134 amino acids. Pre-pro BNP modification results in the formation of a signal peptide and the 108 amino-acid pro BNP, that is stored in the ventricles.¹⁰¹ During secretion from the cardiomyocytes, pro BNP undergoes post-translational modification and splits into the physiologically active form of BNP (32 amino acids) and a biologically inactive fragment, N-terminal pro BNP (NT-pro BNP).¹⁰¹

CNP is structurally homologous to both ANP and BNP, but is mainly produced and secreted from vascular endothelium.¹⁰³ It is also produced as a pre-pro peptide that undergoes cleavages to form first pro CNP then CNP (22 amino acids).¹⁰¹ Compared to ANP and BNP, the plasma half-life of CNP is much shorter, therefore the circulating levels of CNP are extremely low.¹⁰⁴

Natriuretic peptides exert their effects by binding to their common receptors present on the plasma membrane: natriuretic peptide receptor A (NPR1), natriuretic peptide receptor B (NPR2) and natriuretic peptide receptor C (NPR3). NPR1 and NPR2 belong to the members of the cell-surface family of guanylyl cyclase receptors, the enzymes that catalyze the synthesis of the intracellular second messenger, cyclic guanosine monophosphate (cGMP).¹⁰⁵ In contrast, NPR3 is a non-guanylyl cyclase receptor, which is mainly involved in clearance of natriuretic peptides.¹⁰⁶ The NPR1 is activated by physiological concentrations of ANP and BNP, but not by CNP. Conversely, NPR2 is specifically activated by CNP. Whereas, all three natriuretic peptides bind to NPR3 and undergo clearance and degradation.¹⁰⁷

The expression of NPR1 is reported in various organs including heart, kidney, lungs, liver and adipocytes.¹⁰⁸ The net effect of ANP and BNP binding to NPR1 is a decrease in both arterial blood pressure and intravascular volume.¹⁰⁹ In the kidney, ANP and BNP elicits natriuresis (excretion of sodium), diuresis (excretion of water) and inhibition of renin release.¹⁰⁹ These hormones also lower peripheral vascular resistance by decreasing vascular tone and inhibits aldosterone synthesis and secretion by the adrenal zona glomerulosa cells.¹⁰⁹ ANP also elicits effects in the brain, including suppression of vasopressin release from the pituitary and inhibition of the firing of hypothalamic neurons.¹¹⁰

Due to their natriuretic, diuretic and vasorelaxant activities, ANP and BNP play an important role as cardio-protective hormones under conditions of CVD such as heart failure (HF), CAD, hypertension and left ventricular hypertrophy and cerebrovascular accidents.^{111,112} Plasma BNP and NT-pro BNP have become established biomarkers to facilitate the diagnosis and prognosis of HF, and have been associated with cardiovascular mortality as well as all-cause mortality in the general population.¹¹³ Studies have also revealed that acute administration of ANP or BNP can improve left ventricular function in patients with chronic heart failure, suggesting the potential potency of these natriuretic peptides as short-term therapeutic agents.^{101,114} Furthermore, many epidemiological and clinical studies have shown that patients with obesity, insulin resistance and diabetes have reduced levels of circulating ANP and BNP and altered expression in their receptor, suggesting natriuretic peptides may contribute to enhancing susceptibility to CVD via factors involved in metabolic syndrome.¹¹⁵

1.6 Carotid atherosclerosis and carotid ultrasound

Atherosclerosis is a systemic disease with focal manifestations, such as in the coronary arteries causing CAD. Carotid artery is a barometer of atherosclerosis elsewhere in the vascular tree and carotid atherosclerosis is correlated with significant angiographic CAD.¹¹⁶ Ultrasound

has the advantage of being a simple, safe, and inexpensive, making it an ideal tool for screening carotid atherosclerosis.³⁸ Carotid intima-media thickness (CIMT), as measured by ultrasound, is a quantitative trait that strongly correlates with the severity and extent of CAD, and is increasingly used in clinical decision-making.¹¹⁶ CIMT is comprised of intimal layer where early atherosclerosis develops and the media layer which is subject to non-atherosclerotic medial hypertrophy commonly induced by aging and hypertension. Since the larger portion of CIMT is the medial layer, CIMT has not been shown to add to CVD risk prediction. Carotid plaque, on the other hand, represents the atherosclerotic process itself that starts in the intimal layer and thus been shown to predict CVD events far better than CIMT.¹¹⁷ The transition from an increased CIMT to plaque is arbitrarily defined and is debated whether the process is a continuous process,¹¹⁸ or if CIMT and plaques are separate phenotypes.¹¹⁹ Plaque is reported as present when occurring anywhere in the common carotid artery (CCA), bulb or internal carotid artery (ICA).

Some studies defined plaque as a focal thickening of the intima-media greater than 1 mm, protruding into the lumen, which is at least twice as thick as the surrounding normal IMT.¹²⁰ Other studies defined plaque as CIMT greater than 1.2 mm.¹²¹ The European Mannheim consensus is the most commonly used definition, which defines plaque as a focal thickening that encroaches into the lumen by 0.5 mm or by 50% of the surrounding IMT or where IMT is greater than 1.5 mm.¹²²

Most earlier studies reported plaque in a categorical manner. Plaque assessment is qualitative, reported as either “present” or “absent”.¹²³ In the numerical method, total number of plaques is reported occurring anywhere in the CCA, the carotid bulb or the ICA.¹²¹

Plaque thickness is one of the most commonly used parameters, where maximum plaque height (MPH) is measured as seen in any plaque in any imaging plane.¹²⁴ Plaque area is also frequently used, where cross-sectional area of one or multiple plaque is measured and highest plaque area reported.^{125–127}

1.7 Genetic aspect of atherosclerosis

Evidence of atherosclerosis in ancient mummies demonstrate that humans may always have had the genetic propensity to develop atherosclerosis. What is similar between now and then is the human genetic material, our genome, including ancient polymorphisms that were uncovered to predispose the carrier to the development of atherosclerotic CVD. Seminal studies of genetic material obtained from the 5,300-year-old Ötzi showed mutations in the 9p21 chromosomal region identical to variants identified in contemporary humans that are strong predictors for the development of CAD.¹²⁸

Roberts and Stewart¹²⁹ suggested that genetic predisposition accounts for 40% to 60% of human susceptibility to coronary artery disease and thus to atherosclerosis. The recently acquired ability to sequence the entire human genome is having profound influence on our investigations of human diseases, including atherosclerosis. Unlike many of the hereditary cardiomyopathies and dysrhythmias, which are single-gene disorders, genetic predisposition to atherosclerosis is due to multiple common genes, widely distributed in the entire population.^{130,131} Genome-wide association studies have discovered an increasing number of individual single nucleotide polymorphisms (SNPs) risk variants that make up this predisposition.¹³² The risk associated with each SNP has generally been modest, averaging an increased incidence of disease of about 18%,¹³³ but the frequency and number of SNP make their contribution substantial.¹³⁴ The nature of how these common genetic polymorphisms interact with the environment to produce atherosclerosis and clinical coronary artery disease remains unexplained, but is the subject of intense investigation. Most of the known genetic risk factors for atherosclerosis are linked by network analysis to lipid metabolism and inflammation. Bos et al.¹³⁵ have correlated a number of these SNPs to multi-vessel bed calcification. Zink et al.¹²⁸ further explore the current contribution of genetics to atherosclerotic susceptibility and its potential evolutionary changes.

A synthesis of these findings is consistent with a gene-environment interplay as causal for atherosclerosis. While genes create the susceptibility, the environment, diet, lifestyle, and their impact on currently understood risk factors, determine when and if this susceptibility results in manifestations of atherosclerosis that are clinically apparent.¹³⁶

A polymorphism in the *NPPB* gene that encodes for BNP, rs198389 has been established as a functional variant in the promoter region of this gene. The minor allele G was previously shown in cultured cells to be associated with a 1.8-fold higher level of promoter activity compared to the major allele A.¹³⁷ Numerous human population studies have demonstrated that the G allele of rs198389 is associated with elevated levels of NT-proBNP and/or BNP.¹³⁷⁻¹⁴⁴ The same minor allele has been associated with a lower risk of developing type II diabetes mellitus,^{137,145,146} reduced systolic BP,¹⁴¹ decreased risk of ventricular dysfunction following coronary artery bypass grafting (CABG),¹⁴⁷ reduced hospital readmissions, and less left ventricular diastolic dysfunction in patients after MI.¹⁴³ In a recent study, Seidelmann et al.¹⁴⁴ not only confirmed the association between rs198389 G allele and elevated levels of NT-pro-BNP and reduced hypertension, but also showed that the minor allele is associated with lower CV mortality and increased lifespan.

1.8 Hypothesis and Objectives

Genetic polymorphisms contribute significantly to CAD risk and outcome. Even though a great number of SNPs have been discovered to associate with atherosclerosis in large population studies, the extent to which SNPs affect physiological systems is unclear. In order to be able to use genetic variants in diagnosis and prognosis of CAD, cohort studies with complete demographic and clinical data are needed. *The main HYPOTHESIS of this thesis is that SNPs of the vasoactive systems contribute to the development of atherosclerosis and can be used to predict CAD outcomes.* The main objectives of this thesis is to identify SNPs that are associated

with atherosclerosis in both the coronary arteries and the carotid arteries. In the following research chapters, we approached the manifestation of atherosclerosis longitudinally, highlighting all three stages of CAD presentation: pre-clinical phenotype in the form of ultrasound-detected carotid atherosclerosis, clinical diagnosis with coronary angiography, and prognosis of adverse outcomes through follow-up. The specific hypotheses and objectives for each chapter are summarized below.

1.8.1 Chapter 2: Sex Differences of the Natriuretic Peptide Polymorphism Associated with Angiographic Coronary Atherosclerosis

It is well understood that the natriuretic peptide system plays an important role in hemodynamic regulation in CVD. SNP of the NP genes have been associated with clinical outcomes of CVD, but there has been no attempt in finding an association between NP SNPs and angiographic CAD. Furthermore, no previous study has compared the effect of these genetic variants between men and women. Here we examined the association between SNPs of NP genes and coronary atherosclerosis and *HYPOTHESIZE that these SNPs affect males and females differently.*

Main Objectives:

1. To determine if SNPs of NP genes, *NPPA* rs5065, *NPPB* rs198389 and *NPR2* rs10758325, are associated with coronary atherosclerosis evaluated by angiography.
2. To assess the sex-specific associations between SNPs and CAD in men and women separately.

1.8.2 Chapter 3: Single Nucleotide Polymorphism of B-Type Natriuretic Peptide is Associated with Atherosclerosis in both Coronary Arteries and Carotid Arteries

Carotid artery is a barometer of atherosclerosis elsewhere in the vascular tree. Carotid intima-media thickness (CIMT) and carotid plaque size, as measured by ultrasound, correlate with significant CAD and CV events. Association between NP genetic variants and carotid atherosclerosis has never been investigated. Because ANP and BNP influence CVD systemically, we *HYPOTHESIZE that SNPs of natriuretic peptides are associated with atherosclerosis measured by carotid ultrasound.*

Main objectives:

1. To corroborate the correlation between carotid atherosclerosis evaluated by ultrasound and coronary atherosclerosis assessed by angiography.
2. To determine if NP SNPs, *NPPA* rs5065 and *NPPB* rs198389, associate with CIMT and carotid atherosclerotic plaque size.

1.8.3 Chapter 4: Single Nucleotide Polymorphism of B-Type Natriuretic Peptide as a Predictor for Increased Risk of Cardiovascular Events

Previous studies have shown that *NPPB* rs198389 was associated with elevated circulating levels of BNP, as well as reduced hypertension and lower CV mortality. We *HYPOTHESIZE that rs198389 genotypes are associated with major adverse cardiovascular events, including MI or ACS, stroke, undergoing CABG or PCI, and cardiac death.*

Main objective:

1. To determine if *NPPB* rs198389 is associated with life time major adverse cardiovascular event (MACE) and premature MACE before the age of 65 in a retrospective study design.
2. To determine if *NPPB* rs198389 is associated with recurrent MACE during a five-year follow-up in a prospective cohort design.

Chapter 2

Sex Differences of the Natriuretic Peptide Polymorphism Associated with Angiographic Coronary Atherosclerosis

This chapter has been modified from the original publication: Li TY, Tse MY, Pang SC, McLellan CS, King WD, Johri AM. Sex Differences of the Natriuretic Peptide Polymorphism Associated with Angiographic Coronary Atherosclerosis. *Cardiology Research* 2017, 8(1): 1–6.

2.1 Abstract

Background and Aims:

Polymorphisms within natriuretic peptide (NP) genes have been associated with clinical outcomes for cardiovascular disease, but no previous study has compared the effect of these polymorphisms between men and women. This study aimed to investigate the association between single nucleotide polymorphisms (SNPs) in key genes of the NP system and coronary angiographic outcomes, with the focus on the sexual dimorphism in the effects of these SNPs.

Methods:

Patients undergoing clinically indicated coronary angiography (n=513, 328 men and 185 women) were consented and genotyped for *NPPA* rs5065, *NPPB* rs198389 and *NPR2* rs10758325. Patients were stratified into having normal coronaries, non-obstructive CAD or obstructive CAD, based on the highest stenosis in any epicardial artery. Average luminal narrowing across all four arteries was derived to represent the overall atherosclerotic burden.

Results:

The frequency of *NPPB* rs198389 AA genotype was significantly higher in women with obstructive CAD (p=0.014). The same association was not observed in males. With respect to atherosclerotic burden, an association was found between the AA genotype and average luminal narrowing in women (p=0.005), but not in men.

Conclusions:

The current study identified an association between a SNP of the *NPPB* gene and coronary atherosclerotic burden through angiographic evidence in women but not in men. These results suggest that B-type natriuretic peptide (BNP) may have more important involvement in the development of CAD in women compared to men, and as such, genotyping of the *NPPB* gene may serve as a potential biomarker to identify women with high risk for CAD.

2.2 Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide. Although our understanding of the pathogenesis of atherosclerosis is not fully determined, CAD is well-known to result from buildup of plaque in coronary arteries, leading to narrowing of these vessels.

Previous studies have demonstrated significant differences in diagnosis and development of CAD between men and women. Compared to men, women were found to have a lower prevalence of obstructive CAD, but those women who were diagnosed with obstructive CAD had higher rates of adverse outcomes than men¹⁴⁸⁻¹⁵⁰. In the presence of non-obstructive CAD, microvascular dysfunction, endothelial dysfunction and other processes that predict adverse outcome appear more frequent in women than in men¹⁵¹. It is unknown if these striking differences in CAD prevalence and outcomes are related to differences in the underlying genetic expression of hormonal circulatory systems involved in myocardial and vascular regulation.

The natriuretic peptide system plays an important role in hemodynamic regulation and its relation to heart disease has become of increasing importance lately with the advent of novel pharmaceuticals¹⁵². The natriuretic peptide system consists of three members, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), encoded by *NPPA*, *NPPB* and *NPPC* genes, respectively. They exert their effects by binding to high-affinity receptors that are expressed on the surface of target cells. Natriuretic peptide receptors A and B mediate the majority of the cardiovascular effects of the natriuretic peptides. Natriuretic peptide receptor C is the clearance receptor, binding all three natriuretic peptides for internalization and degradation. These receptors are encoded by *NPR1*, *NPR2* and *NPR3* genes, respectively.

Circulating levels of ANP and BNP are elevated in patients with ventricular hypertrophy and congestive heart failure¹⁵³, making them useful tools in assessing the degree of cardiac

dysfunction and severity of left ventricular failure. Levels of BNP and N-terminal BNP (NT-pro BNP) have consistently been found to be independent risk markers for both morbidity and mortality in post MI and heart failure patients. On the other hand, newly approved heart failure medications work through the natriuretic peptide system to promote vasodilation and natriuresis, stimulating further interest in response to therapies that modify this system ¹⁵².

Numerous single nucleotide polymorphisms (SNPs) of *NPPA* and *NPPB* have previously been found to be associated with cardiovascular outcomes. Ellis et al. (2011) reported that minor alleles of *NPPA* rs5065, *NPPB* rs632793, rs198388, rs198389 and *NPR2* rs10758325 are associated with a lower rate of hospital readmission related to all cardiovascular diseases ¹⁴³. However, no previous study has examined the association between SNPs and angiographic CAD specifically, nor differences between males and females.

The current study aims to investigate three SNPs within *NPPA*, *NPPB* and *NPR2* genes, namely rs5065, rs198389 and rs10758325, for their association with the severity of CAD through angiographic evidence. More importantly, we were interested in determining whether the effects of these SNPs on CAD differ between males and females.

2.3 Patients and Methods

2.3.1 Patient cohort:

Written, informed consent was obtained from each participant recruited. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the Health Sciences and Affiliated Teaching Hospitals Research Ethics Board at Queen's University (DMED-1365-11). The inclusion criteria for this study comprised of patients undergoing an elective coronary angiogram at the Kingston General Hospital (KGH) Cardiovascular Lab (Kingston, Ontario). Patients were excluded only if there was a contra-indication for the blood sample collection. Patients were referred to undergo angiography due to

one or more of the following indications: angina, positive ECG test, positive (stress) imaging test, and previously known CAD.

2.3.2 Angiography:

Coronary angiography was performed according to the standard Judkins method using a GE System 2000 (GE Healthcare) by one of four experienced interventional cardiologists.

Percentage luminal diameter narrowing in each of the four major coronary arteries (left main, left anterior descending, circumflex and right coronary artery) was recorded. Patients were stratified into three categories: normal coronary (luminal narrowing $<20\%$ in all epicardial arteries), non-obstructive CAD (luminal narrowing $\geq 20\%$ and $<70\%$ in any epicardial artery or $\geq 20\%$ and $<50\%$ in the left main artery) and obstructive CAD (luminal narrowing $\geq 70\%$ in any epicardial artery or $\geq 50\%$ in the left main artery). Average luminal narrowing across all four vessels was also acquired to create a continuous variable that represents the overall coronary plaque burden.

2.3.3 SNP selection:

SNPs of the natriuretic peptide system were selected using the following criteria: 1) have been identified in previous studies as associated with circulating natriuretic peptide level or cardiovascular outcomes and 2) have a minor allele frequency $>10\%$. The SNPs selected were *NPPA* rs5065, *NPPB* rs198389 and *NPR2* rs10758325.

2.3.4 DNA extraction and genotyping:

Whole blood was collected during the angiography procedure. White blood cells were isolated from the whole blood samples and digested using cell lysis buffer and proteinase K (5-PRIME, VWR Canada). Genomic DNA was precipitated using isopropanol, isolated and

rehydrated with Tris buffer. DNA samples were diluted to 5ng/ μ L prior to genotyping. Genotyping was performed using TaqMan SNP genotyping assays according to the manufacturer's protocol (Applied Biosystems, Foster City, California, US) on a LightCycler[®] 480-II Real-Time PCR System (LC480-II; Roche Applied Science, Laval, Quebec, Canada).

2.3.5 Statistical analysis:

Associations between natriuretic peptide SNP genotypes and CAD severity category were tested using a log-binomial regression model controlling for age and sex, where the relative risk (RR) was reported. Associations between natriuretic peptide SNP genotypes and average luminal narrowing were tested using a linear least squares regression model controlling for age and sex. The effect estimate (β) was reported to represent the adjusted difference in overall plaque burden. The differences in SNP effects between sexes were tested with an interaction term in aforementioned models. The log-binomial regression model was also used to test for associations between natriuretic peptide SNPs and clinical characteristics including hypertension, type II diabetes and history of MI. A p-value of ≤ 0.05 was deemed to be statistically significant. All analyses were performed using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, North Carolina).

2.4 Results

2.4.1 Power calculation

A priori, a patient sample size of 500 was planned and prevalence of obstructive CAD was estimated to be 50% according to study definitions (luminal narrowing $\geq 70\%$ in any epicardial artery or $\geq 50\%$ in the left main artery). SNPs will be dichotomized with at risk genotype prevalence ranging from 25% to 65%. This sample provides 80% power to detect relative risks of 1.3 and greater for each SNP (two-tailed significance of 0.05)¹⁵⁴. A higher

proportion of males than females were expected in the sample and detectable relative risks are 1.4 in males (n=320) and 1.5 in females (n=180).

2.4.2 Participant characteristics

Characteristics of the study cohort (n=513) are described in Table 2.1. The mean age of participants was 65.3 years, 64% of them were male. The majority of participants were Caucasians, with four Asian descendants, two African descendants, two Canadian First Nation individuals and one mixed race. Just over half of the participants (56.1%) were diagnosed with obstructive CAD, while 22.6% were diagnosed with no or minimal disease (normal coronary arteries).

The distribution of average luminal diameter narrowing is presented in Figure 2.1. It is heavily skewed to the left because of the large number (124) of patients with 0% narrowing in all four vessels. Thus, only patients diagnosed with CAD (luminal narrowing $\geq 20\%$ in any epicardial artery) (n=387) were included in the linear least squares model to assess overall coronary plaque burden. The mean of average luminal narrowing among these patients was 39.5% (± 22.3).

2.4.3 Genetic Statistics:

Genotype frequencies for each natriuretic peptide system SNP are shown in Table 2.2. In our study population, all three SNPs were in Hardy Weinberg equilibrium (p -values were 0.09, 0.23 and 0.78 for rs5065, rs198389 and rs10758325, respectively); rs5065 and rs198389 were found to be in linkage disequilibrium. There was no association between genotype and age or sex for all three SNPs.

For all three SNPs, heterozygous genotype and homozygous minor allele genotype were combined in further analyses using regression models, because 1) relatively small proportions of homozygous minor allele genotype, and 2) heterozygous and homozygous minor allele genotypes have been shown to behave similarly in terms of their effects in cardiovascular outcomes.¹⁴³

Table 2.1. Participant characteristics (n=513).

Characteristics	Mean (\pm SD) or Percent
Age (years)	65.3 \pm 11.1
Sex male	63.94%
Caucasian	98.2%
BMI (kg/m ²)	30.3 \pm 6.3
Current Smoker	18.8%
Hypertension	65.1%
Diabetes	31.3%
Previous MI	28.0%
Family history of heart diseases	67.7%
CAD categories	
Normal coronary	22.6%
Non-obstructive CAD	21.3%
Obstructive CAD	56.1%

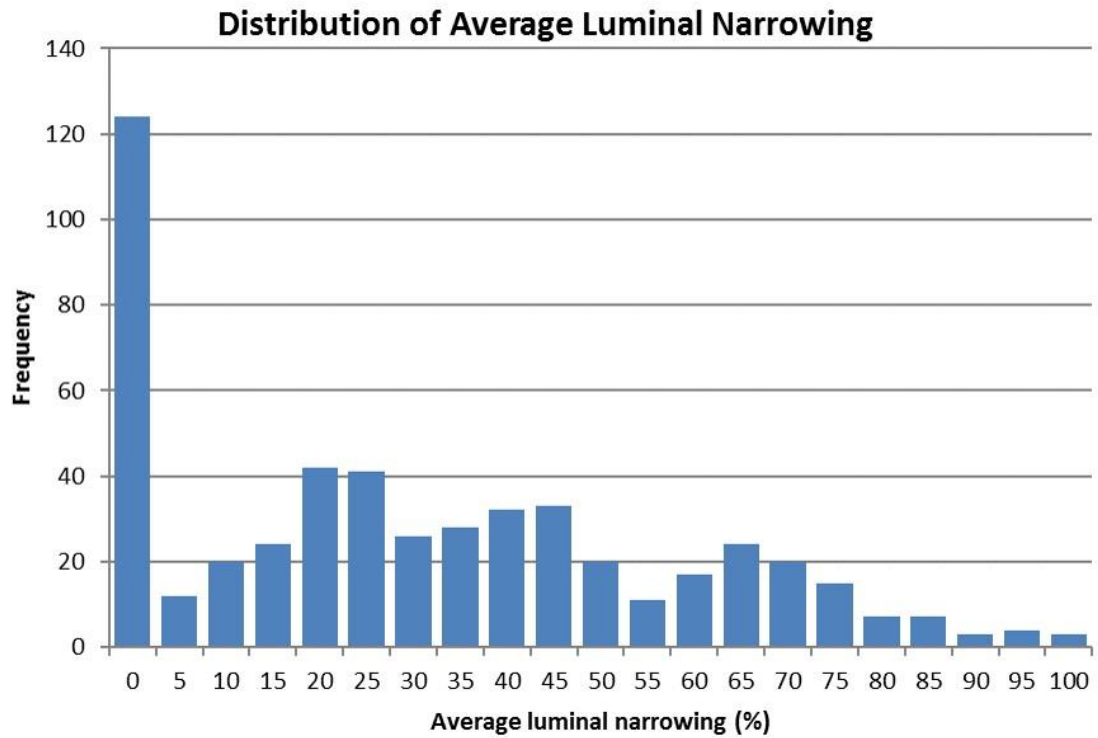


Figure 2.1. Distribution of average luminal diameter narrowing across four major coronary arteries (n=513).

Average luminal narrowing is heavily skewed to the left due to the great number of participants with no-to-minimal coronary stenosis. These participants were excluded to produce a normal distribution for the analysis with average luminal narrowing.

Table 2.2. Natriuretic peptide system SNP genotype frequencies.

SNP ID (major allele/minor allele)		n	Genotypes			MAF
			Homozygous major allele (n)	Heterozygous (n)	Homozygous minor allele (n)	
<i>NPPA</i> rs5065 (A/G)		510	73.5% (375)	23.3% (119)	3.1% (16)	0.15
<i>NPPB</i> rs198389 (A/G)		490	34.2% (174)	50.5% (257)	15.3% (78)	0.42
<i>NPR2</i> rs10758325 (G/A)		507	34.7% (176)	49.0% (249)	16.3% (83)	0.41

MAF, minor allele frequency.

2.4.4 Association between basic characteristics, obstructive CAD and average luminal narrowing:

Patients' age ($p<0.01$, RR=1.20/10 years), male sex ($p<0.01$, RR=1.52), hypertension ($p=0.03$, RR=1.23), type II diabetes ($p<0.01$, RR=1.31) and history of MI ($p<0.01$, RR=1.39) were associated with obstructive CAD. No association was found between obstructive CAD and smoking or family history of heart diseases.

Age ($p<0.01$, $\beta=+5.39$), the male sex ($p<0.01$, $\beta=+9.40$), hypertension ($p=0.02$, $\beta=+6.12$) and type II diabetes ($p<0.01$, $\beta=+8.78$) were associated with increased average luminal narrowing. No association was found between average luminal narrowing and smoking, history of MI or family history of heart diseases.

2.4.5 Association between natriuretic peptide SNPs and obstructive CAD:

No association was observed between any natriuretic peptide SNPs and obstructive CAD (any one of four major coronary arteries with luminal narrowing of 70% or more) in the overall cohort. When sexes were divided, however, *NPPB* rs198389 homozygous major allele genotype (AA) was found to be associated with obstructive CAD in women only, with a relative risk of 1.50 (CI 1.09-2.07, $p=0.014$) compared to AG or GG genotypes. The effect of rs198389 was also observed to be significantly different between the sexes ($p=0.015$), as no association was observed in the male cohort (Table 2.3).

2.4.6 Association between natriuretic peptide SNPs and average luminal narrowing:

Among patients diagnosed with CAD (non-obstructive or obstructive; $n=387$), *NPPB* rs198389 homozygous major allele genotype (AA) was found to be associated with increased average luminal narrowing ($\beta=4.91\pm 2.25$, $p=0.030$) compared to AG or GG genotypes. When sexes were divided, this association was stronger in women ($\beta=11.05\pm 3.92$, $p=0.0051$), but no association was observed in men (Table 2.4).

Table 2.3. Association of SNPs of the natriuretic peptide system and obstructive CAD.

Obstructive CAD					
SNP	Genotype	Overall RR (95% CI)	Male RR (95% CI)	Female RR (95% CI)	<i>p</i> -value sex difference
<i>NPPA</i>					
rs5065 (n=510)	AA	1.04 (0.88- 1.22)	0.99 (0.83-1.16)	1.31 (0.88-1.95)	0.199
	AG/GG	Reference	Reference	Reference	
<i>NPPB</i>					
rs198389 (n=509)	AA	1.00 (0.87- 1.16)	0.93 (0.79-1.10)	1.50 (1.09- 2.07)*	0.015
	AG/GG	Reference	Reference	Reference	
<i>NPR2</i>					
rs10758325 (n=508)	GG	1.02 (0.88- 1.18)	1.04 (0.90-1.22)	0.90 (0.63-1.28)	0.443
	GA/AA	Reference	Reference	Reference	

* $p < 0.05$.

Adjusted for age and sex.

Table 2.4. Association of SNPs of the natriuretic peptide system and average luminal narrowing (excluding patients with 0% luminal narrowing).

Average luminal narrowing					
SNP	Genotype	Overall β (95% CI)	Male β (95% CI)	Female β (95% CI)	<i>p</i> -value sex difference
<i>NPPA</i>					
rs5065	AA	1.39 (-3.43-6.21)	-1.37 (-7.25-4.51)	6.95 (-1.39-15.30)	0.110
(n=385)	AG/GG	Reference	Reference	Reference	
<i>NPPB</i>					
rs198389	AA	4.91 (0.50-9.32)*	1.92 (-3.44-7.28)	11.05 (3.37-18.73)†	0.057
(n=385)	AG/GG	Reference	Reference	Reference	
<i>NPR2</i>					
rs10758325	GG	0.65 (-3.84-5.14)	-0.37 (-5.79-5.05)	2.88 (-5.14-10.91)	0.511
(n=383)	GA/AA	Reference	Reference	Reference	

* $p < 0.05$.

† $p < 0.01$.

Adjusted for age and sex.

2.5 Discussion

In the current study, we did not find any association between *NPPA* rs5065 and *NPR2* rs10758325 and CAD categories or overall coronary burden. However, we did find novel gender-specific association between *NPPB* rs198389 and CAD. *NPPB* rs198389 AA genotype was significantly associated with obstructive CAD and overall coronary burden in women but not men.

Minor allele of rs198389 affects *NPPB* promoter binding activity, resulting in higher circulating levels of BNP and NT-proBNP ¹⁴². Despite of the research on *NPPB* rs198389 in hypertension, diabetes, left ventricular function and general cardiovascular outcome ^{137,143}, no previous study has directly tested the association between rs198389 and CAD. In the current study, we have investigated this association using coronary angiographic evidence, which is currently the clinical standard for determining CAD and quantifying the severity of atherosclerosis. In the overall cohort, none of rs198389 genotypes was associated with obstructive CAD category, but minor allele carriers (AG or GG) had significantly lower atherosclerotic burden than individuals with AA genotype based on average luminal narrowing. It suggests that while the AA genotype for rs198389 may aid in the development of atherosclerosis, its effect may not be enough to produce clinically significant single-vessel disease, which the CAD categories are based on.

BNP's role in atherosclerosis pathophysiology is unknown. One of the possible pathway is through blood pressure regulation since hypertension is a prominent risk factor for CAD. However, current results do not support this as no association was found between rs198389 genotypes and hypertension.

More importantly, rs198389 genotypes had significantly different effects on CAD between sexes. Among women, the AA genotype was associated with higher risk of obstructive CAD and increased atherosclerotic burden, whereas no association was found in men. It suggests

that the AA genotype for rs198389 may be related to both overall atherosclerosis development and severe single-vessel disease. Furthermore, we may propose that BNP is significantly more involved in the pathophysiology of CAD in women than in men. It is possible that coronary atherosclerosis develops via different pathways in different sexes.

These sex-specific findings support the concept that CAD pathophysiology in women differs from that in men. Currently, diagnosis and prognosis of CAD in women are based on traditional risk scoring models, which were developed with studies where majority of subjects were men. This lead to a greater frequency of misclassification in women, who present CAD in sex-specific patterns. For example, non-obstructive CAD is more prevalent and has a poorer prognosis in women than in men ^{150,151}. The current findings suggest that integration of genetic markers may help to better stratify female CAD patients in the future.

Previously, numerous studies have attempted to establish an association between *NPPA* rs5065 and CAD, but have yielded opposing results. Some researchers found that the minor allele G was associated with higher risk of acute coronary syndrome ¹⁵⁵ and greater severities of CAD ¹⁵⁶, while other studies reported that the major allele A was more prevalent in CAD patients ¹⁵⁷ and was associated with a higher risk of hypertension, more adverse ventricular remodeling and a higher rate of hospital readmission ¹⁴³. The discrepancy could be explained by diverse patient populations and different outcomes defining CAD. With regards to whether major allele or minor allele of *NPPA* rs5065 is deleterious in CAD, the current study could not support these findings either way, as we did not find any association between *NPPA* rs5065 genotypes and CAD categories or atherosclerotic burden.

Study Limitations

The current study is limited by its relatively small sample size, especially of the female cohort. Future endeavors should focus on recruiting a much larger female cohort or a female-only population. Other angiographic scoring system, such as the Gensini score, could be incorporated

in future studies to confirm current results and validate the method of average luminal narrowing. Lastly, natriuretic peptide SNPs, especially *NPPB* rs198389, could be added into traditional risk stratification and diagnostic tools in order to test whether they would improve predicative values.

2.6 Conclusion

In summary, the current study investigated the association between three natriuretic peptide SNPs and CAD through angiographic evidence in a Canadian population. We identified *NPPB* rs198389 minor allele associated with a lower coronary atherosclerotic burden. This polymorphism was also shown to be associated with a reduced risk of obstructive CAD only in women, suggesting that BNP has different involvements in the pathophysiology of CAD between sexes. These findings provide further evidence for an important protective role of BNP in CAD development, and support the notion that cardiovascular diseases in women differ from those in men at the molecular level.

Chapter 3

Single Nucleotide Polymorphism of B-Type Natriuretic Peptide is Associated with Atherosclerosis in both Coronary Arteries and Carotid Arteries

Results in this chapter is currently being prepared for publication: Li TY, Tse MY, Pang SC, Héту MF, McLellan CS, King WD, Johri AM. Single Nucleotide Polymorphism of B-Type Natriuretic Peptide is Associated with Carotid Plaque Revealed by Ultrasound. To be submitted April, 2019.

3.1 Abstract

Single nucleotide polymorphisms (SNPs) of *NPPA* and *NPPB* genes, rs5065 and rs198389, respectively, have previously been associated with increased risk of cardiovascular outcomes. Carotid artery is a barometer of atherosclerosis elsewhere in the vascular tree. Carotid intima-media thickness (CIMT) and carotid atherosclerotic plaque size, as measured by ultrasound, both correlate with significant CAD and CV events. The present study was designed to examine the association between genotypes of natriuretic peptide SNPs and carotid atherosclerosis.

Two-hundred and four patients who underwent coronary angiography received carotid ultrasound and were genotyped for *NPPA* rs5065 and *NPPB* rs198389. Patients were stratified into having normal coronaries, non-obstructive CAD or obstructive CAD. Carotid ultrasound images were analyzed to quantify CIMT, maximum plaque height (MPH) and total plaque area (TPA).

Increased MPH and TPA were both found to correlate with greater severity of coronary atherosclerosis. The AA genotype of *NPPB* rs198389 was associated with increased average luminal narrowing in coronary arteries, as well as greater MPH in carotid arteries.

These findings suggest that this particular BNP SNP may function at the systemic level and influence the manifestation of atherosclerosis in both coronary and carotid arteries. Carotid plaque quantified by ultrasound serves as an intermediate pre-clinical phenotype between the BNP genetic variant and the clinical diagnosis of CAD, which further implicates the involvement of BNP in the pathophysiology of atherosclerosis.

3.2 Introduction

Atherosclerosis is a complex systemic disease with focal manifestations that lead to potentially fatal events such as myocardial infarction (MI) and stroke. Natriuretic peptides, namely atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), play critical roles in hemodynamic regulation and their relation to cardiovascular diseases has become of increasing importance lately with the advent of novel pharmaceuticals. Both ANP and BNP have been shown to be involved in the development of hypertension. Plasma BNP and N-terminal proBNP (NT-proBNP) have become established biomarkers to facilitate the diagnosis and prognosis of heart failure and have been associated with cardiovascular morbidity and mortality in the general population.¹¹³

Several common single nucleotide polymorphisms (SNPs) have been considered as functional variants in *NPPA* and *NPPB* genes that encodes for ANP and BNP, respectively. The SNP of *NPPA*, rs5065, constitutes a T-to-C nucleotide substitution in the stop codon of exon 3, leading to the extension of ANP by two additional arginines, ANP-RR.¹⁵⁸ ANP-RR functions similarly to ANP, but in vitro studies suggested that ANP-RR may lead to enhanced susceptibility to vascular damage.¹⁵⁹ There is, however, contradictory evidence in the association between rs5065 genotypes and cardiovascular outcomes. Ellis et al.¹⁴³ found a reduced rate of cardiovascular readmission among patients with the minor allele (C) of rs5065, whereas other studies have shown that the C allele was associated with increased risk of ischemic stroke¹⁶⁰ and higher prevalence of MI.^{158,161}

The SNP of *NPPB*, rs198389, is a functional variant in the gene's promoter region. The minor allele (G) is associated with a higher level of promoter activity compared to the major allele (A).¹³⁷ Numerous studies have shown that the minor allele is associated with elevated levels of NT-proBNP and/or BNP. The minor G allele has also been associated with reduced blood pressure, hypertension and cardiovascular mortality.¹⁴⁴

Due to the complexity of cardiovascular diseases, it has been suggested genetic investigations of cardiovascular endpoints are potentially confounded by heterogeneity of the disease pathogenesis. The “distance” between genotype and phenotype potentially obscures the identity of causative variants. Investigations using intermediate phenotypes linked to a disease pathway may represent a more powerful approach.¹⁶²

The carotid artery is a barometer of atherosclerosis elsewhere in the vascular tree and carotid atherosclerosis is correlated with significant angiographic coronary artery disease (CAD).¹¹⁶ Carotid intima-media thickness (CIMT), as measured by ultrasound, is a quantitative trait that strongly correlates with severity and extent of CAD and is increasingly used in clinical decision-making. Carotid plaque height and plaque area have been shown to predict stroke and coronary events even better than CIMT. The association between carotid plaque quantification and natriuretic peptide genetic variants has not been studied.

In the present study, we investigated functional variants of *NPPA* and *NPPB*, rs5065 and rs198389, respectively, in order to characterize carotid atherosclerotic plaque measured by ultrasound, as well as angiographic coronary atherosclerosis. We hypothesized that rs5065 and rs198389 genotypes may be differentially associated with the degree of atherosclerotic diseases. The additive effects of both SNPs were also examined to elucidate potential interactions between ANP and BNP.

3.3 Patients and Methods

3.3.1 Study Population

Outpatients referred for a coronary angiograph between May and October 2013 were recruited at the Kingston General Hospital (KGH) Cardiovascular Lab (Kingston, Ontario). Patients meeting the following inclusion criteria were eligible: males or females aged >18 years, non-emergency outpatients referred for a clinically indicated angiogram for one of: nonspecific

chest pain evaluation; stable or unstable angina pectoris; positive stress test; preoperative assessment; old or recent MI (>2days). Patients were excluded only if there was a contraindication for blood sample collection. All participants provided informed consent and agreed to have blood drawn and a carotid ultrasound performed. Demographic and medical data was collected from the participant's medical chart, hospital information database, and from participant interviews. The study was approved by Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (DMED-1365-11). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

3.3.2 Angiographic Score

Coronary angiography was performed according to the standard Judkins method using a GE System 2000 (GE Healthcare) by one of four experienced interventional cardiologists in Kingston General Hospital. Percentage luminal narrowing in each of the four major coronary arteries (left main, left anterior descending, circumflex and right coronary artery) was recorded. Patients were stratified into three categories: normal coronary (luminal narrowing <20% in all epicardial arteries), non-obstructive CAD (luminal narrowing ≥ 20 and <70% in at least one segment of any epicardial artery or ≥ 20 and <50% in the left main artery) and obstructive CAD (luminal narrowing ≥ 70 % in at least one segment of any epicardial artery or ≥ 50 % in the left main artery). Average luminal narrowing across all four vessels was also acquired to represent the overall coronary plaque burden.

3.3.3 Carotid Plaque Quantification

Carotid ultrasound was conducted using a GE Vivid E9 (GE Healthcare, Milwaukee, WI) vascular ultrasonography device equipped with a 9L-D transducer. All images were stored in Digital Imaging and Communications in Medicine (DICOM) format and analyzed offline using

EchoPAC V113 software (GE Vingmed Ultrasound, Norway) for plaque quantification. Carotid intima media thickness (CIMT) was measured with the auto border detection function and the mean of the right and left side was used in the analysis. Plaque height was measured manually using calipers in the carotid bulb/internal carotid artery (ICA) region and the maximum plaque height (MPH) of either side was recorded for analysis. Plaque area was traced manually and the total area of both sides was added to give a total plaque area (TPA).

3.3.4 SNP Selection

SNPs of the natriuretic peptide system were selected using the following criteria: 1) have been identified in previous studies as associated with circulating natriuretic peptide level or cardiovascular outcomes and 2) have a minor allele frequency >10%. The SNPs selected were *NPPA* rs5065 and *NPPB* rs198389.

3.3.5 DNA Extraction and Genotyping

Whole blood was collected during the angiography procedure. White blood cells were isolated from the whole blood samples and digested using cell lysis buffer and proteinase K (5-PRIME, VWR Canada). Genomic DNA was precipitated using isopropanol, isolated and rehydrated with Tris buffer. DNA samples were diluted to 5 ng/μL prior to genotyping. Genotyping was performed using TaqMan SNP genotyping assays according to the manufacturer's protocol (Applied Biosystems, Foster City, California, US) on a LightCycler[®] 480-II Real-Time PCR System (LC480-II; Roche Applied Science, Laval, Quebec, Canada).

3.3.6 Statistical Analysis

All data was analyzed using SPSS® 24 software (IBM Corp., 2016). Pearson Chi-square test was used to compare nominal variables and the Wilcoxon-Mann-Whitney 2 sample test (Rank Sums) was used for continuous variables. Pearson's correlation coefficient was used to evaluate the bivariate (unadjusted) associations of continuous variables. A backward selection criterion of $p < 0.10$ was used to select independent factors associated with atherosclerosis variables. The candidates included genotypes of SNP, carotid plaque measures: CIMT, MPH, TPA, demographics: age, sex, estimated glomerular filtration rate (eGFR), body mass index (BMI), and traditional cardiac risk factors (tobacco use, diabetes, hypertension, and dyslipidemia). Following selection, log-binomial regression models were used for nominal response variable (CAD categories) and linear least squares regression models were used for continuous response variables (average luminal narrowing and carotid plaque measures). A p-value of < 0.05 was deemed to be statistically significant.

3.4 Results

Two hundred and four participants were recruited and received a carotid ultrasound. Table 3.1 describes the demographic, atherosclerosis variables and SNP genotypes of the sample population. The mean age of participants was 65.5 years, 66% of them were male. All participants were Caucasians, with the exception of three: two Asian descendants and one African descendant. Of the 204 participants, 31% had diabetes, 39% had a $MBI \geq 30$, and the majority had hypertension (75%) and dyslipidemia (82%). One hundred of twenty-five participants (61%) were diagnosed with obstructive CAD; 32 participants (16%) were found to have normal coronary arteries. The majority of participants had various degrees of carotid atherosclerosis, while only 14 participants (7%) were free of any carotid plaque.

Both SNPs of the natriuretic peptide system, rs5065 and rs198389, were in Hardy Weinberg equilibrium. There was no association between SNP genotypes and age or sex. For each SNP, heterozygous genotype and homozygous minor allele genotype were combined in analyses, because 1) homozygous minor allele genotype was rare in the sample population, and 2) heterozygous and homozygous minor allele genotypes have been shown to have similar effects on cardiovascular outcomes.

Table 3.2 summarizes associations between cardiac risk factors and atherosclerosis measures. The presence of diabetes and dyslipidemia was associated with both coronary atherosclerosis (obstructive CAD and increased average luminal narrowing) and carotid atherosclerotic plaques (MPH and TPA). There were no significant differences in CIMT, MPH and TPA between sexes, but males had a higher degree of angiographic CAD.

Table 3.1. Demographic, atherosclerosis variables, and SNP genotypes in the sample population.

		Overall (n=204)
Demographic		
Age (years, mean±SD)		65.5±9.4
Diabetes (n, %)		63 (31%)
Hypertension (n, %)		154 (75%)
Dyslipidemia (n, %)		168 (82%)
Tobacco use (n, %)		32 (16%)
BMI (kg/m ² , mean±SD)		30.1±6.7
BMI ≥ 30 (n, %)		79 (39%)
Angiographic Category (n, %)		
Normal coronary		32 (16%)
Non-obstructive CAD		47 (23%)
Obstructive CAD		125 (61%)
Carotid ultrasound measures (mean±SD)		
Mean CIMT (mm)		0.81±0.15
Maximum plaque height (mm)		2.97±1.35
Total plaque area (mm ²)		55.7±40.6
SNP Genotypes (n, %)		
<i>NPPA</i> rs5065 (A/G)	AA	154 (76%)
	AG	45 (22%)
	GG	5 (2.5%)
<i>NPPB</i> rs198389 (A/G)	AA	65 (32%)
	AG	111 (54%)
	GG	28 (14%)

BMI: body mass index, CAD: coronary artery disease, CIMT: carotid intima media thickness.

Table 3.2. Association between cardiac risk factors and atherosclerosis measures.

	Obstructive CAD	Average Luminal Narrowing (%)	CIMT (mm)	MPH (mm)	TPA (mm ²)
Risk Factors	Relative Risk	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Sex					
Male	2.96 ^b	33.3±25.6 ^c	0.82±0.16	3.01±1.33	59.4±43.0
Female	Reference	21.8±23.5	0.79±0.14	2.88±1.39	48.5±34.8
Diabetes					
yes	2.37 ^d	39.8±25.1 ^a	0.81±0.16	3.41±0.97 ^c	71.0±43.7 _b
no	Reference	24.7±24.2	0.81±0.15	2.77±1.45	48.9±37.3
Hypertension					
yes	NS	31.1±25.4	0.82±0.15	3.07±1.29	58.0±40.4
no	Reference	24.1±24.8	0.78±0.16	2.65±1.49	48.6±40.7
Dyslipidemia					
yes	3.25 ^c	32.4±25.4 ^b	0.82±0.15 ^c	3.17±1.21 ^b	61.1±38.9 _a
no	Reference	15.9±20.6	0.75±0.16	2.01±1.54	30.7±39.7
Tobacco use					
yes	2.57 ^d	33.6±21.7	0.82±0.15	3.14±1.38	63.8±43.0
no	Reference	28.6±26.0	0.81±0.15	2.94±1.34	54.2±40.1
BMI ≥ 30					
yes	NS	31.8±26.6	0.82±0.15	2.96±1.36	59.8±43.1
no	Reference	27.9±24.6	0.81±0.15	2.97±1.34	53.1±38.9

The Pearson Chi-square test was used to compare nominal variables, and the Wilcoxon Test (Rank Sums) was used for continuous variables. ^a $p < 0.0001$, ^b $p < 0.001$, ^c $p < 0.01$, ^d $p < 0.05$. BMI: body mass index, CAD: coronary artery disease, CIMT: carotid intima media thickness, MPH: maximum plaque height, TPA: total plaque area, WC: waist circumference, NS: not significant.

CIMT was not associated with the degree of CAD evaluated by both maximum stenosis and average luminal narrowing. Both carotid MPH and TPA were associated with angiographic obstructive CAD (Table 3.3). There was a moderate linear correlation between MPH and average luminal narrowing, as well as between TPA and average luminal narrowing (Figure 3.1).

Tables 3.4 summarizes associations between natriuretic peptide SNPs and atherosclerosis measures. Although no statistically significant association was found between *NPPA* rs5065 and any atherosclerosis measures (obstructive CAD, average coronary luminal narrowing or maximum carotid plaque height), the homozygous major allele genotype (AA) was trending towards worsen atherosclerosis in both coronary arteries and carotid arteries. The association between *NPPB* rs198389 homozygous major allele genotype (AA) and obstructive CAD was not statistically significant, but this genotype was found to correlate with increased average coronary luminal narrowing and carotid MPH. When two SNPs were combined, the combination of AA for rs5065 and AA for rs198389 was associated with obstructive CAD, increased average coronary luminal narrowing and increased MPH in the carotid arteries. These associations yielded greater relative risk and beta coefficient compared to those of rs198389 alone. They remained statistically significant even after age, sex and other cardiac risk factors were adjusted.

Table 3.3. Association between carotid plaque measures and coronary atherosclerosis.

Carotid plaque measures	Obstructive CAD		Average Luminal Narrowing	
	Relative Risk	95% CI	Beta	95% CI
Maximum plaque height	1.62 ^b	[1.23-2.13]	5.86 ^a	[3.42-8.30]
Sex	3.90 ^a	[1.97-7.74]	11.71 ^b	[5.24-18.17]
Diabetes	NS		11.17 ^c	[4.33-18.00]
Tobacco use	3.03 ^d	[1.11-8.23]	NS	
Total plaque area	1.15 ^c	[1.04-1.26]	2.04 ^a	[1.23-2.85]
Sex	3.41 ^b	[1.73-6.71]	10.18 ^c	[3.68-16.65]
Diabetes	2.57 ^d	[1.13-5.83]	10.14 ^c	[3.26-17.01]
Dyslipidemia	NS		8.51 ^d	[0.24-16.79]

Selection model included age, sex, BMI, diabetes, hypertension, dyslipidemia, eGFR and tobacco use. Only contributors that remain in the model are presented. ^a $p < 0.0001$, ^b $p < 0.001$, ^c $p < 0.01$, ^d $p < 0.05$. CAD: coronary artery disease, CI: confidence interval, NS: not significant.

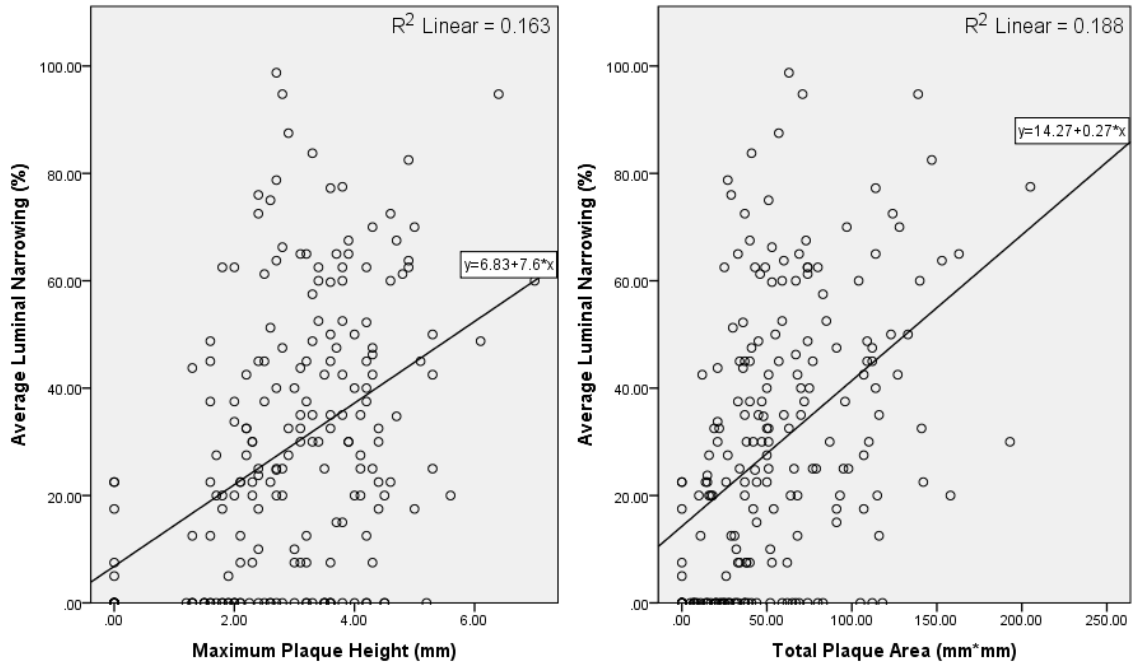


Figure 3.1. Linear correlation between carotid plaque size, as represented by maximum plaque height and total plaque area, average luminal narrowing in the coronary arteries.

$p < 0.05$ for both.

Table 3.4. Association between natriuretic peptide SNPs and atherosclerosis measures.

SNP	Genotype	Obstructive CAD	Average Luminal Narrowing	Maximum Plaque Height
		RR [95% CI]	Beta [95% CI]	Beta [95% CI]
<i>NPPA</i> rs5065	AA	1.49 [0.78-2.84]	5.23 [-2.92-13.37]	0.20 [-0.23-0.63]
	AG/GG	Reference	Reference	Reference
<i>NPPB</i> rs198389	AA	1.84 [0.98-3.46]	8.30 [0.84-15.76] ^d	0.41 [0.01-0.80] ^d
	AG/GG	Reference	Reference	Reference
Combination of two SNPs	AA + AA	2.44 [1.23-4.83] ^d	9.62 [1.90-17.35] ^d	0.49 [0.08-0.90] ^d
	Other combinations	Reference	Reference	Reference
Combination of two SNPs (adjusted for age and Sex)	AA + AA	2.55 [1.25-5.21] ^d	9.22 [1.78-16.67] ^d	0.47 [0.08-0.87] ^d
	Other combinations	Reference	Reference	Reference

^d $p < 0.05$. SNP: single nucleotide polymorphism, CAD: coronary artery disease, CI: confidence interval.

3.5 Discussion

In the present study, we confirmed the connection between carotid plaque measures and coronary atherosclerosis. Similar to findings from previous studies,¹⁶³ MPH and TPA were strongly associated with having obstructive CAD, which was angiographically determined by any coronary vessel having $\geq 70\%$ stenosis ($\geq 50\%$ in the left main coronary artery). While clinically important, evaluating CAD by the maximum stenosis in any single vessel may mask multi-vessel disease and miss subclinical dispersed atherosclerosis. For this reason, in addition to obstructive CAD, the average luminal narrowing across all four main coronary arteries as a dependent variable to evaluate the extent of coronary atherosclerosis was included. Average luminal narrowing, a continuous variable, provided an opportunity to conduct linear regression, which is statistically more powerful than binomial logistic regression restricted by categorical dependent variable. It was found that a positive correlation between both MPH and TPA, and average coronary luminal narrowing.

Contrary to previous findings,^{163,164} there was no statistically significant association between CIMT and coronary atherosclerosis parameters in the present population. This is perhaps because the present population was of a higher risk for developing CAD as 61% were diagnosed with having obstructive CAD. Another consideration is that the standard to evaluate CAD varies between studies. Patients with any coronary vessel with $\geq 50\%$ stenosis was considered as having CAD, whereas in the present study, the more severe, obstructive CAD, which was represented by $\geq 70\%$ stenosis were considered. Nevertheless, the present study confirmed that MPH and TPA were more robust at predicting CAD compared to CIMT.

In a previous study, 50,000 genetic variants from 2,100 genes were investigated for association with subclinical atherosclerosis measured by CIMT.¹⁶⁵ A single SNP related to natriuretic peptide, rs10082235 of NPR1 (coding for a receptor for ANP and BNP), was associated with CIMT.¹⁶⁵ None of the SNPs of *NPPA* or *NPPB* was examined with respect to

CIMT.¹⁶⁵ In the present study, the lack of association between either *NPPA* rs5065 or *NPPB* rs198389 and CIMT was confirmed. However, it was found that rs198389 AA genotype was associated with both increased MPH and increased TPA, compared to AG/GG genotypes. To our knowledge, this is the first study to report a genetic variant associated with carotid plaque measures. Additionally, rs198389 AA genotype was associated with coronary atherosclerosis represented as increased average luminal narrowing. This association was found previously in a female cohort of the same population.¹⁶⁶ Findings of the current study show that this particular *NPPB* variant is associated with systemic atherosclerosis manifested in both coronary and carotid vasculature. This information adds to the existing knowledge on *NPPB* functional variants, indicating higher levels of NT-proBNP and/or BNP produced by the G allele of rs198389 may protect against the development of atherosclerosis systemically.

In the present study, the additive effect of two SNPs on atherosclerosis measures was also examined. The combination of AA genotype for rs5065 and AA genotype for rs198389 was associated with obstructive CAD, increased average coronary luminal narrowing and carotid MPH. These associations were stronger than those of either rs5065 or rs198389 alone, indicated by the smaller *p* value and greater r-square value. It shows that these genetic variants may function in tandem to elicit greater physiological effects that can promote or protect against atherosclerosis. Previous studies have shown that the minor G allele of rs5065 was associated with higher plasma BNP levels, even though the exact mechanism was unknown.¹⁵⁸ Since G allele of rs198389 also produces higher levels of BNP, it can be hypothesized that the combination of AA and AA genotypes for both SNPs may result in lower levels of BNP than the effect of either SNP functioning alone, leaving patients especially vulnerable to atherosclerosis development.

Limitations

The main limitation of the present study was the small sample size. We found association between rs198389 genotypes and coronary atherosclerosis in women but not in men among a larger cohort of the same population.¹⁶⁶ The present sample size was not sufficient to conduct any sex-specific analyses. Another limitation of the study is the lack of information on plasma BNP levels, which would allow us to determine if the effect of natriuretic peptide variants was mediated via circulating BNP.

3.6 Conclusion

The extent and severity of coronary atherosclerosis correlated with carotid MPH and TPA, but not with CIMT. The *NPPB* rs198389 AA genotype is associated with increased average coronary luminal narrowing, carotid MPH and TPA. The combination of AA and AA genotypes for both *NPPA* rs5065 and *NPPB* rs198389 was associated with a greater quantity of plaques in coronary arteries as well as carotid arteries, suggesting these two functional variants may have additive effect on the pathogenesis of atherosclerosis. A larger sample size with plasma BNP analysis will be required to verify the physiological pathways between these genotypes and atherosclerosis.

Chapter 4

Single Nucleotide Polymorphism of B-Type Natriuretic Peptide as a Predictor for Increased Risk of Cardiovascular Events

Results in this chapter is currently being prepared for publication: Li TY, Tse MY, Pang SC, Héту MF, McLellan CS, King WD, Johri AM. Single Nucleotide Polymorphism of B-Type Natriuretic Peptide Predicts Increased Risk of Cardiovascular Events. To be submitted April, 2019.

4.1 Abstract

B-type natriuretic peptide (BNP) plays a critical role in lowering blood volume and pressure, whose circulating levels have been established as a biomarker for heart failure and cardiovascular (CV) outcomes. Single nucleotide polymorphism (SNP) of the BNP gene, rs198389, was associated with elevated BNP circulating levels, as well as reduced hypertension and lower CV mortality, but CV events such as myocardial infarction (MI) were never explored. The present study was designed to investigate the association between rs198389 and CV events, especially premature events and recurrent events.

Two-hundred and four patients who underwent coronary angiography were genotyped for *NPPB* rs198389. Medical records were reviewed in retrospective from birth to the recruitment, as well as in prospective during the follow-up period (median of 4.8 years). Major adverse cardiovascular events (MACE) recorded include MI or acute coronary syndrome, stroke, undergoing coronary artery bypass grafting or percutaneous coronary intervention, and cardiac death.

Patients with homozygous major allele genotype (AA), compared to minor allele carriers (AG and GG), were found to be more likely to experience MACE in the life-time and before the age of 65. They also had higher risk of developing recurrent MACE during the follow-up period. In multivariate adjusted models, the effect of rs198389 genotype was greater than traditional risk factors.

These findings demonstrate the protective effect of the rs198389 G allele against MACE, particularly premature events, which correlate with a longer period of medical treatment and greater risk of complications. The rs198389 variant is a significant predictor for recurrent MACE, which can potentially be targeted in secondary prevention among patients with known cardiovascular disease.

4.2 Introduction

Ischemic heart disease is the leading cause of the death in the world and it is primarily caused by atherosclerosis in the coronary arteries, also known as coronary artery disease (CAD).¹ The traditional diagnostic evaluation, prevention and treatment strategies mainly target the top five cardiovascular (CV) risk factors: hypertension, diabetes mellitus, dyslipidemia, obesity and smoking. Estimation of the risk of CV events based only on the presence of traditional risk factors is commonly insufficient.^{55,167,168} Approximately 50% of patients admitted to a hospital with acute coronary syndrome (ACS) or myocardial infarction (MI) do not possess these traditional risk factors.^{169,170} Additional CAD biomarkers and predictors of CV events are in demand more than ever.

B-type natriuretic peptide (BNP), a member of the natriuretic peptide (NP) family, plays a critical role in the regulation of circulatory volume, plasma renin-angiotensin-aldosterone levels, natriuresis, and ultimately the maintenance of blood pressure (BP).¹⁷¹ Elevated circulating levels of BNP are able to counter-balance the negative effects of intravascular volume expansion by stimulating natriuresis and diuresis as well as promoting peripheral vasodilation. Plasma BNP and N-terminal prohormone of BNP (NT-proBNP) have become established biomarkers to facilitate the diagnosis and prognosis of heart failure (HF), and have been associated with CV mortality as well as all-cause mortality in the general population.¹¹³

A single nucleotide polymorphism (SNP) in the *NPPB* gene that encodes for BNP, rs198389 has been established as a functional variant in the promoter region of this gene. The minor allele G was previously shown in cultured cells to be associated with a 1.8-fold higher level of promoter activity compared to the major allele A.¹³⁷ Numerous human population studies have demonstrated that the G allele of rs198389 is associated with elevated levels of NT-proBNP and/or BNP.¹³⁷⁻¹⁴⁴ The same minor allele has been associated with a lower risk of developing type II diabetes mellitus,^{137,145,146} reduced systolic BP,¹⁴¹ decreased risk of ventricular dysfunction

following coronary artery bypass grafting (CABG),¹⁴⁷ reduced hospital readmissions, and less left ventricular diastolic dysfunction in patients after MI.¹⁴³ In a recent study, Seidelmann et al.¹⁴⁴ not only confirmed the association between rs198389 G allele and elevated levels of NT-pro-BNP and reduced hypertension, but also showed that the minor allele is associated with lower CV mortality and increased lifespan. However, previous genetic association studies have not expanded to all major adverse cardiovascular events (MACE), including MI, ACS, stroke, undergoing CABG or percutaneous coronary intervention (PCI) and cardiac death.

In the present prospective cohort study, we investigated the association between the *NPPB* functional variant rs198389 and MACE, particularly premature events and recurrent events in a population undergoing elective angiography. We hypothesized that rs198389 genotypes may be associated with different risks of developing CV events.

4.3 Patients and Methods

4.3.1 Study Population

The study was approved by Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (DMED-1365-11). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All participants provided informed consent to participate in the study.

Two-hundred and four outpatients referred for a coronary angiograph at the Kingston General Hospital (KGH) Cardiovascular Lab (Kingston, Ontario, Canada) participated in this study and agreed to have blood drawn and medical records reviewed. Patients meeting the following inclusion criteria were eligible: males or females aged >18 years, non-emergency outpatients referred for a clinically indicated angiogram for one of: nonspecific chest pain evaluation; stable or unstable angina pectoris; positive stress test; preoperative assessment; old or recent MI (>2 days). Patients were excluded if there was a contra-indication for blood sample

collection. Participants were initially recruited between May and October 2013 and were interviewed by phone or in person in 2014. A final medical record review was conducted in June 2018.

4.3.2 Identification of Cardiovascular Outcomes

Outcomes evaluated in this study were major adverse cardiovascular events (MACE) from birth to the recruitment, as well as during the follow-up period between May 2013 and June 2018. We defined MACE as one of the following: clinically confirmed myocardial infarction (MI) or acute coronary syndrome (ACS), stroke, undergoing coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI), and cardiac death. The date and nature of each event was extracted from the hospital information database.

4.3.3 SNP Selection

SNPs of the natriuretic peptide system were selected using the following criteria: 1) have been identified in previous studies as associated with circulating natriuretic peptide level or CV outcomes and 2) have a minor allele frequency >10%. The SNPs selected were *NPPA* rs5065 and *NPPB* rs198389.

4.3.4 DNA Extraction and Genotyping

Whole blood was collected during the angiography procedure. White blood cells were isolated from the whole blood samples and digested using cell lysis buffer and proteinase K (5-PRIME, VWR International, Mississauga, Ontario, Canada). Genomic DNA was precipitated using isopropanol, isolated and rehydrated with Tris buffer. DNA samples were diluted to 5 ng/μL prior to genotyping. Genotyping was performed using TaqMan SNP genotyping assays according to the manufacturer's protocol (Applied Biosystems, Foster City, California, USA) on

a LightCycler[®] 480-II Real-Time PCR System (LC480-II; Roche Applied Science, Laval, Quebec, Canada).

4.3.5 Statistical Analysis

All data was analyzed using SPSS[®] 24 software (IBM Corp., 2016). Baseline characteristics are summarized using descriptive statistics for continuous variables, and number counts and percentages for categorical variables. Log-binomial regression was used to test for a relationship between risk factor variables and rs198389 genotype. Risk factor variables included age, sex, estimated glomerular filtration rate (eGFR), body mass index (BMI), tobacco use, diabetes, hypertension, and dyslipidemia. Three models investigating the association between rs198389 genotype and MACE were created: 1) the unadjusted model only included the genotype; 2) intermediate model adjusted for age and sex; and 3) the fully adjusted model accounted for age, sex and all the other risk factor variables. Life-time MACE, MACE before age of 65 and PCI or CABG at recruitment were associated with rs198389 genotype using log-binomial regression models, where relative risks (RR) were calculated. Hazard ratios (HRs) for MACE during the follow-up period were calculated using Cox proportional hazards regression. The Kaplan-Meier hazard curve was calculated for MACE during the follow-up period. A p-value of <0.05 was deemed to be statistically significant.

4.4 Results

Tables 4.1 describes the demographic, atherosclerosis variables and SNP genotypes of the sample population. The mean age of participants was 65.5 years, 66% of them were male. The majority of participants were Caucasians, with two of Asian descent and one of African descent. Of the 204 participants, 31% had diabetes, 39% had a BMI ≥ 30 kg/m², and the majority had hypertension (75%) and dyslipidemia (82%). There was a total of 253 life-time MACEs among

136 participants, of whom 79 experienced their first MACE before the age of 65. Eighty-eight participants (43%) had either received PCI during the angiography at recruitment or undergone CABG within 30 days of the angiography. After a median of 4.8 years of follow-up, 38 participants (19%) had experienced one or more MACE.

Table 4.2 summarizes associations between CV risk factors and MACE. Male sex, advanced age, dyslipidemia and tobacco use were associated with the presence of one or more life-time MACEs. The male sex and diabetes were associated with PCI or CABG at the time of recruitment. Among all risk factors, only eGFR<60 was associated with developing MACE during the five-year follow-up period.

Table 4.1. Demographic, atherosclerosis variables, and SNP genotypes in the sample population.

	Overall (n=204)
Demographic	
Men (n, %)	134 (66%)
Age (years, mean±SD)	65.5±9.4
Diabetes (n, %)	63 (31%)
Hypertension (n, %)	154 (75%)
Dyslipidemia (n, %)	168 (82%)
Tobacco use (n, %)	32 (16%)
BMI (kg/m ² , mean±SD)	30.1±6.7
eGFR (mL/min/1.73m ² , mean±SD)	82.1±23.2
Major Adverse Cardiac Event (MACE)	
Life-time MACE (n, %)	136 (67%)
Number of MACE in life-time (mean, CI)	1.2 (1.1-1.4)
MACE before age 65 (n, %)	79 (39%)
PCI or CABG at recruitment (n, %)	88 (43%)
MACE during five-year follow-up (n, %)	38 (19%)
<i>NPPB</i> rs198389 Genotypes (n, %)	
AA	65 (32%)
AG	111 (54%)
GG	28 (14%)

SNP: single nucleotide polymorphism, BMI: body mass index, PCI: percutaneous intervention, CABG: coronary artery bypass graft.

Table 4.2. Association between cardiac risk factors and presence of MACE.

Risk Factors	Life-time MACE RR (95% CI)	PCI/CABG at Recruitment RR (95% CI)	MACE during five- year follow-up HR (95% CI)
Men	2.52 (1.37-4.63)**	2.12 (1.15-3.89)*	2.03 (0.93-4.44)
Age (divided by tertiles)			
≤ 62	Reference	Reference	Reference
> 62 and ≤ 70	2.51 (1.24-5.09)*	1.61 (0.82-3.15)	1.05 (0.45-2.48)
>70	2.75 (1.32-5.75)**	1.21 (0.60-2.42)	1.79 (0.81-3.95)
Diabetes	1.37 (0.72-2.62)	1.89 (1.04-3.44)*	1.24 (0.63-2.44)
Hypertension	1.65 (0.85-3.18)	1.48 (0.77-2.86)	0.89 (0.43-1.84)
Dyslipidemia	2.92 (1.41-6.04)**	1.31 (0.63-2.72)	1.15 (0.48-2.75)
Tobacco use	3.12 (1.14-8.51)*	1.61 (0.76-3.44)	0.64 (0.23-1.80)
BMI ≥ 30 kg/m ²	0.65 (0.36-1.18)	0.77 (0.44-1.37)	1.07 (0.55-2.06)
eGFR < 60	1.61 (0.68-3.80)	0.89 (0.41-1.91)	2.36 (1.17-4.78)*

The Pearson Chi-square test was used to compare nominal variables. * $p < 0.05$, ** $p < 0.01$.

MACE: major adverse cardiac event, BMI: body mass index, eGFR: estimated glomerular filtration rate, PCI: percutaneous intervention, CABG: coronary artery bypass graft, RR: relative risk, HR: hazard ratio, CI: confidence interval.

The BNP functional variant rs198389 was in Hardy-Weinberg equilibrium in the study population. There was no association between *NPPB* rs198389 and age, sex or any other traditional risk factors. Heterozygous (AG) and homozygous minor allele (GG) genotypes of rs198389 were combined in analyses, because 1) the homozygous minor allele genotype was rare in the sample population, and 2) heterozygous and homozygous minor allele genotypes have been shown to have similar effects on CV outcomes.

Table 4.3 summarizes associations between *NPPB* rs198389 genotypes and MACE. The homozygous major allele (AA) genotype was found to be associated with a higher risk of having at least one life-time MACE versus AG and GG genotypes in the unadjusted model (RR, 3.41; $P=0.001$) as well as in the multivariate adjusted models (RR, 3.74; $P=0.001$). Patients with the AA genotype were more likely to experience their first MACE before the age of 65 compared to AG and GG genotypes in both unadjusted and multivariate adjusted models (RR, 1.90; $P=0.04$ and RR, 2.24; $P=0.03$, respectively). The AA genotype was also associated with a higher risk of receiving a PCI or CABG at the time of recruitment as demonstrated in the unadjusted model (RR, 1.89; $P=0.04$) and the age-sex adjusted model (RR, 1.99; $P=0.03$), but not in the fully adjusted model ($P=0.13$).

During the follow-up period, inheritance of the AA genotype incurred increased risk of experiencing at least one MACE compared with the AG and GG genotypes (HR, 2.55; $P=0.004$), as shown by the Kaplan-Meier hazard curve (Figure 4.1). This association remains statistically significant after adjusting for age and sex (HR, 2.61; $P=0.004$) as well all risk factors (HR, 2.92; $P=0.002$).

Table 4.3. Association between *NPPB* rs198389 genotype and MACE.

rs198389 Genotype	AG/GG	AA	<i>P</i> -value
Life-time MACE			
Unadjusted, RR (95% CI)	Reference	3.41 (1.64-7.09)	0.001
Age and sex, RR (95% CI)	Reference	3.90 (1.80-8.42)	0.001
All risk factors, RR (95% CI)	Reference	3.74 (1.66-8.44)	0.001
MACE before 65			
Unadjusted, RR (95% CI)	Reference	1.90 (1.04-3.46)	0.04
Age and sex, RR (95% CI)	Reference	2.28 (1.19-4.38)	0.01
All risk factors, RR (95% CI)	Reference	2.24 (1.10-4.57)	0.03
PCI/CABG at recruitment			
Unadjusted, RR (95% CI)	Reference	1.89 (1.04-3.44)	0.04
Age and sex, RR (95% CI)	Reference	1.99 (1.08-3.66)	0.03
All risk factors, RR (95% CI)	Reference	1.65 (0.87-3.13)	0.13
MACE during follow-up			
Unadjusted, HR (95% CI)	Reference	2.55 (1.34-4.86)	0.004
Age and sex, HR (95% CI)	Reference	2.61 (1.37-4.98)	0.004
All risk factors, HR (95% CI)	Reference	2.92 (1.48-5.74)	0.002

MACE: major adverse cardiovascular event, RR: relative risk, disease, HR: hazard ratio, CI: confidence interval, PCI: percutaneous intervention, CABG: coronary artery bypass graft.

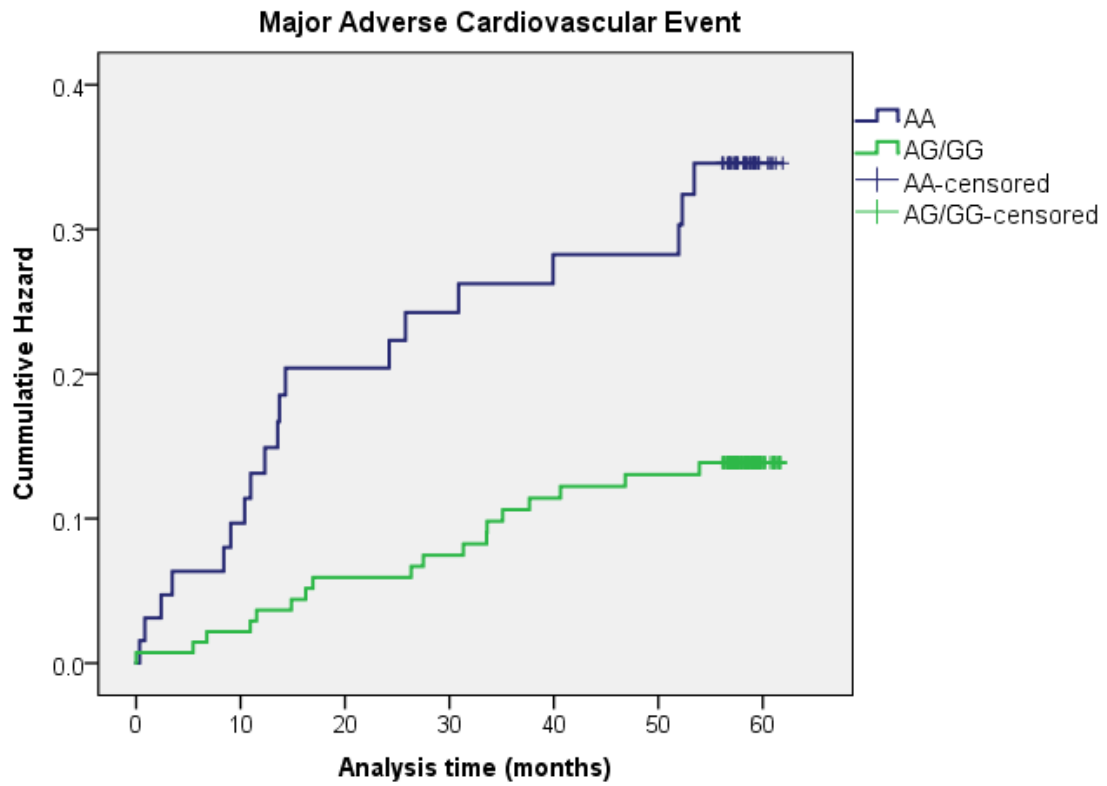


Figure 4.1. Kaplan-Meier hazard curve for major adverse cardiovascular events during five-year follow-up stratified by rs198389 genotype (N=204). $P < 0.01$.

4.5 Discussion

In the present study, we showed an association between the *NPPB* promotor functional variant rs198389 and major adverse cardiovascular events including: MI, ACS, stroke, and cardiac death. Patients with the AA genotype were 3.7 times more likely to have at least one event in their life-time and 2.2 times more likely to have their first event before the age of 65, compared with AG and GG genotypes. Our data, therefore, indicates that the G allele of this particular SNP has protective effects against cardiovascular events. These results are in accordance with findings from a much larger population where the G allele was associated with reduced CV mortality.¹⁴⁴ Unlike the previous study, however, we included all major events rather than just death. The timing of a participant's first event was of particular focus. Premature events before the age of 65 usually result in early diagnoses of CV disease, which may correlate with a longer period of medical treatment, higher risk of complications, and increased CV morbidity.

In the prospective portion of the study, we followed the participants for nearly five years and found a significant difference in the rate of events between *NPPB* rs198389 genotypes. Patients with AA genotype at any time point during the follow-up were 2.9 times more likely to experience a MACE than G allele carriers (AG and GG). The Kaplan-Meier hazard curve shows that approximately 35% of patients with AA genotype experienced at least one event after five years, whereas only 13% of G allele carriers experienced only one event. Because 84% of participants had been diagnosed with CAD by the time of recruitment, the majority of MACE during follow-up was considered as recurrent events. In a previous study where patients were followed for 2.8 years post-MI, the G allele of rs198389 was associated with a reduction in hospital readmission due to unstable angina, MI, stroke and heart failure.¹⁴³ The present findings significantly corroborate existing evidence of cardioprotective effects of the rs198389 G allele. The exact mechanism behind this important finding remains unclear.

The rs198389 polymorphism is a functional variant as it is located in a regulatory region of the expression of BNP. The G allele disrupts a putative binding site for the Atp1a1 regulatory element binding factor 6 (AREB6) transcription factor and yields a higher promoter activity than the A allele.¹³⁷ It has been confirmed by numerous population studies that the G allele of rs198389 is associated with elevated blood levels of NT-proBNP and BNP.¹³⁷⁻¹⁴⁴ In the circumstance of cardiac dysfunction, secondary to an insult such as MI, circulating BNP levels increase several-fold to compensate for increased preload and afterload, decreased cardiac output, and activation of the sympathetic nervous system and renin-angiotensin-aldosterone system. In this sense, an elevation in BNP is a well-established clinical marker of hemodynamic stress. A plethora of studies have demonstrated that higher circulating levels of both BNP and NT-proBNP are predictive of recurrent adverse events among patients with heart failure,¹⁷² ACS,^{173,174} and stable CAD.¹⁷⁵⁻¹⁸⁰ Additionally, elevated BNP and NT-proBNP levels are strong predictors for first CV events in asymptomatic populations.¹⁸¹⁻¹⁸³

On the other hand, a growing body of evidence from animal studies and human population studies is demonstrating that elevated basal levels of BNP may play a beneficial role. Transgenic mice overexpressing BNP are hypotensive in comparison to wild-type littermates.¹⁸⁴ In a large prospective cohort study, Seidelmann et al.¹⁴⁴ showed that the G allele of *NPPB* rs198389 conferred higher NT-proBNP levels, reduced BP and hypertension, and lower CV mortality. The release of BNP in response to elevated BP is a normal physiological response to restore fluid balance and hemodynamic homeostasis. In this sense, genetically augmented BNP could potentially be protective as the system is able to respond distinctively to stressful stimuli.

Whether an elevation in BNP is compensatory or genetically determined, previous evidence suggests that the protective effect revolves around BNP's role in hemodynamic regulation. However, the current study did not detect any correlation between rs198389 genotype and BP or hypertension. It is likely because the majority of the current population is hypertensive

(75%) thus the statistical power was insufficient to detect the association. Nonetheless, physiological mechanisms other than hemodynamic regulation may be at play. *In vitro* studies have demonstrated the anti-inflammatory properties of natriuretic peptides. In particular, atrial natriuretic peptide (ANP) directly reduces the production of tumor necrosis factor- α (TNF- α) by macrophages and interferes with the expression of endothelium adhesion molecules such as E-selectin and ICAM-1.¹⁷¹ These molecules are essential for inflammatory processes involved in the development of atherosclerotic plaque, which is the culprit of CAD and ischemic cerebral vascular disease.²⁵ Although the anti-inflammatory effect of BNP has yet to be elucidated, functional variants of *NPPB* gene have been shown to be associated with elevated ANP levels due to close proximity with the *NPPA* gene (code for ANP) on the distal short arm of human chromosome 1.¹⁴⁴ It is possible that the rs198389 polymorphism may protect against atherosclerosis through the anti-inflammatory effect of either BNP directly or upregulation of ANP.

The current data demonstrates that rs198389 genotype is a strong predictor for major adverse cardiovascular events, specifically premature first events and recurrent events. In the multivariate adjusted models, the effect of this genotype is greater than all traditional risk factors, including sex, age, diabetes, and dyslipidemia. Although commonly used, these traditional risk factors are inadequate in the estimation of CV risk, limiting our ability to reduce the prevalence and severity of CV diseases. Our study supports the utility of *NPPB* rs198389 polymorphism as a predictive genetic marker, which could be especially targeted in secondary prevention among patients with known CAD.

Study Limitations

Firstly, the current cohort size was relatively small and insufficient for sex-specific analyses. We have shown previously in the same population, rs198389 is associated with the severity of CAD based on angiographic data in women but not in men.¹⁶⁶ Second, the follow-up

period was relatively short, hence the cumulative hazard rate did not reach 50% of the cohort. Nevertheless, the short period it took to differentiate risk between genotypes did highlight the potential predictive power of the SNP. Finally, we did not measure circulating levels of BNP or ANP in order to show the direct phenotypic effect of the rs198389 functional variant and link it to MACE. The association of the SNP with MACE eludes to the potential participation of inflammatory pathways. Therefore, inflammatory markers should be investigated in conjunction with BNP SNP in future studies with larger sample sizes and longer-term outcomes.

4.6 Conclusion

The rs198389 AA genotype is associated with greater chance of premature major adverse cardiovascular events before the age of 65, and increased risk of developing recurrent events in the current cohort.

Chapter 5

General Discussion

5.1 Major Findings of the Current Studies

The continuing epidemic of atherosclerotic cardiovascular disease demands a more thorough understanding of the atherogenesis process and the discovery of novel biomarkers to better detect and stratify coronary artery disease (CAD). As shown by archeological and epidemiological evidence, genetic factors play a crucial role in the development of atherosclerotic diseases. Previous studies, including large GWAS, have extensively shown the association between a multitude of single nucleotide polymorphisms (SNPs) and the presence of CAD. However, SNPs are generally absent from clinical practice due to a lack of understanding of their physiological involvement in the manifestation of atherosclerosis. The studies in this dissertation aimed to investigate the role of SNPs of vasoactive systems in three stages of pathological presentation: pre-clinical phenotype, clinical diagnosis, and adverse outcomes. The following is a brief summary of the studies and principle findings.

1. In the first part (Chapter 2), the AA genotype of *NPPB* rs198389 was found to be associated with significant coronary atherosclerosis in women, but not in men. The association stands true for both increased average luminal narrowing and severe CAD category, as determined by coronary angiography.
2. In the second part (Chapter 3), the correlation between carotid plaque size measured by ultrasound and coronary atherosclerosis evaluated by angiography was confirmed. The AA genotype of *NPPB* rs198389 was found to be associated with increased plaque height in carotid arteries.
3. In the third part (Chapter 4), the AA genotype of *NPPB* rs198389 was found to be associated with higher risk of experiencing major adverse cardiovascular events (MACE)

in the life-time and before the age of 65, as well as recurrent MACE during the five-year follow-up period.

5.2 Association between SNP genotypes and clinically diagnosed CAD

The natriuretic peptide system plays an important role in hemodynamic regulation. It consists of three hormones, ANP, BNP and CNP, encoded by genes *NPPA*, *NPPB* and *NPPC*, respectively. They exert vasodilating, natriuretic and diuretic effects by bind to high-affinity surface receptors expressed by numerous cell types. Natriuretic peptide receptors A and B mediate the majority of the cardiovascular effects of NPs, while receptor C is responsible for clearance of these peptides from the circulation. The receptors are encoded by genes *NPR1*, *NPR2* and *NPR3*, respectively.

Circulating levels of ANP and BNP are both elevated in patients with ventricular hypertrophy and congestive heart failure. Levels of BNP and NT-pro-BNP have consistently been found to be independent risk markers for both morbidity and mortality in post MI and heart failure patients. Numerous SNPs of the NP system have previously been found to be associated with cardiovascular outcomes, including *NPPA* rs5065, *NPPB* rs198389, and *NPR2* rs10758325. However, the association between these SNPs and angiographic results has never been examined, even though coronary angiography is the clinical standard for the diagnosis of CAD. Additionally, sex-specific analysis on these SNPs has not been previous reported.

To evaluate the presence and extend of coronary atherosclerosis, we utilized angiographic results in two ways. First, a nominal variable was established based on the coronary artery that contains the most severe lesion. The variable contains three categories: normal coronary (luminal narrowing <20% in all epicardial arteries), non-obstructive CAD (luminal narrowing \geq 20 and <70% in any epicardial artery or \geq 20% and <50% in the left main artery), and obstructive CAD (luminal narrowing \geq 70% in any epicardial artery or \geq 50% in the left main artery). Previously

published works by our group have included this CAD category variable and others have used the same or similar stratification schemes. It discerns individuals with severe coronary lesions that most likely require percutaneous intervention (PCI) or coronary artery bypass graft (CABG), but masks moderate lesions that are not significant for intervention, yet contributes to the overall atherosclerotic burden. In effort to supplement the CAD category variable, a second continuous variable was created by averaging luminal narrowing across all four epicardial arteries. This variable is less established in the literature and it diffuses significant single-vessel disease, which makes it less clinically relevant. However, it is a good representation of the overall coronary atherosclerotic burden, especially in the case of multiple-vessel diseases. Additionally, continuous response variables are generally more sensitive to the effect of predictive factors (SNP genotype in this case) compared to nominal response variables. By including both CAD category and average luminal narrowing in the same study, we expected to elucidate the effect of NP SNP genotype with respective emphases of both variables.

We found that the average luminal narrowing was significantly greater in individuals with the AA genotype for *NPPB* rs198389 than in minor allele carriers (AG or GG genotype), despite a lack of association between rs198389 genotype and CAD category. It suggests that while having two copies of the A allele may aid in developing a greater overall coronary atherosclerotic burden, its effect may not be sufficient to produce clinically significant lesions that require intervention. On the other hand, the discrepancy could be due to the fact that the continuous variable of average luminal narrowing has greater statistical sensitivity than that of the CAD category variable. A separate, larger sample is needed to confirm these results and validate the average luminal narrowing variable.

5.3 Sex-specific effects of the BNP SNP on the diagnosis of CAD

The most important novel finding of the first study is the sex-specific effect of the BNP functional variant on coronary atherosclerosis. Among women, the AG and GG genotypes for rs198389 was associated with lower risk of obstructive CAD as well as less overall coronary atherosclerotic burden compared to the AA genotype, whereas no association was found in men. It suggests that the protective role of the G allele is augmented in the female physiology. It is possible that the development of coronary atherosclerosis involves different pathways in women as compared to men. A study in mice demonstrated that in response to hemodynamic stress, males relied on ANP while nitric oxide (NO) expression was more dominant in females.¹⁸⁵ Although there was no difference in BNP expression, it nonetheless demonstrated that mechanisms of cardiovascular physiology and pathophysiology were not identical between sexes.

Differences in CVD morbidity and mortality between sexes have been well recognized in addition to the alarming increase in CVD incidence in women.¹⁸⁶ The presentation and prognosis of CVD follow distinct patterns in the female population. Compared to men, women were found to have a lower prevalence of obstructive CAD, but those women who were diagnosed with obstructive CAD had higher rates of adverse outcomes than men.^{148–150} Moreover, women are at greater risk of non-obstructive CAD, in the presence of which, microvascular dysfunction, endothelial dysfunction and other processes that lead to poor prognosis appear more frequent in women than in men.¹⁵¹ However, the female sex is under-represented in both animal and human studies.^{187,188} The use of male-only animal studies has traditionally dominated the scientific literature, and as a result, the knowledge used for the development of therapeutics for treatment of the population has been largely tailored to the male gender. The dosage of therapeutics is then weight-adjusted for use in the female population. This approach inherently overlooks the influence of ovarian hormones on underlying mechanisms of disease development and vasoactive regulation in the female segment of the population.¹⁸⁹ Failure to account for the presence of and

the cyclical changes in female sex hormones limits the scope of our understanding of development of CVD.¹⁹⁰ Fortunately, sex-specific differences in disease prevalence and progression have gained considerable attention in the recent decade. A special effort has been put on improving diagnosis and treatment of CAD in women. The current findings elucidate a genetic marker that can be particularly useful in clinical decision making for CAD in the female population.

5.4 Carotid atherosclerosis evaluated by ultrasound as the pre-clinical phenotype

Due to the complexity of cardiovascular diseases, it has been suggested that genetic investigations of cardiovascular endpoints are potentially confounded by heterogeneity of disease pathogenesis. The “distance” between genotype and clinical phenotype potentially obscures the identity of causative variants. Investigations using intermediate phenotypes linked to a disease pathway may represent a more powerful approach.¹⁶² Carotid atherosclerosis is an ideal candidate to serve as such a pre-clinical phenotype for CAD.

Because atherosclerosis is a systemic disease, the carotid artery is a barometer of the entire vascular tree. Carotid atherosclerosis signals significant atherosclerotic lesions in the coronary arteries.¹¹⁶ Due to its advantage of being safe, noninvasive and inexpensive, carotid ultrasound has been established as an important tool in diagnosis and prognosis of CAD.³⁸ Two parameters are commonly evaluated by two-dimensional carotid ultrasound: carotid intima-media thickness (CIMT) and carotid plaque quantification as represented by maximal plaque height (MPH) or total plaque area (TPA). Both CIMT and carotid plaque have been shown to correlate with the severity of angiographic CAD and predict CVD events.¹¹⁶

Research on the effect of SNPs on carotid atherosclerosis is sparse; very few significant results have been published thus far. In a multi-ethnic population study, Lanktree et al.¹⁶⁵ investigated 50,000 genetic variants from 2,100 genes for association with subclinical atherosclerosis measured by CIMT. A single SNP related to natriuretic peptide, *NPR1*

rs10082235 (natriuretic peptide receptor A), was associated with CIMT variation. None of the SNPs of *NPPA* or *NPPB* was discovered with respect to CIMT. Carotid plaque was not examined in this study.

In the second study of this dissertation (Chapter 3), we set out to explore the association between genetic variants of natriuretic peptides and carotid atherosclerosis in terms of both CIMT and carotid plaque. *NPPB* rs198389 was significantly associated with carotid plaque height and area, but not with CIMT. This is likely because carotid plaque quantification is a superior representation of atherosclerosis compared to CIMT. CIMT is comprised of tunica intima where early atherosclerosis develops and the tunica media which is subject to non-atherosclerotic medial hypertrophy, commonly induced by aging and hypertension. Since tunica media is the larger portion, CIMT is readily confounded by non-atherosclerotic phenomenon, therefore it has not been shown to add to CVD risk prediction. Carotid plaque, on the other hand, represents the atherosclerotic process itself that starts in the intimal layer and has thus been shown to predict CVD events far better than CIMT.¹¹⁷

The current study found that minor allele carriers of rs198389 (genotypes AG and GG) had smaller MPH and TPA, compared to individuals with the AA genotype. Additionally, the G allele of rs198389 was associated with reduced average luminal narrowing in the coronary arteries, as the same case as in the overall cohort in the first study. These results suggest that the effects of this particular *NPPB* SNP function at the systemic level, protecting against the manifestation of atherosclerosis in both coronary arteries and carotid arteries. Carotid plaque quantified by ultrasound serves as an intermediate pre-clinical phenotype between the BNP genetic variant and the clinical diagnosis of CAD, which further implicates the involvement of BNP in the pathophysiology of atherosclerosis.

5.5 The capability of SNPs in predicting major adverse cardiovascular events

The final stage of manifestation of atherosclerosis is through the occurrence of major adverse cardiovascular events (MACE), such as MI, the lead cause of mortality in the world, and acute coronary syndrome (ACS), which burdens one's life with disability, medications and surgical interventions (morbidity). The traditional diagnostic evaluation, prevention, and treatment strategies mainly target the top five cardiovascular (CV) risk factors: hypertension, diabetes mellitus, dyslipidemia, obesity and smoking. Estimation of the risk of CV events based solely on the presence of traditional risk factors is commonly insufficient, resulting in a "CAD gap".^{55,167,168} Approximately 50% of patients admitted to a hospital with ACS or MI have normal levels of these traditional risk factors.^{169,170} Incorporation of genetic markers into prediction and diagnosis may help bridge this gap.

The minor G allele of rs198389 has previously been associated with a number of benefits related to hemodynamic stress and cardiac remodeling, including reduced systolic blood pressure,¹⁴¹ decreased risk of ventricular dysfunction following CABG,¹⁴⁷ and less left ventricular diastolic dysfunction after MI.¹⁴³ In a recent study, Seidelmann et al.¹⁴⁴ showed that the G allele was associated with lower CV mortality and increased lifespan. In the final part of this dissertation (Chapter 4), we expanded the search to all MACE, including MI, ACS, stroke, undergoing CABG or percutaneous coronary intervention (PCI), and cardiac death. Our study design consisted of two portions. The retrospective portion surveyed all MACE from birth to initial recruitment and focused on premature events before the age of 65. The prospective portion followed participant for a five-year period, during which the majority of MACE were recurrent events.

It was found that rs198389 G allele carriers (AG and GG genotypes), compared to those with AA genotype, were less likely to experience a MACE in their life-time and had reduced risk in having recurrent events during the follow-up period. These results concur with previous

findings where the G allele was associated with reduced CV mortality¹⁴⁴ and less hospital readmission.¹⁴³ In our cohort, the G allele was also associated with lower chance of developing the first MACE before the age of 65. In fact, the association was stronger in younger patients, as age became a larger factor in older patients. This provides strong evidence to support the involvement of the *NPPB* polymorphism in the pathophysiology of atherosclerosis. More importantly, in our multivariate adjusted models, the effect of genotype is greater than all traditional risk factors, including sex, age, diabetes and dyslipidemia. It suggests that rs198389 is possibly a better predictor for CV events than traditional risk factors. Although it is still far from being involved in clinical decision making, this BNP genetic variant has the potential to complement the traditional diagnostic evaluation, prevention and treatment strategies, and help to bridge the “CAD gap”. Especially in secondary prevention, patients with AA genotype for *NPPB* rs198389 could warrant closer monitoring and more aggressive treatments.

5.6 The protective role of *NPPB* rs198389 in atherosclerotic cardiovascular disease

Among the many SNPs of vasoactive systems we explored in this dissertation, *NPPB* rs198389 was the only one that consistently showed association with pre-clinical marker, diagnostic standard and clinical outcome of CAD. It is most likely because rs198389 was one of the few functional genetic variants that definitely alters its protein product, whose effects were extensively studied.

The single nucleotide polymorphism *NPPB* rs198389 is located in a regulatory region of the BNP gene that alters its expression. The minor allele G disrupts a putative binding site for the AREB6 (Atp1a1 regulatory element binding factor 6) transcription factor, yielding a 1.8-fold higher level of promoter activity in cultured cells than the A allele.¹³⁷ It has been confirmed by numerous population studies that the G allele of rs198389 is associated with elevated levels of NT-proBNP and BNP.^{137–144} Circulating BNP promotes diuresis, natriuresis and vasodilation. In

the circumstance of cardiac dysfunction, secondary to an insult such as MI, BNP levels increase several-fold to compensate for increased preload and afterload, decreased cardiac output, and activation of the sympathetic nervous system and renin-angiotensin-aldosterone system. In this sense, elevation in BNP and NT-proBNP levels is a well-established clinical marker of hemodynamic stress. A plethora of studies have demonstrated that higher circulating levels of both BNP and NT-proBNP are predictive of recurrent adverse events among patients with heart failure,¹⁷² ACS,^{173,174} and stable CAD.¹⁷⁵⁻¹⁸⁰ Additionally, elevated BNP and NT-proBNP levels are strong predictors for first CV events in asymptomatic populations.¹⁸¹⁻¹⁸³

On the other hand, a growing body of evidence from animal studies and human population studies has been demonstrating that elevated basal levels of BNP may play a beneficial role. Transgenic mice overexpressing BNP are hypotensive in comparison to wild-type littermates.¹⁸⁴ In a large prospective cohort study, rs198389 G allele carriers consistently displayed higher circulating levels of NT-proBNP while having reduced BP and lower CV mortality.¹⁴⁴ The release of BNP in response to elevated BP is a normal physiological response to restore fluid balance and hemodynamic homeostasis. In this sense, genetically augmented BNP could potentially be protective as the system is able to respond distinctively to stressful stimuli.

Whether an elevation in BNP is compensatory or genetically determined, previous evidence suggests that the protective effect revolves around BNP's role in hemodynamic regulation. However, we did not detect any correlation between rs198389 genotype and BP or hypertension in the current cohort. It is likely because the majority of the current population is hypertensive (75%) thus the statistical power was insufficient to detect the association. Nonetheless, physiological mechanisms other than hemodynamic regulation may be at play. *In vitro* studies have demonstrated the anti-inflammatory properties of natriuretic peptides. In particular, ANP directly reduces the production of tumor necrosis factor- α (TNF- α) by macrophages and interferes with the expression of endothelium adhesion molecules.¹⁷¹ These

molecules are essential for inflammatory processes involved in the development of atherosclerotic plaque, which is the culprit of CAD and ischemic cerebral vascular disease.²⁵ Although the anti-inflammatory effect of BNP is yet to be elucidated, functional variants of the *NPPB* gene has been shown to associate with ANP levels due to its close proximity with *NPPA* gene on chromosome one (specifically 1p36).¹⁴⁴ It is possible that the rs198389 polymorphism may protect against atherosclerosis through the anti-inflammatory effects of either BNP directly or upregulation of ANP.

5.7 Limitations

This dissertation has several limitations that are worth noting. First, the overall sample size (513) would generally be considered too small for genetic association studies. As its name suggests, SNP is a variant of a single nucleotide and it has to have very subtle effects on an organism's fitness to maintain a consistent allele frequency in a population. In order to detect small effects caused by SNPs, similar studies usually recruit thousands of participants. Small sample size could be a reason why we failed to find significant results in several SNPs (notably, *NPPA* rs5065) that were established by previous research. Although a larger sample size could potentially reveal more associations, a prolonged recruitment period was not feasible for this dissertation research.

Second, the population we recruited from is not representative of a general population. In order to have readily available coronary angiography results, we recruited patients who were scheduled to undergo the procedure, and therefore had at least one of the following clinical indications: nonspecific chest pain evaluation, stable or unstable angina pectoris, positive stress test, preoperative assessment, old or recent MI (>21 days). While the demographic led to a great variety of atherosclerotic phenotypes, it is biased towards the high-risk population with a larger proportion of men. As a result, it would be difficult to apply the current findings on a low-risk

population or a female population. Nonetheless, the sampling bias may be a necessary compromise, since subjecting healthy individuals to coronary angiography could be very difficult and arguably unethical.

Third, the latter two parts of this dissertation research comprised of only a portion of the overall sample recruited. In the first part, we discovered association between *NPPB* rs198389 and CAD specific to women. However, only 204 of the original 513 participants agreed to take part in the carotid ultrasound study and the five-year follow-up. Consequently, the number of women left in the cohort was insufficient to conduct a sex-specific analysis. This limitation could only be overcome by expanding the initial recruitment.

The final limitation is the lack of information on circulating proteins of the SNPs in question. Measuring plasma levels of BNP and ANP would allow us to determine if the protective effect of rs198389 was mediated via these proteins or other mechanisms. Our findings elude to potential participation of inflammatory pathways. Therefore, inflammatory markers should also be investigated in future studies with larger samples and long-term outcomes.

5.8 Overall Conclusions

Despite extensive research on the association between SNPs and CAD, genetic markers are largely absent from clinical practice because of a lack of understanding of their physiological involvement in the development of atherosclerosis. The present dissertation research focused on functional genetic variants of vasoactive systems and approached the manifestation of atherosclerosis in a longitudinal fashion (summarized in Figure 5.1). We elucidated the protective role of rs198389, a SNP of the gene coding for BNP, in all three stages of CAD presentation: pre-clinical phenotype in the form of ultrasound-detected carotid atherosclerotic plaque, clinical diagnostic evaluation using coronary angiography, and prediction of adverse outcome through follow-up. The highlights of the research findings include a female-specific association between

the SNP and angiographic CAD, a novel association between the SNP and carotid plaque quantified by ultrasound, and the SNP showing greater effect in predicting premature and recurrent adverse events than traditional risk factors. These results provide strong evidence to support the involvement of BNP in the pathophysiology of atherosclerosis and demonstrate the potential of genetic markers in the prediction of CAD.

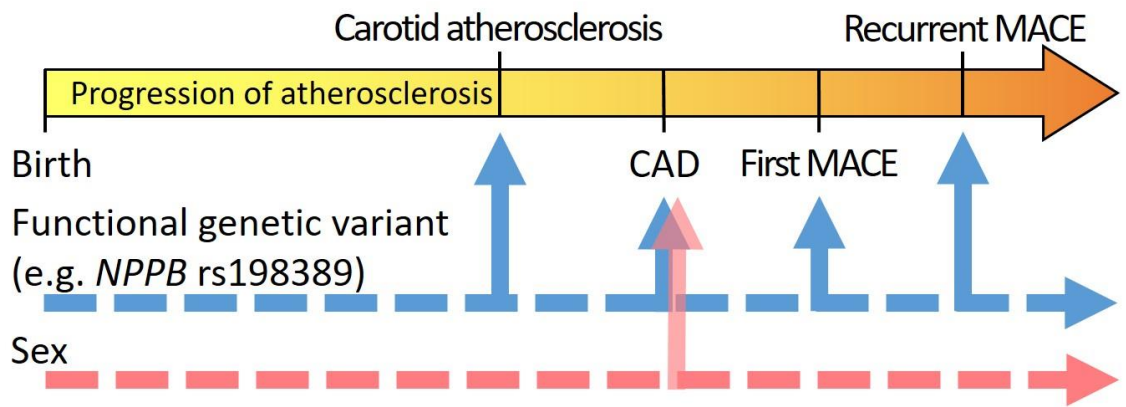


Figure 5.1. Flow diagram showing the longitudinal approach on the manifestation of atherosclerosis, including three stages: pre-clinical phenotype, clinical diagnosis and adverse outcomes.

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Appendix A

Ethics Approvals



QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Renewal of Ethics Clearance

October 18, 2016

Dr. Amer Johri
Department of Medicine
Kingston General Hospital

ROMEO/TRAQ #: 6010614

Department Code: DMED-1627-13

Study Title: The Three-Dimensional Carotid Ultrasound Combined with Stress Echocardiography (3D CSE) for the Assessment of Cardiovascular Disease

Review Type: Delegated

Date Ethics Clearance Effective: October 22, 2016

Ethics Clearance Expiry Date: October 22, 2017

Dear Dr. Johri,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application. This study, including all currently approved documentation has been granted ethical clearance until the expiry date noted above.

Prior to the expiration of your ethics clearance, you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented below.

Yours sincerely,

A handwritten signature in cursive script that reads "Albert J. Clark".

Chair, Health Sciences Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federalwide Assurance Number: FWA#: 00004184, IRB#: 00001173

HSREB members involved in the research project do not participate in the review, discussion, or decision.



**QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS
RESEARCH ETHICS BOARD (HSREB)**

HSREB Renewal of Ethics Clearance

January 24, 2018

Dr. Amer Johri
Department of Medicine
Kingston Health Sciences Centre – KGH Site

ROMEO/TRAQ #: 6005751

Department Code: DMED-1365-11

Study Title: Storm Preparations: Use of the Carotid Ultrasound for Assessment of Atherosclerotic Disease

Review Type: Delegated

Date Ethics Clearance Effective: February 04, 2018

Ethics Clearance Expiry Date: February 04, 2019

Dear Dr. Johri,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application. This study, including all currently approved documentation has been granted ethical clearance until the expiry date noted above.

Prior to the expiration of your ethics clearance, you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented below.

Yours sincerely,

A handwritten signature in cursive script that reads "Albert Z. Clark".

Chair, Health Sciences Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federalwide Assurance Number: FWA#: 00004184, IRB#: 00001173

HSREB members involved in the research project do not participate in the review, discussion, or decision.

Appendix B

Table of Single nucleotide polymorphisms investigated in this thesis.

Protein	Gene	SNP	Major Allele	Minor Allele	HapMap Minor Allele Frequency	KGH Cath Population Frequency (n)
ANP	<i>NPPA</i>	rs5065	A	G	0.14	0.15 (510)
BNP	<i>NPPB</i>	rs198389	A	G	0.44	0.42 (490)
NPR-B	<i>NPR2</i>	rs10758325	G	A	0.36	0.41 (507)
eNOS	<i>NOS3 (e)</i>	rs1799983	G	T	0.34	0.33 (492)
iNOS	<i>NOS2 (i)</i>	rs2297518	G	A	0.17	0.20 (507)
Endothelin	<i>EDNI</i>	rs5370	G	T	0.24	0.18 (510)
Angiotensinogen	<i>AGT</i>	rs699	A	G	0.41	0.42 (458)
AT1	<i>AGTRI</i>	rs5186	A	C	0.38*	0.30 (511)
ANP, atrial natriuretic peptide BNP, B-type natriuretic peptide NPR-B, natriuretic peptide receptor B eNOS, endothelin nitric oxide synthase iNOS, inducible nitric oxide synthase AT1, angiotensin receptor 1 KGH, Kingston General Hospital						

Appendix C

Schematic Diagram of the Process of Single Nucleotide Polymorphism Genotyping Using Polymerase Chain Reaction

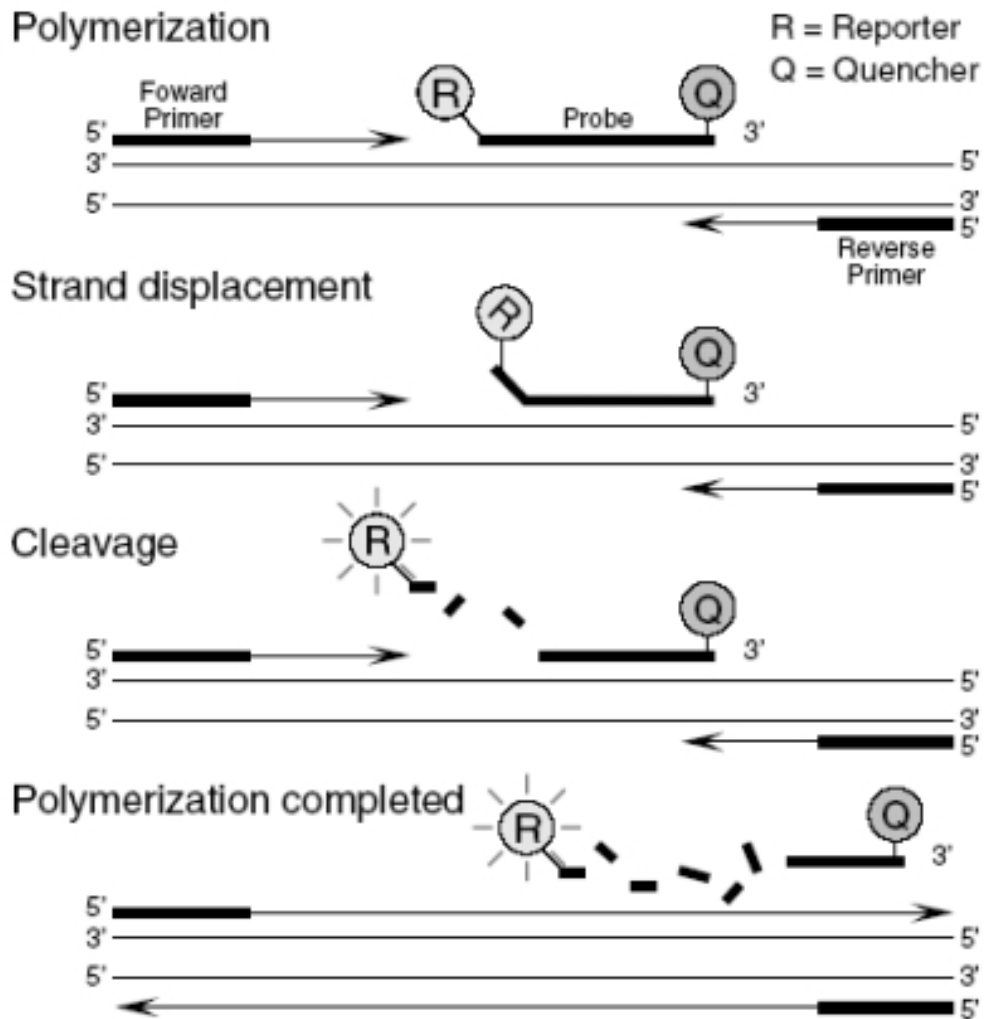


Figure C. Schematic diagram of the process of single nucleotide polymorphism genotyping using polymerase chain reaction.

Appendix D

Example of Single Nucleotide Polymorphism Genotyping Result

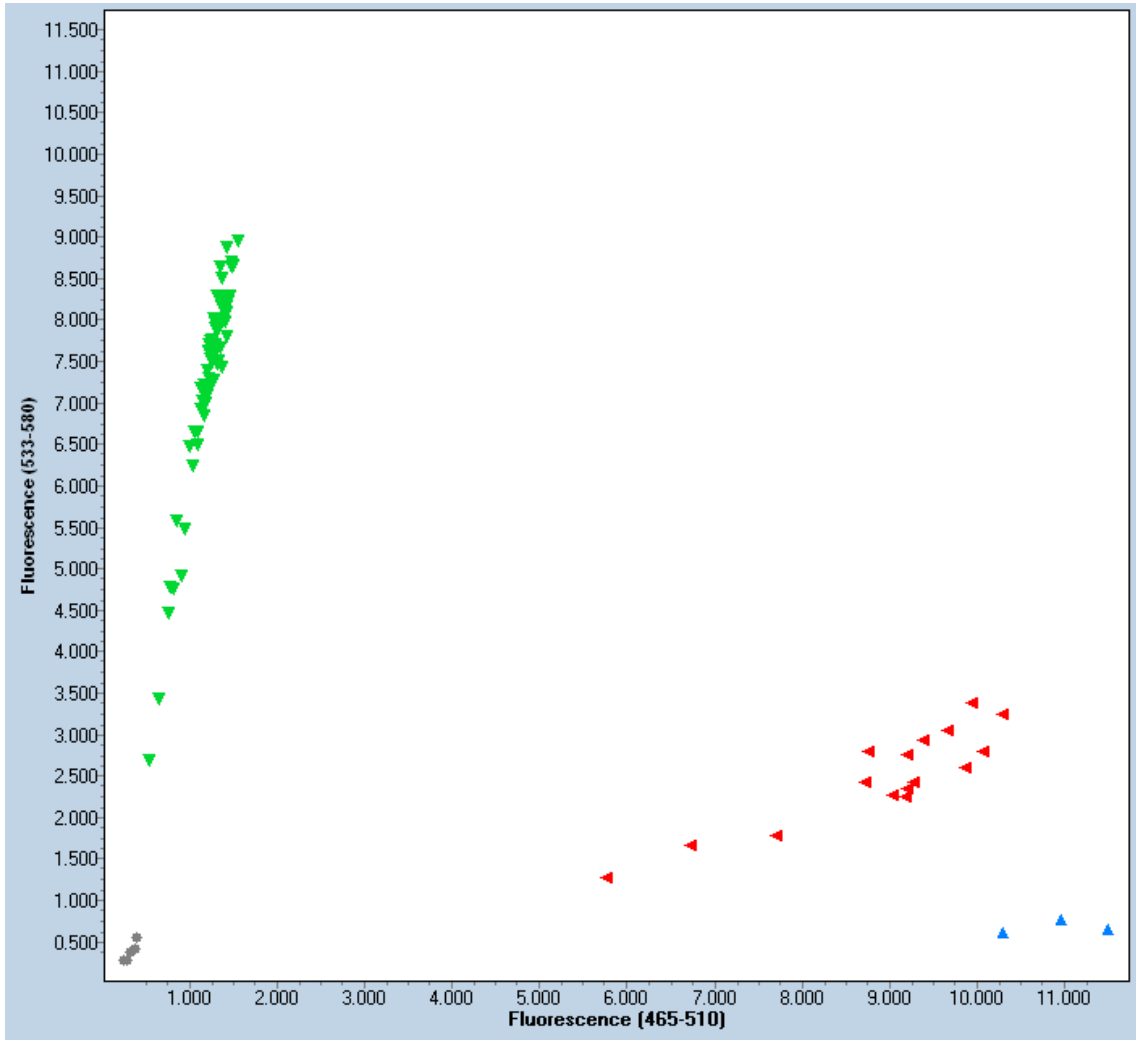


Figure D. Example of single nucleotide polymorphism genotyping result of *NPPA* rs5065.