AN INVESTIGATION OF THE PROGNOSTIC UTILITY OF RANTES LEVELS IN PREDICTING MORTALITY IN AN ANGIOGRAPHY POPULATION

by

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B.Sc., The University of British Columbia, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2014

ABSTRACT

RANTES (Regulated upon activation, normal T cell expressed and secreted) is a chemokine involved in the recruitment and transmigration of leukocytes across the vascular endothelium. A large body of experimental evidence suggests the role of RANTES and its receptor, CCR5, in atherosclerosis and has prompted the study of RANTES as a biomarker of coronary artery disease (CAD).

We sought to investigate baseline plasma RANTES as a marker of mortality in a cohort of patients referred for angiography. RANTES levels were measured in 831 patients who were classified by angiogram as positive or negative for CAD. At the time of recruitment, blood samples were collected and later assayed using a commercial ELISA kit for RANTES. Mortality data was collected in 2009 to identify deceased patients and the cause of death.

RANTES levels were significantly different between men and women (27.7 ng/mL and 34.2 ng/mL, p=0.001) and between subjects with and without family history of CAD (30.3 ng/mL and 28.1 ng/mL, p=0.006). The predictive value of RANTES levels for CAD was assessed using a multivariate logistic regression model adjusting for traditional markers of CAD. Triglycerides and apolipoprotein B levels were independent predictors, with odds ratios of OR 2.65 [95% CI: 1.05-6.69] and OR 2.65 [95% CI: 1.01-6.93], p<0.001 respectively. RANTES levels were associated with CAD, OR 1.67 [95% CI: 1.00-2.76], p=0.047, although this association was moderate. When divided into quartiles, subjects with a RANTES level above the fourth quartile were associated with a greater risk of CAD, OR 1.73 [95% CI: 1.05-2.86], p=0.031. After a mean follow-up of 11.1 years, 256 of the 831 subjects were deceased. Using a Cox regression model,

we assessed the ability of RANTES to predict cardiovascular and all-cause mortality. Only age and smoking status independently predicted death due to any cause (p<0.001 and p=0.015, respectively).

Our results suggest that RANTES levels do not enhance cardiovascular risk prediction in a population of patients with stable CAD. Further studies to explain the association between RANTES, family history of CAD and female gender could determine whether risk prediction would be improved for these groups.

PREFACE

This dissertation is an original intellectual product of the author, S., Di Palma. The work reported in Chapters 2-3 was covered by UBC-Providence Health Care Research Institute, Ethics Certificate number H02-50103.

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ABBREVIATIONS

ACS	Acute Coronary Syndrome
ARIC	Atherosclerosis Risk in Communities study
ATP III	Adult Treatment Panel III
AUC	Area under the receiver operating characteristic curve
BMI	Body Mass Index
CAD	Coronary Artery Disease
CRP	C-reactive Protein
CV	Coefficient of Variation
DARC	Duffy Antigen Receptor for chemokines
DBP	Diastolic Blood Pressure
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EPC	Endothelial Progenitor Cell
FHS	Framingham Heart Study
FRS	Framingham Risk Score
LDL-C	Low density lipoprotein cholesterol
Lp-PLA2	Lipoprotein-associated phospholipase A2
HDL-C	High density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
ICAM-1	Intracellular adhesion molecule-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IP-10	Interferon y-induced Protein of 10kDa

IRAS	Insulin Resistance Atherosclerosis Study
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NCEP	National Cholesterol Education Program
RANTES	Regulated Upon Activation, Normal T cell Expressed and Secreted
ROC	Receiver Operator Characteristic
SBP	Systolic Blood Pressure
SCA	Selective Coronary Angiography
SDF-1	Stromal Derived Factor-1
SR-PSOX	Scavenger Receptor PSOX
TC	Total Cholesterol
TG	Triglycerides
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1

ACKNOWLEDGEMENTS

My supervisors:
Drs. Jiri Frohlich & John Hill for their guidance, support and above all, their patience.

My committee:
Drs. Jiri Frohlich, John Hill, John Mancini, Greg Bondy, Haydn Pritchard, Sammy Chan & Chun Seow

The Atherosclerosis Specialty Laboratory:
Dr. Kenny Lee and Dr. Claire Heslop who contributed data from the original cohort that supported this study, as well as my lab colleagues for their friendship and support, in particular Dr. Catalin Taraboanta.

Others:
Aleta Allen, Dr. David Walker.

DEDICATION

To my parents, for their unconditional love and continual support.

1 INTRODUCTION

1.1 Atherosclerosis

Atherosclerosis is a chronic, progressive inflammatory disease characterized by the formation of lesions in the walls of medium and large-sized arteries. Early on, these lesions form in the subendothelial space and consist of lipid-filled macrophages known as foam cells (1). Foam cells form the first stage of lesion development, the fatty streak, and though reversible, typically progresses to a more advanced lesion.

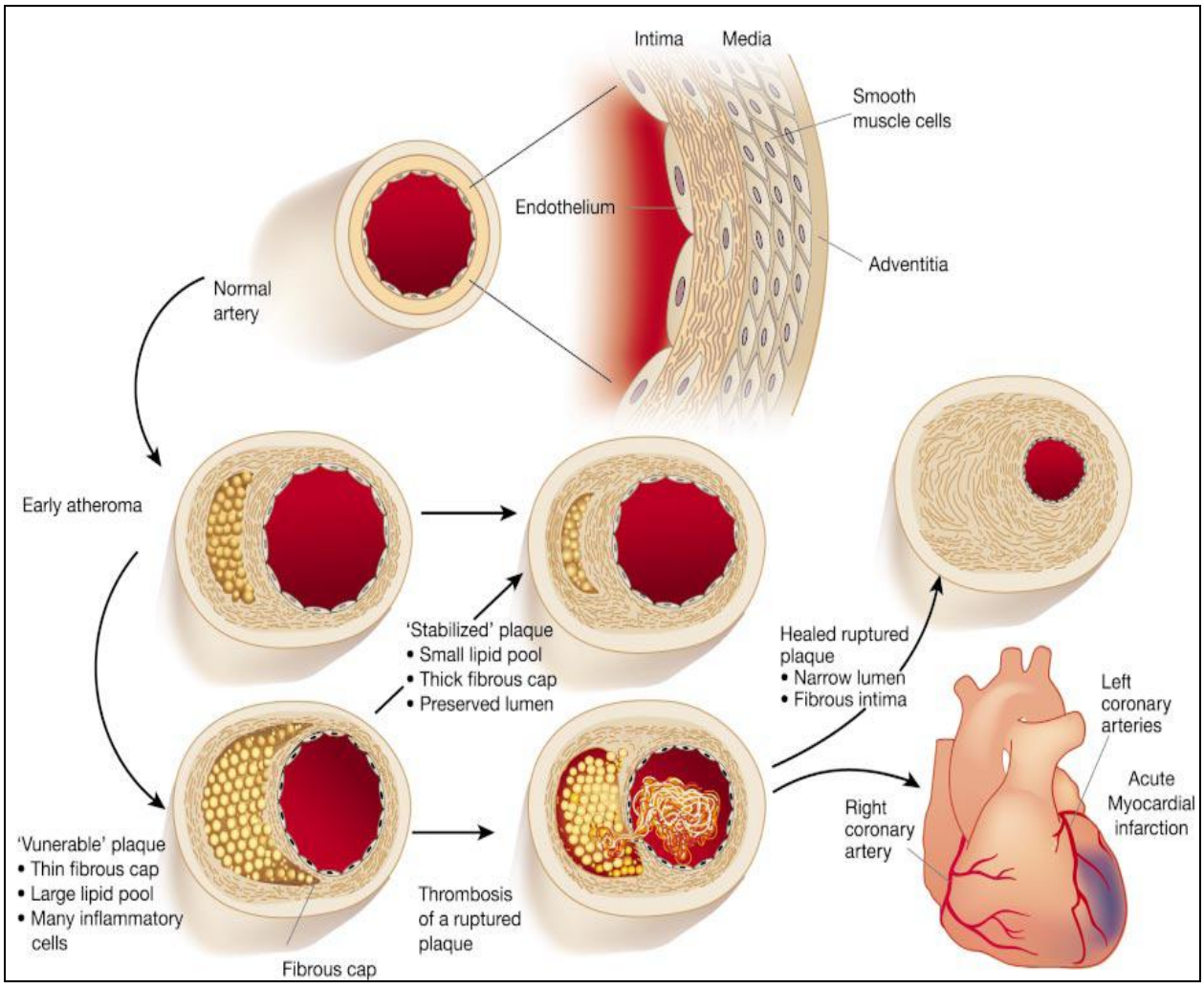
Because high plasma levels of cholesterol are one of the main risk factors for atherosclerosis, the process of atherosclerosis was believed at one time to be simply a build-up of lipids, particularly from low density lipoprotein cholesterol (LDL-C), in the arterial wall (2). It is now understood to be a complex inflammatory process which involves a powerful immune response involving a host of cellular and molecular mechanisms (3-5). Endothelial dysfunction is the initiating factor in atherogenesis. Dysfunction and activation is a result of damage to the vascular endothelium from high levels of circulating modified LDL particles, high blood pressure, free radical production from cigarette smoking or diabetes. The endothelium responds to injury by increasing its permeability and adhesiveness to leukocytes, and upregulating the production of inflammatory cytokines, chemokines, growth factors and adhesion molecules.

The altered permeability of the endothelium allows LDL particles to enter the subendothelial space and propagate the inflammatory response. Retention of LDL in the intima stimulates endothelial cell activation and triggers the expression of adhesion molecules which allow circulating leukocytes to be recruited from the circulation and transmigrate across the

endothelium. Once inside the subendothelial space, LDL particles are modified by lipolysis, glycation, proteolysis, aggregation and oxidation as a result of the action of reactive oxygen species, lipases, and other enzymes in the arterial wall, and are taken up by macrophages to form foam cells.

Initially, the inflammatory response is an attempt to clear the offending agent – the LDL particles. However, if the cause of endothelial dysfunction continues, the inflammatory response becomes a chronic one, and repeated influx of monocytes and T cells occurs along with the migration and proliferation of smooth muscle cells to the site of the lesion. The inflammatory infiltrate causes the arterial wall to thicken and dilate, leaving the diameter of the lumen initially unaffected. Continued inflammation leads to further leukocyte recruitment and release of inflammatory cytokines, smooth muscle cells continue to migrate to the lesion, and fibrous tissue forms. The lesion becomes larger and its fibrous cap continues to thicken over the underlying core of lipids, leukocytes and necrotic cells. This represents a healing response – the deposition of fibrous material to the lesion prevents the lesion from intruding the lumen and provides stability. Calcification of the plaque and neovascularization are also common. This is considered an advanced, complicated lesion and eventually may occlude the arterial lumen leading to vessel stenosis and altered blood flow. The fibrous cap may also be degraded over time, leading to rupture, clot formation and obstruction of blood flow (Figure 1.1) (6).

Figure 1.1 Life History of an Atheroma



Libby, P. Nature 2002

The clinical outcome of atherosclerosis is myocardial infarction (MI), defined as an occlusion of the arteries to the heart, resulting in injury or necrosis of the heart tissue. Clinical symptoms of atherosclerosis emerge when a weakened fibrous cap ruptures and precipitates formation of an occlusive thrombus, or when the plaque progressively grows until the vessel is obstructed and blood flow to the heart is compromised. It was originally thought that progressive luminal narrowing from continued growth of the plaque was primarily responsible for myocardial

infarction and the presence of clinical symptoms. It is now understood that lesions develop for the most part outwardly, away from the lumen (7, 8), without causing significant stenosis and that the composition of the plaque is a better indicator of its vulnerability to rupturing (9).

1.2 Coronary Artery Disease

Coronary Artery Disease (CAD) is, in the vast majority of cases, the disease caused by atherosclerosis. Cardiovascular disease is the leading cause of death globally with The World Health Organization reporting that in 2005, 17.5 million deaths were attributed to cardiovascular disease and 8 million of these deaths were due to coronary artery disease (CAD). Furthermore, the WHO predicts that cardiovascular diseases will continue to be a burden on our healthcare system, estimating that by 2015, 20 million people will die from a heart attack or stroke.

Coronary artery disease develops over many years, eventually causing cardiac ischemia. The heart muscle requires a continuous supply of oxygen and nutrients to survive, and obstruction of blood flow in the coronary arteries as a result of atherosclerosis rapidly leads to serious problems. Clinical symptoms of coronary artery disease include chest pain (angina pectoris), when the cardiac muscle is not receiving adequate oxygen. Over time, chronic ischemic conditions may result in damaged heart muscle leading to heart failure. Myocardial infarction is an acute event that occurs when blood flow to the heart muscle is suddenly blocked. The ischemic conditions cause necrosis of cardiac muscle cells, leading to irreversible damage to the heart muscle.

1.3 Risk Assessment for CAD

1.3.1 Identification of Risk Factors

Before late 1940s, the origins of CAD were not well understood and with the burden of CAD to the US healthcare system growing, the need to investigate the causes of CAD became apparent. The now well established concept of risk factors, essential to the prediction and prevention of CAD, originated from the Framingham Heart Study (FHS) initiated in 1948 (10). The original cohort of this study consisted of 5209 men and women, aged 30 to 62 years old, from Framingham, Massachusetts. Through long-term follow up of these individuals, the FHS identified raised blood cholesterol, raised blood pressure and smoking as the major factors of CAD. As more evidence accumulated over the years, obesity, diabetes and physical inactivity were also identified as independent risk factors (11).

Another major contribution of the FHS is the development of the Framingham Risk Score (FRS). This equation calculates the ten-year risk of having a cardiovascular event (fatal or non-fatal MI) based on age, blood pressure, total cholesterol (TC) or low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), smoking status and the presence of diabetes mellitus (Figures 1.2 and 1.3) (10, 12). Other global risk assessment models have been developed in the United States and European countries in an effort to build on the success of the FRS and improve its applicability to different populations. These scores were designed using comparable epidemiological methods and include PROCAM (13) and the European Society of Cardiology SCORE (14), among others.

Figure 1.2 Framingham Risk Score for Men

Step 1

Age	
Years	Points
30-34	-1
35-39	0
40-44	1
45-49	2
50-54	3
55-59	4
60-64	5
65-69	6
70-74	7

Step 2

LDL - Cholesterol		
(mg/dl)	(mmol/L)	Points
<100	≤2.59	-3
100-129	2.60-3.36	0
130-159	3.37-4.14	0
160-189	4.15-4.91	1
≥190	≥4.92	2

Key	
Color	Risk
green	Very low
white	Low
yellow	Moderate
rose	High
red	Very high

Step 3

HDL - Cholesterol		
(mg/dl)	(mmol/L)	Points
<35	≤0.90	2
35-44	0.91-1.16	1
45-49	1.17-1.29	0
50-59	1.30-1.55	0
≥60	≥1.56	-1

Step 4

Blood Pressure					
Systolic (mmHg)	Diastolic (mmHg)				
	<80	80-84	85-89	90-99	≥100
<120	0				
120-129		0 pts			
130-139			1		
140-159				2	
≥160					3 pts

Note: When systolic and diastolic pressures provide different estimates for point scores, use the higher number

Step 5

Diabetes	
	Points
No	0
Yes	2

Step 6

Smoker	
	Points
No	0
Yes	2

Risk estimates were derived from the experience of the NHLBI's Framingham Heart Study, a predominantly Caucasian population in Massachusetts, USA

Step 7 (sum from steps 1-6)

Adding up the points	
Age	_____
LDL Cholesterol	_____
HDL Cholesterol	_____
Blood Pressure	_____
Diabetes	_____
Smoker	_____
Point Total	_____

Step 8 (determine CHD risk from point total)

CHD Risk	
Point Total	10 Yr CHD Risk
≤-3	1%
-2	2%
-1	2%
0	3%
1	4%
2	4%
3	6%
4	7%
5	9%
6	11%
7	14%
8	18%
9	22%
10	27%
11	33%
12	40%
13	47%
≥14	≥56%

Step 9 (compare to man of the same age)

Comparative Risk		
Age (years)	Average 10 Yr CHD Risk	Low* 10 Yr CHD Risk
30-34	3%	2%
35-39	5%	3%
40-44	7%	4%
45-49	11%	4%
50-54	14%	6%
55-59	16%	7%
60-64	21%	9%
65-69	25%	11%
70-74	30%	14%

*Low risk was calculated for a man the same age, normal blood pressure, LDL cholesterol 100-129 mg/dL, HDL cholesterol 45 mg/dL, non-smoker, no diabetes

Figure 1.3 Framingham Risk Score for Women

Step 1

Age	
Years	Points
30-34	-9
35-39	-4
40-44	0
45-49	3
50-54	6
55-59	7
60-64	8
65-69	8
70-74	8

Step 2

LDL - Cholesterol		
(mg/dl)	(mmol/L)	Points
<100	≤2.59	-2
100-129	2.60-3.36	0
130-159	3.37-4.14	0
160-189	4.15-4.91	2
≥190	≥4.92	2

Key	
Color	Risk
green	Very low
white	Low
yellow	Moderate
rose	High
red	Very high

Step 3

HDL - Cholesterol		
(mg/dl)	(mmol/L)	Points
<35	≤0.90	5
35-44	0.91-1.16	2
45-49	1.17-1.29	1
50-59	1.30-1.55	0
≥60	≥1.56	-2

Step 4

Blood Pressure					
Systolic (mmHg)	Diastolic (mmHg)				
	<80	80-84	85-89	90-99	≥100
<120	-3 pts				
120-129		0 pts			
130-139			0 pts		
140-159				2 pts	
≥160					3 pts

Note: When systolic and diastolic pressures provide different estimates for point scores, use the higher number

Step 5

Diabetes	
	Points
No	0
Yes	4

Step 6

Smoker	
	Points
No	0
Yes	2

Risk estimates were derived from the experience of the NHLBI's Framingham Heart Study, a predominantly Caucasian population in Massachusetts, USA

Step 7 (sum from steps 1-6)

Adding up the points	
Age	_____
LDL Cholesterol	_____
HDL Cholesterol	_____
Blood Pressure	_____
Diabetes	_____
Smoker	_____
Point Total	_____

Step 8 (determine CHD risk from point total)

CHD Risk	
Point Total	10 Yr CHD Risk
≤-2	1%
-1	2%
0	2%
1	2%
2	3%
3	3%
4	4%
5	5%
6	6%
7	7%
8	8%
9	9%
10	11%
11	13%
12	15%
13	17%
14	20%
15	24%
16	27%
≥17	≥32%

Step 9 (compare to women of the same age)

Comparative Risk		
Age (years)	Average 10 Yr CHD Risk	Low* 10 Yr CHD Risk
30-34	<1%	<1%
35-39	1%	<1%
40-44	2%	2%
45-49	5%	3%
50-54	8%	5%
55-59	12%	7%
60-64	12%	8%
65-69	13%	8%
70-74	14%	8%

*Low risk was calculated for a woman the same age, normal blood pressure, LDL cholesterol 100-129 mg/dL, HDL cholesterol 55 mg/dL, non-smoker, no diabetes

The investigation of cardiovascular risk factors has had a huge impact on the evolution of risk prediction scores and on elucidating the mechanisms of CAD development. The ability to predict risk in patients has also influenced the guidelines used by physicians in clinical practice. Both the National Cholesterol Education Program (NCEP) Expert Panel (15) and Canadian Cardiovascular Society (16, 17) align their treatment targets with the FRS categories. By placing patients in low (<10%), intermediate (10-20%) or high risk (>20%) categories, the FRS allows physicians to implement a personalized prevention or treatment strategy.

1.3.2 Non-modifiable Risk Factors

Sex, age, family history of premature CAD and ethnicity are non-modifiable risk factors for CAD. Age is clearly a critical component in any risk prediction algorithm. With advancing age, the risk of developing coronary artery disease increases significantly. The age of an individual is a reflection of the progressive accumulation of coronary atherosclerosis and the cumulative exposure to risk factors over the years.

Men are at higher risk of developing coronary artery disease and develop the disease earlier in life than women. Women are typically 10 years older than men when any coronary disease manifestations first appear, and myocardial infarction occurs as much as 20 years later in life. However, one in 9 American women aged 45-64 years has clinical evidence of coronary heart disease and after age 65 years this increases to 1 in 3 women (18). At this age, the difference in risk seen between genders is abolished due to an increase in women's risk of CAD following menopause (19).

A positive family history of coronary artery disease is considered an independent risk factor for developing CAD. Results from the Framingham Offspring Study show that after

correction for known risk factors, parental cardiovascular disease led to a 1.7- to twofold increase in overall risk. Family history of premature CAD is defined as having a first degree relative diagnosed with CAD before the age of 55 and 65 years, for men and women respectively.

Certain ethic groups are at increased risk of coronary artery disease. African Americans have the highest overall CAD mortality rates and the highest coronary death rates of any ethnic group in the United States, particularly at younger ages. This is in part, a result of the high prevalence of coronary risk factors in the population, in particular hypertension, left ventricular hypertrophy, type 2 diabetes mellitus, cigarette smoking, obesity, and physical inactivity which occur more frequently in African Americans than in Caucasians (20). Traditional risk factors have similar predictive value for African Americans, however, the association between hypertension and diabetes with mortality is stronger in this population relative to white Americans.

The frequency of type 2 diabetes, obesity, higher triglycerides and lower HDL levels is increased in Hispanic populations as well. Interestingly, mortality due to CAD is approximately 20 percent lower in this population, despite poorer cardiovascular risk profiles for these individuals. Similarly, South Asians, Native Americans and Asian/Pacific Islanders are at increased risk of developing CAD (21). These ethnic groups have higher incidence of CAD risk factors, in particular insulin resistance, metabolic syndrome and type 2 diabetes.

1.3.3 Modifiable Risk Factors

1.3.3.1 Hypertension

Increased blood pressure is an independent risk factor for CAD and is strongly related to both cardiovascular and overall mortality. At age 40—69 years, each increase of 20 mm Hg in systolic blood pressure is associated with more than a two-fold difference in death due to stroke, and with twofold differences in the death rates from coronary artery disease and from other vascular causes. Furthermore, increases in blood pressure above 115/75 mm Hg show a proportional increase in risk for CAD (22).

The comparative importance of diastolic blood pressure (DBP), systolic blood pressure (SBP) and pulse pressure (PP) as indicators of cardiovascular disease risk has undergone several changes. Initially, DBP was thought to be the best measure of risk, but using early follow-up from the Framingham Heart Study, Kannel et el. concluded that SBP was in fact more important than DBP. This publication, confirmed by various other studies has been influential in shaping medical opinion that SBP, in addition to DBP, is a more significant risk factor. Currently, the Framingham Risk Score includes SBP in its equation for risk prediction, but cardiovascular disease risk is increased by the elevation of DBP and/or SBP, with the higher of the two measurements indicating severity.

1.3.3.2 Dyslipidemia

Hypercholesterolemia usually refers to elevated low density lipoprotein levels, and is a principal independent risk factor for CAD. The development of atherosclerosis and coronary artery disease was at one time believed to be simply an accumulation of lipids in the arterial wall. Circulating plasma LDL are modified by oxidation, causing injury to the vascular endothelium. The LDL particles enter the subendothelial space where they are further oxidized

and taken up by macrophages to form foam cells. Modified LDL also attract monocytes and amplify the inflammatory response in the arterial wall promoting atheroma formation. Modified high-density lipoproteins particles have anti-atherogenic properties and are considered a negative risk factor for CAD (23). HDL promotes the movement of cholesterol from the peripheral tissues to the liver for excretion. Both LDL-C and HDL-C levels can be modified by lifestyle changes in diet and physical activity or by pharmacotherapy. Statins effectively lower LDL levels by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis. This leads to upregulation of LDL receptors by the liver increasing the uptake of LDL particles from the circulation, and lowers LDL cholesterol levels.

Evidence for triglyceride (TG) levels as a risk factor for CAD is less powerful. The Framingham Heart Study demonstrated that triglycerides were an independent predictor of risk for women age 50-69 and in type II diabetics, however, studies have not shown that triglyceride levels are a robust predictor of risk across broad populations (24). Triglycerides do appear to act synergistically with both LDL and HDL levels and patients with high LDL, low HDL and high TG stand to benefit most from lipid-reducing pharmacotherapy (25).

1.3.3.3 Diabetes

Type 2 diabetes mellitus is a disorder that results from increased insulin resistance; cells do not utilize insulin and glucose uptake by the peripheral tissues in impaired glucose leading to elevated blood glucose levels. Type 2 diabetes is associated with a 2 to 4-fold higher risk of coronary artery disease (26); individuals who have diabetes are usually considered high risk even in the absence of established CAD. The Insulin Resistance Atherosclerosis Study (IRAS) demonstrated that diabetic patients without CAD were shown to have carotid atherosclerosis as shown by B-mode ultrasonography, and had findings similar to non-diabetic patients with

CAD (26). According to the Adult Treatment Panel III (ATP-III) guidelines, type 2 diabetics are considered equivalent in risk to individuals who have already had a cardiovascular event and should be treated aggressively. Furthermore, the FRS was not developed for diabetic patients and thus may underestimate cardiovascular risk for those with diabetes, further warranting an aggressive prevention and treatment strategy (27).

1.3.3.4 Obesity

The underlying cause of obesity is lack of physical activity and excess caloric intake. It is closely associated with multiple co-morbidities of CAD including hypertension, dyslipidemia, stroke and diabetes and is a major risk factor and determinant of CAD. The incidence of obesity has been rising; globally there are 1 billion overweight adults and at least 300 million of them are obese (28). Definitions of overweight and obesity are based on Body Mass Index (BMI), a ratio of body weight in kilograms to height in meters squared. Individuals with a BMI between 18.5-25 kg/m^2 are considered of normal weight, those overweight have a BMI between 25-30 kg/m^2, and obese patients have a BMI greater than 30 kg/m^2.

BMI is typically used to assess weight, however, the calculation does not take into consideration the variation that occurs in the distribution of adipose tissue and lean muscle mass between individuals. For example, BMI is not accurate for individuals with extremely high or low percentages of lean body mass (i.e. athletes or the elderly). Also, because the BMI categories were developed using Caucasian populations they are not accurate for other ethnic groups. Most importantly, BMI is a measurement of peripheral adipose tissue and not visceral adipose tissue which has been shown to be more atherogenic (29). Some studies have indicated that peripheral body fat is negatively associated with insulin resistance, whereas central fat is positively associated with insulin resistance (30). BMI does not differentiate between these two

body fat distribution profiles. For this reason, waist circumference is measured as an indicator of visceral adipose tissue and numerous studies have shown that waist circumference cutoffs for men (>102 cm) and women (>88 cm) are associated with increased risk of CAD and are a better predictor than BMI (31).

1.3.3.5 Metabolic Syndrome

The Prospective Cardiovascular Munster (PROCAM) study shows that for men aged 40–65 years the incidence of myocardial infarction is increased 2.5-fold when either hypertension or diabetes is present, and 8-fold when both risk factors are present (13). The presence of an abnormal lipid profile in addition to hypertension and diabetes increased the frequency of MI 19-fold. Numerous studies have suggested that the presence of multiple risk factors increases cardiovascular risk over the simple addition of risk attributed to each individual risk factor. Metabolic syndrome refers to the clustering of risk factors that are concomitantly present in an individual. The NCEP-ATP III guidelines define it as having three or more of the following risk factors present: abdominal obesity, elevated blood glucose, hypertriglyceridemia, low HDL levels and hypertension (15). The major determinants of metabolic syndrome, however, are insulin resistance and abdominal obesity. These factors contribute to dysfunctional adipose tissue and a pro-inflammatory state leading to an increased risk for type 2 diabetes and CAD.

1.3.3.6 Smoking

Tobacco usage is a modifiable risk factor for cardiovascular disease. It is the second leading cause of death in the world. If the current smoking rates continue, over 9 million people will die annually as a result of this habit by 2030. Smoking affects the body systemically and in

addition to its obvious role in pulmonary diseases, numerous other extrapulmonary diseases are tobacco-related including cardiovascular disease and many cancers.

A large body of population-based studies has revealed that smokers have elevated levels of C-reactive protein (CRP) (32), fibrinogen (33), and interleukin-6 (34) indicating a persistent low-grade systemic inflammatory response. The activation and release of inflammatory cells into the circulation and increase in circulating inflammatory mediators such as acute phase proteins and proinflammatory cytokines promote the atherosclerotic process. In addition to the pro-inflammatory effects of smoking, other mechanisms by which smoking may contribute to CAD are by promoting vascular endothelial dysfunction, and hemostatic and coagulation disturbances (35).

The degree of risk conferred by smoking is linearly related to the number of cigarettes smoked. Even low levels of smoking (eg. five cigarettes per day) are associated with an increased risk of myocardial infarction. The INTERHEART Study showed that each additional cigarette smoked per day increases the risk of non-fatal MI by 5-6%. Exposure to tobacco, even for non-smokers in the form of second-hand smoke, is also associated with an increase risk of myocardial infarction (36)

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1.3.3.7 Physical Activity

A sedentary lifestyle is associated with coronary artery disease and more than 25% of the population-attributable risk for MI is due to lack of physical activity (37). Observational studies have reported decreased numbers of CAD events in subjects who perform regular physical activity and there is a dose-response relationship between CAD and physical exercise. Even one hour of walking per week decreased CAD risk. Physical activity reduces body fat,

improves glucose tolerance and insulin sensitivity, and reduces the risk of high blood pressure, diabetes and dyslipidemia making it a very effective way to reduce overall cardiovascular risk. It has been estimated that the risk of cardiovascular mortality can be reduced by 25% with regular exercise (38).

1.3.4 Limitations of Current Risk Assessment Models

The FHS was a milestone for cardiovascular epidemiology. It allowed for the understanding of the mechanisms of atherosclerosis and drove the identification of risk factors for CAD. The development of the FRS had an impact on clinical guidelines and helped to shape primary prevention strategies for CAD and stroke. Recommendations for reducing risk include physical exercise, healthy diet, controlling blood pressure and cholesterol levels.

The FRS however was designed using a Caucasian population, during a time when CAD incidence was at its highest. For these reasons, the FRS does not provide accurate risk prediction for non-Western population groups, and it tends to overestimate the risk CAD in certain populations (39). Due to the success of the Framingham Risk Score, other scores were developed throughout the United States and Europe in an attempt to improve the generalizability of a risk score for use with diverse populations. Other scores developed include PROCAM (13), SCORE (40) and CUORE (41).

Despite the advances made as a result of the Framingham Heart Study and its impact on risk prediction, current risk models are not perfect. Current risk assessment algorithms are centered around the lipid profile. Although lipids levels are a powerful predictor of atherosclerotic risk, it does not form a complete picture. In fact, many cardiovascular events occur in individuals with cholesterol levels below the recommended threshold. Moreover, in up to 50% of individuals, traditional risk factors alone do not provide accurate risk prediction and

the first sign of CAD is a myocardial infarction or cardiac death (42). This has prompted the search for novel cardiovascular biomarkers that will improve risk assessment.

1.3.5 Biomarkers of Inflammation

Until recently, atherosclerosis was believed to be a progressive accumulation of lipids in the arterial wall. We now understand that atherosclerosis is an inflammatory disease and that traditional risk factors (dyslipidemia, hypertension, obesity, diabetes) act as pro-inflammatory stimuli causing damage to the vascular endothelium. This leads to upregulation of adhesion molecules and the release of inflammatory cytokines, proteases and acute proteins into the circulation (3). Inflammation not only affects the atherosclerotic phase of CAD, but also the vulnerability of plaques to rupture, resulting in myocardial infarction. This concept of inflammation in the development of atherosclerosis has shed new light onto the mechanisms of this disease and because more than half of vascular events occur in individuals with cholesterol levels below average, has lead to the investigation of inflammatory biomarkers to improve cardiovascular risk prediction.

By far the most robust and validated inflammatory marker is C-Reactive Protein (CRP). CRP is an acute phase reactant protein whose secretion from the liver is stimulated by interleukin-6 in response to inflammation (43). Results from more than 25 different prospective studies have been reported, that clearly demonstrate a significant and independent association between increased CRP levels and future cardiovascular events. These prospective studies have shown that CRP is an independent predictor of cardiovascular events, including MI and sudden cardiac death and is useful as a marker of CAD for both apparently healthy individuals and those with established CAD, independent of traditional risk factors (44, 45). Most importantly,

CRP measurements add prognostic information to the Framingham Risk Score with risk prediction being enhanced at all levels of LDL-C and all other levels of the FRS. This makes the measurement of CRP especially useful for individuals who are at intermediate risk for cardiovascular disease as indicated by traditional risk factors, and whose LDL-C levels are low. The Centers of Disease Control and Prevention and the American Heart Association named CRP a marker of cardiovascular risk with guidelines for clinical practice being <1mg/L for low-risk individuals and >3mg/L for those at high risk (46).

Further studies have evaluated whether the reduction of CRP levels is associated with improved clinical outcomes. It is known that the benefits of statins reach far beyond the ability to reduce LDL-C levels. The Cholesterol and Recurrent Events (CARE) trial was the first to show that statin therapy reduces plasma CRP levels in addition to lowering LDL-C (47). Other trials have shown that lowering CRP levels reduces cardiovascular risk and the REVERSAL trial showed that lowering CRP levels in individuals with established CAD results in decreased atherosclerotic lesion progression (48).

Interleukin-6 (IL-6) is a pro-inflammatory cytokine and increased plasma levels are associated with cardiovascular risk. IL-6 independently predicts cardiovascular mortality in elderly patients with established disease (49), and mortality in patients with unstable angina (50). IL-6 is the main inducer of hepatic production of acute-phase proteins and is associated with obesity, insulin resistance, hypertension and increased LDL levels, lifestyle risk factors known to increase systemic inflammation. For this reason, IL-6 is not as robust a marker as CRP, with most of its ability to predict CAD risk being attenuated after adjustment for other risk factors. The role that IL-6 plays in the development of atherosclerosis is largely as a mediator of immune cell recruitment to the lesion and activation of the inflammatory cytokine cascade

including tumor necrosis factor (TNF)-alpha, interleukin-1 and IL-6 can challenge the homeostasis of the vascular wall, leading to accumulation of cells and cholesterol.

1.3.6 The Search for New Biomarkers

Although one cannot ignore the utility of the established risk factors for CAD, clearly there is room for improvement in assessing the risk of atherosclerosis. A new understanding of the pathophysiology of atherosclerosis which includes the concept of inflammation triggered the investigation of inflammatory biomarkers, but even still, risk assessment and prediction is not ideal. For this reason, the scientific community continues to search for novel biomarkers that will aid in the prediction of atherosclerotic events and thus improve primary prevention of CAD.

1.3.7 Emerging Biomarkers

1.3.7.1 Adhesion molecules

Increased expression of adhesion molecules occurs upon endothelial cell activation and both intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) facilitate the leukocyte arrest on inflamed endothelium (51). Based on current studies, ICAM-1 does not appear to be useful as a biomarker for CAD, however, a prospective study of patients with non-ST elevation acute MI showed that soluble VCAM-1 was significantly higher at presentation in patients who has an adverse cardiovascular event at 6 months (52). Likewise, von Willebrand factor levels are increased in patients with acute coronary syndrome (ACS) and independently predict future events (53). E-selectin is another adhesion protein that is highly expressed upon endothelial cell activation. Several studies suggest that adhesion molecules of

this kind may be more informative in patients with stable CAD compared to those with ACS (52).

1.3.7.2 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent proteins with the ability to degrade collagen and/or gelatin. In advanced stages of atherosclerosis, smooth muscle cell recruitment to the lesion and increased collagen content provide stability to the atheroma and prevent rupture of the fibrous cap (52). MMPs are highly expressed within atherosclerotic plaques, in particular in the shoulder region, and degradation of collagen by MMPs promotes plaque rupture. High levels of MMP-10 are found within atheromas and are associated with inflammation and subclinical atherosclerosis (54).

MMP-9 levels are increased in patients with acute MI and unstable angina, as well as type 2 diabetics with established CAD (55). MMP-9 is also associated with decreased TIMP metallopeptidase inhibitor 1 (TIMP-1) levels, a MMP tissue inhibitor associated with premature development of atherosclerosis. Recent studies demonstrate a decrease in atheroma progression by tissue inhibitors of MMPs (56).

1.3.7.3 Oxidative Stress molecules

Oxidative stress is a significant contributor to the development of atherosclerosis, in part by promoting the modification of LDL particles. Recent studies suggest that oxidative stress molecules may be useful as prognostic markers (57).

The generation of oxidized LDL causes the production of reactive oxygen species which further modify the LDL particles and facilitate foam cell formation. Circulating levels of oxidized

LDL are increased in patients with ACS, and with the presence of CAD in a population of subjects undergoing elective angiography. Also, some prospective studies report that increased oxidized LDL levels predict unstable angina, non-fatal MI and cardiac death.

Myeloperoxidase (MPO) is secreted by neutrophils and monocytes in atheromas and its activity generates a host of reactive species which cause oxidative damage to vascular tissue and the atheroma. A prospective study of ACS patients showed that patients with elevated MPO levels were at increased risk of death or MI independent of troponin or CRP (58). Also, another study found that MPO was highly expressed in human plaques from patients who died of sudden cardiac death. These findings suggest that MPO may be involved in plaque destabilization and rupture. Furthermore, MPO independently predicted CAD in a population of patients undergoing selective coronary angiography and top tertile MPO levels predicted a 2.4-fold risk of cardiovascular mortality compared with patients in the lowest tertile (59).

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a serine lipase associated with circulating LDL-C levels. It is produced by macrophages and is found at high concentrations in atherosclerotic plaques (60). The GRACE study showed that independent of traditional risk factors of CAD, elevated Lp-PLA2 expression was associated with a 3-fold increased risk of death or MI in patients with ACS, independent of traditional risk factors (61).

1.3.7.4 Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are derived from bone marrow and give rise to mature endothelial cells. They are involved in the maintenance and repair of the vascular endothelial wall. In cases of decreased EPC mobilization from the bone marrow, endothelial dysfunction occurs and arteries are more prone to atherosclerosis. Low circulating EPC levels are associated with a higher frequency of thrombosis and vascular occlusion and Hill et al.

found that in healthy individuals, the number of EPCs negatively correlated with the Framingham Risk Score and was positively associated with vascular function, as measured by flow-mediated brachial artery reactivity (62).

1.3.7.5 Chemokines

1.3.7.5.1 What are chemokines?

Chemokines are a family of 8-10 kD homologous proteins that direct the migration of leukocytes from the bloodstream to sites of inflammation (63). Over 50 chemokines have been identified to date. They have been divided into four families based on the placement of the cysteine residues in the structure of the mature protein (63, 64). The CXC and CC families are the most extensively characterized, with the majority of the known chemokines falling into these two groups. The CXC and CC chemokines are known to contain 4 cysteines in their structure, with the CXC chemokines, containing two cysteine residues separated by another amino acid ("X"), and the CC chemokines containing two cysteine residues that are adjacent to each other. The third class of chemokines contains one member lymphotactin, which contains only 2 cysteines in its structure. The last chemokine family, CXXXC, also has as single member, fractalkine, which is one of two chemokines capable of acting in both a soluble and transmembrane form.

Chemokines are characterized by their ability to induce cell migration and activation by binding to G-protein-coupled receptors that are expressed by their target cells. Binding of a chemokine to its receptor activates multiple intracellular signaling events that collectively allow the cell to move in response to a chemokine concentration gradient (65). Receptor activation activates the cell leading to inositol triphosphate generation, release of intracellular calcium

and activation of protein kinase C (66). Chemokine-receptor binding also activates proteins of the Ras and Rho families which are involved in cell motility mechanisms (67). Although there are over 50 known chemokines only 8 chemokine receptors have been identified so far. Most chemokines are able to bind to more than one receptor and some chemokines receptors can bind to more than one chemokine. In addition to various chemokine-receptor binding interactions, some receptors are restricted to expression by only one cell type, whereas other receptors are expressed by multiple cell types (63). This means that one chemokine can act on more than one target cell. For example, Monocyte Chemoattractant Protein-1 (MCP-1) binds to CCR1 which is expressed by both monocytes and eosinophils; therefore, MCP-1 has the ability to induce chemotaxis of both cells. Furthermore, chemokine receptors can be constitutively expressed on some cells but expression of the same receptor may be inducible on others. For example, CCR1 and CCR2 are continuously expressed on monocytes, but expression of these receptors by lymphocytes occurs only after induction by interleukin-2 (68). For these reasons, the expression of a given chemokine receptor is a key regulator in the selectivity and amplification of chemokine activity.

Chemokines also bind to two other receptors that are not involved in cell signaling and migration: the Duffy antigen receptor for chemokines (DARC), and heparan and sulfate proteoglycans. The DARC receptor is expressed by red blood cells and endothelial cells. Binding to these receptors facilitates chemokine removal from the bloodstream, maintaining the recruitment of leukocytes by sustaining a chemokine concentration gradient between the bloodstream and the tissue (69). Heparan sulfate proteoglycans are carbohydrate structures that are found in the extracellular matrix and are attached to the surface of endothelial cells lining the blood vessels (70). They also function to maintain a chemokine concentration

gradient across the endothelial wall by binding to chemokines, therefore removing them from the bloodstream, and “presenting” them to circulating leukocytes (71, 72).

1.3.7.5.2 Role in Atherosclerosis

Atherosclerosis is an inflammatory disease in which a multitude of inflammatory cytokines and factors are secreted into the bloodstream. Chemokines are one of the many molecules that are overexpressed and secreted in inflammatory states. They play a significant role in the initiation and progression of the atheroma.

Activation of the vascular endothelium occurs in the initial stages of atherosclerosis. Normally, endothelial cells resist leukocyte recruitment and attachment, however, cardiovascular risk factors, in particular, the minimally oxidized LDL that has accumulated in the subendothelial space stimulates the overlying endothelial cells to produce chemokines, cytokines, cellular adhesion molecules and growth factors (54). This results in monocytes and T cells rolling along the vascular endothelium and attaching to the endothelial surface. Cellular adhesion molecules that are involved in the movement of leukocytes include ICAM-1, PCAM-1, VCAM-1, P-selectin and E-selectin (73-75). They mediate leukocyte arrest by binding to integrins on activated monocytes. The leukocytes migrate across the vascular wall into the subendothelial space, where they differentiate into macrophages and take up oxidized LDL to become foam cells (76). Within the intima, the lesion continues to develop with local production of pro-inflammatory cytokines and chemokines, promoting continuous infiltration of immune cells, and in later stages, the recruitment of smooth muscle cells from the media.

Increased levels of various chemokines have been reported in the circulation of patients with CAD, and were related to clinical outcomes. This suggests that chemokines are involved not only in plaque initiation and progression, but also in its destabilization and rupture. Of the

50 chemokines that have been described, previous investigations have found approximately 20 that are involved in atherogenesis. The potential roles of these chemokines are summarized in Tables 1.1 and 1.2, with the most studied chemokines described below.

1.3.7.5.2.1 MCP-1 (CCL2)

A key mediator of monocyte recruitment into the arterial wall is Monocyte Chemoattractant Protein-1 (MCP-1). It binds to chemokine receptor CCR2 which is expressed by monocytes and T cells. High levels of MCP-1 have been found within atherosclerotic lesions (77-79). Genetic deletion of either MCP-1 or CCR2 in atherosclerotic mouse models confirmed its involvement in the progression of atherosclerosis. MCP-1 deletion in an LDL-receptor deficient model showed a decrease in atheroma development (80) and deletion of CCR2 in apo E deficient mice prevented macrophage infiltration and lesion formation (81). Also, the administration of a CCR2 blocker decreased atherosclerosis (82, 83). Human studies also support a role for MCP-1 in CAD as shown by elevated serum levels found in patients with established CAD or at risk for CAD (84). Consistent with this is the discovery that a genetic variation in the MCP-1 gene, the -2578G allele, is associated with increased MCP-1 serum levels and an increased risk of MI (85). A genetic variation in the CCR2 gene has also been described, but there is conflicting evidence regarding the association of this polymorphism and the risk for MI (86-88). Associations between age, sex, hypertension, diabetes and MCP-1 have also been reported and MCP-1 was associated with subclinical CAD as measured by coronary artery calcium score although this relationship was lost after adjustment for traditional risk factors (89).

1.3.7.5.2.2 CCL19 and CCL21

Both CCL19 and CCL21 play a role in the recruitment of T cells and dendritic cells to lymphoid tissue and share the same receptor, CCR7. More recent studies have shown that these chemokines are involved in the inflammatory response in non-lymphoid tissues including vascular tissue. Both CCL19 and CCL21 were found at high levels in mouse and human atherosclerotic lesions and also in the plasma of patients with established CAD (90). Furthermore, increased expression of corresponding receptor, CCR7, was found in T cells localized to the plaque while decreased expression of CCR7 was found in the plasma of angina patients. The authors suggest that this difference in CCR7 expression may reveal that CCR7+ T cells are preferentially distributed to the lesion during plaque progression, leaving a higher frequency of CCR7- T cells in the circulation.

1.3.7.5.2.3 SDF-1 (CXCL12)

Stromal Cell Derived Factor-1(SDF-1) is a chemoattractant for monocytes and T cells. It is highly expressed in smooth muscle cells, endothelial cells and macrophages in atherosclerotic plaques, but not in healthy arteries (91). SDF-1 induces platelet aggregation, and in synergy with eotaxin induces the migration of smooth muscle cells from the internal elastic lamina into the intimal space (92). SDF-1 also triggers the proliferation of smooth muscle cells by activating matrix metalloproteinase-2 which is known to cause proliferation of SMC and contribute to later stages of plaque progression and instability (93).

1.3.7.5.2.4 IP-10 (CXCL10)

Interferon (INF) γ-induced Protein of 10kDa (IP-10) is expressed by all 3 cells types found in plaques, and very high levels of IP-10 were found in human atheromas (94). As its name

implies, expression of IP-10 is induced by INF-γ. It acts primarily on T cells and binding of IP-10 to T cell-expressed CXCR3 causes arrest on the endothelium and subsequent transmigration across the vascular wall. Deletion of CXCR3 in ApoE -/-deficient mice caused reduced atheroma formation, and lead to an increase in anti-inflammatory molecules Il-10, IL-18 and endothelial nitric oxide synthase (95). Also, IP-10 deficiency resulted in reduced lesion formation and reduced number of T cells in the lesions (96).

1.3.7.5.2.5 SR-PSOX (CXCL16)

CXCL16 is also known as Scavenger Receptor PSOX (SR-PSOX). It is expressed by dendritic cells, macrophages and smooth muscle cells. SR-PSOX acts in both a soluble and transmembrane form, and was shown to mediate uptake of oxidized LDL by macrophages (97, 98). It also directs migration of activated T cells by binding to CXCR6. High levels of SR-PSOX are found in human atheromas although, interestingly, decreased SR-PSOX levels are associated with CAD (99). Studies where SR-PSOX was deleted showed accelerated atherosclerosis in LDLR-/- mice (100). This was associated with increased macrophage recruitment, increased MCP-1 and TNF-α levels in atheromas suggesting that SR-PSOX may have an atheroprotective role.

1.3.7.5.2.6 Fractalkine (CX_3CL1)

Fractalkine is the only member of the CX_3C family that has been identified to date. Like SR-PSOX, it is found in both soluble and transmembrane forms which allow it to act as a chemoattractant and an adhesion molecule (101). In its transmembrane form it is found on activated endothelial cells, where it acts as an adhesion molecule mediating arrest of CX3CR1-expressing cells such as T cells, monocytes and natural killer cells. As a soluble protein,

fractalkine induces the migration of these same cell types. Fractalkine has been found in atherosclerotic lesions, but not in healthy vessels (102, 103). Disruption of the gene coding for fractalkine, or its receptor, CX_3CR1, showed decreased lesion formation and reduced monocyte infiltration to the lesion in an Apo E-/- deficient mouse model (104, 105). Furthermore, a polymorphism in the fractalkine receptor, V249I, was associated with a lower risk of CAD (106, 107). This finding is controversial as another study found no association of the V249I polymorphism with disease but identified another fractalkine polymorphism, T280M, to be protective for ACS (108).

1.3.7.5.2.7 RANTES (CCL5)

RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES), is a soluble chemokine of 7.8 kDa (109). It is secreted from a variety of cell types including activated endothelial cells, smooth muscle cells, T cells and monocytes/macrophages (110, 111). RANTES is also secreted by activated platelets to mediate monocyte arrest. This action can be amplified by co-secretion and interaction with platelet factor 4 (112). RANTES is a chemoattractant for CCR1 and CCR5-expressing T cells and monocytes. Interaction with its receptors triggers leukocyte arrest on the vascular endothelium and migration into the subendothelial space leading to atheroma development. RANTES is highly expressed in human atheromas (113). Numerous *in vitro* studies have shown that CCR1 and CCR5, despite sharing the same ligand, have distinct functions. Studies have reported that CCR5 does not affect early stages of lesion formation in atherosclerosis but rather is involved in late-stage disease (114, 115). By using nonpeptide antagonists or antibodies to block each receptor, it has been shown that RANTES-induced leukocyte arrest is mediated primarily by CCR1, while CCR5 plays a larger role in spreading of the leukocytes and their eventual transmigration across the vascular endothelium

(116, 117). The involvement of CCR5 in plaque destabilization and rupture is also speculated. The unique role of these receptors is further supported by animal studies that utilize atherosclerotic mouse models deficient in either CCR1 or CCR5. In an apolipoprotein E-deficient (ApoE -/-) and CCR5-deficient mouse model, CCR5 deficiency resulted in a decrease in lesion formation and macrophage and T cell accumulation, and increased smooth muscle cell content in the lesion. Increased expression of anti-inflammatory cytokine interleukin-10 (IL-10) in the lesion was also noted (118). Similar results were seen in *in vivo* studies using LDL receptor-deficient (LDLR -/-) mice transplanted with either CCR5 +/+ or CCR5 -/- bone marrow. After 8-12 weeks on a high fat diet, there was a 30% reduction in macrophage plaque content, albeit there was no change to the overall size of the lesion. A 52% reduction in the expression of MMP-9, a 2-fold increase in collagen accumulation and an increase in the IL-10 expression was seen in the CCR5 deficient model compared to LDLR -/- CCR5+/+, although by 35 weeks these differences were not sustained (119). Weber et al. compared the effects of CCR5 deficiency and CCR1 deficiency in an ApoE -/- model and found that like other studies, CCR5 deficiency lead to decreased lesion formation, decreased macrophage and T cell accumulation, and increased plaque stability via increased smooth muscle cell content, IL-10 expression and decreased MMP-9 levels (120). They also demonstrated that ApoE-/-CCR1-/- mice showed an *increase* in overall plaque area, increased T cell content and expression of pro-inflammatory INF-γ (120). Another study showed decreased lesion formation after blocking RANTES signaling *in vivo* via injection of Met-RANTES, a chemokine antagonist for CCR1 and CCR5, into LDLR-/-mice (121). Although both CCR1 and CCR5 are involved in the development of atherosclerosis, they have opposite effects and CCR5 is recognized as the more important of the two RANTES receptors, from the point of view of lesion progression and as a potential therapeutic target.

Further supporting the role of RANTES/CCR5 in atherogenesis are the polymorphism studies which have revealed associations between RANTES/CCR5 and clinical outcomes of CAD. A 32-base pair deletion in the human CCR5 gene (CCR5Δ32), causing complete loss of function, was associated with a reduced risk of early MI and CAD (88, 122). However, in the Nurses' Health Study, no association between CCR5Δ32 and CAD risk was found (123). Two polymorphisms in the RANTES gene have also been identified: -28 C to G and -403 G to A in the promoter region. Simeoni et al. report that -403A was associated with the presence of CAD, cardiovascular independent of traditional risk factors and CRP (124). Furthermore, Boger et al. reported that the -403A allele is associated with all-cause mortality in type 2 diabetes mellitus patients undergoing hemodialysis (125).

The large body of experimental evidence supporting the role of RANTES and CCR5 in atherosclerosis has prompted the study of RANTES as a biomarker of CAD. A few case-control studies have evaluated the prognostic utility of RANTES to predict CAD and cardiovascular mortality though results of these studies are conflicting particularly in populations of patients with stable disease. Increased serum RANTES levels in acute coronary syndromes compared to healthy controls, or in patients with established disease have been repeatedly been reported (126-128). This is discussed in relation to increased platelet activation and inflammation commonly found in ACS patients, resulting in increased secretion of RANTES into the circulation. In one study, after adjustment for traditional risk factors, RANTES levels predicted future cardiovascular events after 18 month follow up of ACS patients. The ability of RANTES to independently predict the presence of stable CAD and cardiovascular mortality is less clear. In a study comparing RANTES levels between 27 patients with ACS, 20 patients with stable CAD and 15 healthy controls, RANTES levels were the highest in the individuals with ACS, however there

was no difference between patients with stable CAD and healthy controls (129). Another study investigated RANTES levels and its association with coronary intima-media thickness (IMT) score among 60 patients with stable CAD and 30 age-matched healthy controls and found that RANTES levels were not different between the two groups (130). Interestingly, a Korean study reported increased serum RANTES levels in 151 men with stable CAD compared to healthy controls. In this study, RANTES was associated with other risk factors for CAD including CRP (131). Lastly, Rothenbacher et al. reported that serum levels of RANTES were lower in patients with stable CAD compared to healthy controls and were associated with a lower Odds Ratio (OR) for CAD (132). Most recently, Covusoglu et al. reported that low RANTES plasma levels were predictive of mortality in a population of subjects with angiographically-confirmed stable CAD after 24 month follow up (133). Furthermore, in this same study, baseline RANTES levels were predictive of MI in patients with stable CAD.

Table 1.1 Chemokines Involved in Atherosclerosis

Chemokine	Synonyms	Receptor	Source	Cell type Recruited
CCL2	MCP-1	CCR1 CCR2	MΦ, TL	Mc, MΦ
CCL3	MIP-1 alpha	CCR1 CCR4 CCR5	MΦ, TL, NK, P	MΦ, Eo, N, BL
CCL4	MIP-1 beta	CCR1 CCR5 CCR8	TL, NK	Mc, MΦ, Eo, TL
CCL5	RANTES	CCR1 CCR3 CCR4 CCR5	TL, P	Mc, TL, Eo
CCL11	Eotaxin	CCR2 CCR3 CCR5	EC	Mc, E, SMC
CCL13	MCP-4	CCR1 CCR2 CCR3 CCR5	EC	Eo, TL, Mc
CCL17	TARC	CCR4 CCR8	TL, EC, P, BL	TL, Eo
CCL18	MIP-4, PARC	CCR3	Mc/MΦ, DC, Eo	TL, BL
CCL19	MIP-3 beta	CCR7	TL, SMC, DC	TL, BL, DC
CCL22	MDC, STCP-1	CCR4	MΦ, TL, EC, DC, BL	TL, DC, Eo, NK
CXCL1	GRO-alpha	CXCR2	MΦ, P	N
CXCL4	PF-4	CXCR3	P	L
CXCL8	IL-8	CXCR1 CXCR2	EC, Eo, MΦ, SMC	N, Eo, TL, BL
CXCL10	IP-10	CXCR3	TL, SMC	Mc, TL, SMC
CXCL11	IP-9, ITAC	CXCR3	EC, Mc, N	TL, NK
CXCL12	SDF-1	CXCR4	B, SMC, EC	CD34+, Mc, TL, BL, MC
CXCL16	SR-PSOX	CXCR6	MΦ, TL, SMC, DC	TL
CX_3CL1	Fractalkine	CX_3CR1	EC, NC	Mc, SMC, N, MC

BL: B Lymphocyte, B: Basophil, DC: Dendritic cell, EC: Endothelial cell, Eo: Eosinophil, MΦ: Macrophage, MC: Mast cell, Mc: Monocyte, NK: Natural Killer cell, N: Neutrophil, P: Platelet, SMC: Smooth Muscle cells, TL: T Lymphocyte

Table 1.2 Chemokine Plasma/Serum Levels in CAD

Chemokine	Expression
CCL2	Increased levels in AMI, unstable angina and ischaemic stroke
CCL5	*Conflicting evidence* Increased levels in AMI and ACS Decreased levels in stable CAD Low levels predict mortality in stable CAD
CCL11	Contradicting evidence on increased/decreased levels
CXCL4	Increased levels in PVD and CAD
CXCL8	Increased levels in stable CAD and AMI
CXCL10	Increased levels in stable CAD
CXCL12	Decreased levels in unstable angina
CXCL16	Decreased levels in CAD
CX_3CL1	Increased levels in ACS

AMI: Acute Myocardial Infarction, ACS: Acute Coronary Syndromes, CAD: Coronary Artery Disease, PVD: Peripheral Vascular Disease

1.4 Hypotheses

In light of the data supporting the role of chemokines in atherosclerosis and their use as potential biomarkers for CAD, we chose to evaluate RANTES levels in a large, long-term follow up cohort of patients with stable CAD to clarify its utility as a biomarker for stable CAD.

We hypothesized that:

1. RANTES levels are associated with the presence of CAD, and the severity of CAD as indicated by the number of vessels affected.
2. RANTES levels independently predict all-cause and cardiovascular mortality.
3. RANTES levels are associated with traditional and non-traditional markers of CAD.

2 METHODS

2.1 Study Design

The study population consisted of 1075 consecutive individuals referred to Vancouver General Hospital and Saint Paul's Hospital for Selective Coronary Angiography (SCA) recruited between 1993 and 1995. At the time of the angiography, fasting blood samples were collected in EDTA, centrifuged and stored at -70°C for future use. This study was approved by the Saint Paul's Hospital Ethics Board, and all subjects signed an informed consent form.

2.1.1 Selective Coronary Angiography

Angiograms were assessed semi-quantitatively by a cardiologist and were scored as having no lesions, a lesion of less than 50% or greater than 50% in one, two or three coronary arteries. Those with greater than 50% obstruction were considered positive for coronary artery disease and subjects with less than 10% obstruction were classified as negative. The extent of diseased was indicated by the number of diseased coronary arteries ranging from 1-3.

2.1.2 Lifestyle and Clinical Variables

At the time of angiography, all subjects were requested to complete a two-page questionnaire addressing ethnicity, and lifestyle factors (smoking, drinking and exercise habits). Weight, height, waist circumference and blood pressure were measured at this time. The subjects were also asked for any personal and family history of cardiovascular disease, diabetes, hypertension or renal insufficiency. Medications were recorded off of their chart.

2.2 Biochemical Markers of Coronary Artery Disease

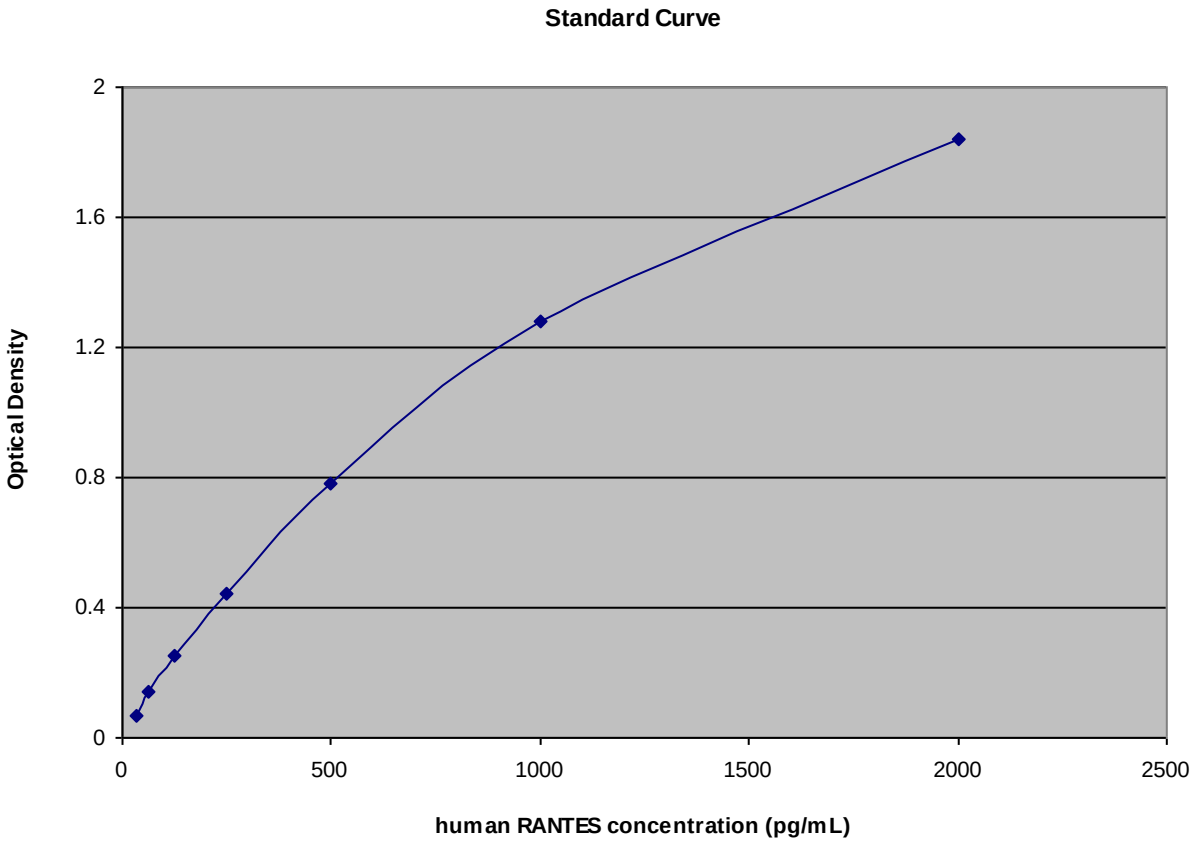
2.2.1 Lipid and Apoproteins

Immediately after blood collection, lipid and lipoprotein analyses were performed on each blood sample. Total cholesterol, triglycerides, high density lipoprotein, apolipoprotein B and apolipoprotein A1 were measured as previously described (ref). Low density lipoprotein cholesterol was calculated using the Friedewald equation for individuals with triglycerides less than 4mmol/L.

2.2.2 RANTES/CCL5

In 2007, previously frozen plasma samples were thawed and aliquoted into smaller volumes to avoid multiple freeze-thaw cycles. Each sample was centrifuged at 4°C for 10 minutes at 10,000g to ensure complete removal of platelets, and then diluted appropriately to ensure the sample would fall within the linear range of the standard curve for the RANTES assay. All samples were assayed in duplicate using a commercial sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit for RANTES/CCL5 purchased from R&D Systems. The sensitivity of this assay was 2 pg/mL, and it had an intra-assay coefficient of variation (CV) of 3.2%, and inter-assay CV of 9.1%. Assay precision was calculated using pooled control plasma that was assayed on each microplate as quality control. Each plate was read using the Tecan Safire absorbance reader, and a standard curve was created by generating a four parameter logistic curve-fit using GraphPad 4.0 software (Figure 1). Concentrations for each sample were obtained from the standard curve and then multiplied by the dilution factor to attain the final plasma concentration.

Figure 2.1 RANTES Standard Curve



Concentration (pg/mL)	Absorbance
31.25	0.069
62.5	0.139
125	0.25
250	0.443
500	0.78
1000	1.281
2000	1.842

2.3 Follow-up Study

In 2008, mortality data were collected from the British Columbia Vital Statistics Agency to identify those subjects who died and the cause of death. The mean follow up time was 11.1

years, and mortality data includes all deaths that occurred from the time of recruitment to the end of 2007.

2.4 Ethics Approval

A research proposal to measure inflammatory markers in the previously collected plasma of these patients was submitted to Research Services at Saint Paul's Hospital. Approval was granted by the Ethics Committee.

2.5 Statistical Analyses

All statistical analyses were performed using SPSS 12.0 software. Because the distribution of RANTES was found to be skewed, logarithmically transformed values were used for all parametric tests. Summary statistics for continuous variables were presented both as means with standard deviations, as well as medians with inter-quartile ranges, and comparisons between groups were performed using the Mann-Whitney test. Categorical data were summarized as frequencies and percentages, and comparisons between groups were performed with Chi-square test. Univariate Pearson correlation was performed to assess associations between RANTES and other markers of CAD. Logistic regression analyses were used to determine whether RANTES levels could independently predict the presence of angiographic CAD, and Cox regression analysis was used to evaluate the ability of RANTES to predict all-cause or CAD-related death. Death was considered CAD-related if the underlying cause of death was caused by ischemic heart disease according to the International Classification of Disease, 10th revision (ICD-10-CM codes I20–I25). Kaplan-Meier curves with the log-rank test were used to assess differences in survival rates across quartiles of RANTES. Significance for all tests was defined as $p \leq 0.05$ and all tests were two-tailed.

3 RESULTS

3.1 Characteristics of the Study Population

This population was a predominantly male population, with 754 males (%) and 285 (%) females. The average age was 59.7 years and 63.4 years for men and women, respectively. The majority of this population is Caucasian (n=852, 74.9 %), and the remaining subjects are of Chinese descent (n=101, 8.9%) or South Asian descent (n=86, 7.6%).

3.1.1 Characteristics of patients based on the presence of CAD

The presence of coronary artery disease was defined as having one or more vessels with greater than or equal to 50% stenosis, and 74.3% of the population (n= 691) satisfied this criterion. Subjects with less than 10% stenosis in one or more vessels were considered negative for CAD (n= 239, 25.7%).

General characteristics of the subjects as stratified by the presence of CAD are shown in Table 3.1 and include age, gender, smoking status, drinking habits, diabetes, hypertension, lipid profile and other biochemical markers. As expected, a greater proportion of the patients with CAD were male. Of 691 patients with CAD, 550 (74%) were male, and 141 (26%) were female. The mean age for subjects with CAD was 60.9 years for men, and 64.9 years for women.

Subjects with CAD also had lipid measurements that put them at increased risk: they had significantly lower HDL cholesterol levels (mean 0.94 ± 0.01 mmol/L), higher triglyceride levels (median & interquartile range, 1.61 [1.19-2.22] mmol/L) and higher levels of apolipoprotein B (0.98 ± 0.01 g/L) compared to those without CAD. There was not a significant difference in LDL cholesterol and total cholesterol levels between the two groups.

Subjects with CAD also had higher levels of inflammatory markers, namely CRP, interleukin-6 as well as total homocysteine. RANTES levels were not significantly different between the two groups. Because of the lack of normal distribution of inflammatory marker levels in this population, plasma levels are reported as median and interquartile range.

Smoking and drinking habits were self-reported at the time of recruitment. Subjects were considered non-smokers or smokers/ex-smokers. There were more smokers/ex-smokers in the group of subjects with CAD, and a greater proportion of the smokers were male. Furthermore, the incidence of type 2 diabetes mellitus in the diseased group was also greater. 19% of the patients with CAD also had diabetes, compared to 8% in the group of patients without CAD. There were no significant differences in the incidence of hypertension or drinking habits between the groups.

Table 3.1 Baseline characteristics of patients by the presence of CAD

	Patient Group; mean (SD)*		
Variable	**CAD - N = 239**	**CAD + N = 691**	***p* value**
Age	57.5 (0.80)	61.8 (0.40)	<0.001
TC, mmol/L	5.06 (0.08)	5.17 (0.04)	0.193
LDL-C, mmol/L	3.69 (0.07)	3.54 (0.04)	0.051
HDL-C, mmol/L	1.03 (0.02)	0.94 (0.01)	<0.001
Triglycerides, mmol/l, media (IQR)	1.31 (0.96-1.96)	1.60 (1.19-2.22)	<0.001
Apolipoprotein B, g/L	0.91 (0.02)	0.98 (0.01)	0.001
CRP, mg/L, median (IQR)	1.96 (0.8-4.6)	2.00 (0.9-5.4)	0.039
SAA, ug/L, median (IQR)	250 (99-579)	279 (106-775)	0.185
IL-6, ng/L, median (IQR)	2.14 (1.25-3.49)	2.37 (1.47-3.96)	0.028
tHcy, umol/L, median (IQR)	13.3 (10.5-16.8)	14.3 (11.5-17.8)	0.004
RANTES, ng/mL, median (IQR)	21.8 (12.7-35.5)	23.0 (13.9-40.0)	0.508
Categorical Variables, no. (%)			
Women	112 (47)	141 (26)	<0.001
Men	127 (53)	550 (74)	
Smoking status			
Has never smoked	101 (43)	165 (25)	<0.001
Has smoked or does smoke	134 (57)	508 (75)	
No diabetes	220 (92)	560 (81)	<0.001
Diabetes	19 (8)	131 (19)	
No hypertension	141 (62)	394 (61)	0.841
Hypertension	88 (38)	256 (39)	
Alcohol consumption			
Never	69 (29)	174 (25)	0.439
1-5 drinks/week	114 (48)	365 (53)	
6-10 drinks/week	40 (17)	117 (17)	
>10 drinks/week	16 (7)	35 (5)	

Note: SD= standard deviation, TC= total cholesterol, LDL-C= low density lipoprotein cholesterol, HDL-C= high density lipoprotein cholesterol, IQR= interquartile range, CRP= C-reactive protein, SAA= serum amyloid A protein, tHcy= total homocysteine.
*Unless stated otherwise.
† 831 plasma samples were available for RANTES measurement
‡ Percentages are based on row totals.

3.1.2 Characteristics of patients based on outcome

Mortality data for this population were obtained from the BC Vital Statistics Agency. After a median follow-up of 12.9 years, 313 deaths occurred of which 132 were cardiovascular-related. Of the 831 patients for whom plasma RANTES levels were measured, 256 died (30.8%) and 110 of these deaths were due to ischaemic heart disease.

Comparison of the baseline characteristics of the survivors with those deceased at follow-up with patients is shown in Table 3.2. The deceased patients were significantly older (p<0.001), and the frequency of diabetes was higher in this group (15% versus 21 %, p=0.041). There were very few differences in lipid parameters, although triglyceride levels were found to be significantly different, with levels being increased in patients alive at follow-up. There were, however, significant differences between levels of other inflammatory markers. Baseline levels of CRP, serum amyloid A, IL-6 and total homocysteine levels were significantly higher in deceased patients compared to those alive at follow-up (p<0.001 in all cases). In a previous study using the same population, increased levels of IL-6 and total homocysteine were independent predictors of CAD-related death (45).

Univariate analysis of plasma RANTES levels showed no difference between patients who were alive and dead at follow-up. Patients who were alive at the time of follow-up had median plasma levels of 22.9 ng/mL (13.2-39.2) while those who were deceased had median levels of 21.7 ng/mL (12.9-33.2), (p=0.206).

Table 3.2 Baseline characteristics of patients by outcome

	Patient Group; mean (SD)*		
Variable	**Alive N = 824**	**Dead N = 313**	***p* value**
Age	58.7 (0.39)	65.6 (0.62)	<0.001
TC, mmol/L	5.20 (0.04)	4.97 (0.07)	0.05
LDL-C, mmol/L	3.59 (0.04)	3.48 (0.06)	0.116
HDL-C, mmol/L	0.96 (0.009)	0.95 (0.02)	0.510
Triglycerides, mmol/l, media (IQR)	1.55 (1.13-2.19)	1.53 (1.06-2.04)	0.094
Apolipoprotein B, g/L	0.97 (0.01)	0.94 (0.01)	0.057
CRP, mg/L, median (IQR)	1.73 (0.79-4.53)	2.87 (1.14-6.49)	<0.001
SAA, ug/L, median (IQR)	234 (95-633)	385 (141-994)	<0.001
IL-6, ng/L, median (IQR)	2.05 (1.29-3.35)	2.90 (1.93-4.69)	<0.001
tHcy, umol/L, median (IQR)	13.5 (10.9-16.5)	15.8 (12.4-19.7)	<0.001
RANTES, ng/mL, median (IQR)	23.1 (13.2-39.6)	21.8 (12.9-36.5)	0.206
Categorical Variables, no. (%)			
Women	189 (26)	96 (32)	0.56
Men	547 (74)	207 (68)	
Smoking status			
Has never smoked	219 (30)	76 (26)	0.129
Has smoked or does smoke	501 (70)	221 (74)	
No diabetes	632 (86)	235 (76)	0.002
Diabetes	104 (14)	68 (24)	
No hypertension	471 (64)	173 (57)	0.041
Hypertension	265 (36)	130 (43)	
Alcohol consumption			
Never	194 (27)	82 (28)	0.995
1-5 drinks/week	383 (53)	153 (52)	
6-10 drinks/week	122 (17)	52 (18)	
>10 drinks/week	21 (3)	9 (2)	

Note: SD= standard deviation, TC= total cholesterol, LDL-C= low density lipoprotein cholesterol, HDL-C= high density lipoprotein cholesterol, IQR= interquartile range, CRP= C-reactive protein, SAA= serum amyloid A protein, tHcy= total homocysteine.
*Unless stated otherwise.
† 831 plasma samples were available for RANTES measurement
‡ Percentages are based on row totals.

3.2 RANTES and patients characteristics

Associations between RANTES levels and patient characteristics were investigated using the nonparametric Mann Whitney U test. Patients with a family history of CAD were found to have higher plasma levels of RANTES than did patients without family history (30.3 ng/mL versus 28.1 ng/mL; p=0.006). A difference in RANTES levels between genders was also observed. The mean RANTES level for women was significantly higher than the mean level observed in men; 27.7 ng/mL and 34.2 ng/mL for men and women respectively, p<0.001.

No relationship between RANTES levels and the presence of diabetes was found. Similarly, a relationship between RANTES levels and premature development of CAD was not significant.

3.3 RANTES and other markers of CAD

Associations between RANTES levels and other known markers of CAD were investigated using a bivariate Pearson correlation analysis. A significant correlation between RANTES and a number of other markers was found, although the strength of these associations was weak. Table 3.3 lists the markers for which there was a significant relationship, as well as the correlation coefficient, r. The strongest correlation was found between RANTES and oxidized LDL cholesterol levels. Although this correlation was statistically significant with a *p* value of <0.001, the correlation coefficient was only 0.145. Correlation coefficients with values ranging from 0 to 0.20 indicate negligible correlation, suggesting that RANTES plasma levels are not related to the other markers of CAD measured in this population.

Table 3.3 Correlations of RANTES levels with other markers of CAD

Variable	Correlation Coefficient	*p* value
Oxidized LDL-C	0.145	<0.001
Total cholesterol	0.123	<0.001
Apolipoprotein B	0.116	0.001
CRP	0.107	0.002
HDL-C	0.103	0.003
MPO	0.097	0.005
Family history of CAD	0.096	0.005
SAA	0.092	0.008
Triglycerides	0.087	0.012
History of PVD	0.071	0.042

3.4 RANTES and the presence of coronary artery disease

The predictive value of RANTES plasma levels for angiographic CAD was assessed using a multivariate logistic regression model adjusting for traditional markers of CAD. Table 3.4 lists the odds ratios and 95% confidence intervals for the markers included in the model. Angiographic CAD was defined as the presence of one or more lesions with greater than or equal to 50% stenosis. Age, gender, smoking status and diabetes were independent predictors of CAD. Triglycerides and apolipoprotein B levels were the only plasma biomarkers that were independent predictors of CAD, with odds ratios of OR 2.65 [95% CI: 1.05-6.69] and OR 2.65 [95% CI: 1.01-6.93], respectively. RANTES levels were associated with angiographic CAD, OR 1.67 [95% CI: 1.00-2.76], p=0.047, although this association was moderate. Interestingly, when divided into quartiles and using the first quartile as the reference category, RANTES level above the fourth quartile were associated with a greater risk of angiographic CAD, OR 1.73 [95% CI: 1.05-2.86], p=0.031.

Table 3.4 Odds Ratios for the presence of CAD as indicated by a multivariate logistic regression model

Variable	Odds Ratios (95% CI)	*p* value
Age	1.05 (1.03-1.07)	<0.001
Gender	4.59 (3.05-6.90)	<0.001
Smoking status	1.96 (1.36-2.84)	<0.001
Diabetes mellitus	1.68 (1.03-2.74)	0.039
TC/HDL-C	1.01 (0.90-1.14)	0.842
Triglycerides*	2.65 (1.05-6.69)	0.039
Apolipoprotein B	2.65 (1.01-6.93)	0.047
RANTES*	1.67 (1.00-2.76)	0.047

*Analysis was performed using log transformed values due to a non-normal distribution of these variables

3.5 RANTES and extent of disease

The ability of RANTES levels to predict the extent of CAD, as defined by the number of diseased vessels, was investigated using a multivariate logistic regression model adjusting for traditional markers of disease. Table 3.5 shows the distribution of the number of vessels affected in the cohort. Approximately 21% of the subjects for whom RANTES was measured did not have CAD, and the number of diseased vessels for 2.2% of this subgroup remains unknown. The remaining subjects were almost evenly distributed between single, double and triple vessel disease, with triple vessel disease being slightly more predominant.

Table 3.5 Distribution of the number of diseased vessels in the group of subjects for whom RANTES levels were measured

No. of diseased vessels	N	Percent (%)
0	171	20.6
1	214	25.8
2	179	21.5
3	249	30.0
Unknown	18	2.2
TOTAL	**831**	**100**

Predictors of the number of vessels affected by CAD are listed in Table 3.6. Gender, age and smoking status were highly significant predictors of single, double and triple vessel disease. Apolipoprotein B was an independent predictor of single and triple vessel disease ($p=0.007$ and $p=0.0029$, respectively), while triglyceride levels independently predicted both single and double vessel disease, $p=0.001$ and $p=0.003$, respectively). Similarly, diabetes was a predictor of single and triple vessel disease, $p=0.016$ and $p<0.001$, respectively). RANTES plasma levels did not predict the number of diseased vessels in this study population.

Table 3.6 Odds ratios for the number of diseased vessels as indicated by a multivariate logistic regression model

	Odds Ratio (95% CI)	*p* value
1 vessel disease		
triglycerides*	8.87 (2.35-33.5)	0.001
apoB	6.28 (1.67-23.6)	0.007
gender	5.37 (2.9-9.7)	<0.001
age	1.03 (1.01-1.06)	0.007
smoking status	2.83 (1.65-4.86)	<0.001
diabetes	2.56 (1.20-5.49)	0.016
RANTES*	1.25 (0.61-2.57)	0.548
2 vessel disease		
triglycerides*	7.65 (1.96-29.94)	0.003
gender	8.08 (4.26-15.33)	<0.001
age	1.06 (1.03-1.09)	<0.001
smoking status	3.12 (1.77-5.51)	<0.001
RANTES*	1.03 (0.49-2.18)	0.931
3 vessel disease		
apoB	4.31 (1.17-15.92)	0.029
gender	10.83 (6.02-19.47)	<0.001
age	1.08 (1.05-1.10)	<0.001
smoking status	1.94 (1.18-3.21)	0.009
diabetes	4.27 (2.07-8.80)	<0.001
RANTES*	1.32 (0.66-2.64)	0.432

*Analysis was performed using log transformed values due to a nonnormal distribution of these variables

3.6 Predictive value of RANTES for all-cause and cardiovascular mortality

After a mean follow-up of 11.1 years, 256 of the 831 subjects for whom RANTES plasma levels were measured were deceased. 110 of these deaths were due to ischaemic heart disease. Using a Cox regression model adjusted for confounding variables, we assessed the ability of RANTES to predict cardiovascular and all-cause death.

Table 3.7 lists the variables that were included in the Cox regression model for both all-cause and cardiovascular mortality. Only age and smoking status were independent predictors

of death due to any cause (p<0.001 and p=0.015, respectively). Being a smoker/ex-smoker was associated with a significant increase in the risk of death indicated by an odds ratio of 1.55 [95% CI: 1.09-2.21]. RANTES levels were analyzed as both a continuous and categorical variable, and in neither case were they significant predictors of all-cause mortality.

In a second Cox regression model investigating predictors of death due to cardiovascular disease, age was found to be the only variable able to independently predict mortality (p<0.001). RANTES levels were not a significant predictor of cardiovascular death (p=0.669), and performing the analysis using quartiles of RANTES as a categorical variable did not change these results.

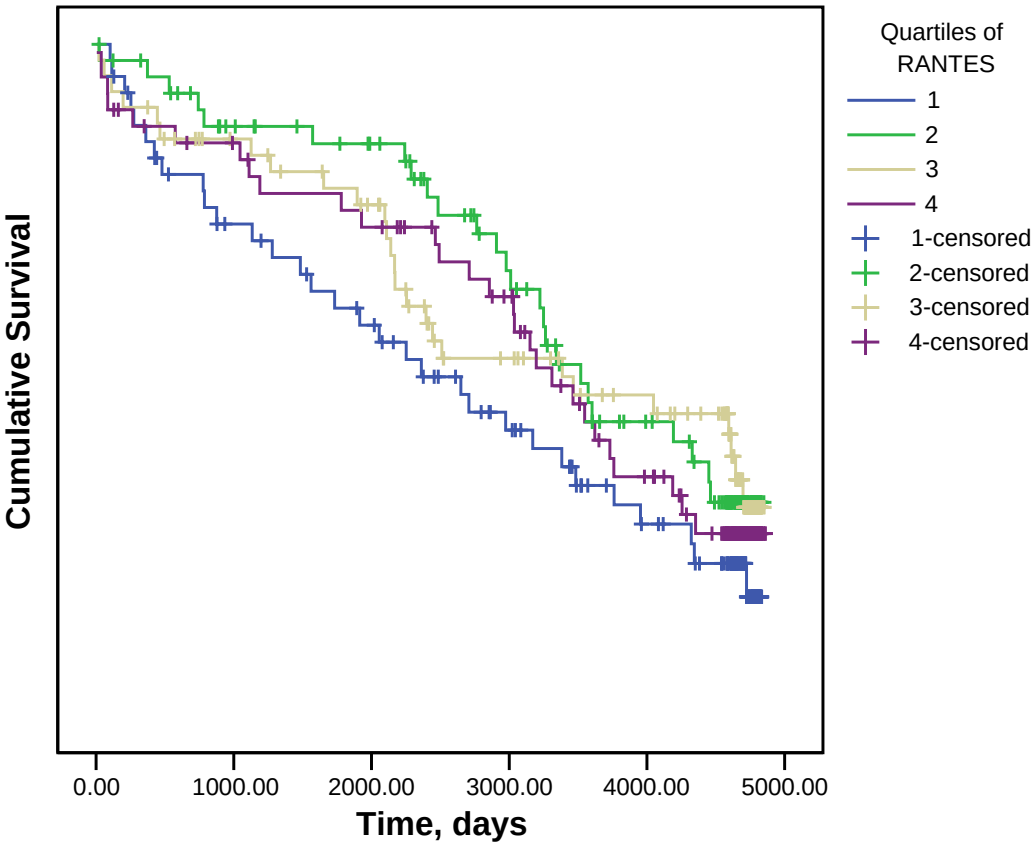
Table 3.7 Cox regression analysis indicating predictors of all-cause and cardiovascular mortality associated hazard ratios

Variable	Hazard Ratio (95% CI*)	*p* value
Predictors of all-cause mortality		
Age	1.06 (1.04-1.07)	<0.001
Gender	1.08 (0.80-1.45)	0.630
Smoking status	0.73 (0.54- 0.98)	0.034
Diabetes mellitus	0.68 (0.51- 0.92)	0.013
Triglycerides*	0.62 (0.34-1.12)	0.111
RANTES*	0.87(0.60-1.26)	0.469
Predictors of cardiovascular mortality		
Age	1.07 (1.04-1.09)	<0.001
RANTES*	0.96 (0.56-1.64)	0.869

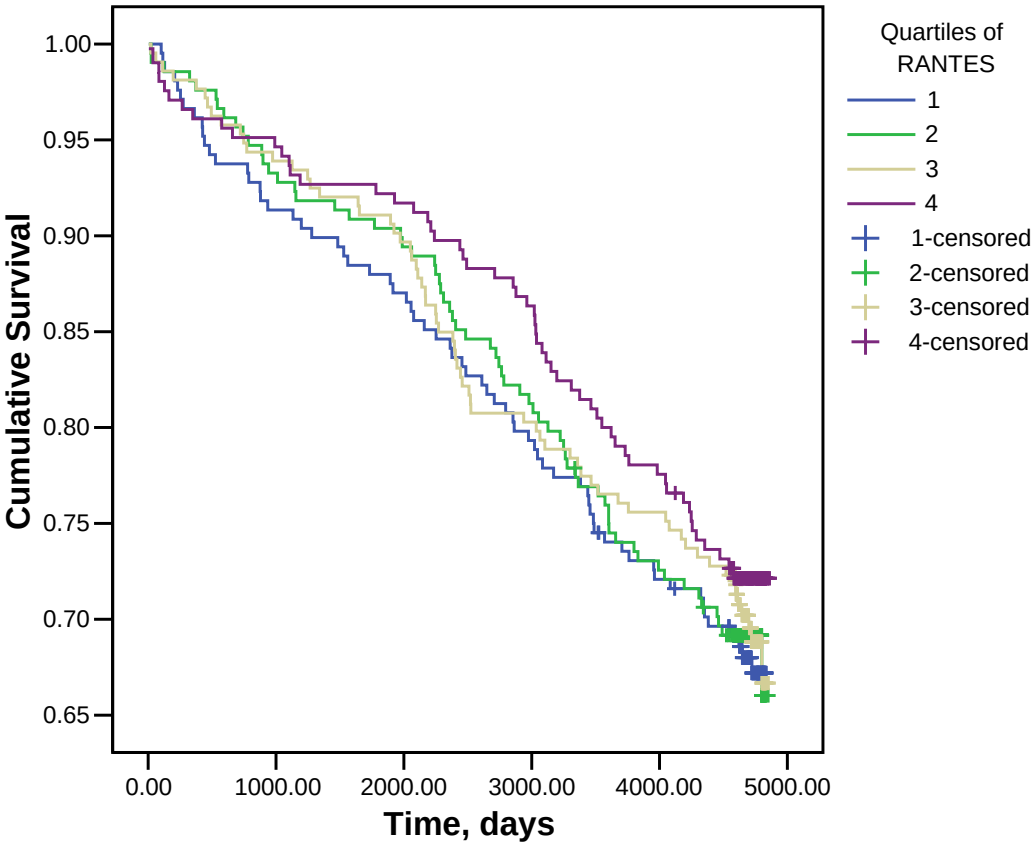
Survival analysis was performed to compare survival rates between groups of patients stratified by quartiles of RANTES levels. Figure 3.2 shows Kaplan-Meier curves for all-cause and cardiovascular mortality according to quartiles of RANTES levels.

Figure 3.2 Kaplan-Meier survival curves for all-cause mortality (A) and cardiovascular mortality (B) across quartiles of RANTES levels

(A)



(B)



The trend in both sets of curves suggests that patients in the lowest quartile of RANTES had increased survival, although there is considerable overlapping of the survival curves. To test for equality between survival rates the log rank test was performed using the first quartile as the reference group. Significant differences between survival rates based on quartiles of RANTES levels were not found as indicated by *p* values 0.056 and 0.062 for all-cause and cardiovascular mortality, respectively.

4 DISCUSSION

Based on the extensive literature documenting the association between inflammatory disease and chemokine levels, we chose to investigate the utility of RANTES plasma levels in predicting cardiovascular mortality in a cohort of patients with stable CAD after 13 years of follow-up. The rationale for this work was based on numerous studies that suggest the involvement of RANTES in atherosclerosis. It has been shown that RANTES mediates the arrest and migration of monocytes to inflamed vascular endothelium and is highly expressed within atheromas themselves. A large body of *in vivo* studies involving mouse models deficient in the RANTES receptor CCR5 showed a reduction in the development of atherosclerosis. Despite this, data defining the utility of RANTES as a biomarker for coronary artery disease is limited and existing studies present conflicting results, particularly in populations of patients with stable disease.

We hoped that by using a large cohort of patients that includes both men and women with stable CAD and by following them for a longer period of time than previously published work that we would be able to provide some clarification on the utility of RANTES as a biomarker for CAD. We expected that plasma RANTES levels would be associated with the presence of CAD and the extent of disease as indicated by the number of diseased coronary arteries and also hypothesized that RANTES levels would predict mortality related to cardiovascular disease and all other causes. Our results are in line with other studies that did not find a difference in RANTES levels between patients with (23.0 ng/mL) and without (21.8 ng/mL) stable CAD, p=0.508. RANTES is believed to play a role in the early stages of atherosclerotic lesion development when activated platelets deposit RANTES on the vascular endothelium triggering monocyte arrest. It is possible that differences in RANTES levels were

not reflected in patients with stable CAD relative to patients without disease (as indicated by negative angiograms) in our cohort because they represent patients at a more advanced stage of disease. Also, high levels of RANTES in ACS is documented repeatedly, however, upregulation/downregulation of RANTES in the circulation of patients with stable disease is still controversial. As is with the case of other non-traditional biomarkers of CAD, chemokine expression is likely different between ACS and stable CAD which reflects the difference in pathological processes in these two clinical situations (134).

We also did not find a difference in RANTES levels when the population was stratified by outcome; RANTES levels did not differ between patients who were deceased (21.8 ng/mL) at follow-up compared to patients who were alive (23.1 ng/mL), p=0.206. Furthermore, RANTES levels were not strongly associated with traditional risk factors for CAD, although a significant but weak positive correlation was found between RANTES and oxidized LDL cholesterol levels. This finding is supported by other studies showing that RANTES is not associated with traditional risk factors for CAD. Interestingly, we did find a significant difference in RANTES levels between men (27.7 ng/mL) and women (34.2 ng/mL), p<0.001, and between patients with a positive family history of disease (30.3 ng/mL) and those without family history of CAD (28.1 ng/mL), p=0.006. This is the first time these findings have been reported. To date, an association between RANTES and female sex hormones has not been determined however, an effect of sex hormones on the secretion of other cytokines/chemokines has been shown (135). If levels of estrogen do modulate RANTES production *in vivo,* in particular, the changes in hormone levels that occur post-menopause, this may be a possible explanation for our results that higher RANTES levels were found in women compared to men.

The ability of RANTES to predict mortality in a population of patients with stable CAD has been investigated so far in only one study. Cavusoglu et al. measured baseline RANTES levels in an all-male population of 389 patients undergoing coronary angiography. Patients were followed up prospectively after 24 months for cardiac death and the incidence of myocardial infarction (133). This cohort included subgroups of ACS and non-ACS/stable CAD subgroups. They reported that higher RANTES levels were found in the ACS group compared to the non-ACS group, although this difference was not statistically significant. When analyzed as a continuous variable they found that RANTES was only of borderline significance for predicting cardiac mortality and was not a significant predictor for MI. However, when RANTES was analyzed as a categorical variable, RANTES was found to be predictive for both of these outcomes. They reported that *low* RANTES levels predicted cardiac mortality (p= 0.0176) and MI (p=0.0339) in the non-ACS subgroup. In our study, we also analyzed RANTES levels as both a continuous and categorical variable but our results did not support the data reported by Cavusoglu et al. In our study, a Cox regression model assessing whether or not RANTES predicted cardiac mortality showed that age was the only independent predictor of CAD-related death and in a separate Cox regression model for all-cause mortality, only age and smoking status were found to be independent predictors. RANTES levels were not a significant predictor of cardiac or all-cause mortality and even when analyzed as quartiles, RANTES remained an insignificant predictor. Kaplan-Meier survival curves were derived to compare survival rates for cardiac and all-cause mortality across quartiles of RANTES levels. A significant difference in survival rates was not found, however, the trend in both sets of curves suggests that patients in the lowest quartile for RANTES had increased survival. Our finding that low RANTES levels are associated with increased survival seems intuitive and supports the underlying role of

inflammation and monocyte recruitment in the development of CAD. Higher RANTES levels would suggest a state that promotes the development of atherosclerosis and increases the chance of death.

When RANTES was analyzed in a multivariate logistic regression model adjusted for traditional risk factors of CAD, RANTES was a borderline significant predictor of CAD ($p = 0.047$) and when divided into quartiles, RANTES levels in the highest quartile were associated with an increased risk of CAD ($p = 0.031$). This finding that subjects with higher RANTES levels have a greater risk of disease is in agreement with our findings that patients with lower RANTES levels have increased survival rates.

RANTES levels were not significantly different between patients stratified by the presence of CAD or outcome, nor did they predict mortality in our cohort which is in contrast to the data presented by Cavusoglu et al. that low, rather than high, levels of RANTES predicted adverse outcomes in their population. Our findings are consistent with the published evidence showing that RANTES levels are not significantly different between patients with and without stable CAD, however, reasons for the discrepancy between our study and that of Cavusgolu remain unclear. The primary difference between our studies was the length of time the patients were followed (24 months in Cavusgolu's study versus 13 years in ours). Knowing that RANTES plays a more active role in the early stages of atheroma development by recruiting monocytes to the lesion, it is possible that RANTES predicts death that occurs early in the atherosclerotic process. As the authors suggest, increased deposition of RANTES on the vascular endothelium initially may result in lower levels detected in the circulation. RANTES levels were only measured at the time of collection and we do not know how RANTES levels change over time as atherosclerotic burden increases. It is possible that as a result of following our subjects for a

longer period of time, our population reflected patients at a later stage of CAD when RANTES is less involved.

Increased serum RANTES levels in acute coronary syndromes (ACS) compared to healthy controls, or in patients with established disease have been repeatedly been reported. This is discussed in relation to increased platelet activation and inflammation found in ACS patients which results in increased secretion of RANTES into the bloodstream. The ability of RANTES to independently predict the presence of stable CAD has been less clear. In a study comparing RANTES levels between patients with ACS, stable CAD and healthy controls, RANTES levels were the highest in the individuals with ACS, however there was no difference between patients with stable CAD and healthy controls. Another study found that RANTES levels were not different between patients with stable CAD and age-matched healthy controls, while a Korean study reported higher serum RANTES levels in 151 men with stable CAD compared to healthy controls. In this study, RANTES was associated with other risk factors for CAD including CRP. Lastly, Rothenbacher et al. reported that serum levels of RANTES were lower in patients with stable CAD compared to healthy controls and were associated with a lower Odds Ratio (OR) for CAD.

4.1 Study Strengths and Limitations

There are several limitations to our study that should be considered. First, the population was a cohort of high-risk patients as evidenced by their risk factor profiles for coronary artery disease. We used the subgroup of patients that were negative for CAD as the "control" group; however, these patients do not truly represent a healthy population of patients as they were referred for coronary angiography. The normal range of RANTES and factors that influence its expression in healthy populations remains unknown and whether or

not RANTES would predict adverse outcomes in lower risk groups has yet to be determined. Lastly, we only measured RANTES levels in plasma collected at baseline and cannot account for changes in RANTES levels over time at different stages of disease and how this may affect cardiovascular outcomes.

Our study also has a number of strengths that help to differentiate it from similar studies that have been previously published. To date, our study uses the largest cohort of patients to investigate the utility of RANTES as a biomarker of CAD. While other cohorts contained a smaller number of patients of only males or females (N <500), our population included both genders. Most importantly, our study had a follow up time of 13 years versus 24 months for the only other study that investigated the ability of RANTES to predict future adverse outcomes. Because the utility of RANTES levels varies in significance between patients with stable CAD and patients with ACS, we recognized the importance of accurately categorizing the patients in our population. While other studies relied solely on patient angiograms we reviewed medical charts for each of the patients for whom RANTES levels were measured. Subsequent review of the patients' charts confirmed that >95% of patients in our cohort did in fact have stable CAD and were not referred for angiography as a result of an acute event.

4.2 Biomarkers and Cardiovascular Risk Prediction

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal or pathogenic processes or pharmacologic response to a therapeutic intervention. Criteria for an ideal biomarker include having a long half-life, stable expression levels and minor circadian variation allowing it to be easily and accurately measured by a method that is standardized and ideally inexpensive (54). Established cardiovascular risk

factors, including dyslipidemia, smoking, hypertension and diabetes have been incorporated into risk algorithms and although effective, fail to fully explain cardiovascular risk. For this reason, there has been a substantial effort made to identify new biomarkers of CAD that improve risk assessment, however, the validity and clinical usefulness of emerging biomarkers of CAD has been questioned. New biomarkers are often statistically associated with CAD but there is a continuing discussion on whether or not emerging biomarkers contribute to risk prediction beyond that of the established risk factors. Much of the skepticism regarding novel biomarkers was generated as a result of the INTERHEART study that was published in 2004. In this large case-control study which studied 15,152 cases and 14,820 controls in 52 countries, the authors concluded that over 90% of myocardial infarction is due to 9 traditional risk factors: smoking, dyslipidemia, hypertension, abdominal obesity, diabetes, psychosocial factors, alcohol consumption, diet and exercise habits (37). The results of this study suggested that modification of these risk factors would prevent the majority of cases of MI, leaving little room for emerging markers to have an impact on risk prediction. Supporting this finding were the results of a cycle of the Framingham Heart Study. Ten emerging biomarkers (CRP, B-type natriuretic peptide, N-terminal pro-atrial natriuretic peptide, aldosterone, rennin, fibrinogen, D-dimer, plasminogen-activator inhibitor type 1 and homocysteine) were investigated in 3209 subjects for their ability to predict death and cardiovascular events (136). While some of these biomarkers were found to be statistically significant for predicting mortality and/or cardiovascular events, the overall conclusion was that the use of multiple biomarkers did not substantially improve risk prediction based on traditional risk factors of CAD.

It is well understood that a significant statistical association of a new marker with CAD that is independent of the traditional risk factors is not enough to determine whether or not it

will be clinically useful. Prognostic utility is considered valuable when adding a novel marker to an existing risk algorithm improves predictive values. The receiver operator characteristic (ROC) curve is a graph that plots the potential false positive and false negative proportions across the range of a given variable. The area under the receiver operating characteristic curves (AUC) or c-statistic assesses how well a test or risk algorithm separates individuals into two classes (i.e. presence or absence of CAD). While statistical significance (i.e. $p > 0.05$) suggests that biomarker levels merely differ between persons with and without CAD, the c-statistic describe the ability of a biomarker to discriminate between those who will and who will not develop a disease. Ideally, the c-statistic equals 1 which would mean that discrimination between cases is perfect, without false positives or false negatives. In general, good risk prediction models have a c-statistic of 0.7 or greater (137). Skepticism of novel markers of CAD is also a result of a series of publications that suggest that cardiovascular risk models do not improve risk prediction when a novel marker is included in an algorithm with conventional risk factors. Another example of this is the Atherosclerosis Risk in Communities (ARIC) study that investigated 19 novel risk markers of CAD and their utility in risk prediction (60). Although many of these markers were associated with the presence of disease and significant hazard rate ratios, Folsom et al. studied how risk prediction was affected as indicated by the AUC when each novel marker was added individually to the traditional risk factors. They reported that only lipoprotein-associated phospholipase A_2 ($LpPLA_2$) resulted in a significant increased to the AUC, from 0.774 to 0.780, although this increase was minimal and did not provide a clinically relevant improvement in risk prediction. In general, novel biomarkers that contribute significantly to risk prediction are expected to increase the AUC by 0.05 or more, and ultimately, a novel marker of CAD is considered useful if it is able to reclassify individuals to a new risk category thus

changing the treatment strategy or clinical course of action. In North America, this would prove most valuable for patients who are classified as intermediate risk (10 year event risk is 10-20%) by the Framingham Risk Score for whom risk prediction is not optimal and for whom a more aggressive treatment strategy may be needed. Whether or not the c-statistic is optimal for determining the accuracy of risk prediction models has recently been challenged (137). The c-statistic represents the likelihood that the predicted risk is higher for a case than a non-case. It is not the probability that an individual will be classified correctly or that an individual with a high score will develop disease. For this reason, it has been suggested that rather than using the c-statistic, calculating the proportion of patients who are reclassified correctly by a new risk algorithm would be more useful when assessing the prognostic utility of a novel biomarker (137).

4.3 Conclusions

Although chemokines appear to play a role in the pathogenesis of atherosclerosis, most chemokines have failed as useful biomarkers. This may be because they are involved in only one pathway of a disease involving many pathways, or they reflect an upstream inflammatory response but are not central to the disease process. The redundancy between various chemokines may also explain why levels of a single chemokine have yet to be included when calculating CAD risk in clinical practice. Based on the results of our study, it appears that RANTES falls into this group. RANTES was not a significant predictor of mortality due to any cause and does not enhance cardiovascular risk prediction in a population of patients with stable CAD who were referred for angiography. RANTES levels may provide prognostic information in other groups and future directions for studying RANTES would include investigation in study populations of healthy individuals. Further studies to explain the

association between RANTES, family history of CAD and female gender are also warranted to determine whether risk prediction could be improved for these individuals.

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