EMERGING ENVIRONMENTAL, MOLECULAR, AND GENETIC RISK FACTORS IN STABLE CORONARY ARTERY DISEASE

by

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Honors BMSc with distinction, The University of Western Ontario, 2004

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

COMBINED DOCTOR OF MEDICINE AND DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies
(Pathology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

July 2009

Abstract

Both environmental and inherited risk factors make significant contributions to coronary artery disease, however susceptibility and age of disease onset for individuals with similar risk profiles varies widely. Novel biomarkers may yet be found which could improve detection of high-risk individuals, and highlight new areas of research for treatment discovery.

This thesis explores risk factors for coronary artery disease and cardiovascular mortality. The first study investigates one specific environmental variable—neighbourhood socioeconomic status—in a cohort of patients who underwent selective coronary angiography. In patients with coronary artery disease, neighbourhood-level disparities contributed to risk of non-cardiovascular mortality, particularly to deaths from cancer, but did not influence cardiovascular mortality risk. Although disparities in health and access to care may persist, these findings suggest other risk factors should be explored to improve cardiovascular patient risk assessment.

Inflammation and oxidative stress contribute to all stages of atherosclerosis, and subsequent chapters focus on contributions of these pathways to cardiovascular risk. Interleukin-6 and C-reactive protein haplotypes were compared to plasma concentrations for prediction of coronary artery disease and cardiovascular mortality. Significant relationships observed between haplotypes, plasma concentrations, angiographic disease, and cardiovascular mortality did not demonstrate causality, which underscores the challenge of distinguishing causal from confounding pathways.

Plasma oxidative stress biomarkers were measured to evaluate their utility for risk prediction, compared to conventional cardiovascular risk factors. Elevated plasma myeloperoxidase predicted coronary artery disease and cardiovascular mortality risk, independent of conventional risk factors and disease severity. Polymorphisms in candidate

oxidative stress genes were also explored for associations with coronary artery disease, and effects on plasma biomarkers. A compound genotype of five polymorphisms predicted angiographic coronary artery disease and elevations in plasma myeloperoxidase. Following validation, these polymorphisms may be useful markers of lifetime oxidative stress burden and cardiovascular disease risk.

Novel cardiovascular risk markers are explored in this thesis, and tested for association with angiographic coronary artery disease, conventional risk factors, and risk of mortality. New questions are raised regarding how disease susceptibility is influenced by environmental and inherited factors, and ideas for future research are discussed.

Table of Contents

List Of Tables

List Of Figures

List of Abbreviations

FHS	Framingham Health Study
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
NCEP-ATP III	National Cholesterol Education Program-Adult Treatment Program III
BMI	Body Mass Index
AHA	American Heart Association
SES	Socioeconomic Status
CRP	C-reactive Protein
hsCRP	High Sensitivity (measurement of) C-reactive protein
IL-6	Interleukin-6
NAD(P)H oxidase	Nicotinamide Adenine Dinucleotide (Phosphate) Oxidase
SOD	Superoxide Dismutase
NO	Nitric Oxide
$ONOO^-$	Peroxynitrite
H_2O_2	Hydrogen Peroxide
OH^-	Hydroxyl Radical
GPX	Glutathione Peroxidase
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
SNP	Single Nucleotide Polymorphism

tagSNP	Tagging Single Nucleotide Polymorphism
rsID	dbSNP Reference SNP Identifier
LD	Linkage Disequilibrium
ROC	Receiver Operator Characteristic
AUC	Area Under ROC Curve
HL	Hosmer-Lemeshow
NRI	Net Reclassification Index
MPO	Myeloperoxidase
N-tyr	Nitrotyrosine
oxLDL	Oxidized LDL
AOC	Antioxidant Capacity

List of Gene Names

ALOX5	arachidonate 5-lipoxygenase
FLAP	arachidonate 5-lipoxygenase-activating protein
CRP	C-reactive protein
CYBA	superoxide-generating NADPH oxidase light chain subunit p22phox
GPX1	glutathione peroxidase 1
GPX3	glutathione peroxidase 3
IL6	interleukin-6
MPO	myeloperoxidase
HMOX1	inducible heme oxygenase
NOS3	endothelial nitric oxide synthase
PON1	paraoxonase 1
PON2	paraoxonase 2
PON3	paraoxonase 3
SOD1	cupper/zinc superoxide dismutase
SOD2	manganese superoxide dismutase

Acknowledgements

I would like to express my gratitude to the health research agencies who have made this work possible: first, the Heart and Stroke Foundation of BC and Yukon for funding these studies, and also the Canadian Institutes of Health Research/ Providence Health Care, and Michael Smith Foundation for Health Research for investing in my education and research. I am also grateful to the cohort of patients made these studies possible.

Throughout this odyssey, I have received encouragement and assistance from faculty, staff and students whom I would like to sincerely thank. First of all, I would like to express enormous gratitude to my supervisor, Dr. John Hill, for his tireless encouragement and support of my work. Through his boundless optimism, Dr. Hill has taught me to enjoy my discoveries, and to keep going even when faced with challenges. I owe Dr. Hill a debt of gratitude for all that I have learned and accomplished, and I wish him every success with his future research.

Several other researchers have contributed to my research and training. Thanks to Dr. Greg Miller for inspiring our study of socioeconomic status and health. Enormous thanks also to Dr. Scott Tebbutt for patiently teaching me about APEX genotyping, and for tirelessly guiding my genotyping experiments towards success. I would also like to thank Jian Ruan for teaching me to perform APEX, and Ben Tripp for his help with the genotyping software. Finally, I extend enormous gratitude to fellow ASL students (past and present) for their helpful discussions and cheerful camaraderie, and for making the lab a great place to work.

I would like to sincerely thank my graduate supervisory committee—Dr. Jiri Frohlich, Dr. Peter Pare, Dr. Keith Walley, and Dr. Haydn Pritchard—for keeping me focused on my research, and for providing the training I needed to do the work well. I would also like to thank my program directors, Dr. Lynn Raymond and Dr. Torsten Neilsen, for cheering me on for all these years, and my graduate advisor Dr. David Walker for his sage advice.

Finally, I wish to extend my deepest gratitude to my family for their unconditional love and support throughout this intense chapter of my life, to my friends for keeping me grounded, and to Five for all his love and kind encouragement.

Co-Authorship Statement

Publications presented in this thesis are based on work carried out for the completion of my PhD program. Citations follow the titles of each chapter, and individual contributions to the carrying out of this research are specified here.

Chapter 2: Dr. Greg Miller inspired this study, provided neighbourhood socioeconomic data for our cohort patients, and assisted with the analysis and literature review. Dr. John Hill was principal investigator for this study, and he provided critical comments and reviewed the manuscript. I performed the experiments, completed statistical analyses, drafted the manuscript, and finalized it for publication.

Chapter 3 and Chapter 5: Dr. John Hill was principal investigator for these manuscripts, provided reviews of the work, and contributed to the manuscripts. Jian Ruan and Dr. Scott Tebbutt trained me in APEX genotyping, and assisted with quality control procedures and the study design. Dr. Mohua Podder generated genotypes through LDA. I designed the genotyping experiments, from selection of gene targets and SNPs, to design of primers and probes for APEX genotyping. I also performed all genotyping experiments, accuracy calculations, and statistical analyses, and I drafted the manuscripts and finalized them for publication.

Chapter 4: Dr. John Hill was principal investigator for this study, and provided analyses and reviews of the work, and contributed to the manuscript. Dr. Jiri Frohlich contributed to cohort patient recruitment, taught me important concepts of cardiovascular patient care, and reviewed the manuscript. I performed all the plasma biomarker measurements and data analysis, as well as statistical analyses of our results. Furthermore, I drafted and prepared the manuscript for publication.

Chapter 1. Introduction and Literature Review

1.1 Definition of Atherosclerosis

Atherosclerosis is a subtype of arteriosclerosis (literally, hardening of the arteries), characterized by the formation of lesions in the arterial intima, which obstruct the vascular lumen, and are prone to rupture. These lesions develop from fatty streaks composed of lipid-filled foam cells, and although not all fatty streaks progress to become advanced lesions, the appearance of fatty streaks has been documented as early as the first decade of life in all human populations (1).

Previously considered a disease of cholesterol storage, atherosclerosis is currently understood as the result of complex interactions between cells in the walls of medium- and large-sized arteries, and the components of circulating blood. Inflammation plays a pivotal role in local and systemic complications of atherosclerosis (2). The arterial endothelium, a primary barrier between the circulation and the artery wall, is susceptible to damage by molecules that are generated by atherosclerotic risk factors, such as vasoconstrictor hormones, products of elevated blood glucose, pro-inflammatory cytokines, or modified low density lipoprotein (LDL) particles. When damaged or disrupted, endothelial cells increase production of adhesion molecules, which leads to recruitment of leukocytes from the circulation.

Leukocytes recruited are mainly mononuclear phagocytes and T-lymphocytes, and these cells transmigrate in response to chemoattractant cytokines generated below the endothelial layer. As they do they exchange inflammatory messages, which perpetuate the formation of an atheroma, the hallmark lesions of atherosclerosis (Figure 1.1). Mature atheromas are lipid-rich deposits which contain cholesterol-rich macrophages (foam cells), surrounded by smooth muscle cells which form a fibrous cap within a thickened extracellular matrix.

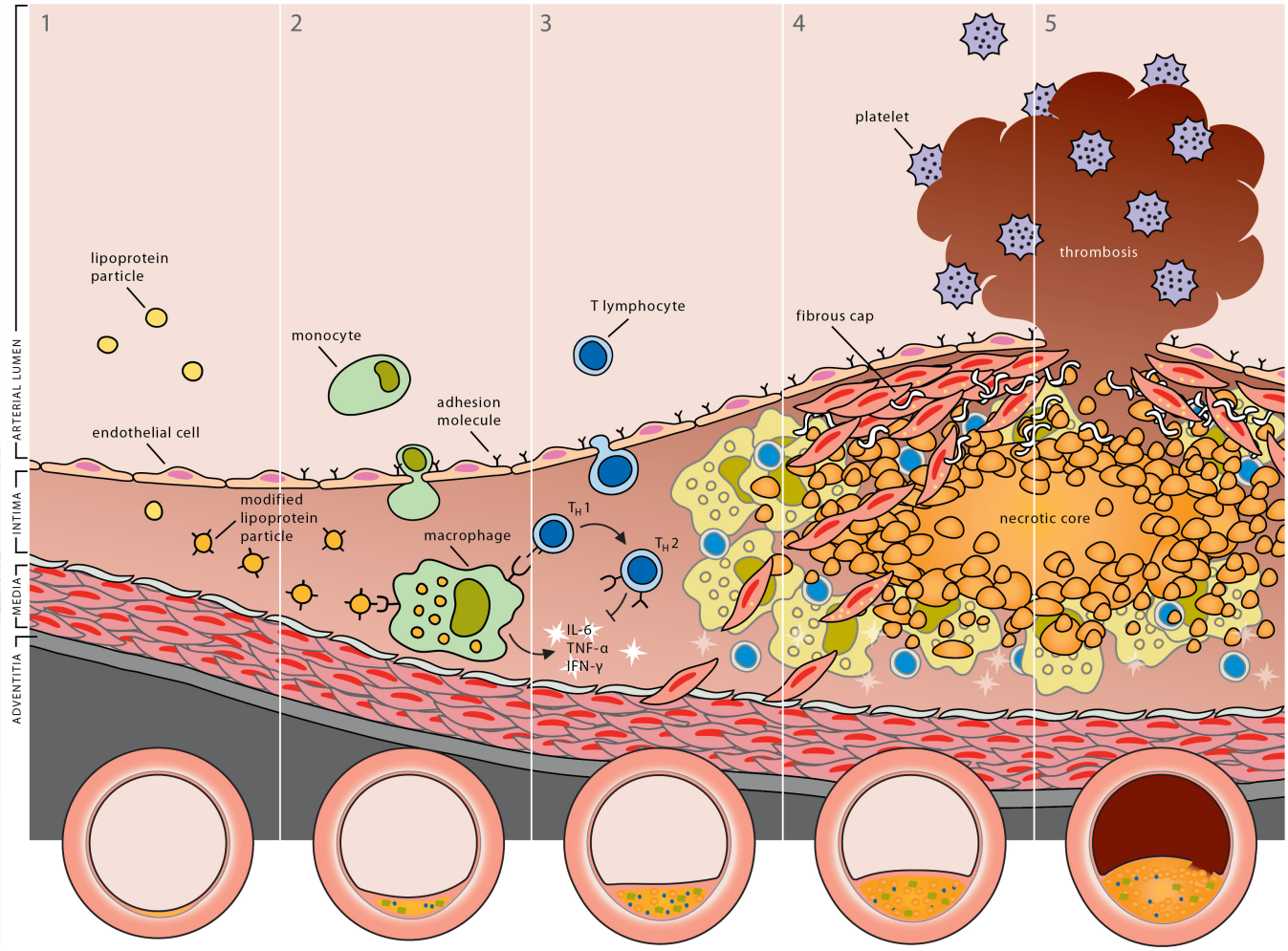


Figure 1.1 Stages of atherosclerosis

Schematic depiction of stages of atherosclerosis from lipoprotein modification (1), recruitment and differentiation of monocytes, and formation of foam cells (2), local inflammatory processes (3), and development of a mature atheroma (4). Risk of plaque rupture (5) and exposure of the thrombogenic lumen contents to the circulation depends on the stability of the fibrous cap surrounding the atheroma, and integrity of the endothelial layer.

Clinical symptoms of atherosclerosis result from acute diminishment of arterial perfusion by plaques that are disrupted and precipitate occlusive thrombi, or by chronic expansion of plaques that obstruct blood flow. For much of its development, the atherosclerotic lesion grows outwardly away from the lumen (3,4), thus, a substantial burden of atherosclerosis can develop before clinical signs are detected from stenosis (5) (Figure 1.2).

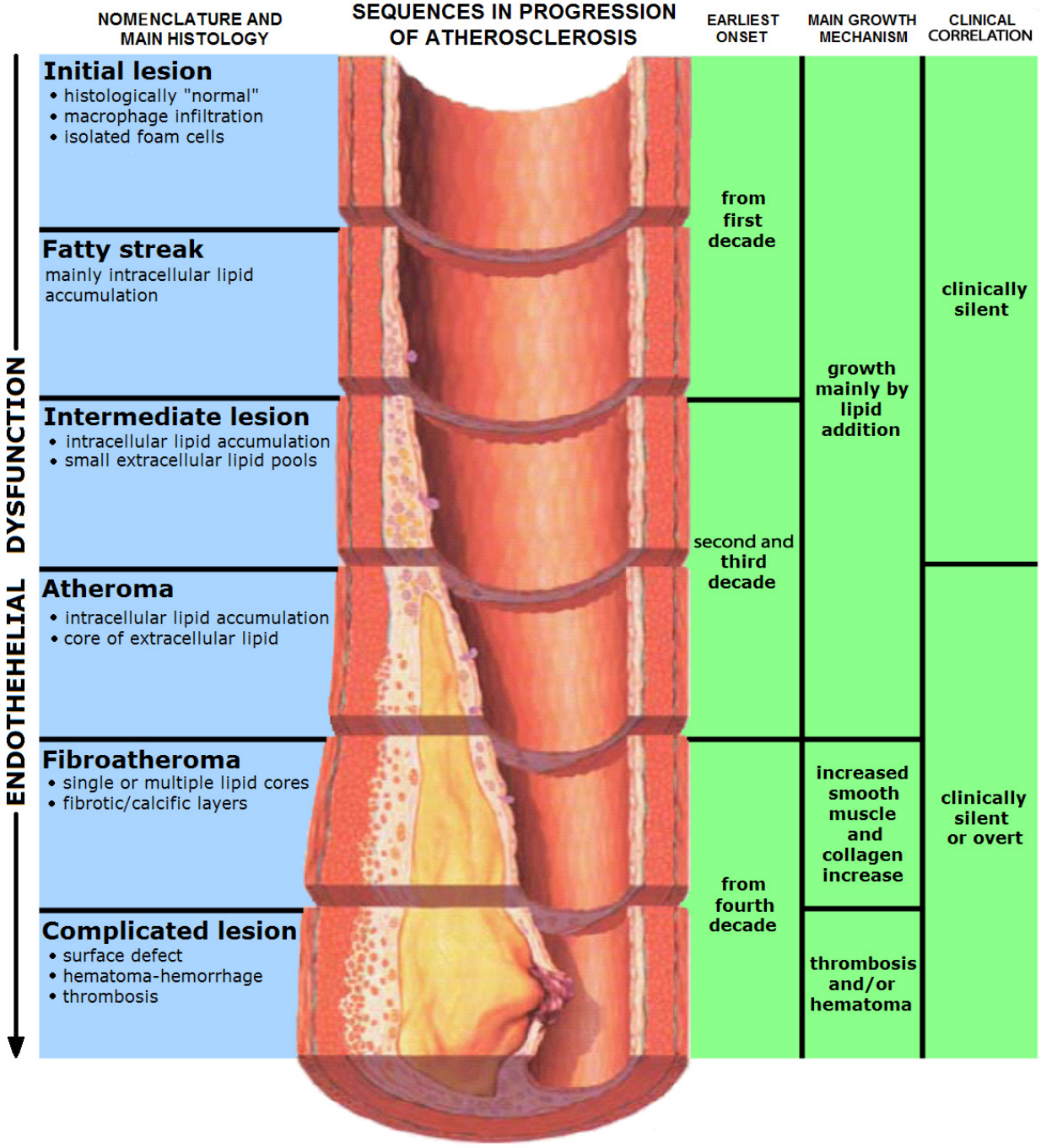


Figure 1.2 Relationship between atherosclerosis and clinical symptoms

As lesions progress across decades to obstruct the vessel lumen, they also change in composition. Complicated lesions, characterized by smooth muscle and extracellular matrix, as well as a lipid rich core, may remain clinically silent or become overt depending on vessel obstruction by plaque growth or lesion rupture. *Image made available without copyright under GNU free documentation license use.*

1.2 Coronary Artery Disease

Coronary artery disease refers to a group of syndromes resulting from myocardial ischemia; the state where there is not sufficient blood perfusion to meet oxygen demand or metabolite removal from the myocardium. In most cases, myocardial ischemia is caused by atherosclerotic lesions that are obstructing coronary vessels and generating thrombi.

Cardiovascular diseases have been the main cause of death in Canada in the last 25 years (6). Although their share declined by >30% between 1979 and 2004, coronary artery disease is still the most expensive chronic disease in the Canadian health care system (7).

The clinical manifestations of coronary artery disease caused by atherosclerosis are as follows:

1) Myocardial infarction—the extent and duration of ischemia is sufficient to cause death to cardiac muscle,
2) Angina pectoris—ischemia causing clinical symptoms of chest pain due to inadequate oxygen to heart muscle, but without cardiac muscle death,
3) Chronic ischemic heart disease—myocardial damage induces cardiac structural remodeling and precipitates heart failure, or
4) Sudden cardiac death.

In most individuals (65% of men, and 47% of women) the first symptoms of coronary artery disease is myocardial infarction (8). However clinical manifestations are not entirely predicted by atherosclerotic lesion burden, nor by degree of vessel stenosis, and the natural history is variable onset, with heterogeneity in presentation patterns and prognoses.

Because angina occurs when coronary perfusion is reduced past a critical level, symptoms manifest whenever demand for perfusion is increased. For patients with coronary artery disease, angina may be classified as stable or unstable. Patients with stable angina symptoms may

experience no changes to their clinical condition for many years. However, if angina increases in frequency, is precipitated by less effort or at rest, or persists in duration, it is referred to as "unstable angina". Discontinuous progression of plaques causing unstable angina reflects episodes of lesion disruption or thrombosis in situ, which can change the degree of vessel obstruction quite rapidly (9). For many patients, unstable angina is the prodrome of myocardial infarction (10).

1.3 Risk Factors for Coronary Artery Disease

1.3.1 Identification of Risk Factors Through Epidemiological Studies

Factors that predict risk of coronary artery disease and cardiovascular mortality have been energetically researched in large-scale populations over many decades. The most famous example is the Framingham Heart Study (FHS), which began in 1948 with a cohort of 5,209 men and women recruited from the town of Framingham, Massachusetts (11), and now also includes the second and third generations of the original cohort.

Cardiovascular risk factors first described in the FHS—high blood pressure, high blood cholesterol, smoking, obesity, diabetes, and physical inactivity—are now understood to be major markers of cardiovascular disease risk. These factors were not only associated with prevalence of coronary artery disease, but also predicted incidence in previously healthy patients (12). Thus, the concept of risk factors was developed, and translated into the Framingham Risk Score (11,13), which is currently used to assess 10 year risk of cardiovascular events (Figure 1.3).

Step 1: Add points for age, and either for total cholesterol or low-density lipoprotein (LDL) cholesterol values

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

Low Density Lipoprotein Cholesterol (LDL)	
mmol/L	**Points**
<2.59	-3
2.60–3.36	0
3.37–4.14	0
4.15–4.92	1
≥4.92	2

OR

Total Cholesterol	
mmol/L	**Points**
<4.14	-3
4.15–5.17	0
5.18–6.21	1
6.22–7.24	2
≥7.25	3

Step 2: Add points for patient blood pressure measurements.

Blood Pressure	
mm Hg	**Points**
<120/<80	-3
120–129/80–84	0
130–139/85–89	1
140–159/90–99	2
≥160	3

Step 3: Add 2 points if the patient has diabetes, and add 2 points if they are a smoker.

Step 4: Use total points to calculate 10-year risk of cardiovascular events.

10-Year Cardiovascular Events Risk		
Points if using LDL	**Points if using Total Cholesterol**	**10-Year Risk***
<-3		1%
-2		2%
-1	<-1	2%
0	0	3%
1	1	4%
2	2	4%
3	3	6%
4	4	7%
5	5	9%
6	6	11%
7	7	14%
8	8	17%
9	9	21%
10	10	26%
11	11	32%
12	12	39%
13	13	46%
≥14	≥14	55%

* Approximate risk for men, based on the Framingham experience in men 30–74.

Figure 1.3 The Framingham risk score

The Framingham risk score applied to determining 10-year cardiovascular disease risk. Separate score sheets exist for men and women; the version for men is presented here, with score values calculated from risk estimates derived from the Framingham Heart Study, a predominantly Caucasian population in Massachusetts, USA.

The risk score test is applied to determine 10 year cardiovascular event risk, and to calculate patient treatment guidelines, according to the US National Cholesterol Education Program Adult Treatment Program (NCEP-ATP) III recommendations (14). Other global risk assessment scores that have been developed using similar epidemiological methods include the PRO-CAM score (15), and the European Society of Cardiology SCORE (16). The study of cardiovascular risk factors has therefore not only enhanced our understanding of disease processes, but has also equipped clinicians with evidence-based tools for calculating risk for individual patients and determining how aggressively to treat them. This is vital in the current reality of limited healthcare resources (17). Current risk assessment methods are compared to the Framingham Risk Score in Table 1.1.

Risk Category	Canadian Cardiovascular Society (2006)	Adult Treatment Panel-III-R (2004)	European Society of Cardiology (2007)
High: 10-year Framingham Risk Score ≥20%, cardiovascular disease, peripheral vascular disease, diabetes	**Treatment Targets:** ▪ LDL <2.00 mmol/L ▪ TC:HDL <4.00 mmol/L Goal ApoB< 0.85 g/L	**Treatment Targets:** ▪ LDL <2.60 mmol/L (optional target goal <1.80 mmol/L) ▪ Non-HDL <3.37 mmol/L	**Treatment Targets:** ▪ LDL <2–2.50 mmol/L ▪ TC <4–4.50 mmol/L
Moderate: 10-year Framingham risk score 10–20%	**Treat When:** ▪ LDL ≥3.50 mmol/L ▪ TC:HDL ≥5.00 mmol/L Goal ApoB< 1.05 g/L	**Treatment Targets:** ▪ LDL-C <3.4 mmol/L ▪ Non-HDL <4.15 mmol/L	**Treatment Targets:** ▪ LDL <3.0 mmol/L ▪ TC <5.00 mmol/L
Low: 10-year Framingham Risk Score <10%	**Treat When:** ▪ LDL ≥5.00 mmol/L ▪ TC:HDL ≥6.00 mmol/L Goal ApoB< 1.25 g/L	**Treatment Targets:** ▪ LDL-C <4.2 mmol/L ▪ Non-HDL <5.0 mmol/L	

Table 1.1 Comparison of lipid guidelines

Comparison of lipid treatment guidelines from the Canadian Cardiovascular Society, Adult Treatment Panel (ATP)-III, and European Society of Cardiology, across risk categories.

LDL: Low density lipoprotein cholesterol HDL: High density lipoprotein cholesterol
TC:HDL: Total:high density lipoprotein cholesterol ratio ApoB: Apolipoprotein B

1.3.2 Non Modifiable Risk Factors

Age, sex, and family history are major non-modifiable cardiovascular risk factors. Age is a key component of the Framingham Risk Score, and results from the FHS demonstrated that between 40 and 60 years of age, incidence of myocardial infarction increases 5-fold, and death from cardiovascular disease also increases each decade (13).

Males are more prone to develop atherosclerosis than women, and men develop coronary artery disease at an earlier age. However, following menopause the incidence in women increases, and risks equalize in the seventh decade (18). Estrogen may contribute to protection from cardiovascular disease in women, but unfortunately estrogen replacement therapies have not succeeded in reducing risks to women after menopause (19).

Family history is also a major contributing factor for cardiovascular risk. Individuals who have family history of premature cardiovascular disease (a first degree relative male ≤45 years old, or female ≤55 years old) have an estimated 2-fold risk compared to individuals without a family history (20). Although there are monogenic disorders that cause premature cardiovascular disease, most cases of cardiovascular disease follow polygenic inheritance patterns, with many inherited risks contributing to an individual's overall risk. Furthermore, susceptibility factors for ischemic heart disease are influenced by heritable genetic variations, thus direct and indirect genetic factors interact in complex ways to contribute to inherited cardiovascular risk.

Ethnicity also influences cardiovascular disease risk, although the degree of risk conferred for different ethnic groups has not been fully elucidated. Until recently, most major epidemiological studies included primarily Caucasian patients of European origin, and findings have not always been applicable for other populations. Cardiovascular disease burden differs for other ethnic groups, including South Asians (21), black Americans (22), and Hispanics (23).

Although the Framingham Risk Score has inspired most contemporary models of cardiovascular risk prediction in North America, it discriminates risk best in white Western females, and least accurately in non white African males (24).

The nature of ethnicity as a risk factor is further complicated by environmental factors. Conventional cardiovascular risk factors that differ across ethnic groups, such as smoking, blood pressure, and cholesterol, do not fully account for differences between groups. Furthermore, risks in certain ethnic groups are augmented by adoption of a Westernized lifestyle (a diet high in saturated fats and refined sugars, with low physical activity) (25), and immigration itself has been suggested to pose a cardiovascular risk, regardless of ethnicity (26). Differences in metabolism, insulin resistance, or visceral adiposity also contribute to disparities (27). Marked differences across ethnic groups are most likely due to interacting genetic, environmental, and cultural factors which influence disease presentation, therapeutic needs, and response to treatment (28), and it remains a priority to resolve these patterns to improve the equity of cardiovascular care.

1.3.3 Modifiable Risk Factors

1.3.3.1 Hypertension

Elevated blood pressure is the single most important cause of cardiovascular disease, and is responsible for an estimated 62% of strokes and 49% of cardiovascular deaths (29). There is a continuous graded relationship between blood pressure and cardiovascular risk, above 115/75 mm mercury (30). Although obesity coupled with lack of physical activity is an important factor in the development of high blood pressure, there is much stronger evidence that salt intake contributes to hypertension, especially to the rise in blood pressure seen in age (31). Consuming

a diet rich in fruits and vegetables reduces blood pressure, which further supports this relationship, as the potassium provided by such a diet offsets effects of sodium intake (32,33).

1.3.3.2 Dyslipidemia

Hypercholesterolemia, elevation of serum cholesterol, causes atherosclerotic lesion development even in the absence of other risk factors (34). The risk is primarily attributed to low-density lipoprotein (LDL) cholesterol, because LDL delivers cholesterol to tissues. High-density lipoproteins (HDL) mobilize cholesterol from peripheral tissues for excretion via the liver, thus HDL cholesterol particles are considered beneficial.

Although LDL particles are not atherogenic on their own, LDL undergoes modification by endothelium-derived free radicals, and becomes pro-atherogenic modified LDL (35,36). Modified LDL is recognized by receptors on the macrophage, and its uptake contributes to foam cell formation and atheroma growth (37,38).

Dietary interventions may be employed to lower cholesterol, but the treatment of choice for many patients is 3-hydroxy-3-methyl-glutaryl(HMG)-CoA reductase inhibitors (statins). By inhibiting the rate limiting enzyme in cholesterol synthesis, statins cause upregulation of LDL-receptors on the liver, and substantially lower circulating LDL cholesterol levels. However, a certain portion of plasma lipoprotein profiles are inherited, and dyslipidemia may persist despite pharmacotherapy. Also, elevated triglycerides are also a risk for cardiovascular disease, although the association is considerably less strong (39).

1.3.3.3 Diabetes

Type 2 diabetes mellitus is a metabolic disorder caused by metabolic resistance to the normal actions of insulin, leading to increased glucose production from the liver, and inadequate uptake of glucose by peripheral tissues. The greatly increased mortality and morbidity associated with type 2 diabetes are mainly attributed to accelerated cardiovascular disease (40). Also, diabetic individuals carry a greater burden of other cardiovascular risk factors (41). Within the Framingham study, subjects with diabetes were nearly four times more likely to have an additional cardiovascular risk factor than subjects without diabetes (13). Diabetes is considered a cardiovascular risk equivalent by ATP-III guidelines, thus all patients with diabetes are treated to aggressive treatment targets, as if cardiovascular disease was already documented (14).

1.3.3.4 Obesity

Body mass index (BMI) is the ratio of body weight to height, and BMI acts as a standard measurement tool for defining overweight and obesity. Normal BMI falls within the range of 18.5–25 kg/m^2, and both overweight (BMI 25–30) and obesity (BMI >30) are associated with higher cardiovascular disease risk compared to normal BMI (40).

Obesity is a major contributor to metabolic disturbances related to cardiovascular disease, including hyperglycemia (42), dyslipidemia (43), oxidative stress (44), chronic inflammation (45,46), and dysfunctional vascular endothelium (47). Obesity is linked to chronic inflammation, thrombotic risk, and complications of cardiovascular disease (48). Estimated relative risk values for obese individuals compared to non obese individuals for developing hypertension, dyslipidemia, insulin resistance, and diabetes are >3, and the relative risk (RR) for coronary artery disease is 2–3x (49). Obesity is now the most prevalent metabolic disorder in the Western

world, and as it is increasing in prevalence in the developing world, it may soon represent the risk factor with the greatest global influence on coronary artery disease risk and mortality (40).

Although it is widely utilized, BMI is a crude measurement that is neither sensitive to lean mass, nor to adipose tissue distribution. Adipose tissue deposited in the visceral compartment is more metabolically atherogenic than adipose tissue deposited subcutaneously (50), and waist circumference, as a measurement of visceral adipose tissue, is associated with cardiovascular risk. Investigation of waist circumference in a cohort of 5,453 cardiology clinic patients demonstrated that patients with an elevated waist circumference (>102 cm in men and >88 cm in women) had higher rates of cardiovascular disease and diabetes, and higher waist circumference increased the risk for mortality (51).

1.3.3.5 Metabolic Syndrome

Metabolic syndrome refers to the clustering of certain cardiovascular risk factors—hypertension, dyslipidemia, hyperglycemia, and abdominal adiposity—and the metabolic disturbances they confer. Insulin resistance and abdominal obesity, major characteristics of metabolic syndrome which result from dysfunctional adipose tissue, lead to increased inflammation and endothelial dysfunction, two other cardiovascular risk factors (52,53). Patients with metabolic syndrome are at increased risk for diabetes (54,55) and cardiovascular disease (56–58). In a study of 840 female and 1002 male patients, carotid atherosclerosis was more prevalent in individuals with metabolic syndrome across all weight categories (59), which suggests effects of metabolic syndrome may be conferred by adipose tissue dysfunction, rather than body composition alone.

1.3.3.6 Smoking

Tobacco use is a major avoidable causes of cardiovascular disease, and an estimated 1/6 of the world's population continues to smoke despite the known serious impact of smoking on health (60). Fresh cigarette smoke contains more than 4,500 components, including toxins (carbon monoxide, ammonia, acetone, nicotine, hydroquinone), direct carcinogens (acroleine, methylcholanthrene), and reactive oxidants (superoxide and nitric oxide) (61). The condensate of cigarette smoke also activates phagocytic lung cells, releasing both pro-inflammatory and pro-thrombotic signals into the circulation (62).

Cigarette smoking is associated with endothelial dysfunction (63), and smoking not only accelerates progression of coronary atherosclerosis, but also promotes formation of new lesions (64). In patients with established coronary artery disease, smoking can trigger myocardial infarction, and is a risk factor for sudden cardiac death (65). Tobacco use from any source, including second hand tobacco smoke exposure, is associated with an increase in risk of myocardial infarction (66).

1.3.3.7 Physical Activity

A sedentary lifestyle is associated with coronary artery disease, with risk for sedentary individuals almost double that of persons with an active lifestyle (67). Regular exercise reduces the risk of hypertension, diabetes, and hypercholesterolemia, improves glucose tolerance and insulin sensitivity, and increases HDL levels (68). Myocardial oxygen uptake and coronary artery diameter are increased by exercise training, which may alter the progression of coronary atherosclerosis and heart failure (69). Because of challenges to quantifying exercise, reviews

range as far as the effects on cardiovascular disease, but cardiovascular mortality may be reduced by >25% by an active lifestyle (7).

1.3.4 Shortcomings in Global Risk Models

Characterization of major modifiable and non modifiable cardiovascular risk factors has led to the recommendation that for primary prevention of coronary artery disease, abstention of cigarette smoking, control of hypertension, weight reduction, and regular exercise should be employed. Increasing HDL-cholesterol while reducing total and LDL-cholesterol levels is also recommended to reduce cardiovascular risk (70).

Despite what is currently known about cardiovascular risk, there is room for improvement in the tools that are used for risk prediction. A considerable number of patients at risk for coronary artery disease cannot be identified on the basis of traditional risk factors alone (71). Also, not all patients with risk factors develop disease; an estimate 10–20% of patients with cardiovascular events have no traditional risk factors (72), and more than 60% of patients have two or fewer risk factors (73).

Although the Framingham Risk Score is widely used in North America, a recent British study identified that risk scoring methods derived from the Framingham Health Study significantly *overestimate* absolute coronary risk assigned to individuals living in the United Kingdom (74). Longer term risk stratification also remains suboptimal even with available global risk scores, therefore the search for novel markers that could improve understanding of disease and accuracy of patient risk assessment continues.

1.3.5 Surrogate Markers of Atherosclerosis

Waiting for coronary artery disease to manifest as artery blockages or myocardial ischemia means missing a critical window for therapy. Surrogate markers which non-invasively estimate cardiovascular disease burden have been investigated for their value in cardiovascular disease research. Coronary calcium scoring, carotid intima-media thickness, and endothelial dysfunction are examples of surrogate markers of cardiovascular disease. Methods for measuring these surrogate disease markers differ by availability, cost, and reproducibility, and evidence for their value in risk prediction or treatment discovery also varies.

Presence and extent of coronary artery calcium is strongly associated with cardiovascular events, independent of traditional risk factors (73,75). Diabetes patients with elevated oxidative stress show higher coronary calcium scores, even after adjusting for other cardiovascular risk factors (76). The most recent American Heart Association (AHA) Scientific Statement supports measuring atherosclerosis burden by coronary calcium scoring in intermediate-risk patients, to improve accuracy of clinical risk prediction, and to determine if more aggressive targets for lipid lowering therapies are warranted (77).

Carotid artery intima-media thickness measured by ultrasound is related to risk of stroke and myocardial infarction (78,79). Furthermore, carotid plaques visualized by ultrasound are associated with several cardiovascular risk factors, such as dyslipidemia, oxidative stress, and inflammation (80).

Endothelial function is most commonly measured by Doppler assessment of flow in the brachial artery, with and without stimuli that induce artery dilation, including measures that increase nitric oxide. Endothelium derived nitric oxide not only contributes to vasorelaxation, but also inhibits leukocyte adhesion, vascular SMC migration, and platelet aggregation (81).

Reduced endothelial function predicts major cardiovascular risk factors and future disease development (82).The role of endothelial dysfunction in coronary artery disease is well accepted (83), yet no therapies that specifically treat endothelial dysfunction have been developed.

Surrogate biomarkers of cardiovascular disease have the potential to augment risk stratification by aiding in patient screening, diagnosis, and assessment of prognosis (84,85). They may also be useful for assessing the strength of association between novel risk factors and cardiovascular disease burden, and effectiveness of new cardiovascular therapies.

1.3.6 Socioeconomic Status

Evidence that individuals with higher socioeconomic status (SES) enjoy better health than those with lower SES has been widely demonstrated, with the general agreement that effects of SES on health are complex and multifactorial (86). Most research has focused on specific SES indicators, such as education, income, and occupational social class. For example, study investigators of the European Prospective Investigation of Cancer and Nutrition in Norfolk (EPIC-Norfolk) cohort considered occupation and education based measures of SES, and found that not all socioeconomic differentials in mortality were explained by modifiable risk factors (87). Although the indicator that best assesses socioeconomic gradients in health has not been agreed upon, different indicators may be more or less relevant to health outcomes and different stages of the life course.

Most studies reviewing SES and health status measure individual level data, however recent studies suggest neighbourhood SES can independently influence health, above and beyond effects of individual SES variables. Indeed, associations between neighbourhood socioeconomic disparity and all-cause mortality, infant mortality, suicide, long term illness, coronary artery

disease, disability, and depression have been demonstrated (88–91). Studies of neighbourhood SES find only modest changes to these relationships with adjustment for individual level SES data (92–95).

Cities across Canada are becoming more segregated by income, and investigation into health disparities across neighbourhoods may help identify and quantify health disparity within urban areas. One study comparing neighbourhoods across Saskatoon revealed significant differences in acute and chronic disease health care service utilization, and in infant mortality and all-cause mortality rates, between lowest and highest-income neighbourhoods (96). Lower socioeconomic status is associated with greater cardiovascular risk factor burden, and higher cardiovascular risk, across Canadian communities, regardless of ethnic groups (97).

Neighbourhood SES also predicts all-cause mortality risk in patients with coronary artery disease. A study of more than 51,000 Canadian patients hospitalized for acute myocardial infarction and followed for one year found a 10% increase in all-cause mortality for each $10,000 decrease in median neighborhood income (98). However, specific causes of mortality were not investigated, and the value of neighbourhood SES for predicting cardiovascular outcomes remains to be explored.

1.3.7 Biomarkers of Inflammation

Inflammation plays a key role in the development and progression of atherosclerosis (2). Plasma biomarkers of inflammation have been investigated as a strategy for primary prevention (99), and to estimate outcome risk in patients with coronary artery disease (100). Among biomarkers of inflammation, the most well established is C-reactive protein (CRP).

CRP is an acute phase protein synthesized in the liver in response to cytokines, especially interleukin-6 (101,102). Increased plasma CRP predicts risk of cardiovascular disease, independent of major risk factors (103), and also predicts mortality risk in patients with coronary artery disease (104). Data from the Cardiovascular Health Study indicated CRP was strongly predictive for incident cardiovascular disease and total mortality, and elevated CRP was associated with atherosclerosis detected by carotid intima media thickness (105).

Although CRP is becoming widely accepted as a disease risk marker, whether it is a causal factor in the pathogenesis of atherosclerosis remains under debate (106). Support for a causal role of CRP in coronary artery disease comes largely from epidemiological evidence, with statistical strength at least as robust as other major risk factors (107). However, CRP is highly susceptible to confounding influence from risk factors, including smoking, hypertension, obesity, and even low socioeconomic status (108,109).

Polymorphisms in the *CRP* gene have been investigated with the goal of demonstrating the causality of CRP in cardiovascular disease, and determining the degree of influence contributed by inherited variations in the gene. Polymorphisms in *CRP* have been associated with risk of cardiovascular events, but these variations have not yet been associated with changes in plasma CRP concentrations (110). Indeed, although *CRP* genotypes have been associated with cardiovascular deaths and total mortality (111), polymorphisms are associated with changes in plasma CRP concentrations do not influence outcome risk (112,113). These findings suggest the underlying inflammatory processes which are predictive of coronary events are not captured solely by genetic variations. Furthermore, genetic variation may influence lifetime risk of disease not addressed by a single plasma biomarker measurement.

Measurements of CRP concentrations have recently been applied to detect patients who do not have elevated plasma cholesterol but who benefit from statin therapy for cardiovascular risk reduction (114). Current AHA guidelines recommended measurement of CRP in asymptomatic subjects at intermediate risk for future coronary events, at the discretion of the physician (99).

Interleukin-6 (IL-6) is another pro-inflammatory marker that is strongly associated with cardiovascular risk (115), and plasma IL-6 concentrations have prognostic value in unstable angina patients (116). Blood concentrations of IL-6 also predict development of heart disease (117). The role of IL-6 in cardiovascular disease is partly attributed to upregulation of CRP in hepatocytes and immune cells within the atherosclerotic lesions, where IL-6 expression is detected at 10- to 40-fold higher levels than non-atherosclerotic vessels (118). IL-6 also contributes to systemic inflammatory processes, leukocyte recruitment, and coagulation, and is the only cytokine to stimulate the synthesis of all acute phase proteins of the inflammatory response (102).

Promoter polymorphism −174G/C is believed to influence regulation of IL-6 production (119), and may affect survival after myocardial infarction (120). Recently, Sanderson and colleagues demonstrated a relationship between polymorphisms in IL-6 and social position that influences IL-6 levels in a cohort of 10,308 individuals in London, England (121). Other variations in the IL-6 gene exist, however they have not been associated with changes to IL-6 or CRP, or risk of cardiovascular disease.

1.4 Oxidative Stress

1.4.1 What is Oxidative Stress?

The original oxidative stress hypothesis emerged 20 years ago (36), with evidence from experiments by Goldstein and Brown suggesting oxidation could modify lipoprotein particles and encourage their unregulated update by macrophages, by the yet undiscovered scavenger receptor (122).

Oxidative stress is understood as an imbalance between production of reactive oxygen species, and their breakdown (Figure 1.4). Free radicals with one or more unpaired electrons, as well as non radical derivative chemical entities capable of similar oxidation reactions, both contribute to oxidative stress. Free radicals may be more reactive, however both have function in low concentrations as signaling molecules, and produce harmful effects when present in excess. Reactive oxygen species act as signaling molecules in pathways related to cellular homeostasis and growth factor-stimulated proliferative processes, and contribute to host defense mechanisms (123). However, excess oxidative stress causes damage to cell components and tissues (124), and production of reactive oxygen species is, unlike overall metabolic rate, strongly correlated with reduced lifespan (125).

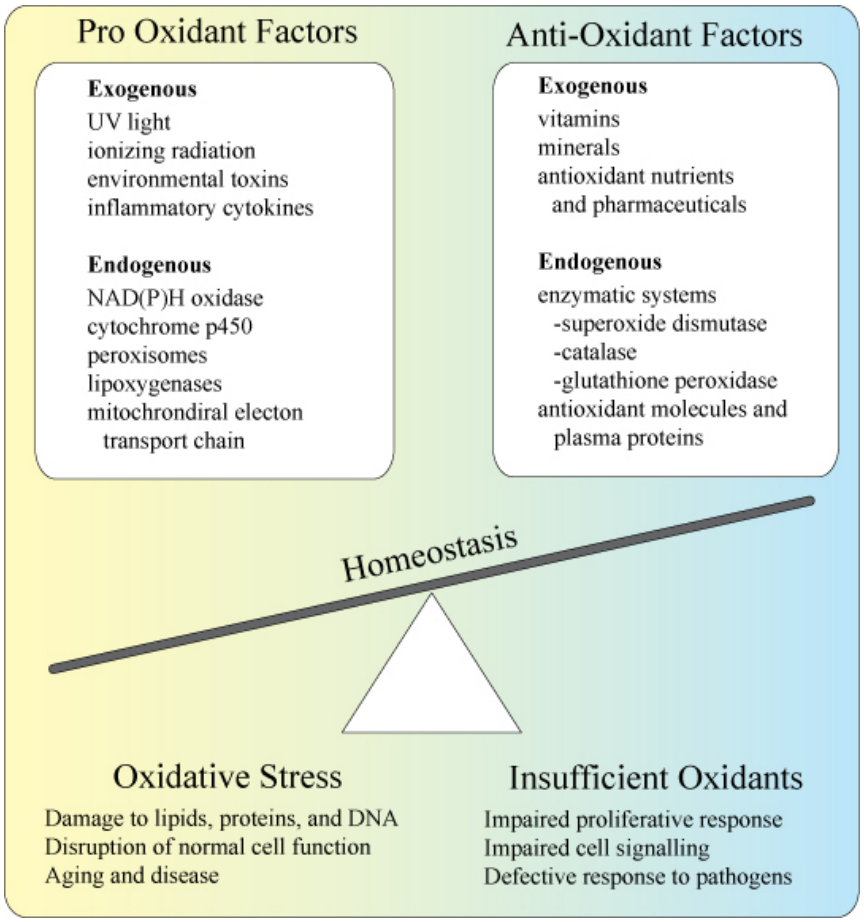


Figure 1.4 Oxidative stress balance

Relative balance of pro- and anti-oxidant factors from exogenous and endogenous sources is important for homeostasis. Insufficient oxidants may impair normal cell growth and signaling, while an excess can lead to cell damage and disease.

1.4.2 Oxidative Stress Molecules and Pathways

Superoxide: Electron leak from the mitochondrial electron transport chain, and auto-oxidation reactions of various cellular substrates, produce the reduced form of oxygen called superoxide (126). Superoxide participates in cellular growth regulation, acts as a signaling molecule (127), and contributes to phagocytic defense mechanisms (128). Dismutase enzymes convert superoxide to the less reactive oxidant hydrogen peroxide, and superoxide can also generate hydroxyl radicals through reactions with cations such as Fe or Cu (129).

Superoxide contributes to hypertension and atherosclerosis by reacting with nitric oxide (NO), to yield peroxynitrite ($ONOO^-$), a potent mediator of lipoprotein oxidation. Because this reaction uses available NO, it impairs NO-mediated, endothelium-dependent relaxation of

smooth muscle cells in the vasculature (130). Although the mitochondrial electron chain generates superoxide through cellular respiration, a major source of superoxide in the vasculature is NAD(P)H oxidase, and other pro-oxidant enzymes.

Hydrogen Peroxide: Dismutation of superoxide by superoxide dismutase (SOD) enzymes generates hydrogen peroxide (H_2O_2). Although H_2O_2 is less reactive than superoxide, it is capable of passive diffusion between cells, and can therefore act at sites different from where it was generated. Activated neutrophils generate H_2O_2, and use it to produce hypochloride, by the pro-oxidant enzyme myeloperoxidase. Hypochloride also reacts with H_2O_2 to form chloride ions and singlet oxygen (131).

Hydroxyl Radical: Although it has the shortest half life and very limited diffusion capacity, hydroxyl radical (OH^-) may be considered the most reactive species in the reactive oxygen species family. Hydroxyl radicals are generated from water molecules by ionizing radiation, or within biological systems through the Fenton or Haber-Weiss reactions. Hydroxyl radicals initiate lipid peroxidation, and cause damage to cellular proteins, carbohydrates, lipids and DNA (132)

Nitric Oxide (NO): NO is important to cardiovascular homeostasis, because it participates in endothelium dependent relaxation and vascular tone regulation, and inhibits platelet aggregation and adhesion molecule expression (133). Imbalances in vascular oxidative stress, either by enhanced superoxide production or decreased NO production or bioavailability, leads to production of peroxynitrite (134) which has vasoconstrictive and atherogenic effects (130,135).

Peroxynitrite is short lived, but can exerts cellular damage by reactions with thiols, metals, or carbon dioxide, or by production of secondary oxidants such as hydroxyl radical, carbonyl radical, or nitrogen dioxide (136).

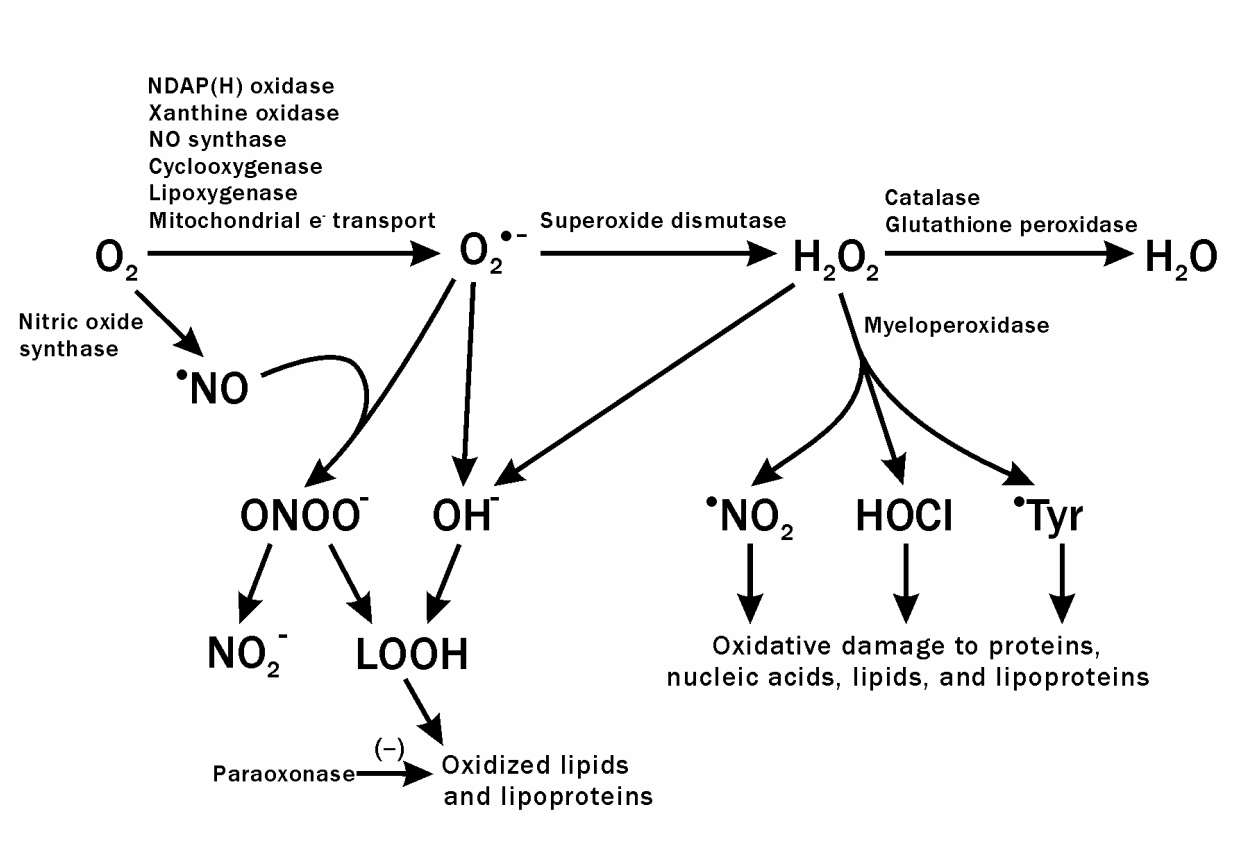


Figure 1.5 Oxidative stress pathways

Pathways and enzymes involved in the production and catabolism of reactive oxygen species, and the targets of oxidative damage.

1.4.3 Antioxidant Molecules and Enzymes

Intracellular Antioxidants: Antioxidant defense systems include enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), as well as nonenzymatic molecules, such as vitamins, flavonoids, coenzyme Q, uric acid, and thioesters. Antioxidant enzymes are important to neutralizing oxidative stress within and outside of cells. SOD catalyzes the reaction of superoxide into H_2O_2 and O_2, and exists in two isoforms; mitochondrial manganese-SOD and cytosolic copper/zinc-SOD both contribute to reducing intracellular oxidative stress. In the cytoplasm, H_2O_2 is hydrolyzed by GPX, except a minority which is handled in the peroxisome by catalase. The antioxidant activity of GPX is also responsible for keeping lipids within the cell in a reduced state, which is accomplished by oxidation of glutathione. The cellular ratio of reduced to oxidized glutathione is considered an oxidative stress marker (137).

Extracellular Antioxidants: Plasma proteins are responsible for binding free iron and copper, and for protecting lipids from the oxidative effects of these cations in the circulation. Plasma proteins include albumin, transferrin and ceruloplasmin, which not only bind cationic metals, but also scavenge superoxide radicals (138,139). Alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C) have antioxidant effects in the cytosolic and extracellular environments, respectively. Both vitamins protect against lipid peroxidation, and may have biological importance to supporting endogenous antioxidant systems (140).

1.4.4 Oxidative Stress in Cardiovascular Disease

As discussed above, reactive oxygen species have normal physiological functions, and are essential for transmitting signals within and between cells, and for phagocytic cell defense systems (141). However, excessive oxidative stress can disrupt normal cellular processes, and cause damage to DNA, lipids, and proteins, leading to changes in membrane permeability and protein function (142).

Oxidation of polyunsaturated fatty acids in the membrane lipid bilayer produces unstable aldehydes, and other cytotoxic products. Most of the damage involved with protein oxidation is at the site of sulfhydryl groups, but protein oxidation can also generate protein hydroperoxides, alkoxyl radicals, and protein-bound reductants, which become susceptible to proteolysis due to conformational modifications or polymerization changes.

Several lines of evidence implicate oxidative stress in cardiovascular disease (143). The oxidative stress hypothesis of atherosclerosis proposes that the oxidation of lipids and lipoproteins promotes foam cell formation and development of atherosclerotic lesions (144). The location and mechanisms of these processes *in vivo* remain unclear, however the proatherogenic effects of oxidative stress likely extend beyond lipoprotein oxidation, to include all stages of atherogenesis (145,146).

Increased production of reactive oxygen species in the vascular wall has been demonstrated in atherosclerosis, and oxidative stress triggers pathological processes in the endothelium, smooth muscle cells, and inflammatory cells (141). Associations have been demonstrated between oxidative stress and known cardiovascular risk factors including obesity, diabetes, hypertension, cigarette smoking, and dyslipidemia (147–150). Obesity is associated both with elevated oxidative stress levels and with lower antioxidant protection (151,152). A

study of the cohort of Framingham offspring found direct relationships between BMI and visceral adiposity with markers of oxidative stress (153). Increased reactive oxygen species production in cardiovascular disease influences risk through common mechanisms with hypertension and hypercholesterolemia (154,155).

Certain pharmacological therapies that address cardiovascular risk factors have been demonstrated to also ameliorate oxidative stress levels. Combination therapy with antihypertensive medications and statins improves endothelial function, and reduces inflammation and oxidative stress (156). The pro-survival effect of statins may be partly conferred through reduction of oxidative stress by inhibition of superoxide anion formation through reduced NAD(P)H activity (157), and increased nitric oxide synthesis (158–161). Medications that lower blood pressure also may have antioxidant effects. Angiotensin II produces superoxide anions, hydrogen peroxide, and hydroxyl radicals, which may adversely affect the cardiovascular system through hypertension and cardiovascular hypertrophy (162). The beta-adrenergic receptor blocker Propranolol has demonstrated anti-peroxidative and anti-radical activity (163,164), and calcium channel blockers may also scavenge peroxyl radicals to an extent that can inhibit lipid peroxidation (165). Finally, the angiotensin-converting enzyme inhibitor Captopril has sulfhydryl content that may enable hydroxyl radical scavenging (166), but its ability to scavenge superoxide radicals is not proven (167).

1.4.5 Dietary Antioxidant Studies

Descriptive epidemiologic studies have supported the hypothesis of an inverse relationship between intake of foods rich in antioxidants and risk of cardiovascular disease (168–172). Not only are rates of cardiovascular disease lower in populations that consume diets rich in

antioxidants and antioxidant vitamins (173), but the benefit is directly reflected by changes in plasma antioxidant levels (174).

The Nurse's Health Study is the largest analytic observational study to investigate antioxidant vitamin intake and cardiovascular disease. A prospective cohort of 121,700 U.S. female nurses aged 30–55 made up the initial cohort, and dietary intake data for 87,245 participants indicated those individuals who consumed the highest levels of β-carotene and vitamin E through diet and vitamin supplements were at reduced risk of myocardial infarction and fatal cardiovascular disease over 8-years of observation (175). Similar analyses performed among a follow-up study of 39,910 male participants in the Health Professional Study showed a lower risk for myocardial infarction and fatal cardiovascular events for subjects with the highest β-carotene and vitamin E intakes (176). Subanalyses from both studies found evidence for vitamin E consumption and cardiovascular risk reduction.

Results from 34,486 post menopausal women with no history of cardiovascular disease who were followed as part of the Iowa Women's Health Study also supported these findings. Although supplemental vitamin E was not observed to reduce cardiovascular risk, dietary vitamin E was strongly associated with reduced risk of cardiovascular mortality (177). Finally, the National Health and Nutrition Examination Survey (NHANES 1) evaluated vitamin C intake among 11,348 adults aged 35–74 over a median of 10 years. Lower rates of cardiovascular death were observed among individuals with the highest dietary and supplemental vitamin C intake (178). However, the use of other supplements was not considered separately, and vitamin C intake was highly correlated with the use of other vitamins in both the Nurses Health Study and the Health Professionals Study, so vitamin C may not be the sole factor responsible for these findings.

1.4.6 Clinical Trials of Antioxidant Therapies

Randomized control trials inspired by these epidemiological observations have been conducted to explore the potential benefit of antioxidant supplements in cardiovascular disease (179). One of the earliest trials was the Cambridge Heart Antioxidant Study (CHAOS) which randomized 2002 patients to receive either 800 IU α-tocopherol or placebo. CHAOS investigators observed reduced rates of the combined endpoint of cardiovascular death or non-fatal myocardial infarction by 50% in the treatment group (180).

Not all antioxidant trials observed similar benefits, however. Male physicians from the Physician Health Study (PHS) who were enrolled in a long term placebo-controlled trial of β-carotene did not show reduced risk of cardiovascular death or myocardial infarction over the 12 years of supplementation. PHS investigators concluded that β-carotene produced neither benefit nor harm in terms of the incidence of cardiovascular disease, or death from all causes (181).

The Alpha-Tocopherol, Beta Carotene (ATBC) Cancer Prevention Study actually found increased CV deaths with beta carotene, and no overall benefit with vitamin E or C (182). However, this study was designed to determine the effects of daily supplementation with antioxidant vitamins on incidence of lung cancer and other cancers among 29,133 Finish male smokers, not for studying cardiovascular disease risk.

The Women's Health Initiative, which included 161,808 post menopausal women followed for cardiovascular events over 8 years, found no evidence for cardiovascular protection with multivitamin use, nor did they find any protection from incidence of common cancers or reductions in total mortality (183).

The Heart Protection Study (HPS), which was designed to investigate risk reduction of lipid lowering therapies, also evaluated antioxidant supplementation and found that no added

benefit was conferred for either cardiovascular or non-cardiovascular outcomes (184). Recent studies have suggested antioxidant supplements may even interfere with the degree of atherosclerotic lesion reduction derived from lipid lowering therapy (185), or may add no benefit treatment with fish-oil, ACE-inhibitors, or aspirin (186–188).

Study design may be among the reasons these trials have not observed a beneficial effect from antioxidant vitamins. Studying high risk populations permits reducing sample size, because event rates are anticipated to be high. However, higher risk patients may be less amenable to the positive effects of supplements, because more advanced stages of atherosclerotic lesion development may not benefit from reductions in oxidative stress (140). Improvements might be more easily observed at earlier stages of lesion development (189), as suggested by the moderate improvements in endothelial function observed in children with hypercholesterolemia given vitamin E and C (190). However, not all secondary prevention trials reported negative findings, and many primary prevention trials did not find any benefit for antioxidant vitamins (review (191)).

The paucity of cardiovascular risk reduction observed from most vitamin trials could also be attributed to vitamin preparations used. Synthetic all-*rac*-α-tocopheral used in most trials (including the PHS, the HPS, and ATBC) is not as effective at increasing plasma vitamin E, and degrades at 3–4x the rate of natural vitamin E (192). Synthetic vitamin E may also have detrimental side effects, by interfering with absorption of other fat-soluble antioxidants, such as γ-tocopherol, and by inhibition of glut-S-transferases, enzymes responsible for drug detoxification (193). Also, antioxidant doses used may not have been optimal; individual variability in absorption and response to antioxidant supplementation is wide, and giving the same dose to all patients may do more harm than benefit.

Unlike synthetic vitamin E, natural vitamin E is composed of a potentially more potent blend of tocopherols. Studies using natural vitamin E (including ASAP, CHAOS, and SPACE) were more likely to report positive results. However, despite changes in circulating levels of vitamin E with natural supplements, no changes to plaque oxidative stress levels have been demonstrated (194).

Furthermore, subpopulations of patients may derive benefit from antioxidant supplements that has not observed in larger studies. Patients who suffer from pathophysiological processes under disproportionate influence by oxidative stress may benefit from interventions with antioxidants. For example, the rate of transplant-associated atherosclerosis was found to be reduced by vitamins C and E given in addition to pravastatin (195). The antioxidant probucol provided prior to angioplasty reduced coronary artery restenosis and repeat angioplasty rates (196). These findings suggest there may be a benefit for antioxidant supplements in particular settings.

Another explanation for the overall negative findings in vitamin studies is that the protective forms of antioxidants present in foods were not provided. Health benefits observed in dietary studies could have be due to plant-derived flavonoids rather than antioxidant vitamins (197). Flavonoids are a form of antioxidant found in certain food products, and include epicatechin and catechin (flavonols in fruit), and procyanidins (tannins, present in wine and cocoa) (198). Certain foods rich in flavonoids are demonstrated to improve endothelial function in patients with coronary artery disease, with results observed for both short- and long-term consumption of black tea (199) and red wine (200,201). Moderate amounts of flavonoid-rich dark chocolate consumed over two weeks improved endothelium dependent flow mediated

dilatation in healthy adults, although no changes in oxidative stress measures, blood pressure, or lipid profiles were observed (202).

Reduced endothelial function, either by reduced expression of endothelial nitric oxide synthase and/or bioavailability of NO, is associated with atherosclerotic disease, but how flavonoids improve endothelial function is still not well understood. In vitro studies indicate flavonoids may diminish NO inactivation by free radicals, by inhibiting of NAD(P)H oxidase, or increasing endothelial nitric oxide synthase expression (203,204). A 12 week randomized placebo controlled trial of isoflavones, a type of phytoestrogen related to flavonols, reduced inflammation and improved endothelial function in subjects with prior history of ischemic stroke (205). Flavonoids may also interfere with low density lipoprotein oxidation (206), and increase total antioxidant capacity of plasma (207).

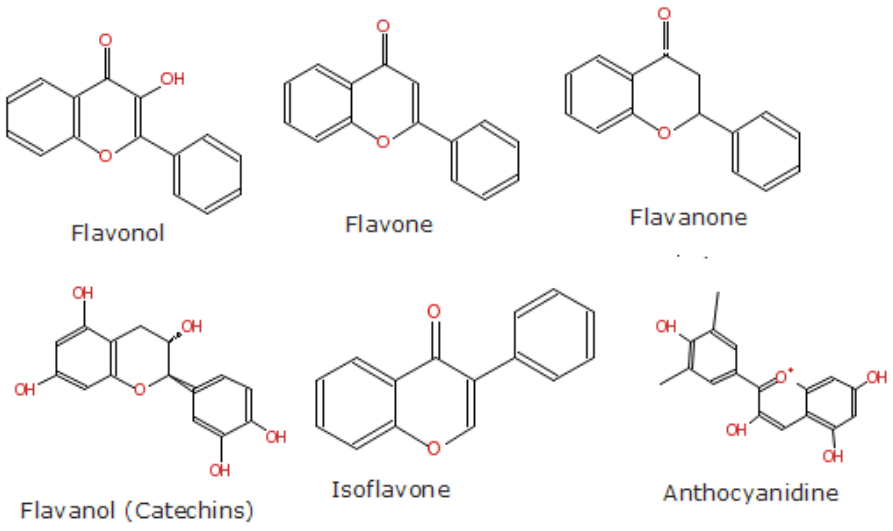


Figure 1.6 Molecular structures of major classes of flavonoids

Larger epidemiological vitamin supplementation studies have not investigated the potential of flavonoids to reduce cardiovascular disease risk, either alone or in combination with classical antioxidant vitamins. In order to determine the potential health benefits of antioxidants derived from vitamins and foods, a better understanding of the specific effects of antioxidants, including rates of absorption, tissue distribution, metabolism and activity in relevant microenvironments, is necessary in order to select the correct formulations and doses that would most improve vascular disease risk and provide long term cardiovascular protection (208).

1.4.7 Oxidative Stress and Plaque Stability

Oxidative stress may not only play a role in plaque development, but may also influence stability of atherosclerotic lesions. Reactive oxygen species production in human coronary artery plaques varies by plaque components and plaque stability (209). Stable coronary artery disease is characterized by a thick, fibrous cap with more extra-cellular matrix materials and smooth muscle cells, and lower oxidative stress levels and fewer macrophages (209). Unstable coronary artery disease, defined clinically by acute coronary syndromes, is associated with plaques that have thinner fibrous caps, and larger lipid pools more populated by macrophages. Increased reactive oxygen species production caused by higher proportions of macrophages may lead to lesion instability, by superoxide-induced expression of matrix-degrading proteases (210). Atherosclerotic specimens and plaque fragments show spatial association between oxidized LDL and the pro-oxidant enzyme NAD(P)H p22phox subunit (211), the activity of which is associated with atherosclerotic lesion formation (212). Oxidized LDL also promotes smooth muscle cell and endothelial cell apoptosis, leading to plaque erosion and vulnerability to rupture (144).

Production of reactive oxygen species promotes smooth muscle cell proliferation, and may promote lesion growth (211). Smooth muscle cells generate reactive oxygen species, depending on their level of activation. Activated intimal smooth muscle cells produce more superoxide and NAD(P)H oxidase components than do inactive intimal smooth muscle cells (213,214).

Quantitative and qualitative differences in disease activity and oxidative stress levels between stable and unstable coronary artery disease may influence circulating oxidative stress levels. Instability and cellular processes within unstable atherosclerotic plaques may increase circulating oxidative stress levels, and oxidative stress biomarkers may be useful surrogate markers for disease burden and prognosis (215).

1.4.8 Biomarkers of Oxidative Stress

Direct measurement of free radicals by electron resonance spin trapping captures oxidative stress reactions in real time (132), but this method is not appropriate for high throughput use in clinical studies. However, damage to polyunsaturated fatty acids, lipoproteins, and amino acids by reactive oxygen species results in pro-inflammatory and atherogenic particles that are detectable in the circulation. These end products of oxidative stress reactions, as well as the levels and activities of pro-oxidant and antioxidant enzymes, may be used to estimate the degree of free radical-mediated oxidative processes.

Given the evidence linking oxidative stress to cardiovascular risk factors, inflammation, and pathogenesis of coronary artery disease (216), biomarkers of oxidative stress processes have been investigated for the strength of their association with cardiovascular disease. The criteria for the selection of oxidative stress markers was for measurement in our studies was: (i) evidence

that the marker represents an enzyme or metabolite within a validated pro-oxidant or antioxidant pathway; (ii) ability to measure the marker in human plasma by an established reproducible assay; and (iii) previous evidence linking the marker with processes related to atherosclerosis. Based on these criteria, I undertook measurement myeloperoxidase, nitrotyrosine, oxidized LDL, and plasma antioxidant capacity.

1.4.8.1 Myeloperoxidase

Myeloperoxidase is an enzyme of the peroxidase family which plays a pro-inflammatory role in the innate immune system defense against bacteria and parasites (217). Myeloperoxidase is secreted by different types of activated leukocytes, but primarily by polymorphonuclear neutrophils (159), including those undergoing activation in the endothelium of coronary arteries (217,218). In the extracellular environment, myeloperoxidase activity generates pro-oxidant molecules, including hypochlorous acid (219), and byproducts of myeloperoxidase cause protein nitrosylation (159,220). Oxidative stress generated by myeloperoxidase activity also alters lipids and proteins on LDL (221,222), and can render HDL dysfunctional by chlorinating or hydrosylating residues on apolipoprotein A1 (223) and impairing cholesterol efflux to HDL particles (224).

Elevated myeloperoxidase concentrations associates with coronary artery disease (225), and risk of adverse outcome in patients with acute coronary syndromes (226,227), acute myocardial infarction (228), and chronic heart failure (229). Myeloperoxidase levels also predict myocardial infarction in patients presenting with chest pain (226) and future cardiovascular risk in healthy individuals (103,230,231). Elevated plasma myeloperoxidase is also associated with

endothelial dysfunction (232). Reductions in MPO gene expression (233) and circulating MPO levels (234) have been observed in patients treated with atorvastatin, a statin drug.

Not all cohort studies have found association between myeloperoxidase and coronary artery disease. A study of 382 stable coronary artery disease patients followed prospectively for a median time of 3.5 years found plasma levels did not predict risk of all-cause mortality (235). In that study, myeloperoxidase concentrations were slightly higher in non-survivors ($p=0.06$), but were not associated with risk of myocardial infarction or with target-lesion revascularization. This small-sized study did not determine which deaths were cardiovascular, so the value of myeloperoxidase for predicting cardiovascular outcomes in stable coronary artery disease patients remains unclear.

1.4.8.2 Nitrotyrosine

Nitrotyrosine is a marker of protein tyrosine residue nitration, which is a the downstream result of interactions between nitric oxide-derived oxidants and superoxide anions, (236) and myeloperoxidase-generated chlorotyrosine (237). Nitrotyrosine levels are elevated in patients with coronary artery disease, may be lowered by statin therapy to a magnitude similar to reductions observed in total cholesterol and LDL particle number, but independent of reductions in C-reactive protein and LDL cholesterol levels (159).

1.4.8.3 Oxidized LDL

Oxidation of LDL is implicated in pathophysiology of coronary artery disease (238) but the chemical entity of oxidized LDL represents many different immunologic chemical changes to nascent LDL particles (239). Oxidation of LDL occurs in two stages, the first of which involves

the oxidation of lipids within the LDL particle, yielding minimally-oxidized LDL, which has chemoattractant properties for circulating monocytes (240). Further oxidation alters apolipoprotein B, making the particle no longer recognizable to its receptor, and it is instead recognized by macrophage scavenger receptors (38). Unlike nascent LDL uptake, oxidized LDL uptake by the scavenger receptor is not regulated by cellular cholesterol levels (37).

High throughput assay methodology permits measurement of oxidized LDL in clinical studies. Elevated plasma oxidized LDL is associated with angiographic coronary artery disease (241), acute coronary syndromes (242), metabolic syndrome (243), and risk of myocardial infarction (243), as well as presence of sub-clinical atherosclerosis (244). Oxidized LDL is associated with carotid intima media thickness, and progression of carotid plaques, independent of conventional risk factors (245,246). In one study, elevated oxidized LDL predicted increased carotid intima media thickness and echogenicity, while C-reactive proteins levels did not (80).

1.4.8.4 Antioxidant Enzymes and Antioxidant Capacity

Glutathione peroxidases are antioxidant enzymes central to defense against free radicals and oxidative stress. A recent prospective study of coronary artery disease patients found intracellular glutathione peroxidase activity measured from circulating red cells was a strong predictor of cardiovascular events in patients with coronary artery disease (247).

Paraoxonase enzymes were first identified as catalyzing breakdown of organophosphates found in pesticides. There are three paraoxonase isoforms coded for by the paraoxonase set of genes. Paraoxonase 1 is synthesized by the liver and circulates bound to HDL, where it prevents oxidation of LDL (248)**.** Paraoxonase 2 is a expressed ubiquitously and may protect cells against oxidative damage (249)**.** Paraoxonase 3 acts in ways similar to paraoxonase 1 but differs by its

regulation and substrate (250)**.** Paraoxonase 1 and 3 are implicated in lowering cardiovascular risk, by reducing formation of oxidized LDL (250).

Unfortunately, methods do not currently exist for measuring intracellular glutathione peroxidase and paraoxonase in EDTA-plasma, so they were not tested in this thesis. However, levels of plasma antioxidant capacity have shown clinical utility for prediction of atherosclerosis (251–253). Plasma antioxidant capacity was therefore selected for evaluation for disease and outcome risk prediction in coronary angiography patients.

1.5 Statistical Considerations for Biomarkers

Biomarkers applied to cardiovascular risk prediction should offer significant improvements in risk assessment, however mere statistical significance (i.e. $p \leq 0.05$) for an emerging biomarker is not sufficient to determine whether or not it will be useful. Statistical significance suggests biomarker concentrations *differ* between subjects with and without a disease, beyond variation expected from chance, but does not indicate the degree of overlap, or how risk prediction will be affected. False positive and false negative rates are high for biomarkers with large overlaps across much of their potential diagnostic ranges (Figure 1.7).

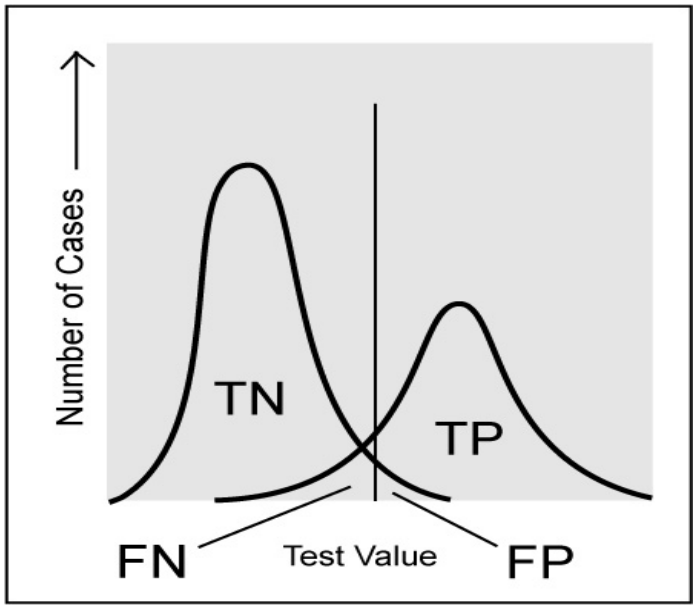


Figure 1.7 Test false positives and false negatives

Illustration of False Positive (FP) and False Negative (FN) ranges for a variable with overlapping values between two groups at a given cut off. The remaining areas of accuracy are denoted as True Positive (TP) and True Negative (TN), where the cut-off will give accurate detection of cases.

Biomarker discrimination, the ability of a marker to distinguish individuals with or without disease, is expressed as the c-statistic, or the area under the receiver operator characteristic (ROC) curve, which ranges from 0.5 (for an uninformative test), to 1.0 (for a perfect test). The ROC curve can be considered a graphical representation of the potential false positive and false negative proportions, across a range of a given variable (Figure 1.8). Generally, good global models for risk discrimination have a c-statistic of 0.7 or greater, and biomarkers that significantly improve risk models are generally expected to increase the c-statistic by 0.05 or greater (254–256), although even modest improvements in the ROC curve can be challenging to make.

Although it is useful for illustrating the value of a marker for discrimination between cases and non-cases, the ROC curve has shortcomings as a model assessment tool. It is not suitable for right-censored survival data, unless the area is adjusted for time to event, and the c-statistic cannot characterize how a marker improves estimation of individual risk.

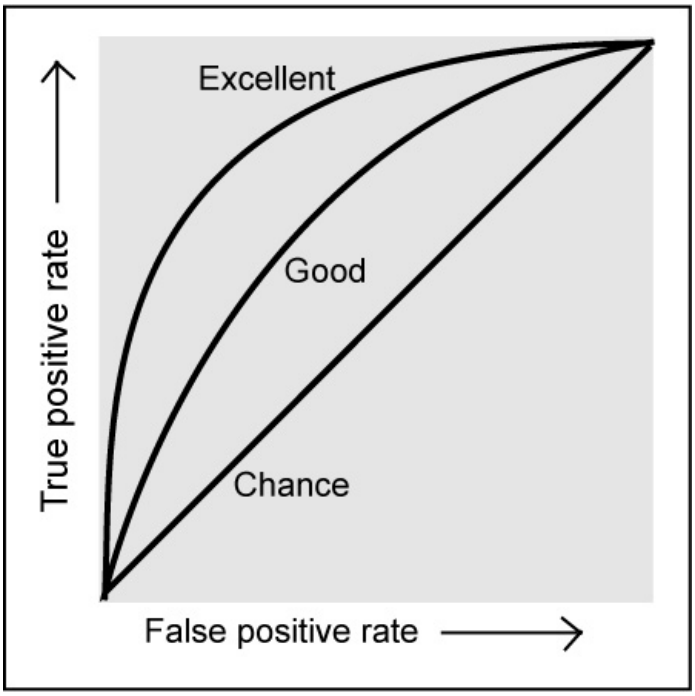


Figure 1.8 The receiver operator characteristic (ROC) curve

Comparison of ROC curves for risk models that prediction no better than chance, with risk models that have good and excellent predictive accuracy. Reduced false positive rates and increased true positive rates improves the area under the ROC curve.

Other tools that measure risk model calibration and patient risk reclassification have been offered recently as adjunct tests to the c-statistic (256). Risk calibration refers to the ability of the model to assign predicted risks that closely match actual observed risks, across a range of low to high risk categories. The Hosmer-Lemeshow test of calibration tests for differences between predicted and observed risks, and reports p-values ≤ 0.05 when predicted risks do not match those observed (257).

Although the Hosmer-Lemeshow test identifies models that are poorly calibrated, it does not necessarily assess where discrepancies in the model calibration may lie, thus finding out how many patients are re-classified across risk categories is another important step (255). After all, the final goal of cardiovascular risk models is to correctly estimate a patient's long term risk, to allow their physician to choose an appropriate treatment target and intensity.

For cardiovascular disease risk reduction in North America, the most commonly used classification scheme is based on the NCEP-ATP III guidelines, which use a scoring scheme based on the 10-year predicted risks for coronary heart disease events of the Framingham Risk Score. Biomarkers that can reclassify an important portion of patients into different risk categories correctly would be valuable for clinical application, particularly if they were able to reclassify those patients who fall in the intermediate risk range (10 year event risk of 10%–20%) (258), where important clinical decisions are more difficult to make. Subjects at intermediate risk would represent good candidates for additional testing, because changes in their risk scores would translate into changes in treatment intensity (70).

Risk reclassification of C-reactive protein was evaluated within the Northwick Park Health Study and the Edinburgh Artery Study participants, to determine its predictive value for cardiovascular events(259). Although higher CRP concentrations were associated with a higher

events risk in both studies, the time adjusted c-statistics for the Framingham-based model (0.62 and 0.68 for the Northwick and Edinburgh studies, respectively) was not significantly improved by including CRP in predictive models (0.66 and 0.67 for the Northwick and Edinburgh studies, respectively). Although risk models with and without CRP did not deviate from good risk calibration by the Hosmer Lemeshow test, net risk reclassification rates offered by CRP did not reflect significant improvement risk estimation. In the Northwick study, overall net reclassification by using CRP in the prognostic model was 8.5% (p=0.09 for improvement from risk estimation without CRP), and similar results were seen in the Edinburgh study. These negative findings were attributed to the wide overlap in CRP concentrations between cases and controls, which made setting a threshold value that distinguishes individuals who suffer events with appropriate sensitivity very difficult.

Novel biomarkers may yet emerge which could be used to improve patient risk estimation from conventional risk scores (260). However, despite increasing availability of biomarker assay methods, and reports that demonstrate associations with cardiovascular disease, consensus regarding how novel biomarkers should be applied to clinical practice are not available. Indeed, indiscriminate testing that wastes resources but does not improve treatment of patients would be the major risk of wide scale biomarker use. In the 35% of adults deemed to be at low risk of cardiovascular events (<5% risk in 10 years), further testing may not justify changing treatments, and might lead to unnecessary concern if a false positive result occurs.

Conversely, high risk patients (>20% risk in 10 years), which make up an estimated 25% of the population, would not likely have their treatments discontinued even if biomarker tests were negative, as aggressive preventive strategies are well supported by current evidence. Indeed, it is the 40% of the population within the intermediate (5–20%) range of risk who are

most likely to benefit from biomarkers, as testing would offer evidence to support their assigned treatment targets, or would reclassify them more accurately to another risk group.

Stable coronary artery disease is one of the earliest and most common presentations of coronary artery disease (261), but it is often poorly diagnosed and managed (262,263). This is chiefly due to variability in clinical presentations and degrees of severity, especially in high-risk groups such as the elderly, and diabetic patients. Biomarkers such as CRP (264) may improve risk prediction in stable coronary artery disease patients, however there is a paucity of studies demonstrating the utility of oxidative stress markers for prognosis in stable coronary artery disease.

New biomarkers may improve risk estimation and risk calculation, and optimize preventive strategies, if they are applied correctly, among populations for whom risk prediction and disease state estimation are not optimal (265). The search for useful risk biomarkers—which are not only relevant to the pathophysiology of atherosclerosis but which actually improve patient risk estimation—continues.

1.6 Genetics of Cardiovascular Disease

1.6.1 Background to Cardiovascular Genetics Research

Since the search for genes that predispose to cardiovascular diseases began >20 years ago, genetic has profoundly altered understanding of atherosclerosis, but not revolutionized how cardiovascular diseases are treated. The expectation of genetic polymorphisms becoming as useful for risk prediction as conventional cardiovascular risk factors has not yet been met, despite years of intensive research.

The key to genetics of cardiovascular disease is based on the premise that individuals differ in their susceptibility, despite similar classical risk factors, and family history plays a key role in these differences. Atherosclerosis is a polygenic disease, with heritability estimated in the range of 40–60% (266). Family history confers a substantially increased risk of coronary artery disease (267). In the Framingham study, family history of cardiovascular disease or stroke or peripheral arterial disease was associated with a 2.4- and 2.2-fold increased risk of disease in men and women, respectively (268). Effects of genetic risk persists beyond correction for risk factors under genetic influence, such as blood pressure and plasma lipoprotein composition (41,269).

Several Mendelian disorders caused by rare mutations demonstrate the effect of high risk mutations on cardiovascular risk. These mutations are rare in most populations and thus contribute negligibly to population-attributable risk for disease, but they demonstrate key principles of lipid metabolism and atherogenesis. The best known example of these mutations is familial hypercholesterolemia, which results from mutations in the LDL-receptor protein, or more rarely in apolipoprotein B (270). These mutations dramatically increase cardiovascular risk for unlucky patients who inherit them, however for most patients genetic susceptibility to

atherosclerosis results from multiple common variants acting each with a relatively small effect, which are complicated by interactions between heritable and environmental factors (271).

1.6.2 Single Nucleotide Polymorphisms

Scattered throughout the human genome are ~11 million single nucleototide polymorphisms (SNPs) that occur at >1% frequency (272). Other types of genetic polymorphisms include tandem replications of short or long sequences, so called mini- and micro-satellites, and more complex sequence alterations such as deletions or insertions. Copy number variations, which are large sections of the genome that appear to have been deleted or duplicated, have also recently been characterized (273–276). Although copy number variations may affect genes and gene regulatory regions, they seem to be associated with less phenotypic effects than SNPs, despite affecting 2–3 times the number of total nucleotides involved in SNPs, and influencing a total of 15% of the genome (276).

SNPs represent a rich soil to test many hypotheses for heritability of cardiovascular disease. SNPs in coding, regulatory or otherwise functional sequences have the potential to influence gene regulation, expression, and translation. Nonsynonymous SNPs are polymorphisms that alter the coding sequence of genes by changing the amino acid sequence, or altering translation. Although nonsynonymous SNPs are the usual suspects in human disease, SNPs in the promoter regions, regulatory regions, introns or intronic splice sites, and even synonymous changes within coding regions, may have functional significance by changing transcriptional and translational processes (277). SNPs that do not lie in coding regions of the genome can be used as markers to detect regions associated with disease, which can be further screened to discover SNPs that cause phenotypic changes.

1.6.3 Linkage Studies and Linkage Disequilibrium

When families with several affected members are investigated for disease mutations, researchers employ linkage analysis. Specific genetic markers are tested for imbalanced patterns of inheritance, or disequilibrium, between affected and unaffected family members.

The methodology behind linkage analysis studies assumes genetic markers that associate with disease are close enough to the disease-causing variation that recombination rates during meiosis are low. When a marker inherited by affected family members is identified, dense mapping is done to look for the disease causing mutation, which is then studied to connect it to the phenotype observed. When these mutations arise spontaneously in families, they are not likely to affect disease risk in whole populations, however they have potential to offer insight into disease pathways by effects they cause.

Linkage studies take advantage of low recombination rates between SNPs on nearby areas of the chromosome, however not all regions of chromosomes have the same recombination frequency. Many regions combine at lower or higher frequency than expected. Linkage disequilibrium (LD) occurs from non-random association of alleles during recombination. LD leads to SNPs associating in groups of alleles on the same chromosome, and being inherited together. In genetic studies, linkage can be quantified by calculated values of D′ (derivation of haplotype frequencies from LD), and by r^2 (correlation between SNPs), with perfect predictive power of knowing one allele from measuring another at $r^2=1$. Recombination hotspots also exist throughout the genome, especially near centromeres or telomeres, and these areas have low LD and fewer correlated SNPs (278).

1.6.4 Haplotypes

A haplotype is a pattern of SNPs in linkage disequilibrium inherited by an individual as a unit on a chromosome donated from the maternal or paternal set. Two categories of tools are used to determine haplotypes unambiguously: direct genotyping pedigrees, which requires testing parents and offspring to determine haplotype structure, and molecular methods applied in combination with genotyping for experiments where pedigree information is not available. The second method, which may involve allele-specific PCR or somatic cell hybrids, is the gold standard method for haplotype construction in unrelated individuals.

Although these methods are well established, it is much less labour intensive for epidemiological researchers to infer haplotypes for unrelated individuals, which can be done very accurately by statistical methods (279). Statistical inference of haplotypes may be done by a number of algorithms, including parsimony (identifying individuals with known haplotypes and searching for combinations consistent with known haplotypes), estimation-maximization likelihood models (making initial guesses on haplotype frequencies based on genotype frequencies and distribution, then assigning unknown haplotypes with high probability), or Bayesian methods (incorporating assumptions or prior information to guide inference of haplotypes not previously observed).

Parsimony algorithms are impaired by the requirement that at least one individual be present for each possible haplotype. Expectation-maximization methods requires accurate genotype frequencies and distributions, which can be skewed in some data sets, and is computationally burdensome. Bayesian algorithms require that the initial assumptions and frequencies given are accurate, and this information is not always available. For any of these methods, a proportion of haplotypes assigned will be incorrect. Singleton haplotypes may never

be resolved, and there may be a loss of power from uncertainty when testing for associations with disease phenotypes (280). However, most algorithms estimate overall haplotype frequencies well for datasets with few or no genotyping errors, and although individual haplotype accuracy varies, researchers can associate haplotype frequencies with disease phenotypes without resolving individual level data, and this approach reduces uncertainty and improves statistical power.

Haplotype blocks are useful for epidemiological studies because they allow SNPs to be identified by tagging SNPs without the need to directly genotype them (281). For example, the 500,000 SNPs in the Wellcome Trust study covered 0.02% of the 3.2 billion bases in the genome, but covered ≈90% of the genome due to associations between SNPs (282).

1.6.5 The International HapMap Project

The International HapMap Project (HapMap) is a public catalog of common SNPs, with linkage disequilibrium structures characterized from analysis of related individuals of African, Asian, and European ancestry (278). Linkage disequilibrium for most of the genome is high. Thus, the combination of alleles across multiple SNPs is not as diverse as expected, and has been shown to be ancestrally conserved (283).

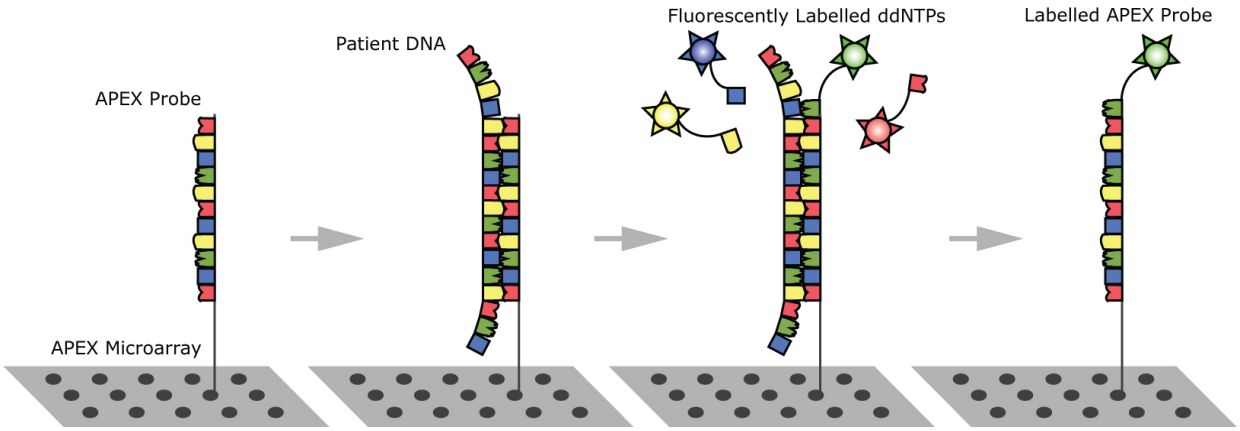
HapMap is an extremely useful resource for designing and interpreting genetic studies. However, it is limited by capturing SNPs only above a certain frequency, which prevents some rarer disease-causing variants of low frequency from being captured by tagging approaches. Up until the most recent version (Phase 3), only four ethnicities were included, thus variation was not necessarily represented for other populations. At the present time, Phase 3 includes 1.5 million markers for 1,115 individuals from 11 populations. However, at 3.1 million SNPs, the

Phase 2 version of HapMap still offers the greatest total number of markers, despite only representing 4 populations.

1.6.6 APEX Genotyping

Genotyping platforms offer high throughput genotyping of multiple SNPs, however costs can be high and customizability varies across the current genotyping platforms, which include Affymetrix GeneChips (284), and Illumina bead-array systems (285). Arrayed primer extension (APEX) is an approach to multiplex genotyping that employs a minisequencing assay on a two-dimensional microarray of oligonucleotide probes (286). These probes are designed to be complementary to 25 bases upstream and downstream of the SNP site. Sanger-based sequencing chemistry is used to genotype SNPs, and during the twenty minutes the sequencing reaction takes to complete, a DNA polymerase carries out a single base extension reaction at the 3'-end of SNP and allele-specific probes immobilized on a glass slide. Where patient DNA has hybridized, the polymerase adds a single dideoxyribonucleotide base labeled with a base-specific fluorescent chromophore tag (287). When scanned, these tags yield SNP-specific genotype information (288).

APEX has been used to detect β-thalassemia (289), and polymorphisms in *BRCA1* (290), and *p53* (291). Also, APEX performs well against other genotyping methods, with up to 100% call rate and 99.99% accuracy rate from automated analysis methods (292). As a customizable research tool for medium-scale genotyping projects, APEX may show utility for point-of-care genetic diagnostic applications because of its relative speed and high genotyping accuracy.



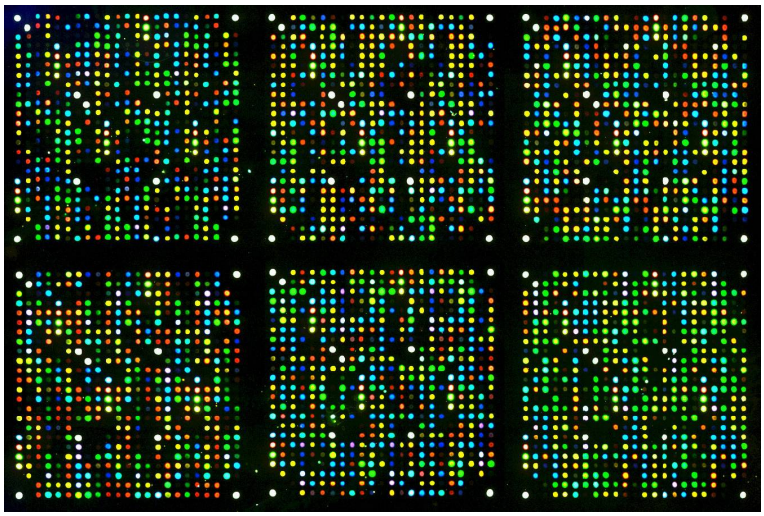


Figure 1.9 APEX genotyping

Above: During APEX genotyping, patient template DNA hybridizes to probes immobilized on a glass slide. A single base extension reaction adds a fluorescent-labeled dideoxyribonucleotide base, which is imaged to reveal genotyping data.

Left: A typical APEX slide, scanned to reveal fluorescence for genotyping.

Although results from APEX experiments may be visualized and interpreted manually using software such as SNPChart (293), larger scale genotyping projects require accurate automated genotyping methods. One tool for automatic APEX genotyping is the Multi-dimensional Automated Clustering Genotyping Tool (MACGT) (294), which employs multi-channel spot intensity data, with SNP-specific oligonucleotide probe information. However, each SNP loci tends to perform differently in APEX reactions, so more accurate automated genotyping results may be obtained using methods that adjust for features unique to each reaction. Linear Discriminate Analysis (LDA) genotyping methods, which were recently developed by Podder and colleagues for application to APEX (295), adjust for probe

performance at both the training and prediction stages of genotyping, which allows for outliers in both known and unknown samples, and increases call and accuracy rates (292).

1.7 Statistical Challenges to Cardiovascular Genetics

New technologies have dramatically increased throughput and reduced cost of genotyping studies. However, the robustness of results from cardiovascular genetic association studies has been criticized as weak, with odds ratios typically <1.3, and few replicated associations (296). Lack of power, which causes true associations to be missed, and spurious ones reported, complicated by the fact that atherosclerosis is a common, multifactorial disease generated from many interacting heritable and non-heritable factors, may explain heterogeneity in the literature. Subpopulation allele frequency variation, computational differences in linkage disequilibrium and haplotype inference, and imprecise clinical phenotyping also contribute to more of the barriers to the emergence of clinically meaningful genetic risk patterns.

Gene-environment interactions and interactions between genes (epistasis) may also contribute to inconsistencies between genetic variation studies, especially because they are poorly characterized or rarely adjusted for (297,298). Several polymorphisms combined across a pathway could have clinically meaningful effects, especially in the context of gene-environment interactions, but the number of possible genotype combinations that would need to be tested poses the statistical challenge of multiple comparisons.

Certain features suggest whether genetic association studies are of potential clinical import. A strong biological hypothesis for selecting genes for evaluation, such as changes in expression with disease, or phenotypes in knockout or transgenic models, and consistency between linkage and whole genome analysis studies, improves the likelihood of meaningful

findings. Association studies must also carefully select tagging SNPs, using appropriate LD measurement for haplotypes, reasonable allele frequency cut-offs, sufficient sample sizes, and include functional variants wherever possible. These efforts would reduce false-positive (type I error) findings, as would adjustment for multiple comparisons, and independent replication (299). Cross-validation and bootstrapping techniques are also used to improve effect estimates (300), however many journals require independent validation before publication of findings, to reduce likelihood of associations being reported that are merely spurious.

Phenotypic biomarkers that associate with disease naturally integrate heritable polymorphisms with disease risk, and may offer more feasible approaches for risk characterization in chronic disease than genetic testing alone. Although phenotypic biomarkers are influenced by both heritable and non-heritable factors, if genetic analyses are used to associated phenotypic biomarkers with disease risk, they may confirm whether a phenotype is primary or secondary to the disease process. For example, elevations in C-reactive protein in patients with metabolic syndrome appear to be secondary to the syndrome, rather than primary to the risk of cardiovascular disease observed (301). However, genetic variations in interleukin-18 which associate with changes to plasma interleukin-18 concentrations, and disease, implicate this marker in a causal pathway (302). This type of study may be more important than basic validation studies to confirm the importance of genetic findings, and to differentiate causal from confounding pathways.

1.8 Candidate Oxidative Stress Genes

There are two major categories of approaches to genetic association studies investigating diseases in unrelated individuals: the genome wide approach, and the candidate gene approach. Although these strategies share basic principles of frequency determination in cases and controls, with replication as an important downstream step to verify preliminary findings, they differ in how genotyping targets are selected.

In a genome wide approach, there is no prior bias, and genomes of cases and controls are scanned using hundreds or thousands of markers. This may generate hundreds of false positives, however stringent statistical methods, and replication of results in independent populations, can improve validity of results. For example, genome wide analysis efforts have recently identified a region on chromosome 9 (9p21) that is associated with increased cardiovascular disease risk in two large populations of European descent (303,304), however no genes have been yet identified within that sequence, and no risk factors associated with the region. Other regions with possible disease genes have been similarly identified (282,305). Because so many markers have been tested, even moderate differences found in genome wide association studies can achieve high significance, even after multiple-comparison adjustment, and false discovery rates must be carefully controlled in linkage and association genome screens (306).

Genes that have a potential role in pathways implicated in atherosclerosis are referred to as candidate genes. The candidate genotyping approach begins with selection of genetic targets on the basis of the specific pathway implicated in disease. Frequencies of polymorphisms in gene within the pathway are then compared between cases or controls. Some candidate genes may influence established risk factors (307,308), however few variants have been discovered so far that consistently associate with coronary artery disease risk (309).

Variance in oxidative stress genes may contribute to the impact of oxidative stress on cardiovascular disease and outcome risk (144). Although common complex diseases are difficult to explain entirely through inherited polymorphisms, association studies that determine the contribution of genetic variants to oxidative stress and coronary artery disease have shown potential (310–312). By their random allocation at conception, variations in oxidative genes are not affected by reverse causation, while plasma markers of oxidative stress may be influenced by cardiovascular risk factors that precede disease development. Furthermore, genotypes are not affected by confounding factors, such as smoking, nor do they change with disease severity. Rather, truly causal pathways in atherosclerosis could be distinguished from confounding pathways if variations in genes that influence oxidative stress biomarkers associate with cardiovascular disease risk.

For the candidate gene study undertaken in this thesis, twelve candidate oxidative stress genes were investigated (Table 1.2). For selection of oxidative stress genes, the following criteria were applied: the gene product participates in a validated pro-oxidant or antioxidant pathway; comprehensive information was available to permit genotyping and haplotype analysis by high-throughput methods; and preference was given to genes or polymorphisms with previously-reported associations with coronary artery disease.

Table 1.2 Candidate oxidative stress genes and polymorphisms selected for genotyping

Gene	Gene Product	Association with Disease	Accession IDs for SNPs Selected for APEX Genotyping
ALOX5	5-lipoxygenase	Increased carotid intima-media thickness(313)	rs3780897 rs3780901 rs11239523 rs1487562 rs3780914 rs1565096 rs2291427 rs745986 rs2115819 rs7894352 rs12762303 rs10900213 rs10751383 rs7089063 rs3824612 rs11239528
FLAP	5-lipoxygenase-activating protein (FLAP)	Myocardial infarction and stroke (314)	rs11147439 rs4072653 rs4468448 rs4769060 rs10507391 rs4073261 rs3885907 rs4076128 rs9671124 rs4769055
CYBA2A	NADPH oxidase p22phox subunit	Coronary artery disease (315), hypertension (316–318)	rs4782308 rs12709102 rs4673 rs3794624
GPX1	Intracellular glutathione peroxidase	Coronary artery disease (319,320)	rs1050450
GPX3	Extracellular glutathione peroxidase	Ischemic stroke (321,322)	rs3763012 rs8177447 rs3792796 rs4958872
HMOX1	Heme oxygenase	Restenosis (323,324)	rs9607267 rs2071748 rs2071749
MPO	Myeloperoxidase	Coronary artery disease (325–327)	rs2333227 rs2071409 rs8082134 rs2759
NOS3	Endothelial nitric oxide synthase	Acute coronary syndromes and coronary artery disease (328)	rs1800783 rs1808593 rs1799983 rs3918188 rs7830 rs179983
PON1	Paraoxonase-1	Endothelial dysfunction(329), ischemic stroke (330), adverse cardiovascular events(331)	rs854560 rs662 rs705379 rs705381
PON2	Paraoxonase-2	Coronary artery disease (332), myocardial infarction (333), and stroke (334)	rs6961773 rs17774346 rs987539 rs730365 rs11981433 rs7785039 rs10487133 rs2299267 rs7802018 rs12534274
PON3	Paraoxonase-3	PON1 level (335) and coronary artery disease protection (murine models) (336)	rs9640632 rs2072200 rs10487132
SOD1	Cu/Zn superoxide dismutase	Oxidative stress in diabetic patients(337)	rs4998557 rs1041740 rs2070424
SOD2	Mn superoxide dismutase	Coronary artery disease and myocardial infarction(338)	rs4880

1.8.1 *ALOX5* and *FLAP*

A microsattelite region in *ALOX5*, the gene that codes for 5-lipoxygenase, has been associated with increased carotid intima-media thickness (313), and early onset cardiovascular disease (339). 5-lipoxygenase produces leukotrienes, which are markers of pro-inflammatory and oxidative processes associated with coronary artery disease (340), and leukotrienes also promote macrophage activity in atherosclerotic lesions (341), and LDL oxidation (342,343).

Haplotypes of 5-lipogenase activating protein gene (*FLAP*) also influence circulating levels of chemokine leukotriene-B4, by activation of 5-lipoxygenase. Associations between haplotypes of *FLAP* and myocardial infarction have been reported in cohorts from Iceland and the United Kingdom (314).

1.8.2 CYBA

NAD(P)H oxidase is a primary source of superoxide in the vessel wall, and the p22phox subunit of NAD(P)H oxidase has been co-localized with reactive oxygen species in atherosclerotic plaques (344). Variants of the NAD(P)H oxidase p22 phox subunit gene (*CYBA*) are associated with endothelial dysfunction (345) and coronary artery disease (316,346). The 242T allele confers lower risk of recurrence of cardiovascular events in patients with angiographic disease (347) and recent myocardial infarctions (348), as well as reduced oxidative stress after intensive exercise training, compared to the 242C allele (349).

1.8.3 *GPX1* and *GPX3*

The intracellular form of glutathione peroxidase is recognized as a key antioxidant enzyme in the endothelium, where it may play an important anti-atherosclerotic role(350), and low intracellular

glutathione peroxidase activity is associated with cardiovascular events in patients with coronary artery disease (247).The Pro197Leu substitution within the *GPX1* gene predicts risk of coronary artery disease (351), increased carotid artery intima-media thickness (352), coronary artery calcification (76), and restenosis after placement of a bare metal coronary artery stent (353).

The plasma isoform of glutathione peroxidase (*GPX3*) is a major extracellular antioxidant enzyme that scavenges reactive oxygen species generated by metabolism and oxidative stress. *GPX3* contributes to nitric oxide availability in the vasculature, by protecting NO from inactivation by reactive oxygen species. Nitric oxide availability is crucial to vasorelaxation and platelet function, and a novel, functional transcription start site in the *GPX3* gene has been associated with NO bioavailability, and thrombosis (321). Risk of cerebral venous thrombosis is also associated with a haplotype of *GPX3* that includes this promoter polymorphism (322).

1.8.4 HMOX1

Heme oxygenase 1 is an inducible, rate-limiting enzyme in heme degradation, which produces carbon monoxide, which can exert anti-inflammatory and antioxidative effects. Promoter variants heme oxygenase-1 (*HMOX1*), have been associated with atherosclerotic lesion restenosis in two studies (323,324).

1.8.5 MPO

Several SNPs have been identified within the myeloperoxidase gene. The majority of research has focused on a promoter polymorphism −463G>A, which is hypothesized to influence gene expression (354). This SNP lies within a thyroid hormone response element, and the A allele is believed to decrease expression of the *MPO* gene (355). This polymorphism has been associated

with atherosclerosis progression in women taking hormone replacement therapy (356), and with prevalence of cardiovascular disease (326,357). Although the promoter polymorphism has also been associated with carotid intima media thickness in non-diabetics, the same association was not significant among diabetics (358). This is consistent with data suggesting the oxidative metabolism of leukocytes is reduced in diabetic patients (359,360).

MPO gene expression is also regulation by peroxisome proliferators activated receptor (PPAR)-gamma at the site influenced by the promoter polymorphism (361). PPAR-gamma agonists such as rosiglitazone are used for treatment in type II diabetes, and they may affect regulation of *MPO*. The promoter polymorphism at −573G>C has also been associated with coronary artery disease in French-Canadians (326), adverse events in patients with coronary artery disease (327) and patients with end-stage renal failure (357).

Another polymorphism in the promoter region of *MPO* has been associated with plasma LDL cholesterol and apolipoprotein B concentrations (362), and as myeloperoxidase has been found to adhere to LDL and apolipoproteinB-100 containing lipoproteins (363), this variant may have significant implications on the atherosclerotic risk conferred by myeloperoxidase activity. There has not been extensive research into other polymorphisms in *MPO*.

1.8.6 NOS3

Endothelial nitric oxide generation is considered atheroprotective, and defects in NO generation cause endothelial dysfunction and promotes atherosclerosis (364,365). The endothelial nitric oxide synthase gene contributes to vascular nitric oxide availability, and polymorphisms in the *NOS3* gene have been linked to coronary artery disease risk (366–368). A meta-analysis of 26 studies found the strongest evidence between *NOS3* variants and coronary artery disease risk for

Glu298Asp and intron-4 polymorphisms (369), however independent prognostic value for cardiovascular death in angiography patients has also been demonstrated for the promoter region SNP −786T>C (370). This mutation reduces endothelial nitric oxide synthesis, which predisposes patients to coronary spasm (371), and may modify anti-inflammatory effects of atorvastatin(372). The −786T>C and 894G>T polymorphisms may contribute in an additive fashion to severity of atherosclerosis (373), however this has not been consistently observed (369).

1.8.7 *PON1*, *PON2*, and *PON3*

The paraoxonase gene family members, *PON1*, *PON2*, and *PON3*, are located adjacently on chromosome 7. Polymorphic variations within the *PON1* gene promoter influence the arlyesterase activity of the enzyme, and severity of coronary artery disease (374). Nonsynonymous SNP Gln192Arg is associated with endothelial dysfunction in diabetic subjects (375), and predicts earlier onset of coronary events (376).

Bhattacharyya and colleagues demonstrated this nonsynonymous polymorphism has a dose dependent effect on serum paraoxonase 1 activity, and patients with the lowest activity genotype had a higher risk for all-cause mortality and major adverse coronary events (331). However, a recent meta-analysis of SNPs in *PON1* indicated only weak associations with risk of coronary artery disease (377), and activity of paraoxonase is argued to be a better disease marker than *PON1* genotype (378), even when haplotypes are used to characterize genetic variation (379).

Activity of paraoxonase 2 is not measurable in serum, but *PON2* is widely expressed in a variety of tissues (380), and several polymorphisms with pathophysiological consequences have

been identified. The Ala148Gly polymorphism has been associated with variations in cholesterol and increased plasma glucose in an aboriginal population (381,382). The Cys311Ser mutation, which has been associated with risk of coronary artery disease (383), shows interactions with smoking for increased myocardial infarction risk (333).

PON3 is yet poorly understood and expressed in less abundance compared to *PON1*, but polymorphisms have recently been identified that influence *PON1* activity (335).

1.8.8 *SOD1* and *SOD2*

Superoxide dismutase (SOD) enzymes catalyze conversion of superoxide radicals to hydrogen peroxide. Copper/zinc-SOD (*SOD1*) is responsible for cytosolic antioxidant defense, and protects smooth muscle cells against oxidative injury. Over-expression of human *SOD1* suppresses graft atherosclerosis and ischemia-reperfusion injury in a mouse model (384). Manganese-SOD (*SOD2*) is present in the mitochondria, and a variant has been identified within the gene coding region that affects a mitochondrial targeting sequence. The higher activity allele of that polymorphisms has been associated with improved diabetes control and reduced vascular complications in diabetics (385), and reduced carotid intima media thickness (386).

1.9 Combinations of Plasma and Genetic Biomarkers

Multiple novel plasma biomarkers measured in 3209 routine physical examination patients followed for a median duration of 7.4 years found cardiovascular disease biomarkers had a moderate ability to predict cardiovascular events, but their value was increased when used in combination (387). Multiple biomarkers of oxidative stress have not yet been studied in combination, or in comparison with traditional cardiovascular risk factors.

Few association studies have analyzed relationship between polymorphisms in multiple oxidative stress genes and atherosclerosis. Haplotypes may be more informative than individual SNPs if functional gene variants are not known with certainty, because they capture patterns of genetic inheritance (388). Recent results from association studies suggest that multiple uncommon alleles tagged by haplotypes may be able to accurately determine variations in coronary risk factors (389).

Testing multiple polymorphisms in oxidative stress genes may uncover patterns of oxidative stress modulation that act in concert to promote or prevent disease. Risk genotypes could be combined as a compound genotype score, which would incorporate functional SNPs or haplotypes within a biologic pathway together. Combining weaker SNP into a compound genotype score could enhance predictive value of genetic discoveries (390). In a study with a similar design, a combination of SNPs was found to influence cholesterol when combined as a genotype score, and significantly predicted cardiovascular disease risk (390). This genotype score also offered meaningful patient risk reclassification for cardiovascular outcomes. Another study investigating four relatively common variants in *MPO*, *PON1*, *CYBA*, and the modifier subunit of glutamate-cysteine ligase (*GCLM*) also used compound SNP scores. The combination of four "pro-oxidant alleles" was independently associated with intima-media thickness (391) in

patients with type 2 diabetes. These results suggests that oxidative stress genes may work in combination to influence coronary artery disease. Their relative and additive value for predicting coronary disease severity and outcome, however, remains to be established.

Whether oxidative stress biomarkers can enhance risk estimation will determine their utility for tailoring primary and secondary prevention strategies, and for detecting patients with higher oxidative stress burden, who might benefit from antioxidant therapies. Although clinical trials for antioxidant protection have not yielded consistent positive results, potential target pathways for antioxidant intervention may yet emerge.

1.10 Objectives, Rationale and Hypotheses

This thesis includes separate studies examining environmental, molecular, and genetic risk factors for risk prediction in a cohort of patients undergoing selective coronary angiography.

Chapter 2 presents a study that was undertaken during the primary collection and analyses of plasma and genetic biomarkers, and is therefore presented first in this document. Collaborations with Dr. Greg Miller and Human Early Learning Partnership at UBC permitted collection of neighbourhood socioeconomic status data for a subset of our cohort, which enabled us to study how environmental stress may affect survival in patients with coronary artery disease. I hypothesized that socioeconomic disparities would contribute to elevated cardiovascular mortality risk in coronary artery disease patients, and that risk factors associated with cardiovascular disease would vary with markers of neighbourhood disparity.

As discussed above, inflammation plays a key role in the pathogenesis of atherosclerosis. The next section of the thesis explores relationships between haplotypes and plasma concentrations of interleukin-6 and C-reactive protein. Haplotypes and plasma concentrations are

compared for prediction of angiographic coronary artery disease and cardiovascular mortality. I hypothesized that haplotypes of *IL6* and *CRP* would influence plasma concentrations of interleukin-6 and C-reactive protein. Furthermore, because plasma inflammation biomarkers predict coronary artery disease and cardiovascular outcomes, I expected that haplotypes that influence plasma levels would be associated with disease risk and outcome. Results from these analyses are presented in **Chapter 3**.

The remaining two chapters concern oxidative stress. As discussed above, oxidative stress pathways may account for the contribution of common cardiovascular risk factors to coronary artery disease (147,392). Despite a wealth of evidence linking oxidative stress with progression of coronary artery disease, the specific mechanisms and relative magnitude of these effects, remain incompletely elucidated. Chapters 4 and 5 present analyses of plasma and genetic markers of oxidative stress, respectively. In **Chapter 4**, the relative and combined utility of plasma biomarkers of oxidative stress were investigated for prediction of coronary artery disease and cardiovascular mortality. I hypothesized that oxidative stress biomarkers would predict angiographic disease, and cardiovascular mortality risk. Furthermore, I hypothesized that biomarkers associated with disease would have additive predictive value when combined. In **Chapter 5**, inherited polymorphisms in oxidative stress genes are explored for association with coronary artery disease, plasma biomarker levels, and cardiovascular mortality risk. I hypothesized that polymorphisms associated with disease risk would also associate with plasma oxidative stress, and have independent predictive value when combined.

Conclusions from these studies are discussed in **Chapter 6**, the final chapter of this thesis. Limitations and strengths of the study design and analyses are also discussed, and guidelines are offered for future research.

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Chapter 2. Neighbourhood Socioeconomics Status Predicts Non-Cardiovascular Mortality in Cardiac Patients with Access to Universal Health Care[1]

2.1 Introduction

Personal socioeconomic status (SES) is an influential determinant of prognosis in coronary artery disease. Patients who have lower family incomes, limited education, and work in lower-prestige occupations are 1.5–2.0 times more likely to die in the years following diagnosis of angina pectoris and myocardial infarction (1–7).

Recently, it has become evident that the SES of the neighborhood in which a patient resides also contributes to coronary artery disease patient outcomes, and does so above and beyond the effects of his/her personal SES (8,9). For example, in a sample of more than 51,000 Canadian patients hospitalized for acute myocardial infarction, Alter *et al.* found that each $10,000 decrease in median neighborhood income was associated with a 10% increase in all-cause mortality over one year (10). Tonne and colleagues studied 3423 patients hospitalized for myocardial infarction in Worcester, Massachusetts, and reported a 1.5-fold increase in mortality among those living in educationally disadvantaged neighborhoods (11).

These studies focused on the outcome of all-cause mortality. Thus, it remains unclear whether patients from economically disadvantaged neighborhoods are dying from cardiovascular diseases versus other conditions. Additionally, although neighbourhood unemployment is associated with coronary artery disease risk (12), effects of neighbourhood unemployment on survival in coronary artery disease patients have not yet been adequately characterized.

In this article, we draw upon a >13 year follow-up of 485 patients in British Columbia with angiographically documented coronary artery disease, and examine socioeconomic

[1] A version of this chapter has been published. Heslop CL, Miller GE, Hill JS. Neighbourhood Socioeconomics Status Predicts Non-Cardiovascular Mortality in Cardiac Patients with Access to Universal Health Care. PLoS ONE 2009;4: e4120.

disparities in mortality. Our objective was to investigate variations in total mortality, cardiovascular mortality, and deaths from other chronic diseases, across neighbourhood SES gradients.

Research of this nature is becoming especially relevant to developed countries which have medical systems that provide universal access to most basic care, such as Canada. The distribution of personal income in Canada has widened, which has contributed to increases in neighbourhood inequality in urban areas (13). The impact of this trend on health outcomes, in the context of the ongoing expansion of private healthcare services, has not yet been clearly characterized.

2.2 Methods

Objectives: We sought to investigate whether mortality, from cardiovascular disease or other causes, varies by neighbourhood socioeconomic gradients in a cohort of stable coronary artery disease patients who accessed the same universal healthcare system but resided in different neighbourhoods within British Columbia, Canada.

Cohort Patients: This article reports on 485 patients (383 men and 102 women) who were referred for selective coronary angiography at two Vancouver teaching hospitals between 1993 and 1995. These patients are a subset of a larger cohort of 1019 selective coronary angiography patients recruited to study novel risk factors for coronary artery disease and cardiovascular outcomes. Previous biochemical and genetic analyses of this cohort by investigators in our research group have been reported elsewhere (14–16). To be included in the current analyses, patients had to provide a home postal code within British Columbia, and have coronary angiography results indicating coronary artery disease. Of the 1019 patients, 780 had angiographic evidence of coronary artery disease. Patients who did not provide a postal code, or provided a home address outside of British Columbia, were excluded (n=295), leaving 485 patients eligible for this study.

A questionnaire regarding clinical and lifestyle variables was administered to every patient by a nurse or attending cardiologist. Information was obtained on smoking status (ever, current and never), and alcohol consumption (never, 1–5, 6–10, and >10 drinks per week). Patient body weight, height, and blood pressure were measured. Patient history of diabetes and hypertension were obtained by self-report. In analyses, body mass index (kg/m^2) was treated as a

continuous variable, and diabetes and hypertension were categorized as no (0) and yes (1). Ever and current smoking status were combined to compare effects to patients who had never smoked.

Ethics: All patients gave written informed consent; this research was approved by the Research Ethics Board of St. Paul's Hospital, Vancouver (Approval Form Attached as Appendix 1).

Coronary Angiograms: Each angiogram was assessed semi-quantitatively by a cardiologist blinded to any experimental results. Each lesion was assessed for percent diameter stenosis rounded to the nearest 10%. Patients in this group had one or more lesions of ≥10% stenosis. This definition was chosen because there is increasing evidence that small plaques may contribute more than large plaques to future risk of cardiovascular morbidity and death (17), and we hoped to avoid misclassifying patients due to subjective differences around the 50% stenosis mark.

Mortality Data: In May, 2008, patients' identifying data were linked with the British Columbia Vital Statistics database to determine whether they had died prior to the end of 2007, the latest data available. Deaths occurring outside the province of British Columbia were not identified, thus these patients were treated as censored in survival analyses. Underlying cause of death codes for deceased patients were provided according to the World Health Organization International Classification of Disease, 10th revision (ICD-10) (18). Bridge coding from ICD-9 to ICD-10 codes was completed by British Columbia Vital Statistics Agency prior to data provision.

Categories employed by the Canadian Centre for Chronic Disease Prevention and Control (19) were used to identify cardiovascular and non-cardiovascular chronic disease deaths. The advantage of this approach is that categories are specific enough to permit disease-specific analyses, but broad enough to minimize variations in mortality coding that may have occurred across the follow-up time. Cardiovascular causes of mortality included ICD-10 codes I00–99. Non-cardiovascular chronic disease deaths included causes due to cancers (Neoplasm ICD-10 C00–97), chronic respiratory diseases (Respiratory Disease ICD-10 J00–98 minus Pneumonia, Influenza, and Acute Bronchitis ICD-10 J10–28), diabetes (ICD-10 E10–E14), mental disorders (ICD-10 F00–F89 and G20–30) or other chronic disease deaths (Chronic Liver Diseases ICD-10 K70, K73–74, Chronic Renal Failure ICD-10 N17–19, and Musculoskeletal Diseases ICD-10 M00–99). Deaths from non-chronic disease causes were included only for analyses of all-cause mortality.

Neighbourhood Socioeconomic Information: Socioeconomic status data were derived from postal code information provided by cohort patients at baseline. Using Statistics Canada's 2001 Census of the Population (20), Semi-Custom Area Profile data, we derived median family income, as well as rates of education (percent without high school education), and unemployment (percent of adults over 25 seeking work), for each patient's neighbourhood. For each SES category, patients were placed in one of five quintiles, according to their neighbourhood's standing relative to the 469 neighbourhoods in the province of British Columbia. Higher scores represent increasing quintiles of neighborhood socioeconomic deprivation or disadvantage. SES data were compiled by the Mapping Unit of the Human Early Learning Partnership (HELP) at the University of British Columbia (21) using neighbourhoods

defined by local populations in collaboration with research group mapping teams, and SES information obtained from a customized disaggregation of Statistics Canada 2001 census data for the 469 neighbourhoods.

Statistical Analysis: Data were analyzed using SPSS version 14, and R version 2.7.2. Relationships between baseline covariates and mortality were assessed using Mann-Whitney U-tests for continuous variables, Pearson chi-square tests for categorical variables, and Mantel-Haenszel tests for linear trends in scaled categorical variables. The magnitude of associations between neighborhood SES and cardiovascular risk factors was assessed by Pearson correlations for continuous variables, and point biserial correlations for categorical variables.

To assess relations between SES disadvantages and mortality, a series of Cox regression survival analyses were completed. Each SES indicator was entered as a continuous variable in each model, without adjustment, and then following forced-entry covariate adjustment for potential confounding variables age, sex, body mass index (BMI), diabetes, smoking status, and alcohol consumption. Model covariates were chosen to represent factors that are associated with risk of mortality, and/or may vary with neighbourhood composition.

Separate models were estimated for all-cause mortality, cardiovascular mortality, and for non-cardiovascular chronic disease mortality. Models were also generated for mortality from cancer, but due to the reduced number of deaths from this cause, only age, sex, BMI, and smoking were included as covariates to avoid over-adjustment (22,23). SES indicators were also entered as categorical variables in covariate adjusted models to permit comparison across quintiles, with the highest level of SES (quintile 1) used as reference. Linearity across SES quintiles were also tested using repeated contrasts to compare each quintile except the first

category to the quintile that precedes it. Validity of the proportional hazards assumption for the survival models was verified using Schoenfeld residuals correlated with time, and partial residual plots for all survival models and covariates (24).

To measure survival model improvements offered by SES indices, area under the curve (AUC) values from time-adjusted receiver operator characteristic curves were generated from covariate-adjusted Cox regression models (25), using nearest neighbour kernel smoothing (26). Models were tested for goodness of fit using Hosmer Lemeshow (HL) tests, which report significance values of $p \leq 0.05$ for risk models with significant deviation from accurate calibration across a range of risk (27).

2.3 Results

Differentials between Surviving and Deceased Patients: For the current study we focused on 485 patients who had valid postal code data and angiographic evidence of coronary artery disease. Of these patients, there were 148 total cases of mortality, 64 of which were attributed to cardiovascular causes, and 66 of which were non-cardiovascular chronic disease deaths. There were also 18 cases of deaths not caused by chronic diseases. Average and total follow-up times were 11.1 and 13.3 years.

The group of patients represented all SES quintiles, with roughly equal numbers of patients in each of the 5 quintiles of income, education, and unemployment (Table 2.1). Table 2.2 displays patient baseline medical and risk factor covariate characteristics. At recruitment, the mean age of the patients was 61 years. The study group was 79% male, 72% were current or former smokers, and 19% had diabetes. Eighty-one percent of the patients reported European descent, with another 10% reporting either Chinese or South Asian ancestry.

Table 2.1 Mortality by neighbourhood socioeconomic status quintile

Neighbourhood SES Category	Quintile 1=high SES 5=low SES	Patients (n)	Proportion (%)	Deceased (n)	Cumulative Mortality Rate (%)	p-value*
Education	1	146	30 %	37	25 %	
	2	86	18 %	28	32 %	
	3	65	13 %	17	26 %	0.06
	4	93	19 %	30	32 %	
	5	95	20 %	36	38 %	
Unemployment	1	63	13 %	17	27 %	
	2	114	24 %	33	29 %	
	3	129	27 %	41	32 %	0.26
	4	91	19 %	24	26 %	
	5	88	18 %	33	38 %	
Median Family Income	1	105	22 %	29	28 %	
	2	86	18 %	21	24 %	
	3	131	27 %	45	34 %	0.10
	4	76	16 %	18	28 %	
	5	87	18 %	35	24 %	

Cumulative mortality rates for coronary artery disease patients across neighbourhood quintiles of socioeconomic status, for indices of education, unemployment, and median family income.

* $P \leq 0.05$ denotes significant differences in mortality rates across neighbourhood socioeconomic status quintiles by Manzel-Haenszel χ^2 tests.

Table 2.2 Baseline characteristics of cohort

Variable	Categories or Units	Patients	Alive	Deceased	p-value*
Age	Years	61.0±10.5	59.3±10.1	64.9±10.3	<0.01
Sex	Male	383 (79)	270 (71)	113 (29)	0.35
	Female	102 (21)	67 (66)	35 (34)	
Ethnicity	European	392 (81)	266 (68)	126 (32)	
	Chinese or South Asian	48 (10)	40 (83)	8 (17)	0.09
	Other	45 (9)	31 (69)	14 (31)	
Smoking status	Never	122 (25)	91 (75)	31 (25)	0.16
	Ever or current	348 (72)	236 (68)	112 (32)	
Hypertension	No	183 (40)	193 (71)	80 (29)	0.67
	Yes	273 (60)	126 (69)	57 (31)	
Diabetes	No	393 (81)	284 (72)	109 (28)	<0.01
	Yes	92 (19)	53 (58)	39 (42)	
Alcohol	Never	118 (24)	86 (73)	32 (32)	
consumption	Occasional (1–5)	268 (55)	183 (68)	85 (32)	0.81
(drinks per week)	Moderate (6–10)	75 (15)	53 (71)	22 (29)	
	Heavy (>10)	12 (3)	9 (75)	3 (25)	
BMI	kg/m^2	28.1±4.5	27.8±4.1	28.7±5.3	0.08

Baseline variable means±standard deviation, or counts (%) for patients with coronary artery disease, and for patients alive or deceased after 13.3 years follow-up time.

*P≤0.05 denotes significant differences between alive and deceased patients within baseline variables, as tested by Mann-Whitney U tests or Pearson chi-square tests for continuous and categorical variables, respectively, and Manzel-Haenszel χ^2 tests for alcohol consumption.

A review of angiography reports for this cohort suggested that approximately 90% of the patients with angiographic coronary artery disease presented with stable disease, and the remaining patients presented with acute coronary syndromes (data not shown). As Table 2.2 shows, patients who were older at study entry were more likely to die over the follow-up, as were patients with diabetes. No other significant differences were observed among baseline variables. Missing response rates were 5% for smoking, 2.5% for alcohol consumption, and 0–2% for all other variables.

Relationships between Neighbourhood SES and Patient Characteristics: Quintiles of SES variables were evenly distributed across cohort patients. Few significant relationships were observed between SES indices and patient characteristics. However, individuals with lower neighbourhood education levels were more likely to have a higher BMI (correlation of 0.09, $p<0.05$), and lower alcohol consumption (correlation of 0.11, $p=0.02$). No other significant correlations were observed.

Survival Analyses: The first wave of analyses tested for differences in all-cause mortality according to neighborhood disadvantage among individuals with coronary artery disease. There was no consistent effect of education, unemployment or family income on mortality. The unadjusted hazard ratios (95% confidence intervals (CI), significance value) were 1.11 (1.00–1.23, $p=0.05$) for education, 1.08 (0.95–1.22, $p=0.24$) for unemployment, and 1.10 (0.98–1.24, $p=0.10$) for income.

The next wave of analyses examined SES disparities in cause-specific mortality. None of the indices of neighborhood disadvantage was associated with mortality from cardiovascular

disease over the follow-up time. Unadjusted hazard ratios (95% CI, and significance values) were 1.03 (0.87–1.21, p=0.75) for education, 0.96 (0.79–1.17, p=0.69) for unemployment, and 1.02 (0.86–1.23, p=0.80) for income.

However, there were significant disparities in non-cardiovascular chronic disease mortality rates by SES. Each one quintile increase in neighbourhood unemployment was associated with a 30% greater risk of non-cardiovascular chronic disease death, following adjustment for risk factors (age, gender, BMI, diabetes, smoking, and alcohol consumption). Quintiles of median family income and neighbourhood education levels were both associated with 21% increases in risk, following adjustment for the same risk factors. Linearity of SES effects for education, income and unemployment were confirmed in covariate-adjusted models as described above for all neighbourhood SES indices ($p<0.05$ for all SES measures). Results from unadjusted survival models, and forced-entry covariate adjusted survival models, are displayed in Table 2.3. Survival curves from covariate adjusted models are depicted graphically in the panels of Figure 2.1

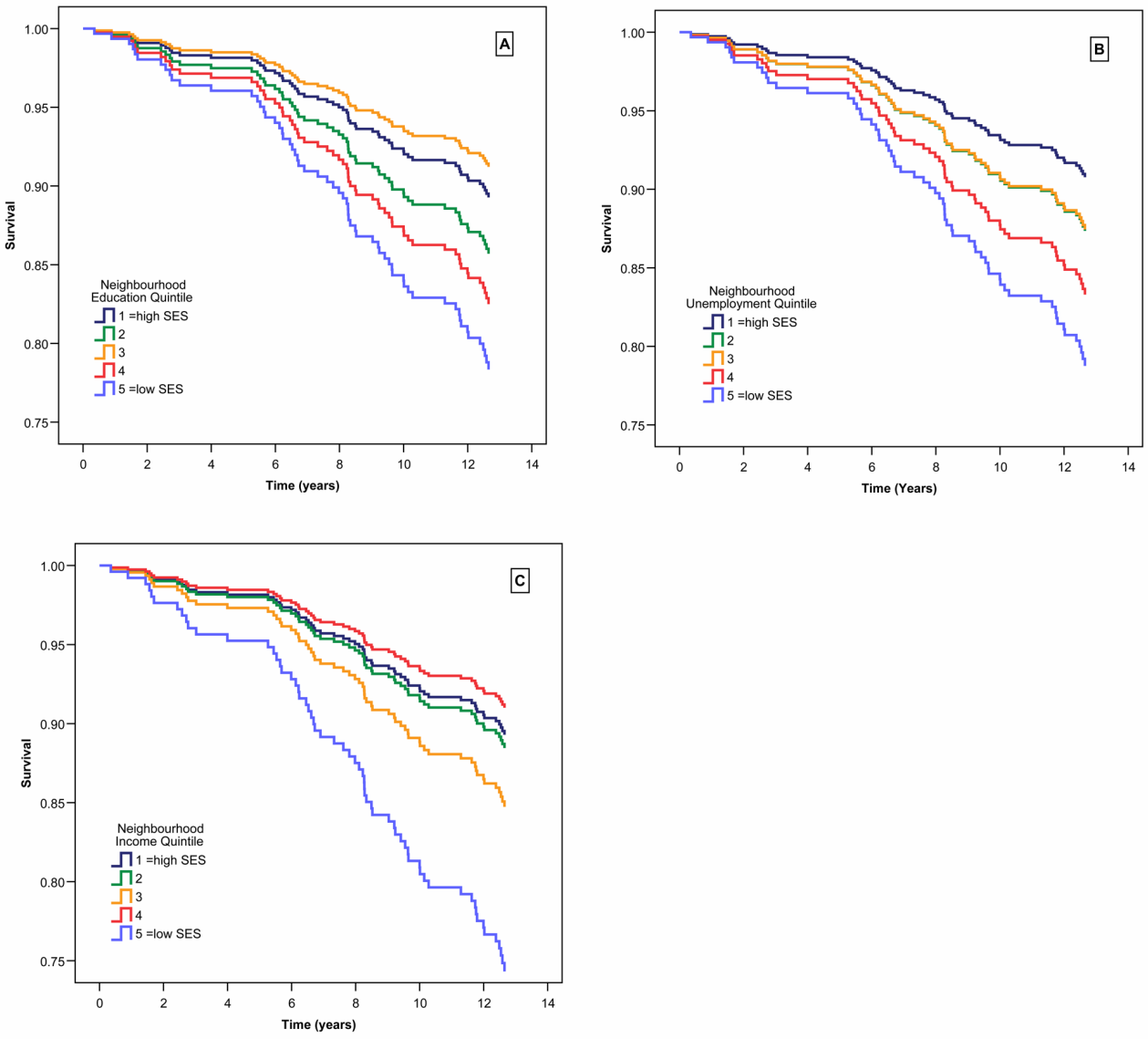
Table 2.3 Survival analyses for non-cardiovascular chronic disease mortality

		SES Index		
	Covariates	**Education**	**Unemployment**	**Median Family Income**
Survival model without covariates	SES quintile	1.20 (1.02–1.42)*	1.23 (1.01–1.50)*	1.24 (1.03–1.48)*
Survival model with covariates: Step 1	Age (years)	1.07 (1.04–1.10)†	1.07 (1.04–1.11)†	1.07 (1.04–1.10)†
	Male sex	0.92 (0.49–1.71)	0.95 (0.51–1.77)	0.97 (0.52–1.82)
	BMI	1.02 (0.96–1.08)	1.02 (0.97–1.08)	1.03 (0.97–1.08)
	Diabetes	1.76 (0.99–3.13)	1.92 (1.08–3.43)*	1.82 (1.03–3.22)*
	Smoking (ever or current)	2.21 (1.13–4.31)*	2.12 (1.08–4.13)*	2.13 (1.10–1.16)*
	Alcohol consumption	1.06 (0.68–1.39)	0.89 (0.61–1.31)	0.93 (0.63–1.36)
Step 2	SES quintile	1.20 (1.02–1.42)*	1.30 (1.06–1.60)*	1.21 (1.01–1.46)*

Hazard ratios, 95% confidence intervals, and significance values for each quintile increase in SES indices are given from Cox regression models for risk of non-cardiovascular chronic disease mortality. Covariates listed were force-entered in adjusted Cox regression models.

* $p \leq 0.05$ †$p \leq 0.01$

Figure 2.1 Survival curves for non-cardiovascular chronic disease mortality by neighbourhood socioeconomic quintile



Quintiles of A) education, B) unemployment, and C) median family income are relative to 469 neighbourhoods in the province of British Columbia mapped from the Human Early Learning Partnership mapping project. Higher scores represent increasing deprivation or disadvantage. Survival curves are derived from Cox regression survival analyses with adjustment for age, sex, BMI, diabetes, smoking history, and alcohol consumption.

Predictive Model Evaluation: Time-adjusted AUC values for non-cardiovascular chronic disease mortality with and without SES indicators were generated from adjusted Cox regression survival analyses. AUC values obtained were 0.600 for covariates age, sex, BMI, diabetes, smoking, and alcohol consumption without SES indicators. The AUC values improved to 0.712, 0.715, and 0.720 for the model with addition of indices for education, unemployment, and income, respectively.

HL tests of model calibration, for which $p \leq 0.05$ indicates poor model calibration across a range of risk, show good risk model fit for prediction of non-cardiovascular mortality for the covariate-adjusted adjusted models tested above. Models including SES variables education, unemployment and income, yielded HL χ^2 test statistics of 9.17 (p=0.33), 8.16 (p=0.42), and 6.94 (p=0.54), respectively, and the basic model statistic was 5.71 (p=0.68). These results suggest the model did not deviate from good predictive fit when neighbourhood indices were included.

Disease Specific Survival Analyses: To discern which causes of death were underlying these associations, we carried out secondary analyses of mortality from specific non-cardiovascular chronic diseases. Cancer was the most common cause of death in this category, with 31 patients (6.4% of the cohort) having died from neoplastic diseases. We did not carry out further analyses for death from other types of chronic diseases, due to insufficient numbers in each category.

As Table 2.4 shows, Cox survival analyses revealed significant neighborhood disparities in cancer mortality for unemployment and family income. These relationships withstood adjustment for the covariates age, sex, BMI, and smoking history. For neighbourhood unemployment, risk of death from neoplastic causes increased 62% per quintile ($p<0.01$). This

relationship is displayed graphically in Figure 2.2. A parallel finding was evident for median family income, with a 42% increase in cancer mortality per quintile (p=0.01). Linearity of SES effects for unemployment and income were confirmed in covariate-adjusted models (p=0.01 for unemployment, p=0.04 for income).

Table 2.4 Survival analyses for cancer deaths by neighbourhood SES indices

		SES Index		
	Variable	**Education**	**Unemployment**	**Median Family Income**
Survival model without covariates	SES quintile	1.11 (0.88–1.41)	1.56 (1.16–2.11)†	1.40 (1.07–1.83)*
Survival model with covariates:	Age (years)	1.05 (1.01–1.09)*	1.05 (1.01–1.10)*	1.04 (1.00–1.09)*
	Male sex	0.99 (0.40–2.43)	1.01 (0.41–2.48)	1.05 (0.43–2.59)
	BMI	0.97 (0.89–1.05)	0.97 (0.89–1.06)	0.97 (0.89–1.06)
Step 1	Smoking (ever or current)	3.25 (1.11–9.51)*	2.98 (1.02–8.76)*	3.19 (1.06–9.33)*
Step 2	SES quintile	1.18 (0.94–1.48)	1.62 (1.20–2.19)†	1.42 (1.09–1.84)†

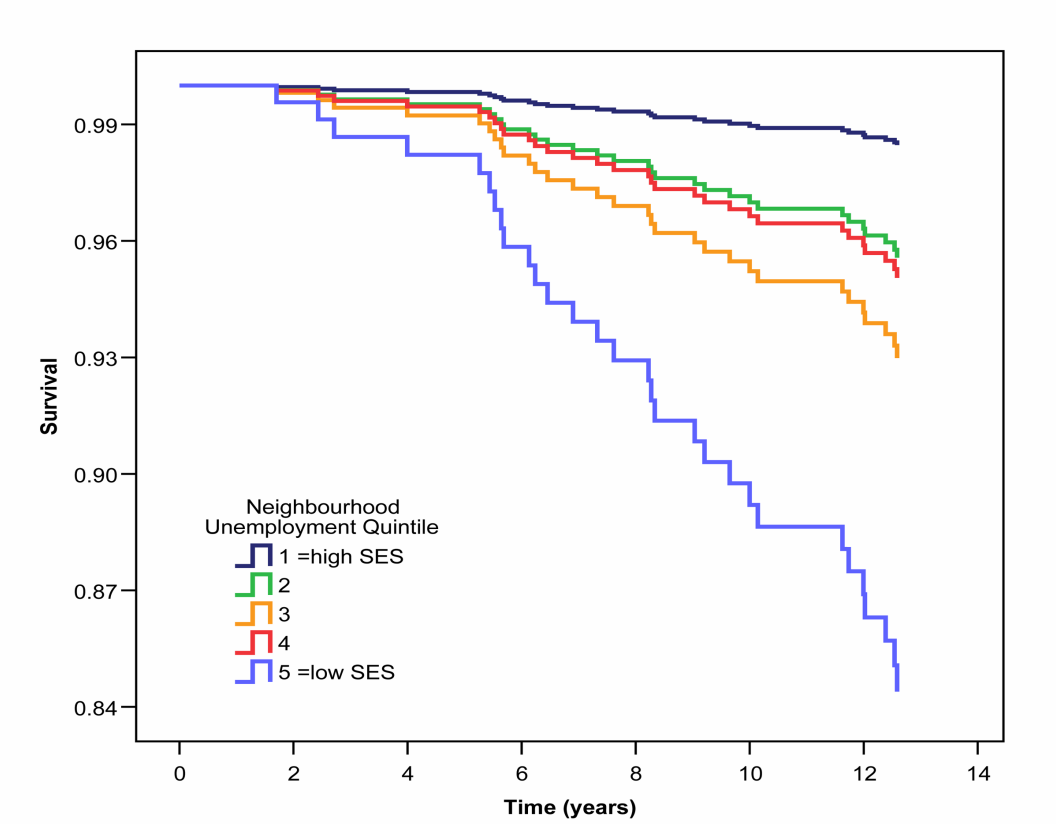
Hazard ratios, 95% confidence intervals, and significance values for each quintile increase in SES indices are given from Cox regression models for risk of cancer mortality. Covariates listed were force-entered in adjusted Cox regression models.

* $p \leq 0.05$ †$p \leq 0.01$

Comparing patients in the lowest versus highest quartiles of SES, there was a 10-fold disparity in cancer mortality from the highest to the lowest quintile of neighbourhood unemployment (95% CI 1.31–76.33, p=0.03). There was a 5.2–fold increase in cancer mortality from the highest to lowest quintile of neighbourhood median family income (95% CI 1.47–18.5, p=0.01). No significant relationship was observed for education.

Despite the limited number of covariates included, the AUC value for the cancer mortality model with age, sex, BMI and smoking, improved from 0.651 to 0.720 and 0.712 for SES indices of unemployment and income, respectively. HL tests indicated no significant deviation from model calibration with SES variables. Covariate-adjusted model HL χ^2 test statistics were 4.09 (p=0.85), and became 9.17 (p=0.33), 4.57 (p=0.80), and 8.09 (p=0.43) with the addition of indices for education, unemployment, and family income, respectively, confirming no loss of model calibration with the addition of SES indices.

Figure 2.2 Survival curve for cancer mortality by quintile of neighbourhood unemployment



Survival curves for cancer mortality are derived from Cox regression survival analyses with adjustment for age, sex, BMI, and smoking history, across quintiles of neighbourhood unemployment for cohort patients with coronary artery disease.

2.4 Discussion

We investigated whether neighbourhood socioeconomic conditions predicted mortality among 485 stable coronary artery disease patients living in British Columbia, after >13 years of follow-up time. Based on the extensive literature documenting social gradients in morbidity and mortality from cardiovascular disease (1-7,9,10), we expected that patients with adverse neighbourhood socioeconomic conditions would be especially prone to cardiac death. However, we did not find a gradient in overall or cardiovascular mortality attributable to neighbourhood characteristics. Instead we observed a marked gradient in mortality from non-cardiovascular chronic diseases. For each quintile increase in neighbourhood SES deprivation, estimated risks for non-cardiovascular chronic disease deaths increased between 21–30%, leading to an average 2.4–fold increase between highest and lowest neighbourhood SES quintiles. Although the number of cancer deaths were small, profound effects were observed for rates of cancer mortality; estimated risks for cancer death increased 42% and 62% for each quintile decrease in neighbourhood SES family income and employment, respectively.

Although it would not be appropriate to use neighbourhood SES solely to distinguish which coronary artery disease patients should be screened for other chronic diseases, we demonstrate that neighbourhood SES indices improved risk prediction for chronic disease mortality in these patients. Areas under time-adjusted receiver operator curves for non-cardiovascular and cancer deaths increased when neighbourhood SES was added to a risk model that included age, sex, BMI, diabetes, smoking, and alcohol consumption. Also, calibration tests well suited to assess cohorts of this size (28) show that good model calibration was achieved across range of risk for covariate-adjusted models.

Alongside numerous studies demonstrating increased incidence of coronary artery disease and cardiovascular death in neighbourhoods with lower SES, there is extensive literature documenting higher total mortality among patients living in lower SES neighbourhoods. However, few studies have investigated which specific types of mortality are increased among patients living in lower SES neighbourhoods. We distinguish cardiovascular mortality from deaths due to other chronic diseases, and add new information regarding the nature of these increased risks.

It is not clear why a social gradient in cardiovascular deaths did not emerge in our data. Two other studies have found that coronary artery disease patients from low SES neighborhoods are at increased all-cause mortality risk (10,11). These studies had much larger sample sizes than ours, so reduced statistical power may have masked these effects in our study. The patients in the other studies had recently been hospitalized for myocardial infarction, so these subjects may have had more advanced disease or been less medically stable in comparison to patients in our study. Also, patients in the other studies were drawn from many regional acute care centers, while our cohort patients were investigated by selective coronary angiography and treated in cardiology services at major teaching hospitals, thus similarities of care may have eliminated—or at least diminished—existing social disparities in prognosis. Disparities in care that associate with neighbourhood SES could explain why these studies observed associations between neighbourhood and cardiovascular mortality and we did not. But as we can presume care was cardiac-specific, it would not have had the same effects for other chronic diseases such as cancer. Future research is needed to explore this possibility, and to determine the applicability of our findings to other cardiac populations.

Our findings are consistent with published evidence showing a gradient in health across neighbourhoods, however it remains unclear whether our findings are attributable to community versus individual SES, as the former could simply be acting as a proxy measure for variations in the latter. Both of these factors predict mortality from a variety of causes, and in many cases they do so independently of one another (8,9,29,30). Our findings that non-cardiovascular chronic disease death rates increase with neighbourhood SES deprivation in coronary artery disease patients suggests neighbourhood conditions may independently instigate longer term health effects which persist in the context of a medical system designed to provide care regardless of individual-level SES. Health status of residents of British Columbia do vary markedly according to where they live, with more geographically compact and populous areas of BC showing the best overall health status, and more sparely populated areas showing the worst (31). British Columbians with lower incomes do show more frequent use of general practitioners and acute inpatient care, while higher income is associated with the greater use of specialist and surgical day care service (32). Without having individual SES data for the patients in this sample, we cannot discriminate between these competing explanations for the findings.

Living in a lower-SES neighborhood may increase people's exposure to pollutants, infections, and carcinogens that contribute to the pathogenesis of chronic diseases such as cancer (33). However, these exposures seem unlikely to be the primary mechanism at play here, because the social gradient in mortality we observed were fairly linear in nature. It is difficult to imagine that exposures are distributed in this fashion as well; i.e. that for each increment in neighborhood SES there is a corresponding reduction in contact with toxicants. Our analyses also controlled for key demographic characteristics and lifestyle variables, thus we believe these factors are unlikely to have played a major role.

Another possibility is that daily stress associated with living in an impoverished neighborhood takes a biological toll on the body. There is a roughly linear inverse association between social class and perceived stress (34), and persons residing in low-SES communities release higher levels of stress-related hormones, have more systemic inflammation, and are more likely to display features of metabolic syndrome (35-37). These stress-related biological perturbations may accumulate over time in a manner that contributes to the development and/or progression of chronic diseases such as cancer (38,39), and the findings of our study are consistent with this theory.

Limitations: This study has limitations that need to be considered. Individual-level socioeconomic information was not collected at baseline. Thus, we are unable to take this data into account when evaluating neighbourhood socioeconomic effects, or to compare the magnitude of individual- versus neighbourhood-level socioeconomic gradients. Both patient self report and measurements at baseline were used to gather information about risk factors. These approaches are subject to recall bias and measurement error, respectively. Also, our participants were not asked about personal history of cancer at initial baseline assessment. Thus, despite other studies suggesting no significant difference in rates of cancer between higher and lower socioeconomic status neighbourhoods in Canada (40), we cannot correct for presence of neoplastic disease prior to study entry. The higher risk of neoplastic mortality observed among individuals in lower-SES neighbourhoods may be partly to lower utilization of diagnostic imaging, as has been documented elsewhere in Canada (41). Unfortunately, we cannot ascertain how this factor contributes to our findings. It will be important for future research to include this information. Finally, this study is not sufficiently powered to investigate each cause of death

separately. These limitations need to be considered in light of the project's strengths, including its prospective design, well-characterized patients, long follow-up time, and complete ascertainment of mortality.

In closing, our findings suggest that even in a country with universal healthcare services, there are marked gradients in mortality according to neighbourhood. We demonstrate the predictive strength of neighbourhood SES above and beyond key modifiable and non-modifiable risk factors for mortality, and suggest that broader approaches to recognizing and addressing socioeconomic inequalities are needed. In order to achieve more equitable healthcare and overcome disparities in resources and access, we must create an improved appreciation of social and biological factors responsible for poor health outcomes, and develop the economic, behavioral, and biomedical interventions needed to ameliorate them.

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Chapter 3. Haplotypes and Plasma Levels of Interleukin-6 and C-Reactive Protein, and Prediction of Cardiovascular Risk[1]

3.1 Introduction

Low grade, chronic inflammation plays a pivotal role in the pathogenesis of coronary artery disease (CAD). Acute phase proteins C-reactive protein (CRP) and interleukin-6 (IL-6) are elevated in CAD patients, and predict risk of MI in healthy individuals(1). Although CAD risk factors influence plasma concentrations of IL-6 and CRP (2), a genetic basis may underlie relationships between inflammation and cardiovascular risk.

Emerging consistency in the literature also suggests polymorphisms in the *CRP* gene affect plasma CRP levels (3–6). Promoter region SNPs in *CRP* influence gene regulation (4,7), and effects of other polymorphisms in *CRP* on plasma levels have been suggested by consistent epidemiological data regarding their association, independently or combined as haplotypes, with single timepoint measurements of CRP (8).

Despite extensive research, no consistent associations between cardiovascular disease and SNPs that influence plasma CRP have been demonstrated. The prospective population based Rotterdam study confirmed the relationship of CRP with risk of coronary heart disease, but no associations between C-reactive protein haplotypes and CAD were demonstrated, despite haplotype related differences in CRP levels (9). In the Physician Health Study, two SNPs associated with reduced CRP concentrations actually conferred *higher* risks for MI (3). No clear causal relationship has been characterized between polymorphisms that influence CRP levels, and cardiovascular disease risk. Environmental and lifestyle variables contribute to CRP levels, and may have more influence over plasma CRP levels than genetic variants (6).

[1] A version of this chapter will be submitted for publication. Heslop CL, Podder M, Tebbutt SJ, Hill JS. Haplotypes and plasma levels of interleukin-6 and C-reactive protein, and prediction of cardiovascular risk.

IL-6 promotes secretion of CRP from hepatocytes, and from immune cells within atherosclerotic lesions, where IL-6 expression is detected at 10- to 40-fold higher levels than non-atherosclerotic vessels (10). Single nucleotide polymorphisms (SNPs) in the *IL6* gene influence IL-6 concentrations (11,12), and may therefore affect CRP levels in the circulation, and at sites of atherogenesis. One study of 677 Korean men found CRP concentrations were influenced more strongly by a promoter in the *IL6* gene than by polymorphisms across *CRP* (13). However, this study did not measure or control for plasma IL-6 levels. Among participants of the Cardiovascular Health Study, *IL6* haplotypes influenced both IL-6 and CRP levels, but no association with cardiovascular outcome was demonstrated (14).

Based on the above mentioned evidence, we examined IL6 and CRP haplotypes for effects on plasma IL-6 and CRP levels in a prospective cohort of patients who underwent selective coronary angiography. We investigated predictive value of *IL6* and *CRP* haplotypes for presence of angiographic CAD, and risk of cardiovascular mortality, relative to predictive value of cardiovascular risk factors, and plasma IL-6 and CRP.

3.2 Methods

Angiography Population: The cohort of coronary angiography patients chosen for this study is a population of older adults, 85% percent of whom report Caucasian ethnicity. IL-6 and CRP levels have previously been associated with angiographic coronary artery disease and cardiovascular mortality in this population (15). The original cohort of 1117 coronary angiography patients was recruited from two Vancouver teaching hospitals in 1993–1995, but the current study included only patients with available DNA samples (n=797).

Indications for angiography included stable angina and previous myocardial infarction (MI), as well as aortic stenosis and/or regurgitation, and mitral valve regurgitation. Patients with unstable angina and/or MI within the preceding 2 months were excluded. Cardiovascular disease status at baseline was defined by degree of stenosis on coronary angiograms rounded to the nearest 10%, with presence of coronary artery disease defined by ≥20% in any major epicardial vessel, and severe disease as ≥50% stenosis.

Cohort evaluation at baseline included demographic, lifestyle, medical history and fasting blood collection. Data regarding cigarette smoking history (past or current, or never), self-identified ethnicity, previous diagnosis of hypertension, previous diagnosis of diabetes mellitus, and history of premature CAD in patients' family (males before 45 and females before 55 years of age), were obtained by self-report. Blood pressure was also measured, and hypertension classified by pre-existing diagnosis and/or baseline blood pressure >140/90 mmHg. Height and weight were measured, and body mass index (BMI) calculated. Waist circumference was measured midway between the iliac crest and the bottom of the ribcage. Medication use was recorded from patient's charts. All patients provided written informed consent, this study was

approved by the Research Ethics Board of University of British Columbia/Providence-St. Paul's Hospital.

Mortality Data: Study individuals were linked with the BC Vital Statistics Agency mortality database to determine deaths up to the end of 2007, and attributed causes of death. Cardiovascular deaths were identified by World Health Organization International Classification of Disease (10^{th} revision) mortality codes I20–I25, and I50–I59.

Measurement of IL-6 and CRP: Baseline IL-6 and CRP were measured in our laboratory, using high sensitivity methods described elsewhere (15). Briefly, CRP was measured by high-sensitivity chemiluminescent enzyme-linked immunometric assay, using the IMMUNLITE 2000 automated analyzer (Diagnostic Products Corporation, Los Angeles, CA), with a maximum interassay coefficient of variation of 8.7%. The Quantikine High Sensitivity Human Interleukin-6 Immunoassay (R&D Systems, Minneapolis, MN) was used to measure IL-6, with a maximum interassay CV of 9.6%.

TagSNP Selection: Polymorphisms across the *IL6* and *CRP* genes were selected based on linkage disequilibrium (LD) patterns derived from regional sequences identified in Centre D'Etude du Polymorphisme Humain (CEPH) individuals collected from International HapMap Project (www.hapmap.org, Build 2). For both genes, regions upstream and downstream were chosen based on linkage disequilibrium (LD) patterns. TagSNPs were selected using Haploview (version 3.3.2) with tagging by pairwise methods, with forced inclusion of SNPs previously associated with CAD or CAD risk factors. Gene coverage of $r^2 \geq 0.96$ was achieved for all SNPs

≥5% minor allele frequency (MAF). A total of 4 SNPs in *CRP* (rs1417938 T/A, rs1800947 G/C, rs1205 C/T, and rs2808630 C/T), and 6 SNPs in *IL6* (rs1800795 G/C, rs2069827 G/T, rs1818879 G/A, rs2069837 A/G, rs2069840 C/G, and rs1554606 G/T) were included.

DNA Collection: DNA was isolated from whole blood buffy coats (555 patient samples) or from plasma (242 patient samples) using QIAamp DNA Blood Mini Kit (QIAGEN, Chatsworth, CA), with the manufacturer's modified protocol for plasma samples. Whole blood extracted DNA concentrations were determined by fluorescent signal enhancement of PicoGreen® dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR), and adjusted to 10ng/μL. DNA concentrations extracted from 400μL plasma into 30μL ddH_20 were determined by Nanodrop 8000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE). Median DNA concentrations extracted from plasma [interquartile range (IQR)] were 2.74 [1.85–5.18] μg/μL.

APEX Genotyping: APEX genotyping was performed according to methods developed in our laboratory and described in detail elsewhere (16), as summarized here. *Note: APEX genotyping of IL6 and CRP was done simultaneously with candidate oxidative stress genotyping experiments, as detailed in Chapter 5.*

Microarray Printing: Oligonucleotide 25-mer APEX probes and allele-specific (ASO) APEX probes were manually designed based on validated regional sequence data obtained from Single Nucleotide Polymorphism Database (dbSNP) (17). Two APEX probes were designed, one per strand, to identify the allele at the SNP site. Four ASO APEX probes, two per strand, were

designed to include alleles at the SNP site, which confirm genotyping results from APEX probes. Probe sequences are listed in Appendix 2.

Probe oligonucleotides were synthesized with amino-modified 5′ ends to facilitate microarray printing, at 200pmol/µL concentrations in 150mM sodium phosphate buffer (pH 8.5) by Integrated DNA Technologies (Coralville, IA). Probes were further diluted to 50pmol/µL using a Biomek FX robot (Beckman Coulter, Fullerton, CA). APEX microarray printing on CodeLink Activated Microarray slides (SurModics In Vitro Diagnostic Products, Eden Prairie, MN) was performed by The Microarray Facility of The Prostate Centre at Vancouver General Hospital (Vancouver, BC). A QArrayMax microarray printer (Genetix Ltd, New Milton, Hampshire, UK) equipped with ArrayIt Stealth Technology SMP2 quill pins (TeleChem International Inc.) in a 6-pin tool (2x3 configuration) was used to deposit approximately 0.1 pmoL of oligonucleotide per spot, in a volume of 0.5nL.

Probe oligonucleotides were spotted in 6 subgrids of 23x24 spots, consisting of 532 oligonucleotide spots plus 20 blanks, with a spot pitch of 165 microns, and spot diameter of 60 to 80 µm. Each oligonucleotide was spotted in quintuplicate three times on each array, with three identical arrays (fields) per slide, permitting each slide to be used for 3 patient samples. Each subgrid included Npg1 positive controls, which are plant sequence probes used in APEX (18), and buffer-only negative control spots.

Multiplex PCR: PCR primers were designed manually to amplify a region of 100–200 bases around each SNP. Primers were designed 18–21 bases long with melting temperatures of 65±7°C, with A or AA ends to reduce secondary structure formation. Uniqueness of primer sequences were verified by Basic Local Alignment Search Tool (BLAST,

http://blast.ncbi.nlm.nih.gov/). A linker sequence was also added to primer 5′ ends to improve amplification, and to reduce primer interactions and dimerization. Linker sequences were 5′-TAGGACTCACTTAGGGAG-3′ for left primers, and 5′-CGATGTAGGTGACACTAG-3′ for right primers. PCR Primer sequences are listed in Appendix 3.

Multiplex PCR of each sample was carried out in a single 25 μL reaction containing 20nM (final) of each PCR primer plus 20nM of linker primers, 200 μM dNTPs without dTTP, 160 μM dTTP, 40 μM dUTP, 10 units of HotStar Taq DNA polymerase (5U/μL Qiagen, USA), and PCR reaction buffer providing 1.5mM $MgCl_2$, in a solution of 100mM Tris-HCL, 50mM KCl, 100 μg/mL gelatin, pH 8.3. Template quantities used for PCR were 20ng for whole blood extracted DNA, and varying concentrations in a volume of 12μL for plasma extracted DNA. Amplification reactions were completed in a Bio-Rad mycycler (Bio-Rad, Hercules, CA), beginning with a 15 minute polymerase activation step at 95°C, followed by 40 cycles of denaturation at 95°C for 40s, primer annealing at 55°C for 2 min, 55°C–70°C ramp up for 2.5 minutes, and a 3 minute extension step at 72°C.

Products of PCR amplification (12μL of PCR product from whole blood extracted DNA, and 23 μL of PCR product from plasma extracted DNA) were fragmented using 1 U uracyl-N-glycosylase (UNG, Interscience, Troy, NY) to produce a DNA size optimal for hybridization to the APEX microarray (approximately 50–100 bases) with cleavage of residual deoxyribonucleotides by 1 U shrimp alkaline phosphatase (SAP; Amersham Biosciences/GE Healthcare, USA). Digestion took place at 37°C for 60 minutes, followed by enzyme inactivation for 10 minutes at 95°C. A small volume of PCR products and digested PCR products were verified by agarose gel electrophoresis and visualized by GelRed™ (Biotium, Inc., Hayward, CA) to confirm digestion (a typical gel is depicted in Appendix 4).

APEX Genotyping: APEX reactions took place in a total volume of ~41 μL, containing fragmented PCR product and 2 pmol Npg1positive control template oligonucleotide, as well as 1.25 μM each fluorescently labeled dideoxynucleotide triphosphates (Texas Red-ddATP, Cy3-ddCTP, Cy5-ddGTP, R110-DdUTP; Perkin Elmer Life Sciences, Boston, MA), and 10 U Thermo Sequenase DNA polymerase (Amersham Biosciences/GE Healthcare) diluted in reaction buffer (260 mM Tris-HCl, pH 9.5, 65 mM $MgCl_2$).

Reaction mixtures were applied to APEX microarrays, covered by Parafilm™ sealing film (M type), and allowed to react at 58°C for 20 minutes at agitation level 1 on a Thermo Hybaid HyPor20 incubation plate (Thermo Electron, Waltham, MA). Following washing in 95°C water and 0.3% alcanox, microarrays were scanned using *arrayWoRx*^{e} Auto Biochip Reader (Applied Precision, LLC, Issaquah, WA, USA) with exposure times set at 60–70% positive control spot pixel saturations, and scanned at 10 μm resolution. Image files were imported into SNP Chart (19) for microarray gridding, spot segmentation, and background subtraction, as previously published by our laboratory (20).

Automated Genotyping: A prototype training set was constructed by manual genotyping in SNPChart of unrelated CEPH Coriell subjects and patients samples. The training set included a minimum of 59 genotypes per SNP, with several representations of each allele. Accuracy of manual genotyping was verified by independent replication and by importing multi-channel spot intensity data and SNP-specific oligonucleotide information for manually genotyped patient samples into Multi-dimensional Automated Clustering Genotyping Tool (MACGT) (21).

Automated genotyping of the entire cohort was completed using normalized median probe intensity levels exported from SNPChart for robust linear discriminate analysis (LDA) with dynamic variable selection as previously published by our laboratory(22,23), and applied to APEX genotyping with >99.9% accuracy (16). A threshold for genotyping confidence of 0.500 was applied to LDA results, with non calls for genotypes with scores below this threshold. Details of LDA methods use in this study are described in Appendix 5.

Quality Control: Accuracy of automated genotyping results was estimated by calculating concordance of Coriell sample genotypes with validated genotypes from Genome Variation Server (GVS, http://gvs.gs.washington.edu/GVS/index.jsp), and by comparing cohort patient samples genotyped in duplicate. The concordance rate for Coriell samples was 99.9% between APEX results and GVS (n=18 samples), and 100% between duplicate Coriell samples (n=11).

Statistics: Baseline patient characteristics are presented as proportions (percentages), mean ± standard deviation (SD) or median [interquartile range(IQR)] depending on the measurement scale and distribution. Plasma levels of inflammatory mediators were right skewed, and therefore logarithmically transformed to achieve symmetric distribution. Baseline differences between groups were analyzed by Student's *t*-tests and Mann-Whitney rank sum tests for skewed continuous variables, and χ^2 tests for categorical variables. Associations between log transformed biomarker levels and angiographic CAD were tested by logistic regression, with adjustment for age, sex, and body-mass index.

Deviations from Hardy-Weinberg equilibrium were tested for by χ^2 tests. Linkage disequilibrium was assessed by Lewontin's D′ statistic (24), and Devlin and Risch's squared

correlation coefficient (r^2), and haplotype blocks were constructed based on LD methods described by Gabriel (25), using Haploview version 4.1.

Analyses of SNPs and haplotypes in *IL6* and *CRP* were performed using SimHap version 1.0.2 (26). Multivariate logistic regression analyses were used to detect association between IL6 and CRP haplotypes and angiographic CAD. Multiple linear regression models were used to test effects of haplotypes on plasma biomarkers. All regression models were minimally adjusted for age and sex, with further adjustment for factors associated with biomarker levels in linear regression models. Mean IL-6 and CRP levels (95% confidence intervals) for patients in each haplotype group were calculated from covariate adjusted linear regression models β coefficients and standard errors.

To address the problem of multiple testing, we selected haplotypes as the major genetic variable at the first level of analyses, with two-sided $\alpha<0.05$ considered statistically significant in global tests across haplotypes. However, a total of seven primary genetic tests were performed (each gene was tested for association with its protein level, and presence of CAD, and cardiovascular mortality, and IL6 gene was also tested for association with CRP). Bonferroni correction was therefore applied for calculate an adjusted significance p-value ($P_{corrected} = 0.05/7$ tests = 0.0071).

Because a Bonferroni calculation may not accurately asses the chances for false positive results, false discovery rate (FDR) values were also calculated for all p values for haplotype, SNPs, and biomarkers, as a measure of experiment-wise false discovery rate. Corresponding q values that estimate the risk of false positive for each p value were generated using QValue (27), and are reported in results to permit assessment of false positive findings expected for each reported p-value.

3.3 Results

CAD and Inflammatory Biomarkers: At baseline, 627 of the 797 patients had angiographic evidence of CAD, and 564 of those patients had lesions ≥50% stenosis. Baseline characteristics for patients are presented in Table 3.1.

Patients with CAD were older, and more likely to have hypertension, diabetes, and a smoking history. Furthermore, patients with CAD had higher CRP and IL-6 (Table 3.2). Unadjusted odds ratios (OR) for angiographic CAD were 1.81 [1.11–2.97], p=0.011 for log IL-6; and 1.43 [95% confidence interval 1.06–1.94] for log CRP, p=0.019. These relationships persisted with adjustment for age, sex, BMI, smoking, diabetes, and hypertension. Plasma levels of IL-6 and CRP were strongly correlated in this population. (Spearman correlation coefficient 0.640, p<0.001). Relationships between IL-6 and CRP and baseline risk factors are presented in Table 3.3.

Table 3.1 Clinical characteristics of the study population stratified according to angiographically documented coronary artery disease

Characteristic*	Angiographic evidence of coronary artery disease		p value †
	No (n=170)	Yes (n=627)	
Age, years	58.0±12.3	61.9±10.6	<0.001
Males, %	51.7	81.0	<0.001
Smoking status, %			
Never smoked	42.9	24.8	<0.001
Former or current smoker	57.1	75.2	
Hypertension, %	34.2	38.5	0.335
Diabetes, %	6.0	19.9	<0.001
Family history of coronary artery disease, %	54.4	58.2	0.398
Body mass index, kg/m^2	27.5±5.01	28.1±4.52	0.142
Waist circumference, cm	86.3±28.5	93.3±25.3	0.004
Lipid Variables			
Low-density lipoprotein cholesterol, mg/dL	3.72±1.09	3.52±0.94	0.028
High-density lipoprotein cholesterol, mg/dL	1.01±0.28	0.92±0.24	<0.001
Total cholesterol:high-density lipoprotein cholesterol ratio	5.37±1.96	5.95±2.14	0.002
Mortality Outcome			
Total Mortality, %	21.5	32.1	0.011
Cardiovascular death, %	4.0	15.7	<0.001

CAD is defined as any lesions ≥20% stenosis on baseline selective coronary angiography. Data are presented as mean±standard error, median [interquartile range], or percentage.

***** mean values ±standard deviation, or % are given for baseline characteristics

† p-values calculated from independent samples t-tests, or χ^2 tests for continuous and categorical variables, respectively.

Table 3.2 Association between biomarkers and angiographically documented coronary artery disease

	Angiographic evidence of coronary artery disease		
Characteristic*	**No (n=170)**	**Yes (n=627)**	**p value †**
C-reactive protein, mg/L	1.45 [0.73–3.95]	1.79 [0.85–4.90]	0.001
Interleukin-6, ng/L	2.13 [1.31–3.54]	2.26 [1.35–3.64]	0.02

***** median values are given with interquartile ranges

† p-values calculated from age and sex adjusted multivariate regression analyses

Table 3.3 Baseline predictors of plasma IL-6 and CRP

Covariate	***Coefficient (Standard Error)***	***P value***
Log (IL-6)		
Age	0.008 (0.001)	<0.001
BMI	0.009 (0.003)	0.003
Waist Circumference	0.001 (0.001)	<0.001
Log (CRP)		
Age	0.004 (0.002)	0.022
Male sex	−0.204 (0.050)	<0.001
BMI	0.023 (0.005)	<0.001
Waist Circumference	0.001 (0.001)	0.098

Variables remaining from multiple regression models with backward stepwise elimination: age, sex, smoking history, body mass index, waist circumference, diabetes, and diastolic and systolic blood pressure.

Genotyping Results: The experiment wide genotyping success rate was 99.6%, and all genotypes were consistent with HWE. Haplotypes were predicted from genotype data. Observed linkage disequilibrium for genes, and haplotype structure in this cohort are displayed in Appendices 6 and 7. SNP rs2069837 in *IL6* was not correlated to nearby SNPs, so it was not included haplotypes analyzed for *IL6*, and was instead analyzed separately.

Cardiovascular Mortality: All patients were followed up to the end of 2007 for mortality, and among the patients included in this study, there were 223 deaths, 100 of which were attributed to cardiovascular causes. Patients who died from cardiovascular causes were older, and were more likely to have diabetes, and higher baseline CRP and IL-6 ($p<0.001$ for both biomarkers). Age, sex and BMI adjusted HR for cardiovascular mortality were 4.04 [2.07–7.87], $p<0.001$ for log IL-6; and 1.80 [95% confidence interval 1.27–2.55] for log CRP, $p=0.001$.

Haplotypes and Biomarkers: Associations between haplotypes and biomarkers were calculated by a global score across haplotypes for biomarker inequalities between groups, with further comparisons between each haplotypes and the most commonly observed (reference) haplotype. Table 3.4 shows effects of haplotypes and SNPs in *IL6* on IL-6 and CRP levels, in age and sex adjusted models. Elevated plasma CRP was observed for one haplotype, but this association as well as the global p value across haplotypes was only nominally significant ($p=0.045$, $q=0.18$). The singleton SNP in *IL6* (rs2069837) was also associated with increased CRP levels, but the results were only nominally significant with adjustment for multiple testing ($p=0.037$).

Table 3.4 *IL6* haplotypes and genotypes and plasma IL-6 and CRP concentrations

rsID allele	2069827 G/T	1800795 G/C	2069837 A/G*	2069840 C/G	1554606 G/T	1818879 G/A	Estimated Frequency % (SE)	Δ logIL-6 (SE)	P value	Δ logCRP (SE)	P value
	G	**C**	-	C	**T**	G	28.2 (0.01)	*reference*	-	*reference*	-
	G	G	-	**G**	G	**A**	23.9 (0.01)	−0.037 (0.026)	0.14	−0.050 (0.037)	0.20
	G	G	-	C	G	G	14.5 (0.09)	−0.008 (0.030)	0.77	0.031 (0.043)	0.48
	G	G	-	**G**	G	G	10.0 (0.01)	0.024 (0.034)	0.48	0.055 (0.049)	0.26
	G	G	-	C	G	**A**	8.6 (0.01)	−0.006 (0.033)	0.76	0.091 (0.047)	**0.05**
	T	**C**	-	C	**T**	G	8.6 (0.01)	−0.025 (0.033)	0.45	0.061 (0.047)	0.20
							Rare	−0.080 (0.039)	0.044	−0.034 (0.055)	0.51
							Global Score	-	0.179	-	**0.045**
		Δ Log IL-6	−0.0089								
		(SE)	(0.019)								
		P value	0.640								
		Δ Log CRP	0.060								
		(SE)	(0.029)								
		P value	**0.037**								

Δ denotes changes in regression models adjusted for age, sex, and BMI. *rs2069837 was tested separately from IL-6 haplotypes.

SE = Standard Error LogIL-6 = Log 10 Interleukin-6 LogCRP = Log 10 C-reactive Protein

Table 3.5 displays effects of haplotypes and SNPs in *CRP* on plasma CRP. An effect of *CRP* haplotypes on plasma CRP was observed, with reductions in CRP levels for the TCTC haplotype, versus the reference haplotype (TGCC) (p=0.024, q=0.17). Further testing of *CRP* SNPs associated with this haplotype found only nominally significant reductions in plasma CRP with SNP rs1800947, by 0.103 (p=0.029, q=0.17).

Table 3.5 *CRP* haplotypes and genotypes and plasma CRP concentrations

rsID	**1417938**	**1800947**	**1205**	**2808630**	**Estimated Frequency % (SE)**	**Δ logCRP (SE)**	**P value**
allele	**T/A**	**G/C**	**C/T**	**T/C**			
	T	G	T	T	27.6 (0.011)	*reference*	--
	T	G	**C**	**C**	27.5 (0.011)	−0.017 (0.034)	0.62
	A	G	C	T	27.2 (0.011)	−0.010 (0.034)	0.77
	T	G	C	T	8.66 (0.007)	0.071 (0.049)	0.14
	T	**C**	**T**	C	7.32 (0.007)	−0.119 (0.053)	**0.024**
					Rare	0.103 (0.100)	0.30
					Global Score		0.06

Δ denotes changes in regression models adjusted for age, sex, and BMI.

SE = Standard Error LogCRP = Log C-reactive Protein

Associations between haplotypes in *IL6* and *CRP* with angiographic coronary artery disease are displayed in Table 3.6. Odds ratios (OR) and 95% confidence intervals from logistic regression models adjusted for cardiovascular risk factors (age, sex, BMI, smoking, diabetes, hypertension, and total cholesterol: HDL cholesterol ratio). No associations were detected for haplotypes in *IL6.* However, two haplotypes in *CRP* were nominally associated with CAD risk,

but not at significance levels that exceeded the adjusted threshold (p=0.034, and p=0.011; q=0.17 for both).

Table 3.6 Odds ratio (OR) and 95% confidence interval (CI) for association between haplotypes and coronary artery disease

A: *IL6*

rsID	2069827	1800795	2069840	1554606	1818879		
allele	**G/T**	**G/C**	**C/G**	**G/T**	**G/A**	**OR [95% CI]**	**P value**
	G	**C**	C	**T**	G	*reference*	--
	G	G	**G**	G	**A**	0.96 [0.65–1.39]	0.79
	G	G	C	G	G	1.24 [0.78–1.97]	0.36
	G	G	**G**	G	G	1.00 [0.61–1.65]	0.85
	G	G	C	G	**A**	1.51 [0.86–2.64]	0.15
	T	**C**	C	**T**	G	1.30 [0.76–2.20]	0.33
						Global Score	0.34

B: *CRP*

rsID	1417938	1800947	1205	2808630		
allele	**T/A**	**G/C**	**C/T**	**T/C**	**OR [95% CI]**	**P value**
	T	G	T	T	*reference*	--
	T	G	**C**	**C**	0.67 [0.49–0.97]	**0.034**
	A	G	C	T	0.74 [0.50–1.05]	0.09
	T	G	C	T	0.52 [0.32–0.86]	**0.011**
	T	**C**	**T**	C	1.02 [0.55–1.89]	0.94
					Global Score	**0.035**

All logistic regression models were adjusted for age, sex, bmi, smoking, diabetes, hypertension, and total:high-density lipoprotein cholesterol ratio.

However, because these haplotypes both differed from the reference haplotype at *CPR* SNP rs1205, we tested this SNP individually for association with CAD risk, and found the minor allele conferred a significantly higher risk of angiographic CAD. The risk with adjustment for age, sex, and BMI was 1.54-fold higher with the minor allele (95% CI: 1.14–2.06; p=0.0041), which was significant following adjustment for multiple comparisons (p=0.029). Full analyses for SNPs in *IL6* and *CRP* associated with angiographic CAD are presented in Appendix 8.

IL6 and *CRP* haplotypes were further tested for prediction of cardiovascular mortality (Table 3.7).

Table 3.7 Hazard ratios (HR) and 95% confidence intervals (CI) for risk of cardiovascular mortality across haplotypes

A: *IL6*

rsID allele	2069827 G/T	1800795 G/C	2069840 C/G	1554606 G/T	1818879 G/A	HR [95% CI]	P value
	G	**C**	C	**T**	G	*reference*	--
	G	G	**G**	G	**A**	1.09 [0.68–1.71]	0.72
	G	G	C	G	G	0.57 [0.60–1.10]	0.09
	G	G	**G**	G	G	0.89 [0.48–1.66]	0.70
	G	G	C	G	**A**	1.03 [0.59–1.81]	0.85
	T	**C**	C	**T**	G	1.89 [1.08–2.99]	**0.020**
						Global Score	0.34

B: *CRP*

rsID allele	1417938 T/A	1800947 G/C	1205 C/T	2808630 T/C	HR [95% CI]	P value
	T	G	T	T	*reference*	--
	T	G	**C**	**C**	1.55 [1.02–2.36]	**0.042**
	A	G	C	T	1.21 [0.79–1.85]	0.37
	T	G	C	T	0.78 [0.39–1.58]	0.49
	T	**C**	**T**	C	1.29 [0.67–2.46]	0.46
					Global Score	**0.040**

Adjusted for age, sex, smoking, BMI, diabetes, hypertension, CAD, and log transformed biomarkers (logIL-6 for *IL6*, and logCRP for *CRP*).

Although associations between haplotypes and cardiovascular mortality risk were observed, they did not reach adjusted significance levels. Higher cardiovascular mortality risk was observed for one haplotype of CRP (TGCC) compared to the reference haplotype (TCCC), in cardiovascular mortality models adjusted for CRP (p=0.042, q=0.18), with the risk compared to the most common haplotype conferred by change in SNPs rs1205 and rs2808630.

In models further adjusted for presence of angiographic CAD, the relationship persisted, and risk for individuals with the TGCC haplotype was 1.46-fold higher than the rest of haplotypes combined (95% CI 1.04–2.05; p=0.027, q=0.17).

Further testing of SNPs for prediction of cardiovascular mortality are displayed in Appendix 9. *IL6* SNP rs2069827 increased risk of cardiovascular mortality, but results only reached nominal significance (HR 1.5, 95% CI 1.06–2.36; p=0.034, q=0.18). However, a significant association was observed between *CRP* SNP rs2808630 and cardiovascular mortality (HR 1.36, 95% CI 1.09–1.94; p=0.006, q=0.10). This association was not attenuated by further adjustment for plasma CRP.

3.4 Discussion

We observed higher plasma concentrations of IL-6 and CRP in patients with angiographic coronary artery disease, and plasma levels were independently predictive of cardiovascular mortality. However we did not observe a causal relationship between IL6 and CRP haplotypes and cardiovascular disease.

Nominally significant effects of *IL6* haplotypes on CRP concentrations, coronary artery disease, and cardiovascular mortality were observed, but no relationships between haplotypes and plasma IL-6 concentrations were detected. In particular, a haplotype in IL6 present in 7% of the cohort was associated with a 1.9-fold increased risk of cardiovascular mortality in our patients, independent of risk factors and angiographic CAD. This haplotype is tagged by the minor allele of rs1818879, a SNP located in the 3'UTR of IL6, and no association was observed between this SNP and plasma IL-6 or CRP levels. However, this SNP has also been associated with risk of chronic obstructive pulmonary disease (COPD) (28). Future characterization of this SNP and the haplotype it identifies may help identify the changes responsible for these observations.

Data for *CRP* haplotypes also show inconsistent effects on plasma CRP, angiographic CAD, and cardiovascular mortality; significantly increased risk for CAD observed for rs1205 were independent of plasma CRP levels, and polymorphisms that influenced plasma CRP show no association with disease. Furthermore, rs2808630 predicted risk of cardiovascular mortality, but did not predict coronary artery disease or changes to plasma CRP.

Systemic inflammation in CAD is well demonstrated, and our data underscores findings of other studies suggesting polymorphisms in *IL6* and *CRP* may only explain a small percentage of single time point biomarker levels, compared to environmental factors. However, data

presented here associate SNPs in *IL6* and *CRP* with angiographic coronary artery disease, and cardiovascular mortality risk, suggesting these inflammation genes influence progression of atherosclerosis, despite little influence on single timepoint plasma biomarker levels.

Some limitations of our study must be considered. First, our study is relatively small for genetic association studies, and may be underpowered for less dramatic associations, and more rare haplotypes. Second, cohort patients were recruited from selective coronary angiography subjects, therefore the generalisability of our findings to other populations remains to be demonstrated. Also, we measured markers at a single time point only, and although we adjust for factors that have demonstrated or potential influences on these measurements, we cannot ascertain biomarker changes over time, which may be more subject to heritability. Also, circulating levels of IL-6 and CRP may not portray inflammatory activity at the lesion site, where disease progression occurs.

Our study has several strengths, including ascertainment of confounding risk factors at baseline, long patient follow up time, and complete ascertainment of mortality data. We used a tagging SNP approach to capture variability across CRP and IL6, in a relatively homogenous cohort of stable CAD patients collected from the same medical facilities, and we measured both CRP and IL-6 using high sensitivity methods. With rigorous adjustment for type I error and false discovery rate estimations, two genetic associations reported were significant. Furthermore, false discovery rate q values were <0.200 for most nominally significant associations, which is the cut-off suggested for significance in candidate gene studies with an *a priori* hypothesis (29). Nevertheless, all reported associations require replication in other independent studies.

Conclusion: Common variations in *IL6* and *CRP* may contribute to risk of cardiovascular mortality in CAD patients, but relationships are not reflected in single timepoint plasma concentrations. Genetic variations and biomarkers do not offer redundant information, and causality of IL-6 and CRP have yet to be consistently confirmed by genetic analyses.

3.5 References

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Chapter 4. Plasma Oxidative Stress Biomarkers for Prediction of Coronary Artery Disease and Cardiovascular Mortality in Coronary Angiography Patients[1]

4.1 Introduction

Patients with stable coronary artery disease (CAD) require on-going effective risk stratification, however there remain significant clinical limitations to current risk estimation tools (1). Biomarkers that might add incremental data to cardiovascular risk prediction models have been sought, with the goal of enhancing approaches to long term risk estimation and improving treatment strategies (2).

Based on evidence that oxidative stress is involved in all stages of atherosclerosis (3), oxidative stress biomarkers have been investigated for their potential clinical value. Oxidative modifications of low density lipoproteins and cellular lipids contribute to vascular endothelium dysfunction (4), monocyte invasion (5), foam cell formation (6), and plaque instability (7). Pro-oxidant enzyme myeloperoxidase (MPO) is released by activated neutrophils and macrophages. MPO has been found to predict cardiovascular disease development (8,9), and myocardial infarction in patients with chest pain (10). Nitrotyrosine (N-tyr), a protein residue modification generated by MPO and reactive nitrogen processes, also predicts presence of CAD (11). Elevated plasma oxidized low-density lipoprotein (oxLDL) predicts CAD, and severity of acute coronary syndromes (12). Despite this compelling evidence, the prognostic utility of oxidative stress biomarkers for long term risk assessment in stable CAD has not yet been explored. Finally, circulating antioxidant enzymes and molecules may protect against oxidative stress. Indeed,

[1] A version of this manuscript has been submitted for publication. Heslop CL, Frohlich JJ, Hill JS. Myeloperoxidase and C-reactive protein have combined utility for predicting cardiovascular mortality in coronary angiography patients.

reduced plasma antioxidant capacity (AOC) is associated with coronary artery calcification (13), but has not yet been demonstrated to predict disease development or outcome.

Oxidative stress biomarkers that identify instability in CAD may be useful for screening stable CAD patients to identify individuals requiring more aggressive interventions. Improved risk prediction would permit uptitration of medical therapy and improved compliance in higher risk patients, and a biomarker that could further guide treatment strategies would optimize patient outcome.

On the basis of the above-mentioned evidence, we evaluated whether plasma oxidative stress biomarkers, separately and in combination, predict future fatal cardiovascular events in a large, prospective cohort of non-emergent coronary angiography patients.

4.2 Methods

Coronary Angiography Cohort: Between 1992–1995, a cohort of 1117 patients (797 men and 320 women) was recruited from consecutive selective coronary angiography subjects at two Vancouver teaching hospitals. Indications for angiography included stable angina and previous myocardial infarction (MI), as well as aortic stenosis and/or regurgitation, and mitral valve regurgitation. Patients with unstable angina and/or MI within the preceding 2 months were excluded (n=98). Analyses of this cohort by investigators in our research group are reported elsewhere (14,15). Lesions visualized in major epicardial vessels were assessed semi-quantitatively for percent stenosis, rounded to the nearest 10%. Presence of CAD was defined by the presence of any lesion ≥20% stenosis, and severe CAD was defined by presence of any lesion ≥50% stenosis.

Patient Characteristics: Data regarding cigarette smoking history (past or current, or never), self-identified ethnicity, previous diagnosis of hypertension, previous diagnosis of diabetes mellitus, and history of premature CAD in patients' family (males before 45 and females before 55 years of age), were obtained by self-report. Blood pressure was also measured, and hypertension classified by pre-existing diagnosis and/or baseline blood pressure >140/90 mmHg. Height and weight were measured, and body mass index (BMI) calculated. Waist circumference was measured midway between the iliac crest and the bottom of the ribcage. Medication use was recorded from patient's charts. All participants gave written informed consent, and the Research Ethics Board of St. Paul's Hospital, Vancouver approved this research.

Mortality Data: Identifying data were linked to British Columbia Vital Statistics Agency mortality database to determine deaths to the end of 2007. Cardiovascular deaths were identified by World Health Organization International Classification of Disease (10^{th} revision) mortality codes I20–I25 and I60–I69 (16).

Biochemical Analysis: Fasting blood samples were collected prior to angiography in ethylenediamine tetra-acetate Vacutainer tubes. Following centrifugation, plasma was aliquoted and stored at -70°C, with a single freeze-thaw cycle prior to biomarker analyses. Lipid and lipoprotein measurements were completed immediately, and inflammatory marker C-reactive protein (CRP) was measured in 2002, using high-sensitivity methods described previously (15).

MPO and Ntyr were measured using solid-phase enzyme-linked immunosorbent (ELISA) assays (Hycult, Uden, The Netherlands). OxLDL was measured using a competitive ELISA (Mercodia, Uppsala, Sweden), based on monoclonal antibody 4E6. Antioxidant Capacity was measured by a colorimetric assay (Alpco, Salem, NH) based on reaction of sample antioxidants with hydrogen peroxide. Samples were diluted to obtain measurements in assay linear ranges: MPO 0.4–100 ng/mL; N-tyr 2–1,500 nmol/L; oxLDL 0.5–7.6 U/L; AOC 130–393 µmol/L. Intra- and inter-assay coefficients of variation were: MPO (7.2%, 7.8%), N-tyr (7.9%, 11.1%), oxLDL (5.4%, 4.2%), AOC (7.4%, 7.5%). Stability of biomarkers in stored plasma was verified by comparison with recently collected plasma samples (Appendix 10). All measurements were performed by investigators blinded to patient data.

Statistical Analyses: Baseline continuous variables are presented as mean±standard error (SE), skewed variables as median [interquartile range (IQR)], and categorical variables as number

(percentage). MPO, Ntyr, AOC and CRP had skewed distribution and were therefore log transformed. Baseline characteristics were tested for relationships with angiographic CAD and cardiovascular mortality using Student's *t*-tests for continuous variables, Mann-Whitney rank-sum tests for skewed continuous variables, and chi-square statistics for categorical variables. Conditional logistic regression models were used to calculate odds ratios (OR) and corresponding confidence intervals (CI) for associations between biomarkers and angiographic CAD, with adjustment for the following cardiovascular risk factors: age, sex, total cholesterol:high-density lipoprotein cholesterol ratio (TC:HDL-C), BMI, smoking, diabetes, and hypertension.

Oxidative stress biomarker tertiles were defined within the cohort. Preliminary associations between biomarker tertiles and mortality were explored by log rank tests. Cox proportionate hazards models were constructed to determine hazard ratios (HR) for cardiovascular mortality across biomarker tertiles, with the first tertiles as reference. Covariate-adjusted Cox models included risk factors listed above, with further adjustment for CRP and severe CAD (≥50% stenosis) where noted. The proportionate hazards assumption was confirmed by correlating Schoenfeld residuals with time, and by partial residual plots.

To evaluate biomarker risk discrimination value, area under time-adjusted receiver operator characteristic curves were calculated from covariate-adjusted Cox regression models with and without biomarkers(17). Areas under receiver operator curves (AUC) were compared using non-parametric methods for comparing curves generated from the same patients (18). Risk model calibration was also assessed by Hosmer-Lemeshow statistics (19).

Finally, survival models were assessed for accuracy of patient risk estimation, using reclassification tables (20).Patient outcome risk was calculated from covariate adjusted models,

classified into categories of <5%, 5–10%, 10–20% and >20%. Patient reclassification by including biomarkers in covariate adjusted models was calculated, and significance of model improvement determined by the net reclassification index (NRI), as described by Pencina et al(21). Data were analyzed using SPSS (version 14.0, SPSS Inc., Chicago, Illinois), and R (version 2.8.1, R Foundation for Statistical Computing, Vienna, Austria), with two tailed p values <0.05 considered to be statically significant.

4.3 Results

Biomarkers and Angiographic CAD: Of the patients recruited, 885 had plasma samples available for biomarker measurement. Of those patients, 651 patients had angiographic CAD, and 604 had severe lesions (≥50% stenosis). Baseline characteristics are presented in Table 4.1. Patients with severe lesions had higher MPO (p=0.022), oxLDL (p=0.031), and AOC (p=0.003). MPO predicted angiographic CAD (OR per SD 1.97, 95% CI 1.08 to 3.60; p=0.03) and severe CAD (OR per SD 1.85, 95% CI 1.09 to 3.18; p=0.021) in covariate adjusted analyses. Associations between oxLDL and AOC with CAD did not persist after covariate adjustment.

Table 4.1 Baseline characteristics of selective coronary angiography cohort

Characteristic*	Angiographic evidence of coronary artery disease		
	No (n=180)	Yes (n=705)	p value†
Age, years	58.0±0.91	61.7±0.41	<0.001
Males, %	53.3	81.7	<0.001
Smoking status, %			
Never smoked	44.6	25.0	<0.001
Former or current smoker	55.4	75.0	
Hypertension, %	55.6	65.3	0.129
Diabetes, %	9.4	20.3	<0.001
Family history of coronary artery disease, %	52.2	58.1	0.174
Body mass index, kg/m^2	27.4±0.36	28.2± 0.19	0.073
Waist circumference, cm	86.8±2.13	93.6±0.97	0.004
Lipid Variables			
Low-density lipoprotein cholesterol, mg/dL	145±1.2	137±1.6	0.007
High-density lipoprotein cholesterol, mg/dL	39±0.8	36±0.4	<0.001
Total cholesterol:high-density lipoprotein cholesterol ratio	5.32± 0.14	5.87±0.08	0.002
C-reactive protein, mg/L	1.54 [0.72–4.04]	1.84 [0.88–5.15]	0.030
Oxidative stress biomarkers			
Myeloperoxidase, ng/μL	80.4 [57.9–123.6]	92.0 [61.9–138.0]	0.036
Nitrotyrosine, nmol/L	71.9 [59.5–90.2]	72.1 [59.5–88.4]	0.965
Oxidized low-density lipoprotein, mU/L	70.4 [58.8–82.8]	72.4 [60.9–89.8]	0.070
Antioxidant capacity, μmol/L	286.8 [265.5–314.0]	300.2 [273.3–323.0]	0.006

CAD is defined as any lesions ≥20% stenosis on baseline selective coronary angiography.

* Data are presented as mean±standard error, median [interquartile range], or percentage.

† p-values are calculated from independent samples t-tests, Mann-Whitney U tests for skewed variables, or χ^2 tests for categorical variables.

Biomarkers and Baseline Characteristics: Plasma antioxidant status was lower in female patients, which supports findings reported elsewhere(22). Median AOC for women was 280.2 [256.1 to 303.7] μmol/L, versus 302.7 for men [277.7 to 325.7] μmol/L; $p<0.001$. No other sex differences were observed for biomarkers. No ethnicity differences could be tested, as 87% of patients reported Caucasian ethnicity.

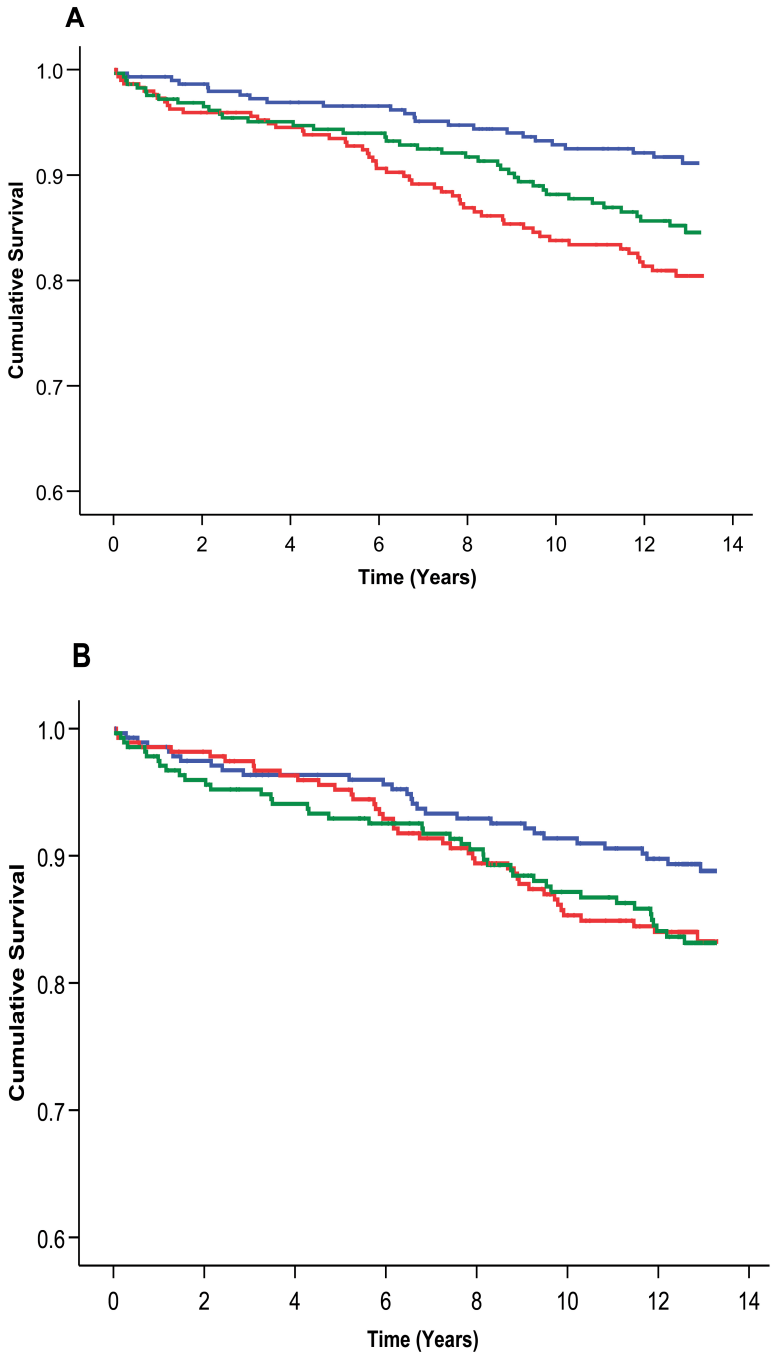
Age correlated slightly with MPO ($r=0.100$, $p=0.01$), and negatively with oxLDL ($r=-0.147$; $p<0.001$). Antioxidant capacity correlated with BMI ($r=0.149$; $p<0.001$), as did oxLDL ($r=0.173$; $p<0.001$), which supports previous research (23). Higher oxLDL levels were observed in patients with a smoking history than in nonsmokers (73.4[61.8 to 91.0] mU/L versus 70.9[58.7 to 85.6] mU/L; $p=0.03$), however these patients also had higher AOC (301.3[274.3 to 328.4] μmol/L versus 295.2[268.6 to 314.7] μmol/L; $p=0.01$). CRP correlated with MPO ($r=0.140$; $p<0.001$), which supports observations reported elsewhere (24). No other relationships between risk factors and biomarkers were observed.

Biomarkers and Cardiovascular Mortality: After a median follow-up time of 12.9 [11.1 to 13.1] years, there were 257 deaths, and 117 were cardiovascular deaths. Patients who died from cardiovascular causes were older ($p<0.001$), and more likely to have diabetics ($p=0.026$). Also, they had higher TC:HDL-C ($p<0.001$), CRP ($p<0.001$), and MPO ($p=0.001$).

Risk for cardiovascular mortality increased across tertiles of MPO ($p=0.007$) and N-tyr ($p=0.029$) (Figures 2.1A and 2.1B). Risk for cardiovascular mortality among patients with severe CAD also increased across tertiles of MPO and N-tyr ($p=0.029$) and N-tyr ($p=0.035$). Ntyr did not predict cardiovascular mortality after covariate adjustment.

The HR for cardiovascular mortality for the top MPO tertile was 2.38 (95% CI 1.47 to 2.98) compared to the bottom tertile ($p<0.001$). The risk remained with covariate adjustment (HR 1.96, 95% CI 1.15 to 3.37; $p=0.012$). With further adjustment for severe CAD (≥50% stenosis) the risk remained high (HR 2.06, 95% CI 1.26 to 3.36; $p=0.004$). Finally, the risk for elevated MPO remained significant with further adjustment to include CRP (HR 1.75, 95% CI 1.16 to 3.10; $p=0.010$), and although the risk for the second tertile was attenuated by CRP, the trend across tertiles was linear ($p=0.036$). Survival models for MPO are displayed in Table 4.2.

Figure 4.1 Cumulative survival curves for cardiovascular mortality by tertiles of myeloperoxidase (A) and nitrotyrosine (B)



Blue lines indicate lowest biomarker tertile, green lines indicate second tertile, and red lines indicate third (highest) tertile. Log rank test: myeloperoxidase p=0.007, nitrotyrosine p=0.029.

Table 4.2 Hazard ratios for cardiovascular mortality by tertiles of myeloperoxidase

Variable	Tertile (range, ng/mL)			p value
	1	2	3	
	(<70.2)	(70.2–118.7)	(<118.7)	
Patients, n	295	295	295	
Cardiovascular deaths, n (%)	24 (8.1)	40 (13.6)	53 (18.0)	
Unadjusted				
Hazard ratio	1.0	1.80	2.38	<0.001
95% CI	–	1.08–2.98	1.47–2.98	
P value	–	0.023	<0.001	
Adjusted for risk factors*				
Hazard ratio	1.0	1.84	1.96	0.014
95% CI	–	1.07–3.14	1.16–3.24	
P value	–	0.026	0.012	
Further adjusted for CAD severity†				
Hazard ratio	1.0	1.52	2.06	0.014
95% CI	–	0.91–2.55	1.26–3.36	
P value	–	0.110	0.004	
Further adjusted for C-reactive protein‡				
Hazard ratio	1.0	1.41	1.75	0.036
95% CI	–	0.84–2.37	1.16–3.10	
P value	–	0.194	0.011	

* Covariate adjustment for age, sex, total:high density lipoprotein cholesterol ratio, body mass index, smoking, diabetes, and hypertension

† Further adjusted for ≥50% stenosis on baseline coronary angiography

‡ Further adjusted for log-transformed C-reactive protein

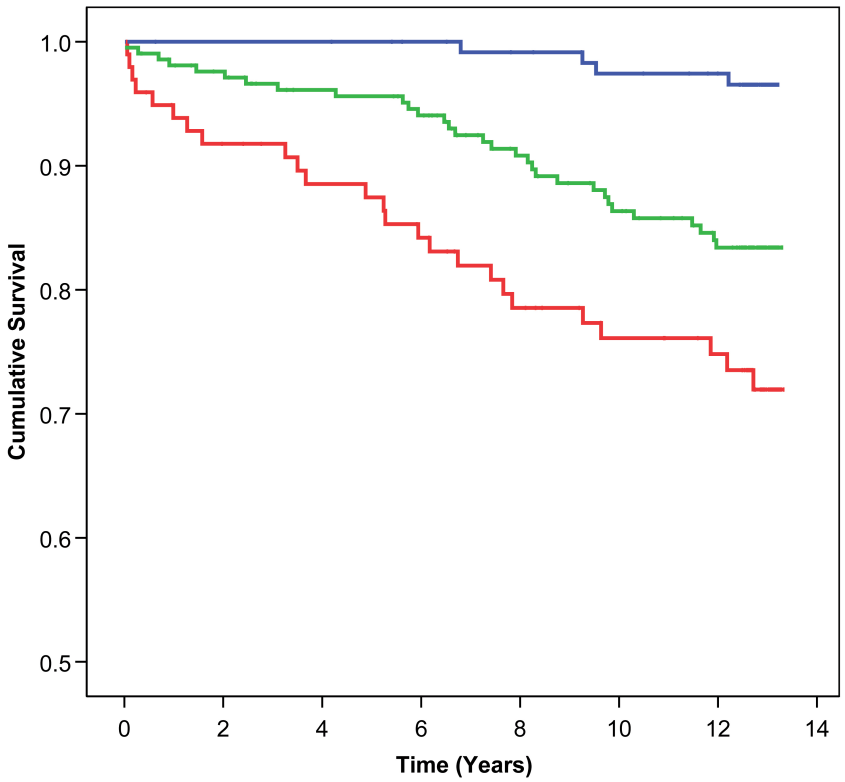
CI = confidence interval

Left ventricular ejection fraction (LVEF) values were collected from coronary angiography reports for a subset of angiography patients (n=415). Decreased LVEF was associated with cardiovascular mortality ($p<0.001$), but LVEF did not correlate with oxidative stress biomarkers. In covariate adjusted survival analyses including adjustment for CAD severity, CRP, and LVEF, risk associated with the highest MPO tertile remained strong (HR 2.88, 95% CI 1.18 to 7.03; $p=0.02$), and the trend remained linear across tertiles ($p=0.030$).

Combined Biomarkers and Cardiovascular Mortality Risk: Oxidative stress scores assigned by adding the number of pro-oxidant markers (MPO, N-tyr and oxLDL) in the highest tertile, with a score for AOC in the lowest tertile, predicted cardiovascular mortality in a linear fashion ($p=0.010$). Patients with the highest scores (3 and 4, n=89) had a 2.8 fold cardiovascular mortality risk (95% CI 1.53 to 5.39; $p=0.001$) compared to patients with the lowest multimarker score (n=262). This relationship persisted with adjustment for covariates and severity CAD ($p=0.037$ for linear trend). However, the trend was attenuated by adjustment for MPO, suggesting no significant benefit was offered by multiple markers.

Combined Value of MPO and CRP: MPO not only predicted cardiovascular mortality independent of CRP, but combining MPO and CRP tertiles predicted cardiovascular mortality risk ($p<0.001$). Patients with either marker elevated had a 5.33-fold risk of cardiovascular mortality compared to patients with both biomarkers in the lowest tertile (95% CI 1.86 to 14.9; $p=0.002$). Patients with both markers elevated had a 4.33-fold risk of cardiovascular mortality compared to patients with either biomarker elevated (95% CI 2.26 to 8.31; $p<0.001$). Cumulative survival curves for this comparison are displayed in Figure 2.2.

Figure 4.2 Cumulative survival curves for cardiovascular mortality by elevations in myeloperoxidase and C-reactive protein



Patients with lowest tertile myeloperoxidase and C-reactive protein (blue line) are compared with patients with highest tertile levels of either marker (green line), and patients with highest tertile measurements of both markers (red line). Log rank test $p<0.001$ for trend.

These observations remained significant after covariate adjustment, including adjustment for either CRP or MPO levels. Among patients with severe CAD, covariate adjusted HR were 3.83 for elevation of one marker (95% CI 1.33 to 11.02; p= 0.013), and 6.41 for both markers (95% CI 2.18 to 18.78; p= 0.001) compared to low levels of both markers. Further adjustment for LVEF did not reduce the relationship.

Evaluation of Survival Models: The covariate adjusted model including CAD severity yielded an AUC of 0.715, which improved to 0.761 with MPO (p=0.031), and to 0.781 with combined MPO and CRP tertiles (p=0.004). No deviations from risk calibration were detected for any risk models. Time-adjusted ROC curves are depicted in Appendix 11.

Finally, covariate adjusted models including CAD severity were used to classify patients into risk categories, and net reclassification indices (NRI) calculated from patient reclassification conferred by biomarkers. Patients reclassified by tertiles of MPO are displayed in Table 4.3. MPO improved risk prediction across all categories, with a NRI of 14.4% (p=0.003). Combined MPO and CRP tertiles also improved patient risk classification over models including risk factors and CRP (NRI 9.6%; p=0.05).

Table 4.3 Risk reclassification table for myeloperoxidase

Risk of cardiovascular mortality for covariate adjusted survival model*		Reclassified risk of cardiovascular mortality with myeloperoxidase in survival models				% reclassified per risk category
		<5%	5–10%	10–20%	>20%	
<5%	Number	144	→ 18			
	Cardiovascular deaths	5	3			11.1
	Mortality rate, %	*3.5*	*16.7*			
5–10%	Number	37 ←	120	→ 34		
	Cardiovascular deaths	0	7	4		37.2
	Mortality rate, %	*0*	*5.8*	*11.8*		
10–20%	Number		50 ←	188	→ 37	
	Cardiovascular deaths		3	27	10	31.6
	Mortality rate, %		*6*	*14.4*	*27*	
>20%	Number			38 ←	153	
	Cardiovascular deaths			5	47	19.9
	Mortality rate, %			*13.2*	*30.7*	

Estimated mortality risk for patients from cardiovascular mortality risk models adjusted for age, sex, body-mass index, total:HDL cholesterol ratio, smoking, diabetes, hypertension, and angiographic stenosis ≥50%. Patients reclassified into different mortality risk categories by adding tertiles of myeloperoxidase to risk models are shown by arrows, with observed mortality rates for each reclassified risk category in shaded table cells.

4.4 Discussion

Myeloperoxidase has been localized to human atherosclerotic lesions, and implicated in pro-atherogenic modifications to lipoprotein particles (25). Also, catabolism of nitric oxide by MPO may impair its vasodilatory and anti-inflammatory functions in the vasculature (26). Indeed, elevated MPO independently predicts reduced endothelial function (27), while CRP has not been consistently found to do so (28,29).

In this prospective study of 885 coronary angiography patients, baseline MPO independently predicted cardiovascular mortality beyond traditional risk factors and degree of angiographic stenosis. Furthermore, MPO enhanced survival model discrimination and improved patient risk classification across meaningful risk categories. Although multiple markers of oxidative stress incrementally influenced cardiovascular mortality risk, the greatest value was offered by evaluation of MPO. Finally, MPO and CRP had independent and combined predictive value, and improved accuracy of patient risk estimations.

MPO has previously been demonstrated to identify patients with acute coronary syndromes and MI who are at higher risk for adverse outcomes (30,31), however one study found risk conferred by MPO was only for events during the initial 72 hours after onset of symptoms (32). Although elevations of MPO secondary to recruitment and activation of neutrophils in ACS may explain these results (33), MPO may also be induce plaque rupture by activating metalloproteases (34) and triggering endothelial cell apoptosis (35). We demonstrate for the first time the value of MPO for long term risk estimation for patients with stable CAD, in comparison to traditional risk factors and CRP. Multiple oxidative stress markers have been associated with CAD in a smaller, cross-sectional study (36), however our study is the first prospective investigation of multiple oxidative stress markers in angiography patients. Our

findings extend and refine the current evidence implicating oxidative stress in cardiovascular disease, and we clarify the relative prognostic utility of oxidative stress biomarkers compared to conventional risk factors.

Not all studies are consistent with our findings. Kubala and colleagues did not find elevated myeloperoxidase in CAD patients (24), and a recent study of 382 stable CAD patients found MPO did not predict total mortality (37). However, we followed our larger cohort of patients for a longer period of time, and determined cause for all cases of mortality. Also, we included patients with moderate lesions, which are more often implicated in MI (38). By investigating patients across a range of risk, we elucidated the prognostic value of MPO, and identified that MPO offers the improvements across meaningful risk categories, including intermediate categories where more challenging therapeutic decisions must be made. Most importantly, we demonstrate for the first time the complementary value of MPO and CRP for identifying patients at risk for cardiovascular mortality.

Limitations: There are limitations to our study that must be considered. We could not ascertain biomarker changes over time, nor could we adjust for baseline renal function, which may confound associations between MPO and cardiovascular mortality. Data regarding treatments following initial assessment were not available. Although there is good evidence from multiple studies that MPO has value for risk assessment in cardiovascular cohorts, the transportability of our results to a more diverse cohort or to lower risk populations has yet to be shown.

Conclusion: Oxidative stress biomarker MPO powerfully enhances cardiovascular risk prediction, and is additive with CRP for predicting risk in selective angiography patients. Future studies are essential to demonstrate whether outcomes may be improved when MPO and CRP measurements are utilized together for prognostic assessment and therapeutic decision making.

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Chapter 5. Combined Polymorphisms in Oxidative Stress Genes Predict Coronary Artery Disease and Plasma Oxidative Stress in Coronary Angiography Patients[1]

5.1 Introduction

Oxidative stress plays a pivotal role in the initiation and progression of cardiovascular disease (1) and plasma biomarkers of oxidative stress have been identified that predict coronary artery disease, and risk of adverse cardiovascular events (2,3). Although oxidative stress burden is influenced by conventional risk factors, including hypertension and diabetes (4), inherited variations in oxidative stress genes may also contribute to oxidative stress levels, and cardiovascular risk. Polymorphisms which have functional effects on oxidative stress pathways may represent non-modifiable, lifelong cardiovascular risk factors, and gene-environment interactions may further contribute to disease risk (5).

Candidate oxidative stress genes have been identified which may contribute to atherosclerosis. Arachidonate 5-lipoxygenase (ALOX5) and its activating protein are critical regulators of leukotriene synthesis(6), and 5-lipoxygenase activity is required for inflammatory and atherosclerotic responses to oxidized low density lipoprotein (LDL) (7). 5-lipoxygenase activity has been implicated in plaque formation (8) and plaque instability (9), and tandem repeat sequence in the *ALOX5* gene promoter region has been associated with carotid intima-media thickness (10). Results from two population-based studies show a six SNP haplotype in the 5-lipoxygenase activating protein gene (*FLAP*) predicts risk of myocardial infarction (11,12).

[1] A version of this manuscript will be submitted for publication. Heslop CL, Podder M, Ruan J, Tebbutt SJ, Hill JS. Combined Polymorphisms in Oxidative Stress Genes Predict Coronary Artery Disease and Plasma Oxidative Stress in Coronary Angiography Patients.

NAD(P)H oxidase is a major source of superoxide in the vasculature, and the p22phox subunit of NAD(P)H oxidase has been spatially associated with oxidized LDL and reactive oxygen species production in atherosclerotic plaques (13). Polymorphisms in *CYBA*, the gene encoding the p22phox subunit, have been associated with coronary artery disease risk (14,15) and recurrent cardiovascular events (16,17).

Myeloperoxidase is a pro-oxidant enzyme secreted by activated neutrophils and monocytes which generates atherogenic modifications to LDL particles (18). Products of myeloperoxidase activity can also modify residues on apolipoprotein A1, impairing cholesterol efflux to high-density lipoproteins (HDL) (19). Polymorphisms in the *MPO* gene may affect plasma LDL cholesterol and LDL particle size (20), and have been associated with coronary artery disease (21,22), and risk of major adverse cardiovascular events (13).

Polymorphisms in antioxidant genes have also been associated with cardiovascular disease risk. Glutathione peroxidases are antioxidant enzymes important for intra- and extracellular oxidative stress protection. A nonsynonymous SNP in intracellular glutathione peroxidase (*GPX1*) is associated with carotid artery intima-media thickness (23), and coronary artery calcification (24). Polymorphisms in plasma glutathione peroxidase (*GPX3)* have been associated with increased risk of ischemic stroke (25).

Heme oxygenase-1 is an antioxidative defense enzyme, which generates carbon monoxide from degradation of heme molecules, and is induced in response to oxidative stress, hypoxia, heavy metals, and inflammatory cytokines (26). A microsattelite polymorphic region in *HMOX1* is associated with atherosclerotic lesion restenosis (27,28), but other polymorphisms in *HMOX1* have not yet been extensively explored.

Endothelial nitric oxide synthase generates nitric oxide in the vasculature, where it contributes to vascular smooth muscle cell function, and inhibits platelet aggregation and leukocyte adhesion to vascular endothelial cells (29). Polymorphisms in the endothelial nitric oxide synthase gene (*NOS3*) also influence coronary artery disease risk (30–32).

The paraoxonase gene family includes *PON1*, *PON2*, and *PON3*, which are located adjacently on chromosome 7, and SNPs in these antioxidant enzymes have been associated with changes to oxidative stress production, and development of atherosclerosis (33–35).

Finally, superoxide dismutase (SOD) enzymes catalyze conversion of superoxide radicals to hydrogen peroxide. Polymorphisms in *SOD1* and *SOD2* may influence oxidative stress burden, and risk of cardiovascular disease (36).

Although genes in oxidative stress pathways have been implicated in cardiovascular risk, the impact of polymorphisms in these genes on oxidative stress phenotypes has not investigated or compared to their effects on clinical endpoints. By their random allocation prior to disease, single nucleotide polymorphisms (SNPs) in oxidative stress genes could distinguish causal from confounded associations between plasma oxidative stress and cardiovascular disease; SNPs which influence oxidative stress phenotypes and contribute to disease burden would causally implicate oxidative stress pathways in disease risk.

To investigate this hypothesis, we genotyped tagging and nonsynonymous SNPs in oxidative stress genes in a cohort of patients who underwent selective coronary angiography. Genotypes were evaluated for prediction of angiographic coronary artery disease, and association with plasma oxidative stress biomarker concentrations. Polymorphisms in oxidative stress genes were also tested for prediction of cardiovascular mortality.

5.2 Methods

Angiography Patients: A cohort of 1117 consecutive selective coronary angiography patients was recruited between 1993 and 1995. Details regarding this angiography cohort have been described elsewhere (37,38). These analyses include patients with DNA available for genotyping (n=797).

Degree of stenosis visualized in major epicardial vessels rounded to the nearest 10% was recorded, and presence of coronary artery disease (CAD) defined as stenosis ≥20% diameter in any vessel, based on previously work demonstrating patients in this cohort with ≥20% stenosis were at an increased risk of cardiovascular mortality (37). Severe CAD was defined as ≥50% vessel stenosis. To reduce influence of acute disease states, patients with acute coronary syndromes or myocardial infarction (MI) in the previous 2 months were excluded at the time of recruitment. All individuals signed an informed consent form; the study was approved by the ethics review board of University of British Columbia/Providence Health-St. Paul's Hospital.

Patient Characteristics: Described in previous chapters.

Mortality Data: Described in previous chapters.

Plasma Biomarkers: Details of plasma biomarker measurement are described in Chapter 4. Briefly, plasma oxidative stress biomarkers were measured using enzyme-linked immunosorbent assays for myeloperoxidase, nitrotyrosine, and oxidized LDL. Plasma antioxidant capacity was determined by colorimetric assay. All measurements were performed blinded to patient disease status, genotypes, and outcome.

DNA Collection: Described in Chapter 3.

Genotyping: A total of 13 candidate genes were chosen based on involvement in pro- or anti-oxidant pathways, and previous implication in cardiovascular disease. For 10 genes (*ALOX5, FLAP, CYBA, GPX3, HMOX1, MPO, NOS3, PON2, PON3, SOD1*), a "tagging" strategy was used to select SNPs that capture genetic variation across the gene region (tagSNPs). To select tagSNPs, data for genetic variation in the CEPH (Centre D'Etude du Polymorphisme Human) population of Utah residents with ancestry from northern and western Europe was downloaded from Build 2 data of the International Hapmap Project (39), including upstream and downstream regions based on regional linkage disequilibrium (LD) structure. TagSNPs were chosen from CEPH data using Haploview (version 3.3.2) with pairwise tagging methods, and forced inclusion of SNPs previously associated with CAD or CAD risk factors. Gene coverage of $r^2 \geq 0.8$ was achieved for all SNPs ≥10% minor allele frequency (MAF). For 3 additional genes (*GPX1*, *PON1*, and *SOD2*), only SNPs with previously demonstrated associations with CAD or CAD risk factors were included.

APEX Genotyping: APEX genotyping procedures are described in Chapter 3. Seven SNPs failed manual genotyping, and were excluded from automated genotyping and further analyses: rs2071748, rs2333227, rs3794624, rs4880, rs705379, rs705381, and rs7785039. The final analyses therefore included 61 SNPs across 12 oxidative stress genes.

Quality Control: Accuracy of automated genotyping results was estimated by comparing genotypes from Caucasian Human Variation Collection samples (Coriell Institute for Medical

Research, Camden, NJ) with validated genotypes collected from the Genome Variation Server (GVS, http://gvs.gs.washington.edu/GVS/index.jsp). Cohort patient samples genotyped in blinded duplicates were also compared. The concordance rate for Coriell samples was 99.9% between APEX results and GVS (n=18 samples), and 100% between duplicate Coriell samples (n=11). Blinded duplicates were 99.3% concordant for whole blood extracted DNA samples, and had a genotyping failure rate of 0.1%. Plasma extracted DNA samples had a genotyping failure rate of 0.7%, and were 98.3% concordant with genotypes from matching whole blood extracted DNA samples.

Statistical Analysis: Chi-square tests were used to test for deviation from Hardy-Weinberg equilibrium. Analyses of SNPs in candidate genes were performed using SimHap version 1.0.2 (40). Multivariate logistic regression analyses were used to test association between SNPs and angiographic CAD, in additive genetic models adjusted for age and sex. SNPs associated with CAD were further evaluated for changes to plasma oxidative stress biomarkers by multivariate linear regression models adjusted for age and sex, with further adjustment where noted. Covariate adjusted mean plasma biomarker levels and 95% confidence intervals (95% CI) were estimated for each genotype group from regression coefficients (β) and standard error values. For tagging SNPs related to each other in haplotype blocks, haplotypes were constructed by estimation-maximization based methods from unphased genotype data, with simulations used to deal with missing data and uncertainty around imputed haplotypes. Haplotypes with frequencies ≥5% were compared for association with angiographic CAD with the most commonly observed (reference) haplotype. Additive genetic models minimally adjusted for age and sex were used to test for associations with CAD. Haplotypes associated with angiographic CAD were further

investigated for changes in plasma biomarkers by multivariate linear regression, adjusted for age and sex, with further adjustment for cardiovascular risk factors where noted. Covariate adjusted mean plasma biomarker levels and 95% confidence intervals (95% CI) were estimated for each haplotype from regression coefficients (β) and standard error values.

Combined SNPs: A combined SNP score was generated by adding together the number of copies of each allele for SNPs associated with CAD risk. Combined SNPs were tested for additive predictive value for CAD and severe angiographic CAD (≥50% stenosis) by logistic regression analyses, with adjustment for cardiovascular risk factors. Combined SNPs were tested for linear increases in predictive value by orthogonal polynomial contrasts. Biomarkers levels across combined SNP scores were compared by ANOVA with Tukey-Kramer method, which is robust for differences in group size.

Survival Analyses: Cox proportionate hazards analyses were performed test only SNPs and haplotypes associated with CAD for prediction of cardiovascular mortality, in models adjusted for cardiovascular risk factors.

Multiple Comparison: All significance values are reported with a two-tailed α of 0.05. A Bonerroni corrected significance value based on linkage disequilibrium was used to determine the number of independent tests (41). Because LD-based methods were used for haplotype definitions (42), a low type I error rate was expected (43), thus type I error rate correction was p=0.05 divided by the number of haplotype blocks tested, plus the number of singleton SNPs not included within haplotype blocks. A total of 32 independent blocks and singleton SNPs were

tested for association with CAD, therefore the corrected p-value threshold for associations with CAD was p=0.0016 (p=0.05/32).

To further address the risk of false discoveries, local False Discovery Rate (FDR) q values are reported for genotypes associated with CAD, calculated from p values for all independent tests. Q-value calculation was performed using QValue (44) based on methods of Benjamini and Hochberg (45). A threshold q-value was not set, but similar candidate gene studies have suggested q values below 0.200 are at low false discovery risk (46).

5.3 Results

Cohort Baseline Characteristics: Baseline characteristics are reported in previous chapters, and included in Appendix 12 for completeness.

Genotypes associated with CAD: Associations between oxidative stress gene SNPs and coronary artery disease are displayed in Table 5.1. Four SNPs were nominally associated with CAD at $p<0.05$, in gene regions of *ALOX5*, *GPX1*, and *MPO*. Only rs1050450 in *GPX1* was associated with angiographic CAD beyond the multiple comparison-adjusted P-value threshold ($p=0.0016$), at $p=0.001$ (q value=0.037).

Oxidative Stress Gene Haplotypes: Haplotype blocks were identified within *ALOX5*, *FLAP*, *CYBA*, *GPX3*, *MPO*, *NOS3*, *PON2*, and *PON3* using CEPH haplotype data in Haploview, and defined by LD confidence intervals between tagSNPs (42). Haplotype blocks consisted of 2 to 6 SNPs, and most SNPs in haplotypes were tagSNPs for polymorphisms not genotyped directly.

Associations between haplotypes and angiographic CAD are displayed for completeness in Appendix 13, with observations at $p\leq0.05$ reported here. Haplotypes in *GPX3* were nominally associated with angiographic CAD ($p=0.006$, $q=0.129$). Haplotypes of *PON2* were also nominally associated with CAD ($p=0.05$, $q=0.487$). Although the significance values observed for these findings did not meet the adjusted P-value threshold, these haplotypes were further tested for changes to plasma biomarker levels (Appendix 14), as were single SNPs associated with CAD (Appendix 15).

Table 5.1 SNPs in oxidative stress genes and associations with evidence of angiographic coronary artery disease (≥ 20% stenosis) among 797 selective coronary angiography patients

Gene	SNP Location	tagSNP	Odds Ratio (95% Confidence Interval)	p value*	q value†
ALOX5	5'-flanking	rs12762303	0.87 (0.61–1.26)	0.476	0.766
	Intron 2	rs745986	0.79 (0.57–1.09)	0.152	0.584
	Intron 3	rs10900213	0.76 (0.59–0.99)	**0.040**	**0.328**
	Intron 4	rs3780901	1.04 (0.78–1.38)	0.780	0.821
	Intron 6	rs1565096	1.04 (0.76–1.41)	0.800	0.821
FLAP	Intron 1	rs4769055	0.98 (0.73–1.32)	0.907	0.821
	Intron 4	rs4769060	1.03 (0.787–1.36)	0.800	0.821
	Intron 4	rs10507391	0.985 (0.73–1.32)	0.919	0.821
GPX1	Pro198Leu	rs1050450	1.89 (1.29–2.76)	**0.001**	**0.037**
GPX3	Intron 1	rs4958872	0.90 (0.65–1.16)	0.343	0.712
	Intron 4	rs10507391	0.985 (0.73–1.32)	0.919	0.821
HMOX1	Intron 2	rs9607267	1.27 (0.95–1.70)	0.115	0.584
	Intron 3	rs2071749	0.56 (0.74–1.24)	0.730	0.821
MPO	Intron 11	rs2071409	0.69 (0.49–0.96)	**0.029**	**0.295**
	Val717Ile	rs2759	0.65 (0.30–1.42)	0.281	0.627
	5'-flanking	rs2107545	0.71 (0.52–0.97)	**0.032**	**0.295**
NOS3	Glu298Asp	rs1799983	1.03 (0.77–1.38)	0.831	0.821
	Intron 14	rs3918188	1.06 (0.79–1.41)	0.712	0.821
	Intron 23	rs1808593	0.95 (0.70–1.28)	0.737	0.821
SOD1	Intron 4	rs1041740	0.88 (0.66–1.17)	0.390	0.733

* P values are from age and sex-adjusted logistic regression models for additive genetic effects.

† Q values represent false discovery rates expected for the corresponding p-value, calculated from the experiment-wide distribution of p-values.

Combined SNPs: A combined SNP score was calculated for each patient, according to the number of risk alleles at the five SNP sites identified at $p<0.05$ for association with CAD. Risk alleles were *ALOX5* rs10900213 G, *GPX1* rs1050450 A, and *MPO* rs2071409 T and rs2107545 T. Also, the rs8177447 T allele which conferred CAD risk observed for haplotypes across *GPX3* was also included as a risk allele.

Six patients had 3 risk alleles, 39 had 4 alleles, 94 had 5 alleles, 178 had 6 alleles, 208 had 7 alleles, 141 had 8 alleles, 60 had 9 alleles, and 1 had 10 risk alleles. Since the number of subjects with 3, 4 and 10 alleles was very low (<5% of the cohort), we combined subjects with 3–5 and 9–10 risk alleles, and compared the 5 groups.

As expected from observations for individual SNPs, combined SNP scores were associated with CAD, and angiographic CAD severity. Results are displayed in Table 5.2. Further testing in covariate adjusted models for angiographic CAD revealed a linear relationship between combined SNP scores and CAD risk, with a 1.5-fold risk from each additional risk allele (95% CI 1.29–1.72; $p<0.001$). Between the lowest and highest combined SNP scores, the odds for severe CAD was increased by 5.8-fold (95% CI 2.38–14.28; $p=0.012$), and the association remained significant following adjustment for cardiovascular risk factors ($p<0.001$).

Because elevated plasma oxidized LDL was associated with CAD ($p=0.015$), models were further adjusted for plasma oxLDL, which did not attenuate the relationship between combined SNPs and CAD risk. Adjustment with myeloperoxidase also did not attenuate the relationships (data not shown).

Table 5.2 Odds ratio for CAD by combined SNP scores

	Risk Increase Per Allele	Lowest Score to Highest Score	P value for trend
Angiographic CAD			
Odds Ratio	1.49	6.23	<0.001
95% CI	1.29–1.72	2.45–17.92	
P value	<0.001	<0.001	
Severe Angiographic CAD*			
Odds Ratio	1.34	5.83	<0.001
95% CI	1.18–1.51	2.38–14.28	
P value	<0.001	0.012	
Severe Angiographic CAD, adjusted for CAD risk factors†			
Odds Ratio	1.34	6.86	0.001
95% CI	1.17–1.57	2.84–18.94	
P value	<0.001	<0.001	
Severe Angiographic CAD, further adjusted for risk factors and oxidized LDL‡			
Odds Ratio	1.34	7.02	0.001
95% CI	1.17–1.55	2.53–19.46	
P value	<0.001	<0.001	

Odds Ratios (OR) and 95% confidence intervals (CI) for multiple oxidative stress alleles combined as a score for prediction of angiographic CAD, and change to plasma myeloperoxidase.

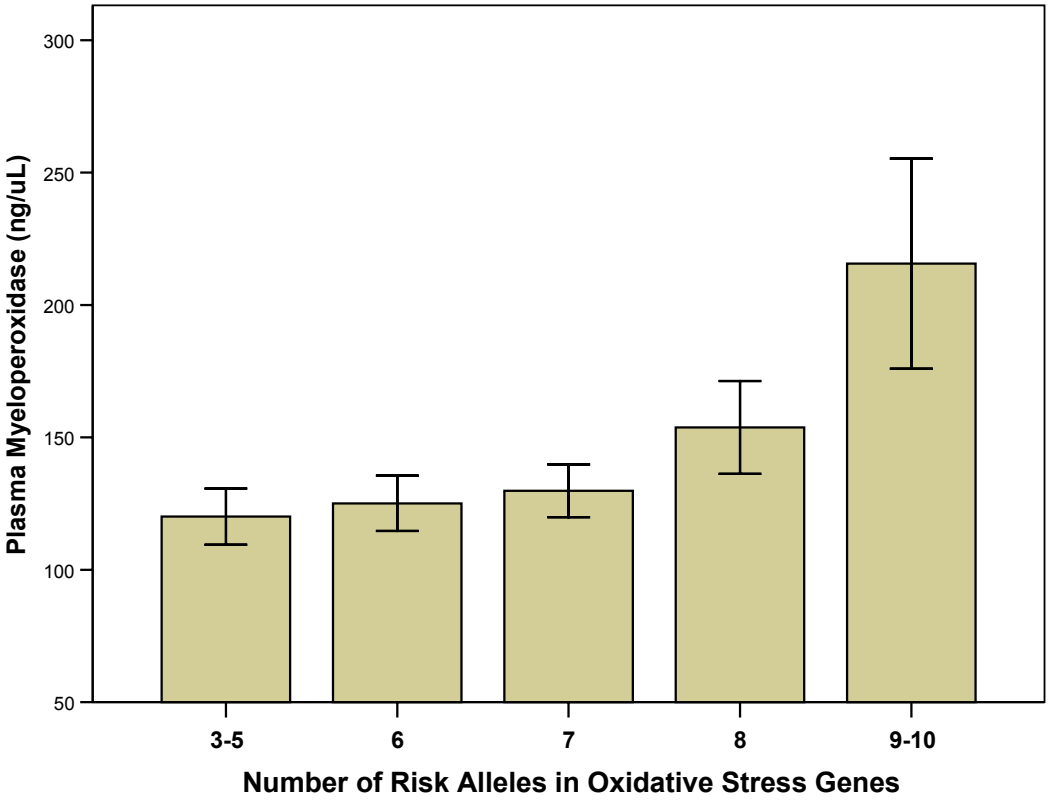
* lesions ≥50% stenosis

† age, sex, bmi, total:HDL cholesterol ratio, smoking, diabetes, and hypertension

‡ further adjusted for log-transformed oxidized LDL levels

Combined SNPs and Biomarkers: Combined oxidative stress SNPs were associated with elevated plasma myeloperoxidase concentrations, with an increase of 14.4 ng/μL (95% CI 4.80–24.0) (p=0.001) per additional risk allele. This relationship persisted following adjustment for potential confounding risk factors (age, sex, smoking, diabetes, hypertension, and total:HDL cholesterol ratio), p=0.004. Effects of oxidative stress SNP scores on plasma myeloperoxidase concentrations are displayed in Figure 5.1.

Figure 5.1 Plasma myeloperoxidase levels for patients by combined oxidative stress SNPs



Data are displayed as mean±standard error. Subjects with 3–5 and 9–10 alleles are combined due to low numbers observed in outlying groups. P=0.003 for linear trend by Tukey-Kramer test, and p<0.001 for comparison between the lowest and highest groups.

Cardiovascular Mortality: After a median follow-up of 13.3 years, there were 223 deaths, 100 of which were cardiovascular deaths, among patients included in these analyses. Plasma myeloperoxidase predicted risk of cardiovascular mortality, with 2.4-fold higher risk per standard deviation increase in plasma myeloperoxidase, independent of adjustment for age, sex, BMI, smoking, diabetes, and total:HDL-cholesterol ratio ($p<0.001$). No other plasma biomarker independently predicted cardiovascular mortality risk.

Combined SNP scores did not predict cardiovascular mortality risk. Haplotypes associated with angiographic CAD at $p<0.05$ were also tested for prediction of cardiovascular mortality. *GPX3* haplotypes did not predict cardiovascular mortality (Appendix 16). However, a haplotype of *PON2* predicted cardiovascular mortality risk (Table 5.3). Carriers of the high risk haplotype had a 2.4-fold risk of mortality compared to the reference haplotype (95% CI 1.57–3.55, $p=3.6x10^{-5}$, $q=0.003$). The relationship persisted with adjustment for factors associated with cardiovascular mortality and oxidative stress (age, sex, BMI, smoking, diabetes, and hypertension) ($p=4.6x10^{-4}$). Also, haplotype carriers had higher myeloperoxidase levels ($p=0.003$), and myeloperoxidase levels differed across all haplotypes ($p=0.014$). Plasma myeloperoxidase concentrations across *PON2* haplotypes are listed in Table 5.4.

Table 5.3 Hazard ratio for cardiovascular mortality by haplotypes of *PON2*

PON2 Haplotype	Hazard Ratio (95% Confidence Interval)	p value*	q value
1-1	--		
1-2	1.39 (0.89–2.16)	0.151	0.584
1-3	1.45 (0.91–2.31)	0.119	0.584
1-4	1.39 (0.79–2.42)	0.251	0.626
1-5	2.37 (1.57–3.55)	**3.61×10^{-5}**	**0.003**
1-6	1.04 (0.40–2.27)	0.849	0.820
	Global	**0.0006**	**0.032**

* P values from Cox survival analyses, with adjustment for age and sex.

Table 5.4 Change in plasma myeloperoxidase by haplotype of *PON2*

PON2 Haplotype	Δ MPO ng/μL	p value*
1-1	--	
1-2	7.31	**0.056**
1-3	−12.19	0.908
1-4	−4.88	0.868
1-5	36.32	**0.003**
1-6	15.26	0.111
	Global	**0.014**

*P value for changes to plasma myeloperoxidase, from linear regression analyses of log transformed myeloperoxidase, adjusted for age and sex.

5.4 Discussion

Several SNPs in oxidative stress genes were nominally associated with coronary atherosclerosis in angiography patients, and a nonsynonymous SNP in *GPX1* was significantly associated with disease. When these SNPs were combined, they had additive predictive value for coronary artery disease, which were independent for risk factors and plasma oxidative stress biomarkers. Combined SNPs were also associated with increased plasma myeloperoxidase, a biomarker of oxidative stress which independently predicts cardiovascular mortality in this angiography cohort.

Polymorphisms contributing to the combined SNP score were in *ALOX5*, *MPO*, *GPX1* and *GPX3*. These SNPs may have moderate influences on oxidative stress pathways, which combine to produce an additive effect on CAD risk, and plasma myeloperoxidase levels. *ALOX5* is believe to promote inflammation and oxidative stress in atherosclerotic lesions, where it is expressed in macrophages and neutrophilic granulocytes (8). Leukotriene B4 synthesis by 5-lipoxygenase induces expression and activation of myeloperoxidase, which subsequently generates potent oxidants (53). An *FLAP* inhibitor tested for MI risk reduction in 191 individuals carrying an at-risk haplotype of *FLAP* showed lowering of plasma myeloperoxidase levels (54), which may have occurred secondary to reduced ALOX5 activity. Replication of our nominally significant findings for ALOX5 in other cohorts is warranted, as are further investigations into biomarkers directly influenced by *ALOX5*, such as plasma leukotriene levels.

Two polymorphisms in *MPO* were included in the combined SNP score. No previous associations have been reported between these SNPs and CAD, and further clinical studies are needed to validate and evaluate the functional importance of these SNPs.

Nonsynonymous SNP Pro197Leu in the *GPX1* gene was associated with a marked increase in angiographic CAD risk within this cohort. *GPX1* exerts an antioxidative role by catalyzing the reduction of hydrogen peroxide and organic peroxides (47). Selenium is present at the active site of glutathione peroxidase as selenocysteine, and a selenium deficient diet results in decreased enzyme activity (48), which predicts cardiovascular events in coronary artery disease patients (49). A comparison of glutathione peroxidase activities across Pro197Leu genotypes in another study revealed the polymorphism may influence how plasma selenium contributes to glutathione peroxidase activity (50). Coronary calcium scores and intima media thickness of carotid arteries are both higher in Japanese type 2 diabetes patients carrying the Pro197Leu variant (23,24), and the nonsynonymous SNP has been associated with coronary disease risk in a Chinese population (51). Results from our study support emerging consensus the Pro197Leu polymorphisms confers a protective phenotype. Cell culture studies have also demonstrate interactions between glutathione peroxidase and 5-lipoxygenase in human monocytes, where glutathione peroxidase acts in a glutathione-independent manner as a 5-lipoxygenase inhibitor (52). Whether meaningful reductions in leukotriene levels result from this effect has not yet been demonstrated.

We observed a relationship between a haplotype in *GPX3* and coronary artery disease. The *GPX3* haplotype extends across the middle of the gene, and the haplotype GGT conferred a reduced risk of disease, which was only nominally significant, and no differences in plasma biomarkers were observed for this haplotype. One haplotype across *PON2* nominally associated with angiographic CAD predicted cardiovascular mortality risk, independent of cardiovascular risk factors and angiographic CAD. Reduced paraoxonase 2 expression in endothelial cells and monocytes/macrophages within carotid lesions versus adjacent tissue and distant vessel sites has

been demonstrated, and oxidative stress marker malonyldialdehyde, a product of fatty acid oxidation, was co-localized to areas of reduced PON2 expression (53). Macrophages from hypercholesterolemic subjects treated with acetylated-LDL and oxidized LDL also show reduced PON2 expression (54). Furthermore, studies of murine monocytes suggest *PON2* deficiency aggravates atherosclerosis (55), whereas adenovirus-mediated over expression reduces plaque area (56).

Without a phenotypic measurement of paraoxonase 2 activity in our subjects, we cannot relate observations regarding the *PON2* haplotype to a specific mechanism of cardiovascular risk. However, elevated myeloperoxidase in risk haplotype carriers suggests they may have an elevated oxidative stress burden. In patients with the risk haplotype, *PON2* function may be compromised, but further studies of *PON2* genotypes are needed to confirm this hypothesis.

This is the first study to combine oxidative stress biomarkers for prediction of coronary artery disease in angiography population. Our findings are consistent with another study investigating functional SNPs in 4 candidate oxidative stress genes (glutamate-cysteine ligase modifier subunit *GCLM*, *PON1*, *CYBA*, and *MPO*) for carotid intima media thickness in a cohort of Japanese type 2 diabetic patients (57). Higher intima media thickness was associated with a promoter polymorphism in *GCLM*, but only weak associations were observed for other SNPs tested. However, a significant relationship between the number of pro-oxidant alleles and intima media thickness was observed. Furthermore, accumulation of pro oxidant alleles influenced serum iso-prostaglandin F2a, a biomarker of oxidative stress.

Strengths and Limitations: Our study has several strengths. We genotyped with a high degree of accuracy a maximally informative subset of tagSNPs (58) across genes related to oxidative

stress and atherosclerosis. Risk factors were characterized at baseline, and follow up time was extensive, with complete ascertainment of cases and attributed causes of mortality. However, there are several limitations to this study that must be considered. Plasma biomarkers were only measured at a single time point, and no information about biomarker changes over time is available. Furthermore, only biomarkers suitable for high-throughput measurement in stored plasma were included, which limits the conclusions we may make about genes not directly related to those markers. Also, although 87% of cohort patients reported Caucasian ethnicity, population stratification may have influenced our results. However, we verified significant findings in the portion of the cohort reporting Caucasian ethnicity (data not shown), which suggests that cohort heterogeneity did not influence findings. Unfortunately, we did not have adequate power to detect OR for CAD in the lower range of expected for complex trait genotype-phenotype associations (i.e. >0.80 or <1.20). Although we followed these patients for >13 years, we observed only 100 cases of cardiovascular mortality, therefore power for detecting associations with cardiovascular mortality was also limited. Finally, we enrolled patients presenting for selective coronary angiography, who were well enough to consent to the baseline assessment. Applicability of our findings to patients with different clinical presentation will have to be established through future studies.

Conclusions: Observations from this study suggest that polymorphisms in oxidative stress genes may influence the pathogenesis of atherosclerosis, and affect plasma oxidative stress levels. Results from this hypothesis-generating study of candidate genes merit further testing in an independent population to confirm the role of oxidative stress pathways in coronary artery disease.

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Chapter 6. Discussion of Findings and Thesis Conclusions

6.1 Review of Topic and Summary

Despite progress in the identification and management of risk factors, cardiovascular disease remains a major cause of morbidity and mortality worldwide, and prevalence continues to increase (1). Both environmental and inherited factors contribute to the development and clinical course of coronary artery disease through complex pathways.

In the present thesis, I evaluated a number of emerging environmental, plasma, and genetic markers for prediction coronary artery disease and cardiovascular mortality in a cohort of coronary angiography patients. Findings from these experiments offer new evidence and inspire new research questions regarding the role of risk factors in coronary artery disease.

6.2 Neighbourhood Socioeconomic Status and Coronary Artery Disease

Neighbourhood socioeconomic status, a disparity marker previously associated with total mortality in patients with coronary artery disease, was tested for association with conventional risk factors and prediction of cardiovascular mortality. Rates of cardiovascular mortality did not differ by neighbourhood socioeconomic status. However, patients were recruited from two major urban teaching hospitals, thus they may have received similar care at baseline despite coming from different neighbourhoods. Non-cardiovascular chronic disease mortality rates significantly increased with neighbourhood level disparity, which may indicate patients from neighbourhoods with lower incomes, education, and employment rates have poorer overall health than patients from less disadvantaged neighbourhoods.

Future studies could explore rates of cardiovascular and non-cardiovascular chronic diseases between neighbourhoods. It has been suggested that patients with chronic diseases may

relocate to disadvantaged neighbourhoods following reductions in income. Although it would be difficult to determine which came first—disadvantaged neighbourhood or chronic disease—a public health study regarding direction of causality could include patient interviews to answer this question. Our analyses did not adjust for individual level socioeconomic status, therefore future studies should compare effects of individual-level socioeconomic variables on the trends observed for neighbourhood socioeconomic status.

Because we observed no association between neighbourhood SES and rates of cardiovascular mortality in our angiography cohort, and only a subset of cohort patients provided postal codes, we did not investigate or compare inflammation or oxidative stress biomarker concentrations across SES quintiles. However, future studies investigating cardiovascular effects of socioeconomic stress in larger cohorts demonstrating clear associations with disease risk should consider the value of biomarkers for assessing the impact of psychosocial factors on the pathogenesis of cardiovascular disease.

6.3 Polymorphisms and Plasma Levels of Interleukin-6 and C-Reactive Protein

Haplotypes across *IL6* and *CRP* genes were tested for association with plasma concentrations of interleukin-6 and C-reactive protein, and for prediction of angiographic coronary artery disease, and cardiovascular mortality. Our results are not suggestive of causality, which is consistent with similar epidemiological studies. In our cohort, plasma concentrations of interleukin-6 and C-reactive protein were strongly correlated, and predicted angiographic disease and cardiovascular mortality. However, haplotypes in the *IL6* gene did not significantly influence plasma interleukin-6 or C-reactive protein levels after adjustment for multiple comparisons, and haplotypes in *CRP* associated with changes in plasma C-reactive protein did not predict disease

or outcome. Finally, polymorphisms in *IL6* and *CRP* that were associated with angiographic coronary artery disease and cardiovascular mortality did not significantly alter plasma biomarker concentrations.

Interleukin-6 and C-reactive protein have been extensively studied with the goal of demonstrating that inflammation is causal to cardiovascular disease. The growing consensus from association and Mendelian randomization analyses suggest that C-reactive protein may not play a causal role in atherosclerosis (2). Instead, confounding factors may lead to elevations in C-reactive protein observed with cardiovascular disease. Even if this is true, C-reactive protein still provides useful information regarding cardiovascular risk, because plasma levels capture information on burden of various risk factors, inflammatory signals including those from dysfunctional adipose tissue, and total inflammatory plaque burden.

However, even if common variations in inflammation genes influence plasma levels only slightly, they may influence plasma levels during states of acute inflammation, or gene expression in sites of atherosclerosis. Additional work is warranted to assess if the predictive utility of C-reactive protein measurement is modified by genotypes in these instances. Studies comparing *CRP* polymorphisms with C-reactive protein concentrations in atherosclerotic plaque fragments, and plasma levels of C-reactive protein at different timepoints, may potentially uncover relationships not detected from single timepoint biomarker studies.

6.4 Plasma Oxidative Stress Biomarkers

Oxidative stress has been implicated in progression of atherosclerotic plaques from stable to unstable states (3), and biomarkers of oxidative stress may be clinically useful for short- and long- term risk prediction in patients with coronary artery disease. The relative and additive

value of plasma oxidative stress biomarkers for predicting cardiovascular disease and mortality, compared to conventional risk factors, was explored in Chapter 4. Patients with angiographic disease had higher baseline myeloperoxidase and nitrotyrosine levels than patients without disease, however only myeloperoxidase independently predicted angiographic coronary artery disease. It was surprising to find that antioxidant levels were higher in patients with disease, however this relationship did not persist after adjustment for sex and smoking status. Smokers had higher antioxidant levels, which is consistent with results reported elsewhere (4). Female patients also had lower antioxidant levels, which has also been reported previously (5).

Although patients who died from cardiovascular causes had higher nitrotyrosine, only myeloperoxidase independently predicted cardiovascular mortality. Myeloperoxidase and C-reactive protein had complementary predictive value, and using both markers together significantly improved patient risk discrimination beyond risk assessment from conventional cardiovascular risk factors.

Unlike C-reactive protein, myeloperoxidase is not on the immediate horizon for widespread clinical use, however its favorable comparison in this study with C-reactive protein for risk prediction and risk category reclassification suggests it may be a candidate for future clinical applications. Using biomarkers for cardiovascular disease risk estimation in the clinical setting requires that biomarkers have appropriate cost, reproducibility of associations, and potential to incrementally improve existing risk parameters (6). Although results from this study indicate myeloperoxidase has value in risk prediction among angiography patients, whether it would be a good candidate for widespread clinical application remains to be established.

If the clinical utility of plasma myeloperoxidase concentration is demonstrated, it could become a useful oxidative stress biomarker for randomized-controlled trials investigating

antioxidants such as flavonols and isoflavonoids for primary and secondary cardiovascular risk reduction. Further studies are warranted to explore the range of myeloperoxidase values in healthy populations, and in patients with confounding disease states associated with cardiovascular disease, such as renal insufficiency, metabolic syndrome, and heart failure.

6.5 Polymorphisms in Oxidative Stress Genes

Polymorphisms in oxidative stress genes which associate with coronary artery disease may implicate key oxidative stress pathways in atherosclerosis, especially if changes to oxidative stress phenotypes are observed. In Chapter 5, polymorphisms in candidate oxidative stress genes were explored for association with angiographic disease, and changes to plasma biomarker concentrations. Combined oxidative stress SNPs predicted coronary artery disease, independent of cardiovascular risk factors and plasma oxidative stress. Combined SNPs were also associated with increased plasma myeloperoxidase concentrations, and the observation was linear across combined SNP scores.

This hypothesis-generating candidate gene study yielded a large quantity of information regarding oxidative stress pathways which may guide future research questions, both basic and clinical. The following is a selection of future studies that could further build upon the observations of this thesis and elucidate the role of oxidative stress pathways in cardiovascular disease.

1) Glutathione peroxidase activity could be compared in patients with different alleles of *GPX1* SNP Pro197Leu, which was associated with increased cardiovascular risk in this cohort. No high throughput assay for assessment of intracellular glutathione peroxidase activity was available for use in our plasma samples, however further validation studies in another cohort

could include glutathione peroxidase measurements from whole blood samples. Measurement of the reduced glutathione ratio by liquid chromatography on a subset of samples, or pooled samples of different Pro197Leu genotypes, could also be informative. A similar validation procedure should be followed for observed associations in *GPX3*, with measurement of plasma glutathione peroxidase activity, or related metabolites.

2) One SNP in *ALOX5* was included in our combined SNP score, and 5-lipoxygenase has been associated with oxidative stress pathways in cardiovascular disease. Measurement of plasma leukotriene levels across *ALOX5* genotypes could help elucidate the relationship between this SNP and coronary artery disease.

3) A *PON2* haplotype predicted cardiovascular mortality and this finding should be validated in another cohort. The importance of paraoxonase 2 in oxidative stress is slowly emerging, and this finding suggests prognosis in coronary artery disease patients may be influenced by variations in the *PON2* gene. Further investigations of this haplotype are warranted to validate this finding, and to determine how it influences paraoxonase 2 activity, and cardiovascular risk. Because reduced *PON2* mRNA and protein levels have been demonstrated in atherosclerotic plaques (7), comparison of *PON2* expression in lesions from individuals with and without the risk haplotype would also help confirm the implications of the *PON2* haplotype on atherosclerosis.

Validation of the reported findings for candidate oxidative stress genes could be pursued using databases of human genome variation that have recently become publicly available. However, due to the numerous factors that influence oxidative stress and inflammation, any cohort studied used for validation would need to be well phenotyped. Phenotyping by multiple

biomarker measurements at different time points, and across confounding risk factors, would be ideal to capture the dynamics of oxidative stress and inflammation pathways. These dynamics may be more influenced by heritability than single timepoint measurements. At the present time, no such publicly available cohort currently exists.

6.6 Limitations to the Study Cohort

Limitations to each study are summarized in the corresponding chapters. However, it is important to mention here that observations made in this thesis could have been influenced by nature of the cohort. While selective coronary angiography has been the gold standard for the assessment of coronary atherosclerosis presence and severity, there is increasing evidence that its sensitivity is low, because diagnosis of percent stenosis replies on comparison with adjacent segments of the artery, and there is a possibility of underestimation due to diffuse development of coronary artery plaques (8). In addition, plaque expansion by eccentric modification may leave the luminal space unchanged and disease undetectable by angiography (9). However, long-term studies of patients with chest pain and normal angiograms have shown no greater cardiovascular morbidity than individuals not referred for angiography (10).

Selective coronary angiography patients with <20% stenosis were considered the non-disease control group, however these patients had still been referred for coronary angiography so they were therefore not *healthy* controls. That only myeloperoxidase was independently predictive of coronary artery disease, despite the previous evidence implicating the biomarkers we selected with disease, suggests the control group may not be representative of a disease-free population. Replication of our findings in a population with matched healthy controls would be helpful, and this would also indicate the range of plasma biomarkers for healthy individuals. As

the overlap between subjects with and without a condition can influence the value of a predictive test, this would be valuable information. Further comparisons of our findings with a control group of significantly older adults with no coronary artery disease could also offer important validation of the genetic and plasma marker associations with cardiovascular risk we observed.

6.7 Conclusions

Atherosclerosis is a multifactorial disease, and although the impact of traditional risk factors has long been demonstrated, emerging risk factors may offer complementary and additive value to patient risk assessment, thereby optimizing the use of diagnostic and therapeutic efforts for higher risk individuals. These emerging markers may be environmental risk factors, such as neighbourhood-level socioeconomic indices, or they may be markers of disease processes, like C-reactive protein.

Although oxidative stress is associated with all stages of atherosclerotic lesion development (3), results from antioxidant trials have inspired skepticism about whether oxidative stress plays a causal role in cardiovascular disease (4). As a plasma marker of oxidative stress, myeloperoxidase identifies patients with coronary artery disease, and predicts risk of cardiovascular mortality. Also, combined polymorphisms in oxidative stress genes predicted coronary artery disease and elevated plasma myeloperoxidase levels. If validated, these associations could implicate oxidative stress has a causal role in cardiovascular disease.

Further studies are warranted to test these emerging risk factors for disease prediction and prognostic value, with the longer term objectives of improving prevention, detection and treatment of cardiovascular diseases.

6.8 References

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Appendices

Appendix 1. Ethics Approval Form

UBC-Providence Health Care Research Institute
Office of Research Services
11th Floor Hornby Site - SPH
c/o 1081 Burrard St.
Vancouver, BC V6Z 1Y6
Tel: (604) 806-8567
Fax: (604) 806-8568

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: ANNUAL RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC-PHC REB NUMBER:
John S. Hill	PHCRI	H05-50045

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Providence Health Care	St. Paul's Hospital

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):

N/A

SPONSORING AGENCIES:

Heart and Stroke Foundation of British Columbia and Yukon

PROJECT TITLE:
Evaluation of the Relative Predictive Value of Biomarkers of Oxidative Stress for Cardiovascular Mortality

EXPIRY DATE OF THIS APPROVAL: February 15, 2010

APPROVAL DATE: February 15, 2009

CERTIFICATION:

1. The membership of the UBC-PHC REB complies with the membership requirements for research ethics boards defined in Part C Division 5 of the Food and Drug Regulations of Canada.
2. The UBC-PHC REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC-PHC REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the principal investigator named above at the specified research site(s). This review of the UBC-PHC REB have been documented in writing.

The UBC-PHC Research Ethics Board Chair or Associate Chair, has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal.

Approval of the UBC-PHC Research Ethics Board or Associate Chair, verified by the signature of one of the following:

Dr. Kuo-Hsing Kuo, Chair

Dr. J. Kernahan, Associate Chair

Dr. I. Fedoroff, Associate Chair

Appendix 2. Probe Sequences for APEX Genotyping

SNP and Probe ID	Probe Sequence
ALOX5	
rs3780897 LEFT	GGCCCATGCTAGCTCACAGGCGGTG
rs3780897 RIGHT	GGCCCATGCTAGCTCACAGGCGGTGC
rs3780897_1 LEFT	GGCCCATGCTAGCTCACAGGCGGTGT
rs3780897_1 RIGHT	AGGGGGTTGCAGGGCACCAGGCCCC
rs3780897_2 LEFT	GGGGGTTGCAGGGCACCAGGCCCCG
rs3780897_2 RIGHT	GGGGGTTGCAGGGCACCAGGCCCCA
rs3780901 LEFT	GTCACCTGCAGGCTCCTTGTTCATC
rs3780901 RIGHT	ACTGTCTTTTTCTCCTCATGTGTCA
rs3780901_1 LEFT	TCACCTGCAGGCTCCTTGTTCATCC
rs3780901_1 RIGHT	CTGTCTTTTTCTCCTCATGTGTCAG
rs3780901_2 LEFT	TCACCTGCAGGCTCCTTGTTCATCT
rs3780901_2 RIGHT	CTGTCTTTTTCTCCTCATGTGTCAA
rs11239523 LEFT	GATGGAGAGATAGATGAGAAATTGA
rs11239523 RIGHT	ATGGAGAGATAGATGAGAAATTGAC
rs11239523_1 LEFT	ATGGAGAGATAGATGAGAAATTGAT
rs11239523_1 RIGHT	CATCCATGAATCCATCCATCCGTAT
rs11239523_2 LEFT	CATCCATGAATCCATCCATCCGTATG
rs11239523_2 RIGHT	CATCCATGAATCCATCCATCCGTATA
rs1487562 LEFT	CAGAGGGTCCTGGGGTTGAGGCTGT
rs1487562 RIGHT	TCGGTAACACAACTGTCCTCGCCAC
rs1487562_1 LEFT	AGAGGGTCCTGGGGTTGAGGCTGTC
rs1487562_1 RIGHT	CGGTAACACAACTGTCCTCGCCACG
rs1487562_2 LEFT	AGAGGGTCCTGGGGTTGAGGCTGTT
rs1487562_2 RIGHT	CGGTAACACAACTGTCCTCGCCACA
rs3780914 LEFT	CCACCGCAGGGCCCTTTACCCCCTC
rs3780914 RIGHT	AGTGACTTTGGAATCAGAAGAGCCT
rs3780914_1 LEFT	CACCGCAGGGCCCTTTACCCCCTCC
rs3780914_1 RIGHT	GTGACTTTGGAATCAGAAGAGCCTG
rs3780914_2 LEFT	CACCGCAGGGCCCTTTACCCCCTCT
rs3780914_2 RIGHT	GTGACTTTGGAATCAGAAGAGCCTA
rs1565096 LEFT	TTTCCCCAGGCCTGATTTTTGGTCC
rs1565096 RIGHT	ATGTGTCCAAATCAGGCTCAGCAGA
rs1565096_1 LEFT	TTCCCCAGGCCTGATTTTTGGTCCA
rs1565096_1 RIGHT	TGTGTCCAAATCAGGCTCAGCAGAT
rs1565096_2 LEFT	TTCCCCAGGCCTGATTTTTGGTCCG
rs1565096_2 RIGHT	TGTGTCCAAATCAGGCTCAGCAGAC
rs2291427 LEFT	CTGGAGAGATGTTCTCAGAGTCAGT
rs2291427 RIGHT	AGCTGTTTTCTTCCTTTAGGGGCAT
rs2291427_1 LEFT	TGGAGAGATGTTCTCAGAGTCAGTA
rs2291427_1 RIGHT	GCTGTTTTCTTCCTTTAGGGGCATT
rs2291427_2 LEFT	TGGAGAGATGTTCTCAGAGTCAGTG
rs2291427_2 RIGHT	GCTGTTTTCTTCCTTTAGGGGCATC
rs745986 LEFT	ACCTCACTTCGAGTCATAACCCTTC

SNP and Probe ID	Probe Sequence
rs745986 RIGHT	TTCCAGGTTTAAACTGAGAGCAAGC
rs745986_1 LEFT	CCTCACTTCGAGTCATAACCCTTCA
rs745986_1 RIGHT	TCCAGGTTTAAACTGAGAGCAAGCT
rs745986_2 LEFT	CCTCACTTCGAGTCATAACCCTTCG
rs745986_2 RIGHT	TCCAGGTTTAAACTGAGAGCAAGCC
rs2115819 LEFT	TGTGTAACACTGGGATGGAAAGGGT
rs2115819 RIGHT	TTGGGTTTCCTCCTTTGCTTAAGAA
rs2215819_1 LEFT	GTGTAACACTGGGATGGAAAGGGTC
rs2215819_1 RIGHT	TGGGTTTCCTCCTTTGCTTAAGAAG
rs2215819_2 LEFT	GTGTAACACTGGGATGGAAAGGGTT
rs2215819_2 RIGHT	TGGGTTTCCTCCTTTGCTTAAGAAA
rs7894352 LEFT	GAGACAAGTCTCAATCATTTTAGGA
rs7894352 RIGHT	GCGTCCTTAACTTTCGCAAATAAAT
rs7894352_1 LEFT	AGACAAGTCTCAATCATTTTAGGAA
rs7894352_1 RIGHT	CGTCCTTAACTTTCGCAAATAAATT
rs7894352_2 LEFT	AGACAAGTCTCAATCATTTTAGGAG
rs7894352_2 RIGHT	CGTCCTTAACTTTCGCAAATAAATC
rs12762303 LEFT	GGGGTGGCCTCCTCCAGGAAGCCCT
rs12762303 RIGHT	GAAGAGCGACCCATGAGAGAATCAG
rs12762303_1 LEFT	GGGTGGCCTCCTCCAGGAAGCCCTC
rs12762303_1 RIGHT	AAGAGCGACCCATGAGAGAATCAGG
rs12762303_2 LEFT	GGGTGGCCTCCTCCAGGAAGCCCTT
rs12762303_2 RIGHT	AAGAGCGACCCATGAGAGAATCAGA
rs10900213 LEFT	CTATGAGACAGCATCAAAGTCACTC
rs10900213 RIGHT	GCAGCCCCTAAACCAGAGCAGGCTC
rs10900213_1 LEFT	TATGAGACAGCATCAAAGTCACTCG
rs10900213_1 RIGHT	CAGCCCCTAAACCAGAGCAGGCTCC
rs10900213_2 LEFT	TATGAGACAGCATCAAAGTCACTCT
rs10900213_2 RIGHT	CAGCCCCTAAACCAGAGCAGGCTCA
rs10751383 LEFT	TTGTGAGTGTGTCTGCATCACTCAG
rs10751383 RIGHT	CCTGGTCCTGATCCCGATCCCGGCT
rs10751383_1 LEFT	TGTGAGTGTGTCTGCATCACTCAGA
rs10751383_1 RIGHT	CTGGTCCTGATCCCGATCCCGGCTT
rs10751383_2 LEFT	TGTGAGTGTGTCTGCATCACTCAGC
rs10751383_2 RIGHT	CTGGTCCTGATCCCGATCCCGGCTG
rs7089063 LEFT	GAAAAAAAAAAAAAAGCTGCACATT
rs7089063 RIGHT	AAATTTGCATTTCCCTGATAACATA
rs7089063_1 LEFT	AAAAAAAAAAAAAAGCTGCACATTA
rs7089063_1 RIGHT	AATTTGCATTTCCCTGATAACATAT
rs7089063_2 LEFT	AAAAAAAAAAAAAAGCTGCACATTC
rs7089063_2 RIGHT	AATTTGCATTTCCCTGATAACATAG
rs3824612 LEFT	CCCGTTTCTGCTCTCTACCCTACAA
rs3824612 RIGHT	AATTCAGAGCAAAAAAGAAGGGAGA
rs3824612_1 LEFT	CCGTTTCTGCTCTCTACCCTACAAC
rs3824612_1 RIGHT	ATTCAGAGCAAAAAAGAAGGGAGAG
rs3824612_2 LEFT	CCGTTTCTGCTCTCTACCCTACAAT
rs3824612_2 RIGHT	ATTCAGAGCAAAAAAGAAGGGAGAA
rs11239528 LEFT	CTATGATTTCAGTCCTAATGAAAAC
rs11239528 RIGHT	GTACCTGGGTCCTGGGCCAACATAC

SNP and Probe ID	Probe Sequence
rs11239528_1 LEFT	TATGATTTCAGTCCTAATGAAAACA
rs11239528_1 RIGHT	TACCTGGGTCCTGGGCCAACATACT
rs11239528_2 LEFT	TATGATTTCAGTCCTAATGAAAACG
rs11239528_2 RIGHT	TACCTGGGTCCTGGGCCAACATACC
FLAP	
rs11147439 LEFT	ACTCTCCAGGGAGTGAGGACTGTGC
rs11147439 RIGHT	CTAAGAGGGTGATCACTGTATGCTG
rs11147439_1 LEFT	CTCTCCAGGGAGTGAGGACTGTGCA
rs11147439_1 RIGHT	TAAGAGGGTGATCACTGTATGCTGT
rs11147439_2 LEFT	CTCTCCAGGGAGTGAGGACTGTGCC
rs11147439_2 RIGHT	TAAGAGGGTGATCACTGTATGCTGG
rs4072653 LEFT	TGGAGGGCACAGGCAGAAAGGCCAC
rs4072653 RIGHT	TTAAACAGTTCATCATGGGACTGAG
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rs4072653_1 RIGHT	TAAACAGTTCATCATGGGACTGAGC
rs4072653_2 LEFT	GGAGGGCACAGGCAGAAAGGCCACA
rs4072653_2 RIGHT	TAAACAGTTCATCATGGGACTGAGT
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rs4468448 RIGHT	TTTCAGAGCCTTCAAAGCTCAGGGA
rs4468448_1 LEFT	CTGACTAGGACCTCTGATTCTTCTC
rs4468448_1 RIGHT	TTCAGAGCCTTCAAAGCTCAGGGAG
rs4468448_2 LEFT	CTGACTAGGACCTCTGATTCTTCTT
rs4468448_2 RIGHT	TTCAGAGCCTTCAAAGCTCAGGGAA
rs4769060 LEFT	GGGTTTTGAGGTCTAGGCTCTCTTC
rs4769060 RIGHT	TCATTGAACTTATTTCAAACCCAAG
rs4769060_1 LEFT	GGTTTTGAGGTCTAGGCTCTCTTCA
rs4769060_1 RIGHT	CATTGAACTTATTTCAAACCCAAGT
rs4769060_2 LEFT	GGTTTTGAGGTCTAGGCTCTCTTCG
rs4769060_2 RIGHT	CATTGAACTTATTTCAAACCCAAGC
rs10507391 LEFT	GTCCAAGCCTCTCTTTGCAATTCTA
rs10507391 RIGHT	TCTATGGTTGCAACATTGAGGTTAA
rs10507391_1 LEFT	TCCAAGCCTCTCTTTGCAATTCTAA
rs10507391_1 RIGHT	CTATGGTTGCAACATTGAGGTTAAT
rs10507391_2 LEFT	TCCAAGCCTCTCTTTGCAATTCTAT
rs10507391_2 RIGHT	CTATGGTTGCAACATTGAGGTTAAA
rs4073261 LEFT	ACTTCTCACTCCCTCCTCCCAATTT
rs4073261 RIGHT	GGGGGACTGACTAATCCCGCAAATA
rs4073261_1 LEFT	CTTCTCACTCCCTCCTCCCAATTTC
rs4073261_1 RIGHT	GGGGACTGACTAATCCCGCAAATAG
rs4073261_2 LEFT	CTTCTCACTCCCTCCTCCCAATTTT
rs4073261_2 RIGHT	GGGGACTGACTAATCCCGCAAATAA
rs3885907 LEFT	AACTTATTTAACTTCTCTGGCCCTC
rs3885907 RIGHT	CACTCCACTGCTCAGTGAAGGAAAC
rs3885907_1 LEFT	ACTTATTTAACTTCTCTGGCCCTCA
rs3885907_1 RIGHT	ACTCCACTGCTCAGTGAAGGAAACT
rs3885907_2 LEFT	ACTTATTTAACTTCTCTGGCCCTCC
rs3885907_2 RIGHT	ACTCCACTGCTCAGTGAAGGAAACG
rs4076128 LEFT	GTGCAGCATATGCATAATTACGGAG

SNP and Probe ID	Probe Sequence
rs4076128 RIGHT	CCCACCCATCCCCTGCTCCCAGCGC
rs4076128_1 LEFT	TGCAGCATATGCATAATTACGGAGA
rs4076128_1 RIGHT	CCACCCATCCCCTGCTCCCAGCGCT
rs4076128_2 LEFT	TGCAGCATATGCATAATTACGGAGG
rs4076128_2 RIGHT	CCACCCATCCCCTGCTCCCAGCGCC
rs9671124 LEFT	TTAAATTCTGAAGGCCTGAGGTGGG
rs9671124 RIGHT	ACATGTTAGAAATGCAAATGTTCAC
rs9671124_1 LEFT	TAAATTCTGAAGGCCTGAGGTGGGC
rs9671124_1 RIGHT	CATGTTAGAAATGCAAATGTTCACG
rs9671124_2 LEFT	TAAATTCTGAAGGCCTGAGGTGGGT
rs9671124_2 RIGHT	CATGTTAGAAATGCAAATGTTCACA
rs4769055 LEFT	CCAGAATGGTAAGGAAAGCCCTTCA
rs4769055 RIGHT	AATCTCCCCTTCTGTTCTTCCCTGA
rs4769055_1 LEFT	CAGAATGGTAAGGAAAGCCCTTCAA
rs4769055_1 RIGHT	ATCTCCCCTTCTGTTCTTCCCTGAT
rs4769055_2 LEFT	CAGAATGGTAAGGAAAGCCCTTCAC
rs4769055_2 RIGHT	ATCTCCCCTTCTGTTCTTCCCTGAG
CYBA	
rs4782308 LEFT	CTGTGAGCCCTGCCTGGGCCCCGCA
rs4782308 RIGHT	TCCTGCTCTGGGGTCTCTCCGGGGG
rs4782308_1 LEFT	TGTGAGCCCTGCCTGGGCCCCGCAA
rs4782308_1 RIGHT	CCTGCTCTGGGGTCTCTCCGGGGGT
rs4782308_2 LEFT	TGTGAGCCCTGCCTGGGCCCCGCAG
rs4782308_2 RIGHT	CCTGCTCTGGGGTCTCTCCGGGGGC
rs12709102 LEFT	GGGTTAATGAGGAAATGCAAGGAAA
rs12709102 RIGHT	TTTGGCGTGTCTAGTGTGTCGGGTC
rs12709102_1 LEFT	GGTTAATGAGGAAATGCAAGGAAAT
rs12709102_1 RIGHT	TTGGCGTGTCTAGTGTGTCGGGTCA
rs12709102_2 LEFT	GGTTAATGAGGAAATGCAAGGAAAC
rs12709102_2 RIGHT	TTGGCGTGTCTAGTGTGTCGGGTCG
rs3794624 LEFT	GGGGCTGAACCGCCTCTTCCCCTGC
rs3794624 RIGHT	GACTCCGCGAAGGAGGAAGTTGCAG
rs3794624_1 LEFT	GGGCTGAACCGCCTCTTCCCCTGCA
rs3794624_1 RIGHT	ACTCCGCGAAGGAGGAAGTTGCAGT
rs3794624_2 LEFT	GGGCTGAACCGCCTCTTCCCCTGCG
rs3794624_2 RIGHT	ACTCCGCGAAGGAGGAAGTTGCAGC
rs4673 LEFT	TCCTCCCTCCCCCAGGGGACAGAAG
rs4673 RIGHT	AACAGCTTCACCACGGCGGTCATGT
rs4673_1 LEFT	CCTCCCTCCCCCAGGGGACAGAAGC
rs4673_1 RIGHT	ACAGCTTCACCACGGCGGTCATGTG
rs4673_2 LEFT	CCTCCCTCCCCCAGGGGACAGAAGT
rs4673_2 RIGHT	ACAGCTTCACCACGGCGGTCATGTA
GPX1	
rs1050450 LEFT	AGGAGGGGCGCCCTAGGCACAGCTG
rs1050450 RIGHT	ATCGAAGCCCTGCTGTCTCAAGGGC
rs1050450_1 LEFT	GGAGGGGCGCCCTAGGCACAGCTGG
rs1050450_1 RIGHT	TCGAAGCCCTGCTGTCTCAAGGGCC

SNP and Probe ID	Probe Sequence
rs1050450_2 LEFT	GGAGGGGCGCCCTAGGCACAGCTGA
rs1050450_2 RIGHT	TCGAAGCCCTGCTGTCTCAAGGGCT
GPX3	
rs3763012 LEFT	TAATATTAATAAGTGCTTATTAGGT
rs3763012 RIGHT	TTAAAAAGCACTTATTAATATTAAG
rs3763012_1 LEFT	AATATTAATAAGTGCTTATTAGGTG
rs3763012_1 RIGHT	TAAAAAGCACTTATTAATATTAAGC
rs3763012_2 LEFT	AATATTAATAAGTGCTTATTAGGTA
rs3763012_2 RIGHT	TAAAAAGCACTTATTAATATTAAGT
rs8177447 LEFT	CCTCAAGCAAGGTTGACACTCCTCT
rs8177447 RIGHT	AGGACAGGAGTTCTAGAGCAGGGAT
rs8177447_1 LEFT	CTCAAGCAAGGTTGACACTCCTCTC
rs8177447_1 RIGHT	GGACAGGAGTTCTAGAGCAGGGATG
rs8177447_2 LEFT	CTCAAGCAAGGTTGACACTCCTCTT
rs8177447_2 RIGHT	GGACAGGAGTTCTAGAGCAGGGATA
rs3792796 LEFT	GAGCCCCAGCAAAGGGGGTCACATG
rs3792796 RIGHT	GCTGGGCTTTGCTGTGTCTCTGATG
rs3792796_1 LEFT	AGCCCCAGCAAAGGGGGTCACATGG
rs3792796_1 RIGHT	CTGGGCTTTGCTGTGTCTCTGATGC
rs3792796_2 LEFT	AGCCCCAGCAAAGGGGGTCACATGC
rs3792796_2 RIGHT	CTGGGCTTTGCTGTGTCTCTGATGG
rs4958872 LEFT	AAAGAGTGACAACTGCTGACGTGCA
rs4958872 RIGHT	AGAAACCAGCAGTTACTAGGATCCC
rs4958872_1 LEFT	AAGAGTGACAACTGCTGACGTGCAC
rs4958872_1 RIGHT	GAAACCAGCAGTTACTAGGATCCCG
rs4958872_2 LEFT	AAGAGTGACAACTGCTGACGTGCAT
rs4958872_2 RIGHT	GAAACCAGCAGTTACTAGGATCCCA
HMOX1	
rs9607267 LEFT	CAGGAAGGAGAATTGTGCCCTGTAG
rs9607267 RIGHT	CTGGCTTTTGACCTCTGCGTAACCA
rs9607267_1 LEFT	AGGAAGGAGAATTGTGCCCTGTAGC
rs9607267_1 RIGHT	TGGCTTTTGACCTCTGCGTAACCAG
rs9607267_2 LEFT	AGGAAGGAGAATTGTGCCCTGTAGT
rs9607267_2 RIGHT	TGGCTTTTGACCTCTGCGTAACCAA
rs2071748 LEFT	ACCTGAGCCAGACATCACCCTGGCT
rs2071748 RIGHT	GCTATCTGTGAGACATGGAACACCG
rs2071748_1 LEFT	CCTGAGCCAGACATCACCCTGGCTA
rs2071748_1 RIGHT	CTATCTGTGAGACATGGAACACCGT
rs2071748_2 LEFT	CCTGAGCCAGACATCACCCTGGCTG
rs2071748_2 RIGHT	CTATCTGTGAGACATGGAACACCGC
rs2071749 LEFT	TCTTAGACTTATAAGGCTTGAGTGA
rs2071749 RIGHT	GCTGTGGGGACTTTACTGCTGTAAA
rs2071749_1 LEFT	CTTAGACTTATAAGGCTTGAGTGAA
rs2071749_1 RIGHT	CTGTGGGGACTTTACTGCTGTAAAT
rs2071749_2 LEFT	CTTAGACTTATAAGGCTTGAGTGAG
rs2071749_2 RIGHT	CTGTGGGGACTTTACTGCTGTAAAC

SNP and Probe ID	Probe Sequence
MPO	
rs2333227 LEFT	AACTCCTGACCTCAAGTGATCCACC
rs2333227 RIGHT	TCCCAGCACTTTGGGAGGCTGAGGC
rs2333227_1 LEFT	ACTCCTGACCTCAAGTGATCCACCC
rs2333227_1 RIGHT	CCCAGCACTTTGGGAGGCTGAGGCG
rs2333227_2 LEFT	ACTCCTGACCTCAAGTGATCCACCT
rs2333227_2 RIGHT	CCCAGCACTTTGGGAGGCTGAGGCA
rs2071409 LEFT	CCTCGTTCTCCCACCAAAACCTGCA
rs2071409 RIGHT	GGCACAGTGTCCATGGGTGTTCCCC
rs2071409_1 LEFT	CTCGTTCTCCCACCAAAACCTGCAT
rs2071409_1 RIGHT	GCACAGTGTCCATGGGTGTTCCCCA
rs2071409_2 LEFT	CTCGTTCTCCCACCAAAACCTGCAG
rs2071409_2 RIGHT	GCACAGTGTCCATGGGTGTTCCCCC
rs2759 LEFT	CGGGGATATGAGTTGGACATGAAGA
rs2759 RIGHT	CATCACCACCGTGTCTAAGAACAAC
rs2759_1 LEFT	GGGGATATGAGTTGGACATGAAGAT
rs2759_1 RIGHT	ATCACCACCGTGTCTAAGAACAACA
rs2759_2 LEFT	GGGGATATGAGTTGGACATGAAGAC
rs2759_2 RIGHT	ATCACCACCGTGTCTAAGAACAACG
rs2107545 LEFT	CCAAAAGCCTCTAAAATTCTAGAGC
rs2107545 RIGHT	TGGTCGAGTGGCTCTTTGTGAATCT
rs2107545_1 LEFT	CAAAAGCCTCTAAAATTCTAGAGCC
rs2107545_1 RIGHT	GGTCGAGTGGCTCTTTGTGAATCG
rs2107545_2 LEFT	CAAAAGCCTCTAAAATTCTAGAGCT
rs2107545_2 RIGHT	GGTCGAGTGGCTCTTTGTGAATCA
NOS3	
rs1800783 LEFT	GCTTAATATGGAAGCCAGACTTGGG
rs1800783 RIGHT	CTACCATGCTGGAGGAGACAACAGA
rs1800783_1 LEFT	CTTAATATGGAAGCCAGACTTGGGA
rs1800783_1 RIGHT	TACCATGCTGGAGGAGACAACAGAT
rs1800783_2 LEFT	CTTAATATGGAAGCCAGACTTGGGT
rs1800783_2 RIGHT	TACCATGCTGGAGGAGACAACAGAA
rs1808593 LEFT	CTATAGCTCCCAGAGCCAGAGCTGG
rs1808593 RIGHT	AAGCCACAGGGCCAGCCGGTTTGAT
rs1808593_1 LEFT	TATAGCTCCCAGAGCCAGAGCTGGG
rs1808593_1 RIGHT	AGCCACAGGGCCAGCCGGTTTGATC
rs1808593_2 LEFT	TATAGCTCCCAGAGCCAGAGCTGGT
rs1808593_2 RIGHT	AGCCACAGGGCCAGCCGGTTTGATA
rs1799983 LEFT	CCCTGCTGCTGCAGGCCCCAGATGA
rs1799983 RIGHT	GGGGCAGAAGGAAGAGTTCTGGGGG
rs1799983_1 LEFT	CCTGCTGCTGCAGGCCCCAGATGAG
rs1799983_1 RIGHT	GGGCAGAAGGAAGAGTTCTGGGGGC
rs1799983_2 LEFT	CCTGCTGCTGCAGGCCCCAGATGAT
rs1799983_2 RIGHT	GGGCAGAAGGAAGAGTTCTGGGGGA
rs3918188 LEFT	TGGGAGCAAGGCACACGTACAAGGG
rs3918188 RIGHT	TCTGGGAACAGGTGCTCTCTCAAAC
rs3918188_1 LEFT	GGGAGCAAGGCACACGTACAAGGGA
rs3918188_1 RIGHT	CTGGGAACAGGTGCTCTCTCAAACT

SNP and Probe ID	Probe Sequence
rs3918188_2 LEFT	GGGAGCAAGGCACACGTACAAGGGC
rs3918188_2 RIGHT	CTGGGAACAGGTGCTCTCTCAAACG
rs7830 LEFT	ACTCCCTTCAGGCAGTCCTTTAGTC
rs7830 RIGHT	ACATTGAGAGCAAAGGTGAGGCTGG
rs7830_1 LEFT	CTCCCTTCAGGCAGTCCTTTAGTCA
rs7830_1 RIGHT	CATTGAGAGCAAAGGTGAGGCTGGT
rs7830_2 LEFT	CTCCCTTCAGGCAGTCCTTTAGTCC
rs7830_2 RIGHT	CATTGAGAGCAAAGGTGAGGCTGGG
rs179983 LEFT	GAAGCTTTCTGGCTTATTTGAACGC
rs179983 RIGHT	TATCTAAACAGTGTACTATGTGTTC
rs179983_1 LEFT	AAGCTTTCTGGCTTATTTGAACGCG
rs179983_1 RIGHT	ATCTAAACAGTGTACTATGTGTTCC
rs179983_2 LEFT	AAGCTTTCTGGCTTATTTGAACGCA
rs179983_2 RIGHT	ATCTAAACAGTGTACTATGTGTTCT
PON1	
rs854560 LEFT	GCCAGTCCATTAGGCAGTATCTCCA
rs854560 RIGHT	TCTGGCAGAAACTGGCTCTGAAGAC
rs854560_1 LEFT	CCAGTCCATTAGGCAGTATCTCCAA
rs854560_1 RIGHT	CTGGCAGAAACTGGCTCTGAAGACT
rs854560_2 LEFT	CCAGTCCATTAGGCAGTATCTCCAT
rs854560_2 RIGHT	CTGGCAGAAACTGGCTCTGAAGACA
rs662 LEFT	TAAACCCAAATACATCTCCCAGGAT
rs662 RIGHT	CACTATTTTCTTGACCCCTACTTAC
rs662_1 LEFT	AAACCCAAATACATCTCCCAGGATT
rs662_1 RIGHT	ACTATTTTCTTGACCCCTACTTACA
rs662_2 LEFT	AAACCCAAATACATCTCCCAGGATC
rs662_2 RIGHT	ACTATTTTCTTGACCCCTACTTACG
rs705379 LEFT	TAGCTGCGGACCCGGCGGGGAGGGG
rs705379 RIGHT	GGGGCAGCGCCGATTGGCCCGCCCC
rs705379_1 LEFT	AGCTGCGGACCCGGCGGGGAGGGGC
rs705379_1 RIGHT	GGGCAGCGCCGATTGGCCCGCCCCG
rs705379_2 LEFT	AGCTGCGGACCCGGCGGGGAGGGGT
rs705379_2 RIGHT	GGGCAGCGCCGATTGGCCCGCCCCA
rs705381 LEFT	CTGCGGTGGGGGCTGACCGCAAGCC
rs705381 RIGHT	TGCGGTGGGGGCTGACCGCAAGCCG
rs705381_1 LEFT	TGCGGTGGGGGCTGACCGCAAGCCA
rs705381_1 RIGHT	GGGCCGACCAGGTGCACAGAAGGCG
rs705381_2 LEFT	GGCCGACCAGGTGCACAGAAGGCGC
rs705381_2 RIGHT	GGCCGACCAGGTGCACAGAAGGCGT
PON2	
rs6961773 LEFT	TGGGGGTAGAGGAAGGAGGCACAGG
rs6961773 RIGHT	CCTGTAGGCAGGTCCTGTTGGTTCT
rs6961773_1 LEFT	GGGGGTAGAGGAAGGAGGCACAGGG
rs6961773_1 RIGHT	CTGTAGGCAGGTCCTGTTGGTTCTC
rs6961773_2 LEFT	GGGGGTAGAGGAAGGAGGCACAGGT
rs6961773_2 RIGHT	CTGTAGGCAGGTCCTGTTGGTTCTA
rs17774346 LEFT	TAATAGACTAGTAGTTAGATTATCA

SNP and Probe ID	Probe Sequence
rs17774346 RIGHT	AAGGAAAGCCAATATTTATTGTTCA
rs17774346_1 LEFT	AATAGACTAGTAGTTAGATTATCAA
rs17774346_1 RIGHT	AGGAAAGCCAATATTTATTGTTCAT
rs17774346_1 LEFT	AATAGACTAGTAGTTAGATTATCAG
rs17774346_1 RIGHT	AGGAAAGCCAATATTTATTGTTCAC
rs987539 LEFT	GTGAAAAGCCATGAACTCAGTGTTG
rs987539 RIGHT	TGTCTTCTTCATGTTTATATCTGCT
rs987539_1 LEFT	TGAAAAGCCATGAACTCAGTGTTGC
rs987539_1 RIGHT	GTCTTCTTCATGTTTATATCTGCTG
rs987539_2 LEFT	TGAAAAGCCATGAACTCAGTGTTGT
rs987539_2 RIGHT	GTCTTCTTCATGTTTATATCTGCTA
rs730365 LEFT	ACTATTCTCATCTCCTGACTTTATG
rs730365 RIGHT	TAGCAAATGGGCCAATTTGAAGGGA
rs730365_1 LEFT	CTATTCTCATCTCCTGACTTTATGC
rs730365_1 RIGHT	AGCAAATGGGCCAATTTGAAGGGAG
rs730365_2 LEFT	CTATTCTCATCTCCTGACTTTATGT
rs730365_2 RIGHT	AGCAAATGGGCCAATTTGAAGGGAA
rs11981433 LEFT	AGACTGCAAAGAAGCCAAGAGAAAA
rs11981433 RIGHT	GGTGTGTCAGGCTCTGAGCAATGCC
rs11981433_1 LEFT	GACTGCAAAGAAGCCAAGAGAAAAC
rs11981433_1 RIGHT	GTGTGTCAGGCTCTGAGCAATGCCG
rs11981433_2 LEFT	GACTGCAAAGAAGCCAAGAGAAAAT
rs11981433_2 RIGHT	GTGTGTCAGGCTCTGAGCAATGCCA
rs7785039 LEFT	AAAAAAAAAGGGAAAAAGGCTGCTC
rs7785039 RIGHT	TATGTGGTCTGTTTCTACTTCTGAA
rs7785039_1 LEFT	AAAAAAAAGGGAAAAAGGCTGCTCC
rs7785039_1 RIGHT	ATGTGGTCTGTTTCTACTTCTGAAG
rs7785039_2 LEFT	AAAAAAAAGGGAAAAAGGCTGCTCT
rs7785039_2 RIGHT	ATGTGGTCTGTTTCTACTTCTGAAA
rs10487133 LEFT	TGTGGAACCCAATTTCTACCTTCAT
rs10487133 RIGHT	CACAGTGTCAGTTCTTTCAATAATA
rs10487133_1 LEFT	GTGGAACCCAATTTCTACCTTCATG
rs10487133_1 RIGHT	ACAGTGTCAGTTCTTTCAATAATAC
rs10487133_2 LEFT	GTGGAACCCAATTTCTACCTTCATT
rs10487133_2 RIGHT	ACAGTGTCAGTTCTTTCAATAATAA
rs2299267 LEFT	CAACTTGCAGAAAAACTGGTGGCTT
rs2299267 RIGHT	AGCTTATTATGTTGTCACTAATGTG
rs2299267_1 LEFT	AACTTGCAGAAAAACTGGTGGCTTA
rs2299267_1 RIGHT	GCTTATTATGTTGTCACTAATGTGT
rs2299267_2 LEFT	AACTTGCAGAAAAACTGGTGGCTTG
rs2299267_2 RIGHT	GCTTATTATGTTGTCACTAATGTGC
rs7802018 LEFT	TAAAAGGTGCGAGGCAGCAAGCAGA
rs7802018 RIGHT	AACCCCCAGAGTACCCATGTGAATG
rs7802018_1 LEFT	AAAAGGTGCGAGGCAGCAAGCAGAA
rs7802018_1 RIGHT	ACCCCCAGAGTACCCATGTGAATGT
rs7802018_2 LEFT	AAAAGGTGCGAGGCAGCAAGCAGAG
rs7802018_2 RIGHT	ACCCCCAGAGTACCCATGTGAATGC
rs12534274 LEFT	AAGCTATTTGCCTTCTCTGTTTTAG
rs12534274 RIGHT	AGAAAAGAAAGGAAAACAAAAGGAA

SNP and Probe ID	Probe Sequence
rs12534274_1 LEFT	AGCTATTTGCCTTCTCTGTTTTAGA
rs12534274_1 RIGHT	GAAAAGAAAGGAAAACAAAAGGAAT
rs12534274_2 LEFT	AGCTATTTGCCTTCTCTGTTTTAGG
rs12534274_2 RIGHT	GAAAAGAAAGGAAAACAAAAGGAAC
PON3	
rs9640632 LEFT	ACCTACCTTGTTCCAGCTGCTGCTA
rs9640632 RIGHT	TCAGACCTGTGCAGAACTGGTTGAA
rs9640632_1 LEFT	CCTACCTTGTTCCAGCTGCTGCTAC
rs9640632_1 RIGHT	CAGACCTGTGCAGAACTGGTTGAAG
rs9640632_2 LEFT	CCTACCTTGTTCCAGCTGCTGCTAT
rs9640632_2 RIGHT	CAGACCTGTGCAGAACTGGTTGAAA
rs2072200 LEFT	TCATCTTCTCCAGGATTTGGGGCAC
rs2072200 RIGHT	GCACGAAGGTCAGGAGACAGGTTCC
rs2072200_1 LEFT	CATCTTCTCCAGGATTTGGGGCACC
rs2072200_1 RIGHT	CACGAAGGTCAGGAGACAGGTTCCG
rs2072200_2 LEFT	CATCTTCTCCAGGATTTGGGGCACG
rs2072200_2 RIGHT	CACGAAGGTCAGGAGACAGGTTCCC
rs10487132 LEFT	ATTAGCCTATGCACAACTATCATTA
rs10487132 RIGHT	TGATTTTAGACTTAGCAGTGGGATT
rs10487132_1 LEFT	TTAGCCTATGCACAACTATCATTAA
rs10487132_1 RIGHT	GATTTTAGACTTAGCAGTGGGATTT
rs10487132_2 LEFT	TTAGCCTATGCACAACTATCATTAG
rs10487132_2 RIGHT	GATTTTAGACTTAGCAGTGGGATTC
SOD1	
rs4998557 LEFT	CATTACCTGAATGGCTATACTGCTT
rs4998557 RIGHT	CCTTTCCACTCTACCAAAATGAAAG
rs4998557_1 LEFT	ATTACCTGAATGGCTATACTGCTTA
rs4998557_1 RIGHT	CTTTCCACTCTACCAAAATGAAAGT
rs4998557_2 LEFT	ATTACCTGAATGGCTATACTGCTTG
rs4998557_2 RIGHT	CTTTCCACTCTACCAAAATGAAAGC
rs1041740 LEFT	GGAACTGCATCTGGTTCTTGCAAAA
rs1041740 RIGHT	GTAAAAGAGAGCCTGTCTACTTGGT
rs1041740_1 LEFT	GAACTGCATCTGGTTCTTGCAAAAC
rs1041740_1 RIGHT	TAAAAGAGAGCCTGTCTACTTGGTG
rs1041740_2 LEFT	GAACTGCATCTGGTTCTTGCAAAAT
rs1041740_2 RIGHT	TAAAAGAGAGCCTGTCTACTTGGTA
rs2070424 LEFT	GGGACATAGCTTTGTTAGCTATGCC
rs2070424 RIGHT	TTACTGAGTTATGCCTGTTAATTAC
rs2070424_1 LEFT	GGACATAGCTTTGTTAGCTATGCCA
rs2070424_1 RIGHT	TACTGAGTTATGCCTGTTAATTACT
rs2070424_2 LEFT	GGACATAGCTTTGTTAGCTATGCCG
rs2070424_2 RIGHT	TACTGAGTTATGCCTGTTAATTACC
SOD2	
rs4880 LEFT	CTGCCTGGAGCCCAGATACCCCAAA
rs4880 RIGHT	AGCACCAGCAGGCAGCTGGCTCCGG
rs4880_1 LEFT	TGCCTGGAGCCCAGATACCCCAAAG

SNP and Probe ID	Probe Sequence
rs4880_1 RIGHT	GCACCAGCAGGCAGCTGGCTCCGGC
rs4880_2 LEFT	TGCCTGGAGCCCAGATACCCCAAAA
rs4880_2 RIGHT	GCACCAGCAGGCAGCTGGCTCCGGT
CRP	
rs1205 LEFT	ACTTCCAGTTTGGCTTCTGTCCTCA
rs1205 RIGHT	CTTGTTTGCCACATGGAGAGAGACT
rs1205_1 LEFT	CTTCCAGTTTGGCTTCTGTCCTCAC
rs1205_1 RIGHT	TTGTTTGCCACATGGAGAGAGACTG
rs1205_2 LEFT	CTTCCAGTTTGGCTTCTGTCCTCAT
rs1205_2 RIGHT	TTGTTTGCCACATGGAGAGAGACTA
rs2808630 LEFT	AGGCCAGAGGCTGTCTACCAGACTA
rs2808630 RIGHT	TTCAGTTGCTTGCATCTTACTATAC
rs2808630_1 LEFT	GGCCAGAGGCTGTCTACCAGACTAC
rs2808630_1 RIGHT	TCAGTTGCTTGCATCTTACTATACG
rs2808630_2 LEFT	GGCCAGAGGCTGTCTACCAGACTAT
rs2808630_2 RIGHT	TCAGTTGCTTGCATCTTACTATACA
rs2794520 LEFT	ATCTGTGTGTATGAAGGGCATAGGA
rs2794520 RIGHT	GGCCTCATTCAGTGTGGACCCGTCT
rs2794520_1 LEFT	TCTGTGTGTATGAAGGGCATAGGAC
rs2794520_1 RIGHT	GCCTCATTCAGTGTGGACCCGTCTG
rs2794520_2 LEFT	TCTGTGTGTATGAAGGGCATAGGAT
rs2794520_2 RIGHT	GCCTCATTCAGTGTGGACCCGTCTA
rs2808629 LEFT	AACATGTGGGCAGCATATTCGAAAC
rs2808629 RIGHT	TTGTAATAGTCCTTTTTCTCTTGCG
rs2808629_1 LEFT	ACATGTGGGCAGCATATTCGAAACA
rs2808629_1 RIGHT	TGTAATAGTCCTTTTTCTCTTGCGT
rs2808629_2 LEFT	ACATGTGGGCAGCATATTCGAAACG
rs2808629_2 RIGHT	TGTAATAGTCCTTTTTCTCTTGCGC
rs1417938 LEFT	CCCCACCCCCATACCTCAGATCAAA
rs1417938 RIGHT	AGGGCCACCCCAGGCTATGGGAGAG
rs1417938_1 LEFT	CCCACCCCCATACCTCAGATCAAAA
rs1417938_1 RIGHT	GGGCCACCCCAGGCTATGGGAGAGT
rs1417938_2 LEFT	CCCACCCCCATACCTCAGATCAAAT
rs1417938_2 RIGHT	GGGCCACCCCAGGCTATGGGAGAGA
rs1800947 LEFT	ATGTGAACATGTGGGACTTTGTGCT
rs1800947 RIGHT	AGATGGTGTTAATCTCATCTGGTGA
rs1800947_1 LEFT	TGTGAACATGTGGGACTTTGTGCTC
rs1800947_1 RIGHT	GATGGTGTTAATCTCATCTGGTGAG
rs1800947_2 LEFT	TGTGAACATGTGGGACTTTGTGCTG
rs1800947_2 RIGHT	GATGGTGTTAATCTCATCTGGTGAC
IL6	
rs2069840 LEFT	TATGTAAATTTCATGAGGAGGCCAA
rs2069840 RIGHT	TAAACTGCCTTTAAAAAAGCTTGAA
rs2069840_1 LEFT	ATGTAAATTTCATGAGGAGGCCAAC
rs2069840_1 RIGHT	AAACTGCCTTTAAAAAAGCTTGAAG
rs2069840_2 LEFT	ATGTAAATTTCATGAGGAGGCCAAG
rs2069840_2 RIGHT	AAACTGCCTTTAAAAAAGCTTGAAC

SNP and Probe ID	Probe Sequence
rs1800795 LEFT	ACTTTTCCCCCTAGTTGTGTCTTGC
rs1800795 RIGHT	TGTGCAATGTGACGTCCTTTAGCAT
rs1800795_1 LEFT	CTTTTCCCCCTAGTTGTGTCTTGCC
rs1800795_1 RIGHT	GTGCAATGTGACGTCCTTTAGCATG
rs1800795_2 LEFT	CTTTTCCCCCTAGTTGTGTCTTGCG
rs1800795_2 RIGHT	GTGCAATGTGACGTCCTTTAGCATC
rs2069827 LEFT	GCCCAACAGAGGTCACTGTTTTATC
rs2069827 RIGHT	TGCTAAGAAGAGATCTCTTCAAGAT
rs2069827_1 LEFT	CCCAACAGAGGTCACTGTTTTATCG
rs2069827_1 RIGHT	GCTAAGAAGAGATCTCTTCAAGATC
rs2069827_2 LEFT	CCCAACAGAGGTCACTGTTTTATCT
rs2069827_2 RIGHT	GCTAAGAAGAGATCTCTTCAAGATA
rs2069837 LEFT	TATCTACTGTGTGCCAGGCACTTTA
rs2069837 RIGHT	TTTGAAGATTAGACACAATATTTAT
rs2069837_1 LEFT	ATCTACTGTGTGCCAGGCACTTTAA
rs2069837_1 RIGHT	TTGAAGATTAGACACAATATTTATT
rs2069837_2 LEFT	ATCTACTGTGTGCCAGGCACTTTAG
rs2069837_2 RIGHT	TTGAAGATTAGACACAATATTTATC
rs1554606 LEFT	TTAGTTCATCCTGGGAAAGGTACTC
rs1554606 RIGHT	GCAGCCAGAGAGGGAAAAGGCCCTG
rs1554606_1 LEFT	TAGTTCATCCTGGGAAAGGTACTCG
rs1554606_1 RIGHT	CAGCCAGAGAGGGAAAAGGCCCTGC
rs1554606_2 LEFT	TAGTTCATCCTGGGAAAGGTACTCT
rs1554606_2 RIGHT	CAGCCAGAGAGGGAAAAGGCCCTGA
rs1818879 LEFT	AGACGAGCTGGGCGCAGTGGCTCAC
rs1818879 RIGHT	CCTCCCAAAGTGCTGGGATTATAGG
rs1818879_1 LEFT	GACGAGCTGGGCGCAGTGGCTCACA
rs1818879_1 RIGHT	CTCCCAAAGTGCTGGGATTATAGGT
rs1818879_2 LEFT	GACGAGCTGGGCGCAGTGGCTCACG
rs1818879_2 RIGHT	CTCCCAAAGTGCTGGGATTATAGGC

Appendix 3. Primer Sequences for Multiplex PCR

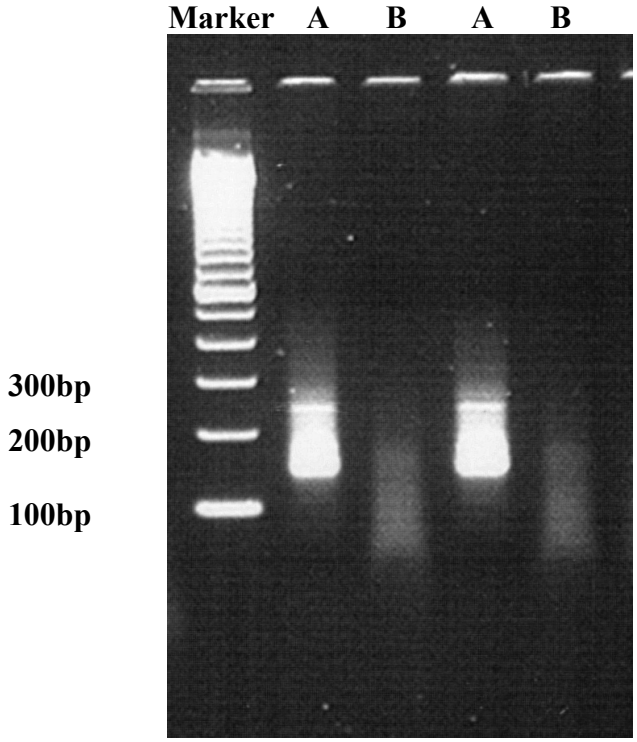
SNP ID	Left Primer	Right Primer
ALOX5		
rs11239523	GGAAGAAGAGGTGGATGGGGGA	CCATCCATTCACCATCCACCCA
rs3780897	TCACGAAGTGGCCGTGACGCA	CCACCAGTGCCCCTTCCTGAA
rs12762303	AGAAGCCCGCGCTGAAGGA	CAGGGGTGCTCCGGGA
rs10900215	GTCTCAGGGTTCCCACTCTGA	GGCTGACCCTGTGTCCAGTAA
rs1487562	CTGTACCAGGTGCATGTTGCA	TCTTGGGAAGTTCCAGAAGTA
rs3780914	GTAATATGGCTGGGACAGGAA	TCTTGGGAAGTTCCAGAAGTA
rs1565096	GAGTCGCAGGAAAGGGAGA	TGCTGCAGGAGGCAGAGA
rs2291427	GTGGGTGGCAGAAAGGGA	GTGTCCTGGGGAACAGCA
rs745986	CTCCTAGTGTGCCTGGCAA	CCTGGCCTTTGAAGGCTTA
rs2215819	TTAGAC AAAATTACGG GGGAA	GTTGCTTTTCGGACAGGAGA
rs7894352	CTAGGACTTGTCATTGAGAA	CACCTTCAGAGAAAGGCATA
rs10900213	CTGCCCATAAATCCTATCCAA	ACCACAAACATGATTTCTGAA
rs10751383	GACACACATATGTGCCAGTGA	GAGAGACCCACAAGGAGGGA
rs7089063	GGAGGTTGCAGTGAGCCAA	TCTCAATTTTGGCCATTCTAA
rs3824612	CCTGCTCCCAGTTCCCTAAA	GTGGCTGGAAGACAGAGTGTA
rs11239528	AGCAGACATAAGATGGCCTGA	CAGCACATCAGGGAGACTTAA
FLAP		
rs11147439	GGGTGCAAAACATCACGGTAGA	AAAGCTGGTGCTCAGAACTTA
rs4072653	ATCGGCATGAACTGGCCA	AGAGCGGGGAGACAACAATAA
rs4468448	AGCATCCCCTTGGGTGGGA	CAAGTCCCCCCAGCTGTA
rs4769060	CAGCAGAGCCCTCGTGGTAA	TTGAAGGGTAGAAGTGTCTCA
rs10507391	AGGCAATCACAAAGATCCAGA	GAAAGGCAGGGTTTTTGCATA
rs4073261	ACAGTCAAGTGCAAGAGTGAA	CTCACAATTATCATGTGGCA
rs3885907	CTATGTGTCATTGCTGACTCA	CCACAGGTGGAGGTTATCAA
rs4076128	TAACTAACCTGTCACACACA	CAGCGACAGGGCTGCGA
rs9671124	TCTGGGCCCCAACACAGA	GTGGCAGTTCTCCAGGCA
rs4769055	AATGTTGTCCTGTTGGCCA	GACTTCCAAACAACCATCAAA
CYBA		
rs4782308	AAACCAAGCTCCACCCAA	ACCATGGAGCGCTGGTGA
rs12709102	ACCCCAGTACCCCTCCCA	GGGCGTGTGCTCACAGCA
rs3794624	CGGTGACCCCGGAAACCA	CCTTGCAGACCCTGGGGA
rs4673	CGAGTGGGAGAGGCCCA	ATGCAGGACGGCCCGA
GPX1		
rs1050450	CCATTGACATCGAGCCTGA	GCAGCACTGCAACTGCCAA
GPX3		
rs3763012	GGGTTCCATAGCACTCACTTA	ACTAGCACTGACGTCTCCTAA
rs8177447	CTGGGAAGGAAGAGGGTCA	TGTACCCAGGAGCTCCGA
rs3792796	ACTCCATCTTACAATGGAGGA	CTGGGAGTCAGGAGACCTGA
rs4958872	CTCTACAAAAGGACTGCAGAA	TCTCTCTGCCAGGTTTAGTCA
HMOX1		
rs9607267	GAGGTGGTGGGGTTCAGAA	GTGCTTAAAGGAGATTGGCCA
rs2071748	CTGTCCCTCACTAGCTACGGA	GCATGTCGCCTCCCTCAA
rs2071749	CTGGGTGATCTTGGGCAA	GCTTCCTCTGTGCCAGACA

SNP ID	Left Primer	Right Primer
MPO		
rs2333227	GAGCCAGTGGCTCATGCCTGTA	TAGCCTCTAGCCACATCATCA
rs2071409	CTGTCGCTGCTGCATGCTGAA	TGCCCTGCCAGCCCAGAA
rs2759	GAAGTGTACTGCAGTTGACAA	CCGGATCATCTGCGACAA
rs2107545	AGTACCCACAATCTCACTTGA	CAGTGACTTGCTGTGTGTGAA
NOS3		
rs1800783	CCTTGGTCATGCACATTTCA	GATGGGATCCAGCCCCTA
rs1808593	TGGCTCTCTCATTCATTTAGA	AGGGACATAGAGAGGAACA
rs1799983	ACGGCTGGACCCCAGGAA	GCTCCAGCCCCACCTCAA
rs7830	CCACCCCTCTGTCCCTAGA	CTGGCAGGAGCGGCTGCA
PON1		
rs854560	GTGGGCATGGGTATACAGA	GGATCCACATCCTGCAATA
rs662	CTGAGCACTTTTATGGCACAA	CTGCCACCACTCGAACTTCA
rs705379	TCTGTGCACCTGGTCGGCCCA	CTGCCTGCCTGCAGCCGCA
rs705381	CCTCCCCGACTGGACTAGGCA	GCCGGGTCCGCAGCTA
PON2		
rs6961773	CTCTTTCACCATTCCTATCCA	GCAGGTCTCACTCAAAGGGAA
rs17774346	GGGTCTCTCTGGTGTGTTTGC	ATTCTGGCCTGGGTGACAA
rs987539	TGTGTGTACAAACCAGTGAGA	GCACCTGCTCTGTTGAGCAA
rs730365	CAAAACCTATCCTTCCCACCA	GGCTAAGGTGGGAAGTTGGAA
rs11981433	AATGACTGTAAGCCTCACACA	GTACCCCCAAAAGCTATTGAA
rs7785039	TCTGCTTGCTGCCTCGCA	CTGACCAGCACACAAGAGTTA
rs10487133	CAGTTAGCTCAAGGAACTCAA	GGCAGTGGAAGAATTCCAATA
rs2299267	GGCAAGACCACCAAAATTCA	AGATTAGTGAGAAGGGGAA
rs7802018	CCACATAGCTTCTTGTGGGAA	GAGACCTAAGCGTGGGCA
rs12534274	CAACCACAGGGAGTCTGTTAA	CAGCGGATACTGTTTGCTGTA
PON3		
rs9640632	AGGCTCCTCTTTAGATCCTCA	GCTGCCCCTCTGGGAAGTA
rs2072200	GAAGGCAATCGAAGCGAA	GCAGCACACTCCGAGGTAA
rs10487132	CCCCAGGATACCTTCCTCTGA	GCAGTAGGCACCTATTCATAA
SOD1		
rs4998557	ACGGAGCACCATTACCTGTCA	TCAAGCACTGAAACCTAGGTA
rs1041740	GTGGGAAGCTGTTGTCCCAA	ACTGAATGTTAATGCCCTCAA
rs2070424	TTTTCTGAAAGCCTTTCAGAA	CTGCACCTGATTTCAGAAGTA
SOD2		
rs4880	CAGCCCAGCCTGCGTAGA	CGCCGTAGTCGTAGGGCA
CRP		
rs1205	CAAGGCTCTGTTGTTTGTCAA	CTGGGACCACCAGTAGCCA
rs2808630	GTTCTCTGAATGCAAATACCA	CAGGGATGTAGGTTGAGCTAA
rs2794520	GATGAGACGTTGGGAGATCA	CCTTAGCCTACTTCTGGTGCA
rs2808629	CTCCATTGACTAGGAGAACCA	CAGTCCAGAAATGTACCACCA
rs1417938	GAGACTGTTCATGCAGTCTTA	CTCATGCTTTTGGCCAGACA
rs1800947	CCTGGTGGGAGACATTGGA	CTGAAGGGCCCGCCAAGA

SNP ID	Left Primer	Right Primer
IL6		
rs2069840	TATAGATCCAGGCAGCAACAA	CTCTGGCCATACCTGTCCAA
rs1800795	CTAGCCTCAATGACGACCTAA	GGGTGGGGCTGATTGGAA
rs2069827	GGGTCCTGAAATGTTATGCA	AACATGGTGTTACCTTCACAA
rs2069837	TGGAACATTCTATGGCTTGAA	GTCTCAGTTTCCTTATCTCCA
rs1554606	ATGGTGCCACTGTGGTGAGA	AGGGAGGGCAGACCTGGA
rs1818879	CAGACTCCTTTCATGACCTAA	AAGTGATCCTCCCGCCTCA
PAF-AH		
rs12408803	GAGGGATTCAGGAGTTCCAGTCAA	TATGTACGCGGAGAAGGGGAA
rs298450	GAGCAAAGGCCCTAAGGCTGCA	TATGTCATGTGGCCCTCCA
rs2257255	GAGCCCAATGCTATGGGACGAA	CAGCTGCTGAGTCTTAGCTTA
rs1932067	GAGATGGGGATTATAGCAAGTGAA	TTTCATTCAGACACGTATGCA
APOE		
rs7412	CTCGCCTCCCACCTGCGCAA	CGCACGCGGCCCTGTTCCA
rs429358	GCTGTCCAAGGAGCTGCA	CCGAGCATGGCCTGCA
ADRB2		
rs12654778	AGCTGGGAGGGTGTGTCTCA	ACCGAGGCACGCACATACA
rs1042717	GTCTGCAGACGGTCACCAA	CGTGACGCACAGCACATCA
rs1042719	CTTATCTACTGCCGGAGCCCA	CTGGGAGGTCTTCACACAGCA
SLC6A4		
rs2066713	GCTCCAATTGCATCACCCA	CCCCTGAGGCAGGAGGGTAA
rs140700	GAGGGAGGTGGGTGAATGGA	CGATCCCTGTGTGACTCCAA
rs4251417	CGTCCTGCAGAGAGGGTAGAA	GGATGCCTTCGGGGCTGA
rs4325622	GCACACTGCAGAGGCTTTGA	GCTTGAAGGCAACTCCAGACA
rs8076005	CCCAGGGGCCCCCAGGAACTA	CAGCTACAAAGAGGTTTGGGGA

PCR primers were designed to amplify a 100–200bp region around the SNP site. A linker sequence was added to the 5’ end of primers to improve amplification. The linker sequence was 5’–TACGACTCACTTAGGGAG–3’ for right primers and 5’–CGATGTAGGTGACACTAG–3’ for left primers.

Appendix 4. Agarose Gel Electrophoresis of Multiplex PCR Products



PCR products separated by 1.5% agarose gel electrophoresis, and visualized by UV light using GelRed™ dye**.**

A: 1 µL volume of product generated from multiplex PCR

B: 1 µL volume of multiplex PCR product fragmented by uracyl-N-glycosylase

Appendix 5. Automated Genotyping by LDA with Dynamic Variable Selection

Detailed descriptions of the algorithms used in simple linear discriminant analysis (LDA) with dynamic variable selection can be found in Podder et al.[1] A brief explanation follows.

Ideally, for variable construction, each genotype call could be based on just one of the four sets of probes: (1) APEX_LEFT; (2) APEX_RIGHT; (3) ASO_1LEFT and ASO_2LEFT; and (4) ASO_1RIGHT and ASO_2RIGHT. (ASO denotes allele-specific apex probes.)

Considering the underlying chemistry, we have developed four sets of classifiers, named: APEX.L, APEX.R, ASO.L and ASO.R. Each of these classifiers consists of a pair of explanatory variables, generically denoted by X and Y, corresponding to two candidate alleles in the SNP position. Since there are five realizations (replicates) for each of the two entries in each classifier, we summarized the information for each allele, by taking a robust average:median of the relevant signals from five spots, for each of the classifiers.

Our automated genotype calling algorithm is based on the simple linear discriminant analysis (LDA), using dynamic variable selection as special criteria for various classifiers related to multiple probes. We start our analysis by fitting the simple LDA-based genotype model using each classifier separately, and at the later stage, we compare the LDA-based predicted genotypes with the other available (e.g., MACGT called or validated) genotypes. Subsequently, we apply dynamic-variable LDA-based genotyping model on these four classifiers. When combining four classifiers together, for each SNP we apply LDA to each pair of variables. For generic alleles X and Y, the possible classes are XX, XY, YY and NN (NN class corresponding to negative controls: generally low signal intensities for all channels throughout all probes). We can ignore the NN class, if we find after initial observation that the data is of good quality (i.e., expected intensity values are large).

[1] Podder M, Welch WJ, Zamar RH, and Tebbutt SJ. Dynamic variable selection in SNP genotype autocalling from APEX microarray data. BMC Bioinformatics. 2006;7:521.

Bayesian posterior probabilities for the possible classes from each of the four possible classifiers are:

LDA/Classes	XX	XY	YY	NN
ASO.L	$P_{xx}^{(ASO.L)}$	$P_{xy}^{(ASO.L)}$	$P_{yy}^{(ASO.L)}$	$P_{NN}^{(ASO.L)}$
ASO.R	$P_{xx}^{(ASO.R)}$	$P_{xy}^{(ASO.R)}$	$P_{yy}^{(ASO.R)}$	$P_{NN}^{(ASO.R)}$
APEX.L	$P_{xx}^{(APEX.L)}$	$P_{xy}^{(APEX.L)}$	$P_{yy}^{(APEX.L)}$	$P_{NN}^{(APEX.L)}$
APEX.R	$P_{xx}^{(APEX.R)}$	$P_{xy}^{(APEX.R)}$	$P_{yy}^{(APEX.R)}$	$P_{NN}^{(APEX.R)}$

The posterior probabilities for the four classifiers are combined using an entropy-based weighting scheme. For example, for the ASO.L classifier, define

$$E_{ASO.L} = -[\log(1/4) + (-\sum_{i \in C} P_i^{(ASO.L)} \log(P_i^{(ASO.L)}))]$$

Analogous quantities are computed for other classifiers

$$E_{ASO.R},\ E_{APEX.L} \text{and}\ E_{APEX.R}$$

Proper weights are obtained by normalizing them, *e.g.*,

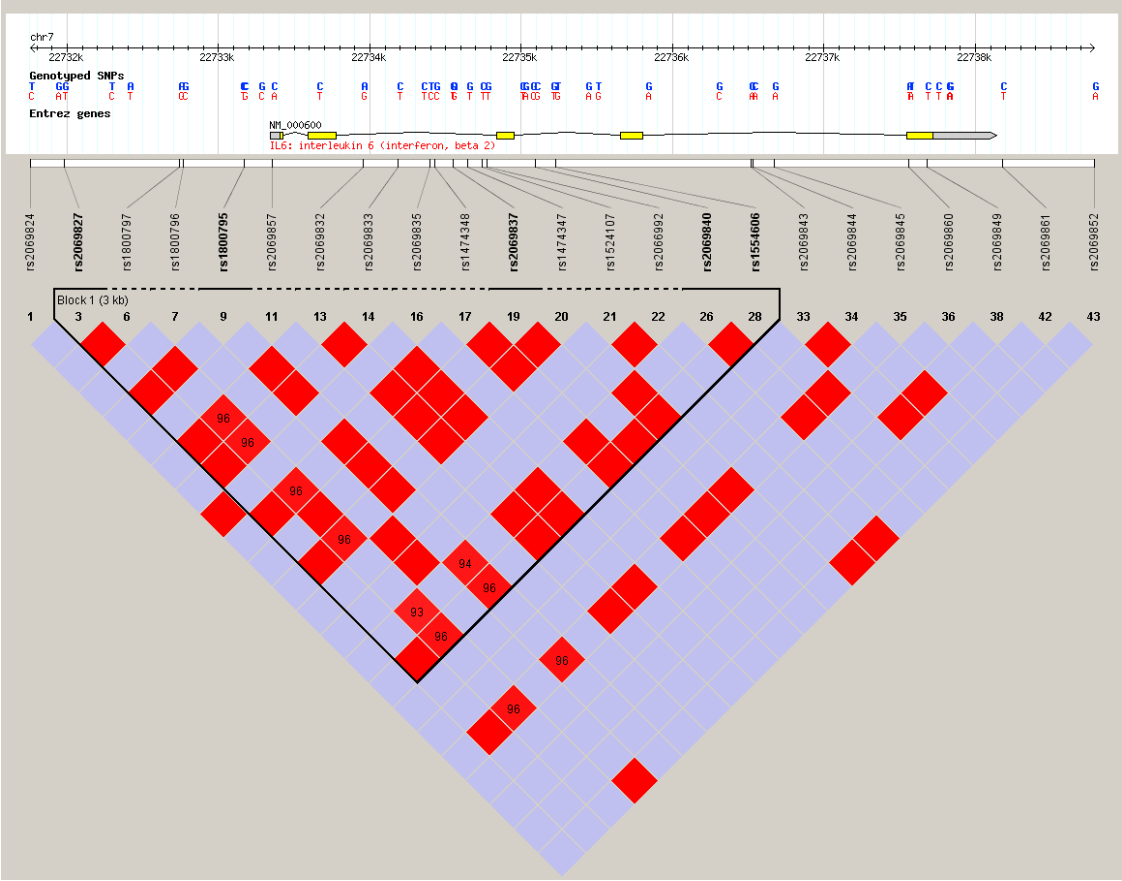
$$W_{ASO.L} = \frac{E_{ASO.L}}{E_{ASO.L} + E_{ASO.R} + E_{APEX.L} + E_{APEX.R}}$$

The weights are applied to the posterior probabilities of the respective class to give the final class posterior probabilities. For example, the final posterior probability for XX class is

$$P_{XX} = W_{ASO.L} P_{XX}^{ASO.L} + W_{ASO.R} P_{XX}^{ASO.R} + W_{APEX.L} P_{XX}^{APEX.L} + W_{APEX.R} P_{XX}^{APEX.R}$$

After obtaining P_{XY}, P_{YY} and P_{NN} in a similar manner, the final genotype call is obtained with highest weighted probability. In the last stage, call rate can be adjusted by applying varying thresholds to the 'final weighted probability' (confidence score), and the concordance with the validated genotype set will vary accordingly.

Appendix 6. Linkage Disequilibrium of Tagging SNPs for Interleukin-6

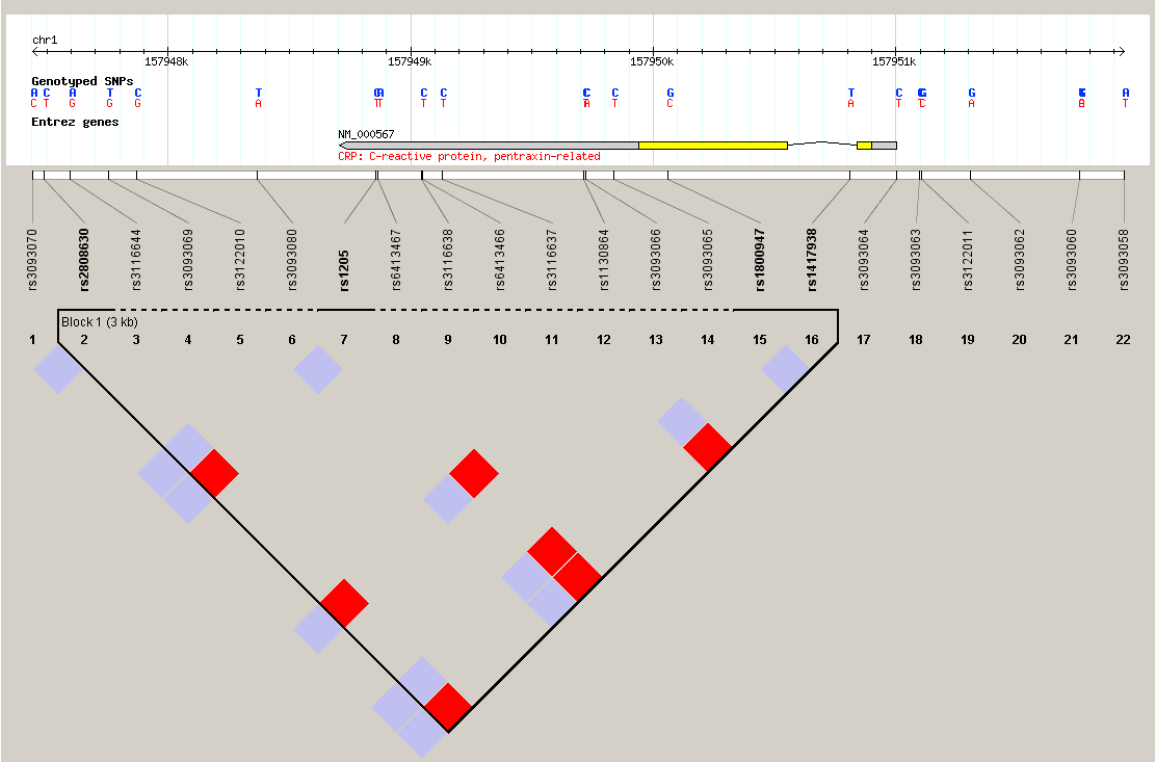


Location of tagging SNPs across the *IL6* gene region: Haplotype structure in Hapmap CEU samples with D′ statistics. Bold numbers are chosen tagSNPs. Not pictured is rs1818879 and with rs1818879, the haplotype block extends into the 3′ flanking region. Non bolded numbers are SNPs at minor allele frequency <5% or tagged by tagSNPs.

	rs1800795	**rs2069827**	**rs2069837**	**rs2069840**	**rs1554606**	**rs1818879**
rs2069827	1	0.92	0.02	−0.88	0.99	−1.00
rs1800795	0.13	1	−0.27	0.96	0.97	−0.95
rs2069837	0.00	0.01	1	−0.19	−0.19	−0.46
rs2069840	0.04	0.31	0.00	1	−0.96	0.59
rs1554606	0.14	0.82	0.01	0.36	1	−0.97
rs1818879	0.05	0.28	0.02	0.32	0.33	1

Observed pair-wise D′ statistics (top corner/white) and r^2 values (bottom corner/grey) for tagSNPs genotyped in angiography cohort patients.

Appendix 7. Linkage Disequilibrium of Tagging SNPs for C-Reactive Protein



Location of tagging SNPs across the *CRP* gene region: Haplotype structure in Hapmap CEU samples. Bold numbers are chosen tagSNPs. Non bolded numbers had minor allele frequencies <5%, except rs1130964, which was tagged directly by rs1205.

	rs1417938	**rs1800947**	**rs2105**	**rs2808630**
rs1417938	1	−1.00	−0.99	−0.94
rs1800947	0.03	1	0.98	−0.85
rs2105	0.21	0.14	1	−0.91
rs2808630	0.14	0.02	0.19	1

Observed pair-wise D′ statistics (top corner/white) and r^2 values (bottom corner/grey) for tagSNPs genotyped in angiography cohort patients.

Appendix 8. Risk of Coronary Artery Disease by *IL6* and *CRP* Genotypes

A: *IL6*

	rs2069827 G/T	**rs1800795 G/C**	**rs2069837 A/G**	**rs2069840 C/G**	**rs1554606 G/T**	**rs1818879 G/A**
OR	1.00	1.25	1.10	0.87	0.89	0.99
[95% CI]	[0.75–1.32]	[0.79–1.97]	[0.82–1.49]	[0.65–1.17]	[0.68–1.17]	[0.75–1.30]
P value	0.980	0.338	0.519	0.359	0.420	0.917

B: *CRP*

	rs1417938 T/A	**rs1800947 G/C**	**rs1205 C/T**	**rs2808630 T/C**
OR	0.90	1.51	1.54	0.91
[95% CI]	[0.66–1.21]	[0.86–2.65]	[1.14–2.06]	[0.68–2.21]
P value	0.467	0.152	0.004*	0.507

Odds ratios (OR) and 95% confidence intervals (CI) for genotype associations with angiographic coronary artery disease. All models are adjusted for age, sex, BMI and log(biomarker) concentrations**.**

*$p<0.05$ with correction for multiple comparisons

Appendix 9. Risk of Cardiovascular Mortality by *IL6* and *CRP* Genotypes

A: *IL6*

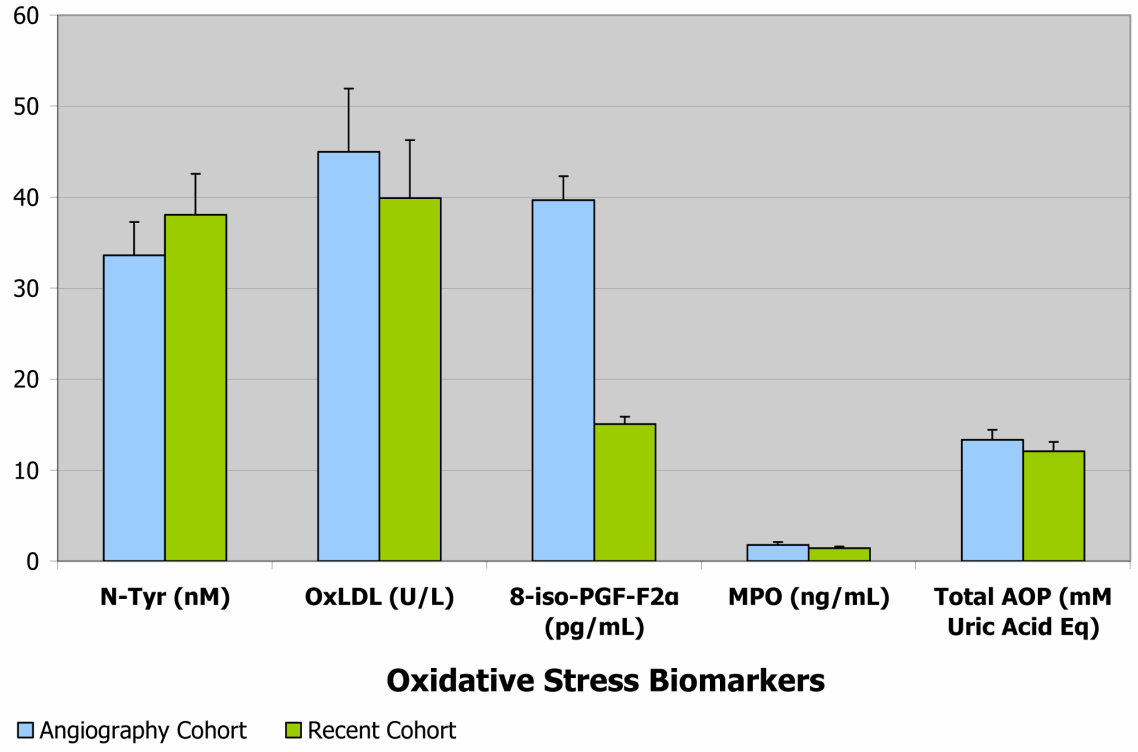
	rs2069827 G/T	**rs1800795 G/C**	**rs2069837 A/G**	**rs2069840 C/G**	**rs1554606 G/T**	**rs1818879 G/A**
HR	1.56	1.15	0.93	1.15	1.19	1.07
[95% CI]	[1.04–2.36]	[0.82–1.60]	[0.66–1.31]	[0.82–1.62]	[0.86–1.65]	[0.77–1.49]
P value	0.034	0.420	0.67	0.42	0.30	0.68

B: *CRP*

	rs1417938 T/A	**rs1800947 G/C**	**rs1205 C/T**	**rs2808630 T/C**
HR	0.971	1.11	0.803	1.36
[95% CI]	[0.69–1.38]	[0.64–1.93]	[0.58–1.12]	[1.09–1.94]
P value	0.87	0.71	0.19	0.006

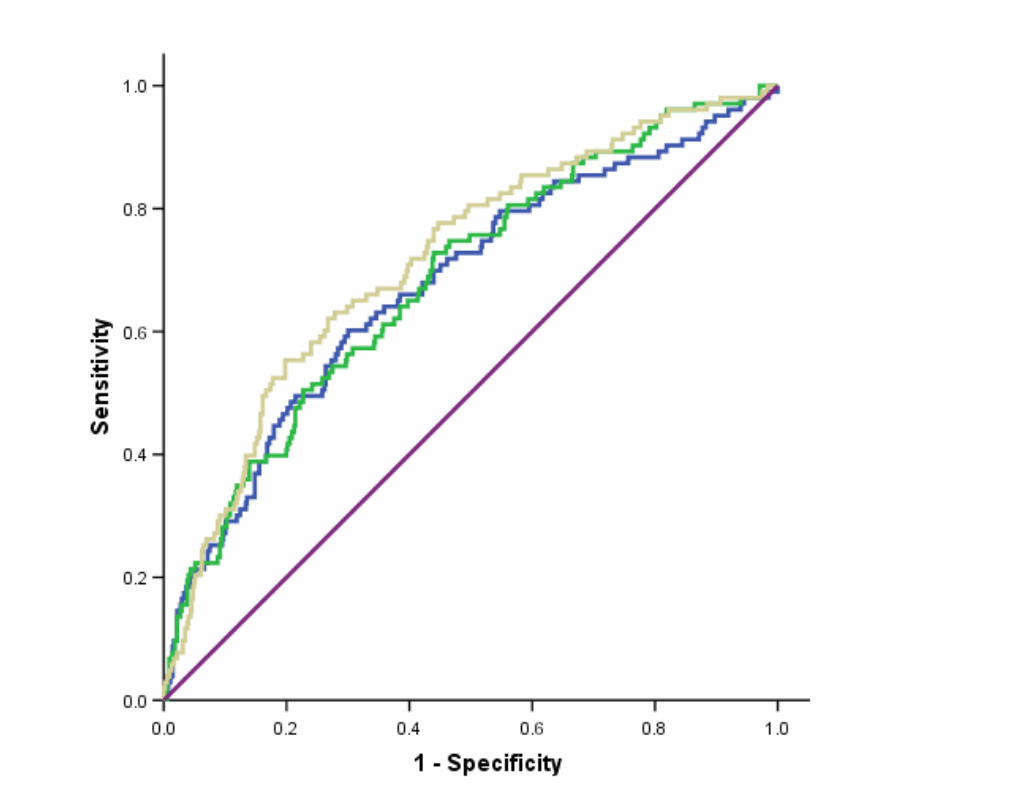
Hazard ratios (HR) and 95% confidence intervals for SNPs in *IL6* and *CRP* associated with cardiovascular mortality in models adjusted for age, sex, bmi, smoking, diabetes, hypertension, CAD, and log(biomarker) concentrations.

Appendix 10. Stability of Oxidative Stress Biomarkers in Stored Plasma



Plasma biomarker measurements of oxidative stress markers Nitrotyrosine (N-tyr), Oxidized LDL (oxLDL), 8-iso-prostaglandin F-2alpha, myeloperoxidase (MPO), and Total Antioxidant Potential (AOP) from the coronary angiography cohort compared to a cohort with blood samples collected more recently. n≥20 for all measurements. Elevations in 8-iso-PGF-F2a with storage are attributed to spontaneous lipid peroxidation. Other biomarkers considered but not shown above were paraoxonase 1, which could not be measured in EDTA-plasma, glutathione peroxidase 1, which can only be measured in erythrocytes, and fluorescent oxidation products, which did not yield robust results in our samples.

Appendix 11. Time Adjusted Receiver Operator Characteristic Curves



Receiver operator characteristic curves and area values (AUC) are based on Cox regression models for the prediction of risk of cardiovascular mortality with conventional risk factors, with or without biomarkers (tertiles of myeloperoxidase, or combined tertiles of myeloperoxidase and C-reactive protein). Conventional risk factors included in the model were age, sex, body mass index, total:high density lipoprotein cholesterol ratio, smoking, diabetes, and hypertension.

The model with conventional risk factors (blue line, AUC 0.715) was compared to the same model with tertiles of myeloperoxidase included (green line, AUC 0.761, p=0.031 for improvement from the basic model). The grey line represents the model with combined tertiles of myeloperoxidase and C-reactive protein (AUC 0.781, p=0.003 for improvement from the basic model). A reference line of 0.500 representing no discrimination power is included (purple line).

Appendix 12. Baseline Characteristics of Coronary Angiography Cohort

1) Baseline clinical characteristics

Characteristic	Angiographic evidence of coronary artery disease		
	No (n=170)	Yes (n=627)	p value
Age, years	58.0±12.3	61.9±10.6	<0.001
Males, %	51.7	81.0	<0.001
Smoking status, %			
Never smoked	42.9	24.8	<0.001
Former or current smoker	57.1	75.2	
Hypertension, %	34.2	38.5	0.335
Diabetes, %	6.0	19.9	<0.001
Family history of CAD, %	54.4	58.2	0.398
Body mass index, kg/m^2	27.5±5.01	28.1±4.52	0.142
Waist circumference, cm	86.3±28.5	93.3±25.3	0.004
Lipid Variables			
Low-density lipoprotein cholesterol, mg/dL	3.72±1.09	3.52±0.94	0.028
High-density lipoprotein cholesterol, mg/dL	1.01±0.28	0.92±0.24	<0.001
Total cholesterol:high-density lipoprotein cholesterol ratio	5.37±1.96	5.95±2.14	0.002
Mortality Outcome			
Total Mortality, %	21.5	32.1	0.011
Cardiovascular death, %	4.0	15.7	<0.001

Mean±standard deviation, or % are given for baseline characteristics. P-values are calculated from independent sample t-tests, or χ^2 tests for continuous and categorical variables, respectively.

2) Association Between Biomarkers and Angiographically Documented CAD

Characteristic	Angiographic CAD		p value*
	No (n=170)	Yes (n=627)	
Myeloperoxidase, ng/μL	84.3 [61.7–137.7]	92.1 [61.6–138.8]	0.030
Nitrotyrosine, nmol/L	73.4 [61.6–89.7]	72.4 [60.4–88.4]	0.349
Oxidized low-density lipoprotein, mU/L	71.1 [58.1–83.5]	72.4 [61.0–90.6]	0.015
Antioxidant capacity, μmol/L	288 [264–314]	300 [273–324]	0.332

Median values for patients with and without angiographic CAD [interquartile ranges] are reported.

*p-values are reported from age-and sex-adjusted multivariate regression analyses.

Patients with angiographic CAD had higher myeloperoxidase (p=0.03) and oxLDL (p=0.015), while nitrotyrosine and antioxidant capacity were not associated with angiographic CAD in age and sex-adjusted models. Further adjustment for cardiovascular risk factors attenuated relationships between oxLDL and angiographic CAD. In particular, smoking history and diabetes were both associated with higher oxLDL concentrations, and adjusting for these risk factors attenuated the relationship between oxLDL and CAD.

Appendix 13. Oxidative Stress Haplotypes and Coronary Artery Disease

Gene and Haplotype Number	Freq. (%)	Polymorphisms				Odds Ratio (95% Confidence Interval)	p value*	q value†
ALOX5		rs3780897	rs3824612					
1-1	47.4	T	C			*reference*		
1-2	37.2	T	T			0.84 (0.63–1.13)	0.258	0.627
1-3	14.9	C	C			0.91 (0.89–1.42)	0.672	0.821
						Global	0.639	0.821
ALOX5		rs11239523	rs7894352	rs21115819				
2-1	33.2	T	G	T		*reference*		
2-2	30.1	T	G	G		1.03 (0.74–1.43)	0.802	0.821
2-3	19.5	T	A	G		1.31 (0.90–1.91)	0.157	0.584
2-4	9.8	C	G	T		1.04 (0.61–1.78)	0.769	0.821
2-5	5.4	C	G	G		1.52 (0.74–3.10)	0.250	0.627
						Global	0.527	0.795
ALOX5		rs1487562	rs3780914	rs2291427	rs10751383			
3-1	49.0	C	T	G	C	*reference*		
3-2	27.1	C	C	A	A	1.05 (0.77–1.45)	0.743	0.821
3-3	15.9	T	C	G	A	0.98 (0.67–1.45)	0.902	0.821
3-4	5.0	C	C	A	C	1.37 (0.67–2.89)	0.385	0.733
						Global	0.740	0.821
FLAP		rs4073261	rs4076128					
1-1	62.9	T	A			*reference*		
1-2	21.6	T	G			1.21 (0.95–1.81)	0.103	0.584
1-3	13.7	C	G			1.18 (0.78–1.80)	0.424	0.750
						Global	0.113	0.584
FLAP		rs11147439	rs3885907					
2-1	42.2	C	C			*reference*		
2-2	40.1	A	A			1.15 (0.85–1.54)	0.367	0.729
2-3	12.1	C	A			0.92 (0.60–1.40)	0.679	0.821
2-4	5.0	A	C			2.02 (0.76–5.21)	0.158	0.584
						Global	0.306	0.669
FLAP		rs9671124	rs4072653	rs4468448				
3-1	32.8	T	G	C		*reference*		
3-2	28.2	C	A	C		0.85 (0.61–1.52)	0.333	0.713
3-3	22.7	C	A	T		1.24 (0.85–1.84)	0.259	0.627
3-4	14.1	T	A	C		0.92 (0.60–1.42)	0.709	0.821
						Global	0.740	0.821

continued

Gene and Haplotype Number	Freq. (%)	Polymorphisms			Odds Ratio (95% Confidence Interval)	p value*	q value†
CYBA		rs4782308	rs4673	rs12709102			
1-1	41.1	A	C	C	*reference*		
1-2	30.3	G	T	T	1.02 (0.75–1.42)	0.865	0.821
1-3	25.2	A	C	T	0.97 (0.69–1.33)	0.788	0.821
					Global	0.915	0.821
GPX3		rs3763012	rs3792796	rs8177447			
1-1	39.1	G	C	C	*reference*		
1-2	21.8	A	C	C	1.27 (0.92–1.76)	0.152	0.584
1-3	21.0	A	G	C	0.97 (0.69–1.33)	0.402	0.743
1-4	10.4	G	G	T	0.60 (0.40–0.90)	**0.011**	**0.168**
					Global	**0.006**	**0.129**
NOS3		rs1800783	rs7830				
1-1	41.1	T	C		*reference*		
1-2	29.1	A	C		0.91 (0.64–1.30)	0.592	0.821
1-3	20.0	T	A		1.08 (0.73–1.61)	0.651	0.821
1-4	14.8	A	A		1.06 (0.70–1.61)	0.782	0.821
					Global	0.760	0.821
PON1		rs854560	rs662				
1-1	40.6	G	C		--		
1-2	29.3	A	C		1.15 (0.86–1.55)	0.346	0.713
1-3	29.1	A	G		1.25 (0.91–1.72)	0.165	0.589
					Global	0.376	0.733

continued

Gene and Haplotype Number	Freq. (%)	Polymorphisms					
PON2		rs987529	rs6961773	rs2299267	rs11981433	rs12534274	rs17774346
1-1	30.6	C	T	A	C	G	A
1-2	15.6	T	G	A	T	A	A
1-3	11.0	T	T	G	T	G	G
1-4	9.0	C	T	A	T	G	A
1-5	8.9	C	T	A	C	G	G
1-6	5.1	T	T	A	T	A	G

Gene and Haplotype Number	Odds Ratio (95% Confidence Interval)	p value*	q value†
PON2			
1-1	*reference*		
1-2	0.80 (0.55–1.17)	0.256	0.627
1-3	0.97 (0.63–1.48)	0.851	0.821
1-4	0.85 (0.52–1.37)	0.500	0.769
1-5	1.27 (0.84–2.48)	0.190	0.627
1-6	0.98 (0.51–1.89)	0.813	0.821
	Global	**0.050**	0.487

continued

Gene and Haplotype Number	Freq. (%)	Polymorphisms			Odds Ratio (95% Confidence Interval)	p value*	q value†
PON2		rs7802018	rs730365				
2-1	52.4	A	C		*reference*		
2-2	24.7	G	C		1.29 (0.98–1.70)	0.066	0.505
2-3	10.3	A	T		1.15 (0.72–1.85)	0.564	0.821
					Global	0.271	0.627
PON3		rs9640632	rs10487132	rs2072200			
1-1	38.5	T	G	G	*reference*		
1-2	25.2	C	A	G	0.82 (0.59–1.13)	0.225	0.384
1-3	21.0	C	A	C	1.00 (0.70–1.45)	0.972	0.929
1-4	13.1	T	A	G	0.93 (0.62–1.41)	0.744	0.821
					Global	0.750	0.821
SOD2		rs4998557	rs2069840				
1-1	54.4	G	C		*reference*		
1-2	31.3	G	G		0.91 (0.66–1.23)	0.563	0.769
1-3	10.5	A	C		1.39 (0.83–2.30)	0.206	0.384
					Global	0.339	0.713

* P values are from age and sex-adjusted logistic regression models for additive genetic effect of haplotypes. Only haplotypes ≥5% frequency were included in analyses.

† Q values represent false discovery rate expectation for the corresponding p-value, calculated from the experiment-wide distribution of p-values.

Appendix 14. Oxidative Stress Biomarkers and *GPX3* and *PON2* Haplotypes

Gene and Haplotype Number	Oxidized LDL (mU/L)		Antioxidant Capacity (µmol/L)	
	Estimated Mean	p value*	Estimated Mean	p value*
GPX3				
1-1	*reference*		*reference*	
1-2	81.35 (78.5–84.2)	0.861	276.9 (269.5–284.3)	0.793
1-3	83.54 (80.3–86.8)	0.223	280.3 (270.2–290.4)	0.306
1-4	83.61 (79.4–87.8)	0.341	284.1 (273.0–295.2)	0.151
PON2				
1-1	*reference*		*reference*	
1-2	83.79 (80.8–87.5)	0.226	281.0 (269.7–292.1)	0.735
1-3	81.79 (77.8–91.8)	0.871	283.9 (272.1–294.3)	0.439
1-4	82.63 (80.0–87.3)	0.630	276.5 (262.3–283.5)	0.837
1-5	80.91 (75.4–86.4)	0.787	281.0 (272.7–296.8)	0.612
1-6	80.69 (74.6–86.8)	0.746	276.0 (261.1–296.6)	0.545

Variations in baseline oxidative stress biomarker concentrations across haplotypes of oxidative stress genes are shown for haplotypes tested. Only haplotypes associated with coronary artery disease at $p \leq 0.05$ were tested for changes to oxidative stress biomarkers.

*Plasma biomarker means (95% Confidence Intervals) are estimated from additive genetic linear regression models adjusted for age and sex, and p-values represent significance in adjusted models.

Appendix 15. Changes to Biomarkers for SNPs Associated with CAD

Biomarker	Gene	SNP	Allele	Estimated Mean*	P value
Myeloperoxidase ng/μL	*ALOX5*	rs10900213	GG	73.75 (60.29–87.21)	**0.016**
			GT	57.15 (43.69–70.61)	
			TT	40.55 (27.09–54.01)	
	MPO	rs2071409	TT	68.39 (48.85–87.93)	0.758
			TG	71.46 (51.92–91.26)	
			GG	74.53 (54.99–94.06)	
	MPO	rs2107545	TT	77.06 (59.47–94.66)	**0.019**
			TC	55.98 (38.39–73.58)	
			CC	34.90 (17.31–52.5)	
Nitrotyrosine nmol/L	*ALOX5*	rs10900213	GG	46.26 (37.64–54.88)	0.079
			GT	38.52 (29.90–47.14)	
			TT	30.78 (22.16–39.41)	
	MPO	rs2071409	TT	39.42 (26.88–51.96)	0.934
			TG	39.94 (27.40–52.48)	
			GG	40.47 (27.93–53.01)	
	MPO	rs2107545	TT	38.21 (26.83–49.59)	0.600
			TC	41.25 (29.87–52.64)	
			CC	44.30 (32.92–55.68)	
Oxidized LDL mU/L	*ALOX5*	rs10900213	GG	82.78 (80.39–85.17)	0.543
			GT	82.04 (79.65–84.43)	
			TT	81.29 (78.91–83.68)	
	GPX1	rs1050450	GG	82.84 (79.38–86.29)	0.912
			GA	82.64 (79.18–86.10)	
			AA	82.45 (78.99–85.90)	
	MPO	rs2071409	TT	82.92 (79.47–86.33)	0.863
			TG	82.61 (79.17–86.03)	
			GG	82.31 (78.87–85.72)	
	MPO	rs2107545	TT	82.72 (79.62–85.82)	0.484
			TC	83.83 (80.73–86.92)	
			CC	84.93 (81.84–88.03)	
Antioxidant Capacity μmol/L	*ALOX5*	rs10900213	GG	270.7 (264.4–276.9)	0.696
			GT	269.4 (263.2–275.6)	
			TT	268.2 (261.9–274.4)	
	GPX1	rs1050450	GG	281.2 (272.1–290.3)	0.820
			GA	280.2 (271.1–289.3)	
			AA	279.1 (270.0–288.2)	
	MPO	rs2071409	TT	280.7 (271.8–289.6)	0.910
			TG	281.2 (272.3–290.1)	
			GG	281.7 (272.8–290.6)	
	MPO	rs2107545	TT	281.5 (273.3–289.7)	0.730
			TC	280.1 (271.9–288.3)	
			CC	278.6 (270.5–286.8)	

*Plasma biomarker means (95% confidence intervals) are estimated from additive genetic linear regression models adjusted for age and sex.

Genotypes and Plasma Biomarkers: SNPs and haplotypes associated with angiographic CAD at unadjusted significance values of $p \leq 0.05$ were investigated for changes to plasma oxidative stress biomarkers. Polymorphisms in *ALOX5* and *MPO* were tested for changes in all biomarkers, and tagSNPs and haplotypes of *GPX1, GPX3* and *PON2* were investigated for changes to oxidized LDL and antioxidant capacity. Effects of rs10900213 and rs2107575 on myeloperoxidase were independent in combined models, but did not show evidence of snp-snp interactions in effects on myeloperoxidase levels (data not shown).

Appendix 16. Hazard Ratio for Cardiovascular Mortality For GPX3 Haplotypes

Gene and Haplotype Number	Odds Ratio (95% Confidence Interval)	p value*	q value
1-1	*reference*		
1-2	1.02 (0.73–1.43)	0.886	0.821
1-3	0.84 (0.55–1.28)	0.411	0.713
1-4	1.01 (0.60–1.68)	0.948	0.929
1-5	1.34 (0.76–2.38)	0.312	0.713
	Global	0.341	0.713

* P values from Cox survival analyses, with adjustment for age and sex