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The Role of Novel Risk Markers in Cardiovascular Disease

PhD

2011

Tora Leong

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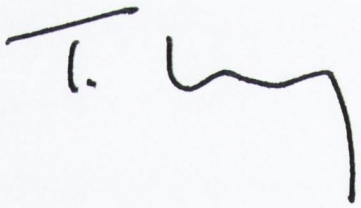
TO MY PARENTS PUN KONG AND CHUI LEE

For their love and encouragement

DECLARATION

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SUMMARY

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in Ireland and globally. Conventional risk factors such as blood pressure, cholesterol and smoking account for most of CVD. A small but significant residual risk is accounted for by novel risk markers. Asymmetric dimethylarginine (ADMA), CD40 ligand and the metabolic syndrome are novel risk markers that are of particular interest because they are strongly associated with endothelial dysfunction and inflammation, both key pivotal processes in atherosclerosis.

QUESTIONS & METHODS

Are these 3 novel risk markers independent of conventional risk factors?

Do they give added value over current systems of CVD risk assessment such as the SCORE equation?

Three studies were used to answer the above questions:

1. ADMA with myocardial infarction and stroke after 24 years follow-up using stored samples (n=880) of *The Population Study of Women in Gothenburg*
2. CD40 ligand with coronary artery disease (n=730) in the *Cath Lab Study* and with carotid intima-media thickness (n=386) in a sub-study
3. The Metabolic Syndrome and CVD in an old multi-centre case-control dataset (n=1,550) of *The European Concerted Action Project: Homocysteinaemia and Vascular Disease*

RESULTS

ADMA: A 0.15μmol/L (1 SD) increase in ADMA resulted in 30% increase in myocardial infarction and stroke after adjustment for traditional risk factors, homocysteine and its covariates in *The Population Study of Women in Gothenburg*. ADMA ≥0.71 μmol/L (top quintile) was also able to re-classify those originally deemed low-risk by the SCORE equation into a category similar to that of the SCORE high-risk group.

CD40 ligand: An unexpected inverse relationship was found between CD40 ligand and coronary artery disease in the *Cath Lab Study* which remained independent and robust after adjustment for traditional risk factors and novel risk markers in combination. However, the independent association of CD40 ligand with carotid intima-media thickness was not inverse.

Carotid intima-media thickness was independently associated with coronary artery disease after adjustment for traditional risk factors factors, in particular when the ≥75th percentile ARIC study cutpoints were used. It had added re-classification ability when used on top of the SCORE equation.

The metabolic syndrome was independently associated with CVD both in the *Cath Lab Study* and the COMAC Study. Of its 5 components, HDL cholesterol and triglycerides were the main drivers of CVD risk.

CONCLUSIONS

Conventional risk factors remain the mainstay of CVD risk assessment. Of the many novel risk markers, P-selectin, homocysteine, white cell count and the metabolic syndrome show promise. The increased CVD risk associated with the metabolic syndrome is driven by HDL cholesterol and triglycerides. CD40 ligand deserves further study.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ADMA	Asymmetric Dimethylarginine
ARB	Angiotensin Receptor Blocker (Angiotensin II AT_1 receptor blocker)
AUROC	Area Under the Receiver Operating Characteristic
BMI	Body Mass Index
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CB	Carotid Bulb
CCA	Common Carotid Artery
CHD	Coronary Heart Disease
CI	Confidence interval
CIMT	Carotid Intima-Media Thickness
CT	Computerised Tomography
CVD	Cardiovascular Disease
HDL	High Density Lipoprotein
HR	Hazard Ratio
hs-CRP	High-sensitivity C-Reactive Protein
IC	Internal Carotid
IMT	Intima-Media Thickness
OR	Odds Ratio
LDL	Low Density Lipoprotein
MACE	Major Adverse Cardiovascular Event
MI	Myocardial Infarction
PCI	Percutaneous Coronary Intervention
PVD	Peripheral Vascular Disease
ROC	Receiver Operating Characteristic
SD	Standard Deviation
VCAM	Vascular Cell Adhesion Molecule

CHAPTER ONE
GENERAL INTRODUCTION & QUESTIONS

Cardiovascular disease (CVD) is the leading cause of death both in Ireland and globally, accounting for 9,980 (35%) deaths in Ireland in 2006, and 17.2 million (29%) globally in 2004. By the time CVD becomes clinically apparent, for example, in the form of a myocardial infarction or 'heart attack', it is associated with high mortality. About half of those with myocardial infarctions in the community die before they are able to make it to hospital for treatment. The background process in the decades leading up to this event is atherosclerosis. Atherosclerosis starts early in life. This is preventable through reduction of risk factors. Therefore, being able to accurately ***identify*** those at risk early so that intervention of risk factors can occur is the key to preventing CVD.

Conventional risk factors such as blood pressure, cholesterol and smoking, and other less proven traditional risk factors such as diabetes, obesity, diet, physical activity, alcohol consumption and psychosocial factors, have been estimated to account for between 50-90% of CVD. Even the most conservative estimate of the residual risk of 10% unaccounted by conventional risk factors is significant because the absolute scale of the problem is so large. In recent times, intense research efforts have been devoted to trying to identify newer risk factors, usually termed ***novel risk markers***, which may account for this residual risk.

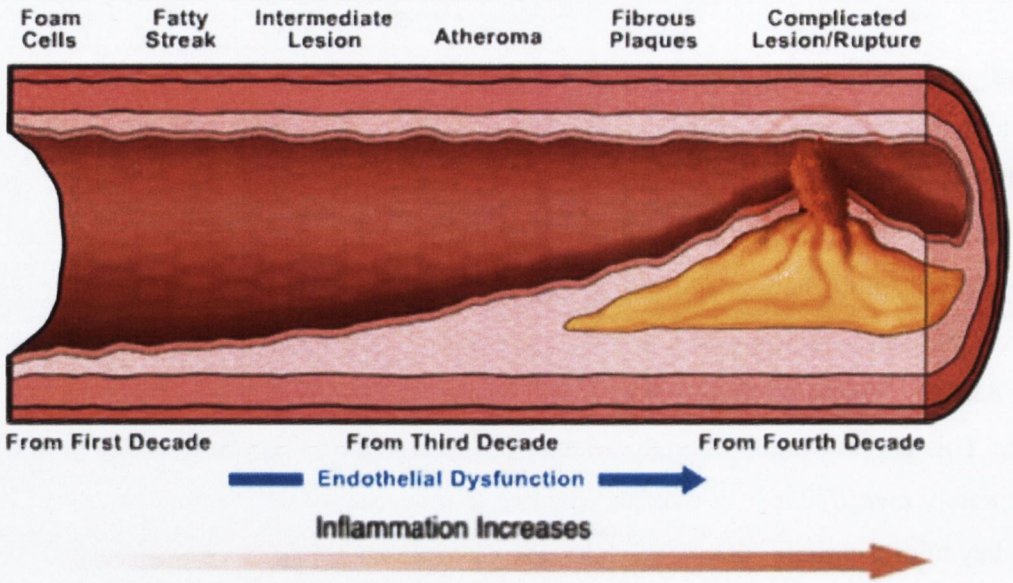
Therefore, the core reasons for this work are to establish:

(a) whether novel risk markers are useful to improve the identification of those at risk of CVD earlier, and

(b) given the large number of proposed risk factors, is it possible to simplify CVD risk estimation to a fewer number of risk factors and markers.

Endothelial dysfunction and inflammation are regarded as key factors in the initiation and development of atherosclerosis. Figure 1.1 illustrates this graphically, and will be discussed in detail in the review of the state of the art.

Figure 1.1 - The role of endothelial dysfunction and inflammation in Atherosclerosis



Asymmetric dimethylarginine (ADMA), CD40 ligand and the metabolic syndrome were identified as novel risk markers of particular interest because they play key pivotal roles in process of atherosclerosis in terms of modulating both endothelial function and the inflammatory process.

ADMA, in particular, is of interest because it directly affects the synthesis of nitric oxide, a key regulator of endothelial dysfunction. Furthermore, ADMA levels are increased by conventional risk factors, raising the possibility that it could represent a final common pathway in the atherosclerotic process.

CD40 ligand plays a pivotal role upstream of the inflammatory process and regulates other markers of inflammation. It has already been shown to be of value to stratify risk in acute coronary syndromes but not yet as a marker of risk in stable patients.

The metabolic syndrome is a clinical state or marker of high CVD risk, and is associated with both inflammation and endothelial dysfunction. As it is a simple and accessible tool based on conventional measurements, this work also examines its role in detecting CVD.

GENERAL QUESTIONS

1. Is ADMA independently associated with CVD or through associations with other risk factors? (Chapter 3)
2. Is CD40 ligand associated with CVD independently of other risk factors? (Chapters 4 and 5)
3. What role does the metabolic syndrome have in relation to CVD risk assessment? (Chapters 4, 5 and 6)
4. Can CVD risk estimation be simplified by consolidating the large number of conventional risk factors and novel risk markers? (Chapters 4 and 6)

QUESTIONS IN DETAIL

CHAPTER THREE: ADMA with Cardiovascular Disease

1. Is ADMA associated with CVD in an initially healthy population?
2. Is the association independent of conventional risk factors?
3. Can ADMA add predictive value to current risk estimation systems such as the SCORE equation and the Framingham risk score?
4. Are the effects of conventional risk factors and homocysteine on CVD risk mediated through ADMA?

CHAPTER FOUR: CD40 ligand with Coronary Artery Disease

1. Is CD40 ligand associated with the presence, extent and complexity of coronary artery disease in a stable population of patients with suspected coronary disease?
2. Is the association independent of conventional risk factors?
3. Of the many better studied novel risk markers shown to have independent associations with CVD such as high-sensitivity (hs-CRP), homocysteine, vascular cell adhesion molecule (VCAM), P-selectin and fibrinogen, how useful are they to predict risk when taken all in combination?
4. What possible important interaction effects do they exhibit?

5. Do they have added predictive value and can they further re-classify patients into a clinically more important category, for instance, qualifying for different therapy?
6. Can we consolidate the large number of conventional risk factors and novel risk markers into a simpler few to predict coronary artery disease?

CHAPTER FIVE: CD40 ligand with Carotid Intima-Media Thickness

1. Is carotid intima-media thickness (CIMT) associated with CD40 ligand and other conventional risk factors and novel risk markers?
2. Of the large number of conventional risk factors and novel risk markers in combination, which ones are still independently associated with CIMT after multivariable adjustment?
3. Can CIMT predict the presence of coronary artery disease?
4. Of the various methods used to measure CIMT, which definitions and cut-points of CIMT are the most predictive of significant coronary artery disease?

CHAPTER SIX: The Metabolic Syndrome with CVD

1. Is the metabolic syndrome associated with CVD?
2. Is the association independent of conventional risk factors and other novel risk markers such as homocysteine, apolipoproteins B & AI and white cell count.
3. Does the metabolic syndrome predict CVD risk better as a whole, or using its individual components in combination?
4. What combination of factors works best in a risk model for predicting CVD?

GENERAL METHODOLOGY

The above questions were examined through:

1. The design and execution of an original research study, the *Cath Lab Study* (n=730), which allowed the examination of the possible association of coronary artery disease on coronary angiography with a large number of conventional risk factors and novel risk markers in combination.

2. The design and execution of a sub-study of Carotid Intima-Media Thickness (n=386) within the *Cath Lab Study* examining the possible association of carotid intima-media thickness with CVD risk factors and coronary artery disease.

3. The analysis of ADMA using stored samples (n=880) of a large prospective cohort study, the *Population Study of Women in Gothenburg*, to examine the possible association of ADMA with CVD.

4. The examination of a dataset of an old multi-centre case-control study, *The European Concerted Action Project: Homocysteinaemia and Vascular Disease* (COMAC) (n=1,550) to determine how the metabolic syndrome and other novel risk markers fit into CVD risk assessment.

CHAPTER TWO
STATE OF THE ART

The Burden of Cardiovascular Disease

Cardiovascular Disease (CVD), which includes coronary heart disease, ischaemic stroke, and peripheral vascular disease remains the leading cause of death in Ireland and countries with developed economies. According to figures published by the Central Statistics Office, CVD accounted for 9,980 deaths (35.0% of total mortality) in Ireland in 2006[1]. Of that, coronary heart disease (CHD) was responsible for 5,017 deaths (17.6% of total mortality) that year. Approximately 22% of premature deaths (under the age of 65) are from CVD in Ireland. Although CVD mortality rates have been on the decline since the peak of 54% during the 1970s, Ireland still has above-average premature deaths from CVD (52 per 100,000) compared to the European Union average of 42 per 100,000[2]. This pattern of death caused by chronic non-communicable diseases such as CVD is typical of countries which have developed economies such as the United States, Western Europe, Canada, Australia and Japan, sometimes described as the fourth stage of epidemiological transition.

Globally, CVD is, by far, the leading cause of death. Figures published by the World Health Organization showed CVD as the leading cause of death which accounted for approximately 17.2 million deaths (29% of total mortality) in 2004[3]. Deaths from CVD accounted for 31.1% of total mortality in males, and 27.7% in females and were about two-fold higher than the next largest cause, infections, and about three-fold higher than the third largest cause, cancer. Indeed, this is true of low- and middle-income countries except for sub-Saharan Africa where infections are still the leading cause.

The Stages of Epidemiological Transition

A model of the epidemiological transition of disease burden had been described by Omran in 1971[4], with further modification by Olshansky and Ault in 1986[5]. Understanding the trends of CVD mortality and risk factors is crucial to implement effective policies for of CVD prevention but this is beyond the scope of this work.

Mortality from disease, using CVD as an example, tends to follow a specific pattern, going through 4 stages of epidemiological transition. Briefly, infectious diseases tend to account for the majority of deaths early on in societal and economical development (first stage), as is currently the case in Sub-Saharan Africa. As economies develop, improvements in nutrition and public health lead to lower mortality (second stage), and the increased calorific intake results in a rise in obesity and CVD risk factor levels such as hypertension and smoking. This leads to a rapid increase in CVD prevalence and deaths (third stage). The fourth stage is the age of delayed degenerative diseases where CVD and cancer are the major causes of morbidity and mortality. At this stage, better treatment and prevention help avoid deaths among those with disease and delay primary events. Age-adjusted CVD mortality rates decline and CVD becomes the disease of older age groups, with increased heart failure as a result. Table 2.1 summarises these stages of epidemiological transition. However, it is estimated that by 2020, CVD will overtake infectious diseases as the leading cause of death in all areas of the world. One would also hope that a utopian 'fifth' stage can happen such as an example in Sweden where progressive reductions in CVD risk factors through aggressive approaches to lower blood pressure, cholesterol levels and smoking lead to a decline in CVD mortality[6].

Table 2.1 - Stages of Epidemiological Transition

Stage	CVD mortality	Causes	Examples
I **Pestilence and famine**	< 10%	Rheumatic Cardiomyopathy (Infectious, Nutritional)	Sub-Saharan Africa South-East Asia (Vietnam, Cambodia)
II **Receding pandemics**	10-35%	Rheumatic Hypertension CHD Stroke	India
III **Degenerative and man-made diseases**	35-65%	CHD Stroke	Former Soviet Union Eastern Europe Latin America Middle East China South-East Asia (Indonesia)
IV **Delayed degenerative diseases**	50%	CHD Stroke Heart failure	USA Western Europe Japan Canada Australia New Zealand

The Rationale for Cardiovascular Disease Prevention

The underlying disease process for CVD is atherosclerosis, a process that begins early in life[7]. The Vietnam[8] and Korean War[9] autopsy studies demonstrated the existence of advanced atherosclerotic lesions in the coronary arteries of healthy young men. Furthermore, studies such as the Pathological Determinants of Atherosclerosis in the Young (PDAY)[10-13], the Bogalusa Heart Study[14] show the association of CVD risk factors with the extent of atherosclerosis present in the coronary arteries at autopsy. The Chicago Heart Association Detection Project showed that in those individuals with cardiovascular risk factors early in life, there is an increased risk of premature coronary heart disease later on[15].

Once established, CVD is difficult to reverse because once it becomes clinically apparent, for example, a myocardial infarction, it is associated with high mortality and morbidity. About half of those with acute myocardial infarctions in the community die before ever reaching the hospital to receive acute care because for many, the first manifestation is a fatal arrhythmia and sudden death. About a third die in the first 24 hours. Even after treatment, the in-hospital mortality of a myocardial infarction is about 10%, with an additional 10% in the first year.

The causes of CVD are now well established and they are known as the conventional CVD risk factors, which are blood pressure, cholesterol and smoking. Modifying these risk factors has been shown to reduce mortality from CVD[16-19]. This is the rationale for adopting a preventive approach to CVD.

The Conventional Cardiovascular Risk Factors

To establish an effective CVD prevention strategy, one must be able to identify those at risk. It was the Framingham Heart Study that established blood pressure, cholesterol and smoking as consistently strong risk factors for coronary heart disease (CHD). The Framingham Heart Study was a cohort study of 5,209 participants started in 1948 in the town of Framingham, Massachusetts in the United States[20]. The Framingham Heart Study, amongst other numerous seminal findings, established smoking[21], blood pressure and cholesterol[22] as consistently strong risk factors for CHD. These conventional risk factors are now established beyond doubt as causal for CVD and reducing the levels of these risk factors has been shown to reduce mortality from CVD.

The Seven Countries Study[23] was initiated by Ancel Keys in 1958 to investigate the variation in CHD mortality in countries with differing mortality rates including Finland, the United States, the Netherlands, Italy, Yugoslavia, Greece and Japan. This would have encompassed countries with high CHD mortality, for example, Finland and low CHD mortality, for example, Japan. Cholesterol was found to be of the greatest importance to explain the different CHD mortality rates between the countries. The mean cholesterol varied significantly between the seven countries and also correlated strongly with CHD rates and also correlated with the fat content of the diet.

The findings of Doll and Hill in the British Doctors Study established smoking as the cause of lung cancer[24] and myocardial infarction, then referred to as coronary thrombosis[25].

All of these seminal findings have been shown time and time again in repeated observations at different times all over the world. Therefore, the role of blood pressure, smoking and cholesterol as causal risk factors for CVD are proven beyond doubt.

Cardiovascular Disease Risk Estimation

One of the ways to estimate an individual's risk of developing CVD risk is using a risk estimation system or equation. Of the many risk estimation systems[26-28], the most well known is the undoubtedly the risk equation based on the Framingham Heart Study. The Framingham equation uses a combination of age, sex, diabetes, smoking, blood pressure, total, HDL and LDL cholesterol to assess CHD risk[29]. While the Framingham equation has been shown to predict risk well in populations with the same high level of risk both in the United States[30] and Northern Europe[31], it can over-predict risk in other European populations[32-34], especially in populations with lower cardiovascular risk such as countries in southern Europe[23,35]. This is thought to be due to the poor applicability of the data derived from the five thousand participants of Framingham, Massachusetts in the United States in the 1950s where the level of CHD risk is high compared to populations in Europe where there is a north-south gradient of risk.

Hence, the Third Joint Task Force in CVD Prevention in Clinical Practice of the European Society of Cardiology (ESC) in their guidelines in 2003[36] introduced the Systematic COronary Risk Evaluation (SCORE) system[37], which is designed to estimate a healthy individual's CVD risk in European populations. The SCORE system uses a combination of age, sex and conventional risk factors such as systolic blood pressure, smoking, total and/or HDL cholesterol to estimate an individual's 10-year risk of fatal CVD. As in the previous recommendations, the risk equation is illustrated in the form of risk charts for ease of use. There are separate risk charts for low and high risk regions of Europe. The SCORE system is based on data derived from 205,178 individuals from 12 European cohorts which is more applicable in estimating CVD risk of European populations.

The Fourth Joint Task Force in 2007[38] recommended the terms *increased* risk rather than *high*-risk to describe individuals with a SCORE 10-year risk of fatal CVD of ≥5%. This is considered roughly the equivalent of the Framingham 10-year risk of 'hard' CHD endpoints of >20% which is the equivalent 'high-risk' category.

Updated qualifiers were given in light of the ability to re-calibrate the SCORE charts using country-specific data because of the choice of mortality endpoints as opposed to total events. The relative risk chart was also introduced to reveal concealed possibly delayed risk in younger individuals who have low absolute risk but significant risk factors. Figures 2.1 and 2.2 show the SCORE charts for high and low risk countries, and Figure 2.3, their instructions for use.

Figure 2.1 – SCORE Risk chart – High Risk countries

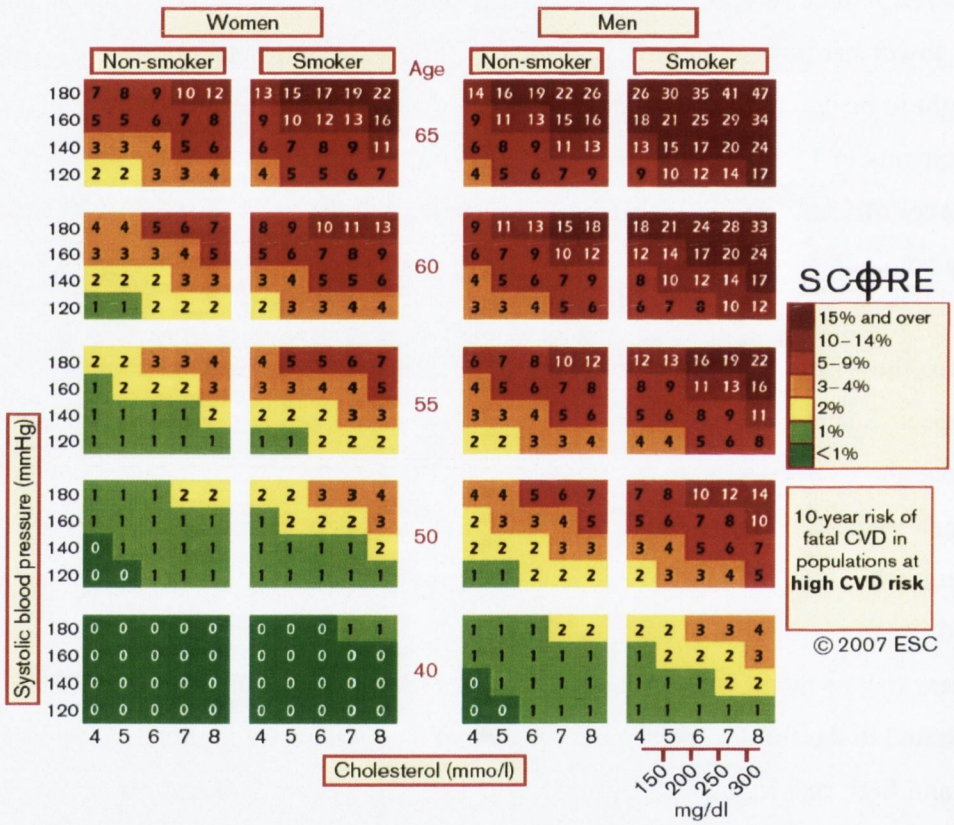


Figure 2.2 – SCORE Risk chart – Low Risk countries

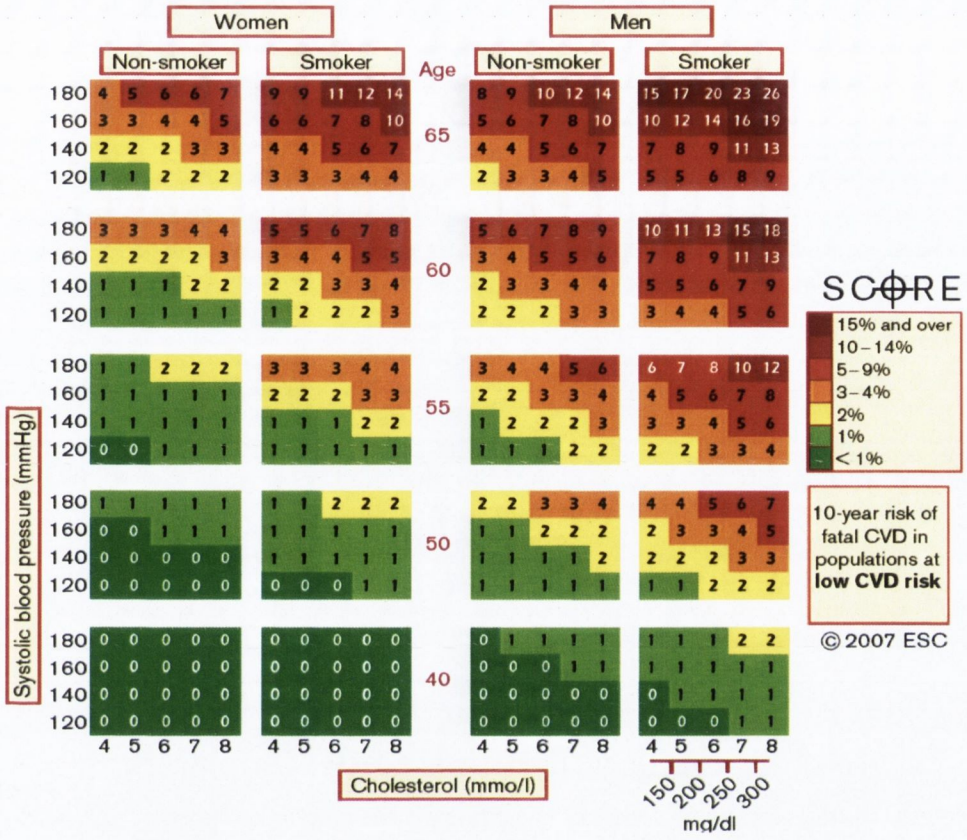


Figure 2.3 – Instructions for use of the SCORE Charts and Qualifiers

How do I use the SCORE charts to assess total CVD risk in asymptomatic persons?

(1) Use the low risk chart in Belgium*, France, Greece*, Italy, Luxembourg, Spain*, Switzerland and Portugal; use the high risk chart in other countries of Europe. *Updated, recalibrated charts are now available for Belgium, Germany, Greece, The Netherlands, Poland, Spain and Sweden.

(2) Find the cell nearest to the person's age, cholesterol and BP values, bearing in mind that risk will be higher as the person approaches the next age, cholesterol or BP category.

(3) Check the qualifiers.

(4) Establish the absolute 10 year risk for fatal CVD. Note that a low absolute risk in a young person may conceal a high relative risk; this may be explained to the person by using the relative risk chart. As the person ages, a high relative risk will translate into a high absolute risk. More intensive lifestyle advice will be needed in such persons.

Risk estimation using SCORE: Qualifiers

(1) The charts should be used in the light of the clinician's knowledge and judgement, especially with regard to local conditions.

(2) As with all risk estimation systems, risk will be overestimated in countries with a falling CVD mortality rate, and underestimated if it is rising.

(3) At any given age, risk appears lower for women than men. This is misleading since, ultimately, more women than men die from CVD. Inspection of the charts shows that their risk is merely deferred by 10 years.

(4) Risk may be higher than indicated in the chart in:

- (i) Sedentary or obese subjects, especially those with central obesity;
- (ii) Those with a strong family history of premature CVD;
- (iii) The socially deprived;
- (iv) Subjects with diabetes–risk may be 5 fold higher in women with diabetes and 3 fold higher in men with diabetes compared to those without diabetes;
- (v) Those with low HDL cholesterol or high triglycerides;
- (vi) Asymptomatic subjects with evidence of preclinical atherosclerosis, for example a reduced ankle-brachial index or on imaging such as carotid ultrasonography or CT scanning.

Novel Risk Markers

A significant proportion of people develop atherosclerosis and CVD in the absence of these established conventional risk factors of raised blood pressure, raised serum cholesterol and smoking. Historically, it was claimed that about half of acute myocardial infarctions present without discernible risk factors[39]. This has led to the an intense search for newer risk factors for CVD, usually referred to collectively as **novel risk markers** in an attempt to explain this residual risk.

It is very likely that the 'only half' figure is an under-estimation of the role of conventional risk factors[40]. These estimates may depend upon the cut-points chosen – earlier studies used higher cut-points to define raised blood pressure and raised cholesterol. Data from the National Cooperative Pooling Project in 1975 comprising eight US prospective studies with 10-year follow up showed that 66% of CHD events among men aged 30-69 years were attributable to high cholesterol (≥6.5 mmol/L), high blood pressure (diastolic blood pressure ≥90 mmHg) and smoking[41].

Later analyses of large studies such as the Multiple Risk Factor Intervention Trial (MRFIT) with 356,222 individuals aged 35-57 years showed that 75% fewer CHD deaths would have occurred if the 3 major risk factor levels had been optimal. Therefore, 75% of CHD deaths were attributed to raised serum cholesterol (≥4.71 mmol/L), raised diastolic blood pressure (≥90 mmHg) and cigarette smoking. The main analyses of the paper showed that serum cholesterol had a graded and continuous relationship to CHD, and serum cholesterol ≥4.65 mmol/L alone accounted for 46% of CHD deaths[42].

The more recent INTERHEART study, a multi-centre case-control study (15,152 cases, 14,820 controls) showed that 90% of the risk of acute myocardial infarction could be attributed to 9 known risk factors which are lipids, smoking, blood pressure, diabetes, abdominal obesity, psychosocial factors, fruits and vegetable consumption, alcohol and physical activity[43].

Hence, a more conservative estimate is probably appropriate for the role of novel risk markers in CVD. Even so, novel risk markers are still relevant because they

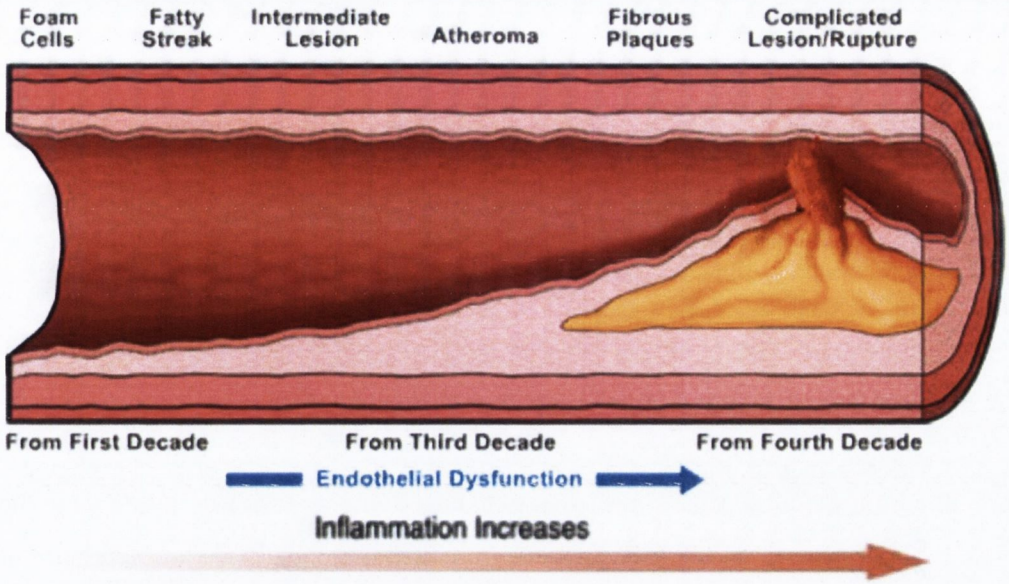
may explain the residual 10% or so of unexplained CVD. In absolute terms, this is still very significant as CVD deaths number 17.2 million in 2004[3]. Novel risk markers may also modify the effect of conventional risk factors and understanding them may be important in effective CVD risk factor intervention. For example, having raised homocysteine multiplies the risk attributed by cigarette smoking and hypertension[44,45], and similarly with C-Reactive Protein and the ratio of total cholesterol to HDL cholesterol[46,47].

Therefore, it is still relevant to investigate the role of potential new risk markers to establish their association, if any, with CVD.

The Vascular Biology of Atherosclerosis

The process of atherosclerosis is no longer thought of as a passive lipid deposition process. It is now thought that an inflammatory process initiates the process of atherosclerosis[48-51]. Three classic stages of atherosclerotic lesions are recognised: the fatty streak, the fibrous plaque, and the advanced complicated lesion[52]. The fatty streak is the earliest atherosclerotic lesion and this has been seen at autopsy in infants and young children suggesting that atherosclerosis starts early in life. The fatty streak is a predominantly inflammatory lesion made up of monocytes and T lymphocytes.

Figure 2.4 - The role of endothelial dysfunction and inflammation in Atherosclerosis



The endothelium does not normally support the binding of inflammatory cells such as monocytes or macrophages. The event which provokes and initiates atherosclerosis is thought to be endothelial injury or denudation[52], or simply endothelial dysfunction (Figure 2.4). Endothelial dysfunction may be caused by numerous factors such as oxidised LDL cholesterol, an atherogenic diet, obesity, the metabolic syndrome, free radicals from cigarette smoking, hypertension, diabetes, genetic alterations, raised homocysteine, infections such as Herpesviruses, *Chlamydia* and *Helicobacter*, and so on.

This endothelial dysfunction results in an increased adhesiveness of the endothelium to leukocytes and platelets, and also increases the permeability of the artery. The adhesiveness of the artery is mediated through the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM) by the endothelial cells. This allows the monocytes and T lymphocytes from the blood to bind with the endothelium. After binding, other factors such as selectins mediate a rolling effect while integrins allow firmer attachment of the monocytes to the inflamed endothelium. The monocytes then migrate into the smooth muscle wall of the artery with the help of monocyte chemoattractant protein, and differentiate into

macrophages which ingest oxidised LDL cholesterol to become foam cells with the help of macrophage colony-stimulating factor. The accumulation of foam cells initially forms the fatty streak.

Fatty streaks initially consist of foam cells together with T lymphocytes which secrete inflammatory cytokines mediated by tumour necrosis factor-α. If the inflammatory response continues, smooth muscle cells migrate into the lesion and proliferate due to stimulation by growth factors such as platelet-derived growth factor. The proliferation of smooth muscle cells which become intermixed with the area of inflammation accumulates to form an intermediate lesion.

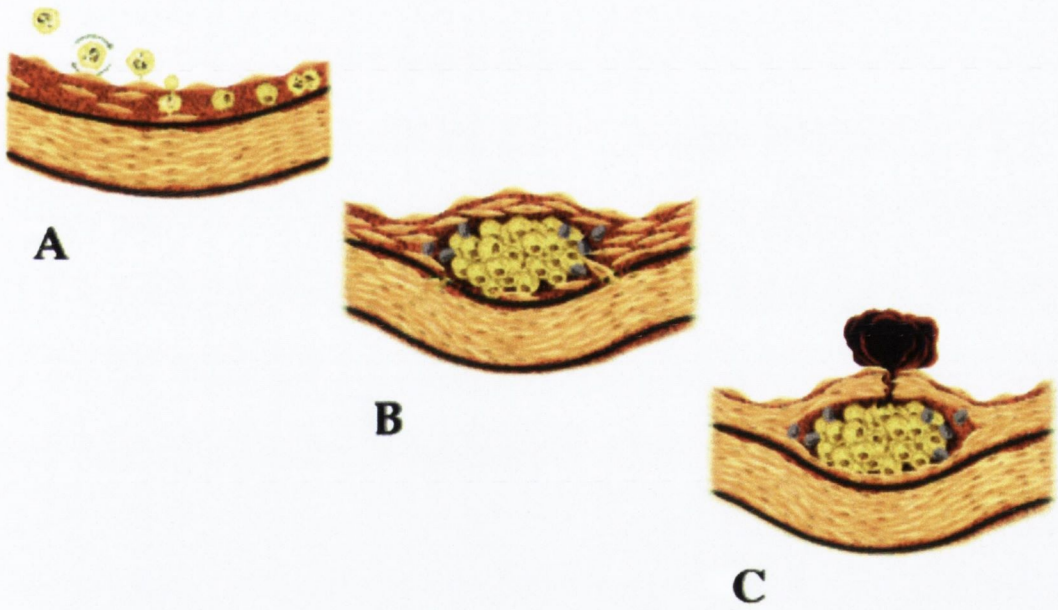
Continued inflammation causes macrophages and T lymphocytes to migrate from the blood and multiply within the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines and growth factors which induce further damage and eventually lead to focal necrosis. Cycles of proliferation of smooth-muscle cells and formation of fibrous tissue lead to further enlargement and restructuring of the lesion so that it becomes covered by a fibrous cap that overlies a core of lipid and necrotic tissue – an advanced, complicated lesion. The arterial wall thickens and compensates by gradual dilation, and the lumen remains unaltered up to a point, a phenomenon known as remodelling[53]. However, in the later stages, this compensatory mechanism becomes overwhelmed and it becomes inevitable that the atherosclerotic plaque will cause luminal narrowing leading to compromise in arterial blood flow. Angina is the result if this happens in the coronary arteries.

Erosion of the fibrous cap leads to rupture, usually at the shoulders of the lesion where macrophages enter, accumulate and are activated. Degradation of the fibrous cap may result from metalloproteinases and other proteolytic enzymes. Activated T lymphocytes may stimulate metalloproteinase production by macrophages in the lesions which promote plaque instability. Stable advanced lesions have uniformly dense fibrous caps. Lesions with high risk of plaque rupture can often be non-occlusive and therefore difficult to diagnose by angiography. However, active inflammation can be detected at autopsy as evident by the accumulation of macrophages. Macrophage accumulation may be associated with C-Reactive Protein

or fibrinogen. Plaque rupture leads to haemorrhage from plaque microvessels (vasa vasorum) and exposure of the lipid core. This leads to thrombosis and sudden occlusion of the arterial lumen results in acute coronary syndromes and myocardial infarctions.

Figure 2.5 shows a summary of the atherosclerotic process leading up to plaque rupture and atherothrombosis. Following that, Tables 2.2 & 2.3 illustrates some of the CVD risk factors, traditional and novel respectively that have been studied for their role in CVD.

Figure 2.5 – Atherosclerosis leading to acute coronary syndromes (reproduced from Libby *et al*[49])



A: Dysfunctional endothelium attracting inflammatory cells onto surface

B: Build-up of lipid core leading to a fibrous plaque

C: Plaque rupture and atherothrombosis

Table 2.2 – Lifestyle and characteristics associated with the risk of cardiovascular events (reproduced from the European Guidelines on CVD Prevention[54])

Lifestyle	Biochemical or Physiological Characteristics	Personal Characteristics
Diet Tobacco smoking Physical activity	Arterial blood pressure Total cholesterol (LDL cholesterol) HDL cholesterol Triglycerides Glycaemia/diabetes Overweight/obesity Thrombogenic factors Markers of chronic inflammation	Age Sex Family history of premature CVD Personal history of CVD Genetic markers

Table 2.3 – Some examples of proposed novel risk markers for CVD

Type	Examples
Cytokines	Interleukin-6 (IL-6) Tumour Necrosis Factor-a (TNF-a) CD-40 Ligand (CD40L)
Markers of Inflammation	High-sensitivity C-Reactive Protein (hs-CRP) Serum Amyloid A (SAA)
Cell Adhesion Molecules	Vascular-cell adhesion molecule (VCAM) Intercellular adhesion molecule (ICAM) Platelet-derived selectin (P-selectin) Endothelial-derived selectin (E-selectin)
Haematological factors	Fibrinogen Plasminogen Von Willebrand Factor (vWF) Plasminogen activator inhibitor-1 (PAI-1)
Platelet-related factors	Platelet aggregation, activity, size, volume
Lipid-related factors	Lipoprotein (a) Apolipoproteins B and AI Small dense LDL particles
Infectious agents	*Helicobacter pylori* (Hp) *Chlamydia pneumoniae* Cytomegalovirus (CMV) Herpes simplex virus (HSV)
Others	The Metabolic Syndrome and Insulin Resistance Homocysteine Asymmetric Dimethylarginine (ADMA) PAI-1 genotype Apolipoprotein E genotype Angiotensin converting enzyme (ACE) genotype
Imaging modalities	Carotid intima-media thickness (CIMT) CT Calcium Score

Causality

The increased understanding of the vascular biology of atherosclerosis has provided most of the clues in the search for novel risk markers. Some markers such as high-sensitivity C-Reactive Protein[46,47,55-58] (hs-CRP), homocysteine[44,45,59], fibrinogen[60] and lipoprotein (a)[61] have been extensively studied and may be causal factors for CVD. High-sensitivity C-Reactive Protein[47,62] and homocysteine have been shown to add prognostic information in terms of predicting CVD outcomes. Other novel risk markers are still in their infancy in terms of determining whether they cause CVD or not.

Apart from causality, other important considerations include whether novel risk markers can also add value to current methods of CVD risk assessment, and if clinically meaningful decisions can be changed by using these risk markers.

Association or correlation does not imply causation. Correlation is necessary for causation and can indicate possible causes or areas for further investigation. The criteria for determining whether or not a risk factor may be causal for disease could be based on suggestions made by Sir Austin Bradford Hill in his seminal essay in 1965[63] which are:

1. **Strength of the association**: The stronger the association, the more likely that it is causal, though a small association does not mean lack of causation.
2. **Consistency**: Causality is more likely if the association has been observed by different individuals at different places and at different times.
3. **Specificity**: If the disease only happens in the presence of the risk factor and not without, then that is a strong argument in favour of causation although lack of specificity does not negate a causal relationship.
4. **Temporality**: The risk factor should predate the disease.
5. **Biological gradient**: If the association has a dose-dependent or graded relationship, it makes a stronger case for causality.
6. **Plausibility**: The mechanism of disease should be biologically plausible but this may be limited by current knowledge.

7. **Coherence**: Findings that are consistent across disciplines strengthen the argument for causation.
8. **Experiment**: If the disease can be altered or prevented by altering the risk factor in question, then it is a strong argument for causation.
9. **Analogy**: In some circumstances, it may be acceptable to judge by past experience of similar factors, especially if the results had been catastrophic and unethical to repeat, for example thalidomide and birth defects.

In the case of novel risk markers, judging by 'Analogy' is not applicable. With the advent of multivariable statistical regression techniques and the availability of computer processing, one should add 'independence of effect' into the list of criteria for causality as it is particularly relevant to the study of novel risk markers.

Criteria for the clinical usefulness of a Novel Risk Marker

Another consideration to make when examining novel risk markers, apart from causality, is whether is can add to current methods of CVD risk assessment which may alter the treatment of a patient. The following criteria are particularly relevant[64]:

1. The diagnostic test for the marker should be standardised, easily and reliably used with accepted population reference values. A relatively high prevalence of abnormal values and a substantial proportion of normal values should be found in intermediate-risk groups.
2. The marker should be an independent predictor of CVD events in intermediate-risk persons who have no history of CVD.
3. The marker should be able reclassify a substantial proportion of intermediate-risk individuals as high-risk.
4. The reclassification process should be to a risk category that will result in a change of management in order to reduce their risk of CVD.
5. Convenience, availability, cost, and safety may be important considerations when there is a choice of markers offering the same prognostic information.

RATIONALE

The review of the state of the art has shown that novel risk markers will likely have a small (approximately 10%) but significant contribution to CVD risk because of the absolute scale of the burden of CVD.

Apart from the established conventional risk factors, there are the other 'traditional risk factors' such as diabetes, HDL cholesterol, triglycerides *et cetera* that are less established. Apart from these, hundreds of factors have been proposed and these include novel risk markers, some of which are more established than others. Clearly, one cannot accommodate all of these in CVD risk assessment. Of the numerous novel risk markers, it felt logical to focus on:

1. **Asymmetric dimethylarginine (ADMA)** because it directly affects the synthesis of nitric oxide, a key regulator of endothelial dysfunction. Furthermore, ADMA levels are increased by conventional CVD risk factors and some novel risk markers, raising the possibility that it could represent a final common pathway in the atherosclerotic process.

2. **CD40 ligand** because it has a proximal role in the inflammatory pathway regulating other pro-inflammatory cytokines such as interleukins, tumour necrosis factor and interferon-gamma. It is also thought to have a particularly prominent role in atherosclerosis because it promotes the expression of pro-atherogenic mediators such as metalloproteinases, which are poorly regulated by other cytokines. CD40 ligand has also been shown to upregulate the production of adhesion molecules in the endothelium which propagate endothelial dysfunction and inflammation early on in the atherosclerotic process.

3. The **metabolic syndrome** because it has been very clearly defined as a major predictor of CVD risk, and is clinically accessible. The metabolic syndrome is also associated with chronic background inflammation and endothelial dysfunction, both of which are pivotal in the development of atherosclerosis

and subsequent CVD. At the very least, the metabolic syndrome is a marker of high CVD risk. This has been shown in numerous epidemiological studies and the relationship is especially strong in coronary heart disease. The biggest advantage of using the metabolic syndrome as a risk marker is that its diagnosis is based on simple, inexpensive and easily accessible clinical tests which are already available during routine CVD assessment but have not been brought into prominence.

Asymmetric Dimethylarginine (ADMA)

Asymmetric dimethylarginine (ADMA) has been proposed as a novel risk marker for cardiovascular disease[65-68] due to its possible role in endothelial dysfunction[69-71], a process thought to be pivotal in the development of atherosclerosis[48]. Both symmetric dimethylarginine (SDMA) and ADMA are structural analogues (methylated forms) of the amino acid L-arginine, the substrate for the synthesis of nitric oxide by the enzyme nitric oxide synthase[72].

ADMA is an endogenous competitive inhibitor of nitric oxide synthase[65,69]. Therefore, raised levels of ADMA lead to reduced nitric acid production. As nitric oxide is the most potent vasodilator known, reduced nitric oxide production leads to endothelial dysfunction, one of the key pivotal events in the initiation of atherosclerosis.

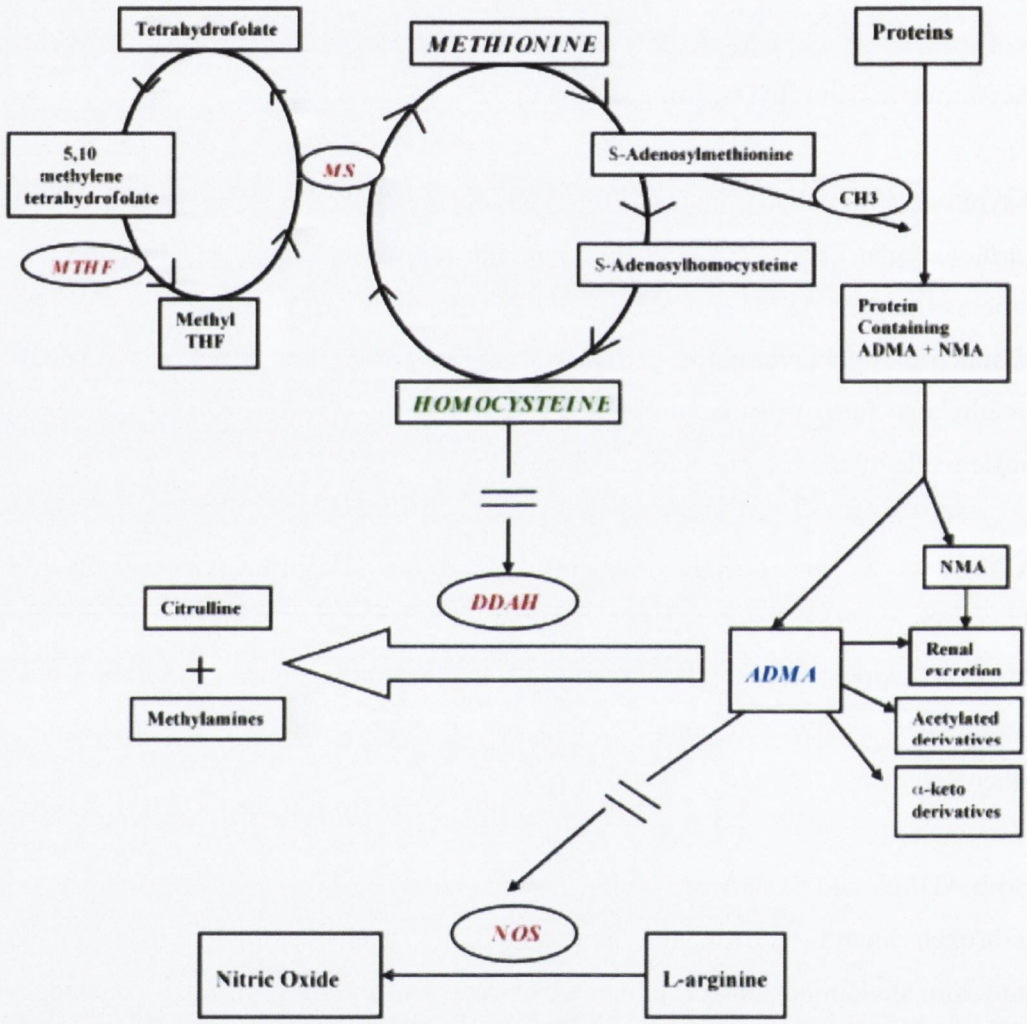
Both ADMA and SDMA are excreted by the kidneys. Most of endogenous ADMA is broken down by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) into dimethylamine and citrulline which are also excreted through the kidneys. Hence, ADMA, as well as SDMA, accumulates in patients with renal failure[69]. However, SDMA does not inhibit nitric oxide synthase, nor is it a substrate for DDAH.

Furthermore, it has been thought that the effect of homocysteine on endothelial function may be mediated through ADMA[73,74]. The association of ADMA with homocysteine has been postulated due to the fact that the methionine-homocysteine

cycle is a major source of methyl groups[74] and that homocysteine may inhibit DDAH, leading to increased ADMA levels, and hence reduced nitric oxide production (Figure 2.6).

Figure 2.6 - Metabolism of ADMA

(Courtesy of Dr Deirdre Ward)



[1] METABOLISM AND INTERACTIONS OF ADMA, HOMOCYSTEINE AND NITRIC OXIDE

NOS – Nitric Oxide Synthase
DDAH – Dimethyldiamino hydrolase
MS – Methionine Synthase
MTHFR – Methylene tetrahydrofolate Reductase

ADMA has been reported to be raised in the presence of other cardiovascular risk factors, as well as in several disease states. These cardiovascular risk factors include traditional risk factors such as blood pressure[72,75,76], serum cholesterol[70], serum triglycerides[77], gestational diabetes[78], insulin resistance[79], smoking, and non-traditional risk markers such as homocysteine[66,74,80], C-Reactive Protein[66] and vascular cell adhesion molecule[81].

Clinical conditions that have been associated with increased ADMA concentrations include peripheral vascular disease[82], increased carotid intima media thickness[66,81,83], chronic heart failure[84], unstable angina[85], stroke[86], hyperthyroidism[87], pre-eclampsia[88], intensive care deaths[89] and coronary artery calcium score[90].

At pathological levels found in end-stage renal failure, ADMA was found to be the second strongest independent predictor of mortality and cardiovascular events after age, and more powerful than other traditional risk factors and C-Reactive Protein[66]. ADMA has also been shown to predict cardiovascular events in patients with Type 2 diabetes[91]. Similarly, ADMA predicted future CVD events in patients with advanced peripheral arterial disease[92]. ADMA, along with N-terminal prohormone Brain Natriuretic Peptide, was also useful in cardiovascular risk stratification in patients with chronic heart failure[93].

There is now an increasing number of studies of ADMA in patients with pre-existing coronary artery disease. One of the earliest is a prospective nested case-control study of middle-aged non-smoking Finnish men which showed ADMA to be an independent predictor of coronary events over traditional risk factors, especially in those who had a history of coronary heart disease[94]. In a Taiwanese population, ADMA levels measured before coronary angioplasty were also found to be an independent predictor of later cardiovascular events, even after allowing for traditional risk factors, homocysteine, C-Reactive Protein, left ventricular function, history of previous cardiovascular disease and medications[95]. The AtheroGene Study, a prospective cohort study on patients with coronary artery disease, showed that ADMA was related with increased CVD events[96]. Similarly, a multi-centre German case-control study also showed ADMA to be associated with increased coronary heart disease risk[97]. Other more recently reported studies include the

Ludwigshafen Risk and Cardiovascular Health Study showing the association of ADMA with all-cause and CVD mortality in an angiographic cohort of patients with coronary artery disease[98], and the population based Monitoring of Trends and Determinants in Cardiovascular Disease/*Kooperative Gesundheitsforschung in der Region Augsburg* study also showed that ADMA predicted future coronary events in non-smoking healthy men using a nested case-control design[99].

ADMA has been inversely correlated with endothelium-dependent vasodilatation in the forearm[100], a measure of endothelial dysfunction. An infusion of L-arginine has been shown to restore endothelial function[100]. There is suggestion that angiotensin converting enzyme inhibitors, angiotensin II receptor blockers and drugs such as metformin, rosiglitazone, oestrogen and statins[101] may also reduce ADMA levels.

CD40 Ligand

CD40 ligand, also known as CD154, is a trans-membrane protein that is a member of the tumour necrosis factor family. It and its receptor CD40 have been implicated in atherothrombosis and atherosclerosis. The gene for CD40 has been located in chromosome 20 (q12-q13.2).

Although initially described on B-lymphocytes and CD4+ T-lymphocytes, CD40 ligand and its receptor CD40, has been found in a wide variety of cells including monocytes, basophils, eosinophils, activated platelets, mast cells, vascular endothelial cells and smooth muscle cells. It is released into plasma as a soluble protein, soluble CD40 ligand. Various cytokines can induce the above cells to express CD40 ligand. In fact, we now know that CD40 ligand can also induce its own expression. The exact mechanism by which the soluble form of CD40 ligand is produced is still unknown but it is believed that it is the membrane-bound CD40 ligand on T-lymphocytes and platelets are shed into the circulation.

The interaction of CD40 and CD40 ligand results in the initiation of several inflammatory processes, indicating its role as an immune-modulator. These include the expression of cytokines, chemokines and growth factors, the upregulation of

adhesion molecules, and the synthesis of matrix metalloproteinases and pro-coagulant factors. These pro-inflammatory mediators include interleukins-1, -6 and -12, and tumour necrosis factor-α. In the endothelial cells and smooth muscle cells, the ligation of CD40 by CD40 ligand results in the production of adhesion molecules such as vascular cell adhesion molecule (VCAM), intercellular cell adhesion molecule (ICAM) and E-selectin. As we understand from our knowledge of the vascular biology of atherosclerosis, these cell adhesion molecules play an important role in endothelial dysfunction in the early stages of atherosclerosis, thus the key importance of both endothelial dysfunction and inflammation are intertwined in the atherosclerotic process.

CD40 ligand has been found to be expressed by T-lymphocytes in human atherosclerotic tissue[102]. Even though the release of CD40 ligand by platelets is associated with the acute plaque rupture of the acute coronary syndromes, CD40 ligand has been implicated in the initiation of atherosclerosis[103].

Soluble CD40 ligand has been shown to be raised in conditions such as hypercholesterolaemia[104,105], especially familial hypercholesterolaemia[106], smoking[107], restenosis after angioplasty[108], post cardiopulmonary bypass[109], lipid accumulation in carotid atheroma[110], diabetes[111], pre-diabetes, diabetes and the metabolic syndrome[112], peripheral arterial disease on angiography[113].

Most of the data relating CD40 ligand with CVD events have been in patients who have had acute coronary syndromes. Rupture prone atherosclerotic lesions, as well as lesions that have already ruptured express the highest levels of CD40 and CD40 ligand. It follows that patients with unstable angina had higher CD40 ligand levels than patients with stable angina and healthy controls[114]. In fact, CD40 ligand levels increased in all 5 of the patients in that study who subsequently developed unstable angina, CD40 ligand levels increased significantly after the development of unstable angina[114]. Similarly, in acute myocardial infarction, levels of CD40 ligand were higher than those who had unstable angina, and in turn higher than those who had stable angina and the controls[115]. This study provided the first indication that CD40 ligand may predict future risk because levels of CD40 ligand were higher in those

patients who needed repeat target vessel angioplasty or had recurrent angina within 30 days[115].

In a re-analysis of a randomised trial comparing abciximab to placebo before coronary angioplasty of 1,088 patients, CD40 ligand were elevated in patients who had acute coronary syndromes at 24 hours, 72 hours, 30 days and 6 months. Among the patients not treated with abciximab, the adjusted HR of death and non-fatal myocardial infarctions at 6 months for raised CD40 ligand levels (>5 μg/L) was 2.71 (95% CI 1.51, 5.35), p=0.001. CD40 ligand also added prognostic value in patients with chest pain where raised soluble CD40 ligand levels identified those with acute coronary syndromes who were at high risk for death or non-fatal MI with an adjusted HR 6.65 (95% CI 3.18, 13.89), p<0.001. This study also identified a sub-group of patients with acute coronary syndromes who have high CD40 ligand levels who have a significant treatment effect by abciximab[116]. The effect of CD40 ligand in this study was independent to hs-CRP and Troponin.

CD40 ligand was examined in 2,908 patients enrolled in the MIRACL study who had aggressive treatment with atorvastatin 80 mg vs. placebo for a 16 week period after an acute coronary syndrome. CD40 ligand levels had a threshold effect on risk with levels >90th percentile associated with recurrent CVD events - OR 1.86 (95% CI 1.25, 2.77) which was abrogated by atorvastatin treatment[117].

A nested case-control study (195 cases and 195 controls) of the patients recruited in the OPUS-TIMI16 trial showed that raised CD40 ligand levels in patients with acute coronary syndromes are associated with an approximately two-fold higher risk of death, recurrent MI and heart failure after multivariable adjustment including hs-CRP and Troponin[118].

A recent Japanese study showed that raised CD40 ligand levels in the culprit arteries of patients with acute MI was associated with an almost nine-fold increased risk of future CVD events after adjustment[119].

There is paucity of data on the role of CD40 ligand in stable populations. The Women's Health Study was the first major study to show the association of CD40 ligand with future CVD events (myocardial infarction, cerebrovascular events and CVD deaths) in a nested case-control design of 130 cases matched by age and smoking with 130 controls. The RR of CVD events after 4 years for CD40 ligand >3.71 ng/mL (>95th percentile) was 2.8 (95% CI 0.9, 8.0), p=0.05 after adjustment for traditional risk factors[120].

Since the *Cath Lab Study* completed recruitment, 3 studies examining the association of CD40 ligand with CVD in stable were reported in the literature. A study of 204 patients from Birmingham, United Kingdom showed that patients with stable coronary artery disease on angiogram had higher levels of CD40 ligand. However, after multivariable regression analyses, the relationship was attenuated. In fact, there was a trend towards a negative association with the presence of coronary artery collaterals that was not statistically significant[121].

An angiographic study of 909 patients from a group in Salt Lake City, Utah, United States using patients from the Intermountain Heart Collaborative Study reported a similar finding of an inverse relationship of CD40 ligand with coronary artery disease on coronary angiography[122]. Their top quartile of CD40 ligand showed an OR of 0.59 (95% CI 0.37, 0.96), p=0.03 of future coronary events defined as combined CHD deaths and acute coronary syndromes within 1 year. They considered the result not statistically significant as they had set the level of statistical significance to p<0.017 from Bonferroni correction. Similar trends were seen in their endpoints of angiographic coronary artery disease alone without events.

Another paper, also by a group in Salt Lake City, Utah, United States who developed a multiplexed immunoassay to simultaneously assess a large number of cytokines, of which CD40 ligand was one, reported no statistically significant difference in CD40 ligand levels between patients who have coronary artery disease compared to those without. The study population consisted of 865 patients with chest pain in whom coronary artery disease was suspected who were enrolled in the Intermountain Heart Collaborative Study [123]. If any, the point estimate of the mean CD40 ligand levels

were higher in the group without coronary artery disease but the result was not statistically significant.

As a potential future target of therapy, the disruption of the CD40-CD40 ligand system has resulted in diminished atherosclerotic lesion formation in hypercholesterolaemic mice[124] and delayed progression of established atheroma and plaque stabilisation in humans[125]. Preliminary intervention studies indicate that soluble CD40 ligand levels can be reduced by drugs such as rosiglitazone[126], troglitazone[127], atorvastatin[106,117,128], simvastatin[106,128], cerivastatin[104,128], pravastatin[104], clopidogrel[129], glycoprotein IIb/IIIa antagonists[130], intensified CVD risk reduction for diabetes[111], ramipril[131].

Therefore, CD40 ligand is implicated in the initiation and progression of atherosclerosis, as well as the acute complications of atherosclerosis leading to acute coronary syndromes.

The Metabolic Syndrome

The metabolic syndrome is characterised by central obesity clustering with several risk factors such as atherogenic dyslipidaemia, hypertension and insulin resistance. Gerald Reaven in his Banting Lecture in 1988 originally proposed insulin resistance as the underlying factor leading this clustering of these risk factors naming it Syndrome X[132]. Other names came, some fanciful, including the insulin resistance syndrome and even the 'deadly quartet'. It is associated with increased cardiovascular risk[133], especially coronary heart disease[134-136], and also the development of diabetes[137,138]. Endothelial dysfunction and inflammation are associated with this syndrome, along with insulin resistance, metabolic disturbances, and impaired fibrinolysis leading to raised levels of glucose, uric acid and C-Reactive Protein.

Central obesity is now thought to be a key driving force for the metabolic syndrome through its active involvement in inflammation leading to endothelial dysfunction and insulin resistance, all which are ingredients for diabetes and cardiovascular

disease. The prevalence of the metabolic syndrome has been previously estimated in various studies to be around 25%[139]. This is worrying public health issue because the incidence and prevalence of obesity worldwide is increasing rapidly due to unhealthy dietary choices and decreased physical activity.

The definition of the metabolic syndrome has evolved over the years from the original syndrome described by Reaven, to various different definitions proposed by organisations such as the World Health Organization (WHO), the European Group for the study of Insulin Resistance (EGIR), American Diabetes Association (ADA), International Diabetes Federation (IDF) and the National Cholesterol Education Program (NCEP). These definitions use different methods to define the various components. For example, the WHO definition uses either body mass index (BMI) or waist-to-hip ratio to define central obesity while the NCEP definition uses waist circumference instead. These original definitions were designed to use clinical definitions to detect the presence of insulin resistance, instead of using the definitive but cumbersome, and potentially high-risk test, the euglycaemic hyperinsulinaemic clamp, during a time when the focus was on insulin resistance. The different definitions have been shown to be useful in predicting an end-point such as diabetes[140,141].

The shift is now towards cardiovascular disease as the primary clinical outcome of the metabolic syndrome[142], and the focus on central obesity as the driver of the metabolic syndrome. The NCEP definition is the easiest to use in the clinical setting and strongly related to CVD as the outcome.

For historical reasons, the main definitions of the metabolic syndrome are listed below as the many definitions were used at some stage by different studies.

The **WHO definition (1998)** of the metabolic syndrome requires:

Diabetes, impaired glucose tolerance, impaired fasting glucose or insulin resistance, clamp glucose uptake (lowest quartile)

AND

2 of the following:

- Abdominal obesity
- Dyslipidaemia
- Hypertension
- Microalbuminuria

Abdominal obesity:

- BMI ≥ 30 or
- Waist:hip > 0.9 (men), 0.85 (women) or
- Waist > 94 cm

Dyslipidaemia:

- HDL < 0.9 mmol/L (men), 1.0 mmol/L (women) and/or
- TG > 1.7 mmol/L

Hypertension:

- BP ≥ 140/90 mmHg (originally 160/90)

Microalbuminuria:

- Urinary albumin excretion rate ≥ 20 mg/min or
- Albumin/creatinine ratio ≥ 30 mg/g

The definition by the NCEP in its Third Adult Treatment Plan (ATPIII) is satisfied when at least 3 of the following 5 characteristics are present:

1. Waist circumference ≥102 cm in Men, ≥88 cm in Women
2. Triglycerides ≥150 mg/dL (1.7 mmol/L)
3. HDL cholesterol <40 mg/dL (1.3 mmol/L) in Men,
   HDL cholesterol <50 mg/dL (1.0 mmol/L) in Women
4. Blood pressure ≥ 130/85 mmHg
5. Fasting glucose ≥110 mg/dL (6.1 mmol/L)

The American Heart Association (AHA) in 2005[143] published a statement after having reviewed the various definitions of the metabolic syndrome, in particular, those that included measures of insulin resistance and ethnicity-based waist circumference cut-points. In conclusion, the AHA recommended the simplicity, clinical utility, and the emphasis on CVD as the outcome of the NCEP ATPIII definition, with only one modification – a reduction of the glucose threshold to 100 mg/dl (5.6 mmol/L). It reflects a shift in emphasis from insulin resistance to central obesity as the primary driver of the metabolic abnormalities associated with the metabolic syndrome.

The AHA-modified ATPIII definition of the metabolic syndrome is satisfied when at least 3 of the following 5 characteristics are present:

1. Waist circumference ≥102 cm in Men, ≥88 cm in Women
2. Triglycerides ≥150 mg/dL (1.7 mmol/L), or on drug treatment
3. HDL cholesterol <40 mg/dL (1.3 mmol/L) in Men,
   HDL cholesterol <50 mg/dL (1.03 mmol/L) in Women, or on drug treatment
4. Systolic BP ≥130 mmHg, or Diastolic BP≥85 mmHg, or on drug treatment
5. Fasting glucose ≥100 mg/dL (5.6 mmol/L), or on drug treatment

NOVELTY OF THE WORK

ADMA is associated with CVD risk factors and adds prognostic information on CVD death in patients with chronic renal failure. Its possible role in predicting myocardial infarction and stroke has not been studied in a population of healthy women as a cohort study with long term follow up.

CD40 ligand has been shown to be useful to stratify risk in patients with acute coronary syndromes. It has also been shown to be a predictor of future CVD risk in women. As CD40 ligand has a role in the initiation and propagation stages of atherosclerosis, we postulate that levels of CD40 ligand could be associated with coronary artery disease severity in patients with chronic stable angina. To our knowledge, this has not been previously examined in patients with stable disease.

Many novel risk markers for CVD have been previously reported and although many studies show their added predictive value individually in CVD risk, this work is unique in that a both conventional risk factors and number of novel risk markers will be studied in combination with the marker of interest, CD40 ligand in the *Cath Lab Study* with objective end-points for coronary artery disease and carotid intima-media thickness.

GENERAL QUESTIONS

1. Is ADMA independently associated with CVD or through associations with other risk factors? (Chapter 3)
2. Is CD40 ligand associated with CVD independently of other risk factors? (Chapters 4 and 5)
3. What role does the metabolic syndrome have in relation to CVD risk assessment? (Chapters 4, 5 and 6)
4. Can CVD risk estimation be simplified by consolidating the large number of conventional risk factors and novel risk markers? (Chapters 4 and 6)

QUESTIONS IN DETAIL

CHAPTER THREE: ADMA with Cardiovascular Disease

1. Is ADMA associated with CVD in an initially healthy population?
2. Is the association independent of conventional risk factors?
3. Can ADMA add predictive value to current risk estimation systems such as the SCORE equation and the Framingham risk score?
4. Are the effects of conventional risk factors and homocysteine on CVD risk mediated through ADMA?

CHAPTER FOUR: CD40 ligand with Coronary Artery Disease

1. Is CD40 ligand associated with the presence, extent and complexity of coronary artery disease in a stable population of patients with suspected coronary disease?
2. Is the association independent of conventional risk factors?
3. Of the many better studied novel risk markers shown to have independent associations with CVD such as high-sensitivity (hs-CRP), homocysteine, vascular cell adhesion molecule (VCAM), P-selectin and fibrinogen, how useful are they to predict risk when taken all in combination?
4. What possible important interaction effects do they exhibit?

5. Do they have added predictive value and can they further re-classify patients into a clinically more important category, for instance, qualifying for different therapy?
6. Can we consolidate the large number of conventional risk factors and novel risk markers into a simpler few to predict coronary artery disease?

CHAPTER FIVE: CD40 ligand with Carotid Intima-Media Thickness

1. Is carotid intima-media thickness (CIMT) associated with CD40 ligand and other conventional risk factors and novel risk markers?
2. Of the large number of conventional risk factors and novel risk markers in combination, which ones are still independently associated with CIMT after multivariable adjustment?
3. Can CIMT predict the presence of coronary artery disease?
4. Of the various methods used to measure CIMT, which definitions and cut-points of CIMT are the most predictive of significant coronary artery disease?

CHAPTER SIX: The Metabolic Syndrome with CVD

1. Is the metabolic syndrome associated with CVD?
2. Is the association independent of conventional risk factors and other novel risk markers such as homocysteine, apolipoproteins B & AI and white cell count.
3. Does the metabolic syndrome predict CVD risk better as a whole, or using its individual components in combination?
4. What combination of factors works best in a risk model for predicting CVD?

GENERAL METHODOLOGY

The above questions were examined through:

1. The analysis of ADMA using stored samples (n=880) of a large prospective cohort study, the *Population Study of Women in Gothenburg*, to examine the possible association of ADMA with CVD.

2. The design and execution of an original research study, the *Cath Lab Study* (n=730), which allowed the examination of the possible association of coronary artery disease on coronary angiography with a large number of conventional risk factors and novel risk markers in combination.

3. The design and execution of a sub-study of Carotid Intima-Media Thickness (n=386) within the *Cath Lab Study* examining the possible association of carotid intima-media thickness with CVD risk factors and coronary artery disease.

4. The examination of a dataset of an old multi-centre case-control study, *The European Concerted Action Project: Homocysteinaemia and Vascular Disease* (COMAC) (n=1,550) to determine how the metabolic syndrome and other novel risk markers fit into CVD risk assessment.

CHAPTER THREE

ASYMMETRIC DIMETHYLARGININE AND CARDIOVASCULAR RISK

This chapter examines the association of ADMA with incident myocardial infarction and stroke. It contains work that has previously been published, as well as unpublished data.

Arteriosclerosis, Thrombosis, and Vascular Biology
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Asymmetric Dimethylarginine Independently Predicts Fatal and Nonfatal Myocardial Infarction and Stroke in Women

24-Year Follow-Up of the Population Study of Women in Gothenburg

Tora Leong, Dimitri Zylberstein, Ian Graham, Lauren Lissner, Deirdre Ward, Jane Fogarty, Calle Bengtsson, Cecilia Björkelund, Dag Thelle; for The Swedish-Irish-Norwegian (SIN) Collaboration

Objective—Asymmetrical dimethylarginine (ADMA) reduces nitric oxide by inhibiting nitric oxide synthase is associated with cardiovascular disease (CVD). Our study examined the association of ADMA with CVD prospectively in a healthy population-based cohort of women.

Methods and Results—We measured baseline ADMA of 880 women in the Population Study of Women in Gothenburg using high-performance liquid chromatography. After adjustment for traditional risk factors, creatinine clearance, and homocysteine using Cox models, the HR (95% CI in parentheses) of CVD end points at 24 years for a 0.15 μmol/L (1 SD) increase in ADMA were: all-cause mortality 1.12 (0.96, 1.32), fatal CVD 1.30 (1.04, 1.62), total CVD events 1.29 (1.09, 1.53). The top quintile (ADMA ≥0.71 μmol/L) compared with the bottom four-fifths, conferred a cumulative risk 22 versus 14%, relative risk 1.75 (95% CI 1.18, 2.59) and population attributable risk 12.7% of total CVD events, and further identified individuals who are at higher than expected risk based on the SCORE and Framingham systems.

Conclusions—A 0.15 μmol/L increase in baseline ADMA levels is associated with approximately 30% increase in incident cardiovascular risk at 24 years in women after adjustment. ADMA levels ≥0.71 μmol/L enhances CVD risk assessment in women. (***Arterioscler Thromb Vasc Biol* 2008;28:961-967**)

Key Words: asymmetrical dimethylarginine ■ cardiovascular diseases ■ myocardial infarction ■ stroke ■ women

INTRODUCTION

Asymmetric dimethylarginine (ADMA) has been proposed as a novel risk marker for cardiovascular disease[65-68] due to its possible role in endothelial dysfunction[69-71], a process thought to be pivotal in the development of atherosclerosis[48]. There is now an increasing number of epidemiological studies of ADMA to examine its association with CVD. The state of the art was reviewed in Chapter 2.

Observational and case-control studies are regarded as less reliable indicators of a possible causal relationship between a risk marker and a disease process than prospective studies in which the marker clearly precedes the occurrence of the disease. Also, there is paucity of studies examining the association of ADMA with incident CVD in healthy populations. In this study, the role of ADMA in predicting cardiovascular risk and its possible relationship with homocysteine and other risk factors was examined in a healthy population based cohort study of women.

METHODS

Study Population

The study population comprised of women recruited to the on-going *Kvinnostudien*, the Population Study of Women in Gothenburg which has been described previously[144-147]. Briefly, the baseline survey of the study took place in 1968/69[144]. The participants were a representative sample of women in Gothenburg, Sweden aged 38, 46, 50, 54 and 60 at entry who were invited from local population registries based on specifically selected days of birth. Of the 1,622 women who were invited, 1,462 (90.1%) underwent the baseline screening survey in 1968/69. For this study, serum samples were not available for the 81 women in the age 60 cohort, making the size of the study population 1,381.

Serum samples

The stored serum samples used in this study were collected over a period of under 1 year at the baseline survey interview and examination in 1968/69. The fasting blood samples were allowed to clot for 2 to 3 hours after collection before being centrifuged at room temperature, and then stored at -20°C in polystyrene cups. The samples used in this study were stored for 28 years at -20°C, before being thawed on 2 separate occasions for other studies. The period between the first[148] and second thaw[149] was 2 years, while that between the second and third thaw (for the current study) was 5 years. After the first thaw, the samples have been stored at -80°C. The samples were transported frozen from Oslo, Norway to Dublin, Ireland packed in dry ice for ADMA determination. ADMA has been previously shown to be stable after 4 freezing and thawing cycles[150].

Laboratory analyses

ADMA levels were determined using a validated high-performance liquid chromatography (HPLC) method[151]. ADMA was converted into fluorescent derivatives with *ortho*-phtaldialdehyde reagent containing 3-mercaptoproprionic acid. The analytes were separated by isocratic reverse-phased HPLC with fluorescence detection. Sample clean-up was performed by solid-phase extraction on polymeric cation-exchange columns using monomethylarginine (MMA) as the internal standard.

The HPLC assay used in the present study was also previously validated in our laboratory in the Department of Clinical Chemistry, Adelaide & Meath Hospital, Dublin, Ireland. The assay was found to be linear over the range 0.1 to 20 μmol/L for ADMA and SDMA which encompasses the normal biological range. In our previous validation study (unpublished), good recovery was achieved for all analytes – arginine (85%), ADMA (99%) and SDMA (97%). The coefficients of variation within-assay were 2.0-5.8% and between-assay were 1.2-7.0% for lower levels and 2.1-2.8% for higher levels. Homocysteine was measured using the Abbot IMX assay in a previous study[149].

Statistical Methods

Follow up data (endpoints) at 24 years was used for this study because for the 24 year follow up, special consideration had been given to participation, representativeness after a long follow-up period, and comparison of mortality between participants and non-participants[147]. Cox's proportion hazards models were used to examine the association of ADMA as a continuous variable and time as the dependent variable with the following cardiovascular end-points at 24 years follow up:

1. Total mortality
2. Non-cardiovascular deaths
3. Cardiovascular deaths (myocardial infarction & stroke)
4. Myocardial infarction deaths
5. Fatal & non-fatal cardiovascular events (myocardial infarction & stroke)
6. Fatal & non-fatal myocardial infarctions

All analyses were stratified by the age cohorts, and accounted for survival time which was up to 24 years. Risk factor levels from the baseline survey were used and were examined as continuous variables. Non-smokers were defined as those who never smoked and ex-smokers. Blood pressure was measured in the sitting position. Creatinine clearance was calculated using the Cockroft & Gault equation.

Analyses involving ADMA stratified by age were followed by multivariable models which consisted of sequentially adding further pre-specified covariates. Model 1 adjusted for established cardiovascular risk factors commonly used in risk assessment (systolic & diastolic blood pressure, serum cholesterol and smoking). Model 2 further adjusted for traditional cardiovascular risk factors which are less established in terms of causality but nevertheless easily obtained and commonly available (serum triglycerides, plasma glucose, waist & hip circumference and body mass index). Model 3 also included creatinine clearance, homocysteine and vitamin B12.

Clinical Utility

In the interest of having a cut-point of clinical utility, the top quintile of the distribution of ADMA was considered. The cumulative hazard of cardiovascular risk of the group in the top one-fifth was compared to the group in the bottom four-fifths using Nelson-Aalen analyses. The relative risk and population attributable risk conferred by this level of ADMA was examined and compared with those of the traditional risk factors. Furthermore, whether this level of ADMA will enhance cardiovascular risk assessment was examined and the CVD risk estimation systems used were the Systematic COronary Risk Evaluation (SCORE) system[37], the risk assessment system recommended by the European Third Joint Task Force for Cardiovascular Disease Prevention in Clinical Practice[54], and the Framingham risk score as recommended by the Third Adult Treatment Plan[152] (ATPIII) of the National Cholesterol Education Program. The software used for the statistical analyses was Intercooled Stata 9.2 for Windows.

RESULTS

Of the total 1,381 in our study population, 998 had stored serum samples available for laboratory analysis. Of these, 28 were unsuitable for laboratory determination of ADMA levels and a further 82 were either duplicated or triplicated for 32 women. All duplicated samples were averaged except for 3 which were excluded due to wide disagreements between ADMA or SDMA levels within the same individual. We excluded 5 women who had ADMA levels which were very far outside the normal distribution ($\geq$ 2.0 μmol/L). The final number of women who had ADMA levels included in the statistical analyses for the current study was 880 (63.7%). The baseline characteristics of the study population are summarised in Table 3.1.

Table 3.1 - Baseline Characteristics of the study population, standard deviations in parentheses unless otherwise stated

	Age cohort at baseline (Numbers recruited at baseline)				**Total Number N=1,381**
	Age 38 cohort (n=372)	**Age 46 cohort** (n=431)	**Age 50 cohort** (n=398)	**Age 54 cohort** (n=180)	
With available samples (n, %)	**182 (48.9%)**	**317 (73.5%)**	**282 (70.9%)**	**89 (49.4%)**	**880 (63.7%)**
ADMA (μmol/L)	0.59 (0.15)	0.60 (0.16)	0.64 (0.14)	0.65 (0.16)	0.62 (0.15)
SDMA (μmol/L)	0.41 (0.14)	0.40 (0.10)	0.40 (0.10)	0.42 (0.13)	0.40 (0.12)
Systolic BP (mmHg)	121 (15)	131 (20)	138 (22)	143 (24)	132 (21)
Diastolic BP (mmHg)	81 (9)	84 (10)	88 (11)	90 (12)	85 (11)
Cholesterol (mmol/L)	6.3 (0.9)	6.8 (1.5)	7.2 (1.1)	7.3 (1.1)	6.9 (1.3)
Smokers (%)	45%	44%	36%	37%	41%
Glucose (mmol/L)	3.9 (0.6)	4.1 (0.7)	4.2 (1.0)	4.3 (1.0)	4.1 (0.8)
Triglycerides (mmol/L)	1.1 (0.4)	1.2 (0.7)	1.3 (0.6)	1.4 (0.7)	1.2 (0.6)
Waist circumference (cm)	71 (8)	72 (7)	75 (9)	75 (9)	73 (8)
Hip circumference (cm)	98 (7)	99 (7)	100 (8)	100 (8)	99 (7)
BMI	23.1 (3.5)	23.5 (3.2)	24.8 (3.9)	24.6 (4.0)	23.9 (3.7)
Homocysteine (μmol/L)	10.8 (4.2)	11.6 (4.1)	11.7 (3.9)	13.7 (4.7)	11.7 (4.2)
Creatinine (μmol/L)	73.6 (13.2)	75.2 (11.7)	76.2 (39.2)	77.9 (13.8)	75.5 (24.8)

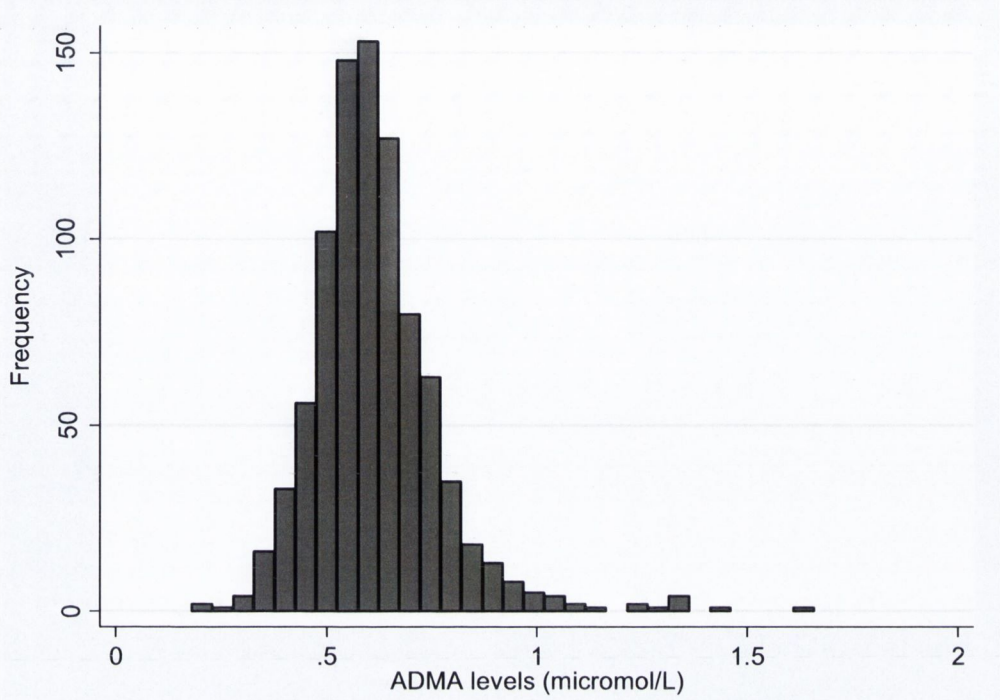
Vitamin B12 (pmol/L)	407 (132)	396 (129)	395 (142)	396 (144)	398 (135)
Previous MI (n, %)	1 (0.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.1%)
Previous Stroke (n, %)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.6%)	1 (0.1%)
Known Diabetics (n, %)	1 (0.5%)	0 (0%)	5 (1.7%)	1 (1.1%)	7 (0.8%)
Premenopausal (n, %)	180 (98.9%)	259 (81.7)	131 (44.9)	2 (2.3)	572 (65.0%)

There were no significant differences in risk factor distributions between those with stored samples available for ADMA determination and those without stored samples (Table 3.2).

Table 3.2 - Comparison of selected characteristics of women with and without stored samples available for ADMA determination

	Women with stored samples for ADMA determination Mean (SD) (n=880)	**Women without stored samples for ADMA determination Mean (SD) (n=501)**	**P value**
Systolic blood pressure (mmHg)	132 (21)	135 (23)	0.19
Diastolic blood pressure (mmHg)	85 (11)	86 (12)	0.51
Serum Cholesterol (mmol/L)	6.9 (1.3)	6.8 (1.1)	0.08
Serum Triglycerides (mmol/L)	1.2 (0.6)	1.2 (0.5)	0.30
Homocysteine (μmol/L)	11.7 (4.2)	11.9 (5.3)	0.48
Creatinine (μmol/L)	75.5 (24.8)	74.2 (15.4)	0.28

Figure 3.1 - Distribution of ADMA in our cohort of healthy women



Spearman's correlations of ADMA with other traditional and non-traditional risk factors generally showed weak univariate association, but were statistically significant for age ($r=0.23$, $p<0.001$), cholesterol ($r=0.11$, $p<0.001$), smoking ($r=0.07$, $p=0.02$), triglycerides ($r=0.12$, $p<0.001$), waist circumference ($r=0.15$, $p<0.001$), hip circumference ($r=0.07$, $p=0.03$), BMI ($r=0.12$, $p<0.001$) and vitamin B12 ($r=-0.24$, $p<0.001$).

Table 3.3 shows the hazard ratios of these endpoints for a one standard deviation increase in ADMA levels (0.15 μmol/L) determined using Cox's proportional hazards models, sequentially adjusting for more traditional cardiovascular risk factors, creatinine clearance, homocysteine and its covariates, using time to event as the dependent variable. After adjustment (Model 3), there was a statistically significant increase in total cardiovascular deaths (myocardial infarction & stroke) HR 1.30 (95% CI 1.04, 1.62), total fatal & non-fatal myocardial infarction & stroke HR 1.29 (95% CI 1.09, 1.53) and total fatal & non-fatal myocardial infarction HR 1.33 (95% CI 1.08, 1.63). This effect of ADMA was independent of traditional

cardiovascular risk factors, creatinine clearance and homocysteine. The magnitude of the increased risk conferred by ADMA was consistent and robust through the sequential adjustments during the multivariate analyses.

Table 3.3 - HR of the various endpoints for a 1 standard deviation (0.15 µmol/L) increase in ADMA (95% CI in parentheses), all analyses stratified by the age cohorts

Endpoints	Univariate	Model 1	Model 2	Model 3
Mortality				
All-cause mortality (138 deaths)	1.14 (0.98, 1.32) p=0.10	1.14 (0.97, 1.32) p=0.11	1.13 (0.97, 1.32) p=0.13	1.12 (0.96, 1.32) p=0.16
MI & Stroke deaths (52 deaths)	**1.28 (1.04, 1.59) p=0.02**	**1.29 (1.05, 1.60) p=0.02**	**1.30 (1.05, 1.62) p=0.02**	**1.30 (1.04, 1.62) p=0.02**
Non-cardiovascular deaths (86 deaths)	1.03 (0.83, 1.27) p=0.77	1.02 (0.82, 1.27) p=0.88	1.00 (0.80, 1.26) p=0.99	0.99 (0.79, 1.25) p=0.91
MI deaths (24 deaths)	1.31 (0.97, 1.78) p=0.08	1.35 (0.99, 1.83) p=0.06	1.32 (0.94, 1.86) p=0.12	1.30 (0.90, 1.88) p=0.16
Stroke deaths (28 deaths)	1.26 (0.93, 1.70) p=0.13	1.25 (0.93, 1.68) p=0.13	1.27 (0.95, 1.70) p=0.11	1.24 (0.92, 1.67) p=0.16
Total Events (Fatal & non-fatal)				
MI & Stroke events (101 events)	**1.32 (1.13, 1.54) p<0.001**	**1.33 (1.13, 1.56) p<0.001**	**1.28 (1.08, 1.51) p=0.004**	**1.29 (1.09, 1.53) p=0.002**
MI events (58 events)	**1.38 (1.15, 1.66) p=0.001**	**1.38 (1.14, 1.67) p=0.001**	**1.31 (1.07, 1.61) p=0.008**	**1.33 (1.08, 1.63) p=0.007**
Stroke events (43 events)	1.20 (0.92, 1.58) p=0.18	1.21 (0.92, 1.59) p=0.18	1.21 (0.91, 1.60) p=0.20	1.23 (0.93, 1.64) p=0.15

The models in Table 3.3 assumed a multiplicative effect of risk factors. There were no statistically significant interactions between ADMA and smoking status, diabetes (glucose ≥7.1 mmol/L) and obesity (BMI ≥30). There were also no statistically significant interactions found between raised ADMA (ADMA ≥0.71 μmol/L) with systolic and diastolic blood pressure, triglycerides, total and HDL cholesterol. There was no evidence of the existence of a non-linear association of ADMA with risk, firstly using ADMA as a higher order term, and also testing for differences in likelihood ratio between multivariate continuous and the categorical models of ADMA.

In order to determine a possible cut-point for clinical use, the cumulative risk of women in the top quintile of the distribution of ADMA levels (≥ 0.71 μmol/L) was compared to those of the bottom four-fifths. Across the quintiles, the risk of incident fatal and non-fatal myocardial infarction & stroke increased only from the fourth quintile. Compared to the bottom fifth, the risk ratios across the quintiles (1-5) were: 1.0, 1.0, 0.7, 1.3, 1.4 (Table 3.4).

Table 3.4 - Relative risk of fatal and non-fatal incident myocardial infarction & stroke for ADMA across the quintiles compared to the bottom fifth

ADMA Quintile	RR compared to Quintile 1	95% CI
1 (<0.51 μmol/L)	1.0	-
2 (≥0.51 μmol/L)	1.0	0.5, 1.9
3 (≥0.57 μmol/L)	0.7	0.3, 1.5
4 (≥0.63 μmol/L)	1.3	0.7, 2.4
5 (≥0.71 μmol/L)	1.4	0.8, 2.6

The ability of ADMA to discriminate fatal and non-fatal incident myocardial infarction & stroke by 24 years was examined using a logistic regression and the areas under the ROC curve for the various models are shown in Table 3.5.

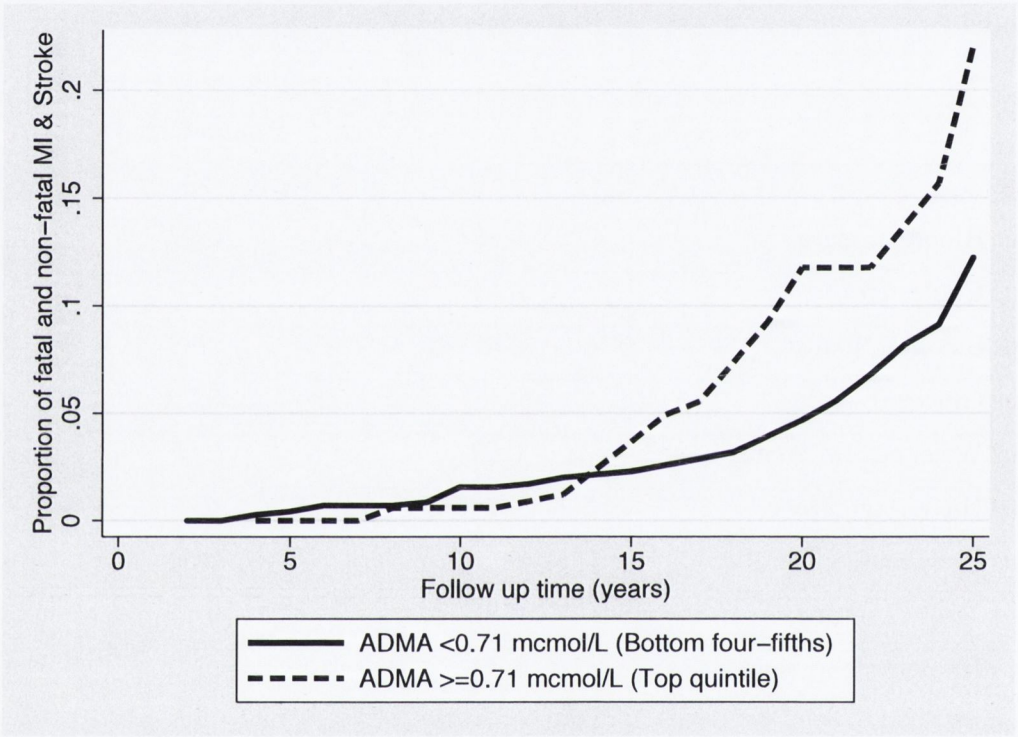
Table 3.5 - The effect on the areas under the ROC curve (*C*-statistic) of incrementally adding the various variables used in our predictive models for incident fatal and non-fatal myocardial infarction & stroke by 24 years, in order of ease of clinical availability after the established risk factors

Model	***C*-statistic**	**P value (compared to previous model)**
Age	0.59	-
Plus Blood pressure	0.60	0.27
Plus Cholesterol	0.61	0.19
Plus Smoking status	0.65	0.08
Plus Glucose	0.65	0.79
Plus Waist circumference	0.69	0.02
Plus Hip circumference	0.70	0.46
Plus Body mass index	0.71	0.59
Plus Triglycerides	0.71	0.91
Plus Homocysteine (incl. vitamin B12)	0.71	0.31
Plus ADMA (incl. GFR)	0.72	0.39

The discriminative ability of ADMA $\geq$ 0.71 μmol/L for incident fatal & non-fatal myocardial infarction and strokes by 24 years was: sensitivity 30%, specificity 82%, positive predictive value 18% and negative predictive value 90%.

A higher cumulative risk of total cardiovascular events (fatal & non-fatal myocardial infarction & stroke) was found for those in the top quintile of ADMA (22% vs. 12%, p=0.004) (Figure 3.2). The relative risk of ADMA (≥ 0.71 μmol/L) for incident fatal and non-fatal myocardial infarction & stroke was 1.75 (95% CI 1.18, 2.59).

Figure 3.2 – Cumulative risk of total cardiovascular events (fatal and non-fatal myocardial infarction & stroke) over the 24 year follow-up period



The population attributable risk of ADMA (≥ 0.71 μmol/L) for incident fatal & non-fatal myocardial infarction & stroke was 12.7% in this study. The following table compares the relative and population attributable risks of ADMA with other traditional and non-traditional risk factors (Table 3.6).

Table 3.6 - Comparison of the relative and population attributable risks of ADMA with other traditional risk factors

Risk factor	**Relative risk (95% CI)**	**Population Attributable Risk**
ADMA ≥ 0.71 μmol/L (top quintile)	1.75 (1.18, 2.59)	12.7%
Blood pressure > 140/90 mmHg	1.52 (1.06, 2.20)	16.3%
Serum cholesterol ≥ 7.8 mmol/L (top quintile)	1.56 (1.04, 2.34)	9.6%
Smoking	1.71 (1.18, 2.47)	22.6%
Serum triglycerides ≥ 1.5 mmol/L (top quintile)	2.01 (1.37, 2.94)	16.4%
Homocysteine ≥ 14.0 μmol/L (top quintile)	1.38 (0.92, 2.08)	7.4%

For the purposes of cardiovascular risk assessment in healthy individuals, most clinicians use a combination of traditional risk factors. The Systematic COronary Risk Evaluation (SCORE) model is the risk assessment system recommended by the European Third Joint Task Force for Cardiovascular Disease Prevention in Clinical Practice. Figure 3.3 illustrates the cumulative CVD events of the women depending on whether they were low-risk (SCORE <5%) or high-risk (SCORE ≥5%) under the SCORE system.

Figure 3.3 – Cumulative risk of total cardiovascular events categorised by SCORE low- and high-risk

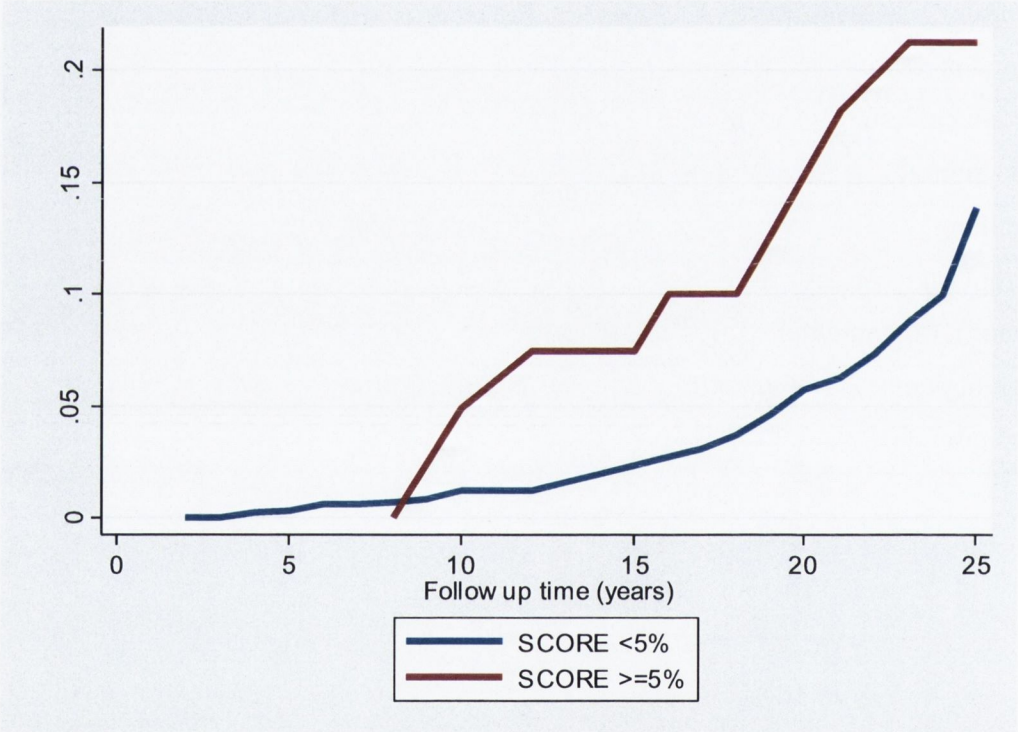
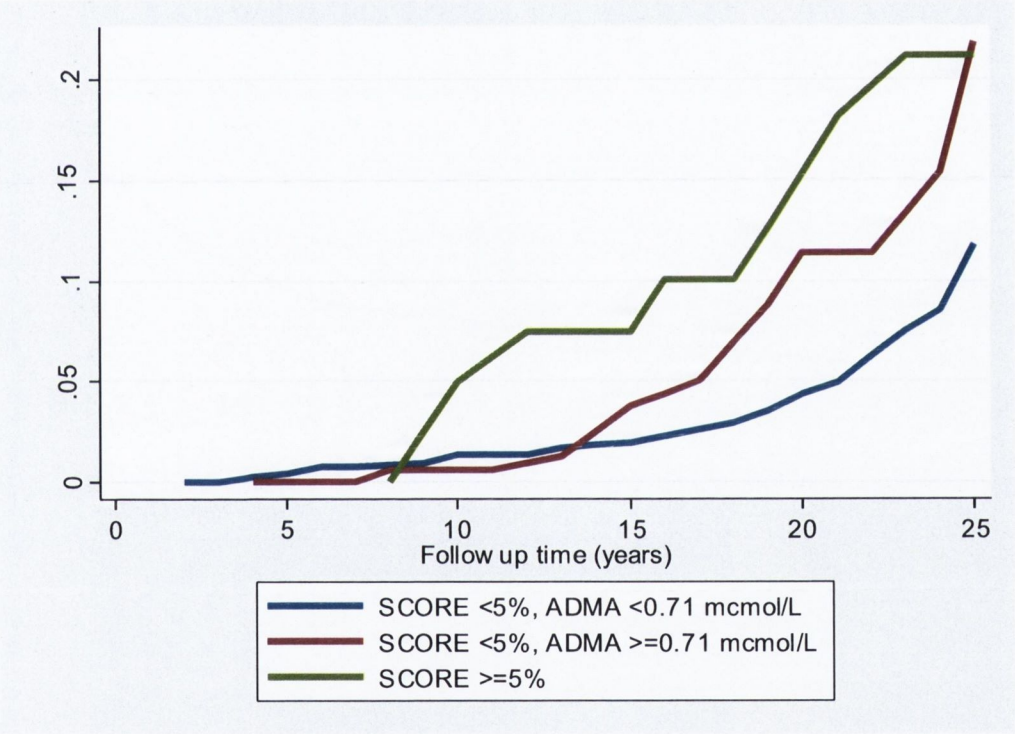


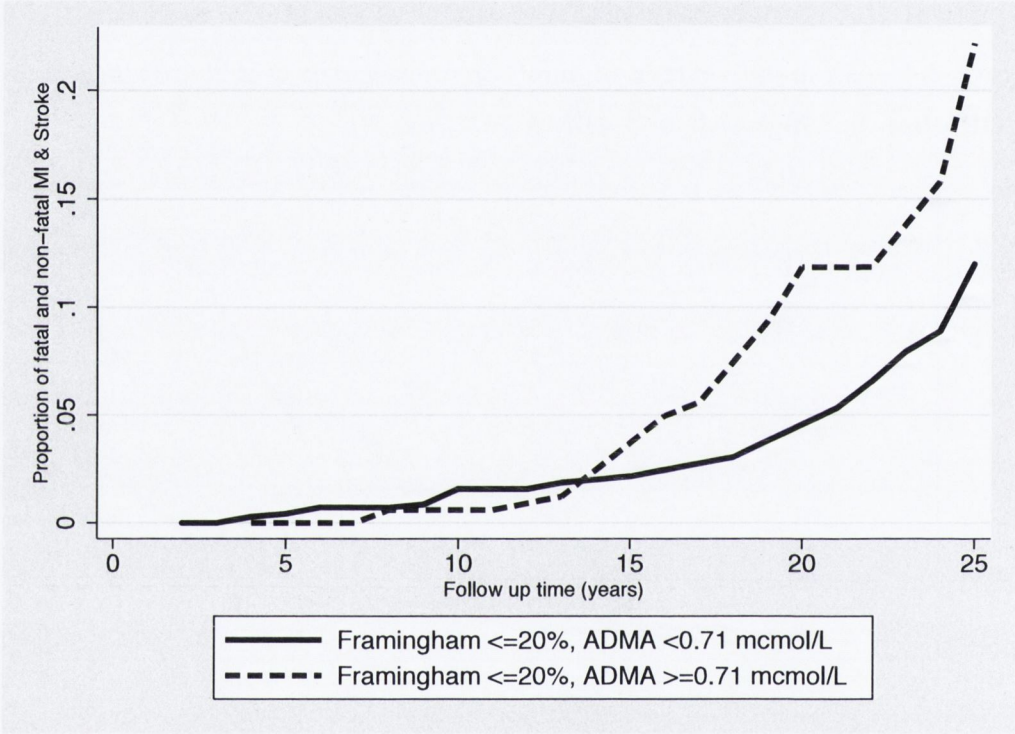
Figure 3.4 shwos that in the 836 women at low-risk estimated by the SCORE system (10-year risk of CVD death of <5% now or projected to age 60), those with levels of ADMA ≥0.71 μmol/L are at higher cumulative risk of incident MI & stroke by 24 years compared to those with ADMA <0.71 μmol/L (22% vs. 12% risk of incident MI & stroke by 24 years, p=0.003). In fact, the low-risk women with ADMA ≥0.71 μmol/L had a cardiovascular risk similar to the women who were identified as high-risk at baseline by the SCORE system by 24 years (22% vs. 21%, p=0.68).

Figure 3.4 – Cumulative risk of total cardiovascular events categorised by SCORE low- and high-risk, and ADMA ≥0.71 μmol/L



Similarly, in Figure 3.5, ADMA ≥0.71 μmol/L also further stratified the 875 women identified by the Framingham risk score as low- and intermediate-risk (10 year risk of coronary heart disease ≤20%) into 2 significant risk categories (22% vs. 12% risk of incident MI & stroke by 24 years, p=0.003).

Figure 3.5 – Cumulative risk of total cardiovascular events in Framingham low-risk women stratified by ADMA



DISCUSSION

This study showed that a 0.15 μmol/L (1 standard deviation) increase in ADMA levels was associated with an approximate 30% increase in incident myocardial infarction and stroke, both fatal and non-fatal, in women after 24 years of follow-up, accounting for time to event. This effect of ADMA was independent of cardiovascular risk factors commonly used for risk assessment. The association with risk also remained robust when adjusted for other traditional risk factors, creatinine clearance and homocysteine, with no statistically significant interactions between ADMA and other traditional cardiovascular risk factors, in particular smoking and those associated with insulin resistance, again supporting the independent effect of ADMA on cardiovascular risk.

The risk for all-cause mortality was increased by approximately 15% for each 0.15 μmol/L increase in ADMA levels. Although this did not reach statistical significance, the hazard ratio was consistent through the adjustments. The neutral effect of ADMA on the non-cardiovascular causes of death implies that the lack of statistical significance of total mortality was due to the dilution of the increased risk of myocardial infarction and stroke deaths, rather than some 'protective' effect of ADMA on other causes of death in this population.

While noting that the relationship of ADMA with risk was continuous, this study indicated the potential of ADMA levels of 0.71 μmol/L or more (the top quintile of distribution) as a possible cut-point for clinical use, as this gave a relative risk of future myocardial infarction and stroke events of 1.75 (95% CI 1.18, 2.59) and a population attributable risk of 12.7%. Separation of the cumulative risk curves for incident myocardial infarction and stroke (Figure 1) was only seen at about 15 years. This finding may be consistent with the gradual nature of the atherosclerotic process in response to endothelial dysfunction, and that perhaps ADMA is a particularly useful marker to identify those at risk for early primary prevention. In particular, for the 24 year follow-up used in the study, special consideration had given to the representativeness of the cohort with the general population.

Several limitations of the study were considered. Serum samples were not available in 36% of subjects. However, the cardiovascular risk factor profile of the missing subjects was indistinguishable from those included, rendering a systematic bias improbable. It is possible that their inclusion, by increasing the sample size, would have strengthened our findings.

The age and history of thawing of the serum samples were also of concern, though we know from previous experiments that ADMA is stable after at least 4 freeze-thaw cycles[150]. Any possible errors that may have arisen would have been systematic as the samples were collected within a short time span (1 year) and underwent exactly the same thawing and refreezing process and conditions. The range and distribution of ADMA levels were very similar to those in a previous study on middle-aged Finnish men, a population based study of ADMA and cardiovascular disease[94], and also similar to a recent study which sought to identify reference values for ADMA in healthy men[153]. While this study population was exclusively women, previous studies had shown no differences in ADMA levels between men and women[151,154].

The possibility of residual confounding by unmeasured risk factors such as C-Reactive Protein has also been considered. This is unlikely as the effect of ADMA remained robust even when adjusted for the established traditional risk factors. Also, other studies have shown the effect of ADMA remains independent when adjusted for C-Reactive Protein as well as other risk factors[66,96,155].

In the statistical models used, the inclusion of ADMA did not yield a significant improvement in discrimination for incident cardiovascular events as a continuous variable using the *C*-statistic. Nevertheless, this was also true of the established risk factors such as blood pressure and cholesterol. However, in cardiovascular risk estimation, the ability of the marker to reclassify subjects into other risk categories requiring a change in management is more clinically important rather than assessments of discrimination using the areas under the ROC curve[156].

A particular strength of this study is its prospective nature, and to my knowledge, the first report of ADMA in association with cardiovascular risk in a true healthy population based cohort of women utilising the entire cohort that had available

serum samples. The robustness of the magnitude of the risk through the numerous adjustments for traditional and non-traditional risk factors also strengthens the association. More importantly, in terms of clinical practice, ADMA enhances cardiovascular risk assessment by further stratifying women who were not identified as high-risk by both the SCORE and Framingham systems, both of which are the predominant cardiovascular risk assessment systems in Europe and North America respectively. This may have important implications as the high-risk category is often used as a threshold for intensive lifestyle and possibly drug intervention. The role of ADMA was not considered to be useful in those who were already identified as high-risk by both the SCORE and Framingham risk assessment system as those individuals are already targeted for intensive management in most guidelines.

In conclusion, ADMA is an independent predictor of fatal and non-fatal myocardial infarction and stroke in women even after allowing for the effects of traditional and non-traditional risk factors of cardiovascular disease. Our study also suggests that the effect of ADMA on cardiovascular disease operates independently of homocysteine and creatinine clearance. It is reasonable to now consider whether reducing ADMA levels would improve endothelial function and reduce cardiovascular disease risk.

CHAPTER FOUR
THE ROLE OF CD40 LIGAND AND OTHER NOVEL RISK MARKERS IN STABLE CORONARY ARTERY DISEASE

INTRODUCTION

Atherosclerosis is an inflammatory process. CD40 ligand is a novel risk marker of interest for CVD because it has a key pivotal role in the inflammatory process and regulates other markers of inflammation. CD40 ligand has already been shown to be of additional value to stratify CVD risk in patients who have had acute coronary syndromes. There is paucity of data on the role of CD40 ligand in stable patients. The rationale for studying CD40 ligand was reviewed in the state of the art in Chapter 2 of this work.

Many previous studies of novel risk markers showing an independent association have studied each novel risk marker singly and adjusted for conventional risk factors. In this study, the *Cath Lab Study*, there is a unique opportunity to examine the association of not only CD40 ligand, one of the novel risk markers of interest, but to examine its association in combination with both conventional risk factors, the other traditional risk factors, and a number of novel risk markers in combination in multivariable analyses using definite endpoints of coronary artery disease as well as carotid intima-media thickness (Chapter 5) in a sizeable population of stable patients.

AIMS

1. To examine the association of CD40 ligand and other novel risk markers with coronary artery disease, and whether there is a relationship to the complexity of disease.
2. To determine if the association is independent and whether it adds predictive value to conventional risk factor and other risk markers.
3. To determine if interaction effects exist in various sub-groups and with other risk factors.
4. To determine what combination of these risk markers offer the best prediction for the presence of coronary artery disease.

METHODS

The *Cath Lab Study* was an original study specifically designed and carried out in order to examine the association between novel risk markers with angiographic evidence of coronary artery disease. The power calculation was based on detecting with 90% power, an odds ratio of 1.8 at the 5% level of statistical significance, of the top quintile of the distribution of CD40 ligand compared with the bottom four quintiles. The final number recruited was 730 patients in total between 2 centres - the Adelaide & Meath Hospital (n=550) and St. James's Hospital (n=180), Dublin, Ireland.

All patients who presented electively on the day case coronary angiography list who satisfy the following inclusion and exclusion criteria were invited to the study. The patients were recruited with the assistance of two research nurses, and informed consent was obtained after verbal explanation of the study and patient information leaflets. The study had the approval of the ethics committee of both recruiting hospitals. Those who satisfied the following inclusion and exclusion criteria were recruited into the study.

Inclusion & Exclusion Criteria

All adults (>18 years) who gave their consent into the study who did not have the following exclusions:

1. Acute Coronary Syndrome, including unstable angina, non-ST segment elevation myocardial infarction and ST segment elevation myocardial infarction in the last 30 days.
2. Active intercurrent infections such as pneumonia, cellulitis, infected ulcers, urinary tract infections, and so on.
3. Known chronic inflammatory conditions such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, chronic renal failure *et cetera.*
4. Neoplastic disease.
5. Any severe acute illness in the last 90 days.
6. Nitrous oxide anaesthesia in the last 90 days.
7. Hypothyroidism.
8. Chronic renal failure.
9. Steroid and anti-inflammatory drugs.
10. Anti-epileptic medications.

These exclusion criteria were set because CD40 ligand as well as hs-CRP, and perhaps other novel risk markers are associated with inflammation. These will be elevated due to acute illnesses and in acute coronary syndromes. As homocysteine is another marker with prognostic value which the CD40 ligand is compared to, conditions which may affect homocysteine and its assay were also in the exclusions criteria and these include drugs such as antiepileptics, nitric oxide anaesthesia, and diseases such as renal failure and hypothyroidism.

Data Collection

Recruitment and data collection was carried out with the assistance of 2 research nurses after informed consent. Assistance was given to the patients in completing the questionnaire. The following information was collected for the study:

1. **Patient Demographics**:
    - Date of birth
    - Sex
    - Occupation
    - Nationality
    - Education completed: Primary/Secondary/Tertiary

2. **Medical History**:
    - Details of usual angina and details of previous acute coronary syndromes
    - Past Medical History
    - Previous CVD including coronary heart disease, cerebrovascular disease and peripheral vascular disease
    - CVD Risk factors:
        - Any Family history, and whether Premature
          Premature Family History (Men <55 years, Women <65 years)
        - Known Diabetes – type, duration and treatment
        - Smoking – current and previous status
        - Known Hypertension
        - Cholesterol
    - Physical Activity (Exercise levels according to CHAIR database):
        - No exercise
        - Light exercise (<20 min up to 4 times/week)
        - Moderate exercise (>20 min >4 times/week)
        - Heavy exercise (>20 min >5 times/week)
    - Alcohol consumption
    - Drug history

- Aspirin
- Clopidogrel
- NSAIDs
- Other Anti-platelet drugs
- ACE Inhibitors
- Angiotensin Receptor Blockers (ARBs)
- Statins
- Steroids
- Nitrates
- Others

- Length of time fasted

3. **Physical Measurements** (see Specific Methodologies):
   - Height
   - Weight
   - Waist circumference
   - Hip circumference
   - Blood pressure

4. **Laboratory Measurements** (see following section on Laboratory Assays for further details):
   - General haematology, biochemistry
   - High sensitivity C-Reactive Protein
   - Homocysteine
   - CD40 ligand
   - VCAM
   - P-selectin

Endpoints

1. **Coronary Angiography**

2. **Carotid Intima Media Thickness** (Chapter 5)

Study Procedure

1. At the start of the coronary angiogram, immediately after insertion of the femoral arterial access sheath, 60 ml of blood was drawn and immediately bottled into the following proportions – 10 ml EDTA, 20 ml citrate, 10 ml heparin and 20 ml clotted samples.

2. The bloods were immediately spun for the vast majority. If not, the bloods were never at room temperature for more than 2 hours. Centrifugation was performed at 2,500 rpm at room temperature for 15 min and then transferred into colour-coded aliquots and bar-coded with a standardised patient study ID number generated for this study.

3. The aliquots were immediately frozen and stored at -70°C in a freezer on the regular laboratory maintenance and servicing schedule.

4. The images of the coronary angiogram were stored in DICOM format on an extra CD-ROM (copy) with the patient identification removed and coded using our standardised patient study ID.

5. The coronary angiograms were all analysed by me off-line using a DICOM viewer, OsiriX 32-bit v.3.7.1 according to the protocol described in the next section (see Coronary Artery Disease Score). The SYNTAX score was calculated using the online SYNTAX score calculator as an additional end-point and protocol addition as the SYNTAX study was only reported after the *Cath Lab Study* has completed recruitment.

6. A sub-set of the study patients had carotid intima-media thickness assessment using ultrasound as a sub-study (Chapter 5). The majority of the carotid ultrasounds were done on the same day as the coronary angiogram. Those who did not were brought back within 90 days to have the carotid ultrasound performed. Carotid intima-media thickness assessment was not done on all patients firstly because the methodology was not fully finalised until the *Cath Lab Study* was underway, and secondly the routine list for coronary angiography included elective transfers from institutions in Naas and Wexford, approximately 30 km and 130 km away respectively, and a significant number had chosen not to have their carotid ultrasounds done.

Specific Methodologies

The following measurements were made according to the methodologies recommended by *Cardiovascular Survey Methods* published by the World Health Organization to standardise methods of epidemiological survey measurements.

Blood pressure was measured prior to the coronary angiogram soon after the patient has checked into the day ward using a validated blood pressure cuff. A minimum of 2 readings were made at least 2 minutes apart. If the difference is more than 10 mmHg, a third reading was taken. The average of 2 blood pressure readings with a difference of <10 mmHg was used as the blood pressure.

Waist circumference was measured at the halfway point between the lowest level of the costal margin and the anterior superior iliac spine. Waist circumference was measured with the patient standing up dressed with only a gown on, both on inspiration and expiration and the 2 measurements averaged.

Hip circumference was taken at the level of the greater trochanter feet together.

Height was measured with shoes off using a wall stadiometer.

Weight was measured with shoes off and any heavy outer garments removed.

Laboratory Assays

Routine haematology, biochemistry and fibrinogen were performed using standardised assays as part of the patients' assessment for their coronary angiograms. The other novel risk markers were analysed at a later stage using the stored blood samples by the Department of Clinical Chemistry at the Adelaide & Meath Hospital, Dublin, Ireland. The laboratory had standard quality control and maintenance schedules in place.

The novel risk markers were analysed using following assays:

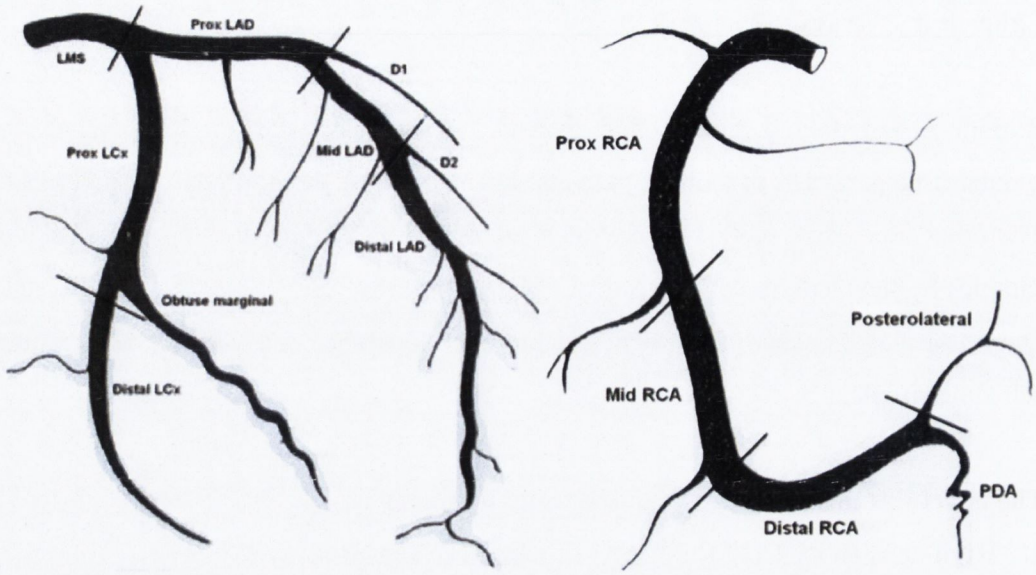
1. **High-sensitivity CRP** (hs-CRP): Roche High sensitivity assay
2. **Homocysteine**: Abbott IMX assay
3. **CD40 ligand**: R&D Systems Human soluble CD40 Ligand Immunoassay
4. **VCAM**: R&D Systems
5. **P-selectin**: R&D Systems

Coronary Artery Disease score

Angiographic images were stored on CD-ROM for off-line analysis. Multiple views according to standard coronary angiography protocol were taken to able to focus on all specific regions of the coronary artery tree and to give as accurate an impression of disease severity as possible. This would also minimise potential errors based on the fact that coronary lesions could be eccentric in nature, where one view may under- or over-estimate the severity due to the fact one is viewing a three-dimensional structure on a two-dimension plane. Figure 4.1 shows how the coronary arteries are divided for our disease scoring system which is based on the Coronary Artery Surgery Study[157] (CASS) used by the Bypass Angioplasty Revascularization Investigation[158] (BARI) investigators and endorsed by the American Heart Association[159], with minor modifications.

Figure 4.1 – Coronary Artery Segments for Disease Scoring

Left Coronary System **Right coronary system**



Referring to Figure 4.1, for each segment, a score was given to the maximum stenosis seen visually in that particular segment as follows:

0: Normal – Completely smooth

1: 1-49%

2: 50-75%

3: 76-99%

4: Totally occluded

The score for all 14 segments was added up to give a score representing total coronary artery disease load, giving a theoretical range of scores from 0 to 56 for the native coronary arteries. If coronary artery bypass grafts were present, they were given a score each for the most severe lesion.

The SYNTAX score

The scoring system described above for coronary artery disease gives a good measure of total coronary artery disease load. However, it does not discriminate haemodynamically significant coronary artery disease (stenoses of ≥50%) which may require revascularisation, be it percutaneous coronary intervention (PCI), or coronary artery bypass grafting (CABG).

The SYNTAX score[160] was an angiographic scoring system used in the SYNTAX study, the design of which was published in 2005. It offers a way of quantifying haemodynamically significant coronary artery disease based on coronary artery segments not unlike that used by our own scoring system.

There are important differences where the SYNTAX score offers advantages over our own scoring system. They include:

1. The SYNTAX score gives a summation of haemodynamically significant coronary artery lesions whereas our own scoring system would give the same lesion a score of 2 or more per segment. However, multiple segments with mild coronary artery disease with scores of '1' which may be insignificant haemodynamically may blur the distinction between patients with mild coronary artery disease and those with critical coronary artery stenoses. Although these patients with mild coronary artery disease may not need revascularisation, it is still clinically important to detect them as these patients require lifelong treatment with aspirin, statins and other drug therapies such as beta-blockers.

2. The SYNTAX score also takes into account the location of the disease. Prognostically important segments such as the left main stem and left anterior descending arteries are given a higher weighting under the SYNTAX score whereas our own scoring system would not discriminate these. The SYNTAX score also takes into account the dominance of the arteries, as well as whether the disease is proximal or distal, accounting for the amount of myocardium in jeopardy.

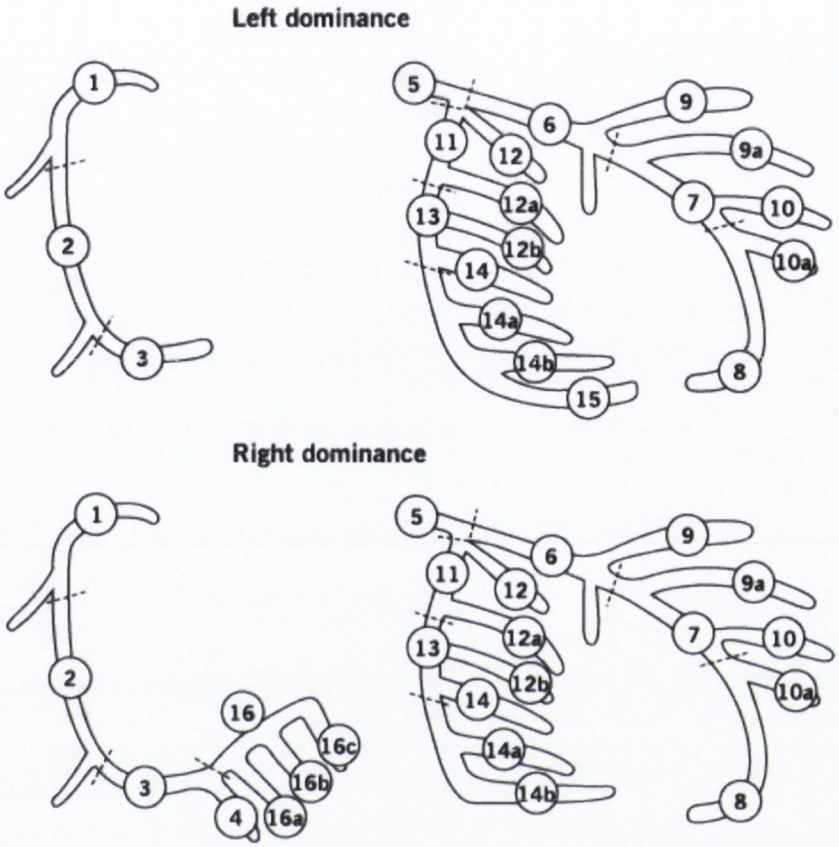
3. The SYNTAX score has also been shown to affect prognosis and helps guide the mode of revascularisation as described below.

SYNTAX categorises disease as low (0-22), intermediate (23-32) and high (≥33). The SYNTAX study was a randomised controlled trial comparing CABG with PCI using drug-eluting (paclitaxel) stents in patients with triple vessel disease or left main stem disease. For the one-year outcomes, there were no statistically significant differences in major adverse cardiovascular events (MACE) between PCI and CABG in those with a low and intermediate SYNTAX scores[161]. Similar results were seen in the 2-year follow up (Kappetein *et al*, ESC 2009). The 3-year follow up (Kappetein *et al*, EACTS 2010) showed that MACE rates were lower in patients who had CABG compared to PCI in both the groups with intermediate and high SYNTAX scores.

SYNTAX Coronary Artery Segments

Figure 4.2 - SYNTAX Definition of the coronary artery segments

(Reproduced from Sianos *et al*[160])



1. RCA proximal: From the ostium to one half the distance to the acute margin of the heart.

2. RCA mid: From the end of first segment to acute margin of heart.

3. RCA distal: From the acute margin of the heart to the origin of the posterior descending artery.

4. Posterior descending artery: Running in the posterior interventricular groove.

16. Posterolateral branch from RCA: Posterolateral branch originating from the distal coronary artery distal to the crux.

16a. Posterolateral branch from RCA: First posterolateral branch from segment 16.

16b. Posterolateral branch from RCA: Second posterolateral branch from segment 16.

16c. Posterolateral branch from RCA: Third posterolateral branch from segment 16.

5. Left main: From the ostium of the LCA through bifurcation into left anterior descending and left circumflex branches.

6. LAD proximal: Proximal to and including first major septal branch.

7. LAD mid: LAD immediately distal to origin of first septal branch and extending to the point where LAD forms an angle (RAO view). If this angle is not identifiable this segment ends at one half the distance from the first septal to the apex of the heart.

8. LAD apical: Terminal portion of LAD, beginning at the end of previous segment and extending to or beyond the apex.

9. First diagonal: The first diagonal originating from segment 6 or 7.

9a. First diagonal a: Additional first diagonal originating from segment 6 or 7, before segment 8.

10. Second diagonal: Originating from segment 8 or the transition between segment 7 and 8.

10a. Second diagonal a: Additional second diagonal originating from segment 8.

11. Proximal circumflex artery: Main stem of circumflex from its origin of left main and including origin of first obtuse marginal branch.

12. Intermediate/anterolateral artery: Branch from trifurcating left main other than proximal LAD or LCX. It belongs to the circumflex territory.

12a. Obtuse marginal a: First side branch of circumflex running in general to the area of obtuse margin of the heart.

12b. Obtuse marginal b: Second additional branch of circumflex running in the same direction as 12.

13. Distal circumflex artery: The stem of the circumflex distal to the origin of the most distal obtuse marginal branch, and running along the posterior left atrioventricular groove. Calibre may be small or artery absent.

14. Left posterolateral: Running to the posterolateral surface of the left ventricle. May be absent or a division of obtuse marginal branch.

14a. Left posterolateral a: Distal from 14 and running in the same direction.

14b. Left posterolateral b: Distal from 14 and 14a and running in the same direction.

15. Posterior descending: Most distal part of dominant left circumflex when present. It gives origin to septal branches. When this artery is present, segment 4 is usually absent.

RESULTS

Altogether, 730 patients were recruited after informed consent over a period of 19 months between April 2004 to October 2005 in two centres, the Adelaide & Meath Hospital (n=550) and St. James's Hospital (n=180). The patients were mostly consecutively recruited from our elective day case coronary angiography list as part of their assessment for suspected cardiac chest pain, or work-up for cardiac surgery.

Our study population comprised of 612 patients who had their coronary angiograms available to me for the scoring of coronary artery disease using the methodology described above, as well as calculating the SYNTAX score.

Baseline characteristics

The study population was predominantly male (66%) with an average age of 59.4 years and about 22% had prior documented coronary heart disease. Average serum cholesterol was relatively low in our population (4.4 mmol/L) which may be attributed to a high level of statin use (71%), as one would expect of a population with high suspicion of having coronary artery disease. Levels of smoking were relatively low at 16% with average lifetime cumulative pack years of 19. However, the population was overall overweight with an average BMI of 28.8 with 12% diabetics and overall high levels of the metabolic syndrome (31%). Table 4.1 shows key baseline characteristics of the study population. Figure 4.3 shows the distribution of the various novel risk markers in the study population.

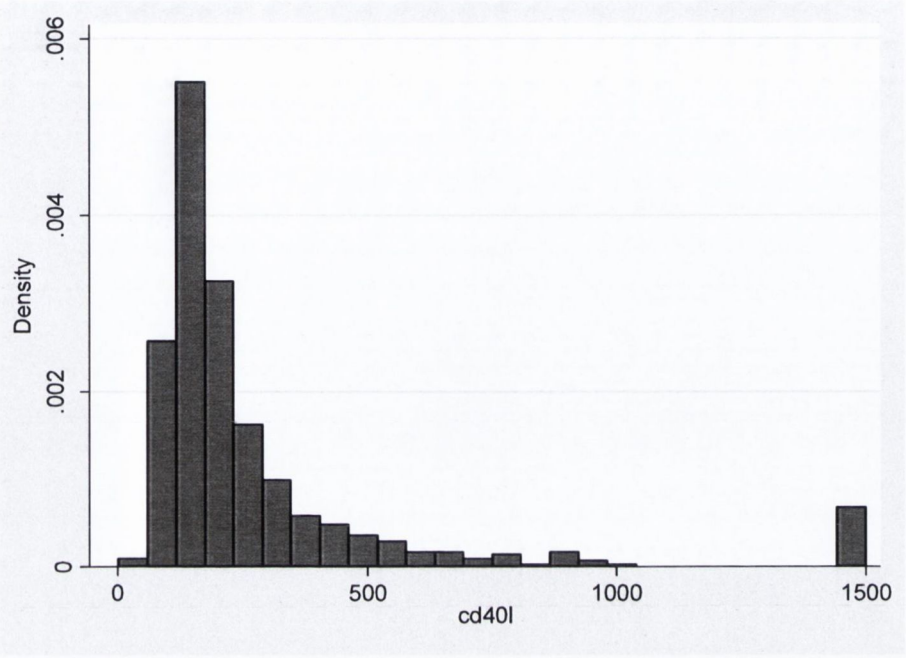
Table 4.1 – Baseline characteristics of the *Cath Lab Study* Population

Characteristic	Mean (Standard deviation)
Age (years)	59.4 (10.1)
Male (%)	66%
Systolic blood pressure (mmHg)	134 (18)
Diastolic blood pressure (mmHg)	74 (10)
Total cholesterol (mmol/L)	4.39 (0.99)

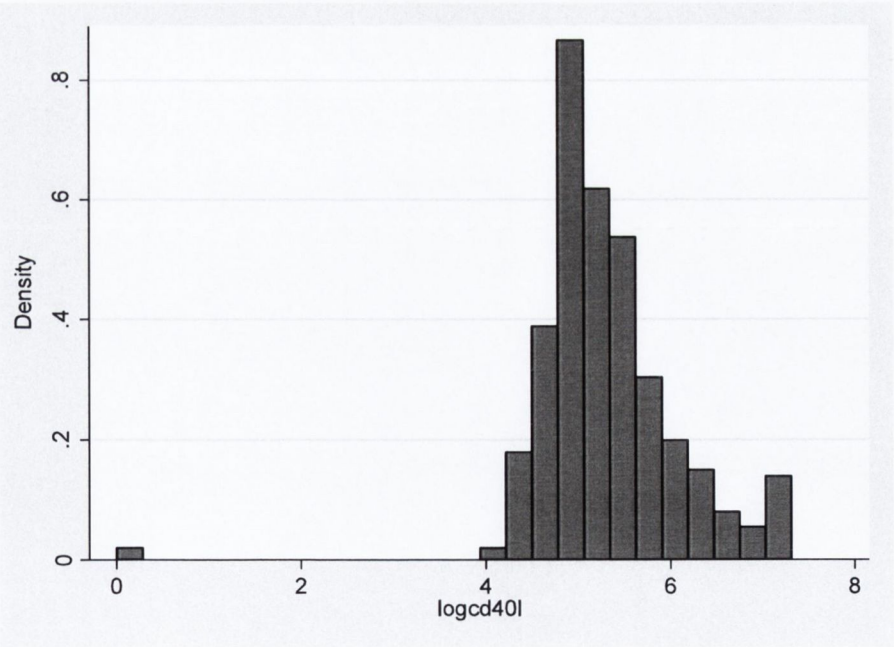
LDL cholesterol (mmol/L)	2.41 (0.86)
HDL cholesterol (mmol/L)	1.30 (0.37)
Current smoker (%)	16%
Lifetime pack years smoked	19 (28)
Diabetic (%)	12%
Previous CABG (%)	5.3%
Previous PCI (%)	16.4%
Height (m)	1.69 (0.96)
Weight (kg)	82.5 (14.7)
Waist circumference (cm)	98 (12)
Hip circumference (cm)	104 (11)
BMI	28.8 (4.7)
Metabolic syndrome (%)	31%
Chest pain (CCS Class)	1.5
Aspirin use (%)	79%
Statin use (%)	71%
Beta-blocker use (%)	47%
ACEI use (%)	27%
ARB use (%)	6.1%
Coronary Artery Disease Score	13.8
SYNTAX Score	7.4
Intima Media Thickness (mm)	1.02
SCORE Risk score (%)	2.9%
CD40 Ligand (pg/ml)	281 (295)
VCAM (ng/ml)	673 (204)
P-selectin (ng/ml)	52 (16)
High sensitivity CRP (mg/L)	3.6 (5.4)
Homocysteine (μmol/L)	10.4 (15.8)
Fibrinogen (g/L)	3.0 (0.6)

Figure 4.3 - Distributions of Novel Risk Markers

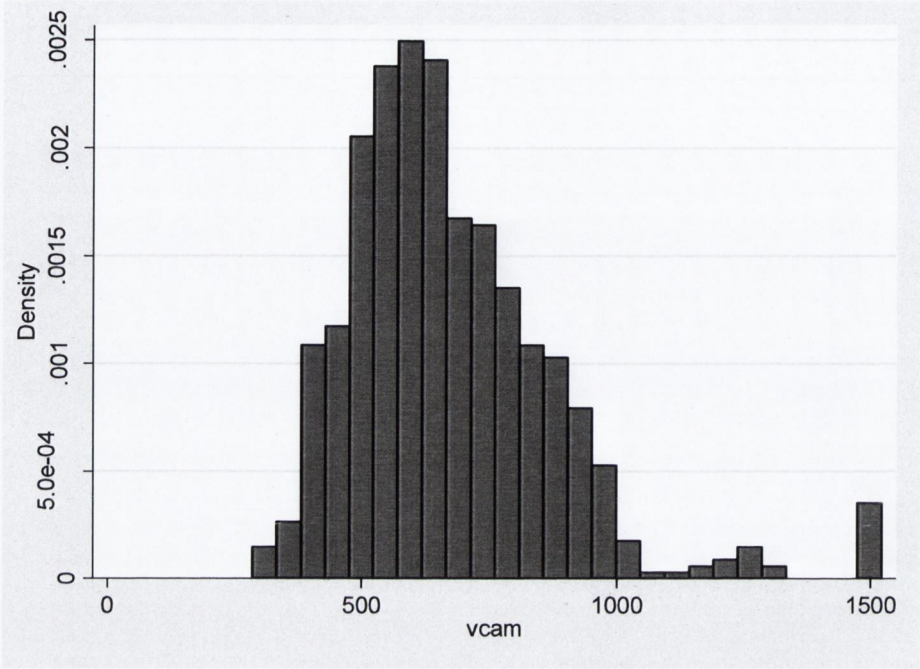
(a) CD40 Ligand



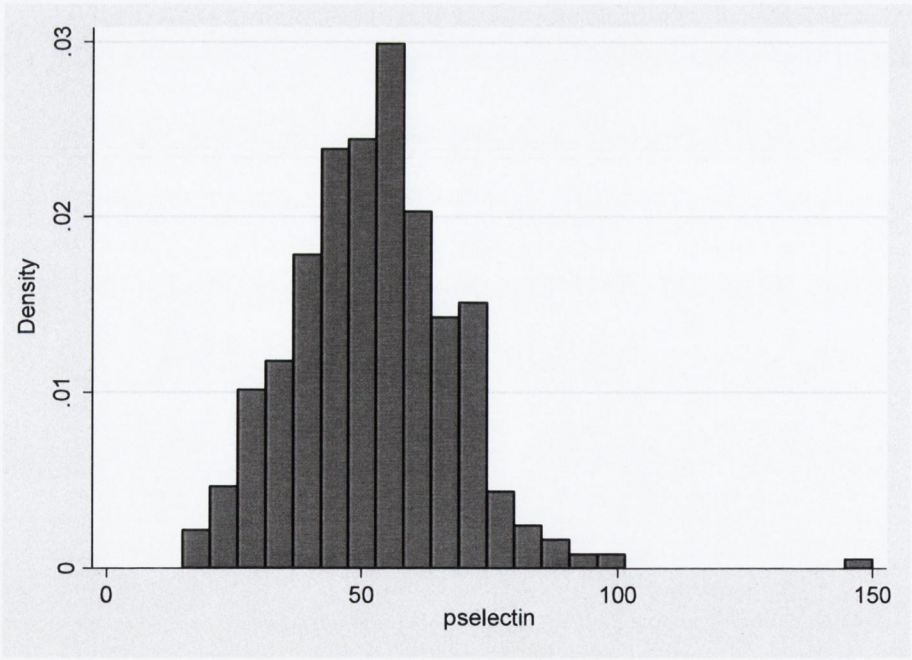
(b) Log-transformed CD40 Ligand



(c) VCAM



(d) P-selectin



Pearson's correlations

There were statistically significant correlations of most risk factor levels with total coronary artery disease load measured with our own scoring system. As expected, HDL was associated with an inverse relationship with coronary artery disease. Unexpectedly however, there were inverse correlations of total cholesterol, hs-CRP and CD40 ligand with coronary artery disease. There was a statistically significant correlation of statin use with coronary artery disease. Table 4.2 shows Pearson's correlations of various risk factors and novel risk markers with coronary artery disease as measured under our scoring system. Similar results were obtained when these risk factors and novel risk markers were correlated with coronary artery disease severity as defined by the SYNTAX score (results not shown).

Table 4.2 - Pearson's Correlations of risk factors and novel risk markers with Coronary Artery Disease under our scoring system

	Correlation (r)	**P value**
Age	0.35	<0.0001
Male Sex	0.36	<0.0001
Systolic blood pressure	0.11	0.007
Total cholesterol	-0.15	0.0002
Statin use	0.18	<0.0001
Pack years of smoking	0.11	0.01
HDL cholesterol	-0.21	<0.0001
Glucose	0.18	<0.0001
Metabolic syndrome	0.20	<0.0001
CCS Chest Pain	0.11	0.03
hs-CRP	-0.09	0.03
Homocysteine	0.13	0.002
Log CD40 ligand	-0.12	0.004
VCAM	0.14	0.0008
P-selectin	0.03	0.50
Fibrinogen	0.07	0.11
SCORE risk score	0.39	<0.0001

As expected, our own scoring system strongly correlates with the SYNTAX score ($r=0.84$, $p<0.0001$) and SYNTAX score categories ($r=0.84$, $p<0.0001$).

Median levels of CD40 ligand and the other novel risk markers and risk factors across categories of total coronary artery disease load and categories of the SYNTAX score are compared in Table 4.3. For the metabolic syndrome, the prevalences in each category are compared instead of the median. Total coronary artery disease load was divided into categories according to tertiles of disease. These categories of the SYNTAX score were as defined as in the SYNTAX study where the baseline are those whose SYNTAX scores were 0 (no haemodynamically significant lesions) and increasing SYNTAX score categories 1 (low SYNTAX score 1-22) and 2 (intermediate and high SYNTAX scores ≥23).

Table 4.3 – Levels of risk factors and novel risk markers across categories of total coronary disease load and the SYNTAX score

(a) Total CAD with novel risk markers

Total CAD	CD40 ligand	VCAM	P-selectin	Fibrinogen	Metabolic syndrome
Tertile 1	188	619	49	2.9	19%
Tertile 2	184	645	55	3.0	41%
Tertile 3	163	685	52	3.0	43%
P-value	0.01	0.008	0.04	0.1	0.0001

(b) SYNTAX Categories with novel risk markers

SYNTAX Categories	CD40 ligand	VCAM	P-selectin	Fibrinogen	Metabolic syndrome
Normal	193	631	50	3.0	21%
Low	172	642	55	3.0	38%
Int & High	157	640	53	3.1	37%
P-value	0.02	0.8	0.01	0.2	0.0001

(c) SYNTAX Categories with traditional risk factors

SYNTAX Categories	Serum Cholesterol	Statin use	HDL Cholesterol	Systolic BP	Smoking
Normal	4.6	61%	1.4	131	19%
Low	4.5	68%	1.3	137	19%
Int & High	4.0	72%	1.2	140	22%
P-value	0.03	0.07	0.005	0.0005	0.6

The association of novel risk markers with coronary artery disease was examined using multinomial logistic regression models with the outcome defined as tertiles of the total coronary artery disease load quantified using our scoring system.

Each risk marker was examined in the multinomial logistic regression models in the following order:

1. **Univariate**: Each risk marker individually
2. **Model 1**: Above with conventional CVD risk factors – age, sex, blood pressure, cholesterol, smoking and statin use,
3. **Model 2**: Above model (Model 1) with HDL cholesterol, glucose, and
4. **Model 3**: Above model (Model 2) and all other novel risk markers, being hs-CRP, homocysteine, CD40 ligand, VCAM, P-selectin and fibrinogen.

Table 4.4 shows odds ratios of total coronary artery disease load measured using our scoring system for the various novel risk markers in multinomial logistic regression models. OR-2 is the odd ratios of the second tertile, while OR-3 is the odds ratio of the third tertile compared with the lowest tertile as the base outcome of total coronary artery disease load.

Similar results were obtained when the SYNTAX score was used as the outcome (Table 4.5). Odds ratios are presented in Table 4.5 from a multinomial logistic regression where OR-B are odds ratios of low SYNTAX score (1-22) compared to SYNTAX score of 0. OR-C are odds ratios of combined intermediate and high SYNTAX scores (≥23) compared to SYNTAX score of 0.

Table 4.4 – OR of tertiles of total coronary artery disease for the various novel risk markers as continuous variables

	Univariate (95% CI) p-value	**Model 1** (95% CI) p-value	**Model 2** (95% CI) p-value	**Model 3** (95% CI) p-value
Log CD40 ligand				
OR-2	0.8 (0.6, 1.2) p=0.2	0.9 (0.6, 1.2) p=0.4	0.8 (0.6, 1.1) p=0.2	0.8 (0.5, 1.1) p=0.2
OR-3	0.7 (0.5, 0.9) p=0.005	0.7 (0.5, 1.0) p=0.03	0.6 (0.4, 0.9) p=0.01	0.6 (0.4, 1.0) p=0.04
VCAM (100 ng/ml increase)				
OR-2	1.1 (1.0, 1.2) p=0.04	1.0 (0.9, 1.1) p=0.8	1.0 (0.9, 1.1) p=0.9	1.0 (0.9, 1.2) p=0.5
OR-3	1.2 (1.1, 1.3) p=0.002	1.0 (0.9, 1.1) p=0.9	1.0 (0.9, 1.1) p=0.6	1.0 (0.9, 1.2) p=0.9
P-selectin (10 ng/ml increase)				
OR-2	1.2 (1.0, 1.3) p=0.03	1.2 (1.0, 1.3) p=0.07	1.1 (1.0, 1.3) p=0.2	1.1 (0.9, 1.4) p=0.2
OR-3	1.1 (1.0, 1.3) p=0.07	1.1 (1.0, 1.3) p=0.1	1.1 (0.9, 1.3) p=0.5	1.1 (0.9, 1.3) p=0.4

hs-CRP (1 mg/L increase)				
OR-2	1.0 (1.0, 1.0) p=0.8	1.0 (1.0, 1.1) p=0.5	1.0 (1.0, 1.0) p=0.03	1.0 (0.9, 1.0) p=0.8
OR-3	1.0 (0.9, 1.0) p=0.05	1.0 (0.9, 1.0) p=0.5	1.0 (0.9, 1.0) p=0.2	1.0 (0.9, 1.0) p=0.1
Homocysteine (5 µmol/L increase)				
OR-2	1.2 (0.9, 1.7) p=0.2	0.7 (0.5, 1.1) p=0.1	0.7 (0.5, 1.0) p-0.07	0.7 (0.5, 1.1) p=0.1
OR-3	1.5 (1.1, 2.1) p=0.005	0.8 (0.5, 1.1) p=0.2	0.7 (0.5, 1.1) p=0.1	0.7 (0.5, 1.1) p=0.2
Fibrinogen (1 g/L increase)				
OR-2	1.4 (1.0, 1.9) p=0.06	1.3 (0.9, 1.9) p=0.2	1.2 (0.8, 1.8) p=0.4	1.3 (0.8, 2.1) p=0.3
OR-3	1.3 (1.0, 1.9) p=0.08	1.3 (0.8, 2.0) p=0.2	1.2 (0.8, 1.8) p=0.5	1.4 (0.8, 2.4) p=0.2
Metabolic syndrome				
OR-2	3.0 (1.9, 4.7) p<0.001	2.2 (1.3, 3.7) p=0.003	1.7 (1.0, 3.1) p=0.06	1.8 (1.0, 3.5) p=0.07
OR-3	3.3 (2.1, 5.2) p<0.001	2.1 (1.2, 3.6) p=0.008	1.5 (0.8, 2.8) p=0.2	1.6 (0.8, 3.2) p=0.2

Table 4.5 – OR of SYNTAX categories for the various novel risk markers as continuous variables

	Univariate (95% CI) p-value	**Model 1** (95% CI) p-value	**Model 2** (95% CI) p-value	**Model 3** (95% CI) p-value
Log CD40 ligand				
OR-B	0.7 (0.5, 1.0) p=0.06	0.8 (0.5, 1.1) p=0.1	0.7 (0.5, 1.1) p=0.1	0.8 (0.5, 1.2) p=0.2
OR-C	0.5 (0.3, 0.8) p=0.002	0.5 (0.3, 0.8) p=0.007	0.4 (0.3, 0.7) p=0.002	0.4 (0.2, 0.7) p=0.003
VCAM (100 ng/ml increase)				
OR-B	1.1 (1.0, 1.2) p=0.3	1.0 (0.9, 1.1) p=0.4	0.9 (0.8, 1.1) p=0.3	0.9 (0.8, 1.1) p=0.2
OR-C	1.0 (0.9, 1.2) p=0.6	0.8 (0.7, 1.0) p=0.01	0.8 (0.7, 1.0) p=0.01	0.8 (0.6, 1.0) p=0.03
P-selectin (10 ng/ml increase)				
OR-B	1.2 (1.1, 1.4) p=0.003	1.2 (1.0, 1.4) p=0.02	1.2 (1.0, 1.4) p=0.03	1.3 (1.1, 1.5) p=0.007
OR-C	1.0 (0.8, 1.3) p=0.9	1.0 (0.8, 1.2) p=0.8	0.9 (0.7, 1.2) p=0.7	1.0 (0.8, 1.3) p=0.9

hs-CRP (1 mg/dL increase)				
OR-B	1.0 (1.0, 1.0) p=0.8	1.0 (1.0, 1.1) p=0.3	1.0 (1.0, 1.1) p=0.4	1.0 (1.0, 1.1) p=0.9
OR-C	1.0 (0.9, 1.0) p=0.8	1.0 (1.0, 1.1) p=0.6	1.0 (0.9, 1.1) p=0.8	1.0 (0.9, 1.1) p=0.7
Homocysteine (5 µmol/L increase)				
OR-B	1.7 (1,2, 2.4) p=0.001	1.2 (0.8, 1.7) p=0.4	1.1 (0.8, 1.6) p=0.5	1.2 (0.8, 1.8) p=0.3
OR-C	1.7 (1.1, 2.7) p=0.02	0.8 (0.5, 1.5) p=0.6	0.8 (0.5, 1.5) p=0.5	1.1 (0.6, 2.0) p=0.8
Fibrinogen (1 g/L increase)				
OR-B	1.2 (0.9, 1.7) p=0.2	1.2 (0.8, 1.8) p=0.3	1.2 (0.8, 1.8) p=0.4	1.2 (0.7, 1.9) p=0.5
OR-C	1.4 (0.9, 2.3) p=0.1	1.3 (0.8, 2.3) p=0.3	1.3 (0.7, 2.3) p=0.4	1.3 (0.6, 2.6) p=0.5
Metabolic syndrome				
OR-B	2.2 (1.5, 3.4) p<0.001	1.3 (0.8, 2.2) p=0.3	1.1 (0.6, 1.9) p=0.7	1.0 (0.5, 1.9) p=1.0
OR-C	2.1 (1.1, 4.0) p=0.02	1.1 (0.6, 2.4) p=0.7	0.9 (0.4, 2.0) p=0.7	0.6 (0.2, 1.6) p=0.3

The magnitude of risk associated with all conventional risk factors and novel risk markers were examined in combination in the following multivariable case control models. Due to their clinical importance, we were interested in examining the outcomes of significant coronary artery disease requiring coronary intervention and prognostically significant coronary artery disease. These outcomes were:

1. Any significant coronary artery disease: SYNTAX score ≥1 compared to those with a SYNTAX score of 0 (Table 4.6),
2. Significant disease (≥50%) in the left main stem, proximal and mid LAD (Table 4.7), and
3. Any significant disease (≥50%) in the proximal coronary arteries (left main stem, LAD, RCA and Circumflex arteries) (Table 4.8).

Table 4.6 – OR of significant coronary artery disease (SYNTAX score ≥1)

	OR	95% CI	P value
Age (10 year increase)	2.3	1.7, 3.1	<0.001
Male sex	2.6	1.4, 4.6	0.002
SBP (10 mmHg increase)	1.2	1.1, 1.4	0.007
DBP (10 mmHg increase)	1.0	0.8, 1.3	0.9
Cholesterol (1 mmol/L increase)	1.1	0.8, 1.4	0.6
Statin use	1.5	0.9, 2.4	0.1
Smoker	1.9	1.0, 3.6	0.04
HDL (1 mmol/L increase)	0.6	0.3, 1.2	0.1
Glucose (1 mmol/L increase)	1.0	0.8, 1.1	0.7
hs-CRP (1 mg/L increase)	1.0	1.0, 1.0	0.2
Homocysteine (5 μmol/L increase)	1.1	0.8, 1.6	0.4
Log CD40 ligand	0.6	0.4, 0.9	0.008
VCAM (100 ng/ml increase)	0.9	0.8, 1.0	0.045
P-selectin (10 ng/ml increase)	1.2	1.0, 1.4	0.048

Table 4.7 – OR of significant disease (≥50%) in either the left main stem, proximal or mid left anterior descending artery

	OR	95% CI	P value
Age (10 year increase)	2.0	1.5, 2.5	<0.001
Male sex	2.0	1.2, 3.4	0.01
SBP (10 mmHg increase)	1.1	0.9, 1.2	0.3
DBP (10 mmHg increase)	1.0	0.8, 1.3	0.8
Cholesterol (1 mmol/L increase)	0.9	0.7, 1.2	0.5
Statin use	1.2	0.8, 2.0	0.3
Smoker	1.3	0.7, 2.2	0.4
HDL (1 mmol/L increase)	0.5	0.2, 0.9	0.01
Glucose (1 mmol/L increase)	1.1	0.9, 1.2	0.4
hs-CRP (1 mg/L increase)	1.0	1.0, 1.0	0.4
Homocysteine (5 μmol/L increase)	0.8	0.6, 1.2	0.3
Log CD40 ligand	0.7	0.5, 1.0	0.03
VCAM (100 ng/ml increase)	0.9	0.8, 1.0	0.3
P-selectin (10 ng/ml increase)	1.0	0.9, 1.0	0.07

Table 4.8 – OR of significant disease (≥50%) in either the left main stem or proximal coronary arteries (LAD, Circumflex or Right Coronary Arteries)

	OR	95% CI	P value
Age (10 year increase)	2.0	1.6, 2.4	<0.001
Male sex	2.3	1.3, 3.9	0.003
SBP (10 mmHg increase)	1.1	1.0, 1.3	0.07
DBP (10 mmHg increase)	0.9	0.7, 1.2	0.5
Cholesterol (1 mmol/L increase)	1.0	0.8, 1.2	0.7
Statin use	1.7	1.1, 2.7	0.03
Smoker	1.8	1.0, 3.0	0.046
HDL (1 mmol/L increase)	0.5	0.3, 0.9	0.03
Glucose (1 mmol/L increase)	1.1	1.0, 1.3	0.2
hs-CRP (1 mg/L increase)	1.0	1.0, 1.0	0.6
Homocysteine (5 μmol/L increase)	0.9	0.6, 1.2	0.4
Log CD40 ligand	0.7	0.5, 1.0	0.03
VCAM (100 ng/ml increase)	0.9	0.8, 1.0	0.2
P-selectin (10 ng/ml increase)	1.0	0.8, 1.1	0.7

No statistically significant interactions were found for logarithmically transformed CD40 ligand in the following subgroups: age<50, sex, smoking status, diabetes, statin use, antiplatelet drug use, and ACE inhibitor/ARB use. The interaction models assumed a multiplicative effect of the risk factors, and included adjustment for the other conventional risk factors.

The diagnostic ability of each variable to predict the presence of haemodynamically significant coronary artery disease defined using the SYNTAX score was assessed by comparing their areas under the receiver operating characteristic (AUROC) curves in Table 4.9. Apart from age and sex, the diagnostic ability of risk markers and even established risk factors for coronary artery disease including blood pressure, cholesterol and smoking appeared to have no significant value when areas under the ROC curve were considered.

Table 4.9 – Diagnostic ability to predict the presence of haemodynamically significant coronary artery disease (SYNTAX ≥1)

	AUROC	**P-value compared with previous**
Age	0.67	Not applicable
+ Sex	0.75	<0.0001
+ Blood pressure	0.76	0.5
+ Cholesterol	0.76	0.4
+ Smoking	0.77	0.3
+ (Negative) HDL cholesterol	0.77	0.8
+ Glucose	0.77	0.8
+ hs-CRP	0.77	0.7
+ Homocysteine	0.78	0.2
+ (Negative) Log CD40 ligand	0.79	0.2
+ VCAM	0.79	0.3
+ P-selectin	0.79	0.1

The discriminative ability of each risk marker which had independent effects in the previous multivariable models was examined in Table 4.10 compared with the SCORE equation, as it is currently the methods of CVD risk assessment in clinical practice, and takes account of the combined effect of the established conventional risk factors.

As these novel risk markers are not yet standardised or in routine use, 'abnormal' the top tertile of these novel risk markers was considered in the study population who had no haemodynamically significant coronary artery disease (SYNTAX score of 0). In the case of CD40 ligand, as the relationship was inverse in this study, it was the lowest tertile that was considered 'abnormal'. These cut-points were CD40 ligand ≤150 pg/ml, VCAM ≥714 pg/ml and P-selectin ≥56 pg/ml. These markers were chosen because they showed independent effects on predicting coronary artery disease in the multivariable models.

Table 4.10 - Tests of Discrimination for Significant Coronary Artery Disease, with 95% CI in parentheses

	Sensitivity	**Specificity**	**Positive Predictive value**	**Negative Predictive Value**
SCORE ≥5%	28% (22%, 35%)	91% (87%, 95%)	73% (61%, 82%)	60% (54%, 65%)
CD40 ligand ≤150 pg/ml	38% (32%, 45%)	70% (63%, 75%)	52% (44%, 60%)	57% (51%, 62%)
VCAM ≥714 pg/ml	35% (29%, 42%)	66% (60%, 72%)	48% (40%, 56%)	54% (49%, 60%)
P-selectin ≥56 pg/ml	45% (38%, 52%)	63% (57%, 70%)	52% (44%, 60%)	57% (50%, 63%)

Table 4.11 examines the ability of (a) CD40 ligand, (b) VCAM and (c) P-selectin to re-classify patients after using the SCORE equation to assess CVD risk. Both CD40 ligand and P-selectin were able to improve re-classification of patients deemed at low risk when used **in conjunction** with the SCORE equation. Emphasis should be given to those who were deemed low-risk because those who were already been classified as high-risk by the SCORE equation would already have been given the highest priority for intervention. Even so, both CD40 ligand and P-selectin do improve re-classification of those deemed high risk already by the SCORE equation.

When used with the SCORE equation, both CD40 ligand levels of ≤150 pg/ml and P-selectin ≥56 pg/ml were able to improve the re-classification of an extra 5% of those who was deemed low-risk by the SCORE equation who actually had significant coronary artery disease, and was able to refine the classification of those who did not have significant coronary artery disease by 3%. In contrast, VCAM ≥714 pg/ml did not improve the re-classification of those who did or did not have coronary artery disease above and beyond the SCORE equation.

Table 4.11 – Reclassification ability of novel risk markers in conjunction with the SCORE system in detecting haemodynamically significant coronary artery disease (SYNTAX ≥1)

(a) CD40 ligand ≤150 pg/ml

SCORE Alone	Actual Coronary Disease	SCORE and CD40 ligand	Actual Coronary Disease
SCORE <5%	40%	CD40 ligand >150	37%
SCORE <5%	40%	CD40 ligand ≤150	45%
SCORE ≥5%	73%	CD40 ligand >150	70%
SCORE ≥5%	73%	CD40 ligand ≤150	79%

(b) VCAM ≥714 pg/ml

SCORE Alone	Actual Coronary Disease	SCORE and VCAM	Actual Coronary Disease
SCORE <5%	40%	VCAM <714	42%
SCORE <5%	40%	VCAM ≥714	37%
SCORE ≥5%	73%	VCAM <714	74%
SCORE ≥5%	73%	VCAM ≥714	73%

(c) P-selectin ≥56 pg/ml

SCORE Alone	Actual Coronary Disease	SCORE and P-selectin	Actual Coronary Disease
SCORE <5%	40%	P-selectin <56	37%
SCORE <5%	40%	P-selectin ≥56	45%
SCORE ≥5%	73%	P-selectin <56	71%
SCORE ≥5%	73%	P-selectin ≥56	79%

DISCUSSION

In this study of stable patients who have suspected significant coronary artery disease, the established conventional risk factors such as age, sex, systolic blood pressure and smoking were independently associated with significant coronary artery disease when considered in a multivariable model. The risk associated with smoking and the male sex was approximately two-fold, while that of each 10 mmHg increase in systolic blood pressure was approximately 20% in our study. This magnitude of risk is in general agreement with most previous studies of these conventional risk factors[162]. As previous studies have found, the effect of diastolic blood pressure became attenuated when systolic blood pressure was examined in combination.

There was an unexpected finding of an inverse relationship of total cholesterol to coronary artery disease in this study. The role of serum cholesterol as a CVD risk factor has been established beyond doubt. This inverse relationship in this study became attenuated and lost statistical significance when adjusted for statin use, the usual treatment of raised cholesterol. The explanation for the inverse relationship of cholesterol with coronary artery disease was very likely due to the fact that those with raised serum cholesterol were already on statins, explaining the 'protective' effect in the model. This was supported by the fact that there was a trend towards increased statin use with more severe coronary artery disease in this study.

The novel risk markers examined in this study included hs-CRP, homocysteine, CD40 ligand, VCAM, P-selectin, fibrinogen and the metabolic syndrome. The distribution of CD40 ligand was highly skewed and was more normalised after logarithmic transformation.

In univariate analyses, VCAM, P-selectin and the metabolic syndrome were related to the severity and complexity of coronary artery disease in a graded manner. CD40 ligand was also related to the severity and complexity of coronary artery disease, but in an inversely graded manner.

In multivariable analyses, of the novel risk markers studied, only **CD40 ligand** (inverse) and **P-selectin** contributed independently to coronary artery disease risk after adjustment for conventional risk factors and all novel risk markers in combination.

Previous studies have shown that all of the studied novel risk markers have been independently associated with CVD in multivariable models which usually only adjusted for conventional risk factors. The *Cath Lab Study* was designed to examine conventional risk factors and all the novel risk markers as well in combination with each other. This is a key strength of this study.

However, the inverse association of CD40 ligand with coronary artery disease was unexpected and is counter-intuitive from our current understanding of atherosclerosis. This warrants further investigation and a reassessment of the potential role of CD40 ligand in coronary artery disease.

Since the *Cath Lab Study* commenced, results of other studies showing inverse associations of CD40 ligand with coronary artery disease had been reported in the literature. An angiographic study of 909 patients from an American group reported a similar finding of an inverse relationship of CD40 ligand with coronary artery disease on coronary angiography[122]. Their top quartile of CD40 ligand showed an OR of 0.59 (95% CI 0.37, 0.96), p=0.03 of future coronary events defined as combined CHD deaths and acute coronary syndromes within 1 year. They considered the result not statistically significant as they had set the level of statistical significance to $p<0.017$ from Bonferroni correction. Similar trends were seen in their endpoints of angiographic coronary artery disease alone without events.

An Israeli study using a nested case-control design on a randomised controlled trial of patients with chronic coronary artery disease, the Bezafibrate Infarction Prevention (BIP) study, showed no statistically significant association of CD40 ligand with future cardiovascular events The OR of ischaemic stroke, recurrent coronary events and sudden cardiac death for the top quartile of CD40 ligand was 0.97 (95% CI 0.82, 1.16) in that study[163]. However, it is interesting to note that although also not statistically significant, when coronary endpoints and ischaemic

stroke were examined separately, the point estimate of the effect of CD40 ligand was reversed in coronary events compared to ischaemic stroke. The OR of coronary artery disease compared to ischaemic stroke for >95th percentile of CD40 ligand was 0.35 (95% CI 0.07, 1.37) vs. 2.22 (95% CI 0.46, 12.5).

How is it possible that CD40 ligand is positively associated with CVD in patients with acute coronary syndromes but negatively in patients with stable coronary artery disease?

Statins have been shown to reduce levels of CD40 ligand. It is possible that the high level of statin use in this study population (71%) and the trend towards higher statin use in those with more severe coronary artery disease may have contributed to the inverse relationship. The multivariable models and interaction analyses assumed a uniform effect of statins on everyone. This is not true in the study population as each patient would have had different drugs and doses of statins and different lengths of treatment. As a result, the model may not have fully adjusted for the effect on statins in the study population. This inverse effect on CVD risk was also exhibited by serum cholesterol, an established risk factor, in this study. The same seeming 'protective' effect of cholesterol had been observed in other observational studies where it was not possible to examine the levels of risk factors before the initiation of cholesterol lowering therapy[164].

There is data to show that other drugs can reduce CD40 ligand levels. Some such as clopidogrel[129], glycoprotein IIb/IIIa antagonists[130] are used more in acute coronary syndromes whereas others such as ACE inhibitors, angiotensin receptor blockers and statins[117] are used more in chronic coronary artery disease. Whether the stress of an acute coronary syndrome, or multiple other drugs used in either conditions with their effect on CD40 ligand yet unknown may be responsible for the discrepant findings in these two conditions.

The Women's Health Study was the first major study to show the association of CD40 ligand with future CVD events (myocardial infarction, cerebrovascular events and CVD deaths) in a stable population. It utilised a nested case-control design of 130 cases matched by age and smoking with 130 controls. The RR of CVD events

after 4 years for CD40 ligand >3.71 ng/mL (>95th percentile) was 2.8 (95% CI 0.9, 8.0), p=0.05 after adjustment for traditional risk factors. This result was actually driven by the 11 cases vs. 1 control who had extreme CD40 ligand levels (>99th percentile)[120]. Incidentally, the median CD40 ligand level in the *Cath Lab Study* who were free of significant coronary artery disease was 193 pg/ml and this is general agreement with the control arm mean of 209 pg/ml in the Women's Health Study.

Finally, another explanation could be that CD40 ligand may be a marker of inflammation and not a cause. If this were the case, it will be up-regulated during unstable coronary syndromes and plaque rupture, and down-regulated during the stable phase.

Both CD40 ligand (lowest tertile) and P-selectin (top tertile) were able to correctly identify patients who actually had significant coronary artery disease but were deemed low-risk by the SCORE equation. It is meaningless to consider the reclassification of those already deemed high-risk by the SCORE equation because prognostically they are already at high-risk from the burden of conventional risk factors whether they have significant coronary artery disease now or not. Therefore, this would identify more individuals at high risk of CVD that would have been missed by the SCORE equation, and have CVD risk factor modification initiated earlier.

CD40 ligand, albeit in reverse, was the only novel risk marker able to independently predict the presence of other pre-specified endpoints, which were the presence of significant left main stem and left anterior descending artery disease, sometimes aptly nicknamed the 'widow maker'. It also was the only marker to be able to identify the presence of any proximal coronary artery disease. However, until we understand further the curious inverse relationship, the clinical utility of CD40 ligand is in question.

The metabolic syndrome was also an independent predictor of risk in univariate analyses and in multivariable models which adjusted for conventional risk factors. However, when HDL cholesterol was added into the model, the effect of the

metabolic syndrome was attenuated and the relationship lost statistical significance. This is consistent with the fact that while the metabolic syndrome may be a marker of high risk for coronary artery disease, the main contributor to this increased risk may be HDL cholesterol, one of the 5 components of the metabolic syndrome. This finding is also confirmed in Chapter 6.

CONCLUSIONS

Of the many novel risk markers studied in the *Cath Lab Study*, CD40 ligand and P-selectin were independently associated with coronary artery disease after adjustment for traditional risk factors and novel risk markers in combination. The multivariable model included age, sex, blood pressure, cholesterol, statin use, smoking, HDL cholesterol, glucose, hs-CRP, homocysteine, CD40 ligand, VCAM and P-selectin.

The unexpected inverse relationship of CD40 ligand with coronary artery disease warrants further investigation. Future studies of risk factors should ideally be done on patients who are treatment naïve which may limit the scope of observational studies.

VCAM and homocysteine were associated with coronary artery disease only in univariate analyses.

The metabolic syndrome was independently associated with coronary artery disease when adjusted for the conventional risk factors. HDL cholesterol appeared to be the driver of this increased risk.

CHAPTER FIVE
CAROTID INTIMA-MEDIA THICKNESS AND NOVEL RISK MARKERS

INTRODUCTION

The focus of CVD Prevention as described by the European Third and Fourth Joint Task Force recommendations should be on all CVD, as opposed to only coronary artery disease because the risk factors are the same – blood pressure, cholesterol and smoking. Atherosclerosis occurs in the entire vascular tree in the same way, whether it is in the coronary arteries, carotid arteries or femoral arteries. The final clinical manifestation is CVD which may be myocardial infarction (heart), stroke (brain) or peripheral vascular disease (intermittent claudication *et cetera*).

The measurement of carotid intima-media thickness (CIMT) is useful because it may be a surrogate measure of total atherosclerotic load in the vascular tree and may be a way to detect underlying atherosclerosis before luminal narrowing is apparent on coronary angiography. Luminal narrowing of the coronary arteries which is detected by coronary angiography only happens late in the process considering that the background atherosclerotic disease process had already started early in life.

CIMT has been shown in studies as an accurate, non-invasive surrogate marker of atherosclerosis and has been shown to predict the risk of future cardiovascular events[165] independent of traditional risk factors[165]. CIMT has also been associated the insulin resistance[166], premature family history of coronary heart disease[167], traditional cardiovascular risk factors[168], low high-density lipoprotein (HDL)[169] and novel risk markers such as C-Reactive Protein (CRP) in women[170], hyperhomocysteinaemia[171] and asymmetric dimethylarginine (ADMA)[172].

The carotid arteries are easily accessible and can be imaged non-invasively using ultrasound. It is possible to visualise on ultrasound, the 3 layers of the artery – intima, media and adventitia, from the lumen towards the outer wall. The layer of tissue between the intima and media first gets thickened as plaque is developing, and

this is measurable on ultrasound. Thus, carotid intima-media thickness (CIMT) has been proposed, studied and used as a surrogate marker for the overall atherosclerotic process in the vascular tree, and even as a surrogate for future events.

Criteria for the validity of surrogate markers as a substitute for clinical endpoints have been proposed[173]:

1. More sensitive and more readily available than the clinical end-point,
2. Easier and more convenient to evaluate, preferably non-invasively,
3. Causal relationship between the surrogate marker and the clinical end-point should be established on the basis of epidemiological, patho-physiological and clinical studies,
4. Specificity, for example patients with and without CVD should exhibit differences in surrogate marker measurements, and
5. Benefit could be determined from observed changes in the surrogate marker in intervention studies.

Surrogate markers may themselves yield patho-physiological information at an early stage of the disease process, and therefore, may have inherent value of their own apart from being a marker for future clinical events. Validated surrogate markers can enable the assessment of new therapies in a relatively short period of time and at relatively low cost compared to the clinical outcome measures, thus allowing the benefits of treatment to be available without having to wait for the long outcome driven by clinical events, potentially bringing the benefit of new therapies earlier to those in need.

Rationale for the examining CIMT

As described in Chapter 4, the *Cath Lab Study* concentrated on determining how novel risk markers contribute to CVD risk with an emphasis on coronary artery disease. Coronary angiography is the gold standard in determining the presence and extent of coronary artery disease. However, the Achilles heel of coronary angiography is that it diagnoses coronary disease based on the extent of arterial lumen obstruction by atherosclerotic plaque. From our increased understanding of the vascular biology of atherosclerosis, we now know that the coronary artery has the ability to compensate and remodel to preserve luminal diameter until late into the atherosclerotic process. Figure 5.1 illustrates how coronary angiography may potentially miss quite advanced atherosclerotic lesions (A to F) as they have not yet encroached into the coronary artery lumen. Thus, as a well-studied surrogate marker of atherosclerosis, CIMT may be useful to detect the earlier part of the atherosclerotic process.

Figure 5.1 – Cross-sectional representation of arterial lumen with varying degrees of plaque

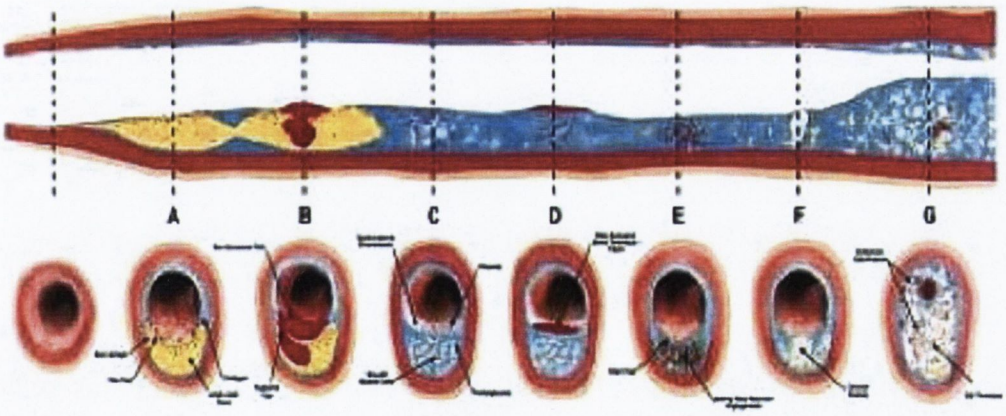
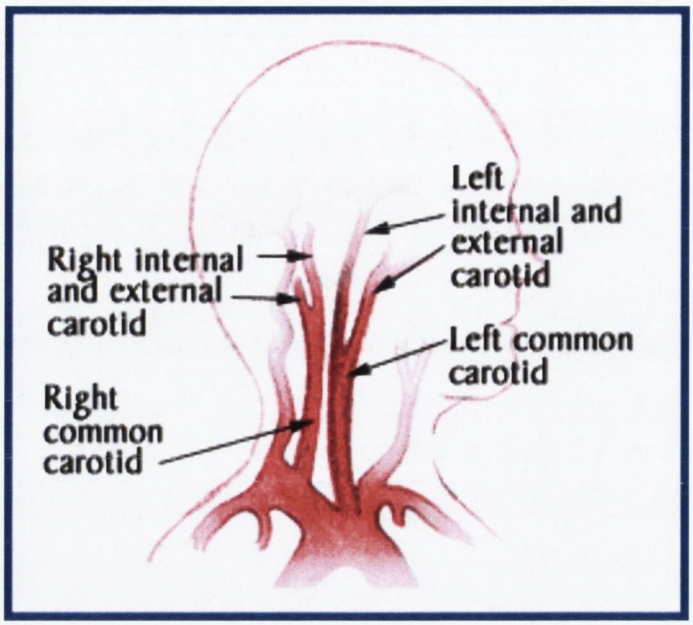


Figure 5.2 - Anatomy of the Carotid Arteries



The carotid arteries (Figure 5.2) are one of the major arterial blood supplies to the brain. The right carotid system comes off the right subclavian artery while the left carotid system originates from the arch of the aorta. The common carotid artery divides at the bifurcation called the carotid bulb into the external and the internal carotid arteries. Prospective outcome studies have been performed using intima-media thickness (IMT) measurements of the carotid artery.

Landmark studies of CIMT

The Atherosclerosis Risk in Communities (ARIC) study[174] studied 12,841 of the 15,792 subjects aged 45-65 years free from CVD in 1987 with a follow-up of 4-7 years. The investigators in the ARIC study used B-mode ultrasound to measure the carotids at 3 points - common carotid artery (CCA), the carotid bulb (CB) and the internal carotid artery (ICA). Both right and left systems were imaged, but only the IMT from far wall of the artery was measured. This gave the ARIC investigators an averaged CIMT measurement based on the 6 sites (right and left far wall CCA, CB and ICA). The ARIC study found that an extreme mean CIMT (≥1 mm) was associated with an increased coronary heart disease risk with unadjusted HR 5.07 for

women and HR1.85 for men. After adjustment for CVD risk factors including age, race, centre, LDL cholesterol, HDL cholesterol, body mass index, sports activity, cigarette-years, hypertension, diabetes, ethanol, and fibrinogen, mean CIMT (0.19 mm increase) was associated with increased coronary heart disease risk HR 1.38 for women and HR 1.17 for men.

A later study with 14,214 subjects of the ARIC population followed up for 6-9 years showed the same extreme mean CIMT was associated with increased ischaemic stroke risk (adjusted HR 2.02 for women and HR 1.78 for men), and mean CIMT (0.18 mm increase) was associated with incident ischaemic stroke (adjusted HR 1.36 for women, HR 1.21 for men)[175].

The Cardiovascular Health Study[176] (CHS) commenced in 1998, examined 4,476 individuals of 65 years of age or more with no clinical CVD with a mean follow-up of 6.2 years. Their methodology for CIMT assessment measured both the common carotid artery (1 longitudinal view) and the internal carotid artery (3 longitudinal views) which included the carotid bulb. Measurements included the maximum IMT thickness of both near and far walls for both carotid systems in this case, producing a maximum possible of 4 measurements for the CCA and 12 measurements for the ICA. Analyses were performed with both CCA and ICA IMT measurements. Briefly, in the CHS, each standard deviation increase in the combined CIMT was independently associated with incident myocardial infarction and stroke RR 1.36 after adjustment for age, sex and conventional risk factors. Also, each 0.55 mm increase in ICA IMT was more strongly associated with myocardial infarction than stroke (adjusted HR 1.34 vs. 1.25).

The Rotterdam Study[177] based in a single centre with 7,983 subjects aged 55 years, was also used to examine the relationship of CIMT with incident myocardial infarction and stroke. The nested case-control design used a study population of 5,130 subjects, of which CIMT was assessed in the 99 subjects with myocardial infractions and 95 subjects with strokes, and in the 1,373 random controls with CIMT data. Carotid ultrasound performed measured both near and far walls of the distal 10 mm of both common carotid arteries. For each location, an average of 3 images was taken. Information on plaques was also examined. The OR for

myocardial infarction and stroke was 1.43 and 1.25 respectively in multivariable models that adjusted for conventional risk factors, BMI, HDL cholesterol and diabetes.

Table 5.1 - Summary of CIMT measurements from the 3 main studies

	Common carotid		Carotid bulb		Internal carotid	
	Near/far	**L & R**	**Near/far**	**L & R**	**Near/far**	**L & R**
ARIC	Far	Both	Far	Both	Far	Both
CHS	Both	Both	Combined ICA		Both	Both
Rotterdam	Both	Both	-	-	-	-

American Society of Echocardiography Consensus Statement

The American Society of Echocardiography in 2008 issued a consensus statement endorsed by the Society for Vascular Medicine, describing recommendations on the methodology of carotid ultrasonography for CIMT assessment as well as cut-points for CIMT based on various large-scale studies of CIMT in various healthy populations[178].

The recommendations for CIMT assessment is for those who have not have any established CVD of any kind or diabetes with an intermediate Framingham risk score (6-20%). This may be pertinent as the Framingham risk score gives 10-year risk while some individuals who are at intermediate risk but because of their age may develop CVD at a later stage well beyond the 10 years, and CIMT may be able to further risk stratify these individuals. The report also recognised that other individuals in whom CIMT assessment may be useful are those who have a family history of premature CVD, those less than 60 years of age with severe abnormalities in a single risk factor who may otherwise not be candidates for drug therapy, e.g. familial hypercholesterolaemia, and women under the age of 60 years of age with 2 or more CVD risk factors.

The carotid ultrasound protocol recommended by this report was to measure only the far wall of the common carotid artery in triplicate and measure the mean IMT for the 1-cm length of the distal CCA using semi-automated edge detection software with validated accuracy. The report also recommended that plaques should be incorporated or 'traced' as part of the CIMT. An option was given to supplement this with a thorough scan of the other parts of the carotid system for the presence of plaques which are defined as the presence of focal wall thickening of >50% of the surrounding vessel wall, or as a focal region with CIMT >1.5 mm that protruding into the lumen distinct from the adjacent boundary.

The consensus statement of the American Society of Echocardiography[178] also suggested that CIMT values ≥75th percentile are indicative of increased CVD risk, those in the 25th to 75th percentile considered average or unchanged risk, while those <25th percentile considered lower risk. The recommendations also discouraged the use of values from clinically referred populations because of high likelihood of referral bias and inaccurate risk estimates. Normograms for large-scale studies with CVD outcome measures such as the ARIC study[174] are available. Our study population is 99.9% Caucasian, with 98.7% of them Irish. The percentile cut-points relevant to our study are illustrated in Table 5.2.

Table 5.2 – Abridged ARIC Study Normogram

	Right CCA (mm)					
	White male (age in years)			White female (age in years)		
Percentile	45	55	65	45	55	65
25th	0.496	0.572	0.648	0.476	0.542	0.608
50th	0.570	0.664	0.758	0.536	0.616	0.696
75th	0.654	0.774	0.894	0.610	0.710	0.810
	Left CCA (mm)					
	White male (age in years)			White female (age in years)		
Percentile	45	55	65	45	55	65
25th	0.524	0.588	0.652	0.472	0.540	0.608
50th	0.598	0.684	0.770	0.538	0.622	0.706
75th	0.690	0.806	0.922	0.610	0.710	0.810

AIMS

1. To examine possible associations of CIMT with conventional CVD risk factors and novel risk markers,
2. To examine possible differences between the various points of measurement of CIMT and its relationship with conventional risk factors, novel risk markers and presence of coronary artery disease,
3. To examine possible additive effects of CIMT on coronary artery disease risk

METHODS

Methodologies of CIMT measurements differ between the major prospective outcome studies. Consideration on the methodology chosen had been based on the following principles. Methodologies that focused only on the common carotid had various advantages. The common carotid artery is the clearest target and automated edge-detection software programs are available for images of the common carotid artery that can help eliminate reader bias and variability. However, focal plaques are found commonly in the internal carotid artery, and seldom in the common carotid. On the other hand, CVD risk factors relate more strongly with the internal carotid artery and some trials such as ACAPS, ASAP, BCAPS would have been negative if common carotid artery progression had been the only measure. It was also recognised that the measurement of internal carotid artery IMT may be more difficult in terms of visualisation, giving potential for missing data. The variability can be two- or three-fold that of the common carotid and automated edge-detection software do not work as well for the internal carotid artery.

The design, execution and closure of this study had already taken place before the above consensus statement had been issued for the measurement of CIMT. However, the study design and how the information was recorded allowed the implementation of the methodology recommended by the American Society of Echocardiography.

Carotid Intima-Media Thickness Assessment

The methodology described below were the result of multi-disciplinary discussion that also included radiologists and vascular scientists. The above background data and principles were discussed and the final methodology used described below was designed to be compatible with the major large prospective studies described above to allow for comparison of findings. The group had chosen not to use automated edge-detection software for the purposes of CIMT assessment, considering that most major studies previously had used manual technique. A similar decision had been made for the analysis of the coronary angiograms described in Chapter 4.

All carotid ultrasounds were to be performed within 3 months of the coronary angiography and blood collection.

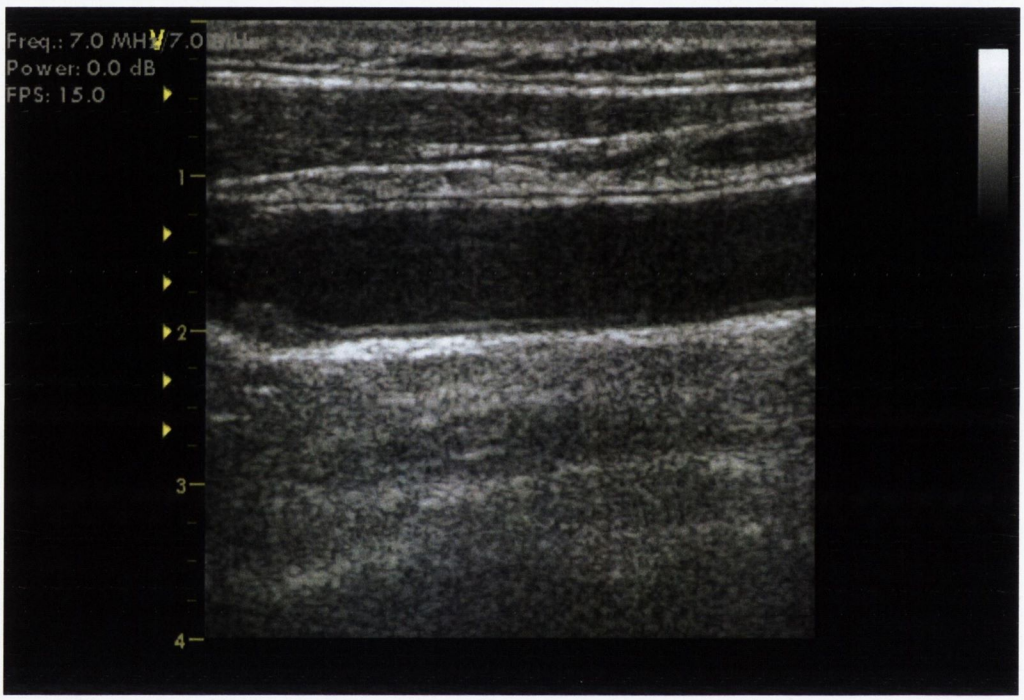
1. Subjects were rested for 10 min with the neck turned slightly.
2. The carotid arteries were scanned with B-mode ultrasound with transducer frequency of 7 MHz.
3. A scout imaging of the carotid system and the best possible view of the IMT will be obtained and stored for off-line analysis. Multiple images were obtained if either the near or far wall IMT is not visualised clearly. IMT measurements were taken from the best possible view.
4. The areas imaged will be (see Definitions below)
    a. Common carotid artery (CCA)
    b. Carotid bulb (CB)
    c. Internal carotid artery (ICA)
5. Images are acquired and stored in DICOM format and analyses will be made off-line on the GE EchoPAC system.

Definitions

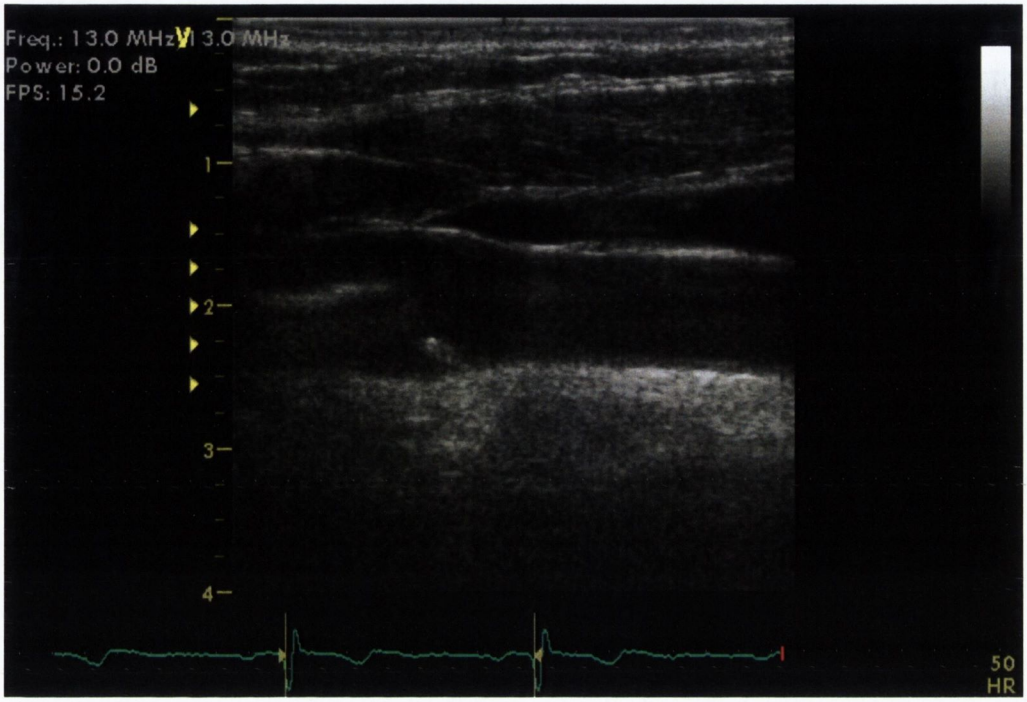
Figure 5.3 (a) and (b) show still images of the carotid ultrasound of a randomly chosen study participant to illustrate the definitions of the various points of IMT assessment.

Figure 5.3 – Ultrasound images of the Carotid Artery

(a) Common carotid



(b) Common Carotid, Carotid Bulb and Internal Carotid Arteries



The **intima-media thickness** (IMT) was defined as the distance between the bright line representing the lumen-intima interface to the bright line representing the media-adventitia interface. The **common carotid artery** (CCA) IMT was the maximum thickness measured for a 1 cm segment proximal to the carotid bulb. The start of the **carotid bulb** (CB) was defined the loss of the parallel wall of the common carotid up until the level of the bifurcation. The **internal carotid artery** (ICA) IMT was the maximum IMT thickness for a 1 cm segment distal to the carotid bulb.

The **far wall** (FW) IMT is the distance between the leading edge of the first bright line (lumen-intima interface) and the leading edge of the second bright line (media-adventitia interface). Similarly, the **near wall** (NW) IMT is the distance between the trailing edge of the first bright line and the trailing edge of the second bright line.

The definition of **combined CIMT** in this study was the mean of the 12 measurements of the maximum IMT of the CCA, CB and ICA near and far walls on both sides.

Endpoints

1. **Combined CIMT**: Mean of the 12 measurements of the maximum IMT of the CCA, CB and ICA near and far walls on both sides
2. **Extreme CIMT**: CIMT ≥1 mm as in ARIC study and used by many clinicians
3. **≥75th percentile ARIC study cut-points**: Far wall CCA IMT at the published ≥75th percentile ARIC study cut-points

RESULTS

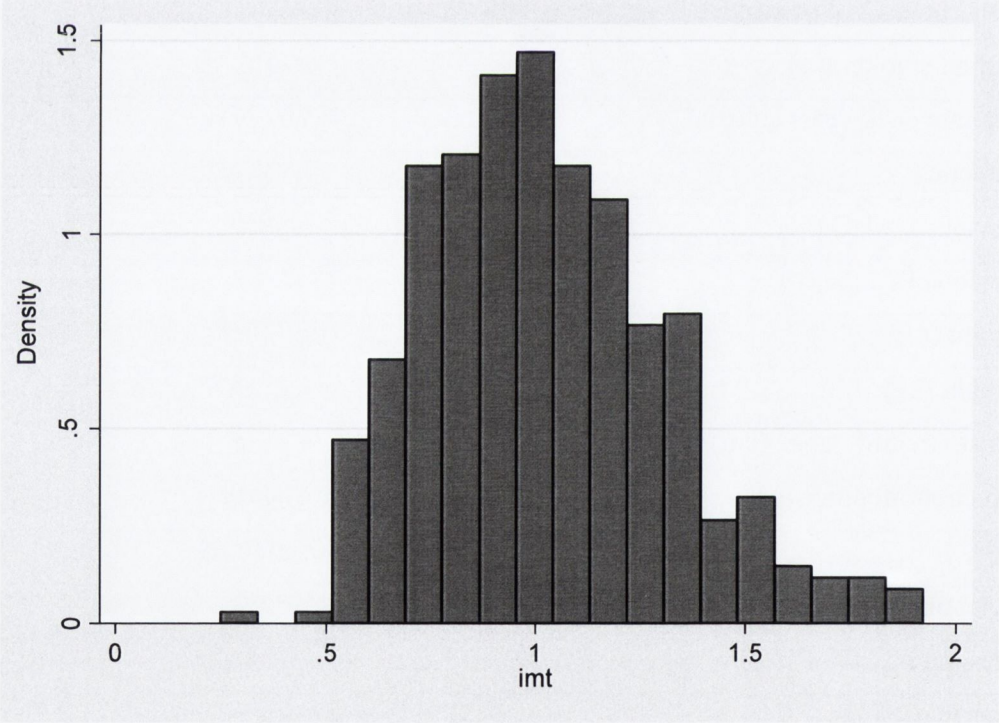
Of the 730 patients enrolled in the *Cath Lab Study*, 386 had carotid ultrasound for CIMT assessment. Baseline characteristics of the population in the CIMT sub-study were similar to the patients in the entire study. Key baseline characteristics of the patients CIMT sub-study are shown in Table 5.3.

Table 5.3 - Baseline characteristics of CIMT sub-study

Characteristic	Mean (Standard deviation)
Age (years)	59.1 (10.0)
Male (%)	69%
Systolic blood pressure (mmHg)	134 (18)
Diastolic blood pressure (mmHg)	74 (10)
Total cholesterol (mmol/L)	4.42 (0.96)
LDL cholesterol (mmol/L)	2.42 (0.82)
HDL cholesterol (mmol/L)	1.34 (0.37)
Current smoker (%)	17%
Lifetime pack years smoked	20 (27)
Diabetic (%)	13%
Previous CABG (%)	6.9%
Previous PCI (%)	22.0%
Height (m)	1.69 (0.94)
Weight (kg)	83.2 (14.8)
Waist circumference (cm)	99 (11)
Hip circumference (cm)	104 (9)
BMI	29.0 (4.6)
Metabolic syndrome (%)	29%
Chest pain (CCS Class)	1.5
Aspirin use (%)	76%
Statin use (%)	68%
Beta-blocker use (%)	49%
ACEI use (%)	28%

ARB use (%)	9.1%
Coronary Artery Disease Score	14.4 (11.3)
SYNTAX Score	7.9 (11.5)
Intima Media Thickness (mm)	1.02 (0.28)
SCORE Risk score (%)	2.9% (3.3%)
CD40 Ligand (pg/ml)	175 (131, 253)
VCAM (ng/ml)	683 (204)
P-selectin (ng/ml)	52 (15)
High sensitivity CRP (mg/L)	3.4 (5.2)
Homocysteine (μmol/L)	9.5 (7.9, 11.5)
Fibrinogen (g/L)	3.0 (0.6)

Figure 5.4 - Distribution of Carotid Intima-Media Thickness



The top tertile of our study population was 1.125 mm. The 25th, 50th, 75th percentiles of our combined CIMT are tabulated in Table 5.4 for men and women.

Table 5.4 – Percentile chart for CIMT in the CIMT Sub-study

Percentile	CIMT Men (mm)	CIMT Women (mm)
25th	0.850	0.727
50th	1.033	0.920
75th	1.133	1.225

Conventional risk factors such as blood pressure, hypercholesterolaemia and smoking was associated with CIMT in a graded manner. As expected, there was increasing statin use across CIMT tertiles. Of the novel risk markers studied, median levels of CD40 ligand, VCAM, P-selectin, homocysteine and the metabolic syndrome increased across tertiles of CIMT, although it was not statistically significant in the case of CD40 ligand.

Table 5.5 - Medians of risk factors and novel risk markers across CIMT tertiles

(a) CIMT with conventional risk factors

CIMT	Systolic BP	Serum Cholesterol	Statin use	High cholesterol	Lifetime Pack yrs
Tertile 1	129	4.4	58%	77%	17
Tertile 2	135	4.3	74%	84%	20
Tertile 3	139	4.5	71%	91%	24
P-value	<0.001	0.51	0.02	0.002	0.05

(b) CIMT with novel risk markers

CIMT	CD40 ligand	VCAM	P-selectin	Homo-cysteine	Metabolic syndrome
Tertile 1	166	646	51	8.7	25%
Tertile 2	171	692	49	9.3	24%
Tertile 3	198	713	55	10.6	41%
P-value	0.08	0.002	0.05	<0.001	0.003

Pearson's Correlations

Combined CIMT strongly correlated with the common carotid ($r=0.72$, $p<0.0001$) and carotid bulb IMT ($r=0.85$, $p<0.0001$) but less so with internal carotid IMT ($r=0.48$, $p<0.0001$). Correlations of CIMT with conventional risk factors and novel risk markers are shown in Table 5.6. Comparisons were made correlating the same factors with IMT measured from the common carotid (CC), carotid bulb (CB) and internal carotid (IC) artery.

Table 5.6 - Univariate Pearson's Correlations of IMT with risk factors and novel risk markers

	CIMT (r) **P-value**	**CC IMT (r)** **P-value**	**CB IMT (r)** **P-value**	**IC IMT (r)** **P-value**
Age	0.37 p<0.0001	0.40 p<0.0001	0.29 p<0.0001	0.12 p=0.03
Male Sex	0.19 p=0.0002	0.12 p=0.02	0.18 p=0.0006	0.05 p=0.41
Systolic blood pressure	0.19 p=0.0002	0.19 p=0.0003	0.13 p=0.01	0.06 p=0.28
Total cholesterol	0.01 p=0.8	0.00 p=0.96	0.05 p=0.32	0.05 p=0.40
Statin use	0.10 p=0.04	0.10 p=0.06	0.09 p=0.08	0.04 p=0.45
Cholesterol ≥5 or on statin	0.12 p=0.02	0.10 p=0.07	0.08 p=0.14	0.05 p=0.43
Current smoker	0.04 p=0.47	-0.07 p=0.04	0.07 p=0.15	-0.01 p=0.84
Lifetime Pack years	0.11 p=0.02	0.07 p=0.19	0.10 p=0.05	0.09 p=0.11
HDL cholesterol	-0.09 p=0.09	-0.07 p=0.18	-0.07 p=0.14	-0.04 p=0.41

Glucose	0.14 p=0.006	0.08 p=0.14	0.14 p=0.007	0.02 p=0.68
Metabolic syndrome	0.18 p=0.0005	0.12 p=0.02	0.13 p=0.009	0.00 p=1.0
hs-CRP	0.01 p=0.9	0.00 p=0.97	-0.00 p=0.93	0.04 p=0.51
Homocysteine	0.20 p=0.0001	0.17 p=0.001	0.17 p=0.0009	-0.01 p=0.92
Log CD40 ligand	0.11 p=0.03	0.12 p=0.03	0.01 p=0.81	0.15 p=0.01
VCAM	0.11 p=0.04	0.12 p=0.02	0.10 p=0.06	-0.05 p=0.31
P-selectin	0.14 p=0.01	0.06 p=0.26	0.13 p=0.01	0.10 p=0.11
Fibrinogen	0.05 p=0.3	0.07 p=0.16	0.03 p=0.59	0.10 p=0.10
SCORE risk	0.32 p<0.0001	0.37 p<0.0001	0.32 p<0.0001	0.21 p=0.002

All of the conventional risk factors and novel risk markers which were significantly correlated with CIMT were examined in a generalised linear model in combination to examine for their possible independent associations with CIMT. Table 5.7 shows the exponential of the beta-coefficients of each risk factor and novel risk marker with CIMT in a multivariable model.

Table 5.7 - Generalised Linear Model of CIMT with risk factors and novel risk markers in combination (multivariable)

	Exp(beta)	95% CI	P value
Age	1.1	1.1, 1.1	<0.001
Male sex	2.5	1.3, 4.9	0.007
Systolic Blood pressure	1.0	1.0, 1.0	0.18
Cholesterol ≥5 mmol/L or on statin	1.9	0.9, 4.1	0.11
Lifetime pack years of smoking	1.0	1.0, 1.0	0.91
Glucose	1.1	0.9, 1.4	0.51
Metabolic syndrome	1.8	0.9, 3.6	0.08
Homocysteine	1.1	1.0, 1.2	0.19
Log CD40 ligand	1.6	1.0, 2.5	0.03
VCAM	1.0	1.0, 1.0	0.73
P-selectin	1.0	1.0, 1.0	0.05

CIMT with Coronary Artery Disease

Table 5.8 shows the association of CIMT with SYNTAX defined haemodynamically significant coronary artery disease in exactly the same multivariable logistic regression models used in the previous chapter to examine novel risk markers. The multivariable models are:

1. **Univariate**: Each CIMT definition individually
2. **Model 1**: Above with conventional CVD risk factors – age, sex, blood pressure, cholesterol, smoking and statin use,
3. **Model 2**: Above model (Model 1) with HDL cholesterol, glucose, and
4. **Model 3**: Above model (Model 2) and all other risk markers, being hs-CRP, homocysteine, log CD40 ligand, VCAM, P-selectin and fibrinogen.

Table 5.8 - OR of Significant Coronary Artery Disease for the various IMT definitions

	Univariate (95% CI) p-value	**Model 1** (95% CI) p-value	**Model 2** (95% CI) p-value	**Model 3** (95% CI) p-value
Combined CIMT (0.1 mm increase)	1.2 (1.1, 1.4) p<0.001	1.1 (1.0, 1.2) p=0.11	1.1 (1.0, 1.2) p=0.13	1.0 (0.9, 1.2) p=0.55
CC CIMT (0.1 mm increase)	1.2 (1.0, 1.3) p=0.004	1.0 (0.9, 1.1) p=0.85	1.0 (0.9, 1.1) p=0.99	1.0 (0.8, 1.1) p=0.64
CB CIMT (0.1 mm increase)	1.1 (1.1, 1.2) p=<0.001	1.1 (1.0, 1.1) p=0.05	1.1 (1.0, 1.1) p=0.05	1.0 (1.0, 1.1) p=0.47
IC CIMT (0.1 mm increase)	1.0 (1.0, 1.1) p=0.28	1.0 (0.9, 1.1) p=0.98	1.0 (0.9, 1.1) p=0.81	1.0 (0.9, 1.1) p=0.80
CIMT ≥1 mm	2.2 (1.4, 3.6) p=0.001	1.1 (0.6, 2.1) p=0.65	1.1 (0.6, 2.1) p=0.70	0.9 (0.5, 1.8) p=0.80
CC CIMT (≥75th percentile ARIC study)	7.8 (2.3, 26.8) p=0.001	6.3 (1.2, 34.0) p=0.03	6.2 (1.2, 33.3) p=0.03	4.5 (0.8, 25.7) p=0.09

For Model 3, when the novel risk markers are added step-wise, the addition of P-selectin attenuated the effect of CC CIMT (≥75th percentile ARIC study cut-points).

Table 5.9 - Discriminative ability for SYNTAX significant coronary artery disease

	Sensitivity	Specificity	Positive Predictive value	Negative Predictive Value
SCORE ≥5%	28% (22%, 35%)	91% (87%, 95%)	73% (61%, 82%)	60% (54%, 65%)
CIMT (≥75th percentile ARIC study)	98% (94%, 100%)	15% (10%, 22%)	52% (45%, 58%)	88% (69%, 98%)

Re-classification by CC IMT (≥75th percentile ARIC study cut-points)

The multivariable models in Table 5.8 suggested that using the CC IMT at the ≥75th percentile ARIC study cut-points was independently associated with the presence of significant coronary artery disease. Table 5.10 examines if this cut-point can re-classify patients better in conjunction with the SCORE equation.

Table 5.10 – Reclassification ability of CC IMT (ARIC Study cut-points) in conjunction with the SCORE system in detecting haemodynamically significant coronary artery disease (SYNTAX ≥1)

SCORE Alone	Actual Coronary Disease	SCORE and CC IMT	Actual Coronary Disease
SCORE <5%	40%	<75th percentile	9%
SCORE <5%	40%	≥75th percentile	85%
SCORE ≥5%	73%	<75th percentile	None
SCORE ≥5%	73%	≥75th percentile	78%

DISCUSSION

Combined CIMT in the CIMT sub-study was taken as a mean of the maximum IMT of both near and far wall of the 3 locations (common carotid, carotid bulb and internal carotid arteries) of both carotid systems. This combined CIMT was associated with conventional CVD risk factors such as age, sex, systolic blood pressure, hypercholesterolaemia, lifetime pack years of smoking, glucose, and also novel risk markers such as the metabolic syndrome, homocysteine, log-transformed CD40 ligand and P-selectin.

Broadly, the IMT based on the common carotid and internal carotid correlate better with conventional risk factors and novel risk markers. The combined CIMT also strongly correlated with common carotid and carotid bulb IMT but less so with internal carotid IMT.

Why would there be a lack of correlation between the internal carotid IMT with CVD risk factors except for age and logarithmically-transformed CD40 ligand? Firstly, it may be that the internal carotid is more difficult to image, thus resulting in more missing data leading to loss of statistical power to show association. However, previous large scale studies such as the ARIC study have found stronger associations of the internal carotid IMT with incident myocardial infarction that ischaemic stroke which may suggest that the internal carotid IMT may be a marker for predicting different aspects of CVD risk. Neither reason fully explains the findings and warrants further investigation.

Although many risk factors and markers described above singly show association with CIMT, this study offers an opportunity to examine if a wide range of conventional risk factors and novel risk markers taken in combination are still independently associated with CIMT. The multivariable generalised linear model in Table 5.7 suggested that only age, sex, logarithmically-transformed CD40 ligand and P-selectin were still independently associated with CIMT when many traditional risk factors and novel risk markers were examined in combination.

Logarithmically-transformed CD40 ligand was positively associated with CIMT in this study which is expected from our current knowledge of atherosclerosis. However, the same marker had an inverse relationship with coronary artery disease (Chapter 4). The contrasting divergent association with the two CVD endpoints is curious and difficult to explain.

Based on this, it is unlikely due to the effect of statins. Statin use in this sub-study was the same as the *Cath Lab Study*, as the blood was drawn at the same time from the same patient under the same conditions.

One may then have to postulate that the effect of CD40 ligand may be different in different vascular beds. This would explain the inverse relationship with coronary artery disease, and the positive one with carotid intima-media thickness. Other groups have findings that may suggest this trend[163]. This will be discussed further in the general discussion in Chapter 7.

Although CIMT is considered a surrogate marker for CVD risk, it may also be a marker of early atherosclerosis. This study offered the opportunity to relate CIMT with coronary artery disease endpoints. IMT measured from all locations except for the internal carotid were associated with SYNTAX-defined significant coronary artery disease in univariate models. However, only carotid bulb IMT and common carotid IMT ≥75th percentile of the ARIC study remained significantly associated with coronary artery disease after multivariable adjustment for conventional risk factors including HDL cholesterol and glucose. Carotid bulb IMT may relate better to coronary artery disease because the carotid bulb is a common location for the build-up of plaques, and the measured IMT incorporates these plaques.

Finally, CC IMT ≥75th percentile of the ARIC study had good discriminative ability and was able to identify more patients who had significant coronary artery disease when used in conjunction with the SCORE equation. Very importantly, CC IMT ≥75th percentile ARIC study cut-points improved detection of significant coronary artery disease in those deemed low-risk by the SCORE equation from 40% to 85%. Clinically meaningful re-classification should be from the low-risk group to one in a higher risk category as that would mean a change in management.

CONCLUSIONS

While CIMT is independently associated with many conventional risk factors and novel risk markers individually, only log-transformed CD40 ligand and P-selectin are associated with CIMT when examined in combination.

The locations for CIMT measurement when it comes to detecting the presence of coronary artery disease were at the carotid bulb and the common carotid using ≥75th percentile ARIC study cut-points.

CD40 ligand was independently associated (positive) with CIMT whereas in the previous chapter, CD40 ligand had an independent inverse relationship with coronary artery disease. This warrants further investigation and study.

CIMT was independently associated with the presence of coronary artery disease after adjustment for traditional risk factors. However, no improvement in detection occurred when novel risk markers were also used.

CIMT measured from the carotid bulb, and also using the 75th percentile cut-points from a large-scale study such as the ARIC study can improve detection of patients with significant coronary artery disease when used in conjunction with the SCORE equation.

CHAPTER SIX

THE ROLE OF THE METABOLIC SYNDROME IN CARDIOVASCULAR RISK ESTIMATION – IS IT MORE THE SUM OF ALL ITS PARTS?

INTRODUCTION

The metabolic syndrome is associated with increased cardiovascular risk[133], especially coronary heart disease[134-136], and also the development of diabetes[137,138]. Endothelial dysfunction and inflammation are considered key players associated with the metabolic syndrome. Of academic interest, and a subject of great debate, is whether the increased CVD risk is associated with the metabolic syndrome is 'more than the sum of its parts'. The state of the art was reviewed in Chapter 2.

The AHA-modified NCEP definition of the metabolic syndrome is satisfied when at least 3 of the following 5 characteristics are present:

1. Waist circumference ≥102 cm in Men, ≥88 cm in Women
2. Triglycerides ≥150 mg/dL (1.7 mmol/L), or on drug treatment
3. HDL cholesterol <40 mg/dL (1.3 mmol/L) in Men,
   HDL cholesterol <50 mg/dL (1.03 mmol/L) in Women, or on drug treatment
4. Systolic BP ≥130 mmHg, or Diastolic BP≥85 mmHg, or on drug treatment
5. Fasting glucose ≥100 mg/dL (5.6 mmol/L), or on drug treatment

AIMS

1. To examine if the metabolic syndrome is associated with CVD independent of conventional risk factors and other novel risk markers.
2. To examine if the metabolic syndrome predicts CVD risk better as a whole component, or its individual components in combination.

METHODS

Study Population

The European Concerted Action Project: Homocysteinaemia and Vascular Disease (COMAC)[179] was a multi-centre case-control study designed to examine the relationship of homocysteine with vascular disease. The patients were recruited from 19 centres from 9 countries in Europe during the 1990s.

The cases were 750 patients with objective evidence of vascular disease. The majority of the cases (about 69%) were recruited within 1 year of diagnosis. All had clinical symptoms and at least one objective evidence of CVD, which were defined as follows:

Coronary heart disease: Evolving ST-segment changes on ECG with ≥2-fold increase of creatinine kinase, pathological Q waves, stenosis of ≥70% of at least one major coronary artery on angiography.

Cerebrovascular disease: Carotid artery disease on ultrasound or angiography (either ≥50% or plaque), CT Brain evidence of cerebral infarction with no demonstrable source of embolism.

Peripheral vascular disease: Obstruction to at least one major peripheral artery on angiography, evidence of vasculitis, fibromuscular dysplasia, large vessel arteritis or dissection, ankle/brachial index <0.9.

The 800 controls were age- and sex-matched individuals without vascular disease from the same geographical background and were derived from random community samples, employee health insurance registers and hospital employees.

Exclusion criteria of the COMAC study included recent systemic illness, pregnancy, hypothyroidism, and conditions which affect homocysteine and its assay which were renal failure, anticonvulsants, nitrous oxide anaesthesia and chronic alcohol abuse.

Definition of the Metabolic Syndrome

The metabolic syndrome was defined using the AHA 2005 modified NCEP definition[143]. As the COMAC study did not examine waist circumference, BMI ≥30 was used as a proxy for the increased waist circumference component of the metabolic syndrome. This has been done previously in other studies[135,138].

Statistical Analyses

Statistical analyses were performed using Intercooled STATA 9.2 for Windows. Means of baseline characteristics of the study population including levels of conventional risk factors, novel risk markers and in particular, the metabolic syndrome and its components were compared using *t*-tests. For homocysteine, because of the known skewed distribution, medians were compared using Kruskal-Wallis test.

Standard logistic regressions were used to examine the effect of the metabolic syndrome on CVD risk, firstly in univariable models followed by multivariable models adjusting for conventional risk factors and also, other novel risk markers in combination. These novel risk markers include homocysteine, apolipoprotein B & AI and white cell count.

The diagnostic ability of the metabolic syndrome to predict CVD risk was examined using areas under the receiver operating characteristic (ROC) curve. This allowed us the opportunity to examine whether a model including the metabolic syndrome as a categorical variable was better at predicting CVD compared to one made up of its components as continuous variables.

RESULTS

Of the total 1,550 individuals in the study, 113 were excluded due to missing data or ambiguity with regards to the diagnosis of the metabolic syndrome. Table 6.1 presents the mean baseline characteristics with standard deviations of the study population, with the exception of homocysteine, where medians are presented.

Table 6.1 – Baseline characteristics of the study population

	Without CVD n=766	**With CVD n=671**	**P-value**
Age	44 (10)	47 (9)	<0.0001
Male sex	71%	73%	0.4
Systolic BP	127 (15)	131 (20)	<0.0001
Diastolic BP	81 (9)	82 (11)	0.06
Cholesterol	5.9 (1.3)	6.4 (1.4)	<0.0001
Smokers	33%	55%	<0.0001
BMI	24.8 (3.4)	25.8 (3.8)	<0.0001
HDL cholesterol	1.3 (0.4)	1.1 (0.4)	<0.0001
Triglycerides	1.3 (0.8)	1.9 (1.3)	<0.0001
Glucose	5.0 (0.7)	5.2 (0.9)	<0.0001
Metabolic syndrome	15%	35%	<0.0001
Homocysteine	9.4	10.8	0.0001
White cell count	6.5 (1.9)	7.3 (2.1)	<0.0001
Apolipoprotein B	1.2 (0.4)	1.3 (0.4)	<0.0001
Apolipoprotein AI	1.3 (0.3)	1.3 (0.3)	0.03
Apo B/AI ratio	0.9 (0.4)	1.1 (0.4)	<0.0001

Table 6.2 examines the association of the metabolic syndrome with CVD by comparing odds ratios of CVD for the metabolic syndrome in various models:

1. Univariate - the metabolic syndrome alone,
2. Adjusted also for the conventional risk factors: age, sex, blood pressure, cholesterol and smoking, and
3. Adjusted for the conventional risk factors and novel risk markers including white cell count, apolipoprotein B/AI ratio, homocysteine and its covariates (creatinine, folate and vitamin B12)

Table 6.2 – OR of CVD for the metabolic syndrome

Metabolic syndrome	OR	95% CI	P value
Univariate	3.1	2.4, 4.1	<0.001
Adjusted for conventional risk factors	2.6	2.0, 3.4	<0.001
Adjusted for above including white cell count, apolipoprotein B/AI, homocysteine and covariates	2.0	1.5, 2.8	<0.001

Table 6.3 shows OR of CVD for selected variables of interest from the above final multivariable model which includes the metabolic syndrome, in combination with conventional risk factors and the novel risk markers.

Table 6.3 – OR of CVD for selected variables (multivariable model)

	OR	95% CI	P value
Metabolic syndrome	2.0	1.5, 2.8	<0.001
Homocysteine (5 μmol/L increase)	1.5	1.3, 1.8	<0.001
White cell count (1 increase)	1.1	1.1, 1.2	<0.001
Apo B/AI ratio (0.1 unit increase)	1.1	1.0, 1.1	0.001

Table 6.4 shows OR of CVD for the same variables but instead showing the OR of CVD for categorical components of the metabolic syndrome separately. This allows us to compare the contributions of each component of the metabolic syndrome to CVD risk after adjustment for all conventional risk factors and novel risk markers. The results suggest that HDL cholesterol is the metabolic syndrome component that contributes the most strongly to CVD risk, followed by blood pressure and triglycerides. It is also interesting to note that the risk associated with apolipoprotein B/AI ratio was attenuated when HDL cholesterol was used in the model.

Table 6.4 – OR of CVD for the components of the metabolic syndrome adjusted for conventional risk factors and novel risk markers

	OR	95% CI	P value
BMI component	1.4	0.9, 2.1	0.1
Triglycerides component	1.4	1.1, 1.9	0.01
Blood pressure component	1.6	1.1, 2.3	0.006
Glucose component	1.2	0.9, 1.7	0.3
HDL component	2.6	2.0, 3.4	<0.001
Homocysteine (5 μmol/L increase)	1.5	1.3, 1.8	<0.001
White cell count (1 increase)	1.1	1.0, 1.2	0.005
Apo B/AI ratio (0.1 unit increase)	1.0	1.0, 1.1	0.7

Table 6.5 compares the diagnostic performance of each risk factor to predict CVD using areas under the receiver operator characteristic (AUROC) curve. Models with risk markers added incrementally were compared with the previous to examine if the new added risk marker added predictive value to CVD risk estimation. Of interest is to examine whether the metabolic syndrome as a categorical variable performs compared to a model containing its individual components as continuous variables.

Table 6.5 – Comparison of the diagnostic ability of the various models for CVD based on their areas under the ROC curve

	AUROC	P-value compared with previous
Conventional risk factors	0.68	Not applicable
+ Metabolic syndrome (categorical)	0.70	0.002
+ Metabolic syndrome components as continuous variables	0.76	<0.0001
+ White cell count	0.76	0.045
+ Apolipoprotein B/AI	0.76	0.7
+ Homocysteine	0.78	0.001

Table 6.6 examines which metabolic syndrome component as continuous variables are the strongest contributors to CVD risk. Each component of the metabolic syndrome was examined by incrementally adding the individual component to a baseline referent model (Model A) consisting of the conventional risk factors. Similar to the comparison by using magnitudes of OR in Table 6.4 previously, HDL cholesterol followed by triglycerides are the strongest contributors to CVD risk.

Table 6.6 – Which metabolic syndrome component contributes the most to CVD risk?

	AUROC	P-value compared with Model 1
Conventional risk factors (Model A)	0.68	Referent
Model A + BMI	0.69	0.15
Model A + Glucose	0.71	0.04
Model A + Triglycerides	0.72	<0.0001
Model A + HDL cholesterol	0.73	<0.0001

Next, consideration was given to whether HDL cholesterol and/or triglycerides could achieve as good a prediction of CVD risk as all the components combined. Table 6.7 examines this with reference to the best prediction model, which was using conventional risk factors and all the metabolic syndrome components in combination as continuous variables.

Table 6.7 – Which minimum set of metabolic syndrome components gives the best CVD prediction?

Conventional Risk factors +	**AUROC**	**P-value compared with best model**
All Metabolic syndrome components as continuous variables (Best prediction model)	0.76	Referent
HDL Cholesterol	0.73	0.002
HDL Cholesterol + Triglycerides	0.76	0.4

DISCUSSION

The metabolic syndrome was associated with approximately two-fold risk of CVD independent of conventional risk factors and other novel risk markers. This magnitude of risk was not dissimilar to other studies of the metabolic syndrome. This study had the added advantage of further adjusting for novel risk markers such as white cell count, apolipoprotein B/AI ratio and homocysteine as well as conventional risk factors in combination. When considering multiple novel risk markers in combination, the metabolic syndrome, white cell count, apolipoprotein B/AI ratio and homocysteine remain independent in multivariable analyses when also corrected for conventional risk factors.

The original COMAC study showed an independent association of homocysteine with CVD risk. This independent risk of homocysteine risk was not attenuated with the addition of the metabolic syndrome and the other risk markers in combination. This study had examined homocysteine as a continuous variable whereas the original COMAC study considered raised homocysteine ($\geq$12.1 μmol/L) which had an odds ratio of 2.2. When considered as the same categorical variable in this study in combination with the metabolic syndrome, the odds ratio was 2.0 (95% CI 1.5, 2.6, $p<0.001$), a similar result. This suggests that both the metabolic syndrome and homocysteine exert independent effects on CVD risk.

There was an interesting finding when the metabolic syndrome was compared with its components in combination in models predicting CVD risk using their areas under the ROC curves. When the outcome of CVD risk was used, the metabolic syndrome did predict risk independently. However, it did not perform as well as a model that used its individual components as continuous variables in combination. It has been debated whether the risk associated with the metabolic syndrome is 'more than the sum of its parts' due to its associations with inflammation, endothelial dysfunction and hypercoagulability. This study suggests that the CVD risk associated with the metabolic syndrome, although independent, is due to the combined effect of its components, not more. In terms of CVD risk estimation, better results could be achieved using the components of the metabolic syndrome individually.

Of the components of the metabolic syndrome, HDL cholesterol, triglycerides and blood pressure were the strongest independent contributors to CVD risk. In fact, further analyses using areas under the ROC curve show that the combination of HDL cholesterol and triglycerides together (2 components) was as good as using all 5 components of the metabolic syndrome to predict CVD risk. This suggests that the increased CVD risk associated with the metabolic syndrome is driven by HDL cholesterol and triglycerides. Interestingly, HDL cholesterol also attenuated the effect of apolipoproteins in multivariable analyses, whether apolipoprotein B, AI or the ratio apolipoprotein B/AI was used.

It implies that rather than using the diagnosis of the metabolic syndrome as a categorical variable, better CVD risk estimation can be achieved using HDL cholesterol and triglycerides as continuous variables, simplifying the process to two variables rather than five. In fact, this is replicated and in agreement with the results of the *Cath Lab Study* in Chapter 4 where the addition of HDL cholesterol into a multivariable model containing the metabolic syndrome attenuated its effect completely.

CONCLUSIONS

The metabolic syndrome, white cell count and homocysteine were independently associated with CVD after adjustment for conventional risk factors in CVD risk estimation. These novel risk markers give added predictive value for CVD risk estimation when examined using areas under the ROC curve.

The effect of apolipoproteins on CVD risk was attenuated by HDL cholesterol.

The CVD risk associated with the metabolic syndrome was mainly driven by blood pressure, HDL cholesterol and triglycerides.

Better CVD risk estimation can be achieved using HDL cholesterol and triglycerides as continuous variables with conventional risk factors rather than the metabolic syndrome as a categorical variable.

CHAPTER SEVEN
GENERAL SUMMARY OF RESULTS & DISCUSSION

This work utilised 3 classical epidemiological study designs to address the questions asked. Similar classical statistical methods were employed in all the studies to examine the association of the 3 novel risk markers of interest with cardiovascular disease which are:

1. Asymmetric dimethylarginine (ADMA) with future myocardial infarctions and stroke in a healthy cohort of women (Chapter 3),
2. CD40 ligand with coronary artery disease and carotid intima-media thickness in a cross-sectional study of stable patients with suspected coronary artery disease (Chapters 4 and 5), and
3. The metabolic syndrome with cardiovascular disease (coronary, cerebrovascular and peripheral vascular disease) in a case-control study dataset (Chapter 6).

Asymmetric Dimethylarginine

Asymmetric Dimethylarginine (ADMA) was predictive of future myocardial infarction and strokes in women in a cohort of women healthy at baseline after 24 years. The association was robust and independent after adjustment for conventional risk factors which included age, blood pressure, serum cholesterol, smoking, triglycerides, glucose, waist and hip circumference, body mass index, as well as homocysteine and its covariates, vitamin B12 and creatinine clearance. There was an approximately 30% increased risk of fatal and non-fatal myocardial infarction and stroke for each 0.15 μmol/L (1 standard deviation) increase in ADMA levels.

More importantly, ADMA at the top quintile (≥0.71 μmol/L) was able to re-classify those who were deemed low risk by conventional risk assessment methods such as the SCORE and Framingham equations into a risk category similar to that of the high-risk category. This is a clinically meaningful change because this category of patients falls into one of the groups identified by the European guidelines as one of

the priorities for CVD prevention. They warrant aggressive risk factor intervention, and the high-risk threshold is commonly used in many countries as a threshold point for drug therapy.

The effect of ADMA was independent of both conventional risk factors and homocysteine, with no significant interaction effects with them. This implies that ADMA is an independent risk marker, and not a medium through which other risk factors act. ADMA also provides additive value to current risk estimation systems.

Most of the previous work on ADMA had been *in vitro* or in patient populations with disease, in particular, renal failure. This study was the first to show the association with CVD prospectively in a free living population of women, healthy at baseline, with a long follow-up and validated methodology, utilising all available serum samples and with special consideration given to the representativeness of the cohort.

The questions asked in Chapter 1 about ADMA can now be answered:

1. Is ADMA associated with CVD in an initially healthy population?
   YES
2. Is the association independent of conventional risk factors?
   YES
3. Can ADMA add predictive value to current risk estimation systems such as the SCORE equation and the Framingham risk score?
   YES
4. Are the effects of conventional risk factors and homocysteine on CVD risk mediated through ADMA?
   NO – ADMA IS AN INDEPENDENT RISK MARKER WITH DISCRIMINATIVE ABILITY

CD40 Ligand

CD40 ligand as a novel risk marker was hypothesised based on its pivotal role in inflammation and in atherothrombosis. CD40 ligand is produced by both T-lymphocytes and platelets and fits into our current knowledge of the vascular biology of atherosclerosis. T-lymphocyte activation occurs early in the inflammatory process during the formation of the atherosclerotic fatty streak. Activated platelets play a key role in atherothrombosis, leading to acute coronary syndromes. Multiple previous studies have shown the association of CD40 ligand with acute coronary syndromes. There is paucity of data on the role of CD40 ligand in stable populations apart from a nested case-control arm of the Women's Health Study, while a cohort study has been reported to be underway.

The association of CD40 ligand with CVD was mixed in the *Cath Lab Study*. CD40 ligand showed a graded, statistically robust but inverse association with coronary artery disease which is unexpected from our current knowledge. With carotid intima-media thickness however, CD40 ligand showed independent graded association of CD40 ligand with CIMT after adjustment for conventional risk factors and other novel risk markers in combination.

These curious unexpected findings cannot be explained fully by this work. However, the following speculations on the results show the need for further study and investigation.

Statins have been shown in most studies to reduce serum levels of CD40 ligand and it would be intuitive to explain the inverse relationship with this. One supporting fact for this is that cholesterol, a well-established CVD risk factor, exhibited the same inverse relationship. Also, in the *Cath Lab Study*, those with more coronary artery disease were more likely to be on statins. However, statistical adjustment did not correct this. More importantly, the inverse relationship was not found in the carotid intima-media thickness sub-study even though they were the same patients on the same medications.

Although the association of CD40 ligand with coronary artery disease was statistically significant, there is still a very small possibility that the finding is due to chance. However, the fact that another similar study in North America also reported a similar statistically significant inverse relationship makes this a chance finding very unlikely.

Does CD40 ligand have a different effect on different vascular beds? This would explain the inverse relationship with coronary artery disease, and the positive one with carotid intima-media thickness. Although not statistically significant, the nested case-control arm of the BIP study also suggested this trend[163]. Although this may initially be counter-intuitive to our current concept of atherosclerosis occurring equally in arteries of the entire vascular system, we do know from clinical practice that this is not the case. Plaques tend to build up more in the branch points or shoulder regions of arteries where there is turbulent flow. Another example is the internal mammary arteries after a coronary artery bypass remain patent, seemingly unaffected by atherosclerosis while it is going on elsewhere.

There are also precedents to risk factors behaving differently in different situations. Take smoking for instance, an established risk factor for CVD. The effect of smoking is particularly strong for peripheral arterial disease, even though it is a strong risk factor for coronary artery disease as well as stroke. Further examples can be seen in findings shown in Table 7.1 which are my own analyses from the COMAC study not formally part of this work. They are summarised in the table below and suggests possible differential effects of various novel risk markers with the different clinical manifestations of CVD.

Table 7.1 – OR of the various sub-types of CVD for selected novel risk markers in a multivariable model suggesting differential effects on different vascular beds

	CHD	Cerebrovascular Disease	PVD
Homocysteine ≥12 μmol/	1.6 (1.1, 2.4) p=0.02	2.6 (1.6, 4.2) p<0.001	1.9 (1.1, 3.4) p=0.03
Apo B/AI ratio	1.1 (1.1, 1.2) p<0.001	1.0 (0.9, 1.0) p=0.4	1.1 (1.0, 1.2) p=0.2
Cysteine	1.4 (1.1, 1.9) p=0.01	0.7 (0.5, 1.0) p=0.04	0.7 (0.5, 1.0) p=0.06
Metabolic syndrome	2.0 (1.3, 3.1) p=0.003	1.5 (0.8, 2.7) p=0.2	2.0 (1.0, 4.0) p=0.047

One is left then with a result that is at variance with findings from current knowledge other scientific disciplines. Is CD40 ligand less associated with inflammation but more with atherothrombosis? Are there gaps in our understanding of atherosclerosis?

The relationship of CD40 ligand with both coronary artery disease and carotid intima-media thickness can be summarised by answering the questions posed.

1. Is CD40 ligand associated with the presence, extent and complexity of coronary artery disease in a stable population of patients with suspected coronary disease?
   YES – BUT INVERSE
2. Is the association independent of conventional risk factors?
   YES
3. Of the many better studied novel risk markers shown to have independent associations with CVD such as high-sensitivity (hs-CRP), homocysteine, vascular cell adhesion molecule (VCAM), P-selectin and fibrinogen, how useful are they to predict risk when taken all in combination?
   P-SELECTIN (AND CD40 LIGAND BUT INVERSELY)
4. What possible important interaction effects do they exhibit?
   INDEPENDENT MARKERS – NO SIGNIFICANT INTERACTIONS
5. Do they have added predictive value and can they further re-classify patients into a clinically more important category, for instance, qualifying for different therapy?
   YES – P-SELECTIN (AND CD40 LIGAND BUT INVERSELY)
6. Can we consolidate the large number of conventional risk factors and novel risk markers into a simpler few to predict coronary artery disease?
   YES – COMBINATION OF CONVENTIONAL RISK FACTORS WITH P-SELECTIN (AND CD40 LIGAND INVERSELY)

Carotid Intima-Media Thickness

Carotid intima-media thickness was associated with most conventional risk factors and novel risk markers both individually and in combination. This strengthens the hypothesis of CIMT as an early marker for atherosclerosis and as a surrogate marker for CVD. More importantly, it improves detection of individuals who have significant coronary artery disease who are deemed low-risk by the SCORE equation.

Another important finding was that CIMT was independent when adjusted for the traditional risk factors but not when novel risk markers were added into the model. This suggests that one would use either novel risk markers or CIMT on top of traditional risk factors in CVD risk assessment.

The common carotid and carotid bulb IMT relate strongly to risk factors. The carotid bulb IMT may relate better to coronary artery disease because the carotid bulb is a common location for the build-up of plaques, and the measured IMT incorporates these plaques.

The questions relating to CIMT have been discussed and summarised below:

1. Is carotid intima-media thickness (CIMT) associated with CD40 ligand and other conventional risk factors and novel risk markers?
   YES
2. Of the large number of conventional risk factors and novel risk markers in combination, which ones are still independently associated with CIMT after multivariable adjustment?
   CD40 LIGAND AND P-SELECTIN
3. Can CIMT predict the presence of coronary artery disease?
   YES. BOTH CIMT AND NOVEL RISK MARKERS IMPROVED CVD RISK PREDICTION ON TOP OF TRADITIONAL RISK FACTORS BUT USING BOTH IS NO BETTER THAN USING EITHER ONE OR THE OTHER
4. Of the various methods used to measure CIMT, which definitions and cut-points of CIMT are the most predictive of significant coronary artery disease?
   COMMON CAROTID AND CAROTID BULB

The Metabolic Syndrome

The metabolic syndrome was associated with an approximately two-fold risk for coronary artery disease in the *Cath Lab Study* (Chapter 4) and similarly with CVD in the COMAC study (Chapter 6). The metabolic syndrome is attractive as a marker of risk because it identifies those at high CVD risk using a combination of simple measures including waist circumference, blood pressure, glucose, HDL cholesterol and triglycerides. Most clinicians would have already measured these traditional risk factors as part of routine CVD risk assessment.

There is academic interest in whether patients with this clustering of risk factors have other underlying risks such as inflammation, endothelial dysfunction, insulin resistance and hypercoagulability, which may lead to an increased CVD risk 'more than the sum of its parts'. Certainly, studies have shown them to be associated with raised novel risk markers such as C-Reactive Protein, homocysteine and so on, which may account for a risk 'over and beyond the sum of all its parts'.

In both the *Cath Lab Study* and the COMAC study, the effect of the metabolic syndrome on CVD risk disappears with the addition of HDL cholesterol into the model. When used to identify the outcome of CVD, which is clinically meaningful, HDL cholesterol and triglycerides were the major drivers and accounts for the CVD risk associated with the metabolic syndrome. This would suggest that the metabolic syndrome is not the sum of all its parts, and the increased CVD risk is as a result of its components, which are themselves traditional CVD risk factors.

Dichotomised variables may under-estimate CVD risk. Consider the two men with characteristics listed in Table 7.2. They are characteristically very similar with levels of traditional risk factors that are close to each other, therefore, implying that their CVD risk is similar. However, Patient 1 qualifies for 2 out of the 5 components of the metabolic syndrome, whereas Patient 2 qualifies (some just) for all 5 components of the metabolic syndrome. Patient 2 would be classified high risk whereas Patient 1 will not. This does not make sense as both their risk profiles are very similar. Therefore, this is the disadvantage of dichotomising variables where

there is a spectrum of risk known. The added advantage is that one conceptualises CVD risk better and a diagnosis can also be made easier when there are goals or 'cut-points' involved.

Table 7.2 – Two patients, one with the metabolic syndrome and one without – is their CVD risk really that different?

	Male Patient 1	**Male Patient 2**
Waist	100 cm	103 cm
Triglycerides	1.6	1.8
HDL cholesterol	1.3	1.1
Blood pressure	150/90	155/95
Glucose	5.5	5.8

The questions asked in Chapter 1 about the metabolic syndrome are now addressed:

1. Is the metabolic syndrome associated with CVD?
   YES
2. Is the association independent of conventional risk factors and other novel risk markers such as homocysteine, apolipoproteins B & AI and white cell count?
   YES
3. Does the metabolic syndrome predict CVD risk better as a whole, or using its individual components in combination?
   ALL 5 INDIVIDUAL COMPONENTS AS CONTINUOUS VARIABLES IN COMBINATION FOR CVD RISK
4. What combination of factors works best in a risk model for predicting CVD?
   USING BOTH HDL CHOLESTEROL AND TRIGLYCERIDES ARE AS GOOD AS USING ALL 5 COMPONENTS

The Conventional Risk Factors and other Novel Risk Markers

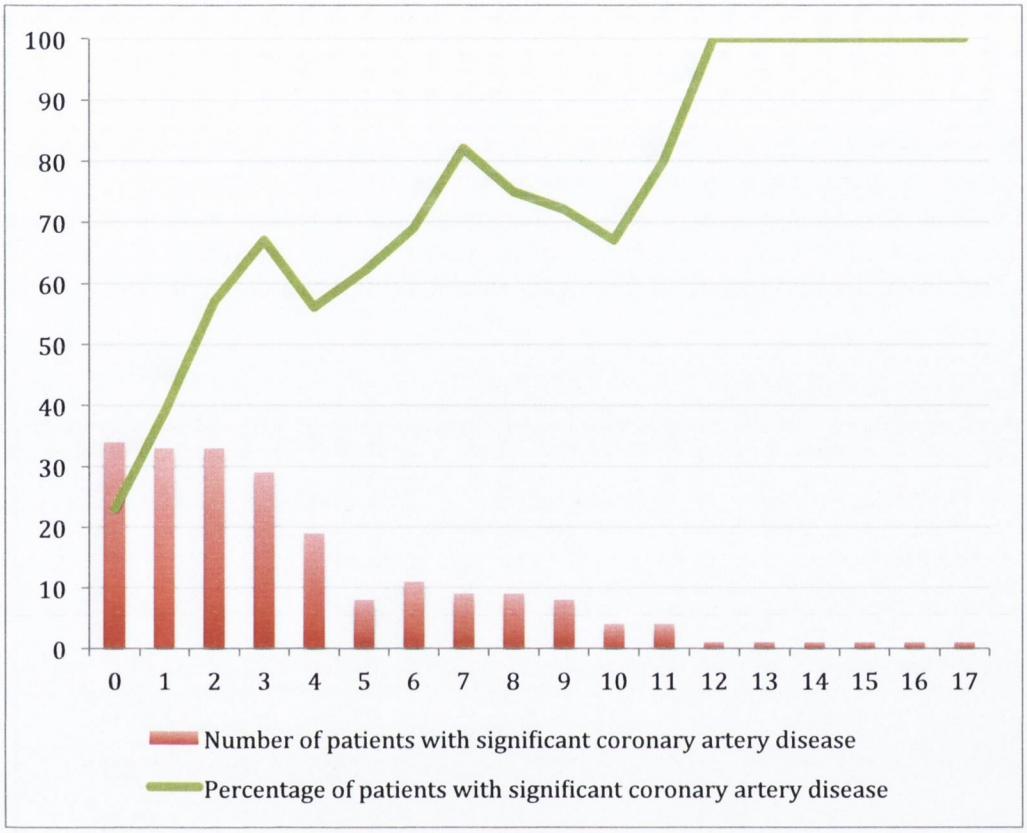
Although the main focus of this work was on the 3 novel risk markers of interest, the established risk factors of blood pressure, cholesterol and smoking were also associated with CVD in this study. The magnitude of the point estimate of CVD risk is in broad agreement with previous large-scale well-established studies of conventional risk factors, implying that the measurement, survey methods and statistical modelling were accurate, adequate and appropriate. The models examining the role of novel risk markers also adjusted for the effect of the other traditional risk factors such as HDL cholesterol and glucose. Although not usually emphasised, non-modifiable factors such as age and sex are the biggest contributors to CVD risk, followed by smoking status.

Other novel risk markers were also found to have independent effects with CVD, most of it already previously reported. Of them, homocysteine, white cell count and P-selectin are also independently associated with CVD.

The Prevention Paradox

CVD risk assessment is currently based on using a combination of age, sex and conventional risk factors in a risk score system such as the SCORE equation. As we know, this brings about a paradox in the field of primary CVD prevention that, although the risk of CVD increases with increasing levels of risk factors, the absolute number of individuals affected by CVD is greatest in the low-risk group by virtue of the larger numbers of individuals in that group. Figure 5.5 illustrates this example.

Figure 7.1 – Overlaid graphs showing the absolute numbers and frequency in each SCORE percentage group of significant coronary artery disease



The horizontal or x-axis shows the risk score given by the SCORE equation which is the 10-year risk of fata CVD deaths in percentage. As previously discussed, high-risk for CVD is when SCORE ≥5%. As one would expect, referring to the data series in green (line graph), as the SCORE risk increases, so does the prevalence of significant coronary artery disease. Referring now to data series in red (bar chart), which are the absolute numbers of individuals with significant coronary artery disease as defined by the SYNTAX score. The low-risk group (SCORE <5%) contained 148 individuals with significant coronary artery disease, while paradoxically the high-risk group (SCORE ≥5%) contained 58 individuals with significant coronary artery disease. Herein lies the Prevention Paradox.

Novel Risk Markers – causal and useful?

In light of the findings of this work, how well do the novel risk markers satisfy the Bradford Hill criteria for causality and the other criteria for clinical utility?

First, the established conventional risk factors:

Table 7.3 – Conventional Risk Factors – causal and clinically useful?

(a) Criteria for causality

	Blood Pressure	**Cholesterol**	**Smoking**
Strength of association	STRONG	STRONG	STRONG
Consistency	YES	YES	YES
Specificity	NO	NO	NO
Temporality	YES	YES	YES
Biological gradient	YES	YES	POSSIBLY
Plausibility	YES	YES	YES
Coherence	YES	YES	YES
Experiment	YES	YES	NO
Analogy	N/A	N/A	N/A

(b) Criteria for clinical utility

	Blood Pressure	**Cholesterol**	**Smoking**
Standardised	YES	YES	N/A
Independent	YES	YES	YES
Ability to re-classify	YES	YES	YES
Change of management	YES	YES	YES?
Convenience/availability	YES	YES	YES

Next, the 3 novel risk markers of interest in this work:

Table 7.4 – Novel Risk Markers – causal and clinically useful?

(a) Criteria for causality

	ADMA	**CD40 ligand**	**Metabolic Syndrome**
Strength of association	STRONG	INVERSE	STRONG
Consistency	YES	NO	YES
Specificity	NO	NO	NO
Temporality	YES	UNKNOWN	POSSIBLY
Biological gradient	POSSIBLY	INVERSE	YES
Plausibility	YES	YES	YES
Coherence	YES	NO	YES
Experiment	NO	NO	NO
Analogy	N/A	N/A	N/A

(b) Criteria for clinical utility

	ADMA	**CD40 ligand**	**Metabolic Syndrome**
Standardised	NO	NO	YES
Independent	YES	YES	YES
Ability to re-classify	YES	YES	YES
Change of management	MAYBE	NO	YES
Convenience/availability	NO	NO	YES

CHAPTER EIGHT
CONCLUSIONS OF THESIS

Conventional risk factors such as age, sex, blood pressure, cholesterol and smoking are well-established risk factors and should remain the mainstay of CVD risk estimation. Global CVD risk can be assessed using a risk estimation system such as the SCORE equation.

Novel risk markers such as ADMA, VCAM, P-selectin, homocysteine, white cell count and the metabolic syndrome showed some promise as independent risk factors for CVD from the various studies in this work. They remained independent not only when adjusted for the effect of conventional CVD risk factors, but also when adjusted in combination with other more established novel risk markers such as C-Reactive Protein, homocysteine and fibrinogen.

In particular, ADMA and P-selectin showed additional ability to correctly re-classify those who were originally deemed low-risk by the SCORE equation who actually have significant coronary artery disease. ADMA directly affects nitric oxide production while P-selectin is pivotal in the adhesion process of inflammatory cells (monocytes and T-lymphocytes) with the arterial endothelium, both of which are key players in endothelial dysfunction.

Although the metabolic syndrome was independently associated with CVD, better CVD risk estimation could be achieved with using its components HDL cholesterol and triglycerides as continuous variables on top of conventional risk factors as opposed to using the metabolic syndrome as a categorical variable. This implies that the metabolic syndrome is not 'more than the sum of its parts'.

Carotid intima-media thickness measured from the common carotid and carotid bulb was associated with CVD risk factors and novel risk markers. Increased carotid intima-media thickness defined using $\geq$75th percentile ARIC study cut-points significantly predicted significant coronary artery disease and could be a useful

alternative to novel risk markers to improve detection of individuals who have significant coronary artery disease.

The inverse relationship of CD40 ligand with coronary artery disease in the study population with stable coronary artery disease was unexpected and deserves further investigation. Although as Huxley put it, it is *the slaying of a beautiful hypothesis by an ugly fact*, it is not *the great tragedy of science* as it illustrates that we cannot be complacent and have to continue to question and push the boundaries of knowledge, a basic tenet of the scientific method.

Future studies of CVD risk factors should be designed with either medication naïve populations, or be prospective in nature where drug treatment has not been initiated before the measurement of the risk factor of interest. It is also reasonable to now consider whether reducing ADMA levels would improve endothelial function and reduce cardiovascular disease risk.

In summary, the conventional risk factors of blood pressure, cholesterol and smoking are well established as causal factors for CVD. Effective and safe treatments are available for these risk factors and CVD can be reduced in that way. Large-scale surveys in both Europe and North America still show poor control of these risk factors in general. A major challenge is to improve this state of affairs. Novel risk markers make a small but significant contribution to CVD. Some such as ADMA, homocysteine, P-selectin and components of the metabolic syndrome show promise, while others such as CD40 ligand need further investigation. The advancement of knowledge through the scientific method is necessary because our understanding of this widespread disease is incomplete.

To conclude, the words of Beaglehole and Magnus seems apt and embodies our current state in CVD Prevention:

Act now, research at leisure

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