The Non-invasive Assessment of Coronary Atherosclerosis

by

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DEDICATION

To Catriona, my Mum and Dad and my late Grandpa

ABSTRACT

Coronary heart disease (CHD) is the biggest single cause of death in the United Kingdom. The development of valid and reliable techniques of non-invasive assessment of coronary atherosclerosis would be invaluable in a number of ways and this thesis examined three techniques (titres of antibodies to heat shock protein 65, ultrasonographic measurement of carotid artery intimal-medial thickening (IMT) and levels of fibrinolytic factors). The second objective was to investigate the role of the immune response to hsp65 and *H.pylori* and of the endogenous fibrinolytic system in the pathogenesis of CHD.

Anti-hsp65 titres correlated with the severity and extent of coronary atherosclerosis, but with insufficient predictive accuracy to be a useful clinical test. *H.pylori* would appear to be an important influence on IgG anti-hsp65 as the eradication of *H.pylori* lead to a significant fall in anti-hsp65 titre (from 25.64 AU/ml to 13.75 AU/ml, p=0.033). Thus an auto-immune response to hsp65 maybe the mechanism by which *H.pylori* lead to increased CHD risk.

Significant correlations between carotid IMT and the angiographic severity and extent of coronary atherosclerosis were demonstrated. However the correlations were quite modest and this casts doubt on the use of carotid IMT as a surrogate marker of CHD mortality and morbidity. There was no evidence of a correlation between plasma PAI-1 antigen or activity or t-PA antigen levels and coronary atherosclerosis. Novel data is presented suggesting that γGT is an important influence on PAI-1 antigen levels in a normotriglyceridaemic population.

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KEY TO ABBREVIATIONS

AMI	acute myocardial infarction
Anti-hsp65	anti-heat shock protein 65
BIF	carotid artery bifurcation
CAD	coronary artery disease
CCA	common carotid artery
CFX	circumflex coronary artery
CHD	Coronary Heart Disease
C.pneumoniae	*Chlamydia pneumoniae*
CRP	C reactive protein
ECG	electrocardiograph
ELISA	enzyme linked immunosorbent assay
HDL	high density lipoprotein
hsp65	heat shock protein 65
H.pylori	*Helicobacter pylori*
ICA	internal carotid artery

IHD	ischaemic heart disease
IMT	intimal-medial thickness
LAD	left anterior descending coronary artery
LDL	low density lipoprotein
Lp(a)	lipoprotein (a)
MI	Myocardial infarction
PM	post mortem
RCA	right coronary artery
ROC	receiver operator curve
PAI-1	plasminogen activator inhibitor 1
t-PA	tissue plasminogen activator
UA	unstable angina

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DECLARATION

None of the work in this thesis has appeared in any other submitted thesis to this or any other University. All of the ideas and the vast majority of the work is my own. I developed, standardised and performed all the anti-hsp65 assays in chapters 3-7. I also personally recruited and venesected all the patients from Chapters 5,6,9 and 11. I analysed all the carotid scans in chapter7. I collated all the data in all the Chapters and performed most of the statistical analysis myself. The writing of the thesis has been solely my efforts. All the books and papers cited were consulted by me personally with the exception references 1,4,8 and 72.

However, in a thesis of this nature, some of the work has to be performed by others: In Chapter 4, the Twins population was recruited by Dr E Holme, the rheumatoid factor assays were performed by Mrs J Veitch, and most of the statistics were performed by Dr IC McKay. In Chapters 6,7,9 and 11, the haematology and biochemistry measurements (cholesterol and subfractions, triglycerides, fibrinogen and lipoprotein, lipoprotein (a) and CRP) were performed by the clinical Departments at the Western Infirmary. In Chapters 7,9 and 11 all the coronary angiograms were analysed and scored by Dr WS Hillis. In Chapter 7, the H*elicobacter pylori* assays were performed by Mrs Ann Cook and in Chapter 11 the fibrinolysis assays were performed by Mrs Norma Moore. In Chapter 7, the *H.pylori* eradication study population was recruited and followed by the M.R.C. funded Dyspepsia Clinic, headed by Professor McColl. In Chapter 9, the carotid

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CHAPTER 1: INTRODUCTION

1.1 HISTORICAL BACKGROUND

1.1.1 Atherosclerosis

Atherosclerosis is the pathological condition that underlies several extremely important disorders including coronary artery disease, cerebrovascular disease, and diseases of the aorta and peripheral arterial circulation. Chronic coronary atherosclerosis maybe asymptomatic or cause stable angina; atherosclerotic plaque rupture with additional thrombosis leads to the clinical events of unstable angina (UA), acute myocardial infarction (AMI), sudden death and heart failure.

It is not a new disease as paleopathologists have shown that some Egyptian mummies have evidence of atherosclerosis (1). Several sixteenth century anatomists including Andreas Vesalius and Gabriel Falloppio, described aneurysms of the aorta and peripheral arteries (2) and Swiss physiologist Abrecht von Heller in 1755 was the first to describe progressive atherosclerotic changes in arteries (3).

Antonio Scarpa in 1804 published a monograph (3) on arterial aneurysms and suggested a link between these and atherosclerosis. He concluded that the most common and important antecedent to aneurysm formation was an ulcerated atheromatous lesion. Scarpa emphasised that an aneurysm was not simply a dilated portion of normal artery; it was the result of

localised disease of the arterial wall (3). In an 1829 paper on pathological anatomy, French pathologist Jeanne Lobstein introduced the term "arterial sclerosis". In 1850 London surgeon Joseph Hodgson claimed that inflammation was the underlying cause of atheromatous arteries. By this time, it was known that arteries consisted of three distinct layers. Hodgson identified atheromatous material between the intima and media and proposed that these changes could be traced to an abnormality of the intima (3). Indeed during the final decades of the nineteenth century, pathologists abandoned the view that atherosclerosis was the result of inflammation and adopted the view that it was a degenerative process.

The modern era of atherosclerosis research began in 1910 when German chemist and Nobel prize-winner Adolf Windaus showed that cholesterol was present in atherosclerotic lesions in humans (3). Antischow produced the first animal model of atherosclerosis in 1913 by feeding cholesterol to rabbits (3). There was a growing interest in the clinical and scientific aspects of atherosclerosis during the early twentieth century. In part this is explained by the gradually declining morbidity and mortality from infectious diseases and thus by the end of the 1930's atherosclerosis had emerged from the position of a medical curiosity to be considered a major cause of death.

1.1.2 Coronary Atherosclerosis and Angina

Renaissance artist Leonardo Da Vinci was the first to describe and illustrate the coronary arteries accurately (4). English physicist and anatomist

William Harvey in 1649 proposed that the coronary circulation nourished the heart (5). English physician William Heberden first described angina pectoris in a talk presented to members of the College of Physicians of London in July 1768 (3). Speaking of the condition he called angina pectoris, Heberden claimed:

> There is a disorder of the breast, marked with strong and peculiar symptoms. The seat of it, and sense of strangling and anxiety with which it is attended, may make it not improperly be called angina pectoris. Those, who are afflicted with it, are seized, while they are walking, and more particularly when they walk soon after eating, with a painful and most disagreeable sensation in the breast, which seems as if it would take their life away, if it were to increase or to continue: the moment they stand still all this uneasiness vanishes. After it has continued some months, it will not cease so instantaneously upon standing still; and it will come on not only when the persons are waking, but when they are lying down and oblige them to rise up out of their beds (3).

Edward Jenner, best remembered for the introduction of vaccination, was the first to conclude that angina was caused by atherosclerosis in 1786. He based his conclusions on autopsy findings, explaining that they revealed "a kind of firm fleshy tube, formed within the coronary vessels, with a considerable quantity of occific matter dispersed irregularly through it" (3).

1.1.3 Coronary Atherosclerosis and Acute Myocardial Infarction

Russian physician WP Obrastzow and ND Straschesko published the first description of myocardial infarction in 1910. They believed that two specific findings were characteristic: prolonged chest discomfort, 'status anginosus' and persistent dyspnoea 'status dyspnoeticus'. After presenting cases

with autopsy correlation, Obrastzow and Straschesko concluded, "the differential diagnosis of coronary thrombosis from angina pectoris is made by the presence of status anginosus with coronary thrombosis and its absence with isolated attacks of angina pectoris" (3,6). Two years later Chicago intern James Herrick published the first description in English of the clinical syndrome of AMI (7). He also provided an explanation of the spectrum of accompanying symptoms "the clinical manifestations of coronary obstruction will evidently vary greatly" Herrick claimed, "depending upon the size, location and number of vessels occluded. The symptoms and end result must also be influenced by blood pressure, by the condition of the myocardium not immediately affected by the obstruction, and by the ability of the remaining vessels properly to carry on their work as determined by their health or disease" (7).

Boston cardiologist Samuel Levine in 1929 first addressed the concept of what we now term cardiac risk factors and claimed that heredity, male sex, obesity, diabetes and hypertension predispose patients to coronary thrombosis (8). American cardiologist Mason Sones performed the first selective coronary angiography in 1958 (9).

During the middle of the twentieth century, some influential clinicians and pathologists began to question the role of coronary thrombosis in acute myocardial infarction. This controversy was finally only resolved in 1980 after the publication of DeWoods seminal work (10). DeWood performed coronary angiograms in patients with AMI and found that 87% of those

studied within 4 hrs of the onset of symptoms had occlusion of the infarct-related artery.

1.2 THE TOLL OF CORONARY ATHEROSCLEROSIS

1.2.1 Mortality

Coronary heart disease (CHD) is the biggest single cause of death in the United Kingdom (11). In 1990 there were nearly 170,000 CHD deaths in the United Kingdom which represents 27% of all deaths (30% for males and 23% for females) and of these 17% were in people under 65 years of age.

Comparison of trends in mortality rates from CHD from the twelve European community countries shows marked differences (see Figure 1.1). Although the rate for the United Kingdom is declining in 1986 (the last year for which data from all the E.C states is available) it was the second highest rate in the European community.

Figure 1.2 shows social class variations in England and Wales and the pattern of variation is similar for both sexes. There is a consistent gradient of increasing standardised mortality ratio rising from lower than the national average in social class I to higher than average for social class V. A similar social class variation has been demonstrated for Scotland.

1.2.2 Morbidity

The 1991 Health Survey for England provided data on the prevalence of CHD based upon individuals self report of having ever been diagnosed by a Doctor. 5% of men and 3% of women reported having had angina (12). The

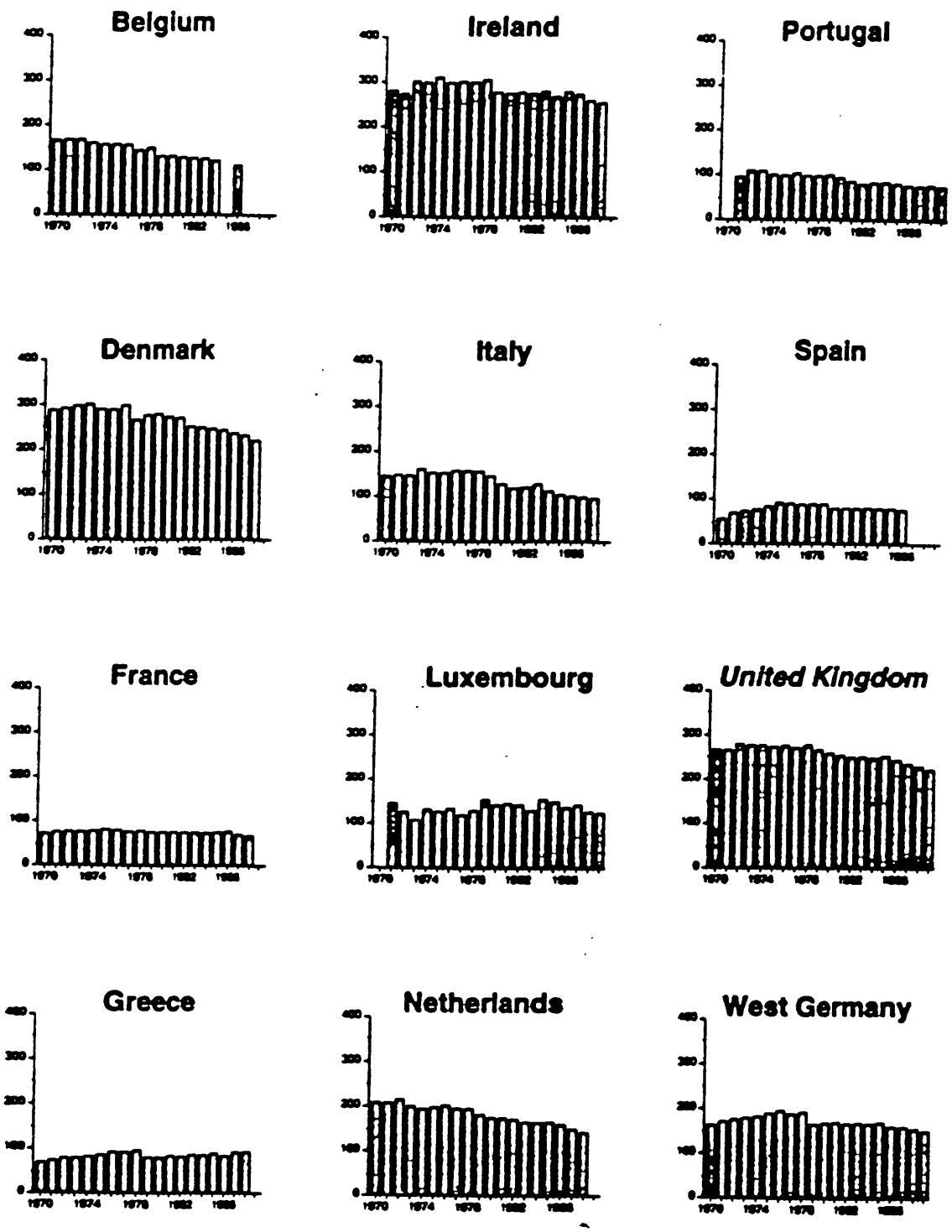


Figure 1.1 Standardised Mortality Rates for Coronary Heart Disease in the European Community. Per 100,000 population, 1970-1989. (Reproduced from 11.)

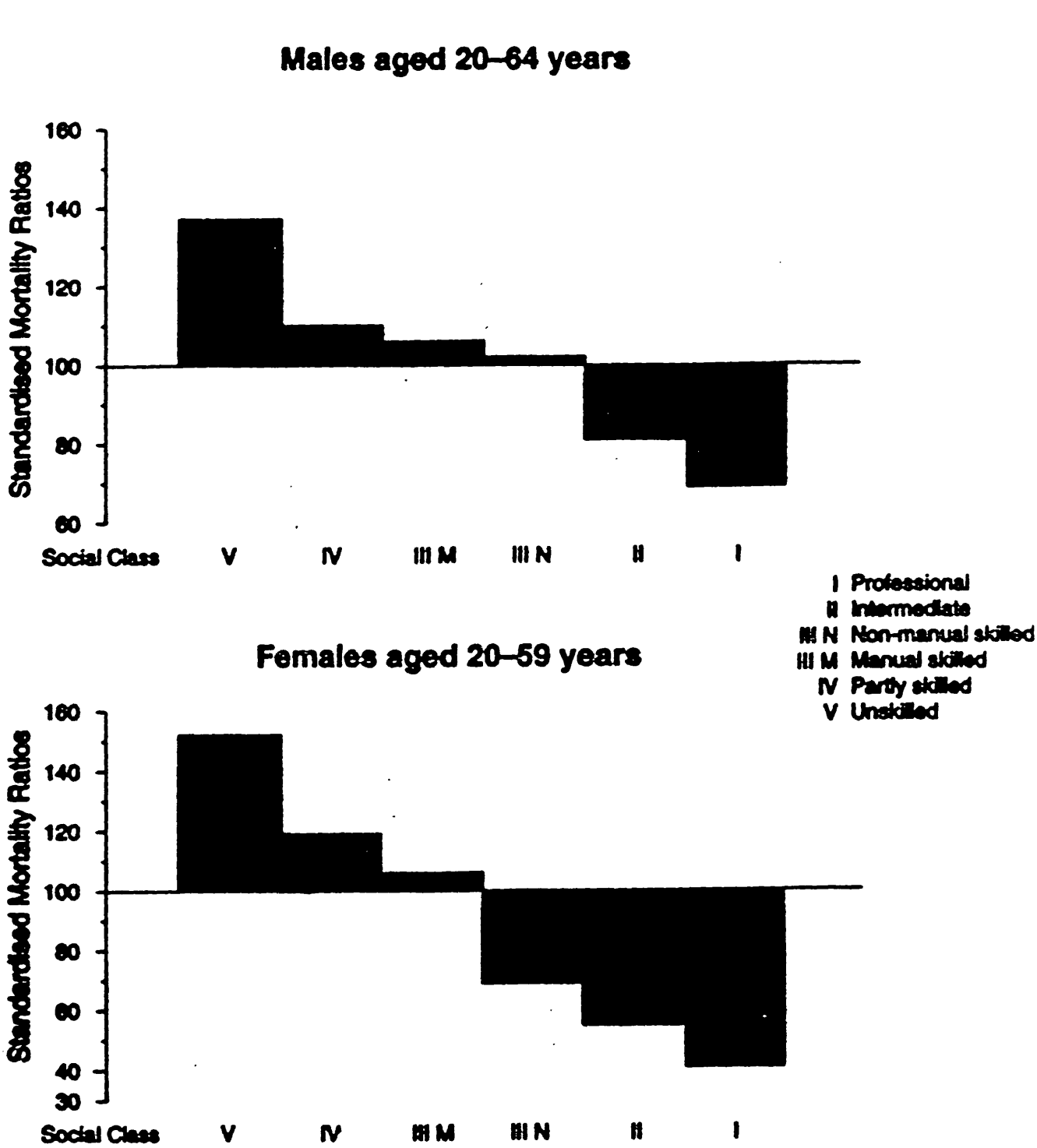


Figure 1.2 Standardised Mortality Rates for Coronary Heart Disease by sex and social class. England and Wales 1982-83. (Reproduced from 11.)

mean weekly incidence of acute (fatal and non-fatal) myocardial infarction in the U.K. is 4 per 100,000 of the population. It is estimated that in 1989/90 CHD attracted about 4% of total NHS expenditure in the U.K. including Community Health Services (11).

1.3 EPIDEMIOLOGICAL RELATIONSHIP BETWEEN VASCULAR TERRITORIES

There is every indication that atherosclerosis is a diffuse condition involving the heart, brain and peripheral arterial circulation, with most risk factors that apply to one arterial bed, also applying to others. It is therefore, not surprising that having one atherosclerosis disease increases the risk of developing the others.

The Framingham Study provides data on the concordance of disease in the different arterial beds (13). The risk of other atherosclerotic events over 30 years of follow up in persons with symptoms of peripheral vascular atherosclerosis (intermittent claudication) as their first manifestation of atherosclerosis was examined. This indicated a two to four fold excess risk of developing overt coronary disease, stroke, TIA or cardiac failure compared to people of the same age without intermittent claudication (14). Following an initial AMI the ten year risk of stroke was 16% in men and 24% in women (13). An initial atherothrombotic cerebrovascular accidents was associated with about a two fold excess risk of cardiac failure and CHD.

The epidemiological data from the Framingham study strongly suggests that development of a clinical atherosclerotic event in one arterial vascular

territory is a hallmark of diffuse atherosclerosis. This suggests a common aetiology or pathogenesis underlying the disease. However, some significant differences in risk factors for atherosclerosis in the different vascular territories have been identified. For example, diabetes has a much greater pathogenic impact on peripheral arterial disease than on coronary disease or stroke (15).

1.4 PATHOGENESIS OF ATHEROSCLEROSIS

There is good evidence from post-mortem studies that the earliest stages of atherosclerosis develop in adolescence and that the atherosclerotic burden increases with age. The initiation and progression of the extent of atherosclerosis and the rupture of chronic plaques with additional thrombosis, are the primary determinants of stable angina and clinical events (UA, AMI, sudden death and heart failure) (16).

Normal arteries consist of intima, media and adventitia. The intima is lined by endothelium on the inner (luminal aspect) of the vessel and consists of a relatively thin layer of connective tissue containing occasional solitary smooth muscle cells and bounded by the internal elastic lamina on its outer aspect. The media is the muscular wall of the artery, bounded by the internal and external elastic laminae. These laminae consist of fenestrated sheets of elastic fibres with numerous openings large enough to permit both substances and cells to pass in either direction. The media of muscular arteries consists of spiralling layers of smooth muscle cells attached to one another and each cell is surrounded by a discontinuous basement membrane

and by interspersed collagen fibrils and proteoglycan. Elastic arteries contain multiple lamellae of smooth muscle cells, each equivalent to a single media in a small muscular artery or arteriole. Each lamella is bounded by an elastic lamina on its inner and outer aspects. The adventitia consists of a dense collagenous structure containing numerous bundles of collagen fibre, elastic fibre and many fibroblasts, together with some smooth muscle cells.

The earliest recognisable lesion of atherosclerosis is the 'fatty streak', an aggregation of lipid-rich macrophages (foam cells) and T lymphocytes within the intima. Data from animal studies have shown that fatty streaks precede the development of 'intermediate lesions' which are composed of layers of macrophages and smooth muscle cells in the intima (17). The intermediate lesions in turn progress to the more advanced, complex, occlusive lesions called 'fibrous plaques'. These fibrous plaques enlarge and impinge in the arterial lumen, thereby obstructing blood flow. They consist of a dense cap of connective tissue with embedded smooth muscle cells overlying a core of lipid and necrotic debris. The fibrous plaques contain monocyte derived macrophages, smooth muscle cells and T lymphocytes many of which are activated. Most episodes of myocardial infarction are due to complete arterial occlusion, secondary to fibrous plaque rupture, followed by superimposed thrombosis.

1.4.1 Hypotheses of Atherogenesis

The current theory of the pathogenesis of the lesions of atherosclerosis have developed from the earlier proposals made by Virchow (18) and Rokatanski

(18) and Duguid (19) and combined in 1973 as the 'response to injury hypothesis of atherosclerosis' (20).

This theory suggests an initial endothelial insult by for example, hypertension or cigarette smoking. One of the most striking features of atherosclerosis is that it does not occur randomly, but rather at specific so-called lesion-prone sites and they usually occur at sites such as flow dividers, areas of curvature and branching sites, suggesting that it is a local haemodynamic environment which is important in the focal initiation of atherosclerotic lesions (21). In addition, it has been shown that these lesion-prone areas are structurally and functionally different from non-lesion prone sites, e.g. their endothelium has increased permeability to plasma proteins such as albumin, fibrinogen and Low Density Lipoprotein (LDL) cholesterol (21) and spontaneous monocyte recruitment and endothelial cell turnover are greater in these areas (21). Whether these changes are due solely to the local haemodynamic upset is unclear, and indeed some lesion-prone sites are not in areas of untoward haemodynamic circumstances. Evidence has accumulated to suggest that oxidised LDL is a key component in endothelial injury and indeed has an important role at many stages of the atherosclerotic process (21). LDL is oxidatively modified by free radicals generated by smooth muscle cells, macrophages and probably endothelial cells.

The second stage of the atherosclerotic process involves monocyte adhesion to injured endothelium. The adhesion is a very active event involving the surface expression of endothelial leucocyte adhesion molecules (ELAMs) and

the secretion of a variety of adhesive cytokines, such as interleukin-1 ß and monocyte colony stimulating factor (22). The attachment is followed by monocyte migration across the endothelium. The monocytes are guided across the endothelium by a number of chemoattractants, the most important of which is considered to be monocyte chemotactic protein-1(22).The monocytes then undergo activation - differentiation, to become macrophages after entering the subendothelial space of the arterial wall (23).

Lipids, in particular LDL cholesterol and Lipoprotein (a) also migrate transendothelially at sites of endothelial injury. Again oxidised LDL has a key role as it is no longer recognised by the normal LDL receptor but is recognised by the so-called macrophage scavenger receptor. Unlike the LDL receptor, the scavenger receptor does not down-regulate with cellular cholesterol accumulation and, thus provides a pathway for the relentless uptake of these chemically modified lipoproteins, with eventual transformation of foam cells to macrophages (20). The combination of foam cells, T lymphocytes which migrate at the same time as monocytes and smooth muscle cells form the fatty streak. The fatty streak then goes through the intermediate stage and, ultimately develops into a fibrous plaque through a combination of three fundamental biological processes as follows:

(1) proliferation of intimal smooth muscle cells together with variable numbers of accumulated foam cells and T lymphocytes;

(2) secretion by the proliferated smooth muscle cells of large amounts of connective tissue matrix, including collagen, elastic fibres and proteoglycans;

(3) accumulation of lipid, principally in the form of cholesterol-esters and free cholesterol within foam cells as well as in the surrounding connective tissues.

It should be noted that there may be a great variability in the relative amounts of tissue formed by each of these processes in the lesions. Consequently, many lesions of atherosclerosis are dense and fibrous, whereas others may contain large amounts of lipid and necrotic debris, with most demonstrating combinations and variations of each of these characteristics (23).

An alternative hyypothesis the Monoclonal hypothesis was proposed in 1973 and suggested that each lesion of atherosclerosis is derived from a single smooth muscle cell that serves as a source of all the cells within a lesion. Benditt and Benditt (24) examined a series of atherosclerotic plaques obtained at autopsy from a small number of black females who were heterozygous for glucose-6-phosphate dehydrogenase (G-6PD). They examined the isoenzyme content of the samples and deduced that each lesion of atherosclerosis represents a clone derived from a single smooth muscle cell. A later version of the monoclonal theory suggests that an atherosclerotic lesion is in fact a benign neoplasm, originating from the

mutation of one cell changed by a virus or other chemical substance. On the whole the Monoclonal Hypothesis has little support today.

1.5 RISK FACTORS FOR CORONARY ATHEROSCLEROSIS

The concept of risk determination by measuring variables associated with the occurrence of disease was formed early in the development of epidemiology. A risk factor is defined by common usage as any measurable trait or characteristic of an individual that predicts that individual's probability of developing clinically manifest disease.

The group that evaluated the evidence relating smoking to health in 1964 developed the criteria to judge the causal significance of a risk factor association (25) and these criteria have been subsequently modified (26). The criteria are usually stated as follows:

1. The relative risk associated with this trait is high.

2. Dose response. The more severe the trait, the greater is the relative risk. Serum cholesterol, blood pressure and smoking meet this criterion.

3. Temporal sequence. The trait precedes the disease.

4. Consistency. The association appears in studies involving different populations, different racial groups and groups living under different conditions.

5. Independence. The trait is associated with increased risk when the effects of other known or suspected causes or risk factors are removed.

6. Coherence. The association is consistent with the results of other sources of evidence: clinical investigation, animal experimentation, or *in vitro* research.

7. Specificity. The trait predicts the occurrence of only one disease for example, the effects of elevated serum cholesterol concentration are limited to the atherosclerotic disease.

8. Reversibility. Reduced incidence of disease when the trait is removed or meliorated provides the most convincing evidence of a causal relationship.

Cholesterol (27,28), hypertension (27,28), smoking (29), male sex and family history are well established risk factors for CHD. In 1981 Hopkins & Williams (30) accumulated a list of 246 risk factors for coronary atherosclerosis. This review used a much looser definition of risk factor than that defined by the 8 criteria listed above. They included traits associated with established risk factors, characteristics associated with atherosclerosis in animal studies, and factors predicted from theoretical considerations. A similar review now would probably include another 100 or so factors fitting this looser definition. A few examples with varying levels of evidence in support of risk factor status include, triglycerides, obesity, lack of physical activity, fibrinogen, leucocyte count, stress and personality, water hardness, homocystinaemia and baldness.

1.6 WHY ASSESS CORONARY ATHEROSCLEROSIS NON-INVASIVELY?

In the past the two approaches to measurement of atherosclerosis in epidemiological and clinical studies has been by PM evaluation and by angiography, and both of these have their limitations.

Coronary angiography is the investigation of choice when atherosclerosis has progressed to clinically significant angina or to clinical events and has a major role in determining those patients who would benefit from surgical treatment of their atherosclerosis (31).

However, angiography has a number of limitations which precludes its widespread use in certain patient groups and in research. The first of these is its invasive nature, for example coronary angiography has a reported mortality of between 0.1 and 0.2% and major morbidity (including MI, stroke, ventricular fibrillation, vascular problems and bleeding) of between 1.7 and 2.6% (32). Hence, it is not ethical to perform angiography on the asymptomatic or serially without good indication on patients.

Also, there is evidence that angiography tends to underestimate the extent of disease and cannot detect early atherosclerosis. This evidence comes initially from the work of Glagov and colleagues (33) who examined diseased and non-diseased arteries at PM. They demonstrated that an artery's response to eccentric accumulation of atheroma within the internal elastic lamina (the early development of atherosclerosis) is to undergo compensatory enlargement with only slow diminution of lumen diameter. Additional evidence showing that angiography underestimates atherosclerosis comes

from studies using intravascular ultrasound. For example St. Goar et al have described the presence of extensive atherosclerosis in angiographically normal appearing arteries (34).

Another problem is how to quantify disease when there is a total occlusion of a coronary artery. Currently the occluded segment is usually scored as severe atherosclerosis but potentially a relatively minor stenosis has ruptured and superimposed thrombus has led to the complete occlusion. In addition, total obstruction of the lumen precludes visualisation of more distally located segments (35).

A fourth limitation is that because angiography is invasive in its use it is restricted to symptomatic populations and hence the generalisability of results obtained from such populations is limited (36). The final limitation is the difficulty with control selection in that angiography controls over-represent related ischaemic or non-coronary cardiac conditions which have their own risk factor association (36).

Notwithstanding these limitations, angiography has been used to a limited extent in epidemiological and interventional atherosclerosis studies. There is little doubt that the angiographic severity of coronary atherosclerosis correlates with prognosis. This evidence comes from prospective studies published in the early 1980's before the routine use of surgical intervention. Mock et al (37) reported data in 1982 from medically treated patients in the large coronary artery surgery study (CASS) and showed 4 year survival of 97,92,84, and 68% for 0,1,2, and 3 vessel disease respectively. Proudfit et al

(38) in 1983 reported 15 year survival rates of 48,28,18 and 9% for 1,2,3 and left main stem disease respectively. In these two studies a vessel was scored as diseased if there was ≥1 stenosis ≥50%. Harris et al (39) in 1980 had shown that stenoses greater than 75% carried a worse prognosis than those measuring 50-75%. However there is a paradox here in that most myocardial infarctions occur as a result of the formation of thrombi on lesions that are not highly stenotic (40). Further, knowledge of the angiographic location of coronary stenosis does not allow one to predict the location of subsequent sites of acute occlusion (41).

Other coronary angiography studies have made an important contribution to our understanding of the association of coronary atherosclerosis, with various risk factors, especially blood levels of cholesterol, smoking, hypertension, alcohol and diabetes. Secondly, data from metanalysis of a number of angiographic studies of lipid-lowering secondary prevention has shown significant results (42). The metanalysis included 688 treated and 593 control subjects with entry and exit coronary angiograms. Analysis demonstrated that regression and stabilisation are 2 and 1.4 times more common in treated than placebo subjects and that progression is reduced by half. These results using the angiographic changes in atherosclerosis as a surrogate for cardiovascular events have been borne out by the much larger Scandinavian Simvastatin survival study (4S study) which used clinical endpoints (43).

It seems unlikely that non-invasive measurement of atherosclerosis will replace angiography for assessing the significantly symptomatic and this is

outwith the scope of this thesis. However, the development of valid and reliable techniques of non-invasive assessment would be invaluable in a number of ways as follows:

1. As an additional diagnostic tool in those with minor or atypical symptoms which are insufficient to justify the risks and costs of invasive investigation.

2. To identify those with a large but still sub-clinical atherosclerotic load, both for intervention to halt the progression of disease and for intensive research. Currently an attempt to identify these high risk individuals is made by assessing their atherosclerotic risk factor profile. However, this has many limitations including inaccuracy and cost. Identifying these high risks individuals has become event more important with the recent publication of the West of Scotland Coronary Prevention Study (WOSCOPS)(44). This study for the first time showed definitive evidence that the cardiovascular benefits of lipid-lowering therapy in primary prevention are not offset by an increase in non-cardiovascular events. The study of 6,595 apparently healthy middle-aged men with mild-to-moderate hypercholesterolaemia (LDL cholesterol 4.5-6 mmol/L) showed a 31% peak ($p=0.0001$) reduction in the combined endpoint of fatal and non-fatal myocardial infarction after an average of 4.9 years of follow up.

In 1986 in the UK 66% of men aged 18-64 had a total cholesterol of greater than5.2 mmol/l and 28% had levels greater than 6.5 mmol/l (45). Therefore the cost implications of offering a significant proportion of them long-term treatment with a relatively expensive drug (Pravastatin) at £31 per

month (46) is enormous. Thus, it seems likely that the use of lipid-lowering therapy in primary prevention will need to be rationed, and currently the WOSCOPS study group have recommended (47) treatment for those with an absolute risk of a cardiac event of greater than20% over the next 10 years based on their entire risk factor profile.

3. Serial measurements of atherosclerosis from an early age in large epidemiological studies would allow evaluation of the natural history of the disease. This would allow examination of which risk factor or factors, e.g. lipid or coagulation measure or lifestyle factor or gene are important for the initiation of atherosclerosis, the progression of atherosclerotic burden and progression of burden to clinical disease. The last point is especially important as the link between progression of coronary atherosclerosis and clinical complications remains unclear (48). For example are subjects with more rapid progression of atherosclerosic burden more likely to suffer clinical complications than those with slower progression? Intuitively the answer would be 'yes' but supporting data is limited. Similarly, why do certain lesions progress to devastating clinical events but other lesions of similar extent and creation remain silent. In addition, constant reassessment of risk factors for atherosclerosis is likely to be necessary in the future. This is because risk factors not previously recognised may be discovered when their prevalence increases or the prevalence of the other factors decrease.

4. If it can be shown that non-invasive techniques of quantification was valid and that the measured atherosclerotic extent correlated with the likelihood

of clinical events, then serial measurements could be used to follow anti-atherosclerotic interventions, either by lifestyle adjustments or pharmacological interventions. Thus, similar statistical power to detect important effects would be achieved by much smaller patient numbers over shorter periods at much less cost than the current epidemiological studies which rely on comparatively rare clinical events as endpoints.

1.7 TECHNIQUES FOR NON-INVASIVELY ASSESSING CORONARY ATHEROSCLEROSIS

1.7.1 Risk Factor Scores

There have been a number of attempts in the U.K. and U.S.A. over recent years to produce multifactorial scoring systems to assess the risk for patients of developing coronary heart disease. However, surveys have shown that these earlier multifactorial scores - for example from Framingham (49), Minnesota (50) and the Regional Heart Study (51) are not widely used.

These earlier scores included unmodifiable risk factors such as age, sex, family history, previous vascular disease and current angina and diabetes, along with the modifiable factors (hypertension, lipids and smoking) to produce a score of absolute risk. Such scores are more accurate predictors but very complex which may explain their poor uptake.

More recently, in 1989, the Coronary Prevention Group and the British Heart Foundation commissioned a new system for scoring risk with an

emphasis on major modifiable risk factors which would be simpler to use and hopefully have wider acceptance.

In response to this Hugh Tunstall-Pedoe produced the Dundee Coronary Risk disk in 1991 (52) which measured coronary risk from smoking history, blood pressure and blood cholesterol. However, again the scoring system has not gained wide acceptance. It has been criticised because the risk factor profile from the Scottish population which was used in its generation differs from that of another area of the UK (53). They also have never been validated in controlled risk factor intervention studies.

1.7.2 Stress Testing

Stress testing using either exercise or pharmacological means, depends upon coronary atherosclerosis being sufficiently severe to produce myocardial ischaemia or reduced coronary blood flow and therefore they cannot detect early coronary atherosclerosis. There are three main techniques used, Exercise Electrocardiography, tress Radionuclide Scan and Stress Echocardiography.

The first of these is most commonly performed and electocardiograph (ECG) ST segment depression is used as a marker of myocardial ischaemia and therefore of significant coronary atherosclerosis.

However there are grave doubts about the specificity of ST segment depression used as the sole indicator of myocardial ischaemia since it may occur during ambulatory ECG monitoring in up to 20% of normal young

subjects and in a number of other conditions (54). False positive tests are notoriously more common in women. In a metanalysis of 12 lead exercise ECG in detecting anatomically significant coronary disease, Gianrossi et al (55) demonstrated a sensitivity of 68% and specificity of 77%. It should be noted that anatomically significant coronary disease was defined as 1 or more stenosis ≥ 50% at coronary angiography.

In patients without a history of chest pain, the predictive accuracy is even less. This is to be expected since the predictive value of any diagnostic test diminishes as the prevalence of the disease diminishes. Hence, for these reasons the exercise ECG is rarely used for screening asymptomatic individuals. Similar problems exist for the other stress testing modalities precluding their use other than in a clinical setting.

1.7.3 Ankle Arm Index

Lower limb atherosclerosis can be measured non-invasively using the ratio of arm blood pressure to ankle blood pressure. This is called the ankle arm index (AAI). The AAI was measured in 5,084 participants ≥65 years of age in the Cardiovascular Health Study (56). Participants were stratified by AAI (< 0.8, 0.8 to 0.9, 0.9 to 1 and greater than 1). AAI was inversely related to a history of clinical cardiovascular events, to cardiovascular risk factors and to other non-invasive measures of atherosclerosis (carotid plaques on ultrasound) and ECG abnormalities. AAI has been proposed as a surrogate marker of coronary atherosclerosis (56).

1.7.4 Computerised Tomography

There has been a recent dramatic increase in the use of electron beam computerised tomography (CT) to screen for coronary calcium phosphate deposits largely in the USA (57). This increase in utilisation seems to be largely profit-driven (a test costs $400 including technical fee and professional interpretation). In 1993 a committee of the American Heart Association (58) concluded that the clinical use of ultrafast CT imaging to screen patients for coronary atherosclerosis is not justified at this time. Major reasons given were firstly, the lack of a precise correlation between the degree of atherosclerosis and coronary calcium and secondly, the unknown prognostic significance of calcification detected by this technology. Indeed there is some evidence that calcified plaques may be less likely to rupture and cause clinical events (59).

Certainly there can be little doubt that electron-beam CT scanning is a highly sensitive (approximating 100%) test for atherosclerosis based on data from autopsy studies (60) and coronary angiography in symptomatic patients (57). However, specificity and positive predictive values were of much lower magnitude (47% and 62%) respectively (57). Indeed greater than 60% of asymptomatic women and 80% of asymptomatic men greater than 60 years old have at least some detectable calcium by this method (61,62)

The current consensus is that the technology holds some promise, but much work remains to be done until it can be a useful test, either for clinical risk stratification or for epidemiological studies.

1.7.5 Arterial Compliance

An index of arterial compliance (stiffness) can be produced by measuring pulse-wave velocity (PWV) at two points in the arterial system, either by use of magnetic resonance imaging (MRI) (63), doppler ultrasound (64) or applanation tonometry (65). It has been suggested that PWV measurements over long arterial pathways, for example the aorta, with consequent termination of the average compliance may give a good assessment of general atherosclerotic load (66).

However, there has only been limited in vivo human work in this area. Hirai et al (67) showed increasing abdominal and carotid artery stiffness in patients with increasing numbers of affected coronary arteries as assessed at angiography. Aortic stiffness increased in adults at increased risk of premature vascular disease as assessed by conventional risk factor profiles (64).

Although much work needs to be done, the technique is very promising as compliance measurements with doppler ultrasound have the necessary ease, safety and reproducibility to be potentially of use in long-term epidemiological studies (66).

1.7.6 Magnetic Resonance Imaging (MRI)

MRI is showing great potential in assessing a number of aspects of the cardiovascular system including left ventricular structure and function and myocardial ischaemia. In addition, investigators have recently shown that MRI can produce high resolution non-invasive coronary angiograms.

Manning et al (68) in 1993 in 39 patients undergoing invasive coronary angiography showed that MRI angiography was 90% sensitive and 92% specific in identifying individual vessels with greater than 50% stenosis (68). Another recent and exciting development has been the accurate quantification of atheroma inside carotid and popliteal arterial wall providing a direct measure of disease extent even when no stenosis has developed (69). However again this is an early preliminary finding and requires much further work.

MRI however is not without its limitations. It is costly and the size of the bore of the magnet precludes the extremely obese and claustrophobic. The magnetic field eliminates those with pacemakers and the technique requires several heartbeats to represent similar cardiac position and hence there is difficulty in those with cardiac dysrhythmia. In addition, the use of vascular clips and stents can create artefacts (70).

1.7.8 Peripheral Blood Markers

The major role to date of peripheral blood markers has been as part of overall risk factor scores as above. There are however examples from other diseases of the use of single blood markers in diagnosis and follow-up. These include proteins secreted from tumours (e.g prostate Specific Antigen), C-Reactive protein (CRP) and specific antibodies in bacterial infections and auto-immune antibodies in connective tissue disease (e.g Rheumatoid Factor (RF) in Rheumatoid Arthritis and Anti-Cardiolipin antibodies in Systemic Lupus Erythematosis).

Thus in this thesis I examine the potential of two peripheral blood markers for the assessment of coronary atherosclerosis, antibody titres to heat shock protein 65 (anti-hsp65) and levels of plasma fibrinolytic factors.

1.7.9 B Mode Ultrasonography of peripheral arteries

This technique is the most widely studied and is largely regarded as providing the best measure of extent of whole body atherosclerosis, and as the most accurate surrogate marker of extent of atherosclerosis (71) and thus we decided to study it.

1.8 SUMMARY AND OUTLINE OF MD

This MD examines three techniques for the non-invasive measurement of coronary atherosclerosis: antibodies to heat shock protein 65 (reviewed in Chapter 2), carotid B-mode ultrasonography (reviewed in Chapter 8) and fibrinolytic parameters (reviewed in Chapter 10).

1.9 AIMS

1.To develop and validate an assay for antibodies to HSP65 and examine the population distribution of titres and factors that influence them.

2.To investigate the potential of anti-hsp65 as a marker of the presence, severity and diffuseness of coronary atherosclerosis.

3.Examine the relationship of anti-hsp65 antibodies with other risk factors for coronary atherosclerosis.

4.Examine the relationship between anti-hsp65 and infection with *Helicobacter pylori.*

5.Investigate the potential B-mode ultrasound of carotid arteries as a marker of presence, severity and diffuseness of coronary atherosclerosis.

6. Compare risk factor profiles for coronary and carotid atherosclerosis.

7.Investigate the potential of endogenous fibrinolytic parameters as markers of presence, severity and diffuseness of coronary atherosclerosis.

8.Examine the relationship of endogenous fibrinolytic parameters with other cardiovascular risk factors especially elements of the Insulin Resistance Syndrome.

1.10 OVERALL OBJECTIVES AND POTENTIAL

1. To investigate, develop and validate methods of non-invasive assessment of coronary atherosclerosis. Potentially these would be of use in diagnosis and prognosis and epidemiological research into the natural history of the disease and to follow intervention.

2. To investigate whether and how the immune response to hsp65 and *H.pylori* and endogenous fibrinolytic factors might be involved in the pathogenesis of coronary atherosclerosis and athero-thrombosis.

CHAPTER 2: INFECTION, IMMUNITY, HSP60/65 AND ATHEROSCLEROSIS

2.1 INFECTION AND ATHEROSCLEROSIS

A causative role for infection in the pathogenesis of atherosclerosis was first postulated by Sir William Osler in 1908 (72). Recent renewed interest has implicated two bacteria *Helicobacter pylori* and *Chlamydia pneumoniae* and a family of viruses, the Herpes viruses.

2.1.1 *Helicobacter pylori*

H.pylori is a chronic bacterial infection of the stomach which causes an active gastritis and is strongly associated with gastric cancer and peptic ulcer disease. 40-60% of middle aged men in the U.K are infected and the prevalence increases with age and lower social class (see Tables 2.1 and 2.2, KEL McColl, personal communication). Mendall et al (73) published the first data linking *H. pylori* infection and CHD in 1994. They compared *H. pylori* seropositivity among 111 men aged 45-65 attending a cardiology out-Patient clinic, compared to a group of 74 men attending a screening clinic in General Practice. The accrued odds ratio for CHD associated with seropositivity was 2.28 (95% confidence interval, 1.25-4.15). The odds ratio however, fell after adjustment for CHD risk factors and socio-economic status to 1.9 (95% confidence intervals, 0.91-3.97).

SOCIAL CLASS						
	1	2	3 NON-MANUAL	3 MANUAL	4	5
Men	26	47	63	75	77	82
Women	33	47	57	69	67	70
Both	28	47	58	74	72	74

Table 2.1 Percentage of subjects with *H.pylori* infection by social class ($p<0.001$).

Age Group						
	25-34	35-44	45-54	55-64	65-74	Total
Men	56	59	65	81	80	70
Women	50	54	61	70	69	62
Both	53	56	63	76	75	66

Table 2.2 Percentage of subjects with *H.pylori* infection by age group ($p<0.001$).

In a second study by from the same centre in 1995 (74), Patel at al reported a cross-sectional study of a population based random sample of 388 white men. Forty-seven men had ECG evidence of myocardial ischaemia previous AMI. Odds ratio for abnormal ECG's was 3.82 (95% confidence interval, 1.6-9.1) for those seropositive for *H.pylori*, after adjustment for a range of socio-economic indicators and risk factors for CHD. Fibrinogen concentration and total leucocyte count in this study were independently positively associated with seropositivity to *H.pylori* and they postulated that *H.pylori* infection leads to increased CHD risk by inducing a hypercoaguable state. However, a study from a different centre in a population based random sample of over 2,000 men and women showed that the association between *H.pylori* infection and ischaemic heart disease (IHD) did not reach statistical significance after adjusting for all possible confounding influences (75). The odds ratio was 1.51 with a 95% confidence interval of 0.93-2.45, p=0.1 (75). In this cohort, there was a weak (p=0.02) negative association between *H.pylori* infection and Fibrinogen.

2.1.2 *Chlamydia pneumoniae*

Chlamydia pneumoniae is an intracellular bacteria which causes pneumonia, bronchitis, pharyngitis and sinusitis. However infections are usually subclinical and up to 60% of the population have evidence of past infection. (76). There have now been several studies examining the association between antibodies to *C.pneumoniae* and CHD. Saikku and colleagues (77) first reported such an association in 1988 when they showed that 68% of 40 males with AMI and

50% of 36 males with chronic CHD had IgG titres ≥ 128 and IgA ≥ 32 to *C. pneumoniae* compared with only 17% of 40 control subjects. In an improved second case-control study sera from 300 subjects from the Helsinki Heart Study (78) were prospectively evaluated for circulating immune complexes to chlamydial Lipo-polysaccharide (LPS) and for antibody to *C. pneumoniae* and results were adjusted for age, smoking and hypertension. The combination of a high serum IgA antibody and high immune complexes to LPS held a relative risk for CHD of 2.6 (95 % CI 1.3-5.2, $p<0.007$).

There have now been data from other groups supporting the association of C. *pneumoniae* infection and coronary (74,79,80) and carotid atherosclerosis (81). *C. pneumoniae* infection has been postulated to cause increased cardiovascular risk either indirectly by producing a rise in Fibrinogen (82) or Triglyceride (83) concentrations or more directly due to infection of atherosclerotic lesion cells (84).

2.1.3 Herpes Viruses

Seven members of the herpes virus family are now known to infect humans. Of these Herpes Simplex Virus type I (HSV-I), Herpes Simplex Virus type II (HSV-II) and human Cytomegalovirus (CMV) have all been associated with atherosclerosis. More than half of children demonstrate antibodies to HSV-I by 10 years of age, and the prevalence of HSV-II infection is variously cited as 0.3-22%. Antibodies to CMV are even more widespread in the population, with cited previous infection rates of greater than 50% of adults

(77).Evidence for involvement of herpes virus with atherosclerosis is accumulating rapidly.

In vivo studies in an animal model of atherosclerosis induced by a herpes virus has been developed by Fabricant et al (85). Pathogen free normo-cholesterolaemic chickens were infected with Marek disease virus and 30 weeks after infection the chickens demonstrated gross atherosclerotic lesions in the large coronary arteries, the aorta and the major aortic branches (85).

In vivo studies of human atherosclerosis by electron microscopic and molecular biological techniques gives further support for a link with Herpes virus infection (86,87). Positive sero-epidemiological studies have been reported linking CMV antibodies with coronary (88) and carotid atherosclerosis (89).

2.2 INFLAMMATION, IMMUNITY AND ATHEROSCLEROSIS

There has been increasing evidence over the last 10 years for the involvement of the immune response in atherosclerosis. Initially this area of research was largely neglected because the immune response was considered to be of a largely secondary nature, but recent evidence suggests that it may have a more prominent pathogenic role both in atherosclerosis and athero-thrombosis.

2.2.1 CRP Is A Risk Factor For AMI

C reactive protein (CRP) is an acute phase reactant and measurement of serum CRP levels reflects whole body inflammation. Using new more sensitive assay techniques, it has recently been shown in stable (90) and

unstable angina (UA) (91) that higher levels of CRP is a risk factor for progression of atherosclerosis to myocardial infarction. In both these studies this association was independent of the angiographic severity of atherosclerosis.

2.2.2 Macrophages And Activated T Cells In Atherosclerotic Plaques.

Immuno-histochemical analysis which has shown macrophages in all stages of atherosclerotic plaques and the lipid-rich region of advanced atherosclerotic plaques are dominated by macrophages (92). T cells are also present in all stages of atherosclerotic plaques (92) and many of these are in an activated state (93). Recent molecular genetic studies have shown that these activated T cells are heterogeneous in terms of their immunological specificity (94). In the early lesions cytotoxic T cells (CD8 positive) dominate over helper T cells (CD4 positive) with a ratio of approximately 2:1. This situation is reversed in the advanced plaque (95) suggesting there may be a switch from a response driven by HLA class I restricted antigen to HLA class II restricted antigen during the evolution of the fatty streak into a fibro-fatty plaque. Also, mechanical testing of aortic fibrous caps indicates that increased macrophage infiltration weakens caps locally, reducing their tensile strength (96). This has been corroborated by immunohistochemical studies showing that there are more macrophages at regions of plaque rupture than at unruptured segments (97).

2.2.3 Immunoglobulin And Complement Component In Plaques

Immunoglobulins are present in high concentrations in the atherosclerotic plaque (98) B-lymphocytes are infrequent in the plaque and immunoglobulins are synthesised elsewhere and accumulate extracellularly and also in the cytoplasm of injured endothelial cells. Their role and antigen specificity remains unclear.

Several complement components have also been detected in the atherosclerotic intima, notably C1 and C3 (99) and C5b-9 (100). The finding of the latter is particularly important since it indicates activation of the complement cascade, however it's role in atherosclerosis is unclear. Muscari et al (101) have shown an association of serum C3 levels with the risk of myocardial infarction. They studied 444 men and 416 women, all asymptomatic, and followed them up for 4 years. Elevated serum C3 was found to be independently associated with ischaemic atherosclerotic events.

2.2.4 Cytokines Are Present In Atherosclerotic Plaques

Pro-inflammatory cytokines including interleukin-1, tumour necrosis factor, interleukin-6 and gamma-interferon are secreted in the plaque (102), probably by T lymphocytes, macrophages, endothelial cells and smooth muscle cells. Pathogenic consequences of such paracrine, cytokine secretion could include activation of macrophages and endothelial cells, stimulation of the immune responses, modulation of cholesterol uptake, and regulation of vascular haemostatic properties(102).

2.2.5 Circulating Antibodies To Plaque Antigens

(i) Antibodies to Oxidised LDL

Oxidised LDL have been proposed to have a central role in the pathogenesis of atherosclerosis (see Chapter 1). Antibodies to oxidised LDL but not native LDL, occur naturally in man. They are not found in young adults, but are detectable in a high proportion of patients with advanced atherosclerosis (103). Salonen et al (104) in a case-control study showed that an elevated titre of antibodies to oxidatively modified LDL was associated with progression of carotid atherosclerosis. In 30 cases with rapid progression of atherosclerosis over two years the mean titre of oxidised-LDL was 2.67 compared to 2.06 in the controls ($p=0.003$). Even after adjusting for potential confounders (smoking, cholesterol and serum copper) and baseline severity of atherosclerosis the difference in antibody titre remained significant ($p=0.031$). Puurunen et al (105) have studied titres in 135 cases and controls from the Helsinki Heart Study. After adjustment for age, smoking, blood pressure and high density lipoprotein cholesterol level, there was a 2.5 fold increased risk (95% confidence interval, 1.3-4.9) of a cardiac endpoint in the highest tertile of antibody level versus the lowest tertile ($p=0.005$ for trend) (105).

(ii) Antibodies To Cytoskeletal Proteins

Nikkari et al (106) found statistically significantly elevated titres of autoantibodies to various cytoskeletal proteins in 10-25% of patients with coronary atherosclerosis compared to normal controls.

(iii) Anti-Cardiolipin Antibodies

The data concerning the relationship between antibodies binding to anionic phospholipids such as cardiolipin and myocardial infarction in subjects without evidence of overt autoimmune disease are conflicting. All published studies have been performed on survivors of myocardial infarction or in patients with established coronary heart disease. Vaarala et al (107) examined a prospective cohort of 133 patients with cardiac endpoints and 133 controls, again from the Helsinki Heart Study. The subjects with antibody levels in the highest quartile of the distribution had an independent relative risk for myocardial infarction of 2.0 (95% confidence interval, 1.1-3.5). Further, there was a correlation between the levels of anti-cardiolipin antibodies and antibodies to oxidised LDL ($r=0.40$, $p<0.001$), and their joint effect was additive for risk. Two other studies have supported this result but three others have not (107).

2.3 HSP60/65 AND ATHEROSCLEROSIS

An offshoot of the research effort into thermal therapy for cancer has been an understanding of the mechanisms of cellular response to heat stress. Exposure of cells to heat stress, leads to the intracellular accumulation of so-called heat shock protein (hsp) (108), it has now been shown that increased hsp expression can also be induced by other cellular stresses such as infection, high temperature, free radicals, mechanical stress and hypoxia. However, many hsps including hsp 60/65 and 70 are constitutively expressed and necessary for normal cell function.

There are 24 heat shock protein 'families' currently known. Members of a given family are defined by their molecular weight and share many features other than size, in particular members of a given family are highly conserved in phylogeny from bacteria to man. They appear to play a crucial cellular homeostatic role. Their cellular function is the intracellular handling of polypeptides, permitting them to reach their appropriate cellular destination, or to transport them across cell membranes, or, simply to prevent incorrect folding. As a result of these roles, the hsp's are often referred to as molecular chaperones (108).

2.3.1 Heat Shock Protein 60/65

Heat shock proteins of the hsp60/65 family (the abbreviation hsp60/65 refers to the whole family) include the GroEL protein of E.Coli, mycobacterial hsp65 (hsp65), hsp62 of *Helicobacter pylori*, Human hsp60 (hsp60) and many others (109). Like all hsp families hsp60/65 is highly conserved between species, for example hsp65 is 75% homologous with hsp60 and hamster and rat hsp60 differ from human hsp60 by only 2 aminoacids (110). Hsp60/65 appears to be present within mitochondria of all cells, although synthesis is cytoplasmic. There may also be cytosolic expression (111) and Xu et al have shown cell membrane expression on endothelial cell, macrophages and smooth muscle cells (112).

Hsp60/65 binds unfolded polypeptides which upon binding are converted to an αhelical structure (113). Hsp 60/65 is also considered to be essential for the correct assembly of unfolded polypeptides. (113)

2.3.2 Hsp60/65, Autoimmunity and Atherosclerosis

Many hsp families have been implicated in various autoimmune diseases (reviewed in 108) including hsp70 in Graves' disease and Hashimoto's thyroiditis. There has also been data suggesting that hsp70 might be involved in atherosclerosis. Berberin et al (114) showed increased expression of hsp70 in the central portion of more thickened atheroma, around the site of necrosis and lipid accumulation. However most of the work relating hsp to auto-immunity generally and atherosclerosis specifically has been with regard to the hsp60/65 family.

Hsp65 of micro-organisms has been shown to be an immunodominant antigen. Up to 40% of the T cell response to mycobacteria is against hsp65 (115) and a variety of T cell types isolated from apparently healthy individuals have been found to recognise hsp60/65 (108). Over 90% of infants have detectable titres of antibodies to hsp60 (116). Further, Jones et al compared the amino acid sequence of hsp60 with a database of all sequenced human peptides and proteins (117). They showed that huhsp 60 shared sequence homology with 86 peptides, of which 19 were known autoantigens including thyroglobulin in myasthenia gravis, cytokeratin in rheumatoid arthritis and 21 Hydroxylase in Addison's disease. A similar analysis of albumin produced 136 peptides with sequence homology but only 4 of these were autoantigens ($p<0.0003$). In addition the sequences of hsp60 with similarity to autoantigens occurred at sites with high antigenic potential (117).

There is increasing evidence to support a role for the hsp60/65 role in various auto-immune diseases including Rheumatoid Arthritis, Diabetes Mellitus, Kawasaki disease, psoriasis and atherosclerosis. The majority of the work has centred on experimental models and clinical studies of Rheumatoid arthritis.

A group of rheumatoid arthritis patients have been shown to have higher mean levels of IgG anti-hsp65 antibodies than controls (118). Bahr et al found a similar result (119) and in addition demonstrated no HLA linkage. Two other papers found no difference between case and controls (120,121). There is also some evidence for cellular immunity as T cells specific to hsp60 are detectable in synovial fluid of rheumatoid patients (122).

In Diabetes Mellitus Elias et al (123) suggested that a β cell target antigen in non-obese diabetic mice is a molecule cross-reactive with hsp65. The development of diabetes was associated with antibodies to hsp65 and T cell clones reactive to hsp65 were able to induce insulitis and hyperglycaemia in young pre-diabetic mice. However, the relationship between diabetes and hsp 60/65 remains controversial (108). Yokota et al (124) showed antibodies to hsp60 and hsp65 in convalescent, but not acute sera of young patients with Kawasaki's disease. Increased antibody to hsp65 has been demonstrated in psoriasis, which may be related to disease severity (125).

There is accumulating evidence for involvement of hsp60/65 in atherosclerosis and this is largely the work of Qingbo Xu and colleagues. They initially injected a panel of antigens into normo-cholesterolaemic New Zealand white

rabbits (126). The antigens included human and rabbit atherosclerotic lesion proteins, ovalbumin, various adjuvants and recombinant hsp65. The animals were sacrificed after 16 weeks and atherosclerotic lesions were found to have developed only in those animals immunised with antigenic preparations containing hsp65, either in the form of whole mycobacteria or purified recombinant hsp65.

Immunohistochemistry and immunofluorescence on serial frozen tissue sections from human atherosclerotic lesions as well as saphenous veins and vena cavae (112), detected hsp60 on endothelium, smooth muscle cells and macrophages in atherosclerotic plaque, whereas vessels of smaller diameter with normal intima showed no detectable expression of the stress protein. The intensity of hsp60 expression correlated positively with the atherosclerotic severity. In addition, a population of T cells in the atherosclerotic lesion were hsp60 antigen specific.

They then selected 15 human serum samples, with either high or low titres to hsp65 and investigated the reactivity of human atherosclerosis lesion components by immunoblotting and immunofluorescence techniques (127). The showed that all 5 high titres reacted with a 60 kD band of atherosclerotic lesion protein and human recombinant hsp60 on western blots. Pooled sera with low antibody titres to hsp65 did not show reactivity with any atherosclerotic lesion and media proteins.

In vitro studies with cultured rat endothelial cells showed that anti-hsp65 antibodies induced complement mediated antibody lysis of the cells (128).

Further culturing the endothelial cells under stressful conditions, including various cytokines produced increased cytoplasmic and surface expression of hsp60 (128). Similarly Heng and Heng working in the USA (129) have demonstrated in vitro that increased hsp60 expression in ischaemic human arterial walls occurs within 30 minutes of external ligation (129).

Finally Xu et al measured titres of antibody against hsp65 in 867 normal inhabitants of South Tyrol (age 40-79) and a significant correlation was found between anti-hsp65 antibody titres and the extent of carotid atherosclerotic plaque ($r=0.23$, $p=0.001$) (130). If other factors including age and sex were taken into account by multiple linear regression, the correlation of antibody titre with lesion size was of only borderline significance ($p=0.083$). However, elevated anti-hsp65 titre was significantly associated with the presence of carotid atherosclerosis after correcting for age, sex and 10 other CHD risk factors in multifactorial logistic regression ($p=0.002$).

In an accompanying Lancet editorial Hansson stated that antibody to hsp65 may prove to be the diagnostic marker of atherosclerosis that clinical medicine has been awaiting for many years (131). I explore this hypothesis in this thesis and examine factors influencing anti-hsp65 titres (chapters 3-7).

CHAPTER 3: DEVELOPMENT OF ELISA FOR MEASUREMENT OF ANTI-HEAT SHOCK PROTEIN 65 ANTIBODIES

3.1 INTRODUCTION

ELISA (enzyme linked immunosorbent assays) has been developed in many configurations and for many applications. A direct ELISA was developed in this study to measure IgG and IgA anti-hsp65 levels. Initially the antigen (hsp65) is adsorbed on to a solid support, the wells of a polypropylene 96 well microtitre plate. The plates are washed between all stages of the ELISA. Unbound sites are then blocked by using an unrelated protein (BSA) which binds to any free sites on the plate. The serum samples are then added to the plates for 1 hour to allow conjugation between specific antibody and the bound antigen. The serum samples are then washed off leaving complexed antibody and antigen. A purified anti-human antibody conjugated with a Horse-radish Peroxidase enzyme is then added. After this the developing solution is added and a colour reaction occurs, which is proportional to the amount of specific bound antibody. The colour is then assayed by spectrophotometry

3.2 MATERIALS

CHEMICALS (Sigma Chemical Co. Limited, Poole, Dorset).

Bovine serum albumin (BSA), Tween 20, O-Phenylenediamine (OPD)

ANTI-SERA (Dakopatts, Glostrup, Denmark)

Horseradish peroxidase conjugated rabbit anti-human IgG

ANTIGEN (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands)

Recombinant hsp65 was produced, purified, aliquoted, and lyophilised with support from the UNDP/World Bank/WHO special programme for research and training in tropical diseases. The protein was obtained from heat-induced *E. coli* K12 strain M1164, which carries plasmid pRIB1300. Cells were lysed by lysozyme treatment and sonication. After centrifugation, the protein was precipitated from the supernatant with ammonium sulphate (20-55% cut). The precipitate was dialysed and further purified by DEAE anion exchange chromatography, dialysed against 10 mM ammonium bicarbonate, aliquoted and lyophilised. There was a total of 0.7mg in each vial provided. On arrival in Glasgow, the protein was dissolved in 1 ml of phosphate-buffered saline (PBS) and stored at -20°C in 50 µL aliquots.

BUFFERS

All buffers were prepared with Analar reagents (British Drug Houses, Poole, Dorset) and deionised water.

(i) Phosphate buffered saline (x 20)

NaCl 320g

K_2HPO_4 48.4g

KH_2PO_4 13.6g

These chemicals were dissolved in deionised water and the solution was made up to 2 litres.

(ii) Coating buffer

Na_2CO_3 0.79g

$NaHCO_3$ 1.46g

These chemicals were dissolved in deionised water and the final solution made up to 500 mls. The final pH was checked to ensure that it was 9.6.

(iii) O-phenyldiamine (OPD) developing solution

Concentrated KOH was added drop-wise to 25 mls of 0.2 M citric acid until the pH was adjusted to pH5. This was then made up to 50 mls total volume with deionised water and 0.025 mg of OPD was added. Immediately prior to use, 25 µL of 30% w/v hydrogen peroxide was added.

3.3 METHODS

3.3.1 Caprylic Acid Purification of IgG

IgG was purified from a serum sample with a high IgG anti-hsp65 concentration according to published methods (132). Briefly 0.1 M Acetic Acid was added to the sample to reduce the pH to 4.5 to 5 and the final sample volume measured. Caprylic acid was then added to give a 5% suspension, which was then mixed vigorously and spun for 7 minutes at 10,000g. The supernatant

was then removed, a 50% saturated ammonium sulphate cut was performed and the solution was mixed at room temperature for 2 hours. It was then spun at 10,000g for 7 minutes and the pellet was re-suspended in PBS and then dialysed overnight into PBS. The subsequent concentration of purified IgG was then calculated by spectrophotometry.

3.3.2 IgG anti-hsp65 ELISA conditions

This was developed and modified from the method described by Xu et al (130). They did not use a standard curve with their test samples and merely expressed their concentrations of anti-hsp65 as OD units. We incorporated a standard curve into the assay to give more accurate and reproducible results. For this we used purified IgG as described in section 2.1 above.

Microtitre plates were coated with 1 μg/ml of recombinant hsp65 in 100μL of PBS per well at 4°C overnight. The plates were then washed with 0.01% Tween in PBS and blocked with 200 μL 0.1% BSA in PBS (PBS-BSA) at room temperature for 1 hr. The plates were washed again and then incubated with 100 μL of serum samples diluted 1:400 with PBS-BSA. After a further wash in PBS-Tween the plates were incubated with HRP-conjugated rabbit anti-human IgG diluted 1:3000 in PBS-BSA. This was left at room temperature for 1 hr and the plates were washed again with PBS-Tween again. Colour was developed using OPD and the reaction stopped with 4N H_2SO_4. The mean absorbence was calculated for each test sample and compared with the serial dilution's of the standard. The unknown values for each test sample were interpolated from the standard curve, and the values expressed as

Arbitrary Units per ml (AU/ml). A representative standard curve is shown in Figure 3.1.

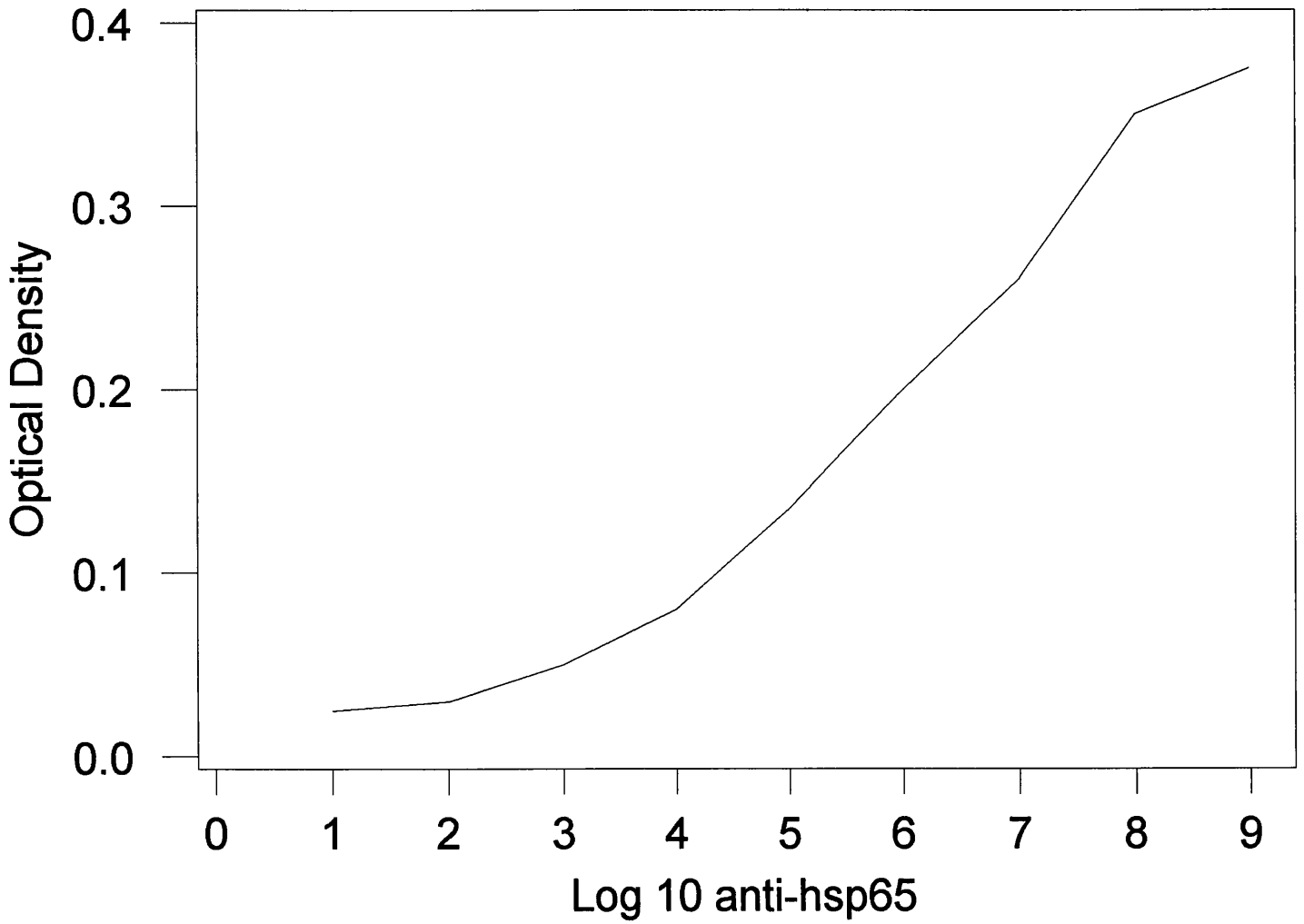
Two coating antigen concentrations (1 μg/ml, 2 μg/ml), 6 serum dilutions (1/25, 1/50, 1/100, 1/200, 1/400, 1/800) and 2 HRP conjugated rabbit anti-human IgG concentrations (1:1000 and 1:3000) were tried in various combinations to optimise assay conditions. The combination of antigen at 1mg/ml, serum dilution of 1/400 and anti-IgG antibody 1:3000 was finally chosen so that most test samples fell within the middle of the standard curve. Optical densities interpolated from nearer the middle of the standard curve give a more precise outcome than optical densities near the boundaries (133). If sample readings were very high, they were repeated at a greater dilution so that their optical density fell within the middle range of the standard curve.

Samples were run in triplicate and if any of the three readings varied by greater than 10% from the mean, the sample was repeated.

3.3.3 IgA anti-hsp65 ELISA condition

The ELISA conditions were exactly as for IgG anti-hsp65 above with three exceptions; the serum samples were diluted 1:100; HRP conjugated rabbit anti-human IgA 1:3000 was used; a serum sample with a high titre of IgA anti-hsp65 was serially diluted from 1/25 to generate the standard curve. Again pre and post samples from a given patient were assayed on the same plate.

Figure 3.1 Standard curve for IgG anti-hsp65



3.3.4 Effect Of Storage Conditions On Anti-hsp65 Measurement

The effect of repeated freeze/thaw cycles on anti-hsp65 titres were investigated by running two control samples with all 13 plates used in chapter 4. The control samples were thawed and refrozen on each occassion and it was noted that there was a significant decrease in control titres with time. Thus, it was concluded that the anti-hsp65 antibody seemed to be particularily susceptible to repeated freeze thaw cycles and in all subsequent experiments the following precautions were taken.

1. Serum samples were thawed for the absolute minimum time possible.

2. Control samples were multiply aliquoted so that each aliquot had been frozen and thawed the same number of times.

3. It was checked that all test serum samples in a given experiment had been frozen and thawed the same number of times prior to assay and that this was not greater than 3.

3.4 REPRODUCIBILITY

3.4.1 Inter-assay variation

Inter-assay variation was established by running aliquots of 2 samples on 10 consecutive plates. The following results for the two samples were obtained:

Sample No.	Mean	S.D	C.V (%)
1	57.9	5.38	9.29
2	40.1	5.62	14.00

3.4.2 Intra-assay variation

Intra-assay variation was established by running 2 samples 8 times each on the same plate (different samples than used in calculation of inter-assay variation).

Sample No.	Mean	S.D	C.V (%)
1	48.00	2.62	5.40
2	92.29	3.10	3.40

3.4.3 Standardisation between chapters

Different control sera were used in chapters 4, 5 and 6. The same controls were used in chapters 6 and 7. Thus, absolute figures for anti-hsp65 titres can only be compared between chapters 6 and 7.

CHAPTER 4: THE DISTRIBUTION OF ANTI-HSP65 IN A POPULATION OF NORMAL TWINS

4.1 INTRODUCTION AND OBJECTIVES

The main objective of this chapter was to establish the population distribution of anti-hsp65 antibody titres. We studied 123 pairs of normal twins for this purpose, this population could also be used to examine the effects of age, sex, genetic and environment on antibody titres.

A secondary objective was to examine the relationship of anti-hsp65 with rheumatoid factor (RF) titres and the rationale for this is as follows.

The part played by RF, (anti-IgG) in the pathogenesis of rheumatoid arthritis (RA) remains unclear. RA is an inflammatory disease of the articular synovium resulting in erosion and deformity of the involved joints. Rheumatoid arthritis is now considered to be an autoimmune disease because of the local infiltration of T and B lymphocytes, its HLA associations, particularly with DR4, and the presence of B lymphocytes in plasma cells synthesising RF within the synovium (134).

Environmental and genetic factors have been implicated as causal factors in the development of RA. Many now accept that experimental RA can be induced by bacterial and viral triggers, and several have been implicated as playing an important role in the induction of RA. In recent years much interest has focused on the antigens derived from

myocobacteria. Studies have shown that normal subjects have B cells capable of producing RF when they have help from appropriate helper T cells (T_H) (135) and in this situation bacterial superantigens have been shown to activate the necessary T_H cells. Mycobacterial heat shock protein (hsp65) is such an antigen and there is some evidence for involvement of this antigen in rheumatoid arthritis (see chapter 2). In addition, patients with rheumatoid arthritis have an mortality from IHD by an unknown mechanism (136). For these reasons we felt it was important to examine the relationship between these two antibodies in this population.

4.2 MATERIALS AND METHODS

4.2.1 Subjects

Following appeals on local radio and in the National and local press, 123 pairs of twins were recruited into the study. The study was approved by the hospital ethical committee and all patents gave written informed consent. Each set of twins were reared together until adulthood was reached. The age range was 14-79 with a mean of 37.3. Their were 8 monozygotic male pairs, 53 monozygotic female, 6 dizygotic male, 35 dizygotic female and 21 dizygotic male-female twin pairs. Zygosity and sex were confirmed by blood group testing (137). A full medical history and examination was performed and twin pairs excluded from further analysis if either twin had any infection or chronic inflammatory disorder at the time of interview. 40 ml of venous blood was taken from each individual and allowed to clot at room temperature for 1 hr and then placed in ice for 30 minutes to allow clot retraction. After centrifugation (2,000g at 2°C for 15 minutes] the serum was removed and

stored in aliquots at -70°C. The serum samples were arranged in a strictly random order for each assay and were analysed blindly for the three RF isotypes and for anti-hsp65.

4.2.2 Anti-hsp65 Assay

Antibody titres were measured as described in chapter 3. The standard was given an IgG anti-hsp65 titre of 2.8 AU/ml based on the estimated IgG concentration purified from the standard sera.

4.2.3 RF Assay

Rheumatoid factor isotypes were measured by ELISA using a modification of the method described by Faith *et al* (138). Briefly, microtitre plates were coated with human IgG (5µg/ml) for IgM-RF and IgA-RF assays and rabbit IgG (5µg/ml) for IgG-RF assay. Between all stages plates were washed extensively in phosphate buffered saline (PBS) containing 0.05% Tween-20 (Sigma). The remaining active binding sites were blocked with 0.1% gelatin (BDH). After incubation with serial dilutions of a standard or with test samples, horseradish peroxidase (HRP) conjugated $F(ab')_2$ anti-IgM or anti-IgA or anti-IgG (Dakopatts) was added to detect bound antibodies.

The colour reaction was developed using OPD (Sigma) and the reaction stopped with 4N H_2SO_4. Absorption was read at 490 nm on an automatic ELISA reader and the mean absorbance was calculated for each test sample and serial dilutions of the standard. The standards were given an RF value of 5,000 Arbitrary Units/ml (AU/ml) 400 AU/ml and 1,000 AU/ml for the IgM, IgA and IgG assays respectively. The AU/ml value of each test sample

was read from the appropriate standard curves and corrected for dilution factors.

4.2.4 Statistical Analysis

There were significant differences between different assay plates, and this was corrected as far as possible by subtracting the corresponding plate mean and adding the grand mean. This left the mean log anti-hsp65 unchanged; i.e. left the geometric mean of anti-hsp65 unchanged. The relationship with age and the familial resemblance between twins was then measured by Spearman's rank correlation coefficient. The effect of sex was examined by Student's T-test.

Correlations between RF and anti-hsp65 titres were examined by rank correlation analysis after separating the data set into two. The first set comprising the first twin of each pair and the second comprising the second twin pair, so as to ensure that the items of data within any one data set were mutually independent.

4.3 RESULTS

4.3.1 Anti-hsp65

The anti-hsp65 titres showed a strong positive skew, and so for descriptive purposes logarithmic data were used. The lower limit of detection was about 0.3 AU/ml, so observations off the bottom of the scale were arbitrarily set at half this value before taking logs. Anti-hsp65 antibodies were detectable in 90.5% of subjects with a geometric mean (SD) of 1.50 (2.57) and range of 0.15-19.7 AU/ml (The distribution is illustrated in Figure 4.1). There

were 3 unrelated twins with very elevated levels of anti-hsp65 at 12.1, 15.0 and 19.7 AU/ml respectively. We calculated the normal range by defining limits so that 2½% of the total number of subjects (i.e. 6) were below the lower limit and 6 subjects were above the upper limit of normal. This gave a normal range of 0.19-8.0 AU/ml. There was no statistically significant correlation of the titres of anti-hsp65 antibodies with age (Figure 4.2). There was a trend for females to have a higher mean titre (2.29 compared to 2.19 AU/ml, see Figure 4.3) but this did not reach statistical significance.

Complete data was available on 57 pairs of monozygotic twins and 57 pairs of dizygotic twins. When anti-hsp65 levels were compared between individuals of a twin pair, the correlation coefficient was 0.015 for the monozygotic twins and 0.025 for dizygotic twins (see Figure 4.4).

4.3.2 RF and Anti-hsp65

There was no significant correlation between anti-hsp65 titres and any of the RF isotypes (see Table 4.1) None of the twins with very elevated anti-hsp65 titres had elevated RF titres or vice-versa.

	IgM RF	IgA RF	IgG RF
First twin anti-hsp65	-0.15	0.086	-0.056
Second twin anti-hsp65	-0.050	-0.023	0.026

Table 4.1 Correlations between anti-hsp65 and RF isotypes (p=ns for all).

Figure 4.1 Distribution of anti-hsp65 titres in a population of normal twins (n=236)

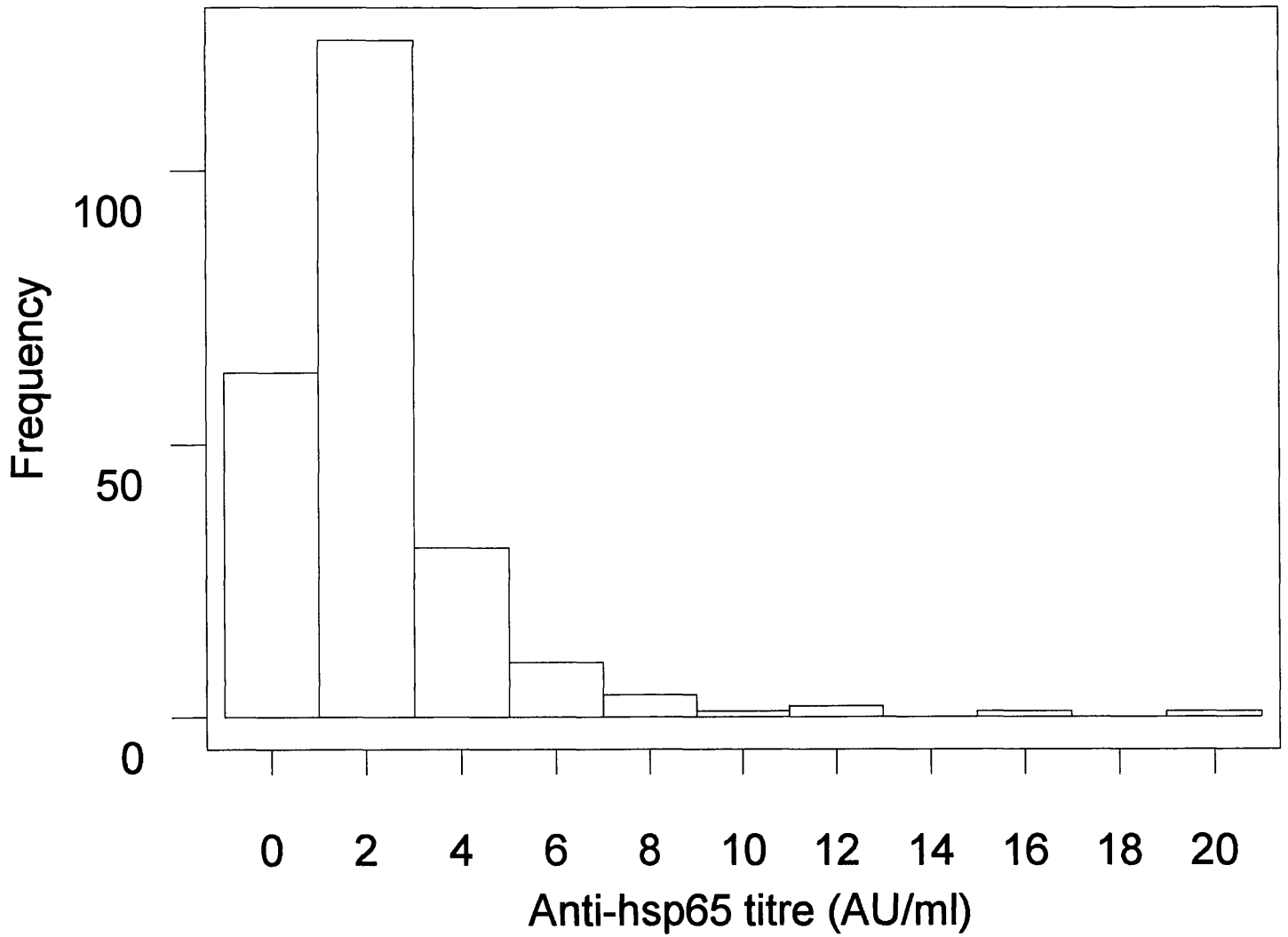


Figure 4.2 Anti-hsp65 titres against age (n=236, r=0.05, p=ns)

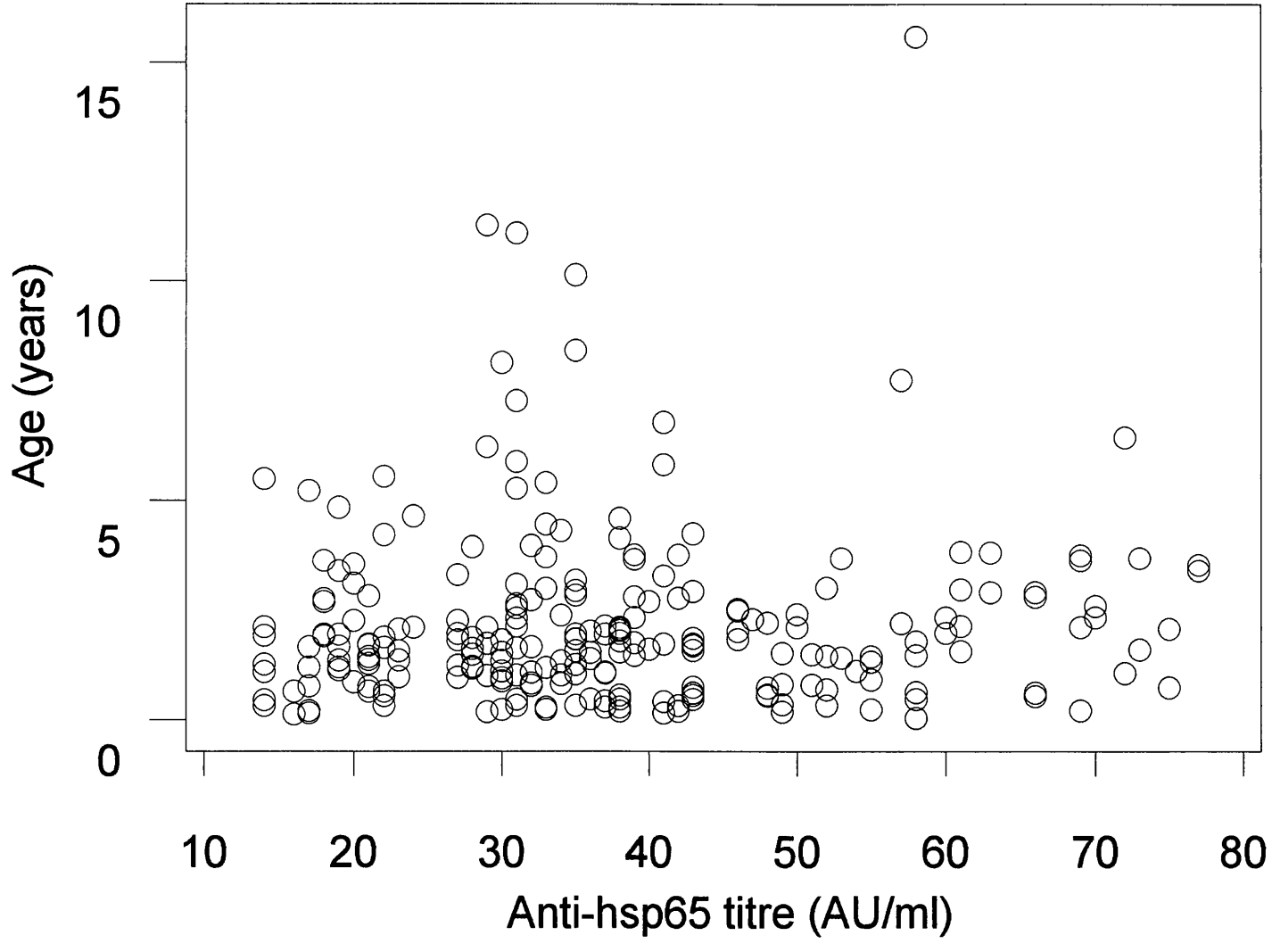
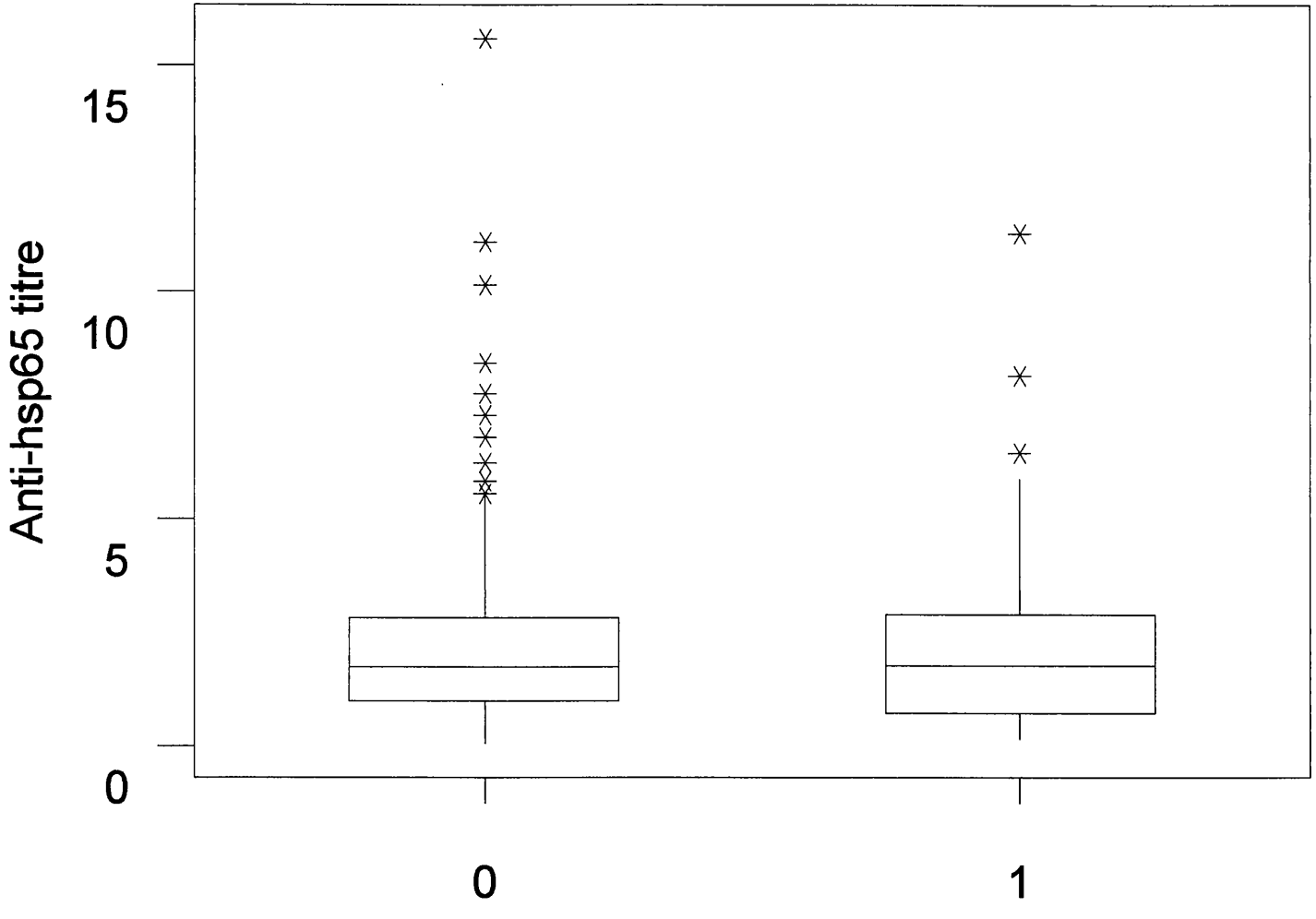
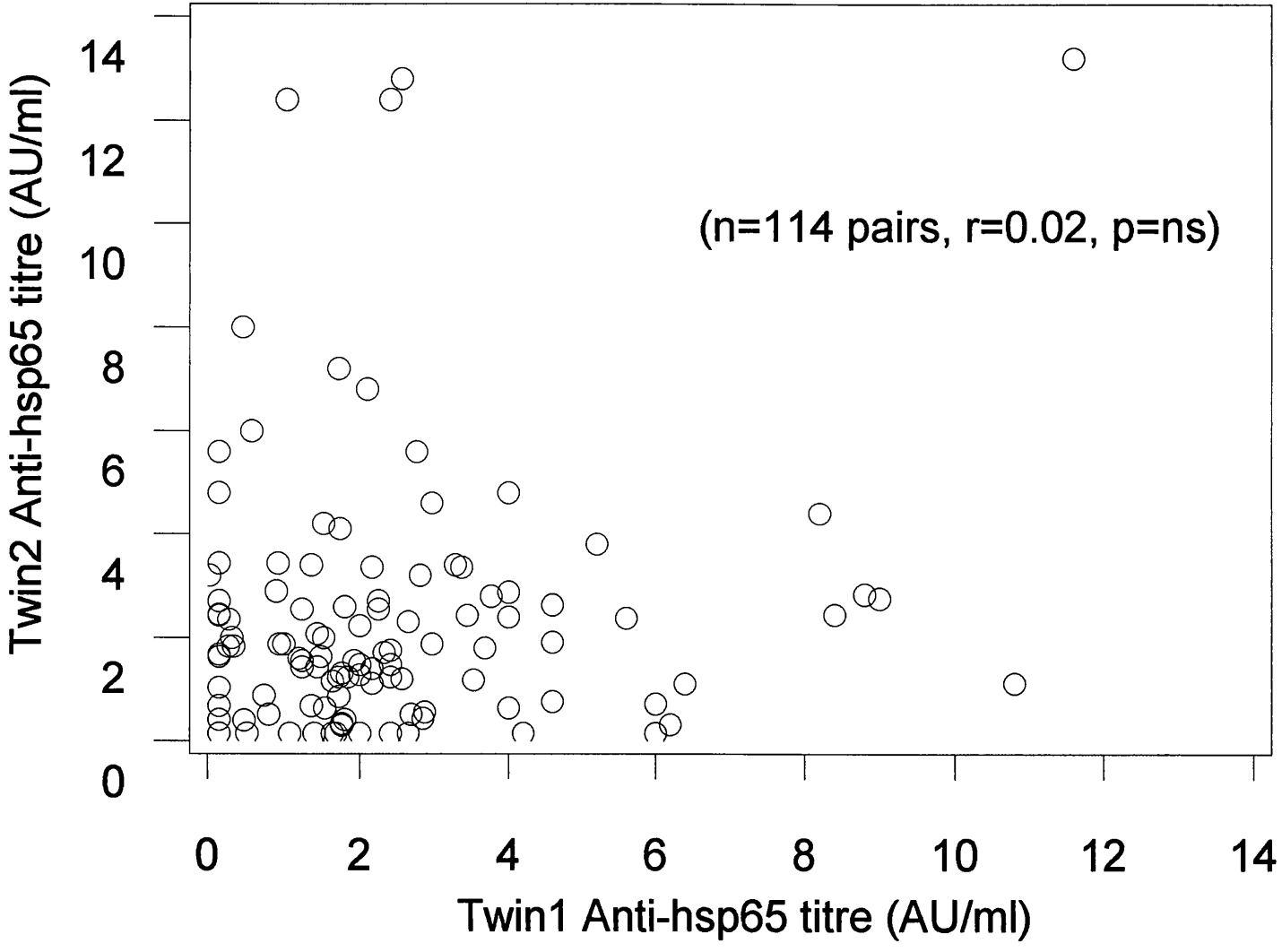


Figure 4.3 Boxplot of anti-hsp65 titre against sex (p=ns).



Line is median value, box is interquartile range and * are outliers.

Figure 4.4 Correlation of anti-hsp65 titres between members of twin pairs



4.4 DISCUSSION

Anti-hsp65 was detectable in 90.5% of this population (normal range 0.15 - 8.0 AU/ml) who had none of the chronic inflammatory diseases commonly associated with these antibodies. This is very similar to the work of Giudice and co-workers (139) who showed that 88.9% of infants produced rising titres of anti-hsp65 after pertussis vaccination. These results together suggest that about 10% of the normal population are non-responders to hsp65 or alternatively may respond initially (IgM or IgA) but do not develop lasting IgG titres.

There was no evidence of an effect of age (Figure 4.2) or sex (Figure 4.3) on anti-hsp65 titre. Pilkington et al (140) also showed no influence of age on anti-hsp65 or anti-hsp60 titre in a population of 173 children aged 6 months to 10 years. Tsoufla et al (118) studied a population consisting of 91 RA cases, 90 Tuberculosis cases and 130 controls with an overall mean age about 35 and could demonstrate no age effect. On the contrary Xu et al (130) did find a significant independent association ($r=0.21$, $p=0.002$) between age and anti-hsp65 titre in a population based random sample of 847 middle aged and older Austrians (mean age about 60). This population was older than ours (mean 37.3 years) and the other two quoted. Thus it may be that in later life age is associated with anti-hsp65 titre. Certainly most auto-antibodies have a least some evidence of a positive relationship with age for example Xu et al (130) showed that RF increased with age.

We demonstrate that females had slightly higher anti-hsp65 titres (2.29 compared to 2.19 AU/ml) but this did not reach statistical significance. Xu et al (130) showed a clear trend in the older age range for females to have higher titres but Worthington et al (120) showed no sex difference. Most auto-antibodies have higher titres in females, for example Xu and colleagues demonstrated a significant positive association between female sex and four auto-antibodies (130).

The correlations of anti-hsp65 antibodies between twin pairs was poor, suggesting that at most there are only minor familial influences on anti-hsp65 titres. In addition, we found the correlation to be greater between dizygotic twins, and hence there is no evidence of a genetic effect on titres.

Similar findings have been shown by Bahr et al (119) who examined the relationship between HLA tissue type in 84 normal individuals with anti-hsp65 titres and found none to exist. Importantly, Thompson et al (141) showed that specific pathogen-free mice (SPF) maintained in an isolator, have negligible levels of anti-hsp65 antibodies, but when exposed to normal animal house conditions, the mice became antibody positive. Thus the evidence strongly indicates anti-hsp65 antibody levels are predominantly a result of environmental conditions. However, Worthington and co-workers (120) suggested that there may be a minor genetic influence in titres. They examined anti-hsp65 antibody titres in a population of twins discordant for RA and showed that the estimate of heredibility was greater in monozygotic twins (r=0.296) compared to dizygotic twins (r=0.201),

suggesting that to a very small extent there may be a genetic role in anti-hsp65 responses. However they did not state whether these estimates were statistically significant. Possible explanations for these contradictory results include because the populations were different as in Worthington's study their twins were discordant for RA, a potentially important confounder. Also they do not state in their methodology whether samples were analysed blindly and in random order. If members of twin pairs were assayed on the same batch or even on the same day then spurious intra-pair correlations may be created due to inter-batch variation.

We found no evidence of a correlation between anti-hsp65 and any of the RF isotypes. This argues against the hypothesis of hsp65 working as bacterial superantigen to provide T_H for RF production. Worthington et al (120) is the only other study to examine the relationship between rheumatoid factor and anti-hsp65 titres. They also showed, albeit in a rather indirect fashion, no evidence of correlation between RF and anti-hsp65 titres; 64% of their subjects with RA were RF positive against 7% of disease free subjects and there was no difference between the groups in anti-hsp65 titre.

In summary, anti-hsp65 titres were detectable in 90.5% of subjects, similar to previous work. There was no evidence of significant familial, age or sex influences on anti-hsp65 titres. In addition, there was no evidence to support the hypothesis that hsp65 functions as a bacterial superantigen to provide helper T cell support for RF production.

CHAPTER 5: ANTI-HSP65 TITRES IN ACUTE CORONARY SYNDROMES

5.1 INTRODUCTION

Acute myocardial infarction is defined as necrosis of cardiac tissue due to occlusion of the infarct artery (10). Unstable angina is defined as angina of increasing frequency and severity, not only induced by effort, but also occurring at rest (142).

The occlusion of a coronary artery leading to AMI appears to be the final common pathway, resulting from a complex and dynamic interaction between chronic atherosclerosis, vasospasm, plaque rupture and platelet activation, ultimately leading to thrombosis and occlusion. Evidence from PM histology (143) and acute angiography (144) suggests that atherosclerotic plaque rupture is a central event with subsequent superimposed occlusive thrombus. Pathological studies of patients with UA are rare, but suggestive of interaction of coronary vasospasm and plaque rupture with non-occlusive thrombus (142). Thus, plaque disruption is the central event in transforming chronic atherosclerosis to acute coronary syndrome. Whether AMI or UA develops depends upon whether the subsequent superimposed thrombotic occlusion is complete for greater than 30-40 minutes.

The risk of plaque disruption is related to extrinsic factors acting on plaques ('rupture triggers') and to intrinsic properties of the individual plaques ('their vulnerability')(97).

Coronary plaques are constantly stressed by a variety of extrinsic biomechanical and haemodynamic forces that may precipitate disruption of plaques.. From Laplace's Law the tension created in fibrous caps of mildly or moderately stenotic plaques is greater than that created in caps of severely stenotic plaque (smaller lumen) with the same cap thickness and exposed to the same blood pressure. This may partly explain the observed findings that less severe stenotic plaques are more prone to rupture (97).

There is some in vivo evidence that haemodynamic rupture triggers may be important as a proportion of acute coronary events do appear to be precipitated by external factors or conditions including emotional stress and vigorous exercise. However the majority of plaque ruptures are not clearly related to an episode of haemodynamic stress and in these cases the plaques intrinsic vulnerability may be more important (although there is likely to be an interaction with triggering factors). Pathological examination of intact and disrupted plaques and mechanical testing of isolated fibrous caps indicate that a plaque's intrinsic vulnerability to rupture depends on three main factors (97).

Firstly, the size and consistency of atheromatous core varies greatly from plaque to plaque and appears critical to the stability of individual lesions based on the work of Gertz & Roberts (145). They reported the composition of

plaques in 5μm segments from 17 infarct-related arteries examined PM and found much larger atheromatous cores in the 39 segments with plaque disruption than the 229 segments with intact surface (32% and 5-12% of plaque area respectively) (145).

Secondly it has been demonstrated in vitro that fibrous cap thinning and reduced collagen content increase a plaque's vulnerability to rupture (146).

Thirdly, mechanical testing of aortic fibrous caps indicates that increased foam cell infiltration weakens caps locally, reducing their tensile strength (96). This has been corroborated by immunohistochemical studies showing there are more macrophages at regions of plaque rupture than at unruptured segments (97). These findings suggest that the inflammatory status of plaques may be an important influence on plaque stability and the chapter examines this hyothesis.

5.2 OBJECTIVES

1. To examine whether AMI leads to a change in anti-hsp65 titres. This could either be secondary to increased hsp65 expression consequent to myocardial ischaemia with oxygen radical generation. Alternatively, the autoimmune response between hsp65 on atherosclerosis cells and host-specific T cells and immunoglobulins might contribute to plaque disruption.

2.To investigate whether patients with acute coronary syndromes have elevated anti-hsp65 titres compared with those with chronic stable atherosclerosis. Elevated titres in unstable syndromes might reflect heightened immunological/inflammatory response in the atherosclerotic

lesions. Potentially anti-hsp65 titres could be useful to risk stratify patients with stable chronic atherosclerosis who are more likely to go on to plaque disruption and acute coronary syndromes.

5.3 MATERIALS AND METHODS

5.3.1 Subjects

The study was approved by the hospital ethical committee and all patents gave written informed consent.

Subjects were excluded if they had a history or clinical evidence of any other disease associated with elevated anti-hsp65 titres (including diabetes, rheumatoid arthritis, on-going inflammation, infection or neoplasm). Only males were recruited in three cohorts a follows:

(i) Chronic atherosclerosis

We recruited 136 consecutive eligible male subjects admitted for elective cardiac catheterisation. Subjects were excluded if they had any other disease associated with elevated anti-hsp65 titre, including diabetes mellitus (fasting glucose of greater than 7.8 mmol/l), rheumatoid arthritis, SLE or had other evidence of active infection, inflammation or malignancy or other significant disease (including more than mild renal or hepatic dysfunction), history of AMI or UA in the previous three months or had had previous coronary angioplasty or coronary artery bypass surgery. A further 18 of the 136 subjects were also excluded as they did not have a history typical of stable angina as assessed by the Rose questionnaire (147). In the majority of cases, the angiography was indicated for the

assessment of chest pain and a smaller group of patients were being investigated for valvular abnormalities. The subjects were recruited over a seven month period and the population demographics are listed in Table 5.1. The study was approved by the hospital Ethical Committee and all patients gave informed consent. Patients were examined including weight and height and excluded if any of the exclusion criteria were revealed. Patients filled in a short health questionnaire, including questions on past and concomitant health problems, history of hypertension, and smoking record. Smoking was treated as a categorical variable (smoker, non-smoker, ex-smoker).

(ii) Unstable Angina

Inclusion criteria-both of the following had to be present

1.History compatible with unstable angina, i.e. recent onset or increase in typical angina, with symptoms occurring on minimal exertion or at rest, but lasting less than 30 minutes.

2.ECG evidence of myocardial ischaemia consisting of at least T wave inversion (greater than 1 mm) in two contiguous limb leads and/or ST depression (greater than 1 mm) in two contiguous leads.

Exclusion criteria-if one was present the patient was excluded

1. Cardiac enzyme rise, Creatinine Kinase (CK) greater than 2 times upper limit of normal) or sequential ECG changes diagnostic of acute MI.

2. AMI within the previous 3 months.

3. Pulmonary oedema or haemodynamic compromise.

4. Other significant on-going hepatic, renal or cardiac disease.

A total of 12 men who met these criteria were recruited over a 4 month period (see Table 5.1).

(iii) Acute Myocardial Infarction

Inclusion criteria- all three of the following had to be met

1. History of significant on-going chest pain lasting greater than 30 minutes.

2. ECG evidence of acute transmural myocardial infarction, i.e. ST elevation of greater than 1 mm in two contiguous limb leads or greater than 2 mm in two contiguous chest leads.

3. A rise in CK to greater than twice the upper limit of normal, confirming the diagnosis of acute MI.

Exclusion criteria-if one was present the patient was excluded.

1. History of episode of unstable angina or myocardial infarction within 3 months of admission.

2. Cardiac enzyme rise (CK greater than 2 times upper limit of normal) but no ECG changes diagnostic of acute transmural MI.

3. AMI within the previous 3 months.

4. Pulmonary oedema or haemodynamic compromise.

5. Other significant on-going hepatic, renal or cardiac disease.

A total of 12 men who met these criteria were recruited over a 4 month period (see Table 5.1).

5.3.2 Blood sampling

In the chronic atherosclerosis group this was performed on the morning of angiography as detailed in Chapter 6. In the unstable angina group, this was performed within 12 hours of admission. In the AMI group three blood samples were drawn from each patient. The first of these was taken as soon as possible after admission and before thrombolytic therapy was administered to the patient. The second was drawn about 72 hours after admission and the third after 120 hours. Serum samples were immediately put on ice and centrifuged within 1 hr and stored at -20°C for subsequent analysis.

5.3.3 Antihsp65 Assays

The assays were performed as in chapter 3. The samples from the three patient groups were analysed together in random order and blinded to the underlying diagnosis.

5.3.4 Statistical Analysis

The anti-hsp65 titres showed a strong positive skew, and so for statistical purposes logarithmic data were used. There were significant differences between assay batches and each log titre was corrected as far as possible by subtracting the mean log titre of the corresponding plate and adding the grand mean log titre. This left the mean log IgG anti-hsp65 level unchanged, i.e. left the geometric mean IgG anti-hsp65 titre unchanged.

One-way Analysis of Variance was used to examined for differences in age and anti-hsp65 titre between the three groups. This technique was also used to examine the serial anti-hsp65 titres following acute MI. Finally, Student's t-test was used to investigate differences in mean titre between stable and unstable coronary syndromes.

5.4 RESULTS

There was no difference in demographics between the three different coronary syndrome groups (Table 5.1). There was a trend for anti-hsp65 titres to fall with time in the acute MI cohort, although this did not reach statistical significance (Figure 5.1). Figure 5.2 illustrates individual patient data. Titres fell in 9/12 patients, stayed the same in two and rose slightly in one. There was no difference in anti-hsp65 titres between the three cohorts (Figure 5.3). In addition, there was no statistical difference between the chronic atherosclerotic group and the combined acute coronary syndrome group (Figure 5.4).

	MI (n=12)	UA (n=12)	SA (n=118)
Age (years)	58.0	58.5	56.4
BMI (kg/m^2)	24.18	26.32	26.47
Hypertension (%)	18.2	27.3	29.3
Smoking - Current	data unavailable	41.6	20.7
- Ex		58.3	63.0
- Never		0	16.3

Table 5.1 Description of cohorts (p=ns for all comparisons)

Figure 5.1 mean (SE) anti-hsp65 titres following MI (n=12, p=ns)

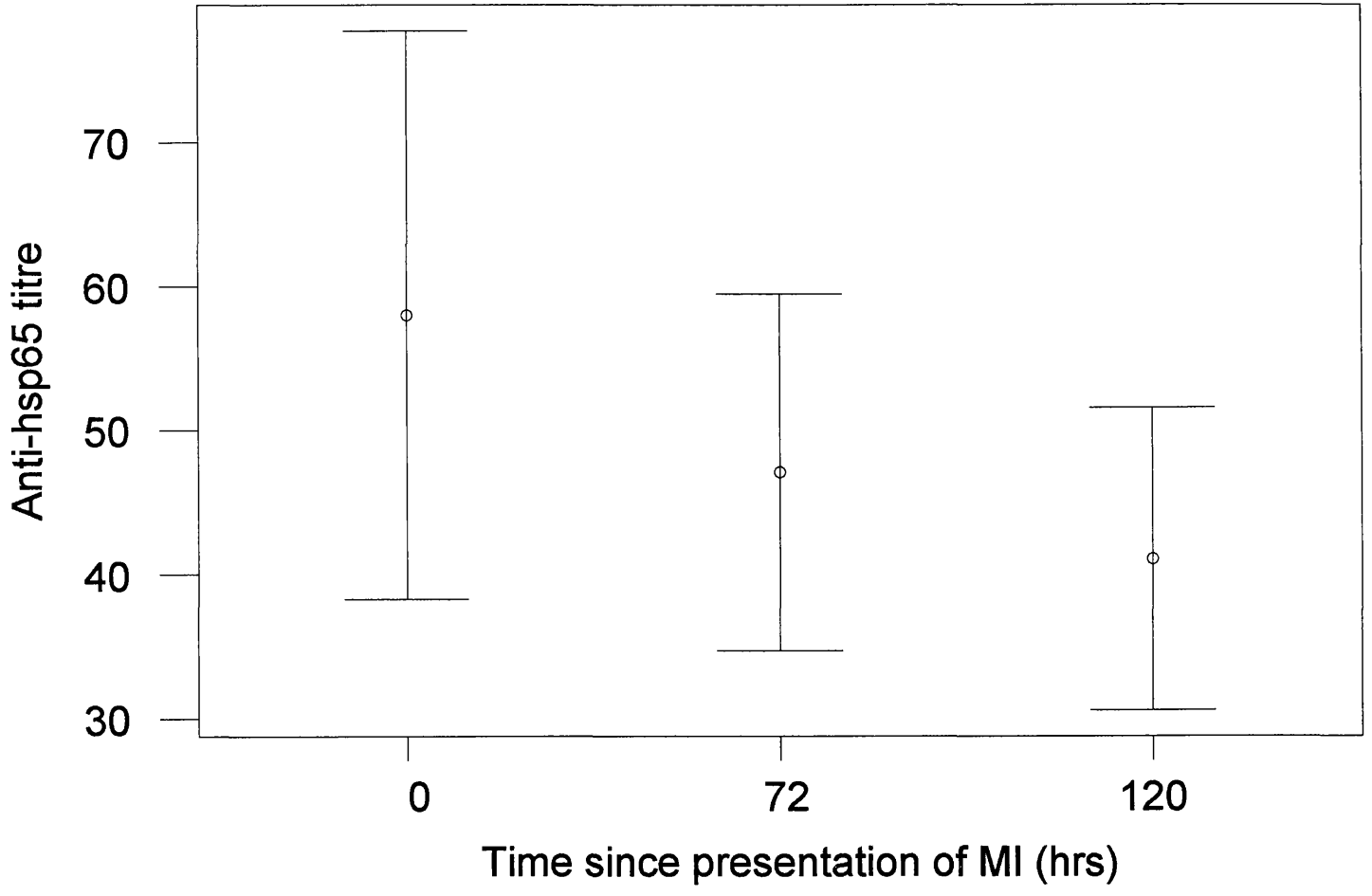


Figure 5.2 Individual anti-hsp65 titres following AMI

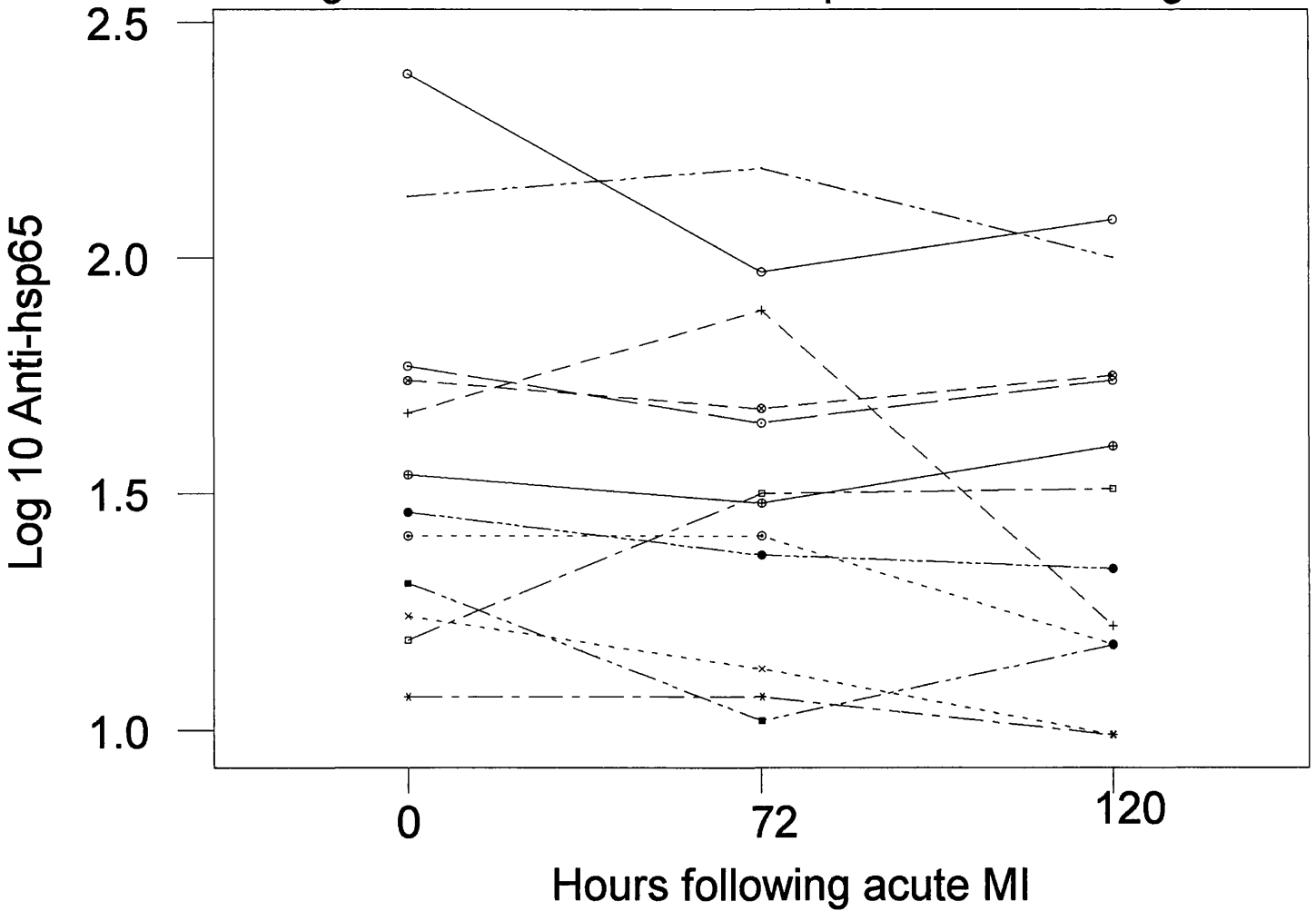


Figure 5.3 Mean (SD) anti-hsp65 titres in coronary syndromes (p=ns)

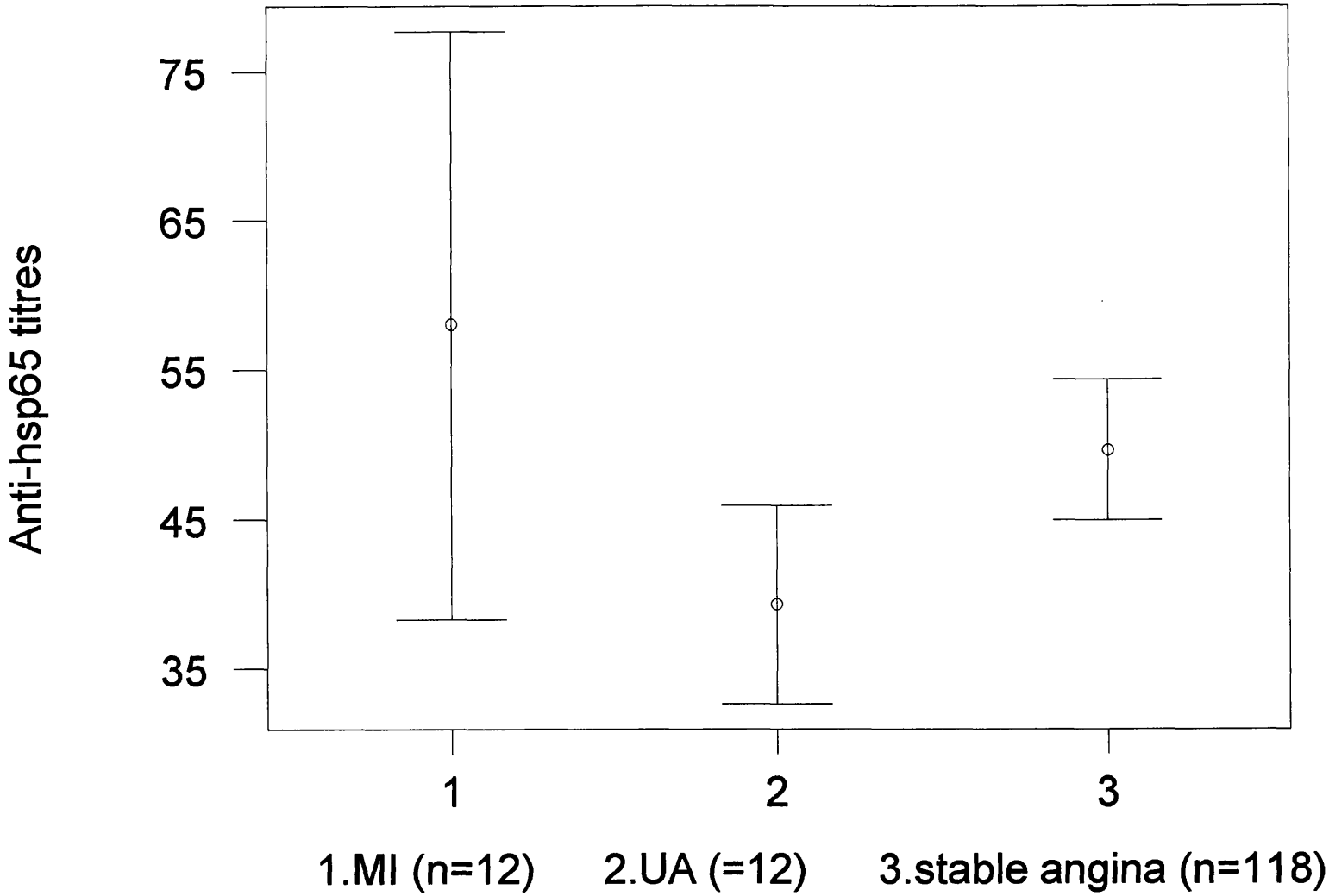
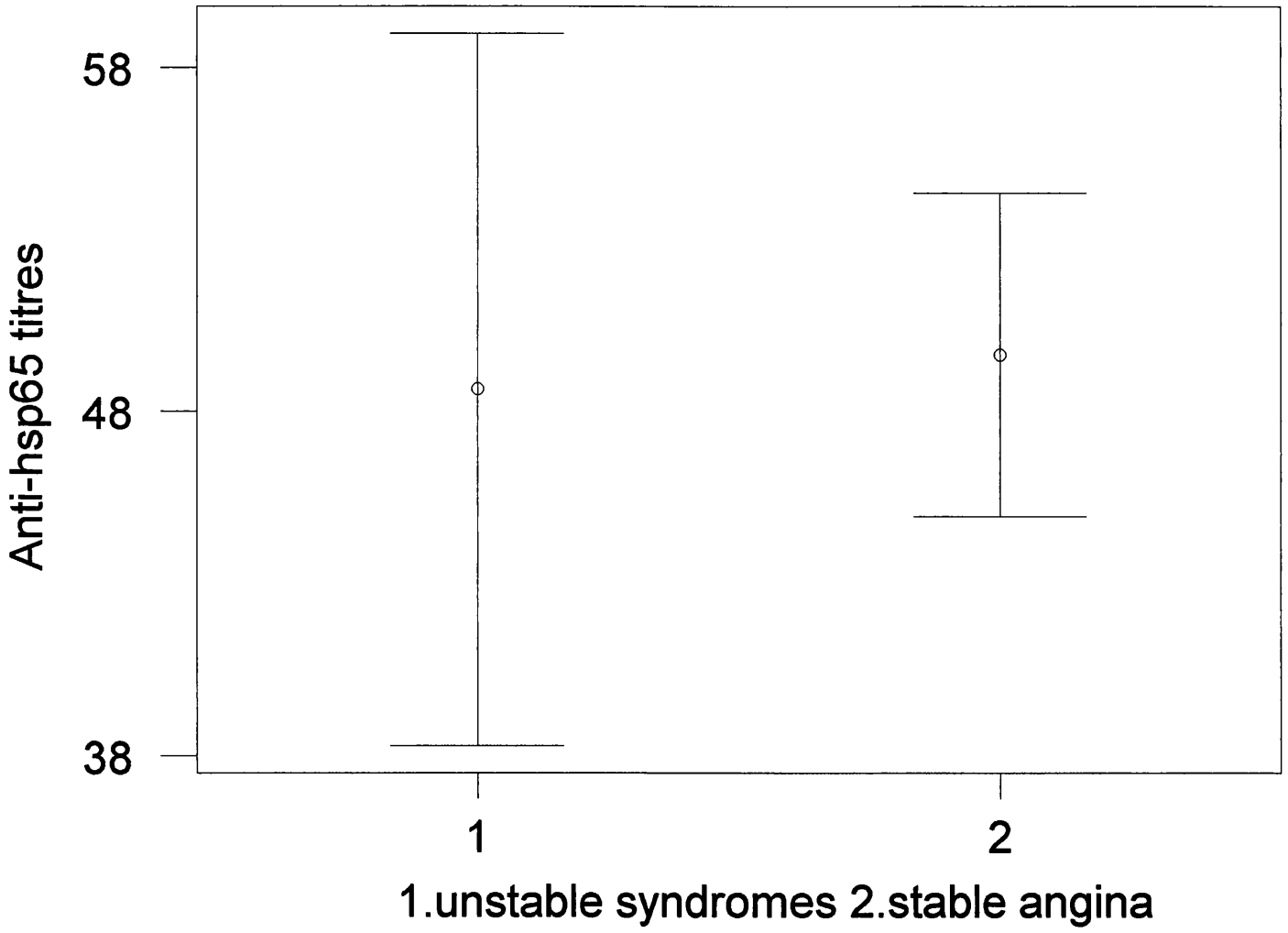


Figure 5.4 Anti-hsp65 titres in coronary syndromes



5.5 DISCUSSION

There was no evidence of a rise in anti-hsp65 titres following AMI. Indeed there was a trend for the titres to fall in the first five days post-infarct (Figures 5.1 and 5.2). This data argues against the autoimmune response with hsp65 on atherosclerosis cells contributing to plaque disruption.

Heng and Heng (129) have recently shown in vitro, increased hsp65 expression on human medial cells within 30 minutes of external arterial ligation. They studied 35 branches of the gastro-duodenal, superior and inferior mesenteric arteries and all 22 ligated arterial specimens reacted positively to monoclonal antibodies against mycobacterial hsp65, whereas all 13 non-ligated arteries were negative. Further, hsp65 was closely associated with activated T cells at sites of arterial injury. Thus it seems probable that there is increased local hsp65 expression at the time of arterial occlusion and injury during AMI, but this is not reflected by a rise in anti-hsp65. This is perhaps because none of the additional hsp65 is released systemically to induce a further immune response. However the trend for the titres to fall suggests the alternative explanation that the additional hsp65 is cleared by the pre-existing antibodies, and there is insufficient new antigen load to stimulate a rise in anti-hsp65 titres.

One potential criticism of our methodology is that we sampled only up to 5 days. However, all patients had detectable antibody titres at base-line, suggesting that any additional antibody rise would be a secondary immune

response and would be obvious within the 5 days. Thus, it appears that acute myocardial infarction does not lead to a serial rise in anti-hsp65 titres.

The second conclusion is that there was no significant difference in titre between acute coronary syndromes and stable chronic atherosclerosis. Indeed, there was a trend for patients with stable symptoms seem to have higher titres than those with acute coronary syndromes. However it is also possible that because of the small numbers in two of the cohorts, that the study had insufficient power to detect a difference in titres.

Nevertheless the data certainly argues against the possibility of anti-hsp65 titres having a discriminant role in risk stratifying patients with stable angina to determine those more likely to go on to plaque rupture and clinical events. These results are disappointing in view of the recent emerging data regarding C-reactive protein as a risk factor for progression of atherosclerosis to myocardial infarction irrespective of the underlying severity of atherosclerosis (90)(see chapter2). It is unclear whether the increased CRP reflects the inflammatory activity in the coronary arteries or parallels the effects of recurrent ischaemia on the myocardium. If the former is true then the inflammatory activity of coronary lesions appears to effect clinical outcome (148). This is in keeping with the in vitro studies of plaque stability detailed above. Prospective studies of anti-hsp65 titres in stable and unstable coronary syndromes are required to fully assess whether anti-hsp65 is a risk factor for progression of progression of a stable coronary atherosclerosis to plaque rupture and unstable coronary syndromes.

CHAPTER 6: RELATIONSHIP BETWEEN ANTI-HSP65 TITRES, CORONARY ATHEROSCLEROSIS AND CHD RISK FACTORS

6.1 INTRODUCTION

Xu et al showed a significant independent relationship between anti-hsp65 titre and the presence of carotid atherosclerosis (130) (reviewed in Chapter 2). This was heralded as potentially the diagnostic marker of atherosclerosis that clinical medicine has been awaiting (131). My study examined titres in coronary atherosclerosis for the first time and had three main objectives.

6.2 OBJECTIVES

1.To examine the correlations between anti-hsp65 titres and severity and extent of coronary atherosclerosis as assessed at coronary angiography.

2. To assess whether anti-hsp65 titre is a useful, clinical diagnostic marker of the presence and significance of coronary atherosclerosis.

3. To compare the relationship of anti-hsp65 titre with CHD risk factors.

6.3 MATERIALS AND METHODS

6.3.1 Subjects

136 consecutive eligible male subjects as detailed in chapter 5, population demographics listed in Table 6.1. Family history was also recorded and regarded as positive if ≥1 first degree relative had an AMI or developed angina

before the age of 60. Smoking was treated as a categorical variable (smoker, non-smoker, ex-smoker) and a continuous variable (estimated lifetime number of packets consumed).

6.3.2 Sample Preparation

The patients fasted overnight and 40 ml of blood was removed the following morning prior to coronary angiography. Samples were centrifuged within 2 hours of collection and the serum and plasma stored at -20°C for subsequent assay.

6.3.3 Assays

The IgG anti-hsp65 titres were measured as outlined in Chapter 3.

In addition, subjects had the following assayed: Total cholesterol was measured using an adaptation of the Abell-Kendall reference method. After ultracentrifugation HDL-Cholesterol was measured by the Heparin/$MnCl_2$ precipitation method and Ldl-cholesterol calculated (149). Triglycerides were assayed by enzymatic hydrolysis with subsequent determination of the liberated glycerol by colorimetry. CRP was measured using specific antiserum to form a precipitate which was measured turbidimetrically and compared with known standards. Lipoprotein (a) and fibrinogen were also quantified by immunoprecipitin analysis.

6.3.4 Coronary Angiography

This was performed using standard techniques and recorded in multiple projections for left and right coronary arteries. Scoring of the angiograms

was performed by a single independent observer. A random 10% were analysed a second time by the same observer blind to the first results to enable calculation of intra-observer variability. The clinical severity of coronary artery disease was assessed on the basis of a modified score of Negri et al (150). Coronary circulation was divided into 8 segments (see Figure 6.1): left main coronary artery; proximal middle and distal segments of left anterior descending (LAD); proximal and distal right coronary (RCA) and proximal and distal circumflex (CFX). Each segment was scored depending upon the severity of stenosis as follows: normal segment scoring 0, < 50% stenosis - 1, 50-90% stenosis - 12.5, 91-99% stenosis - 20, 100% stenosis - 25 and the overall score was the sum of the 8 individual segments. The intra-observer correlation was 0.94.

The extent of atherosclerosis was assessed in three ways. Firstly, by a 'vessel score' from 0 to 3 representing the number of arteries with any evidence of luminal diameter reduction or luminal irregularity. The number of vessels involved was calculated as follows: RCA or Posterior Descending Branch = 1, LAD or either of the first two diagonals = 1, CFX or obtuse marginal or Posterior Lateral Branch or Posterior Descending Branches = 1.The intra-observer and inter-observer correlations were 0.94 and . Secondly a 'clinical vessel score on a scale of 0-3 based on the system of Oberman et al (151) was the number of vessels with a luminal diameter reduction of greater than 50%. The number of vessels involved was calculated as follows: RCA = 1, CFX = 1 and LAD, first diagonal or both= 1. The intra-observer correlation was 0.82. These vessel scores enable calculation of

sensitivity and specificity of anti-hsp65 as a diagnostic indicator of atherosclerosis and clinically significant atherosclerosis (≥1 stenosis >50%) Thirdly, a more detailed 'diffuseness score', as previous work has demonstrated greater correlations between risk factors and these scores than with the simpler vessel scores (152).This score was based on a modification of the method of Negri et al (150)(see Figure 6.1).The coronary circulation is divided into 15 segments and eight of these are first order segments: proximal and middle RCA, left main coronary artery, proximal middle and distal LAD and proximal and distal CFX. In addition, there were 7 second order segments: distal RCA, posterior descending branch (whether arising from circumflex or right coronary artery), obtuse marginal branch, posterolateral branch of CFX and the first two diagonal branches of the LAD. The first order segments received a score of 1 if there was any evidence of atherosclerosis, and the second order segments scored 0.5. The overall diffuseness score was the sum of the individual segment scores and the maximum score attainable was 11.5. The intra-observer correlation was 0.80.

6.3.5 Statistical Analysis

The anti-hsp65 titres showed a distribution with a strong positive skew and so logarithmic data were used for all parametric methods. There were significant differences between assay batches and each log titre was corrected as far as possible by subtracting the mean log titre of the corresponding ELISA plate and adding the grand mean log titre. This left the mean log IgG anti-hsp65 level unchanged, i.e. left the geometric mean

IgG anti-hsp65 titre unchanged. Three of the other variables measured, Lipoprotein (a), triglycerides and CRP were approximately log-normally distributed and thus were expressed logarithmically before calculating confidence intervals. Correlations between continuous variables were examined using Spearman's rank correlation analysis. Relationships between continuous variables and categorical variables were assessed by Mann-Whitney and Kruskal-Wallis tests. Receiver operator characteristic (ROC) curves were used to assess anti-hsp65 as a diagnostic indicator of coronary atherosclerosis. ROC analysis is regarded by many as the best assessment of whether a surrogate test can be used in routine clinical practise (153). The ROC curve represents the overall performance of a diagnostic test in terms of sensitivity and specificity as the ratio of true positives to false positives. The area under this curve quantifies the usefulness of the test. An ideal diagnostic test would inscribe an rectangular line passing from the origin to the top right hand point by way of the top left hand corner (see Figure 6.2); the area beneath such a curve would be nearly 100% of the total area (154) A completely non-discriminatory test would result in a diagonal line from bottom left to top right of the graph (see Figure 6.3), all points on the line representing a ratio of true to false positives of 1:1, the area beneath the curve would be half the total area(154). Multiple linear regression analysis was used to test for residual relationships among interval variables while correcting for possible confounding influences.

6.4 RESULTS

The distribution of the anti-hsp65 titres and other measured serum and plasma variables are listed in Table 6.2. The 'vessel score' counted an artery as affected if there was any evidence of luminal irregularity, and by this score there were 19, 7, 35 and 75 patients with 0, 1, 2 and 3 affected coronary arteries respectively. Using the 'clinical vessel' score which scores an artery as affected if there is ≥ 1 stenosis $\geq 50\%$ there were 19, 7, 35 and 75 patients with 0, 1, 2 and 3 affected coronary arteries respectively. The atherosclerosis severity and diffuseness scores had mean values (SD) of 39.7 (31.4) and 4.1 (2.7) respectively. As expected there were very strong correlations between the four coronary atherosclerosis scores (Table 6.3).

The four coronary atherosclerosis scores correlated significantly although quite weakly ($r=0.18$-0.21, $p<0.05$) with the titres of IgG anti-hsp65 and this is demonstrated in Table 6.3. Table 6.4 lists the sensitivity and specificity of anti-hsp65 levels as a diagnostic indicator of the presence of coronary atherosclerosis (i.e any evidence of luminal irregularity, calculated from the 'vessel score') and of clinically significant coronary atherosclerosis ($\geq$ I stenosis $\geq 50\%$ in ≥ 1 artery, calculated from the 'clinical vessel score'). These are presented graphically in the form of receiver-operator curves (ROCs) in Figures 6.4 and 6.5.

Anti-hsp65 titres correlated with age ($r=0.24$, $p=0.005$) and lifetime cigarette consumption ($r=0.18$ $p=0.049$) but not with any other continuous CHD risk factors (shown in Table 6.5).

Categorical risk factors are compared with continuous risk factors in Tables 6.6-6.8. Again there is evidence that smoking appears to have an influence on anti-hsp65 (Table 6.6) with a trend for an increasing titre through categories of non-smoker, ex-smoker and current smoker. The pattern remained after adjusting for baseline severity and diffuseness of coronary atherosclerosis. A similar pattern is observed for total WBC (p=0.038). A history of hypertension does not affect anti-hsp65 (Table 6.7). However as expected hypertensives were significantly heavier and had trends to higher glucose and triglycerides.

A family history of premature CHD is associated with a higher titre of anti-hsp65 (33.80 AU/ml compared to 23.99 AU/ml, p=0.052, Table 6.8). Subjects with a positive family history had less severe coronary atherosclerosis than those with no family history but after age adjustment the family history group had slightly more severe atherosclerosis (41.6 versus 40.3, p=0.81).

Anti-hsp65 titres were adjusted for the potential confounding influences of age, smoking (packets) and family history and the significance of the association with the coronary atherosclerosis scores was lost (see Table 6.9). However the group with any evidence of coronary atherosclerosis (from the vessel score) had significantly higher adjusted anti-hsp65 than those with no coronary atherosclerosis (27.86 AU/ml compared to 17.10 AU/ml, p=0.012).

Lastly, correlations between other continuous CHD risk factors and the angiographic severity and extent of coronary atherosclerosis are shown in Table 6.10 and 6.11. There were moderately strong correlations between age and all the coronary angiography scores (r=0.26-0.42, p<0.01-<0.001, Table

6.10). There was a weak but significant correlation between LDL-Cholesterol and severity and extent of disease ($r=0.17-0.23$, $p<0.05$). There was a clear trend for current smokers to have more severe and diffuse disease than non-smokers with ex-smokers having an intermediate level (Table 6.11). There was no consistent relationship with hypertension, while a positive family history initially appearing to confer a protective benefit. However this relationship was lost after adjustment for the confounding influence of age.

Age mean (SD)	55.3 (10.4)
Body Mass Index (kg/m^2)	26.7 (3.7)
History of hypertension	30.9%
Family history of coronary atherosclerosis	47.5%
Never smoked	18.5%
Ex-smoker	61.4%
Current smoker	20.1%
Indication for angiography	
Chest pain	95.6%
Valvular abnormality	4.4%

Table 6.1 Description of cohort of 136 male subjects

Variable	Number	Mean	S.D
Total Cholesterol (mmol/l)	133	5.62	1.01
LDL Cholesterol (mmol/l)	119	3.66	0.76
HDL Cholesterol (mmol/l)	127	0.90	0.22
Total/HDL ratio	127	6.63	1.93
Triglycerides (mmol/l)*	133	1.66	1.70
Lipoprotein (a) (mg/dl)*	121	21.87	2.57
Fibrinogen (mg/dl)	123	330.4	76.7
Glucose (mmol/l)	133	5.76	0.66
White Cell Count ($x1O^9$/l)	120	7.07	1.56
Platelets ($x10^{12}$/l)	121	226.2	56.2
CRP (mg/l)	123	9.69	8.76
Anti-hsp65* (AU/ml)	136	26.30	2.29

Table 6.2 Distribution of serum and plasma factors (* variables which were log-transformed before analysis; geometric mean and approximate SD are expressed)

	Correlation with anti-hsp65	Correlation with severity score	Correlation with vessel score	Correlation with clinical vessel score
Severity score	0.21 (p=0.018)	------	------	------
Vessel score	0.18 (p=0.036)	0.81*	------	------
Clinical vessel score	0.21 (p=0.012	0.91*	0.82*	------
Diffuseness score	0.21 (p=0.016)	0.84*	0.80*	0.77*

Table 6.3 Spearman's rank correlation coefficients between severity and extent of coronary atherosclerosis and titres of anti-hsp65. (* $p<0.001$)

	Detection of presence of atherosclerosis		Detection of significant atherosclerosis (≥ 1 stenosis ≥50%)	
Anti-hsp65 titre (AU/ml)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
≥ 5	100	0	100	0
≥ 15	77.8	57.8	78.6	63.2
≥ 30	47.8	84.2	48.6	74.0
≥ 50	22.2	94.7	20.5	84.2
≥ 72.3	13.7	100	13.8	92.6

Table 6.4 Sensitivity and specificity of anti-hsp65 in detecting coronary atherosclerosis

	Correlation with anti-hsp65 titre	
Factor	Correlation	P value
Age	0.24	0.005
BMI	-0.05	0.53
Social class	-0.09	0.28
Packets of cigarettes	0.18	0.049
Lipoprotein (a)	-0.08	0.41
Triglycerides	-0.02	0.17
Total Cholesterol	-0.05	0.55
Total/HDL cholesterol	-0.22	0.81
Fibrinogen	-0.10	0.25

Table 6.5 Spearman's rank correlations between anti-hsp65 titres.

	Never Smoked (n=24)	Ex-smoker (n=84)	Current smoker (n=28)
Age (years)	55.4	56.2	52.9
BMI (kg/m^2)	27.69	26.73	25.59
Cigarette* consumption (pkts)	0	11515	13714
Anti-hsp65***	22.89	28.84	33.88
WBC ($x10^9/l$)**	6.41	7.06	7.62
Neutrophils ($x10^9/l$)	3.93	4.30	4.55
Lymphocytes ($x10^9/l$)	1.92	2.10	2.29
Platelets ($x10^{12}/l$)	208.40	231.7	224.7
CRP (mg/l)	6.76	7.94	7.41
Glucose (mmol/l)	5.76	5.82	5.53
Total Cholesterol (mmol/l)	5.31	5.50	5.75
Ratio LDL/HDL	6.28	6.89	6.20
Triglycerides (mmol/l)	1.58	1.45	1.90
Lipoprotein (a)	26.91	13.49	17.38
Fibrinogen (g/dl)	331.20	329.14	374.08

Table 6.6 Comparison of mean values of cardiovascular risk factors between groups of non smokers, ex-smokers and current smokers (Anti-hsp65, Triglycerides and Fibrinogen age adjusted data, *$p<0.0001$, **$p=0.038$, otherwise p=ns including *** $p=0.29$).

	No hypertension (n= 95)	Hypertension (n=41)	P value
Age (years)	55.3	55.5	0.92
BMI (kg/m^2)	25.77	28.67	<0.00001
Cigarette consumption (pkts)	9972	8646	0.51
Anti-hsp65	26.91	25.12	0.57
WBC ($x10^9/l$)	7.17	6.84	0.20
Neutrophils ($x10^9/l$)	4.27	4.35	0.70
Lymphocytes ($x10^9/l$)	2.19	1.93	0.044
Platelets ($x10^{12}/l$)	230.2	216.8	0.21
CRP (mg/l)	7.76	7.41	0.69
Glucose (mmol/l)	5.71	5.87	0.19
Total Cholesterol (mmol/l)	5.60	5.66	0.74
Ratio LDL/HDL	6.64	6.64	1.0
Triglycerides (mmol/l)	1.58	1.74	0.37
Lipoprotein (a)	20.89	24.54	0.44
Fibrinogen (g/dl)	330.3	330.7	0.98

Table 6.7 Comparison of mean values of cardiovascular risk factors between groups of with and without a history of hypertension.

	No Family History (n=67)	Family History (n=56)	P value
Age (years)	58.25	51.33	0.0001
BMI (kg/m^2)	26.64	27.35	0.27
Cigarette consumption (pkts)	9021	9884	0.64
Anti-hsp65	23.99	33.80	0.052
WBC ($x10^9/l$)	7.18	6.91	0.94
Neutrophils ($x10^9/l$)	4.39	4.22	0.44
Lymphocytes ($x10^9/l$)	2.11	2.10	0.92
Platelets ($x10^{12}/l$)	225.5	229.2	0.73
CRP (mg/l)	8.53	6.76	0.042
Glucose (mmol/l)	5.79	7.78	0.93
Total Cholesterol (mmol/l)	5.55	5.76	0.27
Ratio LDL/HDL	6.64	6.62	0.97
Triglycerides (mmol/l)	1.62	2.00	0.067
Lipoprotein (a)	21.42	22.23	0.83
Fibrinogen (g/dl)	332.8	345.7	0.067

Table 6.8 Comparison of mean values of cardiovascular risk factors between groups with and without a family history of premature coronary heart disease, defined as a first degree relative having symptoms < 60 years of age. (data adjusted for age)

	Correlation before adjustment	Correlation after adjustment
Severity score	0.21*	0.09
Vessel score	0.18*	0.09
Clinical vessel score	0.21*	0.11
Diffuseness score	0.21*	0.07

Table 6.9 Correlation of anti-hsp65 titres with angiographic extent and severity coronary atherosclerosis before and after correction for confounding influences (age, smoking consumption and family history) *$p<0.05$.

	Severity score	Vessel score	Clinical Vessel score	Diffuseness score
Age (years)	0.30***	0.38***	0.26**	0.42***
BMI	0.00	-0.11	-0.08	-0.04
Cigarette consumption	0.02	0.19	0.08	0.04
Glucose	0.12	0.06	0.06	0.19
Lipoprotein (a)	0.02	0.06	0.04	-0.02
Triglycerides	0.05	0.03	0.01	0.02
Cholesterol	0.16	0.14	0.13	0.09
LDL cholesterol	0.17	0.23*	0.17	0.17
HDL cholesterol	0.04	-0.01	0.01	0.11
Ratio	0.08	0.16	0.13	0.01
Fibrinogen	0.06	0.13	0.09	0.09
CRP	-0.19	-0.15	-0.15	-0.18
WBC	-0.12	-0.07	-0.09	-0.05
Platelets	-0.13	-0.01	-0.09	-0.031

Table 6.10 Correlations between other continuous CVS risk factors and the severity and extent of coronary atherosclerosis ($p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

	Severity score	vessel score	Clinical vessel score	diffuseness score
Non- smoker	33.96	1.83	1.42	3.71
Ex smoker	38.53	2.25	1.66	4.01
smoker	48.70	2.43	2.07	4.70
No hypertension	38.4	2.24	1.67	4.17
Hypertension	43.0	2.17	1.78	4.00
No family history	42.8	2.34	1.78	4.36
Family History	38.3	2.04	1.61	3.84

Table 6.11 Relationship between categorical CHD risk factors and the severity and extent of coronary atherosclerosis (p=ns for all)

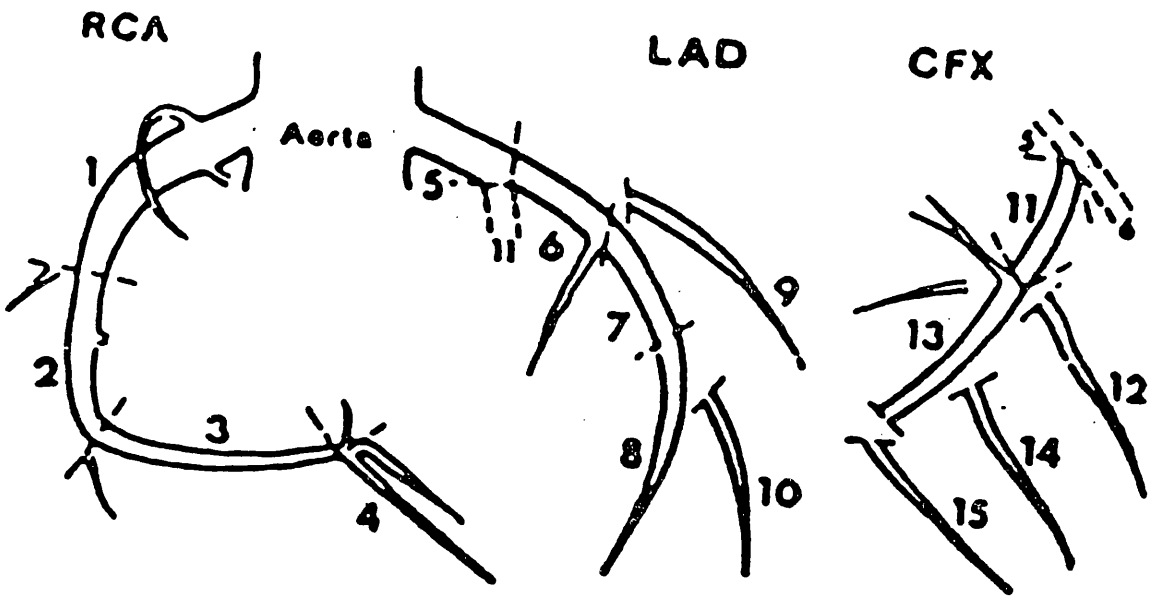


Fig 6.1 The Coronary Circulation. Segments 1-3, 5-8, 11 and 13 were used to calculate the atherosclerosis severity score. All segments were used in the calculation of diffuseness, vessel and clinical vessel scores. Right Coronary Artery (RCA), Left Anterior Descending (LAD), Circumflex (CFX).

Figure 6.2 Hypothetical ROC for an ideal diagnostic test

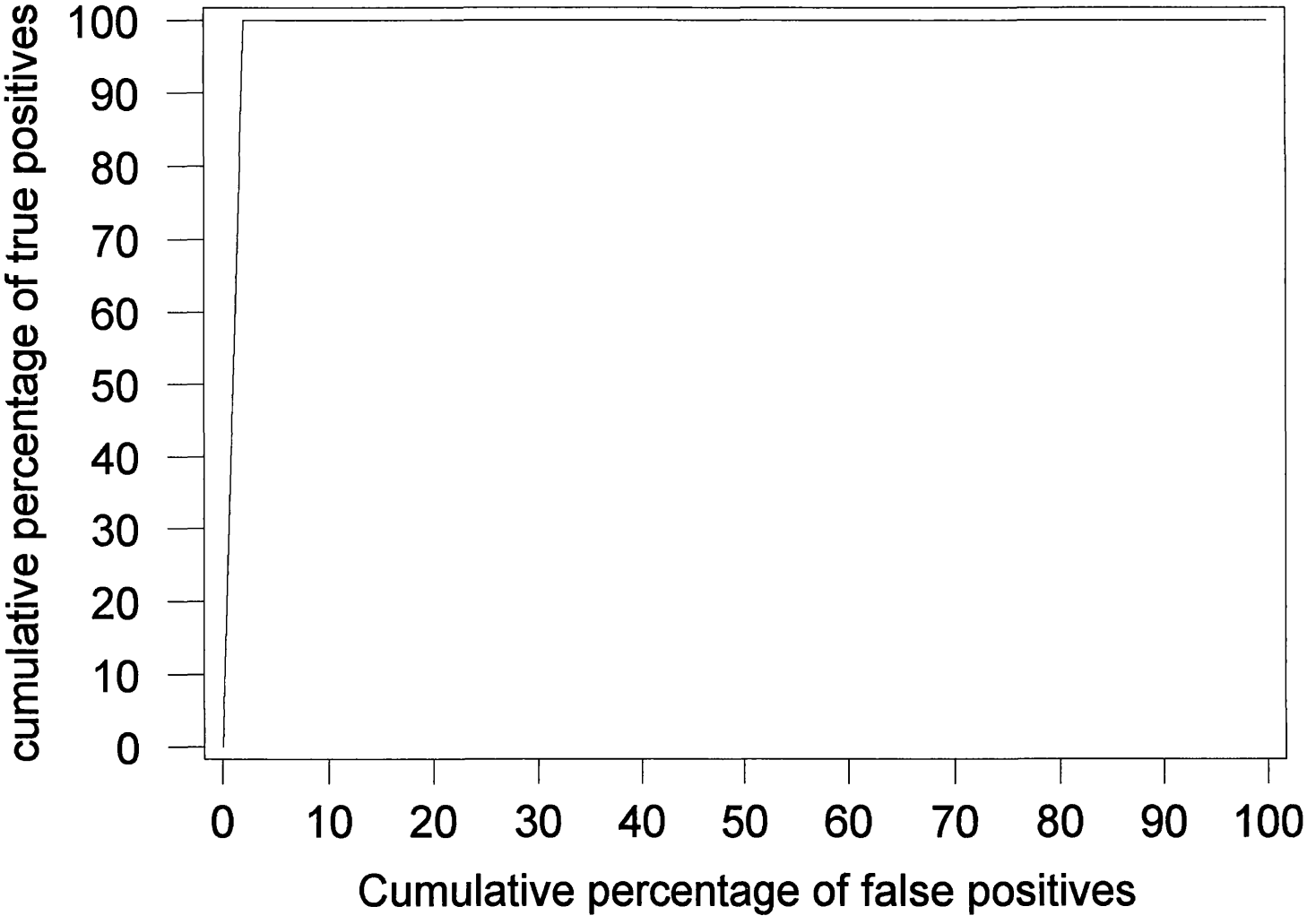


Figure 6.3 Hypothetical ROC for completely non-discrimatory test

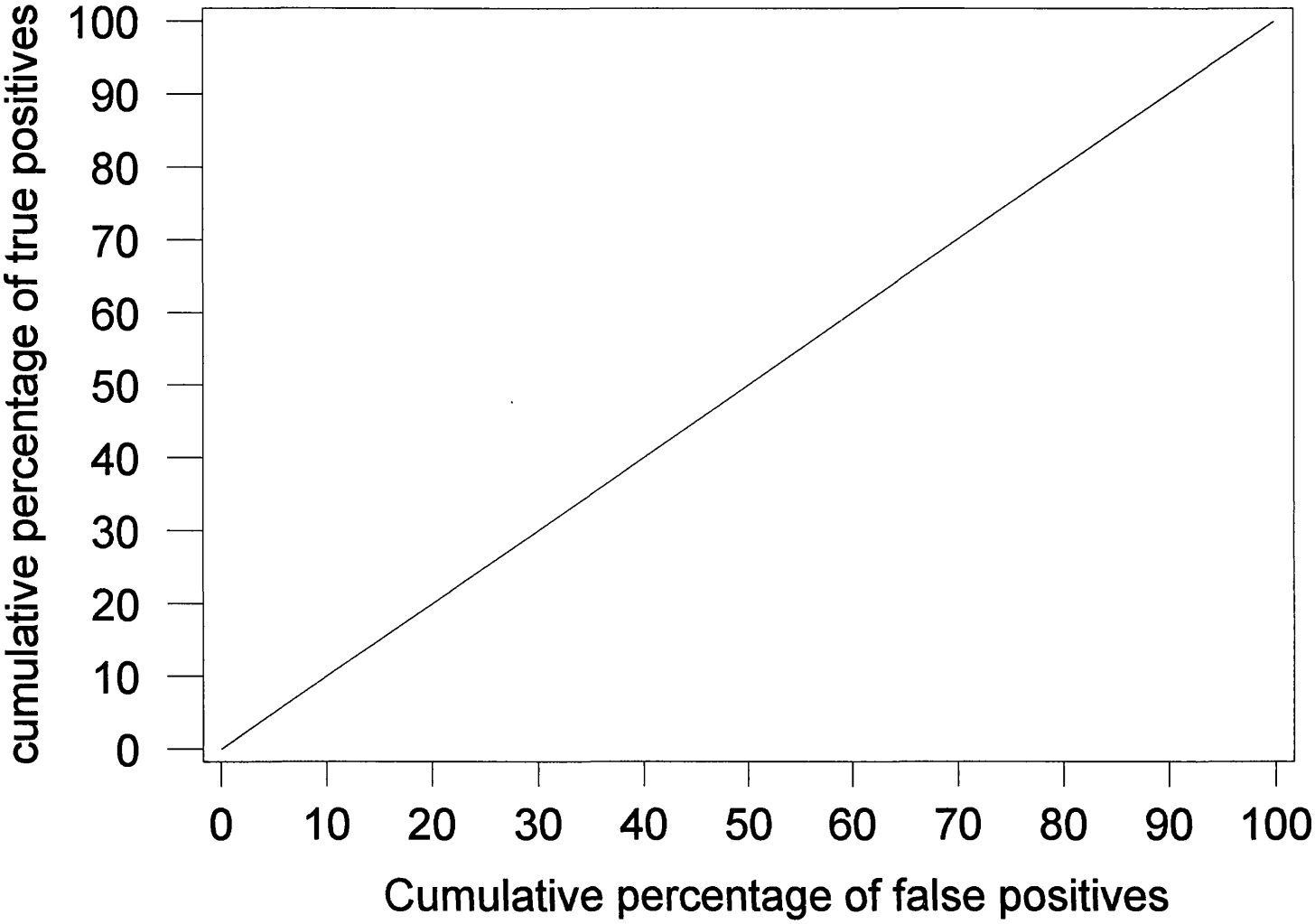


Figure 6.4 ROC for anti-hsp65 as an indicator of coronary atherosclerosis

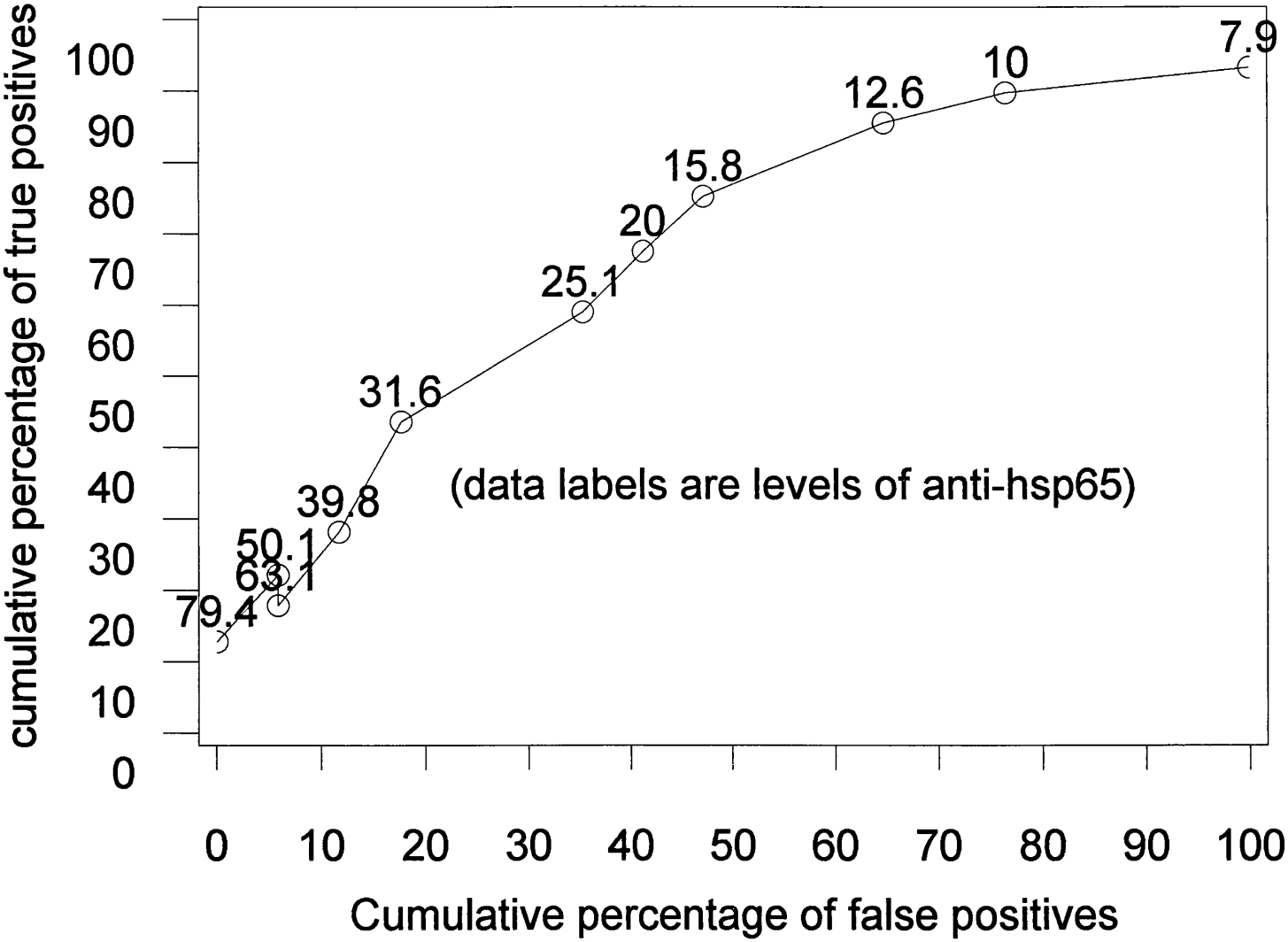
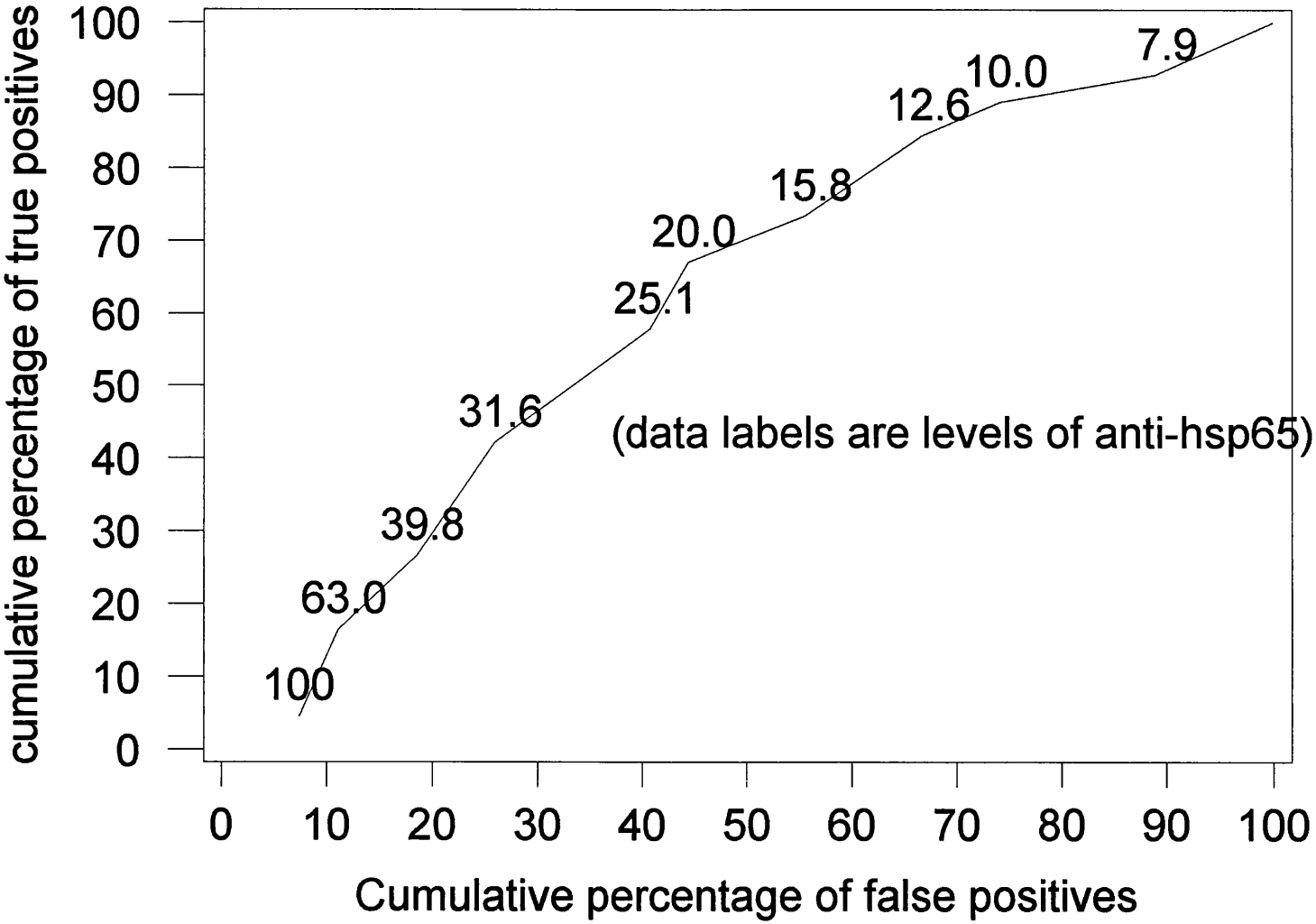


Figure 6.5 ROC for anti-hsp65 as an indicator of significant coronary atherosclerosis



6.5 DISCUSSION

We show clear evidence that anti-hsp65 antibodies are associated with angiographically detected coronary atherosclerosis with statistically significant correlations between titres and severity or extent of disease ($r=0.18$-0.21, $p<0.05$ Table 6.3). However these correlations although statistically significant are quite weak and obviously anti-hsp65 titre will have insufficient predictive accuracy to be a useful clinical test This is shown in Table 6.4 which lists the sensitivity and specificity of anti-hsp65 levels as a diagnostic indicator of the presence of coronary atherosclerosis and of clinically significant coronary atherosclerosis ($\geq$ I stenosis $\geq$ 50% in $\geq$ 1 artery, calculated from the 'clinical vessel score'). At best using a discriminant titre of 15 AU/ml the test has only 77.8% sensitivity and 57.8% specificity for the presence of disease. These are presented graphically in the form of receiver-operator curves (ROCs) in Figures 6.3 and 6.4.

Anti-hsp65 titre also correlated with age ($r=0.24$, $p=0.005$) and with smoking as a continuous variable ($r=0.18$, $p=0.049$) but not with other continuous CHD risk factors (Tables 6.5). Further evidence that smoking has an influence on anti-hsp65 titre is shown in Table 6.6. There was a clear trend for smokers to have higher mean anti-hsp65 titre than lifelong non-smokers with ex-smokers having an intermediate titre (33.88, 22.89 and 28.84 AU/ml respectively). The pattern remained after adjusting for the other possible confounding influence of severity of angiographic coronary atherosclerosis. Xu et al (130) is the only other study to examine the effect of smoking on anti-hsp65 titre and they also

showed a significant positive relationship considering it as a continuous variable.

A similar and statistically significant relationship is observed for total WBC (p=0.038) as has been previously observed (155). This is shown to be due to variations in both neutrophils and lymphocytes (Table 6.6). I also confirm the well established influence of smoking on fibrinogen level. Smokers in our cohort had a mean fibrinogen of 374.0 mg/dl compared to 329.5 mg/dl for non-smokers (p=0.08). Elevating fibrinogen is considered to be one of the major pathogenic processes by which smoking leads to increased CHD risk (156). However there are many other putative mechanisms and our data suggests that anti-hsp65 should potentially be added to the list.

The other CHD risk factor which appeared to influence anti-hsp65 titre is a family history of premature coronary atherosclerosis (Table 6.8). Subjects with a positive family history had a mean titre after age adjustment of 33.80 AU/ml compared to 23.99 (p=0.052). There were no obvious confounders, most importantly those with a positive family history had less severe coronary atherosclerosis than those with no family history. This initially seems paradoxical but can be explained by the different ages of the two groups. After age adjustment the family history group had slightly more severe atherosclerosis (41.6 versus 40.3, p=0.81).

These data suggest that there is a hereditary component to anti-hsp65 titres similar to another study (120) but contrary to that reported in chapter 4. The populations in my two studies are quite different; in chapter 4 we studied a

predominantly female cohort with a mean age of 37.3 while in this chapter our cohort was exclusively male with a mean age of 55.3 years.

The statistical significance of the relationship between titre and severity and extent of disease was lost after adjustment for the possible confounding influences of age, smoking and family history (see Table 6.9). However the group with any evidence of coronary atherosclerosis (from the vessel score) had significantly higher adjusted anti-hsp65 than those with no coronary atherosclerosis (27.86 AU/ml compared to 17.10 AU/ml, p=0.012).

These results are similar to the findings of Xu et al (130) who in a study of carotid atherosclerosis showed that titres of anti-hsp65 correlated with the extent of atherosclerosis and with age and smoking but not other CHD risk factors. However, again similarly to our results, their correlation between titre and extent of disease lost significance after correction for confounders (p=0.083). They concluded that this showed anti-hsp65 was an independent risk factor for the presence of carotid plaque.

Coronary angiography may miss early atheromatous change as discussed in Chapter 1. Thus, in our group with angiographically normal coronary arteries, there may be some with minor atheroma and thus the difference in anti-hsp65 titres between truly atheroma-negative subjects and atheroma-positive subjects may be larger than that indicated.

Two other points of note arose from this study. First it has been shown in stable (90) and unstable angina(91) patients that higher levels of CRP is a risk

factor for progression of atherosclerosis to myocardial infarction. However, there was no evidence of a correlation of CRP with clinical severity or extent of atherosclerosis in our cohort (Table 6.10), suggesting that elevating CRP reflects an additional inflammatory factor in the disease process. The additional factor may at least partly be the anti-hsp60/65 immune response.

Secondly, it has recently been suggested that lipoprotein (a) may exert its adverse affects via an autoimmune mechanism triggered by infection (157). However, we could find no evidence to support this as there was no correlation of lipoprotein (a) with anti-hsp65 titres (Table 6.5).

In summary therefore we have demonstrated that anti-hsp65 titres correlate with the severity and extent of coronary atherosclerosis, but with insufficient predictive accuracy to be a useful clinical test. Three CHD risk factors age, smoking and family history influenced anti-hsp65 titre. After correction for these confounding factors, the elevated anti-hsp65 titre remains statistically significantly associated with the presence of coronary atherosclerosis (27.86 AU/ml compared to 17.10 AU/ml, p=0.012).

CHAPTER 7: *HELICOBACTER PYLORI*, ANTI-HSP65 AND ATHEROSCLEROSIS

7.1 INTRODUCTION

There have been three papers to date AMI examining the relationship between *Helicobacter Pylori* infection and clinically manifest ischaemic heart disease (reviewed in Chapter 2). Two (73,74) support an association which is independent of age, sex, social class and CHD risk factors but the third (75) did not.

Clearly there are difficulties in investigating a residual relationship between *H. pylori* infection and CHD when each individually is strongly related to social class (see Figure 1.2 and Table 2.2) and age. After correcting for age and social class the loss of significance does not exclude a causal relationship between *H.pylori* and CHD, as *H.pylori* infection could be a mechanism by which these factors increase the risk of CHD. Thus, it is important to test the hypothesis of a causal relationship in other ways and one of these is by investigating possible pathogenic processes.

An early postulate suggested that the pathogenic link between *H. pylori* infection and IHD was due to the systemic effects of *H. pylori* on fibrinogen concentration and total white blood cell count (74). Patel et al (74) showed that men seropositive for *Helicobacter pylori* had significantly higher fibrinogen (by 0.175 g/l, 95% confidence interval, 0.039-0.311) and log

white cell count (by 0.123, 95% confidence interval, 0.041-0.205) after correction for age, smoking history, body mass index and social class, than seronegative subjects. However, Murray et al (75) in a larger study a weak negative association between *Helicobacter pylori* infection and fibrinogen (mean(SE) difference in fibrinogen between infected and uninfected -0.09 (0.04) g/l, p= 0.02.)

Thus, it is important to look for alternative mechanisms and in this chapter I investigate the possibility of an auto-immune process provoked by *H.pylori* infection. The immune cells in *H.pylori* gastritis include neutrophils, macrophage/monocytes, plasma cells and predominantly Tcells (mostly T_H cells)(158). There is marked heterogeneity in the T cell responses to purified *H.pylori* antigens (159). In general the seronegative subjects responses were more marked than the seronegative suggesting antigen specific suppression of T cell activation occurs in infected individuals (159). Western blotting of mucosal derived antibodies show that different patients have different response patterns of antibodies to the various antigens of *H.pylori* (160) reflecting differences between the strains of *H.pylori* and also host immune response differences too. The majority of the humoral response is to the surface expressed urease enzyme and the 62 Kd heat shock protein (hsp62) a member of the hsp60/65 family (159,161). *H.pylori* specific serum antibodies of both IgA and IgG isotypes are detectable (162,163) but the relative importance of these isotypes is unclear. Potentially the IgA antibodies are more important as they

have a central role in the mucosal associated lymphoid system (MALT) and can be secreted onto the mucosal surface (164).

7.2 OBJECTIVES

1. To examine whether *H.pylori* infection and titres of antibodies to *H.pylori* are independent risk factors for coronary atherosclerosis.

2. To examine the relationship between anti-*H.pylori* antibodies, infection and CHD risk factors

3. To examine the influence of *H.pylori* infection on anti-hsp65 titre (IgG and IgA isotype, before and after adjustment for confounding influences).

Two studies have been performed, the first uses the same cohort as described in Chapter 6; the second examines the effects of *H.pylori* eradication on anti-hsp65 titre.

7.3 MATERIALS AND METHODS

7.3.1 Subjects

In the first study we used the same cohort as in chapter 6. Briefly, we recruited 136 consecutive eligible men admitted for routine cardiac catheterisation for the investigation of chest pain (95.6%) or valvular abnormalities (4.4%). Social class was assessed using deprivation categories derived from Carstairs scores of postcode sectors. These deprivation categories (DEPCATS 1-7) provide a measure of deprivation or affluence on the basis of a combination of selected 1991 Census variables standardised to their mean (165). The variables used to create the scores were

car ownership, male unemployment, head of household occupation and overcrowding.

In the second study we recruited 100 subjects (age mean 43.1years, range 17-67, 30 males) all with active *H.pylori* infection confirmed by Urea breath test. The subjects were recruited over a three month period and the study was approved by the Hospital Ethical Committee and all patients gave informed consent. The subjects were double blindly randomised to active treatment (n=48) or placebo (n=52). Active treatment consisted of Omeprazole 20 mg bd, Metronidazole 400mg tid and amoxycillin 500mg tid for 14 days.The placebo group received two weeks of Omeprazole 20mg bd plus placebo antibiotics. The subjects returned one year after commencing therapy and had a further breath test. Subjects were excluded if the follow-up breath test result did not confirm successful eradication in the active treatment group or continuing infection in the placebo group. This left 33 in the treatment group and 41 in the placebo group (see Table 7.1).

7.3.2 Sample Preparation

The patients fasted overnight and 40 ml of blood was removed the following morning prior to coronary angiography or breath testing. Samples were centrifuged within 2 hours of collection and the serum and plasma stored at -20°C for subsequent assay.

7.3.3 Assays

IgG and IgA anti-hsp65 were measured by ELISA as described in chapter 3. Pre and post samples from a given patient were assayed on the same plate.

IgG antibodies specific to *H.pylori* were quantified by using the BIO-Rad (California, USA) G.A.P. IgG test kit. This is an ELISA system using plates coated with *H.pylori* antigens. IgG specific *H.pylori* antibodies are quantified in units/ml (U/ml) and a cut-off titre of 12 U/ml has been shown to be 94.9% sensitive and 91.3% specific for culture proven *H.pylori* gastric infection (166).

The samples were all assayed without prior knowledge of the coronary angiography findings.

7.3.4 Coronary Angiography

As chapter 6.

7.3.5 Statistical Analysis

In the first study, anti-*H. pylori* titre was approximately log-normally distributed and thus were expressed logarithmically before calculating confidence intervals. Correlations between continuous variables were examined using Spearman's rank correlation analysis. Relationships between continuous variables and categorical variables were assessed by Mann-Whitney and Kruskal-Wallis tests. Multiple linear regression analysis was used to test for residual relationships among interval

variables while correcting for possible confounding influences. The Chi-square test was used to assess whether the presence of *H. pylori* infection was significantly associated with the presence of atherosclerosis. T-tests were used to compare the means of biochemical risk factors in *H. pylori* seropositive and seronegative subjects, before and after correcting for possible confounding influences.

In the second study, there were no significant differences between IgG anti-hsp65 assay batches but there was differences between IgA assay batches. Thus each IgA anti-hsp65 titre was corrected as far as possible by dividing by the mean titre of the corresponding ELISA plate and multiplying by the grand mean titre. This left the mean IgA anti-hsp65 level unchanged, i.e. left the geometric mean IgG anti-hsp65 titre unchanged. Depending on a variable's distribution either the Mann-Whitney Test or Student's T Test was used to examine for differences between the active and placebo groups. Correlations between IgG and IgA anti-hsp65 titres (pre, post and ratios) were examined by Spearman's rank correlation coefficients.

7.4 RESULTS

In the first study, there were significant correlations between all the atherosclerosis scores and anti-hsp65 titres and between three scores (severity, vessel score and diffuseness score) and antibodies to *H.pylori* (Table 7.2). Generally the correlations with atherosclerosis were stronger for anti-hsp65 than for anti-*H.pylori*. There was a strong and highly statistically

significant correlation between anti-hsp65 titre and antibody titre to *H.pylori* ($r=0.39$, $p<10^{-5}$, see Figure 7.1).

There was a significant correlation between anti-*H.pylori* titres and smoking as a continuous variable ($r=0.28$, $p=0.002$) and between anti-*H.pylori* titre and fibrinogen ($r=0.20$, $p=0.031$) (see Table 7.3). There was no relationship between anti-*H.pylori* titres and categorical variables of hypertension and family history, but there was a positive relationship with smoking (data not shown). Current smokers had a significantly higher mean titre of anti-*H.pylori* than lifelong non-smokers with ex-smokers having an intermediate level (28.71, 12.88 and 25.80 U/ml respectively, $p=0.004$).

An anti-*H.pylori* titre of ≥12 U/ml has been shown to be 94.9% sensitive and 91.3% specific for culture proven *H.pylori* gastric infection (9). Using this discriminant titre in the group with no coronary atherosclerosis, 58% were seropositive for *H. pylori* compared with 71% of subjects with coronary atherosclerosis (difference not statistically significant). However *H.pylori* positive subjects had significantly more severe and diffuse coronary atherosclerosis than seronegative (Table 7.4). However the statistical significance was lost after adjusting for confounders (Table 7.4)

Comparing mean levels of continuous (Table 7.5) and categorical CHD risk factors (Table 7.6) between groups seropositive and seronegative for *H.pylori* infection indicated that *H.pylori* infection was not associated with raised concentrations of fibrinogen, cholesterol, triglycerides or glucose. However the former group had statistically higher mean titres of two potential CHD risk

factors (WBC and anti-hsp65 titre) and borderline significant relationships with social class and smoking consumption. The relationship between social class and *H.pylori* seropositivity is shown in Figure 7.2. Their is also a borderline significant association between seropositivity and smoking as a categorical variable (50, 72 and 79 % of non smokers, ex-smokers and current smokers are seropositive respectively, p=0.06).

Thus based on the above data and those of others (see Tables 2.1 and 2.2) age, social class and smoking appear to be the three most important confounding influences on a possible relationship between IHD and *H.pylori* infection. Thus anti-*H.pylori* titre was adjusted for these possible confounding influences and re-examined and the statistical significance of the relationship between titre and atherosclerosis was lost (see Table 7.2). Similarly although the trends remained there was no statistically significant difference in the severity or diffuseness of coronary atherosclerosis between seropositive and seronegative groups after adjustment for these factors (Table 7.4).

The correlations between the anti-hsp65 titres and antibody titres to *H.pylori* were also re-examined after adjusting for their possible common relationships with age, smoking and social class and the correlation remained significant ($r=0.38$, $p<10^{-4}$). In addition, in the sub-group with likely active *H.pylori* infection (IgG anti-*H.pylori* titre > 12 U/ml) the correlation between antihsp65 and anti-*H.pylori* remained ($r=0.28$, $p=0.006$). There was no elevation of WBC or anti-hsp65 titre after adjustment (Table 7.6)

In the second study, there was no difference in titres between the sexes. In the placebo group (n=41) 27 titres fell, 2 did not change and 12 rose. In comparison in the treatment group (n=34), 27 titres fell, 3 stayed the same and 4 rose. (p=0.066 for comparison of proportion of patients whose titres fell or remained static 29/41 (70.1%) and 30/34 (88.2%) in the placebo and treatment groups, respectively).

Pre-therapy anti-hsp65 titres did not differ significantly between the placebo and treatment groups (Table 7.8). With successful eradication of *H.pylori* IgG anti-hsp65 titre fell from 25.6 to13.8 AU/ml (p=0.033). In comparison with placebo the titre remained essentially static (25.7 Au/ml initially and 22.4 Au/ml at one year, p=0.45).The IgG anti-hsp65 titres pre and post therapy in the placebo group is illustrated in Figure 7.3 and in the treatment group in Figure 7.4.

There was no difference in pre or post treatment IgA anti-hsp65 titres between the two groups (see Table 7.9 and Figure 7.5 and 7.6). The correlations between IgA and IgG anti-hsp65 titres are shown in Table 7.10. Figure 7.7 illustrates pre-treatment IgG anti-hsp65 with pre-treatment IgA anti-hsp65 (r=0.26, p=0.029)

	Placebo	Treatment	Significance
Number	41	33	
Sex	16M 25F	14M 19F	ns
Age Mean (range)	41.7 (17-65)	43.4 (21-62)	0.55
Mean (SD) Pre-treatment Breath Test	184.6 (90.9)	179.8(97.5)	0.83
Mean (SD) Post-treatment Breath Test	177.6 (92.4)	5.9 (4.4)	<0.0001

Table 7.1 Description of groups in *H.pylori* eradication study

Atherosclerosis score	Correlation with anti-*H.pylori*	Correlation with IgG anti-hsp65 (before adjustment)	Correlation with IgG anti-hsp65 (after adjustment)
severity score	0.18 (p=0.040)	0.21 (p=0.018)	0.17 (p=0.08)
Vessel score	0.17 (p=0.036)	0.18 (p=0.036)	0.07 (p=ns)
Clinical vessel score	0.12 (p=0.150)	0.21 (p=0.012)	0.11 (p=ns)
diffuseness score	0.22 (p=0.011)	0.21 (p=0.016)	0.17 (p=0.08)
Anti-*H.pylori*	----------	0.39 ($p<10^{-5}$)	0.38 ($p<10^{-5}$)

Table 7.2 Spearman's rank correlations between severity and extent of coronary atherosclerosis and titres of antibodies to hsp65 and to *H.pylori* (before and after adjusting for confounders: age, social class and smoking consumption).

	Correlation with anti-*H.pylori* titre	
Factor	Correlation	P value
Age	0.139	0.107
BMI	-0.190	0.83
Social class	0.15	0.076
Packets of cigarettes	0.284	0.002*
Lipoprotein (a)	-0.038	0.68
Triglycerides	-0.038	0.68
Total Cholesterol	-0.055	0.53
Total/HDL cholesterol	-0.064	-0.47
Glucose (mmol/l)	0.075	0.932
CRP	0.12	0.199
Fibrinogen	0.196	0.031*

Table 7.3 Spearman's rank correlations between anti-*H.pylori* titres and CVS risk factors (* $p<0.05$).

	Before adjustment			After adjustment		
	H.pylori seronegative	*H.pylori* seropositive	P value	*H.pylori* seronegative	*H.pylori* seropositive	P value
severity score	32.3	43.3	0.049	39.1	39.7	0.79
diffuseness score	3.18	4.54	0.002	3.8	4.39	0.79

Table 7.4 Relationship between *H.pylori* seropositivity and coronary atherosclerosis scores, before and after adjustment for confounding influences (age, social class and smoking consumption).

	H.pylori seronegative	*H.pylori* seropositive
Age (years)	53.6	56.2
BMI (kg/m^2)	26.3	26.8
Cigarette consumption (pkts)*	7172	10534
Social Class (DEPCAT)*	3.55	4.11

Table 7.5 Relationship between *H.pylori* seropositivity and demographic data (*p=0.06).

	Before adjustment		After adjustment	
	H.pylori seronegative	*H.pylori* seropositive	*H.pylori* seronegative	*H.pylori* seropositive
Glucose (mmol/l)	5.75	5.76	5.71	5.77
Lipoprotein (a) (mg/dl)	22.39	21.87	22.91	21.88
Triglycerides (mmol/l)	1.58	1.68	1.62	1.66
Cholesterol (mmol/l)	5.75	5.55	5.64	5.63
LDL cholesterol (mmol/l)	3.82	3.58	3.66	3.68
HDL cholesterol (mmol/l)	0.87	0.91	0.91	0.89
Ratio	6.95	6.50		
Fibrinogen (mg/dl)	3.30	3.30	3.28	3.31
CRP (mg/l)	7.4	7.7	7.6	7.8
WBC ($x10^9$/l)*	6.52	7.35	7.08	7.14
Anti-hsp65 (AU/ml)**	18.75	30.27	25.12	26.30

Table 7.6 Relationship between *H.pylori* seropositivity and continuous CVS risk factors before and after adjustment for confounders (age, smoking and social class) (* p=0.007, ** p=0.0008).

	H.pylori seronegative	*H.pylori* seropositive	% seropositive	P value
Non- smoker	12	12	50.0	
Ex smoker	23	59	72.0	
smoker	6	22	78.6	p=0.06
No hypertension	30	62	67.4	
Hypertension	11	30	73.2	p=ns
No family history	19	45	70.3	
Family History	16	38	70.3	p=ns

Table 7.7 Relationship between *H.pylori* seropositivity and categorical CVS risk factors.

	Pre-hsp65 Mean (SEM)	Post-hsp65 Mean (SEM)	Difference between means	P value difference between means	pre/post therapy titre ratio
Placebo (n=41)	25.70 (4.09)	22.47 (3.89)	3.23	0.450	0.874
Treatment (n=33)	25.64 (5.09)	13.75 (2.47)	11.89	0.033	0.536

Table 7.8 IgG anti-hsp65 titres pre and post therapy (p= 0.38 for comparison of pre-therapy titres, p= 0.030 for comparison of post therapy titres and p= 0.026 for comparison of post/pre titre ratios)

	Pre IgA anti-hsp65 (AU/ml) Mean(SEM)	Post IgA anti-hsp65 (AU/ml) Mean(SEM)	P value
Placebo (n=38)	10.47 (1.3)	9.05 (0.9)	0.38
Treatment (n=32)	7.84 (0.78)	7.19 (0.59)	0.51

Table 7.9 IgA anti-hsp65 titres pre and post therapy.

	IgG Pre	IgA Pre	IgG Ratio	IgA Ratio
IgG Pre		0.26*	-0.34**	0.017
IgA Pre	0.26*		0.13	-0.32***
IgG Ratio	-0.34**	-0.13		0.08
IgA Ratio	0.017	-0.32***	0.08	

Table 7.10 Spearmen's Rank Correlation coefficients for IgG and IgA anti-hsp65 titres pre therapy and ratio of post/pre therapy.(* p=0.029, **p=0.004, *** p=0.008, all other correlations are not significant).

Figure 7.1 Plot of anti-hsp65 against IgG anti-H.pylori

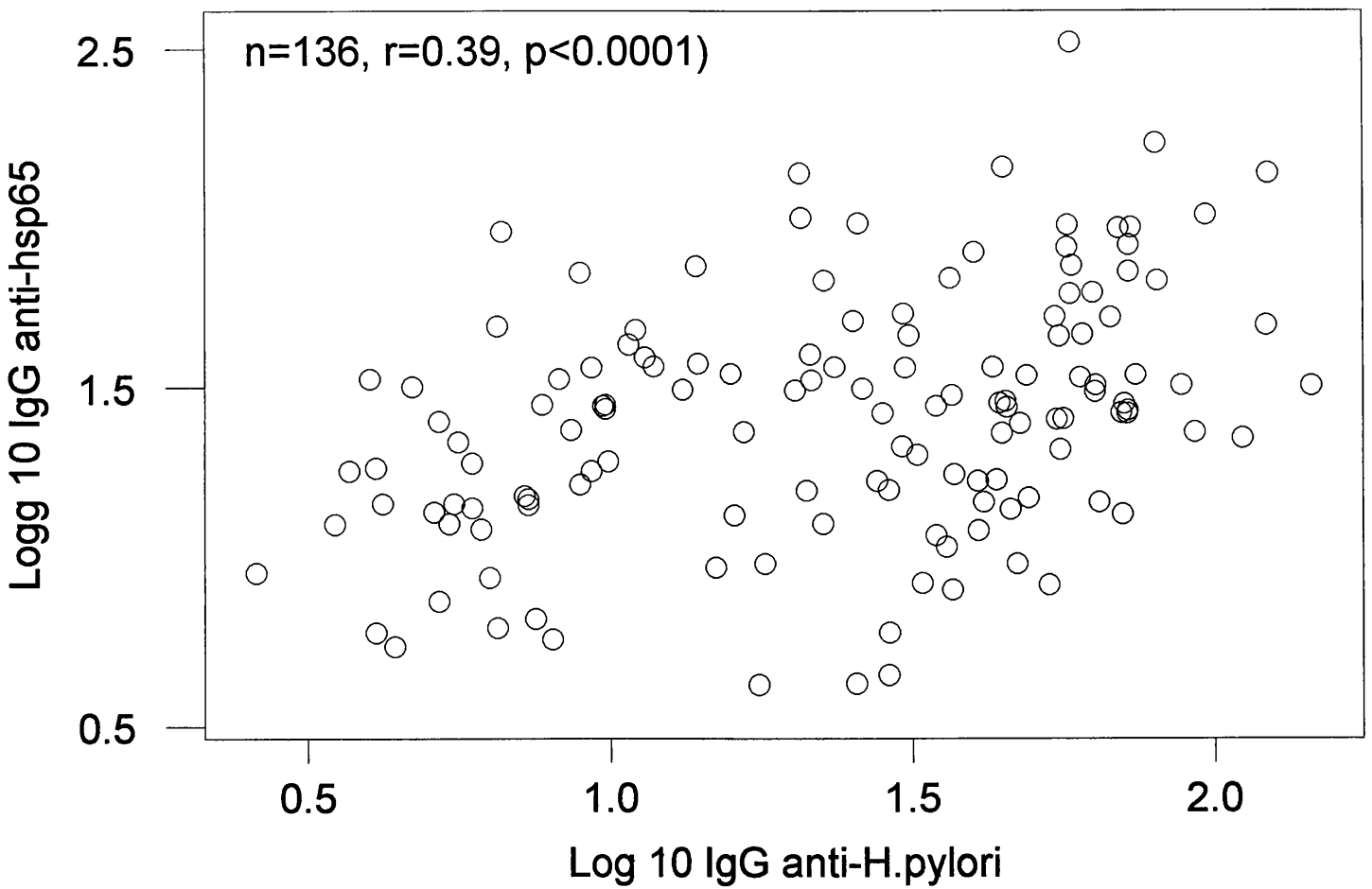


Figure 7.2 H.pylori seropositivity by social class (DEPCAT)

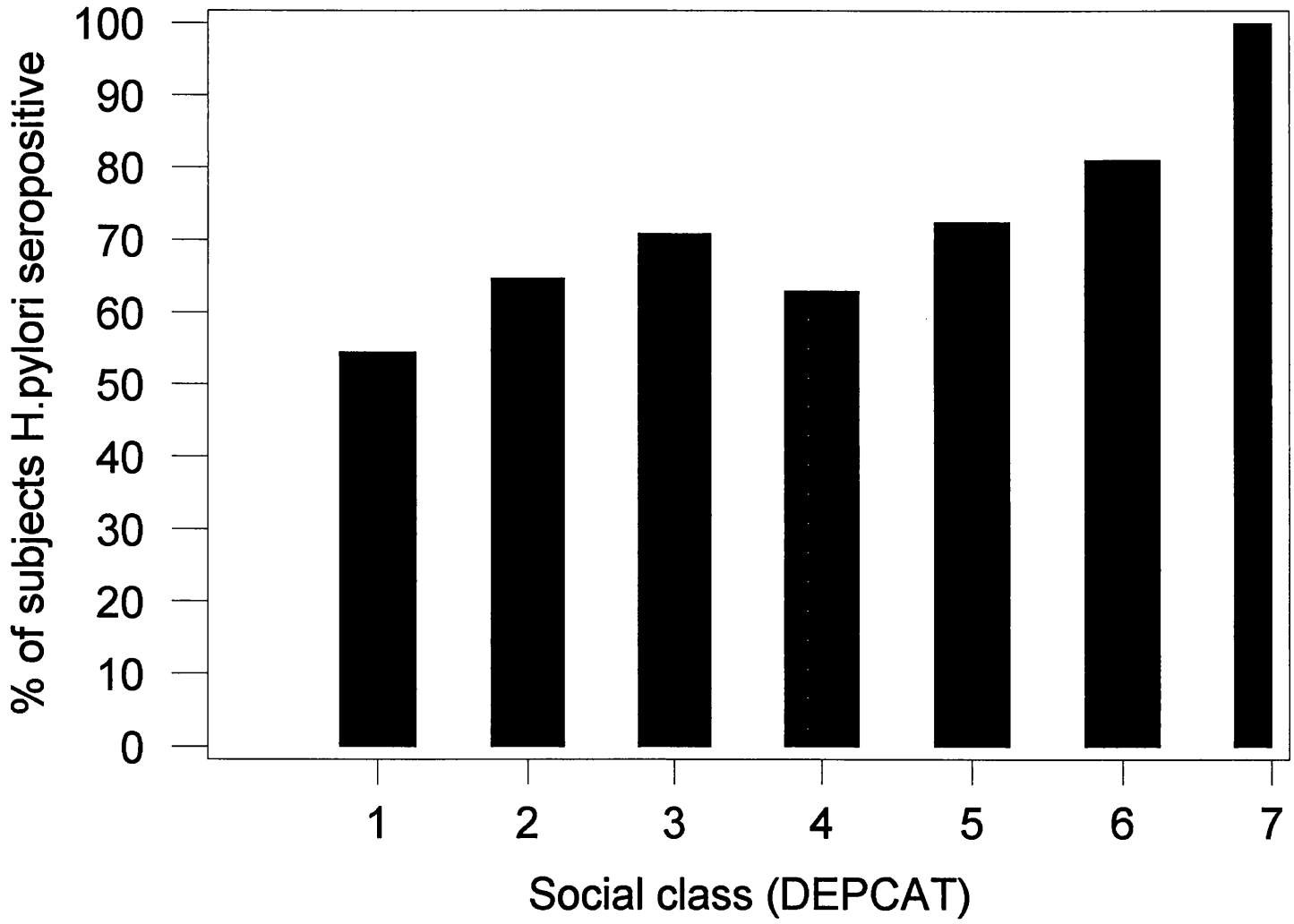


Figure 7.3 pre and post placebo IgG anti-hsp65 (n=41)

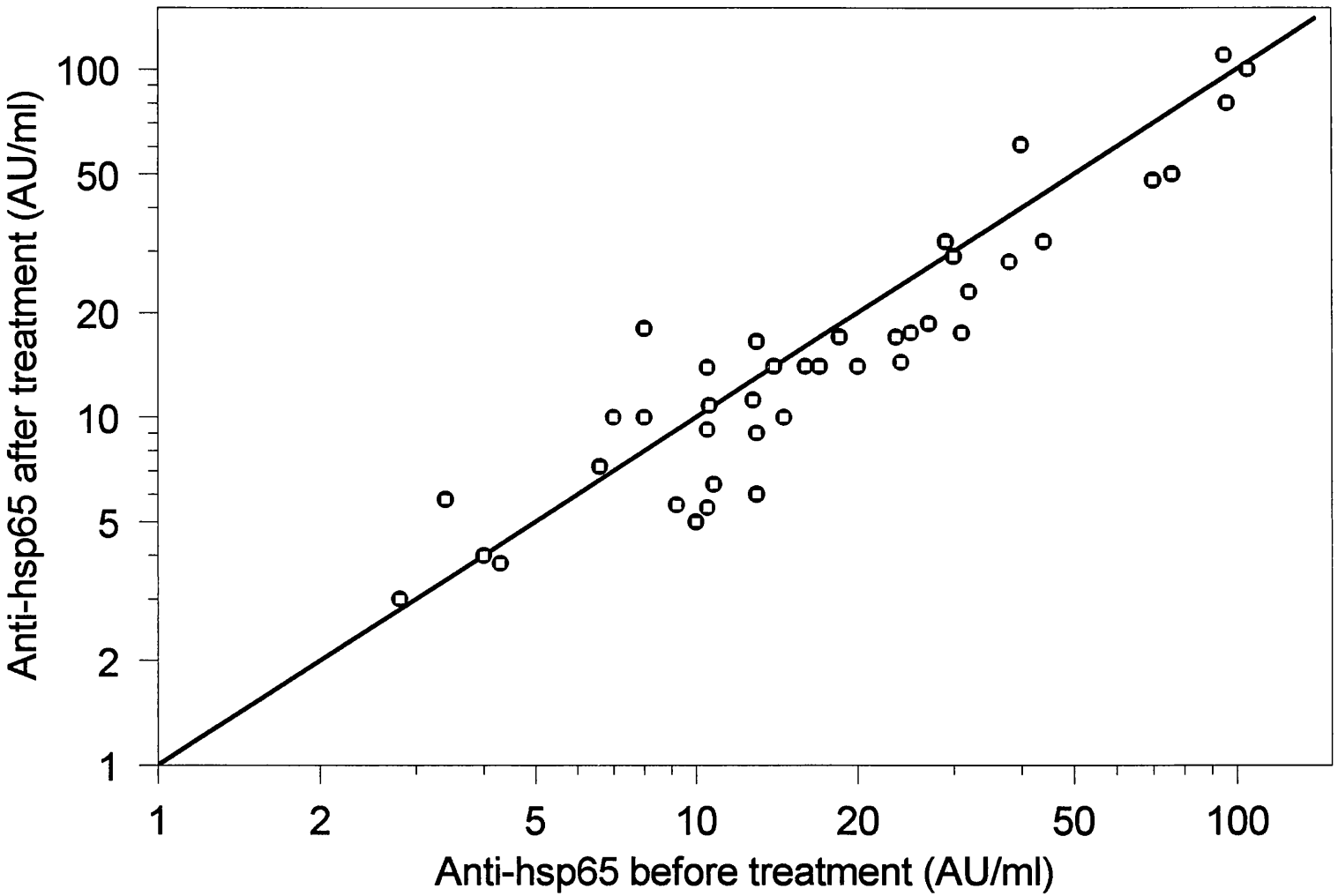


Figure 7.4 pre and post H. pylori eradication IgG anti-hsp65 (n=33)

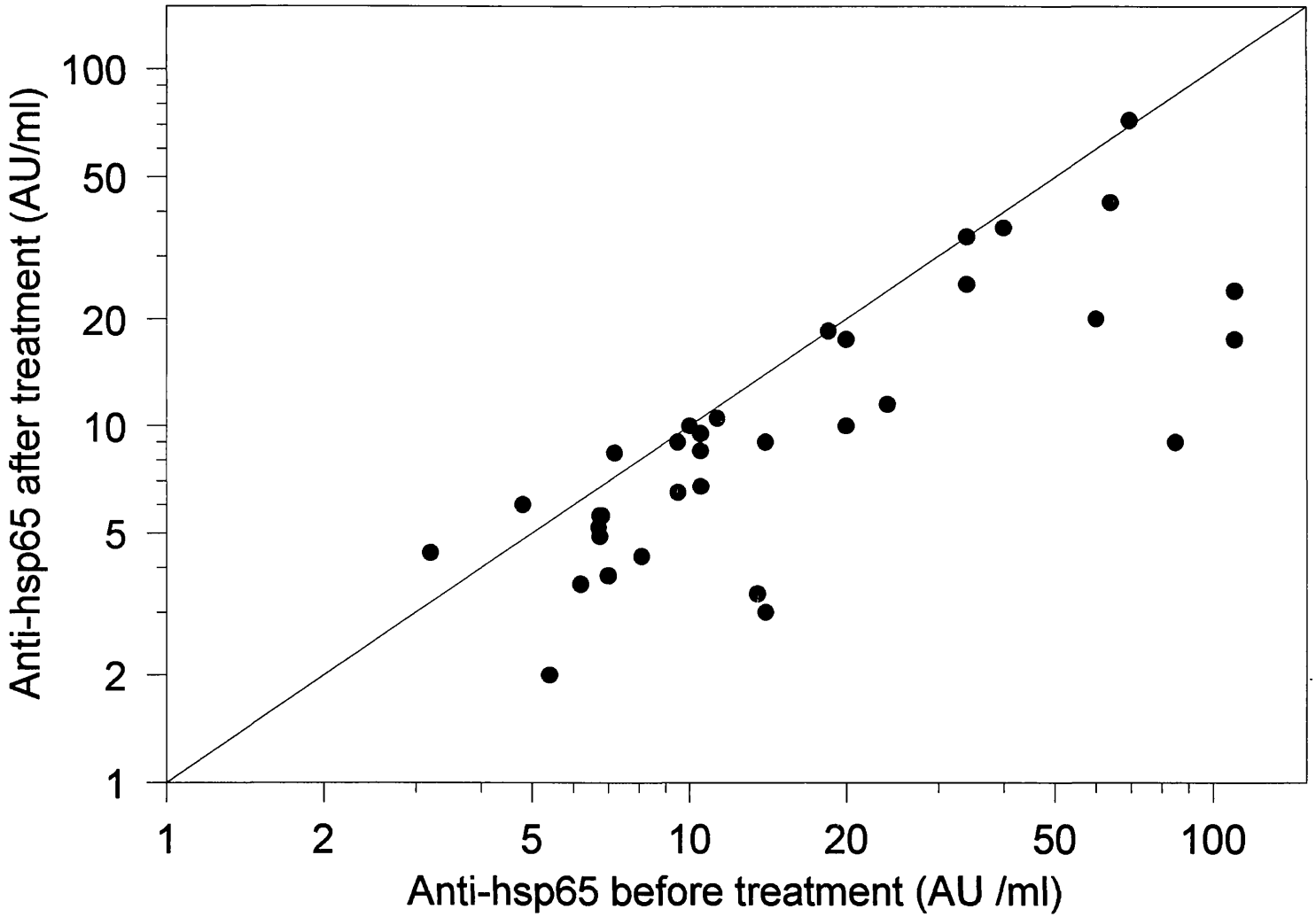


Figure 7.5 Pre and post placebo IgA anti-hsp65 (n=38)

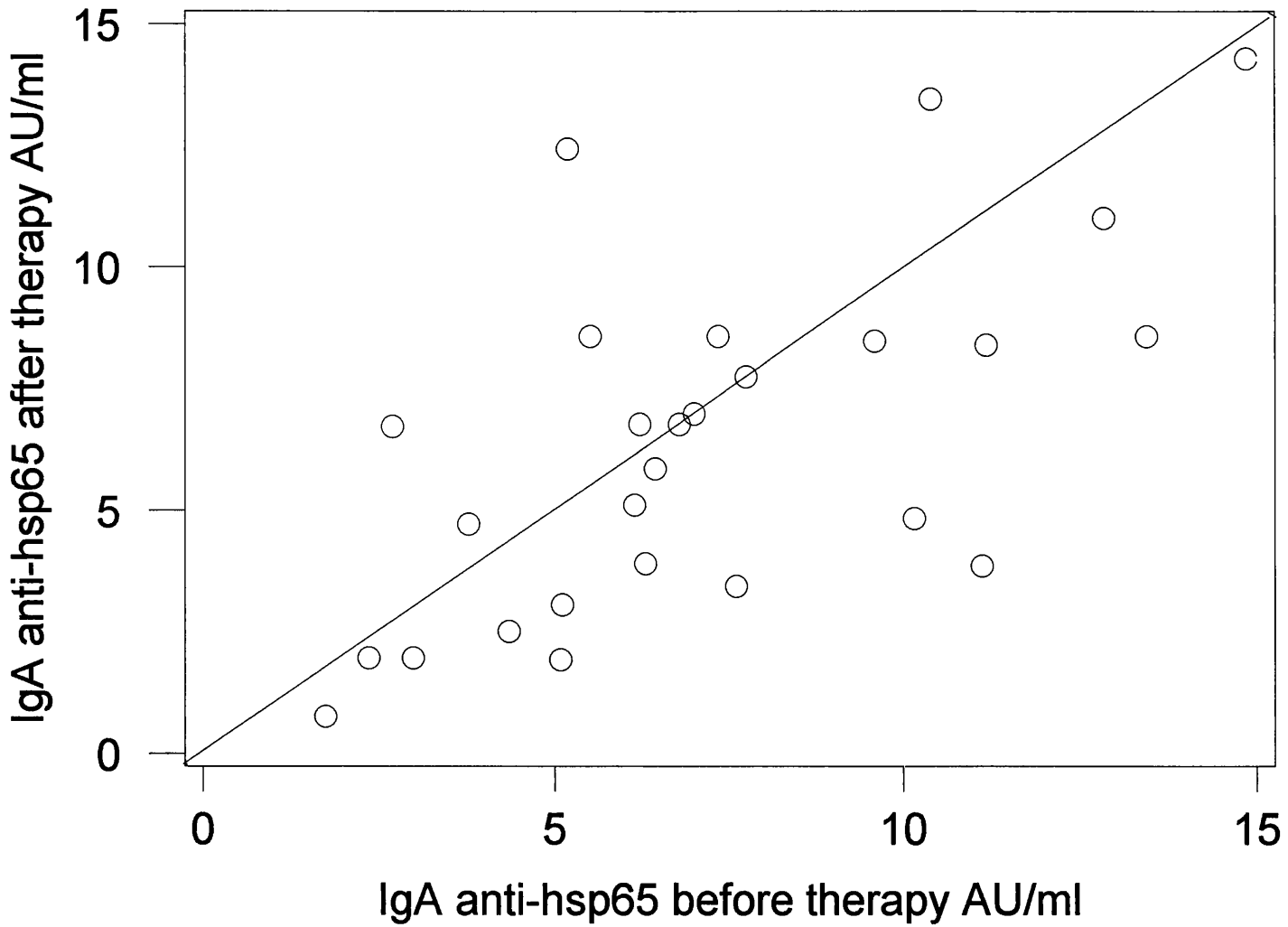


Figure 7.6 Pre and post H.pylori eradication IgA anti-hsp65 titres (n=32)

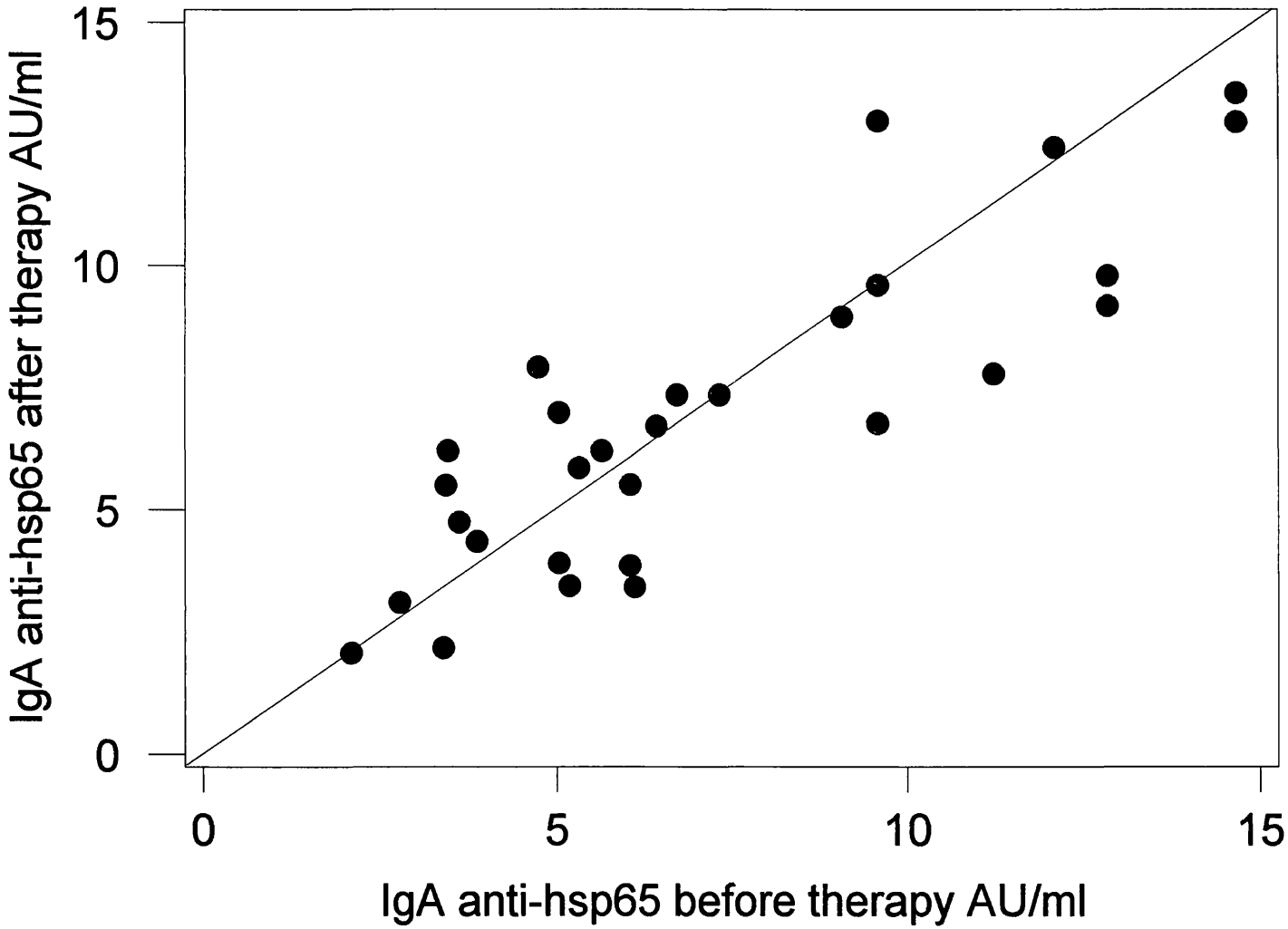
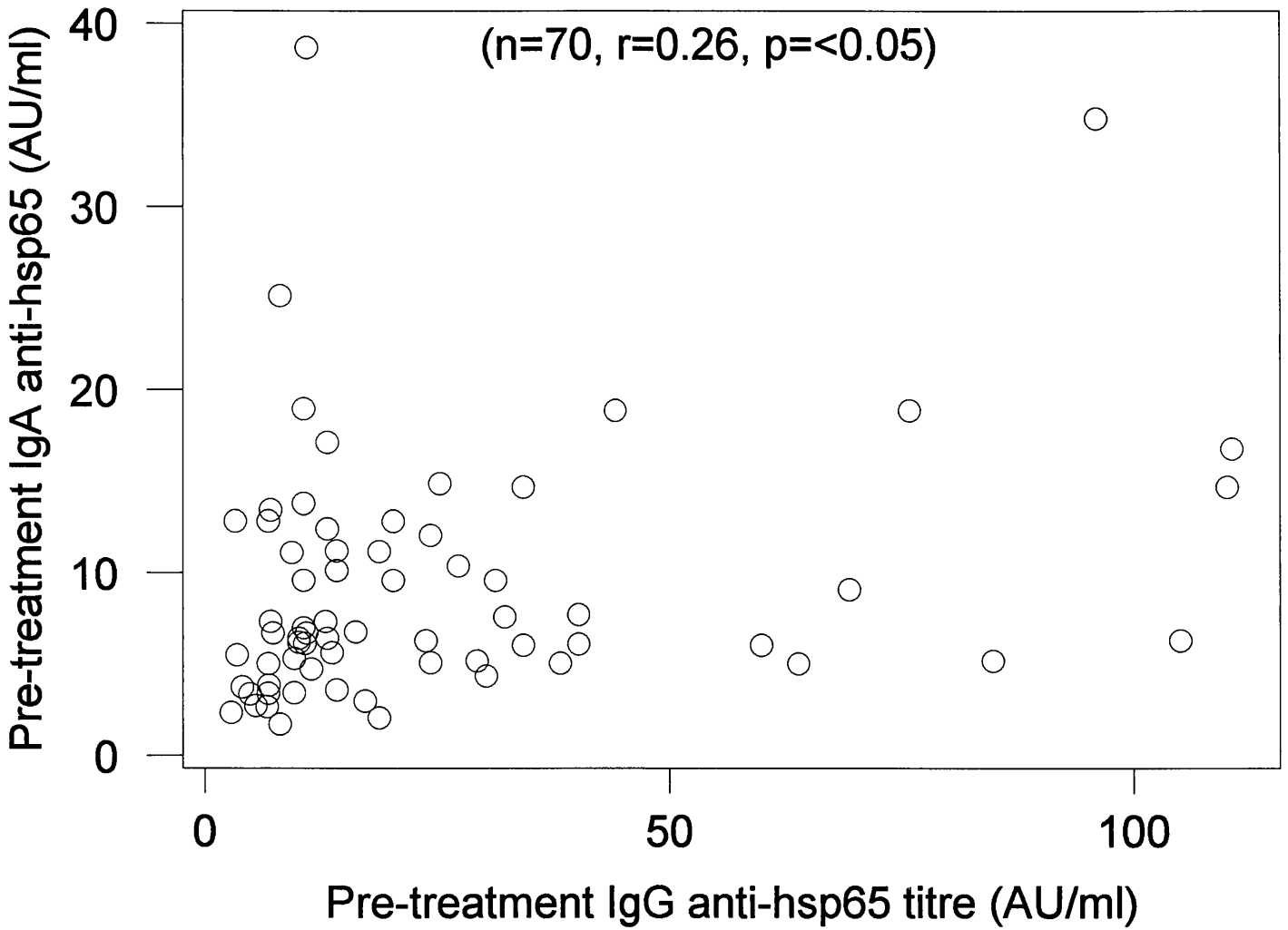


Figure 7.7 Pre-treatment IgA titres against pre-treatment IgG titres



7.5 DISCUSSION

Titres of antibodies to *H.pylori* were shown to correlate positively with the severity and extent of coronary atherosclerosis ($r=0.17$-0.22, $p<0.05$, Table 7.2). Similarly *H.pylori* seropositive subjects (IgG anti-*H.pylori* titre > 12 U/ml) had more diffuse and severe coronary atherosclerosis than seronegative (diffuseness score 4.54 versus 3.18 and severity score 43.3 versus 32.3, Table 7.4) This supports other recent studies linking *H.pylori* infection with clinically manifest coronary artery disease (73,74). In the present study and one previous study (75) the trends were still apparent but they were no longer statistically significant after correction for their common relationships with age, smoking and social class (see Tables 7.2 and 7.4).

Previously it has been postulated that the association between *H.pylori* infection and ischaemic heart disease is due to systemic effects of *H.pylori* infection on fibrinogen concentrations and total white blood cell count (74) but this has been challenged by a larger study (75). Our own data showed no difference in fibrinogen levels between the seropositive and seronegative groups (Table 7.6). However, the seropositive group clearly had elevated anti-hsp65 titres and white cell count compared with the seronegative group (prior to correction for age, smoking and social class) supporting the possibility of an autoimmune process.

Titres of antibodies to hsp65 and to *H.pylori* correlated highly significantly ($r=0.39$, $p<10^{-5}$, Figure 7.1). This relationship remained strong and highly significant even after adjustment for possible confounding influences

($r=0.38$, $p<10^{-5}$). This suggests that *H.pylori* infection might be an influence on anti-hsp65 titres and this was further investigated in the second study.

There is clear evidence that eradication of *H.pylori* infection is associated with a significant fall in anti-hsp65 titres. Mean anti-hsp65 titre fell from 25.64 AU/ml to 13.75 AU/ml (Figure 7.4) with successful eradication of *H. pylori* infection ($p=0.033$). There was a small fall in mean titre from 25.70 AU/ml to 22.47 AU/ml (Figure 7.3) in the placebo control group but this was not significant.

These results are in keeping with other data. *H. pylori* 62 kD heat shock protein (hsp62)(5) is surface expressed and a member of the hsp60/65 family (75% homologous to hsp65 and human hsp60 (167)). Sharma et al (159) demonstrated that this hsp62 antigen provokes an antibody response after *H.pylori* infection. Engstrand et al (161) showed that IgG anti-*H.pylori* hsp62 titres were elevated in 10 of 10 *H.pylori* culture positive individuals. After conventional *H.pylori* eradication treatment, recolonisation was shown by biopsy to have occurred in 8 of 10 people. In these 8, anti-*H.pylori* hsp62 titres stayed the same, whilst in the other two titres fell. This suggests that although hsp62 of *H.pylori* cross-reacts with hsp60/65 of other gut commensals, that it possesses some unique epitopes and is possibly an important influence on anti-hsp65 titres.

Interestingly the post/pre therapy IgG anti-hsp65 titre ratio in the treatment group (13.75/25.64 = 0.536 Table 7.6) is similar to the ratio of sero-negative to sero-positive IgG anti-hsp65 titres in the first study (18.75/30.27 = 0.62, Table

7.6). Indeed perhaps the post/pre ratio should be adjusted for the minor attrition in titre over the year of the study observed in the placebo group. Such an adjustment (treatment group post therapy titre/treatment group pre-therapy titre-difference between pre and post therapy titres in the placebo group) give a ratio of 0.614, even closer to the sero-negative/sero-positive ratio. Taken together these results suggest that about 40% of the anti-hsp65 titre in *H.pylori* infected individuals is due to the *H.pylori* infection.

This inter-individual anti-hsp65 response to *H.pylori* infection maybe due to either host or organism factors. Factors such as the infecting load of *H.pylori* either as a single initial infection or the number of repeated exposures, and the known variations in the immune response to *H.pylori* (159,160) will undoubtedly alter the final antigenic load. Organism factors might be inter-strain variation in either levels of hsp62 expression or variation in expression of epitopes that cross-react with hsp65. Therefore it may be only some strains of *H.pylori* that generate an antibody titre to hsp65. Presumably exposure to other micro-organisms with cross-reacting hsp60/65 will also contribute to the titres. Certainly some of our data suggest this including most importantly from the second study where 7/34 (20.6%) subjects who had successful eradication of *H.pylori*, anti-hsp65 titres stayed the same or rose. There is also supporting data from the first study including the fact that the correlation between antibodies to *H.pylori* and anti-hsp65 was no greater than 0.39 and in addition some subjects who were seronegative for *H. pylori* infection had significant titres of anti-hsp65.

Chlamydia pneumoniae infection has also been implicated in the pathogenesis of atherosclerosis by an unexplained mechanism (74,77-80,82-84). The relationship remained after adjustment for CHD risk factors including social class (74). *C pneumoniae* also expresses an hsp57 which shows close sequence homology with human hsp60 (76). Indeed another interpretation of our data is that the antibiotics given to eradicate *H.pylori* may have eradicated or decreased the load of other chronic bacterial infections and the reduction of these infections may be responsible to a greater or lesser extent for the fall in anti-hsp65 titre. In support of this is our data in from the group who received antibiotics but subsequent breath tests indicated persistent *H. pylori* infection. In this cohort (n=15) there was a trend for anti-hsp65 titre to fall (from 10.3 AU/ml to 9.31 Au/ml). It can at least be concluded that giving antibiotics leads to a fall in anti-hsp65 titre thus indicating that bacterial infections are an important influence on anti-hsp65 titre.

Thus, an alternative hypothesis to explain the association between *H.pylori* and ischaemic heart disease based on the results of this current study, data of Xu et al and the known immunology of *H.pylori* can be summarised as follows: endogenous hsp60 expression is induced on normal arterial intima by stresses such as smoking. Exposure to *H.pylori* and other micro-organisms induces an immune response to bacterial hsp60/62/65 and the antibodies produced cross-react with the human hsp60. This could either initiate or contribute to the local inflammatory and autoimmune process in the arterial intima, leading to initiation or worsening of

atherosclerotic lesions. Thus, it may be the relatively infecting load and extent of the immune response to infection that is important, rather than just infection per se and this may partly explain the contradictory results from studies (73-75) looking at seropositivity for *H.pylori* alone as a risk factor for ischaemic heart disease. Interestingly in this regard Kreuning et al (162) have recently shown a relationship between IgG and IgA antibody titres against *H.pylori* in serum and the severity of gastritis and density of *H.pylori* colonisation in asymptomatic subjects.

There was no significant change in IgA titres with eradication of anti-hsp65 titres (Table 7.6, Figures 7.5 and 7.6). There is no obvious explanation for this finding however two recent papers suggest that systemic IgA *H.pylori* antibodies are less accurate than systemic IgG at diagnosing and quantifying *H.pylori* infection and this may partly be due to the compartmentalisation of IgA into the mucosal associated lymphoid system and thus IgA inconsistently reaches the systemic circulation. In the first study systemic IgA was highly specific (94.4%) but less sensitive for detecting *H.pylori* infection (76.6%) (163). In comparison IgG had specificity and sensitivity of 98.6% and 97.1% respectively. Secondly in Kreuning's study above (162) serum IgG but not IgA *H.pylori* antibody titres correlated with the density of H.*pylori* colonisation of the gastric antrum. In our study there was a minor correlation between IgA and IgG anti-hsp65 titres before treatment ($r=0.26$, $p=0.029$ Figure 7.7). However there was no relationship between the isotypes in change in in titres with treatment (IgG post/pre ratio compared to IgA post/pre ratio, $r=0.08$, p=ns).

Thus it seems likely that *H.pylori* eradication does not influence systemic IgA anti-hsp65 titre because the infection inconsistently provokes circulating antibodies of this isotype either because it does not stimulate any IgA anti-hsp65 or these antibodies do not become systemic.

In summary therefore, titres of antibodies to *H.pylori* were shown to correlate positively with the severity and extent of coronary atherosclerosis ($r=0.17-0.22$, $p<0.05$, Table 7.2). Similarily *H.pylori* seropositive subjects (IgG anti-*H.pylori* titre > 12 U/ml) had more diffuse and severe coronary atherosclerosis than seronegative (diffuseness score 4.54 versus 3.18 and severity score 43.3 versus 32.3, Table 7.4).

Secondly titres of *H.pylori* antibodies correlated with cigarette consumption and subjects seropositive for *H.pylori* were older, smoked more cigarettes and were of lower social class confirming these three as the important confounders of a possible relationship with IHD. Trends for *H.pylori* infected subjects to have more severe coronary atherosclerosis were still apparent after correction for these factors but they were no longer statistically significant.

Thirdly *H.pylori* would appear to be an important influence on IgG anti-hsp65 as the antibody titres correlated ($r=0.38, p<10^{-5}$) and most importantly eradication of *H.pylori* lead to a significant fall in anti-hap65 titre (from 25.64 AU/ml to 13.75 AU/ml).

CHAPTER 8: CAROTID B-MODE ULTRASONOGRAPHY IN THE ASSESSMENT OF CORONARY ATHEROSCLEROSIS

8.1 HISTORICAL BACKGROUND

During the 1970's non-invasive ultrasonic methods evolved to provide 2D images of arterial walls and lumen. This permitted a more detailed study of early non-stenotic atherosclerotic changes in arterial walls before that which could be assessed with angiography (168). Experimental studies on non-human primates (169) and on human subjects (33,170,171), indicate that atherosclerotic lesions may progress without a reduction in the luminal size because of dilatation of the arterial wall. Therefore it was suggested that the correct estimation of the size of atherosclerotic lesions requires simultaneous measurements of arterial wall thickness and residual luminal size (172).

In 1981, a multicentre validation study was initiated to compare B-Mode ultrasound images with angiographic pictures of the carotid system and to compare both methods with pathology. Subsequently Ricotta et al (173) in 1988 reported from this study the first validation that carotid artery lesion width could be measured by B-Mode scanning. They showed that the ultrasound assessment of plaque lesion width correlated better with pathologic measurement of end arterectomy specimens than with the angiographic assessment. They suggested that B Mode ultrasonography

held considerable promise for population based studies of atherosclerosis (177).

Pignoli et al (174) in 1984 performed the first *in vitro* validation study of B-Mode ultrasonography for the direct measurement of arterial wall thickness. They initially studied a limited number of normal or moderately diseased arterial segments. A significant correlation between results of gross pathological evaluations and measurement by B-Mode imaging of arterial wall thickness was found.

In a more detailed second study in 1986 (172) (which has subsequently proven to be a landmark paper) they had three main objectives. Firstly they aimed to determine the anatomic structures involved in ultrasound energy reflection in the arterial wall. They examined 10 aortic specimens in dissection experiments. The specimens were then interrogated by ultrasound and evaluated by microscopy. The findings on beamed scan images were compared with the structural changes on microscopy induced by various dissections in the arterial wall. They found in almost all specimens, a similar "double line pattern" separated by an hypo-echoic or anechoic space The luminal line was more regular and smooth and thinner than the outer one. A similar pattern was found in carotid artery specimens (Figure 8.1).

To delineate the origin of the luminal line on the B-Mode scan, a small incision was made in the luminal surface of the artery. Histology showed that the incision penetrated intima but not the media. B-Mode scanning revealed that there was loss of the luminal line at the site of the incision, indicating

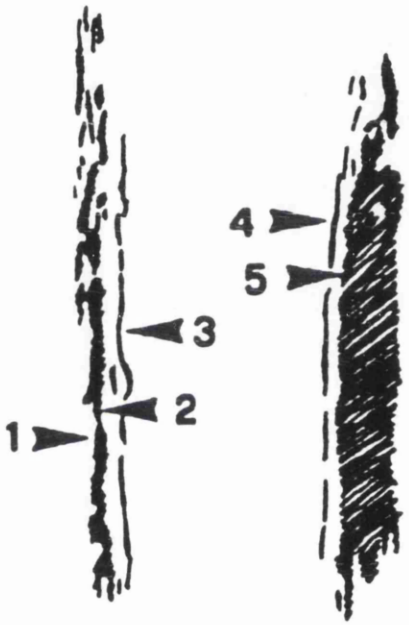


Figure 8.1 B-mode ultrasonographic interfaces of the Common Carotid artery.

1. Periadventitial - adventitia near wall interface
2. Adventitial - media near wall interface
3. Intimal - lumen near wall interface
4. Lumen - intima far wall interface
5. Medial - adventitial far wall interface

that the inner line is generated by the intimal-luminal interface (lines 3 and 4 Figure 8.1). Adventitia was then removed from the media (confirmed by histology) and B-Mode images of these specimens showed disappearance of the outer line in the region without adventitia, indicating that this line was generated by the media-adventitia interface (lines 2 and 5 Figure 8.1).

They then showed B-Mode measures of presumed combined Intimal-Medial Thickness (IMT) correlated with IMT values obtained by histology and gross-pathology. The same B-Mode measures correlated less well with the values for the other components of the vessel wall alone or in combination.

Their second objective was to assess the accuracy of IMT measured on B-Mode images against gross pathology and histology. Quantitative ultrasound was performed in 95 aortic specimens. The specimens were placed in a glass tank filled with water and then scanned. The absolute differences between B-Mode and gross pathological findings was 0.19 ± 0.23 mm and the percentage difference was $11.5 \pm 10.4\%$. Similarly, they scanned segments of carotid artery and showed no significant difference with findings in histology and pathology, but rather surprisingly did not show their data or quote statistics.

Thirdly, they showed the typical B-Mode double line image observed on *in vitro* specimens was also present in both common carotid arteries of 10 live subjects. In addition, they showed that the mean IMT in these subjects did not differ from that assessed *in vitro* by B-mode scan or histology or pathology.

More recently data from intravascular ultrasound experience has challenged the accuracy of quantitative transcutaneous vascular sonography (175-176). In response to this, Wong et al performed a further ultrasonic pathological validation study in 1993 (177). Segments from common carotid and femoral arteries were obtained during the autopsies of 36 male subjects. The segments were suspended on a frame and placed in an imaging tank filled with glycerin and water solution and images obtained using a 7 mH_z linear array probe. The ultrasound readings were then compared with histological measurement and they showed that tissue processing caused only 2.5% shrinkage. Ultrasonography consistently overestimated the thickness of the intima and adventitia and underestimated the thickness of the media. However, for combined IMT of the far wall, the differences between histology and imaging was insignificant, averaging 4% for carotid artery specimens and 9% for femoral artery specimens. In the near wall projection, however, sonographic IMT was consistently about 20% less than that determined histologically. They concluded that ultrasonography is limited mainly by axial resolution in quantifying the dimensions of individual arterial tunica, but is capable of accurately measuring far wall IMT (177).

8.2 METHODOLOGY

One of the problems in comparing results from various studies using B-Mode ultrasound are the different methodologies adopted, including different equipment, different scanning techniques and different reading protocols.

There is now a general move to try and standardise all these factors (72) but there remain a number of areas of contention.

8.2.1 Which Artery Or Arteries To Image And Measure.

Most studies to date have been based on carotid artery measurements, partly because femoral arteries are more difficult to image. In addition, in Wong et al's (177) histological validation study there was a larger variation between histology and sonographic IMT measurement in femoral specimens (9%) than carotid specimens (4%). However, a few studies have used both arteries (178-179). The present study (chapter 9) examined the carotid artery and thus the following discussion does not include femoral arteries.

8.2.2 The Near Wall/Far Wall Controversy.

The near wall/far wall controversy has been debated at length over the last 2-3 years (72). The question is whether it is possible to perform accurate near wall IMT measurements. Theoretically it is not because the anatomical location of a biological structure is always defined by a leading edge of an echo and the thickness of a structure is the difference between the leading edge of two different echoes. This is possible in the far wall, but not in the near wall. This consideration seems borne out by the findings of Wong et al (177) who showed sonographic near wall IMT could not be accurately measured in comparison with the histological measurement of the same IMT. The current consensus is that near and far wall measurements should be presented separately in scientific papers to allow the reader to make his/her

own interpretation, and that further research is required to resolve this issue (72).

Regardless of whether both are to be measured, it has been recommended that frames to be analysed should show both far wall and near wall IMT simultaneously, thus indicating that the ultrasound beam is positioned well into the longitudinal axis of the vessel (180).

8.2.3 Which Arterial Segments To Image And Measure.

There has also been considerable debate about how many points along the carotid artery the IMT should be measured, and whether both arteries should be assessed. The internal carotid artery (ICA) is most prone to plaque formation, with the carotid bifurcation (BIF) next while the common carotid artery (CCA) rarely has plaque involvement until late in life (181). Unfortunately the ease and reproducibility of imaging these segments is inversely proportional to their propensity to plaque formation. For example, in the large ongoing Atherosclerosis Risk In Community (ARIC) study (182) ICA-IMT could only be measured in about 40% of women and 58% of men. Thus, some workers suggested that ICA-IMT should not be routinely measured (72) but others continue to do so.

The three segments, however, all display varying IMT and this brings into question exactly what process we are assessing in measuring IMT. In normal arteries, the media is much thicker than the intima, but the atherosclerotic process usually involves just the intima. It is possible therefore that in assessing increased IMT in the absence of discrete plaque we are measuring

a diffuse thickening of the media (183). Therefore it is unclear whether wall thickening represents an early phase of atherosclerosis or a fundamentally different pathological process from atherosclerosis (36). However, in defence of the technique researchers have found that even in the lower end of the spectrum of IMT, e.g. CCA-IMT 0.6 mm to 0.9 mm, IMT correlated with CHD risk factors (180).

Most investigators now measure IMT at 12 points from each patient (reviewed in 36), consisting of near and far wall measurements at 3 sites in each of the carotid arteries. The boundaries are as defined in the large ARIC study (168) (see Figure 8.2). The extracranial carotid arteries are divided into three segments: the distal 1 cm straight portion of the common carotid artery, the carotid BIF (bulb) and the proximal 1 cm of the ICA. The distal 1 cm length of the CCA is defined by the BIF origin. The second reference, which defines the superior extent of the BIF and the proximal boundary of the ICA, is the tip of the flow divider.

The current evidence would seem to support measurements at multiple sites rather than just at a single site. For example, although IMT between the same site on different sides and between sites on the same side correlate, the correlation is not strong, and there are frequent discrepancies between sites showing the focal nature of atherosclerosis (184). In support of this Crose et al (185) showed that the mean aggregate IMT was the only independent variable associated with the status of CHD in a group of 270

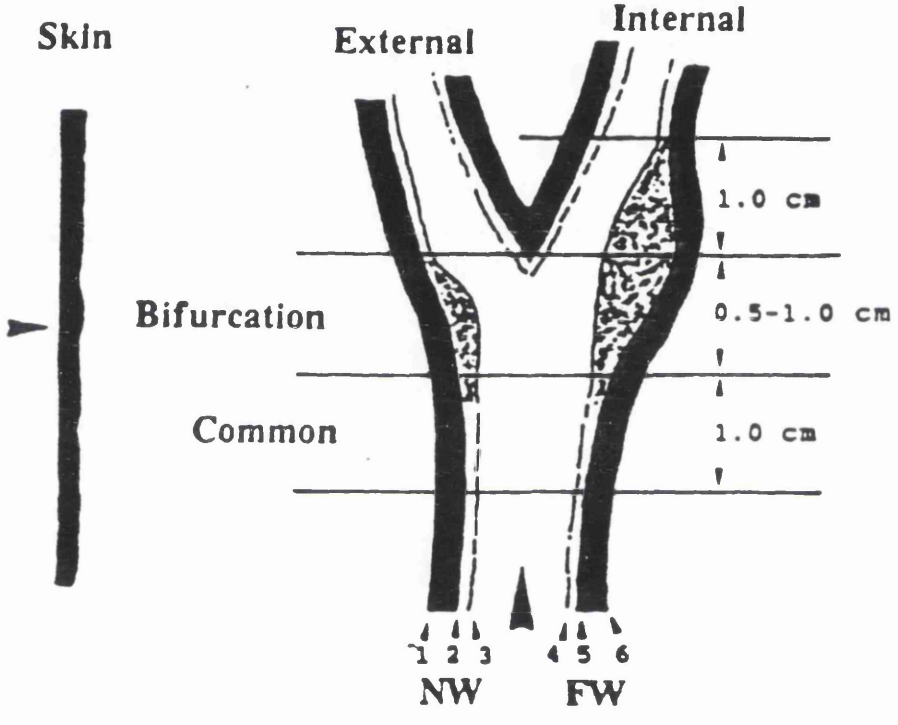


Figure 8.2 Divisions of the Carotid Artery (NW = Near Wall, FW = Far Wall).

patients (185). Their data also supported the use of mean common plus BIF IMT scores.

The ARIC investigators have also argued in support of using data from multiple sites because of the focal nature of the disease and predilection of disease for more distal segments, and in addition because of the greater clinical significance of more distal disease (184). They also raise the possibility that there may be interactive effects of risk factors at different anatomical sites in the carotid circulation (184).

Another approach is to use computerised edge detection to accurately measure IMT in only one arterial segment. For example, Adams et al (186) used an edge detection programme which measures IMT at 150-200 points along a 10 mm segment of left common carotid far wall to produce a mean IMT score for that segment.

Thus, it can be concluded that single maximum or mean maximum IMT from multiple segments or even a single mean from a single segment, may be informative in cross-sectional studies. In longitudinal studies, however, the greater stability associated with mean aggregate measurements is likely to improve analytic power (185). It should also be noted that there has never been any systematic difference in IMT between left and right carotid artery (180).

8.2.4 Which IMT Scoring System To Use.

A large number of different scoring systems have now been developed and what appears to be evolving is the use of different types of score for different situations. Even in studies using measurements from 12 segments there is discrepancy as exactly what is measured in each segment and how the overall score is calculated and basically there are two different approaches.

The first approach measure the IMT at a single point in each individual segment at the point of maximal IMT (185).The overall score is then either the mean of the 12 maximum IMT's (Mean aggregate) or the greatest of the 12 maximum IMT's (Maximum aggregate)(185). Similarily mean or maximum scores can be calculated for various combinations of arterial segments e.g ' Maximum BIF' or 'Mean far wall' etc.

The second approach measures the IMT in a given segment at 5-10 equally spaced points in the segment to produce a segment specific mean IMT (168).These means from any combination of up to 12 segments can then be used to produce mean and maximum scores. There clearly is a lot of potential for confusion between the two approaches. For example, a mean common carotid score by the first system is the mean of the four maximal IMTs (far and near, left and right). Whilst by the second system it means the mean of the four mean IMTs.

Crouse et al (187) have advocated two main scores, a severity measure which is the single largest IMT throughout the carotid bed and an extent score which is the mean of the 12 maximum IMTs but again this issue is largely unresolved.

8.2.5 Miscellaneous Measurement Issues.

The participants of a symposium to discuss IMT measurement issues have recently drawn a number of further conclusions (72). Firstly, that if plaque is localised where IMT measurements should be performed, the plaque thickness should be included in the IMT value. Secondly, that the available data do not allow for a valid decision as to whether adjustment for body size should be undertaken. The issue is a complex one as there are theoretical grounds to suggest that obesity has a causal relationship with atherosclerosis (72).

8.2.6 Methodological Conclusions

More work is required to resolve the above methodological issues. Until they are resolved it is imperative that investigators clearly state exactly what was measured and how and by whom and how the IMT score was calculated. In addition the differing methodologies should at all times be taken into account when comparing results between studies.

8.3 THE POTENTIAL OF CAROTID B-MODE ULTRASONOGRAPHY IN ASSESSING CORONARY ATHEROSCLEROSIS

The potential of carotid ultrasound measurements relies on certain assumptions, some of which are being tested in studies and others that are speculative. One assumption is that the test is sufficiently precise and reproducible. A second assumption is that the images provide a valid

assessment of coronary atherosclerosis. Finally, increased IMT should correlate with CHD morbidity and mortality to make it a useful surrogate endpoint.

8.3.1 Reproducibility

The reproducibility has been assessed in a number of large studies measuring different segments and combination of segments using differing methodologies and figures are quoted for intra and inter-sonographer variability and intra and inter-reader variability whether they are the same or different people performing the scans and reading them.

It is difficult in large studies to have the same person read all the images, however, having several readers introduces problems of reader variability. The difference in mean IMTs between readers can be significant. For example in a combined report (188) from the three atherosclerosis intervention studies, the maximum differences between readers in a pool of 12 was 0.11 mm, four times the annual progression rate (188). The problem can theoretically be overcome by adjustment for reader in the analysis. Another and more attractive solution is to have the same reader perform all measurements in each patient (188).

Figures for intra and inter-reader and intra and inter-sonographer reproducibility in four other studies are listed in Table 8.1. Correlations between repeat measures in all categories ranged from 0.73 to 0.93. In addition, in a single centre population base study using far wall common carotid artery measurements only, Salonen and Salonen (191) showed a

Study	What measured	No of patients	Sonographer		Reader	
			Intra	Inter	Intra	Inter
ACAPS (191)	mean of 12 maximum measurements	858	0.79	0.76	0.95	0.73
combined data from 3 studies (192)	mean of single CCA IMT	615	0.89	0.84		
ARIC (168)*	CCA BIF ICA	807 1066 755		0.74 0.81 0.92		

Figure 8.1 Sonographer and reader variability for measurement of carotid IMT. (*Reliability coefficients are quoted for the ARIC study. These are one minus the total variance attributable to intrasonographer plus reading variance. Pearson's Correlation Coefficients are quoted for the other studies.)

correlation of 0.91 between scans performed 1 week apart by the same sonographer and read by a different reader. It should also be noted that Riley et al in the ACAPS study (189) showed the variability was three to four times for single maximum IMT than a mean maximum of 12 segments, again arguing for a score based on a number of segments.

8.3.2 Validity

The evidence that B-Mode carotid ultrasonography is a valid tool for assessing coronary atherosclerosis is four fold.

(i) Autopsy Evidence

Autopsy evidence demonstrated that the extent of atherosclerosis in carotid vessels correlated with the extent in other beds in monkeys (192) and in humans (193-195). The correlation between carotid and coronary circulations is strongest (Pearson's correlation coefficient r=0.4 to 0.6) (193-195).

(ii) IMT Population Distribution

The population distribution of IMT corresponds with the known population distribution of clinical manifestations of atherosclerosis (182).

(iii) Correlations Between CHD Risk Factors And Carotid IMT

Established CHD risk factors such as age, systolic blood pressure, total cholesterol, HDL cholesterol (inverse), body mass index and smoking are consistently and positively associated with increasing IMT of the carotid artery(196-201) For example in the ARIC study for each 30% increase in LDL cholesterol there was a mean increase in IMT, independent of age, of 0.026

mm for males and 0.029 mm for females (200). Moreover, recent findings from population-based studies have indicated that candidate risk factors including lipoprotein (a) (202), haemostatic factors (including fibrinogen, beta-thromboglobulin, and factor vii) (203-206), fibrinolytic factors (207), antioxidants (beta-carotene, vitamin A) (208), dietary components of supposed atherogenicity (209-210), cardiovascular fitness (211) and angiotensin converting enzyme D/D genotype (212) are all associated with carotid IMT.

Even without the presence of discrete plaque, lower degrees of CCA-IMT may indicate the presence of atherosclerosis in other arteries in which atherosclerosis develops earlier in life. In support of this CCA-IMT ranging from 0.6 to 0.9 mm shows graded association with CHD risk factors and with ankle arm index (a measure of femoral atherosclerosis extent) and with prevalent CHD (180). Age is the most powerful determinant of IMT which increases by about 0.01 to 0.02 mm a year (191).

Finally, studies which reduced risk factors for CHD can influence the progression rates of carotid atherosclerosis as measured by B-Mode or doppler ultrasound (191,212,213).

(iv) Correlation Between Carotid IMT And Coronary Atherosclerosis

The presence of carotid artery plaque has been shown to be associated with coronary atherosclerosis as assessed non-invasively either by the surrogate of a positive exercise test (214) or by ultrafast CT scanning to detect coronary artery calcification (215). However, most of the evidence

showing correlations between extent of coronary and carotid atherosclerosis *in vivo* have used invasive measures, i.e. coronary angiography to assess coronary atherosclerosis and to date there has been 7 of these studies.

Three of these studies have come from the same group at the Bowman Gray Medical School in America. Firstly, in 1990 Craven et al (216) compared a B-Mode score (sum of 12 carotid artery segments) between 343 angiographically proven coronary atherosclerosis positive patients with 167 disease-free controls. In univariate analysis they showed a strong association between coronary status and extent of carotid atherosclerosis. Logistic models including CHD risk factors showed that the extent of carotid atherosclerosis was a strong statistically significant independent variable for the presence of coronary atherosclerosis in people aged greater than 50. The B-Mode score was at least as useful as known CHD risk factors for identifying patients with coronary atherosclerosis.

The second Bowman Gray paper was published in 1995 by Wofford et al (217). They measured extent of carotid atherosclerosis at 9 sites on both sides and summed the results to produce a score. They then divided their cohort of 434 patients into 4 quartiles based on the B-Mode score and showed a significant relationship between quartile and coronary atherosclerosis extent, as assessed by the number of diseased coronary arteries on a scale of 0-4.

The third Bowman Gray paper was published by Crouse et al (185) also in 1995. They tested various B-Mode scores derived from 12 carotid artery

segments for their ability to discriminate between the presence and absence of coronary atherosclerosis in 270 patients. They showed that scores based on mean BIF, mean common plus BIF and meaned aggregate were most strongly related to case control status; however, the predictive power of the mean CCA was also strong.

The fourth paper was produced by a group from London, UK and published in 1994 by Geurolakos et al (218) who measured far wall CCA-IMT on both sides and produced a mean score in 75 male patients undergoing coronary angiography. They showed a significant linear trend between mean IMT and number of diseased coronary vessels ($r=0.44$, $p<0.0001$).

Fifthly, in a sub-group of 57 patients from the CLAS Study at baseline (219) distal common carotid artery far wall IMT correlated with coronary artery average percentage stenosis ($r=0.27$, $p<0.05$) and a similar correlation ($r=0.29$) was also demonstrated after 2 years of intervention.

The sixth, and latest paper comparing extent of coronary atherosclerosis angiography against carotid IMT was published in October 1995 by Adams et al (186) from Sydney in Australia. They measured mean left CCA far wall IMT and compared this with three different coronary angiographic scores in 350 consecutive eligible patients. They used a summary score based on a number of vessels with luminal stenosis $\geq 50\%$; a modified Gensini score which gave a measure of both severity and extent of coronary atherosclerosis and lastly an extent score based on the percentage of affected coronary circulation, as identified by any luminal irregularity.

They showed that the left CCA far wall IMT was weakly, but significantly ($p<0.0001$) correlated with all three scores ($r=0.26$, $r=0.29$ and $r=0.23$) respectively. On the basis of these relatively weak correlations they sounded a cautionary note to all of the epidemiological and interventional studies that are using IMT measurements as a non-invasive marker of coronary atherosclerosis extent. They also demonstrated that CCA-IMT has insufficient predictive accuracy to be a useful clinical test for the exclusion of significant coronary atherosclerosis. They showed that no value of CCA-IMT had both a sensitivity and specificity of greater than 80%. However, it must be noted that they used only a single segment of CCA on only 1 side for their comparisons.

The correlations between a B-Mode score based on a number of segments and a detailed coronary atherosclerosis extent score has not been examined to date.

8.3.3 Carotid IMT As A Risk Factor For CHD Events

The third assumption underlying the use of IMT as a surrogate for coronary atherosclerosis, and in particular, as a surrogate for CHD morbidity and mortality, is that increased IMT should be shown to correlate with CHD morbidity and mortality. To date there has only been one prospective study of the prognostic significance of variation in IMT. Salonen & Salonen (220) reported findings in 1,257 Finnish men who had 36 AMIs over 2 years. They showed that any atherosclerotic lesion in the common carotid artery increased the risk of MI three fold (95% confidence intervals, 1.4-6.5).

Thus, in conclusion, although others are more sceptical (186), the recent consensus symposium (72) concluded that on the basis of the direct validation of the ultrasound measurements against autopsy and indirect validation versus known risk factors, ultrasound IMT is a valid estimate of the degree of early atherosclerosis in humans.

8.4 USE OF CAROID IMT AS A SURROGATE MARKER

The attraction of using IMT as a surrogate endpoint is clear. Power calculations have shown that assuming an IMT progression rate of 0.05 mm per year you would require only 672 patients in a two arm trial followed up for 2 years or 430 patients for 3 years to be 90% sure of detecting a significant difference between the treatments (65). Indeed, the Colestipol-Niacin therapy study (219) has shown statistically significant results with much smaller numbers. These sample sizes are in comparison for example with the recent placebo controlled multicentre 4S study (44) assessing the effects of simvastatin on CHD morbidity and mortality which enrolled 4,444 patients and followed them up over 5 years. To date greater than 40,000 patients have been or are involved in greater than 20 clinical trials and observational studies with carotid IMT as an endpoint (186).

8.5 CONCLUSIONS

B-Mode measurement of Carotid IMT has been shown to be valid and reproducible. Based on evidence from autopsy studies, correlations between B-Mode IMT and CHD risk factors and between B-Mode IMT and extent of coronary atherosclerosis, B-Mode IMT is widely regarded as the most accurate

surrogate estimate of the extent of general and coronary atherosclerosis (65). The use of a continuous variable (IMT) in epidemiological studies affords many practical advantages. Most importantly research can be performed with much smaller numbers of patients over shorter periods of time and consequently less expensively.

However a recent paper (186) has sounded a cautionary note in this regard and better validation of carotid B-Mode IMT against the extent of atherosclerosis in the coronary circulation and in other vascular beds are urgently required. Most importantly the validity of IMT as a surrogate marker of CHD mortality and morbidity requires more prospective studies.

Other goals for the future to strengthen the rationale for using B-Mode ultrasound in the assessment of the natural history of atherosclerosis include; resolution of the remaining technical issues; development of methods including complementary use of both angiographic measurements of lumen diameter and ultrasound assessment of arterial wall; evaluation of new methodology that may allow assessment of the anatomy of lesions and/or their potential for rupture. In this last regard, an exciting recent development has been the use of 3-D ultrasound imaging to measure plaque volume. Delcker et al (221) in a small pilot study of 54 patients showed that diastolic blood pressure and diabetes significantly affected the progression of plaque volume over 1 year.

CHAPTER 9: ASSESSMENT OF CAROTID ARTERY INTIMA - MEDIAL THICKNESS AS A SURROGATE MARKER OF THE SEVERITY AND EXTENT OF CORONARY ATHEROSCLEROSIS

9.1 INTRODUCTION

As discussed in Chapter 8, a large number of epidemiological and interventional studies are using serial carotid artery intimal-medial thickness (IMT) measurements as a surrogate marker for CHD morbidity and mortality. However, a number of questions about this approach remain to be answered, most importantly are serial carotid IMT's a valid surrogate? Other questions include which carotid artery IMT score should be used; Should the IMT score be adjusted for body size? The question of whether the size of a person influences IMT is a complex one and the associations of IMT with height, weight, BMI and BSA should be analysed(72); Also should the IMT score be adjusted for carotid artery lumen diameter as experimental evidence suggests that arteries dilate in response to early atherosclerosis (169-171)?

This present study aimed to produce data towards answering these questions and had four main objectives.

9.2 OBJECTIVES

Firstly, to examine the correlation between the extent and severity of coronary atherosclerosis and the extent and severity of carotid

atherosclerosis. Previous studies have compared a detailed analysis of the extent of carotid atherosclerosis with a very simple quantification of coronary atherosclerosis (185, 216-219) or vice versa (186). Nobody has examined the correlation between detailed analyses of the extent of atherosclerosis in both systems.

Secondly, to investigate which carotid artery IMT score is the best correlation of detailed coronary atherosclerosis severity and extent.

Thirdly, to investigate whether correction for body size or carotid artery diameter improved these correlations.

Fourthly, to compare risk factor profiles for carotid and coronary atherosclerosis.

9.3 MATERIALS AND METHODS

9.3.1 Subjects

Seventy consecutive, eligible white men (age mean 57.7 range 35.0-75.2) admitted for routine cardiac catheterisation for the assessment of chest pain. Exclusion criteria included previous history of coronary artery bypass surgery or PTCA and also history of Diabetes Mellitus (either previously diagnosed or a screening fasting glucose >7.8 mmol/L. We decided to recruit only men and to exclude diabetes as Adams et al (186) have recently shown that these are two out of the three (Age being the other) most important factors predicting more severe coronary atherosclerosis than average for a given level of carotid IMT.

In the majority of cases (65/70, 92.9%) cardiac catheterisation was indicated for the assessment of chest pain and the remainder were being investigated for valvular abnormalities. The subjects were recruited over an 8 month period and the population demographics are listed in Table 9.1. The study was approved by the Hospital Ethical Committee and all patients signed written informed consent forms. Patients completed a short health questionnaire regarding their personal medical history, family history and social history of smoking and alcohol consumption. Patients also underwent a physical examination. BSA was calculated from a validated nomogram (222). There was a minor overlap of this cohort (about 25 patients) with Chapter 6.

9.3.2 Sample Preparation

See Chapter 6.

9.3.3 Assays

Total cholesterol, beta quantification, fibrinogen, lipoprotein (a) and CRP were all assayed as in Chapter 6.

9.3.4 Coronary Angiography

This was performed and analysed as in Chapter 6, with the exception that an additional coronary atherosclerosis score was used, called the modified GENSINI Score. This was adopted to reproduce the methodology of Adams et al (186) and has previously been described and validated (223). This score gives an indication of both the severity and extent of atherosclerosis. The most severe stenosis in each of eight coronary segments

(the same segments used in the calculation of the diffuseness score see Figure 6.1) was graded from 1 to 4. 1, 1 to 49% lumen reduction, 2.5 50-90% stenosis, 3, 90-99% stenosis and 4, 100% occlusion to give a total score of between 0 and 32) The angiograms were again all analysed by a single observer blinded to all other results for the subjects. The distribution of the Severity, diffuseness and Gensini scores are shown in Table 10.2. Using the 'vessel score' there was 7, 2, 18 and 43 subjects with 0, 1, 2 and 3 diseased coronary arteries respectively. The same Figures for the 'clinical vessel score' are 11, 8, 25 and 26.

9.3.5 Carotid Ultrasonography Methodology

These were all performed by a single experienced ultrasonographer using a Biosound 2000 II (Biosound Inc., Indianapolis, USA) with an 8 MHZ transducer. Scans were recorded on super-VHS video tape for subsequent analysis.

The carotid artery is divided into three sections for scanning and analysis, the ICA (ICA), the BIF (BIF) and the CCA (CCA) as previously defined in the ARIC Study (see Chapter 8 and Figure 8.2 for details). The ultrasonographer places the cursor at the level of the delineating landmarks for the guidance of the reader. The scanning protocol was rigid and re-defined and similar to that used in the ARIC Study (168) and took about 20 minutes per patient.

The examination begins on the right side by placing the transducer on the skin in a manner that allows a longitudinal image of the mid portion of the right common carotid artery to be seen on the monitor. The transducer is then

moved cranially, keeping the common carotid artery in focus until the crest at the origin of the carotid bulb is demonstrated. The transducer is then rotated anteriorly and posteriorly until the sonographer has demonstrated and marked with the cursor the position of the crest of the BIF. Usually, this position is either lateral or posterior-lateral. The sonographer then focuses attention demonstrating as clearly as possible, the six interfaces required for measurement of arterial wall thicknesses within the right distal CCA (CCA). The sonographer then searches carefully for the thickest 2-3 and 4-5 sites (See Figure 9.1) present within this region by rotating the transducer anteriorly and then posteriorly. After the transducer has been moved as far posteriorly as possible, it is then rotated anteriorly until the crest at the origin of the bulb is most clearly demonstrated.

The BIF is then positioned within the central region of the monitor. The transducer is then rotated with the aim of demonstrating as clearly as possible the superior V shaped arc of the flow divider. Once this is accomplished the interfaces associated with the near and far arterial walls are optimally imaged. After locating that angle which best demonstrates the flow divider, the transducer is carefully and slowly moved anteriorly while keeping the BIF in focus. When the most anterior angle is reached the transducer is rotated, slowly and carefully as far posterior as possible, then back to that angle which best demonstrates the flow divider.

After the right BIF has been examined, the ICA is examined. The transducer is rotated anteriorly as far possible, keeping the ICA interfaces focused. The

transducer is then rotated posteriorly as far as possible. After this is accomplished the transducer is rotated back to the optimal position which shows the flow divider. After these images have been acquired and recorded on the right side, a similar examination is done on the left side of the neck.

9.3.6 Carotid B-mode Scan Analysis

The carotid artery scans were all read by a single observer blinded to the subject's other results. The reading protocol consisted of an initial viewing of the entire video recording for a given patient. As many as possible of the arterial segments were identified using the cursors and predefined landmarks, as above. However, it became clear that ICA's were not visualised in about 50% of subjects and similarly the near walls of most segments were not reproducibly seen. Thus, these segments were not measured. After the initial viewing, each segment was reviewed again and the end diastolic frame which best showed the maximal IMT in that segment was frozen and digitised by a Digital Time-Base corrector (FA-310 P,FOR-A Company Limited, Tokyo, Japan). The images were then analysed using a quantitative computerised analysis system (Cardiotrace Version 3.03, Cinegraphic Inc, Grand Prairie, Texas, U.S.A.). This system gives an axial resolution of 0.001 mm.

The frames were calibrated against a recorded reference marker of known size. The region containing maximum IMT was enlarged ten times and markers placed at leading edges of echoes from interfaces 4 and 5 (see Figure 9.1 - represents near wall IMT) and at the leading edges of interfaces

2 and 5 (represents carotid artery diameter). This process was repeated twice for each segment and the maximum IMT for that segment was the mean of the two. The process was then repeated for each segment in turn, (although the arterial diameter was only assessed at the level of the CCA on both sides).

9.3.7 IMT Scores

A maximum of four segments were measured for each patient (left and right far wall CCA and BIF and a number of IMT scores were computed. A mean score was the average of the maximum scores at the indicated sites, a maximum score was the greatest of all the maximum IMTs at the indicated sites. For example CCA_{MAX} is the greater of the two CCA measurements and TOT_{MEAN} is the average of the four (2 CCA and 2 BIF) measurements etc. The distribution of the IMT scores are shown in Table 9.2. If a measurement which should have contributed to the calculation of a given IMT score was missing then that IMT score was not calculated.

9.3.8 Statistical analysis

All of the continuous variables were approximately normally distributed with the exception of lipopoprotein(a) which had to be log-transformed. Correlations between continuous variables (coronary atherosclerosis scores, carotid artery measurements, carotid atherosclerosis scores and CHD risk factors), were examined using Pearson's Correlation analysis. Comparison between continuous and categorical variables was performed using student's T-Test (2 categories) and one-way analysis of variance (>2 categories). Multiple linear

regression analysis was used to test for residual relationships among interval variables while correcting for possible confounding influences.

9.4 RESULTS

Table 9.3 shows the correlations between the carotid artery IMT scores and the coronary artery scores. Generally the highest correlations were with CCA_{MEAN} scores (r=0.29 for severity illustrated in Figure 9.1 and r=0.26 for Gensini and r=0.20 for diffuseness). CCA_{MEAN} and BIF_{MEAN} IMT scores increased with successively more diseased coronary arteries (Figure 9.2 and 9.3) however as illustrated there was significant overlap between the groups. Figures 9.4 and 9.5 illustrate this overlap in two patients.

Table 9.4 demonstrates the lack of any correlation between the IMT scores and measures of body size (BMI and BSA). The same Tables show that there was a correlation between arterial size and IMT.

Tables 9.5, 9.6 and 9.7 indicate the correlations between carotid artery IMT scores adjusted for BMI, BSA and carotid artery diameter respectively and coronary angiography scores. Only the adjustment for BMI improved the correlation and at best the correlation between CCA_{MAX} adjusted for BMI and severity of coronary atherosclerosis was 0.32.

Table 9.8 lists the distribution of the continuous CHD risk factors. Tables 9.9 and 9.10 demonstrate univariate correlations between these continuous variables and carotid IMT scores and coronary angiography scores respectively. Table 9.11 repeats a selection of the data from Tables 9.9 and

9.10 to compare univariate correlations between risk factors and carotid IMT with correlations between risk factors and coronary angiography score (Gensini Score). There was no significant relationship between categorical risk factors (family history of IHD, reported history of hypertension and smoking habit) and either carotid IMT or any of the coronary angiography scores (data not shown). The only result which was nearly significant was smoking habit against Carotid IMT. For example the mean TOT_{MAX} score for the never smoked group was 1.41mm (0.36) in comparison to 1.80mm (0.72) for the current and ex-smoker group (p=0.066). Tables 9.12 and 9.13 are multiple linear regression analysis of risk factors for carotid and coronary atherosclerosis.

	Number	Mean	S.D
Age (years)	70	57.70	9.11
Weight (kg)	68	78.93	12.34
Height (cm)	68	173.90	5.82
BMI (kg/m^2)	68	26.08	3.76
BSA (m^2)	68	2.13	1.20

Table 9.1 Demographics of cohort (n=70).

	Number	Mean	S.D
CCA_{MEAN} (mm)	70	0.74	0.18
BIF_{MEAN} (mm)	58	1.48	0.58
TOT_{MEAN} (mm)	47	1.11	0.35
CCA_{MAX} (mm)	70	0.83	0.22
BIF_{MAX} (mm)	57	1.71	0.67
TOT_{MAX} (mm)	57	1.71	0.67
$CAD_{severity}$	67	45.16	31.1
$CAD_{diffuseness}$	67	4.81	2.73
$CAD_{Gensini}$	67	9.59	5.93

Table 9.2 Distribution of Carotid Artery and 3 coronary angiography scores (for the vessel and clinical vessel score's distributions see text).

	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness	CAD Gensini
CCA_{MEAN}	0.29*	0.21	0.28**	0.20	0.26***
BIF_{MEAN}	0.12	0.17	0.06	0.17	0.11
TOT_{MEAN}	0.11	0.18	0.17	0.15	0.13
CCA_{MAX}	0.27****	0.19	0.26****	0.18	0.25*****
BIF_{MAX}	0.12	0.15	0.10	0.12	0.07
TOT_{MAX}	0.12	0.15	0.10	0.12	0.07

Table 9.3 Pearson's Correlations between Carotid IMT scores and coronary angiography scores (*p=0.016, **p=0.019, ***p=0.037, ****p=0.029, *****p=0.045 all other correlations are not significant).

	CCA_{MEAN}	BIF_{MEAN}	TOT_{MEAN}	BMI	BSA
BMI	-0.14	-0.103	-0.16		
BSA	-0.15	0.19	0.01	0.61*	
CCA_{DIAM}	0.47*	0.32**	0.43***	0.18	0.23

Table 9.4 Pearson's Correlations between Carotid IMT scores and Carotid diameter, BSA and BSA (*$p<0.001$, **$p=0.002$, ***$p=0.014$, all other correlations are not significant).

	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness	CAD Gensini
CCA_{MEAN}	0.29*	0.22	0.24*	0.21	0.25*
BIF_{MEAN}	0.13	0.21	0.05	0.22	0.14
TOT_{MEAN}	0.15	0.24	0.15	0.25	0.20
CCA_{MAX}	0.26*	0.19	0.22	0.19	0.24
BIF_{MAX}	0.13	0.18	0.07	0.16	0.09
TOT_{MAX}	0.13	0.18	0.07	0.16	0.09

Table 9.5 Pearson's Correlations between Carotid IMT scores after adjustment for BSA and coronary angiography scores (*$p<0.05$, all other correlations are not significant).

	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness	CAD Gensini
CCA_{MEAN}	0.25*	0.26*	0.32**	0.19	0.21
BIF_{MEAN}	0.10	0.23	0.12	0.18	0.10
TOT_{MEAN}	0.12	0.27	0.25	0.21	0.16
CCA_{MAX}	0.23	0.24	0.31*	0.16	0.21
BIF_{MAX}	0.09	0.19	0.14	0.11	0.04
TOT_{MAX}	0.09	0.09	0.14	0.11	0.04

Table 9.6 Pearson's Correlations between Carotid IMT scores after adjustment for BMI and coronary angiography scores (*$p<0.05$, **$p<0.01$, all other correlations are not significant).

	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness	CAD Gensini
CCA_{MEAN}	0.16	0.21	0.22	0.10	0.11
BIF_{MEAN}	0.03	0.17	0.02	0.12	0.02
TOT_{MEAN}	0.13	0.21	0.22	0.20	0.16
CCA_{MAX}	0.15	0.18	0.20	0.09	0.13
BIF_{MAX}	0.03	0.16	0.06	0.08	-0.01
TOT_{MAX}	0.03	0.16	0.06	0.08	-0.01

Table 9.7 Pearson's Correlations between Carotid IMT scores after adjustment for CCA diameter and coronary angiography scores (all correlations are not significant).

	Number	mean	SD
Age (years)	70	57.70	9.11
Total cholesterol (mmol/l)	62	5.64	1.07
LDL cholesterol (mmol/l)	54	3.60	0.81
HDL cholesterol (mmol/l)	56	0.91	0.22
HDL/LDL ratio	54	6.58	1.44
Log Trigs	62	0.20	0.22
Log Lp(a)	52	1.42	0.43
Fibrinogen (g/dl)	53	331.5	89.6
Cigarettes (lifetime pkts)	61	9253	7947

Table 9.8 Distribution of continuous cardiovascular risk factors.

	CCA_{MEAN}	BIF_{MEAN}	TOT_{MEAN}	CCA_{MAX}	BIF_{MAX}	TOT_{MAX}
Age	0.43*	0.30**	0.36**	0.43*	0.32**	0.30**
Total Cholesterol	0.10	-0.03	-0.02	0.06	-0.09	-0.09
LDL Cholesterol	0.09	-0.09	0.03	0.06	-0.10	-0.10
HDL Cholesterol	0.25	0.24	0.36**	0.23	0.35**	0.36**
HDL/LDL	-0.20	-0.17	-0.31	-0.21	-0.28	-0.28
LogTrigs	-0.07	-0.11	-0.34	-0.09	-0.27	-0.26
LogLp(a)	-0.12	-0.18	-0.19	-0.15	-0.18	-0.18
Fibrinogen	0.02	-0.15	-0.11	-0.1	-0.07	-0.07
Cigarette (pkts)	0.20	0.21	0.27	0.21	0.30**	0.31**

Table 9.9 Pearson's Correlations between continuous cardiovascular risk factors and carotid IMT scores(*$p<0.001$, **$p<0.05$ all other correlations not significant).

	CAD Severity	Vessel score	Clinical Vessel	CAD Diffuseness	CAD Gensini
Age	0.42*	0.47*	0.45*	0.50*	0.41*
Total Cholesterol	0.22	0.23	0.12	0.18	0.25**
LDL Cholesterol	0.17	0.23	0.09	0.07	0.13
HDL Cholesterol	0.10	0.11	0.10	0.12	0.13
HDL/LDL	-0.07	0.08	-0.10	-0.03	-0.04
LogTrigs	0.08	0.04	0.01	0.17	0.20
LogLp(a)	-0.03	0.17	0.09	-0.08	-0.04
Fibrinogen	0.10	0.23	0.19	0.12	0.12
Cigarette (pkts)	0.07	0.10	0.13	0.10	0.08

Table 9.10 Pearson's Correlations between continuous cardiovascular risk factors and coronary angiography scores(*$p<0.001$, $p=0.06$, all other correlations not significant).

	CCA_{MAX}	BIF_{MAX}	TOT_{MAX}	CAD Gensini
Age	0.43*	0.32**	0.30**	0.41*
Total Cholesterol	0.06	-0.09	-0.09	0.25***
LDL Cholesterol	0.06	-0.10	-0.10	0.13
HDL Cholesterol	0.23	0.35	0.36	0.13
HDL/LDL	-0.21	-0.28	-0.28	-0.04
LogTrigs	-0.09	-0.27	-0.26	0.20
LogLp(a)	-0.15	-0.18	-0.18	-0.04
Fibrinogen	-0.1	-0.07	-0.07	0.12
Cigarette (pkts)	0.21	0.30**	0.31**	0.08

Table 9.11 Pearson's Correlations between continuous cardiovascular risk factors carotid IMT and coronary angiography scores(*$p<0.001$, $p<0.05$, $p=0.06$, all other correlations not significant).

Predictor	Coefficient	SD	t-ratio	p
Age (years)	0.29	0.08	3.48	0.001
Cholesterol	0.82	0.79	1.03	0.31
History of hypertension	-1.75	1.82	-0.96	0.34
triglycerides	3.40	4.22	0.81	0.42
smoking history	-0.67	2.01	-0.33	0.74
BMI	0.01	0.27	0.04	0.97
Fibrinogen	-0.01	0.01	-0.02	0.99

Figure 9.12 Best Multivariate model for prediction of GENSINI score (r-sq =29.2%).

Predictor	Coefficient	SD	t-ratio	p
Age (years)	0.01	0.01	3.42	0.001
Fibrinogen	-0.001	0.001	-0.98	0.34
History of hypertension	0.05	0.06	0.95	0.34
Cholesterol	0.02	0.02	0.81	0.42
BMI	-0.001	0.01	-0.8	0.43
triglycerides	-0.05	0.13	-0.34	0.73
smoking history	0.01	0.07	0.04	0.97

Figure 9.13 Best Multivariate model for prediction of CCA_{MEAN} IMT (r-sq = 26.9%)

Predictor	Coefficient	SD	t-ratio	p
Age (years)	0.028	0.01	2.83	0.008
Fibrinogen	-0.001	0.001	-2.81	0.008
smoking history	0.28	0.23	1.24	0.22
History of hypertension	-0.17	0.20	-0.87	0.39
triglycerides	-.39	0.46	-0.86	0.40
Cholesterol	-0.004	0.09	-0.41	0.69
BMI	-0.001	0.03	-0.03	0.98

Figure 9.14 Best Multivariate model for prediction of BIF_{MEAN} IMT (r-sq =28.5%).

Figure 9.1 Correlation between CAD severity and CCAmean IMT score

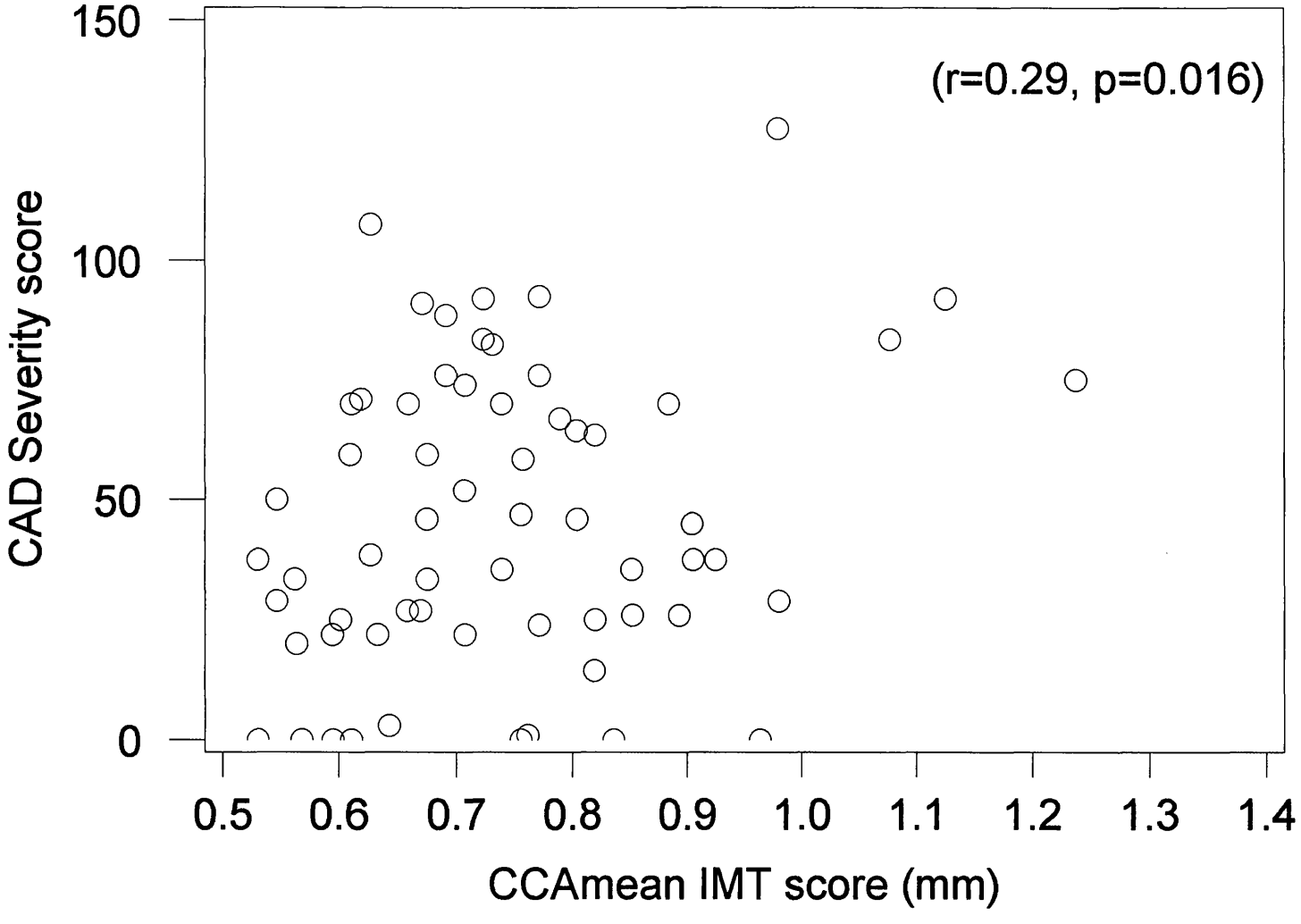


Figure 9.2 CCAmean and number of diseased coronary arteries

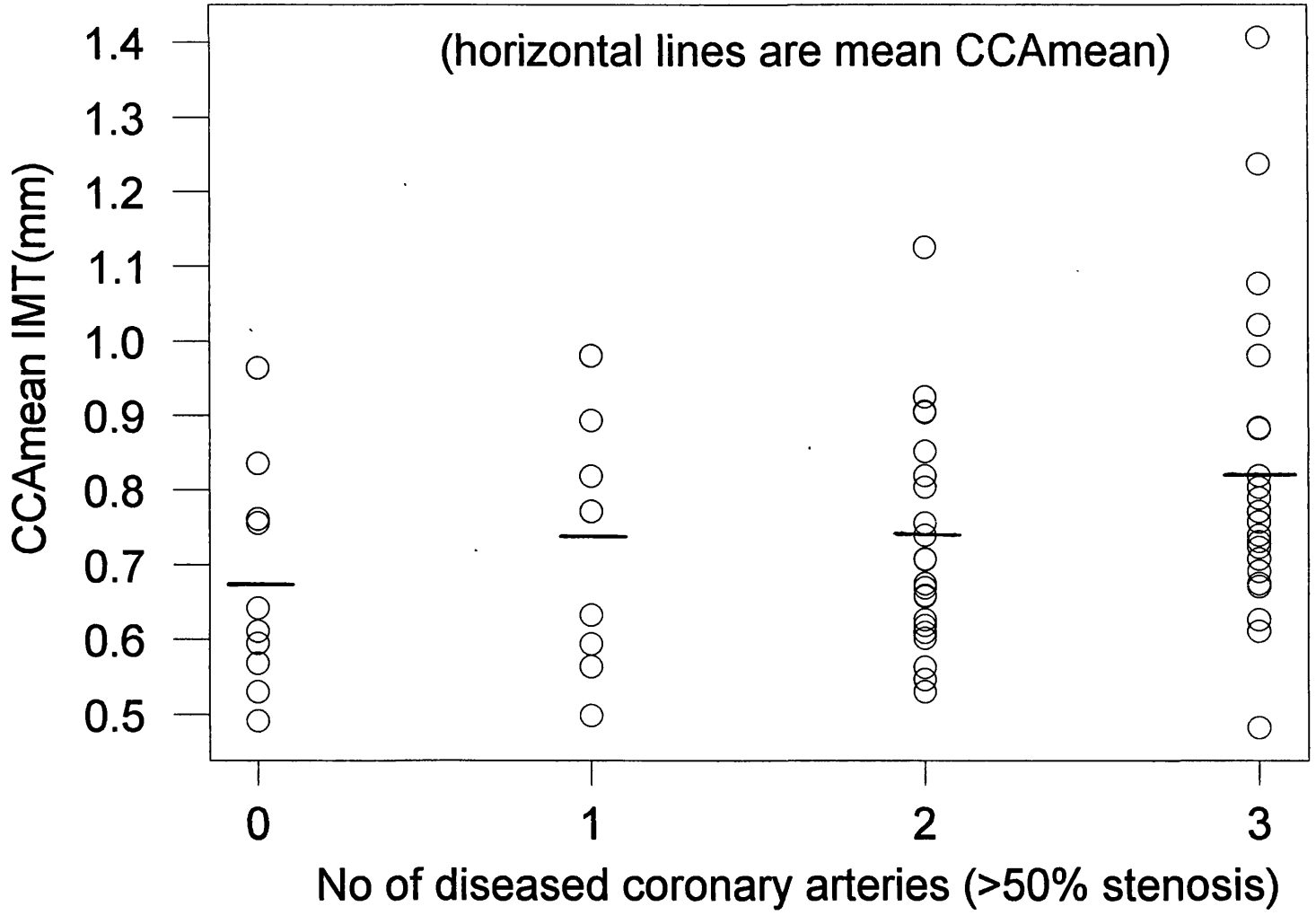
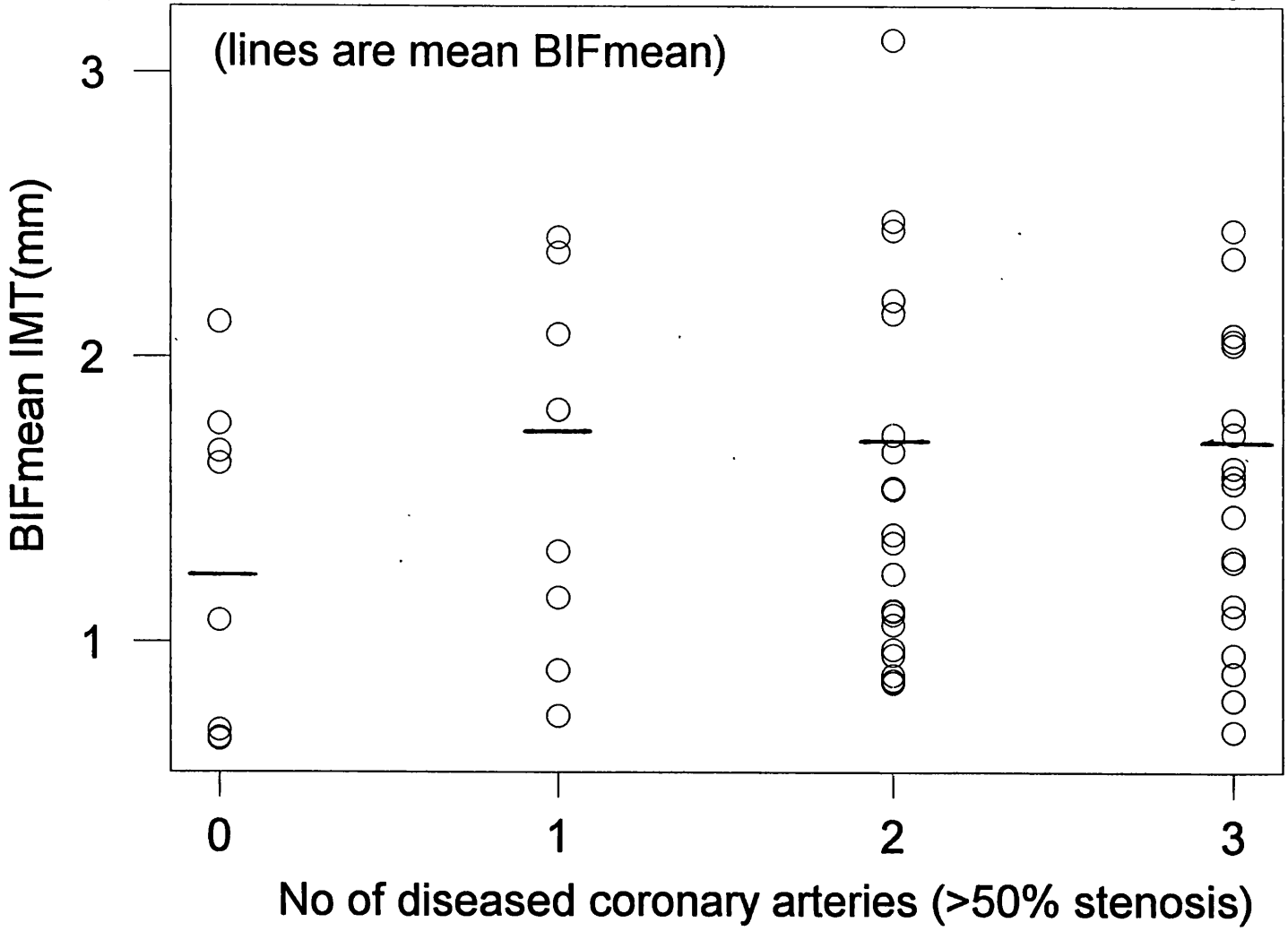


Figure 9.3 BIFmean and number of diseased coronary arteries



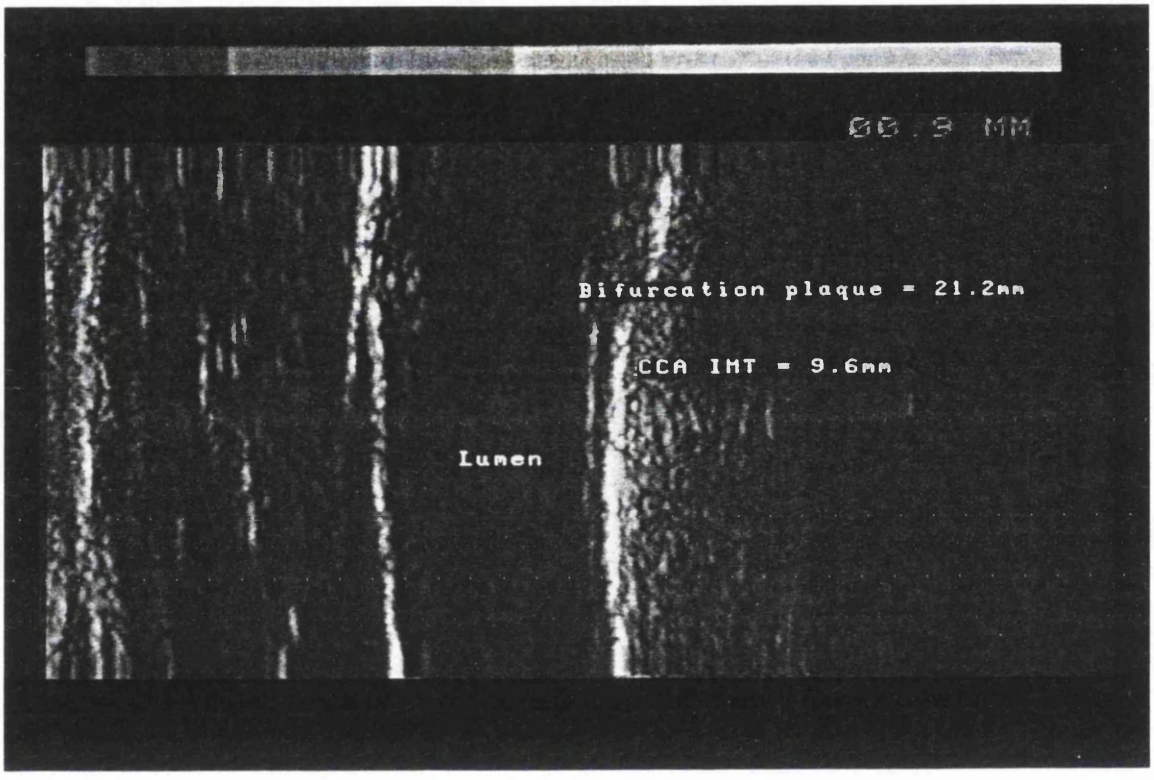


Figure 9.4 Carotid B-mode scan of 59 year old subject showing right far-wall Common Carotid intimal-medial thickening and bifurcation plaque. Patient had no angiographic evidence of coronary atherosclerosis. (note the annotation error, IMTs should be a factor of 10 less).

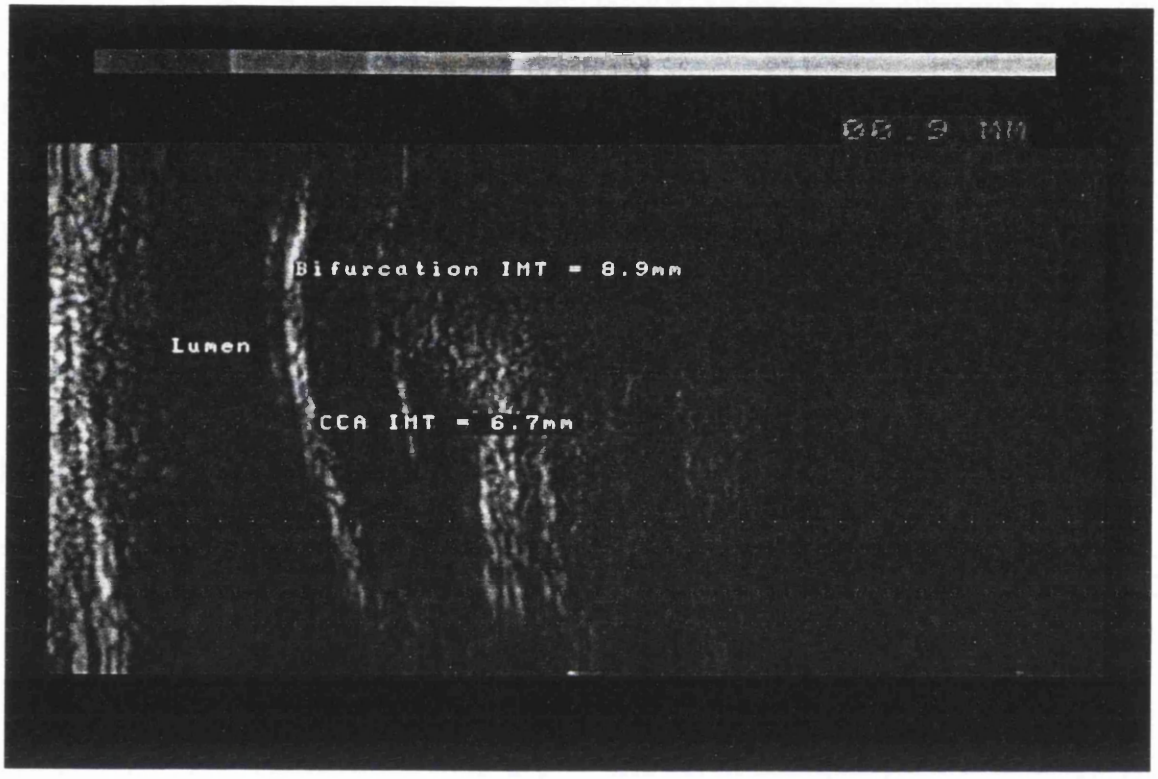


Figure 9.5 Carotid B-mode scan of 63 year old subject with no Carotid artery intimal-medial thickening, right far-wall common and bifurcation illustrated here. Patient had angiographic evidence of severe coronary atherosclerosis (Severity Score 91 compared to mean of 39.7). (note the annotation error, IMTs should be a factor of 10 less).

9.5 DISCUSSION

The correlations between CCA_{MEAN} and CCA_{MAX} IMT scores and the severity and diffuseness of coronary atherosclerosis are disappointingly low (for example, r=0.29 and 0.27 for severity, r=0.20 and r=0.18 for diffuseness and r=0.26 and 0.24 for the Gensini score and see Table 9.3 and Figures 9.1-9.5). These results are very similar to that previously reported in one large recent study (186) using similar coronary angiography scoring (r=0.26 for severity, r=0.23 for diffuseness and r=0.29 for Gensini) and one other smaller studies (219). Rather surprisingly, the addition of further carotid artery segments to the score (ie TOT_{MEAN} and TOT_{MAX} scores) led to a reduction in the correlations (see Table 9.3). Similarly the BIF scores by themselves (BIF_{MAX} and BIF_{MEAN}) correlated less well than the CCA scores.

There are a number of possible explanations as to why the correlations are not of greater magnitude and do not approach the strength of the correlations between carotid and coronary atherosclerosis found at autopsy, r=0.4-0.6 (193-195).

Firstly carotid IMT and coronary angiography scores may be measuring differing pathological processes or different stages or combinations of stages of the same process. Certainly plaque formation is rare in the CCA and the intimal cell layer extremely thin (0.02mm), presumably because it is a straight non-branching artery (186).Thus increased CCA-IMT is mostly due to diffuse thickening of the media. Conversely when coronary arteries develop atherosclerosis sufficiently severe to impinge on the lumen there is usually

significant eccentric intimal thickening. Thus we maybe comparing different processes or different stages of the same process. Plaque formation is more common at the BIF but using BIF IMT measurements is comparing the combination of medial thickening and intimal thickening (plaque) in the carotids with coronary intimal thickening (plaque) alone.

Secondly there are a number of potential problems and inaccuracies with the coronary angiography scores. There are significant inter-individual variation in coronary anatomy. The most common variation is between dominance by either RCA or Cx arteries. None of our scores or those of others (186,219) had any adjustment for this and potentially it could lead to significant inaccuracies. For example if a person had diffuse and severe disease of a tiny non-dominant RCA this would contribute a lot of points to overall scores although there really is very little atherosclerosis present and it is of limited clinical significance. Another problem is that of complete occlusions which again can lead to inaccuracies. Although it is usually stenoses <50% which rupture and occlude our scores and those of others rate an occlusion as the most severe lesion. Also there is the problem of how to deal with the 'missing' segments distal to the occlusion. Most systems give these segments the same score as normal segments.

Thirdly there is accumulating evidence of differential effects of risk factors on the two vascular beds. For example hypercholesterolaemia is more strongly associated with coronary disease than carotid atherosclerosis. Adams et al (186) showed that increasing age , male sex and presence of Diabetes were all

associated with a significantly ($p<0.01$) higher CAD score than average for any level of carotid IMT. In our study cholesterol correlated with CAD Gensini ($r=0.25$, $p=0.06$) score but not carotid IMT(Table 9.11). Conversely life-time smoking consumption correlated with carotid IMT but not coronary disease (see Table 9.11). We made an effort to control for some of the differential effects by including only males and non-diabetics and this may explain the slightly higher correlations in our study than in others.

The fourth problem is the theoretical one that carotid IMT may vary with body size or artery size whist coronary angiography scores by their nature do not. We specifically investigated this by in turn adjusting carotid IMT for two measures of body size, BSA (Table 9.5), BMI (Table 9.6) and for CCA diameter (Table 9.7). However only adjusting IMT for BMI lead to an improvement in the correlations with any of the scores (e.g the correlation between 'clinical vessel score' and CCA_{MEAN} was =0.28 before and r=0.32 after adjusting IMT score for BMI). This improvement might be because bigger people have bigger arteries and thus thicker IMT. Alternatively it could be because obesity is a more important risk factor for coronary atherosclerosis than carotid atherosclerosis although I know of no data to support this.

Fifthly there was selection bias as we studied a consecutive group of patients undergoing elective cardiac catheterisation and therefore our data may not be applicable to the general population. However in our cohort with no coronary stenosis >50% the CCA_{MEAN} IMT was 0.66 ± 0.15 mm (n=11), the BIF_{MEAN} IMT was 1.28 ± 0.58 mm (n=8) and the mean age was 50 ± 9 years. The CCA_{MEAN}

IMT value is similar but the BIF_{MEAN} IMT is greater than that from the large ARIC database (180), (mean CCA-IMT and BIF IMT for healthy 50 year-old white men were 0.65 mm and 0.90mm respectively). It is likely therefore that certainly with reference to the CCA data, our patients without important CAD are similar to age-matched unselected subjects.

Thus there are five broad reasons why the correlations between carotid and coronary atherosclerosis in this study and others (186,219) are not particularily strong. Does this data indicate that carotid IMT is of no use as a non-invasive assessment of coronary atherosclerosis? Certainly the large overlap between subjects with and without CAD (see Figures 9.2-9.5) precludes the use of carotid IMT measurement as an additional diagnostic modality for individuals or as a screening test for those being considered for coronary angiography.

Our data also casts doubt on the validity of the large number of epidemiological and interventional studies using IMT as a surrogate endpoint for CHD mortality and morbidity. The overwhelming need is for further prospective studies to answer this question. These prospective studies should examine the correlation between change in carotid IMT with change in coronary severity and extent score. Most importantly they should investigate whether carotid IMT is indeed a risk factor for cardiovascular events. There has only been one prospective study to date. Salonen & Salonen (191) reported findings in 1,257 Finnish men who had 36 AMIs over 2 years. They showed that any atherosclerotic lesion in the common carotid artery increased the risk of MI three fold (95% confidence intervals, 1.4-6.5).

This is the first study to compare IMT scores including data from the BIF with detailed coronary angiography severity and extent scores. Surprisingly the correlations with these scores were less good than with the CCA scores. Any of the five reasons detailed above may contribute to explain this, perhaps most notably our group with no important CAD did not have typical BIF IMT measurements.

Thus in summary we have demonstrated significant correlations between carotid IMT and the angiographic severity and extent of coronary atherosclerosis. However the correlations were quite modest and there are five possible explanations for this. Simple CCA-IMT scores were better predictors of the severity and extent of disease than more complex IMT scores. There was no convincing evidence to suggest that IMT should be adjusted for body size or carotid arterial size.

CHAPTER 10: ENDOGENOUS FIBRINOLYTIC FUNCTION AND CORONARY ATHEROSCLEROSIS : A REVIEW

10.1. INTRODUCTION

The endogenous fibrinolytic system is a cascade of reactions each catalysed by a Serine Protease. The crucial event in the fibrinolytic cascade is the conversion of plasminogen to plasmin catalysed by tissue plasminogen activator (t-PA) and less importantly urokinase (uKA). The major physiological regular of this reaction is plasminogen activator inhibitor 1 (PAI-1).

t-PA is a 70Kda glyco-protein, with a plasma concentrations of about 5 ng/mL. PAI-1 was first reported in 1954, but was only defined clearly in the early 1980's. PAI-1 is a 48kDa glycoprotein occurring at a low concentration in normal plasma (about 20 ng/ml) and at higher concentrations in platelets (224) and is widely distributed in tissues (225). The endothelium and liver are the likely sites of important plasma PAI-1 synthesis. It is clear that plasma PAI-1 does not arise simply by leakage from platelets. Platelet PAI-1 is present in much higher concentrations (up to 90% of the total blood pool), but with much lesser activity (3-6% of the possible activity). Overall, platelets account for about half the circulating PAI-1 activity (226).

Overall fibrinolytic activity can be measured by techniques such as dilute blood clot lysis or euglobulin lysis and in the last 10 years it has become possible to measure individual components. It is the balance of t-PA and PAI-1 that is most susceptible to fine regulation and therefore most likely to be affected in disease (226).

10.2 REGULATION OF ENDOGENOUS FIBRINOLYSIS

10.2.1 Insulin

Alessi et al (227) showed that insulin induces a dose-dependent increase of PAI-1 secretion by a hepatocyte cell line in culture. Insulin, pro-insulin and split pro-insulin also augment accumulation of functionally active PAI-1 in the media of porcine aortic endothelial cells (228).

Data from a subset (1500 patients) of the large European Concerted Action on Thrombosis (ECAT) study has shown that PAI-1 acidity levels is most closely related to insulin level and that relationships between PAI-1 and body mass, triglyceride level and blood pressure are secondary to this (229). Further, reduction of plasma insulin levels by physiological methods or pharmacological means is accompanied by a decrease in PAI-1 levels in humans (230,231). The evidence has led Juhan-Vague et al (232) to propose that insulin may be the major physiological regulator of PAI-1 activity in plasma and in turn the major regulator of endogenous fibrinolysis (232).

It has recently been proposed that elevated PAI-1 is a further component of syndrome X (233). Reaven in 1988 (234) proposed the term syndrome X for the cluster of a number of CHD risk factors: Insulin resistance, glucose

intolerance, hyperinsulinaemia, increased VLDL triglyceride, decreased HDL cholesterol and increased central (upper body obesity). The syndrome is alternatively known just as the insulin resistance syndrome.

10.2.2 Very LDL (VLDL) and Triglycerides

Stiko-Rahn et al (235) showed that VLDL stimulates PAI-1 secretion from endothelial cells in culture and that hypertriglycerideaemic VLDL is a more potent stimulus than normo-triglycerideaemic VLDL.

10.2.3 Oxidised LDL

The putative central role of oxidised LDL in the pathogenesis of atherosclerosis at a number of levels is discussed in Chapter 1. An additional possible pathogenic process is the influence of oxidised LDL on fibrinolysis. Latron et al (236) showed a dose-dependent increase in PAI-1 secretion from cultured hepatocytes, whereas inactive LDL had no affect.

10.3 RELATIONSHIP BETWEEN ENDOGENOUS FIBIRNOLYSIS AND CHD RISK FACTORS

10.3.1 Smoking

The acute and chronic effects of cigarette smoking on the fibrinolytic system appear to be contradictory. The acute effect is an increase in plasma t-PA. Conversely, endothelial cells seem to produce less t-PA in chronic smokers (237). Further contradictory evidence is provided by the ECAT study, (see Table 10.1) which found higher levels of t-PA and PAI-1 in smokers than non-smokers .

	Non-smokers	Ex-smokers	Smokers	P Value
PAI-1 Activity (U/ml)	13.0	14.1	14.1	<0.004
PAI-1 Antigen (ng/ml)	16.2	18.2	18.5	<0.0001
t-PA Antigen (ng/ml)	9.3	10.3	10.3	<0.0001

Table 10.1 Comparison of fibrinolytic factors (adjusted for age, sex and centre) according to smoking habit in the ECAT study (variable means are shown, P values for differences between smoking categories by ANOVA, reproduced from 238)

10.3.2 Hypertension

Most studies have been inconclusive due to small numbers, the use of non-specific assays of fibrinolytic components and the problems of the complex interactions pertaining to blood pressure, fibrinolysis and metabolic risk factors. In the ECAT study (238) there was a minor relationship between systolic blood pressure and PAI-1 and t-PA but this could largely be explained by BMI.

10.3.3 Obesity

Of various anthropometric variables, the waist to hip circumference ratio has been most strongly related with both t-PA antigen and PAI-1 antigen and activity levels (239,240). The ECAT study (46) reported convincing data for a strong association between fibrinolytic factors and data for BMI (see Table

10.2) Multiple linear regression analysis of a subset of this data has suggested that this relationship might be secondary to that between PAI-1 and insulin (229).

	Quintiles of BMI (kg/m^2)				
	<23.68	23.68-	25.25-	26.67-	28.40
PAI-1 Antigen (ng/ml)	13.9	16.4	17.5	19.0	21.3
PAI-1 Activity (U/ml)	9.9	13.0	13.7	14.9	18.1
t-PA Antigen (ng/ml)	8.9	9.5	10.1	10.7	10.9

Table 10.2 Mean fibrinolytic factor levels* according to quintiles of BMI (*adjusted for age, sex and centre. P value <0.0001 for all, from 238)

10.3.4 Hyperlipoproteinaemia

t-PA and PAI-1 levels do not correlate with cholesterol or Lipoprotein (a) but do quite strongly with triglycerides (241) and this has been confirmed in a number of studies (238). In addition, interventions to reduce triglyceride levels quickly leads to fall in fibrinolytic activity (242). In all studies subjects lost weight which in turn has been related to fibrinolytic function and, thus, it is not possible to deduce a direct causal association between triglycerides and fibrinolysis from this data alone.

However taken together with the in vitro evidence detailed above, all the evidence strongly suggests a causal association does exist.

10.3.5 Diabetes

Patients with type II diabetes have higher values of PAI-1 activity than normal subjects (243) and there is a significant correlation between the levels of plasma insulin and PAI-1 activity in type II diabetes (244).

10.4 RELATIONSHIP BETWEEN ENDOGENOUS FIBRINOLYTIC ACTIVITY AND CHD

10.4.1 Introduction

There is reasonable evidence for a causal relationship between depressed circulating fibrinolytic activity and venous thromboembolism, but evidence for such a relationship with arterial disease is less clear (226). This might partly be because we are measuring fibrinolysis in venous blood and then relating this to events in the arterial circulation. As previously noted, PAI-1 is the major physiological regulator of t-PA and thus most work has concentrated on measuring PAI-1 antigen or activity. Earlier work measured global fibrinolysis and some recent work has also looked at t-PA antigen and activity and t-PA/PAI-1 complex.

10.4.2 Case control studies

Hamsten et al (245) first noted elevated levels of PAI-1 in young survivors of AMI in 1985. Other studies have confirmed the presence of elevated PAI-1 in myocardial infarction or other presentations of CHD (241).

However, it is not possible for such studies to elucidate whether there is causal association or whether the elevated PAI-1 is simply a secondary phenomenon. The additional problem is that PAI-1 is considered by many to be an acute phase reactant and elevation at the time of myocardial infarction might just be as part of a general acute phase response.

10.4.3 Cross sectional studies: Correlations with extent of coronary atherosclerosis

There have been a number of studies showing correlations between plasma levels of fibrinolytic factors and severity and extent of coronary atherosclerosis. This relationship might be causal or alternatively more severe coronary atherosclerosis might lead to more frequent ischaemia, which in turn might lead to PAI-1 release from the endothelium.

The first study in 1985 (246) showed no relationship between PAI-1 activity or t-PA antigen and angiographic severity of coronary atherosclerosis. However, no account was taken of the diurnal variation of the factor and they used an unvalidated angiographic scoring system which included lesion site in the calculation as well as severity.

The second study from Oberoff et al in 1989 (247) also did not show a correlation between the severity of coronary atherosclerosis and levels of fibrinolytic activity. However, again, their study could be criticised in a number of areas. Their patients were not fasting: the samples were not drawn at consistent times of day; some samples were taken before angiography

and others after; and they used a very simplified and unvalidated angiographic scoring system.

The third study from Sakata et al (248) produced results contradictory to the above two studies. They showed that patients with multivessel coronary artery disease had significantly higher PAI-1 activity than those with single vessel disease. In addition, they demonstrated a significant positive correlation between PAI-1 activity in the basal state and the extent of ischaemia ($r=0.47$, $p<0.01$).

Fourthly, in the ECAT study (238), patients with any stenosis of more than 50% had lower fibrinolytic activity in comparison to subjects with normal coronaries. This was assessed by longer clot lysis times and higher PAI-1 antigen (18.0 ng/ml versus 16.4 ng/ml, $p=0.0004$), PAI-1 Activity (14.3 U/ml compared to 12.8 U/ml, $p=0.0008$) and t-PA antigen (10.2 versus 9.5 ng/ml, $p=0.0005$).Factors adjusted for age, sex and centre. No association was found between these factors and the severity of stenosis. It should be noted that they graded the severity very simply on a scale of 0-4, depending on the number of coronary arteries with ≥ 1 stenosis $\geq 50\%$.

Fifthly, Negri et al (150) in a population of 64 non-obese men with angina and angiographically documented coronary atherosclerosis, demonstrated a significant correlation between PAI-1 antigen and coronary atherosclerosis severity ($r=0.55$, $p<0.00001$) and diffuseness ($r=0.27$, $p=0.032$). This was the most recent and probably best conducted study. Most importantly their sampling protocol for PAI-1 was excellent and they used good and well

validated coronary atherosclerosis scores which quantified the severity and extent of disease in great detail. Further, they controlled for the important confounding influence of BMI by excluding the obese. Therefore it is likely that the reasons for their contradictory results with the preceding studies are these methodological details. They did not look at PAI-1 Activity or t-PA antigen.

10.4.4 Prospective Studies

The Northwick Park Heart Study (249) was the first to show a strong and independent relationship between low global fibrinolytic activity and subsequent risk of CHD events (in a cohort of men aged 40-54).

Secondly, the Physician Health Study (250) showed that higher levels of t-PA was a risk factor for later myocardial infarction. This initially seemed to be a paradoxical result, but it is now considered (241) that the elevated t-PA level reflects primarily an increase in circulating complexes formed by t-PA and PAI-1. It therefore seems likely that the association between a high t-PA antigen concentration and coronary artery disease is explained by high plasma PAI-1 activity and reduced overall fibrinolytic activity.

Thirdly, the ECAT study (90) followed 3,000 men and women with stable angina for 2 years. They showed that a higher t-PA antigen (11.9ng/ml versus 10.0 ng/ml, p=0.02) was an independent predictor of myocardial infarction or cardiac death, independent of other CHD risk factors and base-line angiographic severity of coronary atherosclerosis and of left ventricular ejection fraction. However, neither PAI-1 antigen and activity, nor the ratio of PAI-1 antigen to t-PA antigen differed significantly between the

group with coronary events and the event-free, (after adjusting for all confounding factors $p > 0.2$, for all comparisons).

10.5 PATHOGENIC MECHANISMS

The three epidemiological studies suggest that low fibrinolytic activity is an independent risk factor for CHD events (90,249,250). The most likely explanation for the increase in CHD risk is due to decreased clearance of thrombosis after atherosclerotic plaque rupture has occurred.

However, the correlations between the angiographic extent of coronary atherosclerosis and fibrinolytic activity suggests alternative pathogenic mechanisms. Certainly there is some evidence to suggest that altered fibrinolysis may play a role in the pathogenesis of atherosclerosis itself as opposed to thrombosis. Fibrin is consistently found in atherosclerotic lesions (251). In addition, considerable quantities of PAI-1 mRNA and PAI-1 antigen (252) have been noted in plaques but not normal artery.

A third alternative is that levels of PAI-1 antigen and activity reflect the underlying inflammatory state of atherosclerosis. There is increasing evidence that increased plaque inflammatory activity leads to increased chance of plaque rupture and therefore CHD events (see chapter 2).

CHAPTER 11: FIBRINOLYTIC FACTORS IN THE ASSESSMENT OF CORONARY ATHEROSCLEROSIS

11.1 INTRODUCTION

As detailed in Chapter 10, there is considerable contradiction in the published results (150,238,246-249) relating the level of fibrinolytic factors to the severity and extent of coronary atherosclerosis. There were various methodological problems with these studies and in addition, they could be criticised in that they used tridosium citrate as an anticoagulant for PAI-1 assays. Trisodium citrate does not prevent release of platelet PAI-1 antigen and unless the samples are centrifuged immediately a variable amount of platelet PAI-1 will have contaminated the plasma samples. Thus, we performed plasma determination of PAI-1 antigen in blood collected in a mixture of anticoagulant and inhibitors of platelet aggregation (253). Hence, potentially the correlations between PAI-1 antigen and extent and severity of coronary artery disease might be even greater than that observed by Negri et al (150).

11.2 OBJECTIVES

1.To assess the correlation between PAI-1 antigen and severity and extent of coronary atherosclerosis.

2.To assess the correlation between PAI-1 activity, t-PA antigen and severity and extent of coronary atherosclerosis.

3.To examine the predictive accuracy of fibrinolytic factors in the diagnosis of the presence and severity of coronary atherosclerosis.

4.To investigate the relationships of fibrinolytic factors with other CHD risk factors.

5. To compare levels of fibrinolytic factors between patients with and without previous myocardial infarction. In addition, in those with previous MI, to compare a cohort with prior crescendo angina with a group who had no prior angina.

11.3 MATERIALS AND METHODS

11.3.1 Subjects

We recruited 101 consecutive eligible male subjects (age mean 55.17, range 32.07-76.07) admitted for elective coronary angiography for the investigation of chest pain into the 'entire cohort'. Exclusion criteria were diabetes mellitus (previously diagnosed or a screening fasting glucose > 7.8 mmol/L); evidence of active infection, inflammation or malignancy; other significant disease (including more than mild renal or hepatic dysfunction); previous coronary angioplasty or coronary artery bypass surgery; myocardial infarction or UA in the previous 3 months. There was an 8 month recruitment period and Table 11.1 lists the population demographics. The study was approved by the Hospital Ethical Committee and all

patients gave informed consent. Patients were examined, including height, weight, hip and waist measurements and excluded if any of the above criteria was revealed. Patients filled in a short health questionnaire including questions on previous myocardial infarctions, past and concomitant health problems, drug history, history of hypertension, family history of atherosclerosis and smoking record. Family and smoking histories were handled in the same way as in Chapters 6 and 9. There was a small overlap between this cohort and that in Chapters 6 (40 subjects) and 9 (20 subjects).

In addition we created a 'Negri cohort' from the 'entire cohort' with identical inclusion and exclusion criteria to that in Negri et al's paper (150) by excluding individuals who were obese, who had normal coronary arteries and who had elevated CRP. The 'Negri cohort' is described in Table 11.23.

11.3.2 Sample Preparation

The patients fasted and abstained from smoking from 2200 hours the previous evening; 40 mL of blood was removed between 0800 and 1100 hours following a 10 minute supine rest and prior to coronary angiography. Venesection was performed by a single person using a 19 gauge butterfly vacutainer system from a large antecubital fossa vein with minimal use of tourniquet to minimise venous status. Blood was added to two vacutainer tubes containing no additive, two containing trisodium citrate and two Diatubes (Diatube® H, Diagnostic Stago, Asnieres, France). These last tubes contain a mixture of sodium citrate and citric acid,

supplemented with inhibitors of platelet aggregation - theophylline, adenosine, and dipyridamole.

11.3.3 Assays

(i) PAI-1 Antigen

The ELISA used was a slight modification of a previously published assay (254). The technique has been shown to be accurate, reproducible and extremely sensitive, with a detection limit of 1.5 ng/ml (224). Purified endothelial cell PAI-1 was used as a standard in the ELISA. This was serially diluted in "assay buffer" (50 mM sodium phosphate, pH 7.4, 150mM NaCl, 0.1% (w/v) BSA and 0.05% (v/v) Tween 20, supplemented with 5% (v/v) heat inactivated rabbit serum and 10 mM EDTA), to give a range of concentrations from 5 ng/ml - 312pg/ml.

Polystyrene microplates were coated with 100 µl of 10 µg/ml rabbit anti-human PAI-1 IgG in 50 mM bicarbonate/carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were then washed with "wash buffer" (15 mM phosphate buffer, pH 7.4., 150 mM NaCl, 0.05% (v/v) Tween 20), then "blocked" by adding 200 µl of 1.0% (w/v) bovine serum albumin in 50 mM bicarbonate/carbonate buffer to each of the wells and incubated at 37° C for 30 minutes in a closed, humid chamber.

After a further wash the plates were then incubated for 3 hours at 37°C with 100µl of standards (in triplicate) and samples (in duplicate). After a further wash the plates were incubated for 2 hours at 37°C with 100 µl of biotinylated anti-PAI-1 IgG (2 µg/ml in supplemented assay buffer). After further

washing the plates were incubated for an hour with 100 µl of a solution composed of streptavidin-biotin-horseradish peroxidase complex diluted 1:50000 in 50 mM phosphate buffer, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20, 0.1% (w/v) BSA.

Finally after more washing colour was developed by adding 100µl of substrate solution (tetramethylbenzidine, 0.1 mg/ml in 100 mM acetate/citrate buffer, pH 6.0, containing 0.015% (w/v) hydrogen peroxide). After exactly 15 minutes at room temperature the reaction was stopped by the addition of 25 µl 2.5M H_2SO_4, which instantly changes the blue colouration to bright yellow. The absorbance was measured at 450 nm, using a reference wavelength of 620 nm. The mean absorbence was calculated for each test sample and compared with the serial dilution's of the standard and the unknown values for each test sample were interpolated from the standard curve.

(ii) PAI-1 Activity

This assay was developed from that described by Chmielewska and Wiman (255). The central principal is the detection of the extent of neutralisation of known amounts of t-PA activity by test samples, as determined by the relative colour changes produced in a chromogenic plasmin substrate (S-2251). One unit of PAI-1 activity is the quantity that inhibits one unit of t-PA under the conditions of the assay.

Des-AA fibrin (fibrin monomer I) was prepared by treating fibrinogen with bathroxobin (a snake venom that selectively cleaves fibrinopeptide A from fibrinogen) followed by solubilising the fibrin gel using 3.5M urea.

Human plasminogen was prepared from plasma on a lysine-Sepharose column.

Sample dilutions were prepared in phosphate buffer and an equal volume of two-chain t-PA, final concentration 10 U/ml, was added with incubation for exactly 45 minutes at room temperature. The reaction was stopped by adding an equal volume of acetate buffer 15 minutes at room temperature. Samples were then frozen overnight at -70°C.

Purified two-chain t-PA from a melanoma cell line was used as a standard and serially diluted (from 10 U/ml to 0.625 U/ml) by addition of Tris buffer, pH 7.4, containing 0.1% (w/v) Tween 80. An equal volume of 20 mM phosphate buffer, pH 7.3, 0.1M NaCl, 0.1% (w/v) Tween 80 was added and the mixture incubated at room temperature for exactly 45 minutes. After this, an equal volume of 1M acetate buffer, pH 3.9, was added and the tubes incubated for 15 minutes at room temperature and then the standards were frozen overnight at -70°C.

The following day samples and standards were thawed and diluted 50 times by addition of 50 mM Tris buffer, pH 8.8, containing 0.01% (w/v) Tween 80. Residual t-PA activity was then measured in a 96 well microplate by adding equal volumes of standard or sample, and a solution containing 0.05 mg/ml plasminogen 0.3 mM S-2251 and 0.1 mg/ml des-A fibrin monomer (all final concentrations in 50 mM Tris buffer containing 0.01% (w/v) Tween 80) at 37°C in a humid chamber. Plasmin cleavage of S-2251 produces a

yellow/green colour in the substrate. The absorbance of this colour was measured every hour at 405 nm using 620 nm as a reference wavelength.

A calibration curve was plotted and the residual t-PA activity in samples was obtained by extrapolation from this curve. Subtraction of the samples' residual t-PA activity from 10 U/ml (the original quantity added) reveals the amount of t-PA inhibition in the samples, which is assumed to be due to PAI-1 activity.

(iii) t-PA Antigen

A sandwich ELISA technique was used, modified from that described by MacGregor et al (256). Briefly, 96 well microplates were coated with a monoclonal antibody against t-PA at 2.5μg/ml and incubated for three hours at 37°C. Excess binding sites were then blocked by 1% BSA in PBS and incubated for one hour at room temperature. The plates were then washed four times with wash buffer (15 mM sodium phospate pH 7.4 containing 150 mM NaCl and 0.05% Tween 20), and standard and test samples were added. Standard t-PA were diluted serially down to 0.03ng/ml in buffer consisting of PBS-T and 5% heat inactivated horse serum, 0.1% BSA and 10mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.4. Test and serum samples were diluted 1/20 in buffer from which horse serum had been omitted. 200μl of test and standards were doubly aliqouted and incubated overnight at room temperature. The following day after four washes 200μl of anti-t-PA monoclonal antibody conjugated horse radish peroxidase was added to each well and incubated for three hours at room temperature.

After further washing 200μl tetramethylbenzadine substrate was added per well and the reaction was stopped by the addition of 50μl 2.5M H_2SO_4 and the plates were read at 450nm. A calibration curve was plotted and t-PA antigen concentration in samples was obtained by extrapolation from the curve.

(iv) Other Assays

Insulin levels in serum were assayed using an ELISA kit (Incstar Corporation, minnesota, USA). Liver function tests albumin, aspartate transaminase (AST), alkaline phosphatase (ALP), bilirubin (BIL), gammaGT (γGT) and protein were all assayed by standard techniques on an autoanalyser. In addition, subjects had total cholesterol and subtraction, triglycerides, CRP, lipoprotein (a), and fibrinogen all assayed as detailed in Chapter 6.

11.3.4 Coronary Angiography

These were performed and analysed as in Chapter 6. Five coronary angiography scores were developed, the vessel score, the clinical vessel score, diffuseness score, severity score (Chapter 6) and the Gensini score (Chapter 8).

11.3.5 Statistical Analysis

Seven of the variables measured - insulin, PAI-1 antigen and activity, t-PA antigen, lipoprotein (a), and triglycerides were approximately log normally distributed and thus were expressed logarithmically. Relationships between continuous variables were examined using Pearson's Correlation Analysis. Comparison between continuous and categorical variable was performed using

Student's T-Test (2 categories) and one-way analysis of variance (>2 categories). Multiple linear regression was used to devise the best models for predicting the extent of coronary atherosclerosis and also to predict levels of fibrinolytic factors.

11.4 RESULTS

Table 11.2 describes the distributions of all the plasma and serum variables measured and Table 11.3 details the distribution of the coronary atherosclerosis severity, diffuseness and Gensini scores. Using the vessel score there was 12,5,24 and 60 subjects with 0,1,2 and 3 diseased coronary arteries respectively. The same figures for the clinical vessel score was 18,21,24 and 38 respectively. Table 11.4 shows that there was no correlation between any of the fibrinolytic factors and any of the coronary atherosclerosis scores. In Tables 11.5 and 11.6 cohort is divided into two groups, those with and those without significant coronary atherosclerosis (an analysis identical to that performed in the ECAT study (238)). In our study subjects with significant coronary atherosclerosis were older, heavier and had higher fibrinogen levels than those without. There was no difference in the fibrinolytic factors between the two groups (Table 11.5). However, after adjustment for the confounding influences of age and weight, there was a trend for those subjects with atherosclerosis to have higher levels of fibrinolytic factors (Table 11.6).

Table 11.7 demonstrates the correlations between the fibrinolytic factors, age, Insulin and anthropometric measurements. The greatest correlation was

between PAI-1 antigen and Insulin ($r=0.44$, $p<0.001$ see Figure 11.1). PAI-1 antigen correlated next best with weight ($r=0.34$, $p<0.001$) and to a lesser extent with the other measurements. PAI-1 activity only correlated with Insulin ($r=0.31$, $p<0.01$). t-PA antigen correlated best with waist/hip ratio ($r=0.36$, $p<0.001$) and to a lesser extent with the other measurements and with Insulin ($r=0.28$, $p<0.001$).

The correlations between fibrinolytic factors and continuous CHD risk factors are shown in Table 11.8. PAI-1 antigen and PAI-1 activity most strongly correlated with triglycerides ($r=0.47$ (see Figure 11.2) and $r=0.44$ respectively, $p<0.001$ for both) and to a lesser extent with t-PA antigen. PAI-1 antigen and activity also correlated with the LDL/HDL ratio. This, however, can be attributed to a common correlation with triglycerides (correlation between triglycerides and LDL/HDL ratio =0.47, partial correlation between triglycerides and PAI-1 antigen, after adjusting for LDL/HDL ratio =0.36, $p=0.008$; partial correlation coefficient between LDL/HDL ratio and PAI-1 antigen, after adjusting for triglycerides = 0.15, ns). Similarly, for PAI-1 activity there was no residual correlation with LDL/HDL ratio after adjusting for triglycerides.

The effect of history of hypertension is examined in Table 11.9. Those with hypertension had significantly greater body mass index, t-PA antigen, insulin and glucose. The most significant association ($p<0.00001$) with hypertension was BMI and adjusting the other variables for this led to a loss of their relationship with hypertension.

Subjects with a family history of symptomatic CHD (defined as a first degree relative developing symptoms before the age of 60) were significantly younger ($p=0.001$) and had higher PAI-1 activity and antigen and t-PA antigen and lower fibrinogen (see Table 11.10). Fibrinolytic factors were corrected for potential confounders (Table 11.11) and only t-PA antigen remained statistically significant. Fibrinogen correlated with age ($r=0.290$, $p= 0.003$). However, even after adjusting fibrinogen for age, subjects with a family history still had significantly lower fibrinogen (305.4 g/dL compared to 342.3 g/dL, $p=0.009$). In addition, there was no difference between the cohorts in proportions of smokers, non-smokers and ex-smokers (Chi Square = 0.311, $p=0.86$).

Table 11.12 compares mean values of CHD risk factors between groups of non-smokers, ex-smokers and current smokers ($p=$ns for all). In addition, comparing life-long non-smokers with the combination of current smokers and ex-smokers and adjusting for the confounding influence of BMI, again there was no statistically significant differences between group means (Table 11.13).

PAI-1 antigen and activity correlated significantly with γGT ($r=0.38$, $p<0.0001$ and 0.23, $p<0.05$ respectively Table 11.14) and t-PA antigen correlated with albumin and protein ($r=0.24$, $p<0.05$ Table 11.14). When 5 subjects with significant elevation of γGT (>100 IU/ml) were removed from the analysis the correlation between PAI-1 antigen and γGT was 0.46, ($p<0.001$). When patients with mild elevation of γGT were removed as well (10 patients with γGT

above the upper limit of normal) the correlation was 0.31 ($p<0.01$). Similarily the correlations between γGT and PAI-1 antigen remained when the cohort was divided into subsets depending on triglyceride level (Table 11.15). There was no correlation between alcohol intake (units/week) and γGT or PAI-1 antigen or activity.

There was no difference in the mean levels of fibrinolytic factors between groups with/without prior history of myocardial infarction (Table 11.16). However, subjects with a previous MI had significantly higher total cholesterol ($p=0.025$) and lipoprotein (a) ($p=0.014$). There was no difference in mean levels of fibrinolytic factors or indeed any risk factor between groups with/without a history of angina prior to their MI (Table 11.17).

Patients on betablockers had higher levels of all fibrinolytic components than those not on betablockers and this reached statistical significance for PAI-1 antigen (50.0 ng/ml compared to 35.08 ng/ml, $p=0.035$, Table 11.18). However, the significance was lost after correction for the potential confounding influence of triglyceride levels.

Table 11.19 is the best multivariate model for prediction of the extent and severity of coronary atherosclerosis (Gensini score). The addition of t-PA antigen adds little to the predictive ability of the model (multiple r improves from 0.55 to 0.56). The addition of PAI-1 antigen or PAI-1 activity adds nothing to the model (multiple r stays at 0.55). Tables 11.20-11.22 are multiple linear regression analysis to predict PAI-1 antigen, PAI-1 activity and t-PA antigen, respectively. Insulin and γGT are the most significant

predictors of PAI-1 antigen level, insulin is the most significant predictor of PAI-1 activity and PAI-1 antigen is the most important predictor of t-PA antigen level.

Table 11.23 is the description of the "Negri cohort". The results from this cohort were very similar to that from the "entire cohort". There were no significant correlations between fibrinolytic components and coronary atherosclerosis scores (Table 11.24), however insulin did correlate with the scores ($r=0.30$, $p<0.05$); the correlations between fibrinolytic components and anthropomotic measures were slightly stronger than in the entire cohort (data not shown).

	Number with data available	Mean	SD
Age	101	57.17	10.42
BMI(Kg/m^2)	101	27.08	3.48
Waist (cm)	100	96.45	8.80
Hips (cm)	100	100.20	5.85
Waist/Hips	100	0.96	0.05
Waist/Height	100	0.55	0.05
	Number with data available	% with variable present	
Family history of CAD	98	51.0	
History of hypertension	100	32.7	
Non-smokers	100	20.0	
Ex-smokers	100	59.0	
Current smokers	100	21.0	
Previous MI	95	42.1	

Table 11.1 Demographics of cohort (n=101)

Variable	Number	Mean	S.D
Total Cholesterol (mmol/l)	101	5.63	1.00
LDL Cholesterol (mmol/l)	92	3.66	0.80
HDL Cholesterol (mmol/l)	101	0.91	0.23
LDL/HDL ratio	94	6.60	1.85
Triglycerides (mmol/l)*	101	1.66	1.62
Lipoprotein (a)(mg/dl)*	97	22.39	2.57
Fibrinogen (g/dl)	97	323.76	77.81
Insulin (mU/L)*	101	10.71	1.95
Glucose (mmol/L)	99	5.80	0.61
CRP (mg/l)	97	10.20	0.29
PAI-1 Antigen (ng/ml)*	101	45.71	2.09
PAI-1 Activity (U/ml)*	101	15.85	3.98
TPA Antigen (ng/ml)*	101	6.92	1.62
Albumin (g/l)	100	42.77	2.58
ALT (U/l)	100	22.70	12.05
ALP (U/l)	100	175.49	53.17
γGT (U/l)	100	41.48	29.95
BIL(μmol/l)	100	10.49	5.11
Protein (g/l)	100	68.67	3.90

Table 11.2 Distribution of serum and plasma variables. (* for variables which were log transformed, geometric mean and approximate SD are shown in this table and subsequently.)

	Number of patients	mean	SD
CAD severity	101	45.61	33.52
CAD diffuseness	101	4.43	2.72
CAD Gensini	101	8.48	5.49

Table 11.3 Distribution of coronary atherosclerosis scores.

	PAI-1 Antigen	PAI-1 Activity	TPA Antigen	Insulin	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness
PAI-1 Activity	0.57***							
TPA Antigen	0.36***	0.12						
Insulin	0.44***	0.31**	0.28**					
CAD Severity	0.04	0.04	0.11	0.09				
Vessel Score	0.12	0.03	0.15	0.01	0.72***			
Clinical Vessel	0.06	0.04	0.11	0.05	0.84***	0.83***		
CAD Diffuseness	0.01	-0.03	0.10	0.03	0.84***	0.81***	0.77***	
CAD Gensini	0.02	0.01	0.10	0.06	0.96***	0.79***	0.85***	0.94***

Table 11.4 Pearson's Correlations between fibrinolytic factors and coronary angiography scores (n=101, *p<0.05, **p<0.01, *** p<0.001 all other correlations are not significant).

	0 Vessel Disease	1-3 Vessel Disease	P value
No.	18	83	
Age (years)	47.86	56.75	0.0018
BMI (Kg/m^2)	28.97	26.67	0.030
Weight (kg)	89.3	80.50	0.016
Waist/hip Ratio	0.96	0.96	ns
Waist/Ht ratio	0.56	0.55	ns
PAI-1 Activity (U/ml)	18.19	15.48	ns
PAI-1 Antigen (ng/ml)	46.65	45.71	ns
TPA Antigen (ng/ml)	6.31	7.24	ns
Insulin (mU/L)	12.59	10.47	ns
Glucose (mmol/l)	5.83	5.80	ns
Total Cholesterol (mmol/l)	5.35	5.70	ns
Ratio LDL/HDL	6.24	6.68	ns
Triglycerides(mmol/l)	1.79	1.64	ns
Lipoprotein(a)(mg/dl)	19.45	22.96	ns
Fibrinogen (g/dl)	296.20	329.60	0.047

Table 11.5 Comparison of variable means between cohort with no coronary stenosis $\geq$ 50% with cohort having $\geq$ 1stenosis $\geq$ 50%.

	0 Vessel Disease	1-3 Vessel Disease	p value for difference between means
PAI-1 Activity (U/ml)	37.32	47.42	0.31
PAI-1 Antigen (ng/ml)	14.86	16.05	0.81
TPA Antigen (ng/ml)	5.90	7.26	0.11

Table 11.6 Comparison of variable means between cohort with no coronary stenosis $\geq$ 50% with cohort having $\geq$ 1stenosis $\geq$ 50%. (n=101, Variables adjusted for age and weight).

	PAI-1 Antigen *	PAI-1 Activity	TPA Antigen	Insulin	Age
Age	-0.18	-0.14	-0.01	-0.21*	
Weight	0.34***	0.17	0.35***	0.56***	-0.42***
BMI	0.30**	0.19	0.23*	0.56***	-0.31***
Waist/hip Ratio	0.22*	0.04	0.36***	0.44***	0.10
Waist/ height Ratio	0.22*	0.10	0.34***	0.55***	0.03
Insulin	0.44***	0.31**	0.28**		021*

Table 11.7 Pearson's Correlations between fibrinolytic factors and age, Insulin and anthropometric measurements (n=101, *p<0.05, **p<0.01, *** p<0.001 all other correlations are not significant).

	PAI-1 Antigen	PAI-1 Activity	TPA Antigen
Glucose	0.19	0.13	0.27**
Total Cholesterol	0.16	0.18	0.19
HDL Cholesterol	-0.15	-0.12	0.02
LDL Cholesterol	0.02	-0.06	0.03
Ratio LDL/HDL	0.34***	0.20	0.07
VLDL Cholesterol	0.36***	0.34***	0.18
Lipoprotein (a)	-0.07	0.04	0.06
Fibrinogen	-0.01	-0.20*	-0.06
Triglycerides	0.47***	0.37***	0.29**
CRP	-0.04	0.07	-0.09

Table 11.8 Pearson's Correlations between fibrinolytic factors and continuous cardiovascular risk factors (n=97-101, *p<0.05, **p<0.01, *** p<0.001 all other correlations are not significant).

	No Hypertension (n=66)	Hypertension (n=33)	P for difference between means
Age (years)	54.9	55.81	ns
BMI (kg/m^2)	25.96	29.39	<0.00001
PAI-1 Activity (U/ml)	14.79	18.20	ns
PAI-1 Antigen (ng/ml)	42.07	53.33	0.10
TPA Antigen (ng/ml)	6.50	8.32	0.032*
Insulin (mU/L)	9.42	13.87	0.011
Glucose (mmol/l)	5.71	6.00	0.024
Total Cholesterol (mmol/l)	5.69	5.52	ns
Ratio LDL/HDL	6.79	6.23	ns
Triglycerides (mmol/l)	1.64	1.73	ns
Lipoprotein (a)(mg/dl)	20.32	27.16	ns
Fibrinogen (g/dl)	321.50	328.50	ns
CRP (mg/l)	10.2	10.2	ns
CAD severity	45.8	45.30	ns
Cad diffuseness	4.61	4.08	ns
CAD Gensini	8.69	8.06	ns

Table 11.9 Comparison of mean values of cardiovascular risk factors between groups with and without history of hypertension. (* non-significant after correcting for BMI)

	No Family History (n= 50)	Family History (n=48)	P for difference between means
Age (years)	58.76	51.03	0.0001
BMI (kg/m^2)	26.80	27.44	ns
PAI-1 Activity (U/ml)	11.17	22.33	0.013
PAI-1 Antigen (ng/ml)	43.55	47.64	ns
TPA Antigen (ng/ml)	7.73	6.28	0.038
Insulin (mU/L)	9.95	11.89	ns
Glucose (mmol/l)	5.80	5.84	ns
Total Cholesterol (mmol/l)	5.53	5.76	ns
Ratio LDL/HDL	5.57	6.68	ns
Triglycerides (mmol/l)	1.57	1.79	ns
Lipoprotein (a)(mg/dl)	21.28	22.80	ns
Fibrinogen (g/dl)	353.10	300.30	0.0002
CAD severity	47.1	42.60	ns
Cad diffuseness	4.69	4.04	ns
CAD Gensini	8.85	7.86	ns

Table 11.10 Comparison of mean values of cardiovascular risk factors between groups with and without a family history of CAD.

	No Family History (n= 50)	Family History (n=48)	P value
PAI-1 Activity (U/ml)*	12.42	19.63	0.11
PAI-1 Antigen (ng/ml)$^{\phi}$	45.60	45.18	0.80
TPA Antigen (ng/ml)**	7.73	6.28	0.038

Table 11.11 Comparison of variable means between cohorts with and without family history of CAD (Variables adjusted for potential confounding influences *adjusted for age and Fibrinogen, $^{\phi}$ adjusted for age, ** unadjusted.)

	Never Smoked (n=20)	Ex-smoker (n=59)	Current smoker (n=21)
Age (years)	54.91	55.80	53.71
BMI* (kg/m^2)	28.80*	26.73*	26.30*
PAI-1 Activity (U/ml)	14.90	16.25	15.55
PAI-1 Antigen (ng/ml)	47.86	45.71	43.65
TPA Antigen (ng/ml)	6.76	7.41	6.90
Insulin (mU/L)	11.48	10.72	9.33
Glucose (mmol/l)	5.76	5.84	5.7
Total Cholesterol (mmol/l)	5.8	5.6	5.6
Ratio LDL/HDL	6.47	6.52	7.08
Triglycerides (mmol/l)	1.65	1.55	1.74
Lipoprotein (a)(mg/dl)	25.12	22.38	18.62
Fibrinogen (g/dl)	307.32	322.02	340.50
CRP (mg/l)	9.50	10.51	9.86

Table 11.12 Comparison of mean values of cardiovascular risk factors between groups of non smokers, ex-smokers and current smokers (*p=0.030, otherwise p=ns for all).

	Never Smoked (n=20)	Ex or current smoker (n=80)	p Value
PAI-1 Antigen (ng/ml)	46.88	50.82	0.50
PAI-1 Activity (U/ml)	13.03	16.63	0.65
TPA Antigen (ng/ml)	6.03	7.19	0.10

Table 11.13 Comparison of mean levels of fibrinolytic factors between a group of lifelong non smokers with a group of combined current and previous smokers (factors adjusted for BMI).

	PAI-1 Antigen	PAI-1 Activity	TPA Antigen
Alkaline Phosphatase	0.08	0.01	-0.04
Albumin	0.33**	0.04	0.24*
Aspartate Transaminase	0.08	-0.04	0.13
Bilirubin	0.06	0.06	0.14
Gamma GT	0.38***	0.23*	0.15
Protein	0.28**	0.11	0.24*

Table 11.14 Pearson's Correlations between fibrinolytic factors and liver function tests (n=100, *p<0.05, **p<0.01, *** p<0.001 all other correlations are not significant). R=0.56 (p<0.001) for correlation between albumin and protein, no other significant correlation.

	Number of subjects	PAI-1 antigen (U/ml)	PAI-1 activity (ng/ml)
All	101	0.38***	0.24**
Hypertiglyceridaemic	13	0.40**	0.18
Normotriglyceridaemic	88	0.31**	0.18*
Normotriglyceridaemic Normal γGT	73	0.27**	0.17

Table 11.15 Correlations between PAI-1 activity and antigen and γGT in the "entire cohort" and three subsets (see ref 43b) (*$p<0.05$, **$p<0.01$, *** $p<0.001$ all other correlations are not significant).

	No MI (n= 55)	Previous MI (n=40)	P for difference between means
Age (years)	54.50	55.47	ns
BMI (kg/m^2)	27.52	56.74	ns
PAI-1 Activity (U/ml)	18.20	14.45	ns
PAI-1 Antigen (ng/ml)	45.71	46.77	ns
TPA Antigen (ng/ml)	6.62	7.83	0.09
Insulin (mU/L)	10.59	10.62	ns
Glucose (mmol/l)	5.86	5.75	nss
Total Cholesterol (mmol/l)	5.41	5.86	0.025
Ratio LDL/HDL	6.38	6.86	ns
Triglycerides (mmol/l)	1.64	1.67	ns
Lipoprotein (a)(mg/dl)	17.42	27.42	0.014
Fibrinogen (g/dl)	320.70	329.5	ns
CRP (mg/l)	9.81	10.10	ns
CAD severity	39.20	53.59	0.032
Cad diffuseness	4.03	5.09	0.065
CAD Gensini	7.42	9.91	0.030

Table 11.16 Comparison of mean values of cardiovascular risk factors between groups with and without previous MI

	No prior angina (n= 19)	Prior angina (n= 14)
Age (years)	53.52	56.29
BMI (kg/m^2)	27.26	27.15
PAI-1 Activity (U/ml)	17.78	18.62
PAI-1 Antigen (ng/ml)	44.67	47.86
TPA Antigen (ng/ml)	8.13	7.59
Insulin (mU/L)	12.02	12.02
Glucose (mmol/l)	5.88	5.75
Total Cholesterol (mmol/l)	5.71	6.08
Ratio LDL/HDL	6.50	7.03
Triglycerides (mmol/l)	1.61	1.74
Lipoprotein (a)(mg/dl)	28.84	21.88
Fibrinogen (g/dl)	331.20	322.7
CRP (mg/l)	10.50	11.10
CAD severity	55.20	57.60
Cad diffuseness	5.38	5.14
CAD Gensini	10.29	10.43

Table 11.17 Comparison of mean values of cardiovascular risk factors between groups with and withuot a history of angina prior to MI (p=ns for all)

	No B Blocker (n=27)	B Blocker (n=74)	P for difference between means
Age (years)	55.40	55.10	ns
BMI (kg/m^2)	27.26	27.01	ns
PAI-1 Activity (U/ml)	14.82	16.21	ns
PAI-1 Antigen (ng/ml)	35.08	50.00	0.035*
TPA Antigen (ng/ml)	6.22	7.31	ns
Insulin (mU/L)	13.15	9.93	ns
Glucose (mmol/l)	5.89	5.77	ns
Total Cholesterol (mmol/l)	5.65	5.63	ns
Ratio LDL/HDL	6.41	6.67	ns
Triglycerides (mmol/l)	1.43	1.76	0.064
Lipoprotein (a)(mg/dl)	26.24	20.99	ns
Fibrinogen (g/dl)	326.00	323.00	ns
CRP (mg/l)	11.42	9.75	ns
CAD severity	42.00	46.90	ns
Cad diffuseness	3.94	4.61	ns
CAD Gensini	7.71	8.75	ns

Table 11.18 Comparison of mean values of cardiovascular risk factors between groups with and without B Blocker therapy (*significance lost after correcting for Triglycerides.) Similar analyses for groups with and without each of Aspirin, Calcium Antagonist and Nitrate therapy showed no significant results.

Predictor	Coefficient	SD	t-ratio	p
Age (years)	0.287	0.055	5.21	0.000
History of Hypertension	-2.854	1.263	-2.26	0.026
TPA Antigen (ng/ml)	4.243	2.812	1.51	0.135
Cholesterol (mmol/l)	0.722	0.541	1.33	0.186
BMI (kg/m^2)	0.219	0.190	1.15	0.252
Family History of CAD	1.280	1.166	1.10	0.275
Fibrinogen (mg/dl)	0.007	0.007	0.94	0.349
Triglycerides (mmol/l)	-1.940	2.660	-0.73	0.468
Lipoprotein (a) (mg/dl)	0.676	1.237	0.55	0.586

Table 11.19 Best multivariate model for prediction of CAD (modified Gensini Score). Multiple r=0.56. partial r for TPA= 0.16 p=0.16 . If TPA antigen removed from the model, multiple r=0.55 . If PAI antigen exchanged for TPA multiple r=0.55 with probability value = 0.359 (partial r=0.11 p=ns). If PAI activity exchanged for TPA multiple r=0.55 with probability value= 0.576 (partial r=0.07, p=ns).

Predictor	Coefficient	SD	t-ratio	p
Gamma GT (U/l)	0.001	0.001	2.80	0.006
Insulin (mU/l)	0.32	0.12	2.77	0.007
Triglycerides (mmol/l)	0.31	0.15	2.09	0.040
TPA Antigen (ng/ml)	0.26	0.15	1.71	0.091
LDL/HDL Ratio	0.03	0.02	1.61	0.112
Weight (kg)	0.002	0.002	0.59	0.558
Protein (g/l)	0.001	0.008	0.12	0.906

Table 11.20 Multiple Linear Regression to predict PAI-1 Antigen (r^2=44.2%, F=9.51)

Predictor	Coefficient	SD	t-ratio	p
Insulin (mU/l)	0.67	0.19	3.58	0.001
Triglycerides (mmol/l)	0.67	0.31	2.15	0.034
Gamma GT (U/l)	0.003	0.002	1.36	0.179
Fibrinogen (mg/l)	-0.001	0.0001	-1.06	0.293
LDL/HDL Ratio	0.02	0.03	0.63	0.53

Table 11.21 Multiple Linear Regression to predict PAI-1 Activity (r^2=29.9%, F=6.99)

Predictor	Coefficient	SD	t-ratio	p
PAI-1 Antigen (ng/ml)	0.13	0.07	1.85	0.068
Waist/Hip Ratio	0.68	0.38	1.82	0.073
Glucose (mmol/l)	0.052	0.03	1.68	0.097
Triglycerides (mmol/l)	0.11	0.097	1.14	0.258
Protein (g/l)	0.004	0.005	0.90	0.369
Insulin ( )	-0.01	0.08	-0.18	0.856

Table 11.22 Multiple Linear Regression to predict TPA Antigen (r^2=22.5%, F=4.35)

	Number with data available	Mean	SD
Age (years)	68	57.30	10.13
BMI(kg/m^2)	68	25.62	2.55
Weight (kg)	68	7.80	9.77
Waist/Hips	67	0.96	0.05
Waist/Height	67	0.54	0.04
CAD severity	67	54.67	28.89
CAD diffuseness	67	5.32	2.13
CAD Gensini	67	10.19	4.32

Table 11.23 Description of 'Negri' cohort (n=68)

	PAI-1 Antigen	PAI-1 Activity	TPA Antigen	Insulin	CAD Severity	Vessel Score	Clinical Vessel	CAD Diffuseness
PAI-1 Activity	0.71***							
TPA Antigen	0.30**	0.22*						
Insulin	0.40***	0.30**	0.23*					
CAD Severity	0.02	0.00	0.16	0.30*				
Vessel Score	0.12	-0.06	0.16	0.28*	0.53***			
Clinical Vessel	0.07	0.08	0.14	0.27*	0.72***	0.70***		
CAD Diffuseness	-0.05	-0.19	0.18	0.30*	0.69***	0.63***	0.56***	
CAD Gensini	-0.01	-0.08	0.16	0.31*	0.94***	0.62***	0.72***	0.87***

Table 11.24 Pearson's Correlations between fibrinolytic factors and coronary angiography scores for Negri Cohort (n=67-68 *p<0.05, **p<0.01, *** p<0.001 all other correlations are not significant).

Figure 11.1 Correlation between PAI-1 Antigen and Insulin

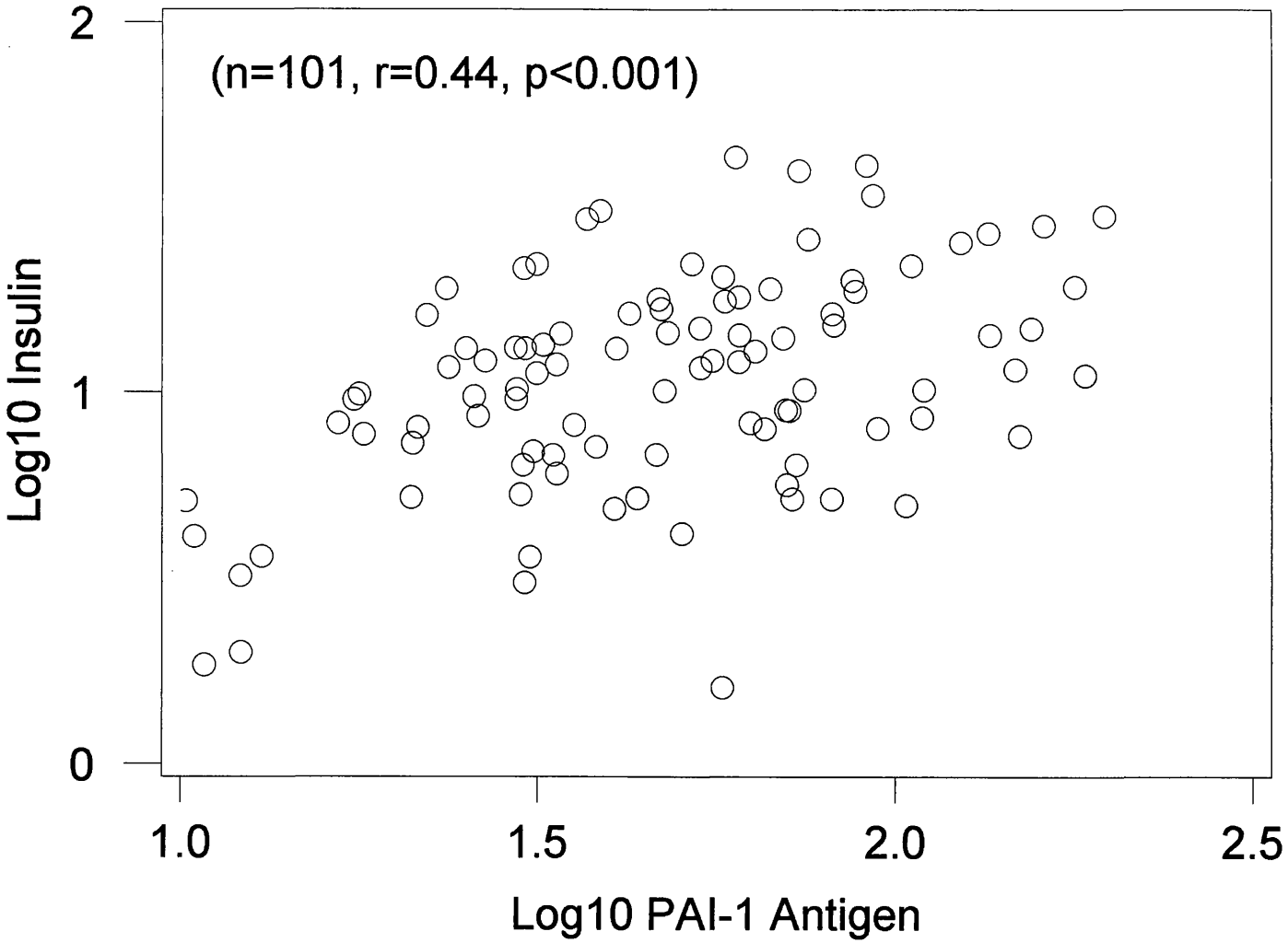
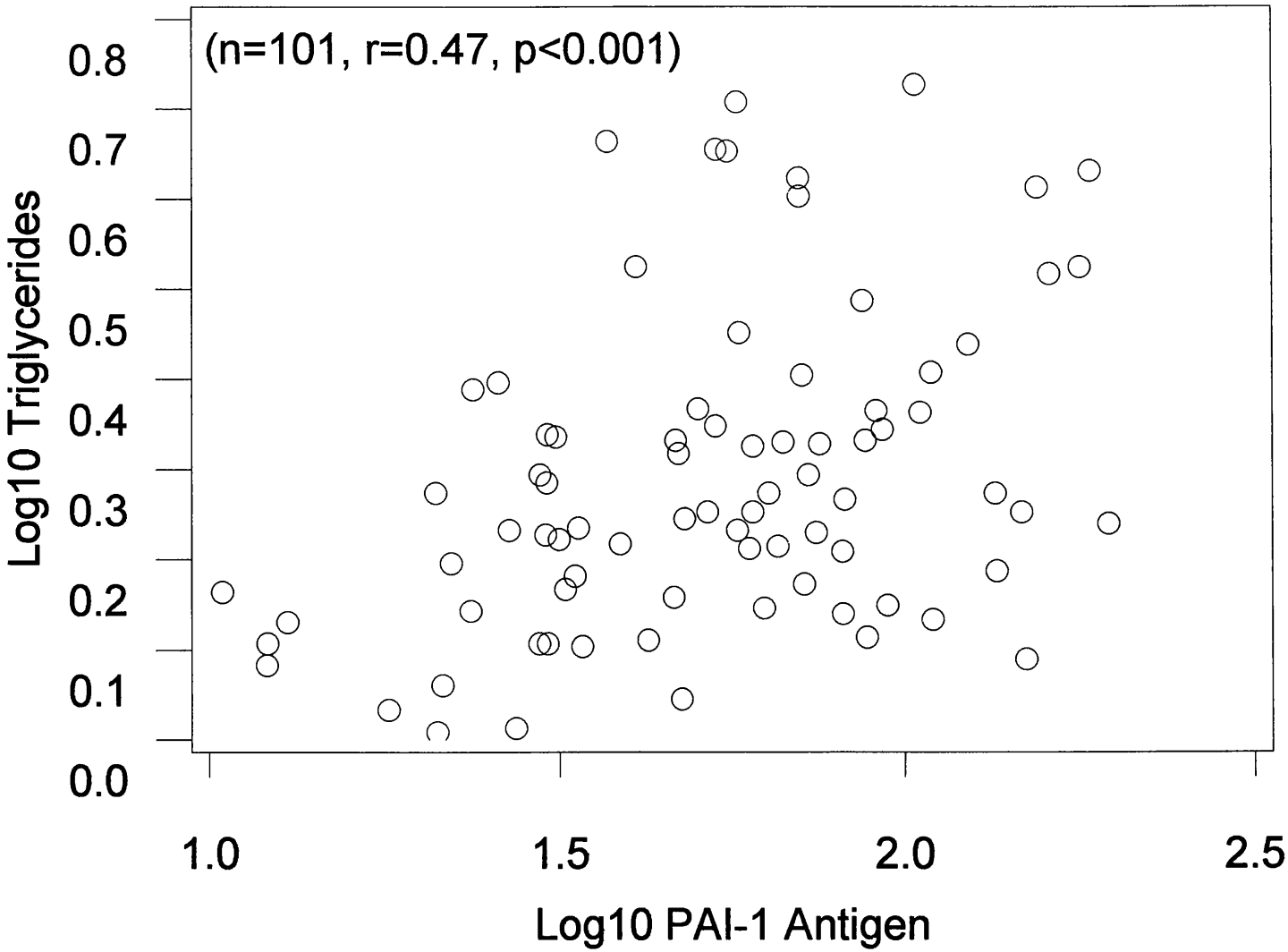


Figure 11.2 Correlation between PAI-1 Antigen and Triglycerides



11.5 DISCUSSION

There was no correlation between PAI-1 antigen and either the severity or diffuseness of coronary atherosclerosis ($r=0.04$ for severity, $r=0.01$ for diffuseness, Table 11.4). This is in agreement with other studies (227,238,256) but in marked contrast to the work of Negri et al (150), who found correlations between plasma PAI-1 antigen and severity of coronary atherosclerosis ($r=0.55$, $p<0.00001$) and PAI-1 antigen and diffuseness score ($r=0.27$, $p<0.032$).

Our entire cohort (Table 11.1) was different from that of Negri et al (29) in that we included individuals who were obese, who had normal coronary arteries and who had elevated CRP. Thus we excluded these subjects to create our "Negri cohort" (Table 11.23). However, again there was no correlation between PAI-1 antigen and severity or diffuseness of coronary atherosclerosis (Table 11.24).

There are a number of possible explanations for the contradictory results. Firstly, our angiography scoring systems were slightly different from that of Negri. The only difference between the severity scores is that they gave a stenosis of 50-75% 10 points and 76-89% 15 points, whereas we treated 50-89% stenosis as one category, gaining a score of 12.5 points. Otherwise the severity scores were identical in all respects. There were more differences between the diffusseness scores in Negri's and our studies. They examined first to third order coronary artery branches whilst we just looked at the first two orders. They graded a stenosis in a given segment as being diffuse or

discrete, whereas we simply looked for presence or absence of stenosis in a segment.

A second possibility is that in Negri's studies some platelet PAI-1 antigen leaked out and contributed to the measured and presumed plasma PAI-1 antigen. We used anticoagulant tubes containing an anti-platelet mixture which prevents platelet secretion of PAI-1 antigen and thus can be confident that we measured only plasma PAI-1 antigen (253).

A third possibility is that populations were different in respect of potential confounding variable(s) and there seems to be at least one important difference between the populations. In Negri's paper there was some association between increasing triglyceride level and severity of coronary atherosclerosis in that those with multivessel coronary disease had significantly higher triglycerides than subjects with only single vessel disease. A similar analysis in our Negri cohort showed a trend in the opposite direction with a higher triglyceride level in those with single vessel disease compared to multivessel disease (1.79 mmol/L compared to 1.56 mmol/L). As discussed below, triglyceride levels are a very important influence on PAI-1 levels.

There were also no correlations between PAI-1 activity or t-PA antigen and the severity and extent of coronary atherosclerosis in the entire cohort (Table 11.4) or the "Negri cohort" (Table 11.24) and this is in agreement with previous work (238,246). Further, there was no statistical difference for mean PAI-1 antigen or activity or t-PA antigen between groups with/without

coronary atherosclerosis (Table 11.5). However, age and weight-adjusted data showed a clear trend for those with atherosclerosis to have higher levels of all three measurements (Table 11.6). The proportional difference between the means of the groups for each measurement is similar to that in the large ECAT study (238) (PAI-1 antigen 18.0 ng/ml versus 16.4 ng/ml, p=0.0004; PAI-1 Activity 14.3 U/ml compared to 12.8 U/ml, p=0.0008; t-PA antigen 10.2 versus 9.5 ng/ml, p=0.0005). The results in the ECAT study were highly significant but this is likely to be because of their larger sample size.

We, however, did show that insulin correlated with both severity and extent of coronary atherosclerosis in our "Negri cohort" ($r=0.30$, $p<0.05$, Figure 11.3). This agrees with the findings of Negri et al (150) who showed a correlation between insulin and severity of $r=0.37$ and between insulin and diffuseness of $r=0.19$. The correlations of PAI-1 antigen and activity with insulin were greater than with any of the anthropometric measurements ($r=0.44$, $p<0.001$ for PAI-1 antigen, Figure 11.1 and Table 11.7). This agrees with data from the ECAT study (229), suggesting that the relationship between PAI-1 and body mass index and waist/hip ratio is generally secondary to the relationship between PAI-1 and insulin. This is further investigated by multiple linear regression in Tables 11.20-22 and discussed below.

t-PA correlated more strongly with waist/hip ratio than other anthropmometric measurements ($r=0.36$, $p<0.001$) as previously documented (239,240,245). However, contrary to this previous work,

PAI-1 antigen correlated more strongly with weight ($r=0.34$, $p<0.001$) than with waist/hip ratio ($r=0.22$, $p<0.05$, Table 11.7). Again, contrary to previous data, PAI-1 activity did not correlate with any of the anthropometric measurements.

PAI-1 antigen and PAI-1 activity and t-PA antigen correlated most strongly with triglycerides ($r=0.47$, $p<0.001$(see Figure 11.1), $r=0.44$, $p<0.001$ and $r=0.29$, $p<0.01$, respectively) of the continuous CHD risk factors (Tables 11.8), as previously noted (241). The apparent correlation between fibrinolytic factors and LDL/HDL ratio is shown to be spurious and due to confounding effects of triglycerides.

Subjects with hypertension had greater BMI and higher PAI-1 antigen, t-PA antigen, insulin and glucose than those without (Table 11.9). However, the strongest association was with BMI and after correcting for this, the significance of the other relationships were lost and this is similar to previous data (238).

A comparison of mean values of CHD risk factors between groups with and without a family history of coronary atherosclerosis produced some surprising results (Table 11.10). Those with a family history were younger, had higher PAI-1 activity but lower t-PA antigen and most surprisingly, highly significant lower fibrinogen (300.3 g/l vs 353.1 g/l, $p=0.0002$). Adjustment for potentially confounding influences led to a loss of significance for PAI-1 antigen, however, there was no obvious confounding factors for t-PA. It is difficult to fit this finding with the epidemiological

evidence regarding higher t-PA antigen levels as a CHD risk factor (90,250). It is also difficult to explain the fibrinogen results as their association remained after adjusting for the potential influence of age. Further there was no difference between the groups regarding smoking the other potential confounding variable. There is known to be a large hereditary component (51%) to fibrinogen levels (257) and elevated fibrinogen is increasingly regarded as a risk factor for CHD mortality and morbidity (258).

Current smokers and ex-smokers had trends towards higher levels of PAI antigen and t-PA antigen than lifelong non-smokers (Table 11.13, after adjusting for the confounding influence of BMI). These trends were in the same direction as the significant results from the ECAT study (see Table 10.1).

There were moderate correlations between PAI-1 antigen and activity and γGT (see Table 11.14). Leiper et al (259) showed increases in the plasma concentration of PAI-1 and t-PA antigens in patients with a variety of hepatic diseases including alcoholic cirrhosis, primary biliary cirrhosis and hepatic malignancy. It should be noted that none of the patients in our study had a history or clinical evidence of past or present hepatic disease.

To my knowledge, Asplund-Carlsson and colleagues (260) have published the only other data relating levels of liver function with fibrinolytic factors. They showed that γGT levels correlated with PAI-1 activity in a population of 29 hypertryglyceridaemic (triglycerides >3.05mmol/l) males who also had elevated γGT ($r=0.50$, $p<0.001$). There was no correlation in cohorts of

normotriglyceridaemic males or hypertiglyceridaemic men with normal γGT and they did not look at PAI-1 or t-PA antigen levels. We divided our cohort using Asplund's criteria (Table 11.15) and there remained significant correlations between γGT and PAI-antigen in all groups but not PAI-1 activity. To my knowledge this is the first description of correlations between γGT and PAI-1 activity in these populations and with PAI-1 antigen in any population.

Why PAI-1 antigen should correlate with γGT is unclear. Elevated γGT is usually related to regular daily consumption of alcohol (260). Experimental studies have fairly consistently shown a decrease in blood fibrinolytic activity with alcohol both acutely and in the long term. Whereas in contrast the epidemiological studies have generally shown a positive association between alcohol consumption and fibrinolytic capacity (see review by Veenstra et al 261). In our study there was no correlation between reported alcohol consumption (units/week) and γGT or PAI antigen or activity. This suggests that γGT varies independently of or inconsistently with alcohol consumption

The endothelium and liver are the likely sites of important plasma PAI-1 antigen synthesis although there relative importance remains unclear (226). Our data suggests that the liver contributes significantly. Indeed γGT was the most important predictor of PAI-1 antigen levels in a multiple linear regression analysis (Table 9). Perhaps γGT is involved with transmembrane transport of PAI-1 and thus increased γGT expression leads to increased PAI-1 excretion into plasma. γGT becomes a less important and Insulin a more important

influence on PAI-1 antigen if patients with elevated γGT are excluded from the analysis (correlation between Insulin and PAI-1 antigen =0.50.)

There was a tendency for subjects with a history of previous myocardial infarction to have higher levels of t-PA antigen compared to those with no history of myocardial infarction (7.83 ng/ml vs 6.62 ng/ml, p=0.09). However, there was no difference in PAI-1 antigen or activity between the two groups (Table 11.16). This is in keeping with the emerging prospective epidemiological data that it is a higher level of t-PA antigen which reflects a decreased fibrinolytic activity and a risk factor for myocardial infarction (90,250). This initially seemed to be a paradoxical result, but it is now considered (241) that the elevated t-PA level reflects primarily an increase in circulating complexes formed by t-PA and PAI-1. It therefore seems likely that the association between a high t-PA antigen concentration and coronary artery disease is explained by high plasma PAI-1 activity and reduced overall fibrinolytic activity.

There was no difference in any continuous CHD risk factor including the fibrinolytic factors between groups with a history of proceeding angina prior to their MI compared to a group with no prior angina (Table 11.17). I had hypothesised that the former group might have better fibrinolytic function and thus be able to resist occlusion and subsequent MI for longer than the latter group, but there were no data to support this.

We examined the effects of four commonly used cardiac medications on fibrinolytic factors (Table 11.18) and the only significant results were for

betablockers. Subjects on betablockers had higher levels of fibrinolytic factors than those not receiving this therapy and this reached statistical significance for PAI-1 antigen. However, patients on betablockers also had higher triglycerides. This is in agreement with one similar study (262) which studied 385 patients with hyperlipidaemia. The 39 patients who were receiving beta blockers had significantly elevated PAI-1 antigen and activity and t-PA antigen than the remainder of the cohort, After adjusting for age, sex, Quetelet index and triglycerides however the trend to higher levels remained but the statistical significance was lost. Our patients on betablockers also had higher triglyceride levels, which is a well documented effect of betablockers (263) and the significant association of PAI-1 antigen and betablockers was lost after adjusting for triglycerides. An adverse effect of beta blockers on fibrinolysis mediated via triglycerides cannot be excluded. However in contrast to the above data, two longitudinal studies (264,265) have demonstrated that patients had improved fibrinolytic activity after commencing betablockers. It seems unlikely that Beta blockers can have an important adverse effect on fibrinolysis as they are of proven mortality benefit post-myocardial infarction (266).

Table 11.19 was the best multivariate model for prediction of the severity and extent of coronary atherosclerosis. As can be seen, the inclusion of any of the fibrinolytic components in the model adds nothing to the predictive accuracy of the model.

Table 11.20 demonstrates that γGT and insulin are the most important factors in determining PAI-1 antigen levels and the next important factor is triglyceride. The latter two of these factors are well documented but this is the first time that the influence of γGT on PAI-1 antigen levels has been demonstrated. γGT is a less important and Insulin a more important influence on PAI-1 antigen if patients with elevated γGT are excluded from the analysis (correlation between Insulin and PAI-1 activity =0.50.)

For PAI-1 activity, insulin and triglycerides are the most important influences (Table 11.21). For t-PA antigen, PAI-1 antigen and waist/hip ratio are the most important factors (Table 11.22).

In summary, there was no evidence of a correlation between plasma PAI-1 antigen levels and the angiographic severity of coronary atherosclerosis and this contradicts recently published work (150). Three possible explanations for this are presented. Also there was little evidence of a relationship between PAI-1 activity or t-PA antigen and the extent of coronary atherosclerosis. The relationship between PAI-1 and the components of the Insulin Resistance Syndrome are confirmed. Novel data is presented suggesting that γGT is an important influence on PAI-1 antigen levels in a normotriglyceridaemic population.

CHAPTER 12: GENERAL DISCUSSION

12.1 INTRODUCTION

CHD is the biggest single cause of death in the United Kingdom (11). In 1990 there were nearly 170,000 coronary heart disease deaths which represents 27% of all deaths and 17% of these deaths in people under 65 years of age. There is also an appreciable morbidity with 5% of men and 3% of women reporting having had angina (12). It is estimated that in 1989/90 CHD attracted about 4% of total NHS expenditure in the U.K (11). Thus there are huge incentives to improve the prevention, diagnosis and treatment of all stages of CHD. This thesis examined one aspect of this, the non-invasive assessment of coronary atherosclerosis.

It seems unlikely that non-invasive measurement of atherosclerosis will replace angiography for assessing the significantly symptomatic who are being considered for surgical intervention and this is outwith the scope of this thesis. However, the development of valid and reliable techniques of non-invasive assessment would be invaluable in a number of ways as follows:

1.As an additional diagnostic tool in those with minor or atypical symptoms which are insufficient to justify the risks and costs of invasive investigation.

2.To identify those with a large but still sub-clinical atherosclerotic load, both for intervention to halt the progression of disease and for intensive research.

3.Serial measurements of atherosclerosis from an early age in large epidemiological studies would allow evaluation of the natural history of the disease.

4. If it can be shown that non-invasive techniques of quantification was valid and that the measured atherosclerotic extent correlated with the likelihood of clinical events, then serial measurements could be used to follow anti-atherosclerotic interventions, either by lifestyle adjustments or pharmacological interventions.

This thesis examined three techniques for the non-invasive assessment of coronary atherosclerosis. The second objective was to investigate the role of the immune response to hsp65 and *H.pylori* and of the endogenous fibrinolytic system in the pathogenesis of atherosclerosis and athero-thrombosis.

12.2 THE IMMUNE RESPONSE TO HSP60/65 AND *H.PYLORI* AND ATHEROSCLEROSIS

Data is presented in chapter 6 showing that levels of anti-hsp65 correlate with both the severity (r=0.21, p=0.018) and diffuseness (r=0.21, p=0.016) of coronary atherosclerosis (Table 6.4). In addition, elevated anti-hsp65 remained significantly associated with the presence of coronary atherosclerosis after adjustment for all confounding factors (27.86 AU/ml

compared to 17.10 AU/ml, p=0.012). Anti-hsp65 has been suggested as the diagnostic marker of atherosclerosis that clinical medicine had been waiting for (131) and this was a major stimulus for this work. However, very disappointingly, levels of anti-hsp65 clearly had insufficient predictive accuracy to be a useful clinical test as at best anti-hsp65 titre had a sensitivity of 77.8% and specificity of 57.8% for the detection of atherosclerosis (see Table 6.4 and Figures 6.2 and 6.3)

Anti-hsp65 does not meet all the eight criteria for causal risk factor status detailed in Chapter 1, although few risk factors do. Nevertheless, our data along with the evidence of Xu et al (112,126-130) suggest that it is likely that the antibodies to hsp60/65 and the immune response to hsp60/65 is involved with the pathogenesis of atherosclerosis.

In Chapters 4-7, the influence of other factors on anti-hsp65 titre are examined. There was no significant familial influences on development of titres (chapter 4). Further, any minor familial influences were likely to be environmental rather than genetic. This is in keeping with experimental evidence in animals which have shown that it is the environmental exposure to micro-organisms which is most important in producing anti-hsp65 titres (141).

There is no difference between the sexes in titres of anti-hsp65 (chapters 4 and 7) and this is in keeping with data from other groups (120,130). This is perhaps surprising in view of the different incidence of coronary heart disease between the two sexes (see Figure 1.3) and therefore you might

expect males to have higher anti-hsp65 levels. We only investigated males in our cohort with coronary atherosclerosis (Chapter 6), but Xu et al (130) showed that elevated anti-hsp65 was associated with carotid atherosclerosis in both sexes.

Thirdly, anti-hsp65 titre increases with advancing age ($r=0.24$, $p<0.005$, Chapter 6). This is in contradiction to the data from Chapter 4 when there was no evidence of an association with age, however, this was in a much younger population. On the whole these results are similar to others published showing no age effect in populations with mean ages <35 (118, 140) but a positive association with age in those with mean age >55 as in Chapter 6 and that of Xu et al (130). Titres of other auto-antibodies, for example RF (130) have been shown to increase with age.

Life-time smoking consumption and smoking habit generally may be a minor influence on anti-hsp65 titre (Chapter 6).

Probably the most important piece of data in this thesis is presented in Chapter 7. There has been suggestions of a link between infection and the pathogenesis of atherosclerosis for many years (reviewed in chapter 2). Mendall's study (73) in 1994 followed in 1995 by a second paper from the same centre (74) both showing an independent association between seropositivity to *Helicobacter pylori* and increased CHD risk, provoked a lot of interest. They postulated (74) that *H.pylori* infection leads to an increase in CHD events by inducing a hypercoaguable state and they presented data indicating an independent positive association

between fibrinogen and *H.pylori* seropositivity. However, a third paper later in 1995 from Belfast (75) showed an odds ratio for CHD events after adjustment for all potential confounders in those with *H.pylori* infection was 1.51, but with a 95% confidence interval of 0.93-2.45 (p=0.1). They did not rule out the possibility of an independent association between *H.pylori* and coronary heart disease but stated that it was unlikely that fibrinogen was the mechanism as they showed a weak negative association with CHD.

The difficulty is in dissecting a possible relationship between two diseases when each is closely related to age and social class (compare Figures 1.2 and 7.2) and it could be that *H.pylori* infection is part of the mechanism by which age and social class lead to increased CHD risk. Thus, it is important to look for a potential causal relationship in other ways and investigation of possible pathogenic processes is one such way (chapter 7).

Firstly, there was a relatively strong and highly significant independent correlation between anti-hsp65 titre and antibodies to *H.pylori* (r=0.38, p<0.00001). Secondly, anti-hsp65 titres were measured serially in a randomised double-blind study of *H.pylori* eradication therapy. In the subjects who received placebo and had continued active *H.pylori* infection, the anti-hsp65 titre remained essentially static over the year of the study. However, in the group who had active therapy and confirmed eradication of *H.pylori* the mean anti-hsp65 titre fell from 25.64 AU/ml to 13.75 AU/ml (p=0.033) (see Figure 7.4).

Further, the data also suggests that about 40% of the anti-hsp65 titre in the population with *H.pylori* infection is due to *H.pylori* infection itself, although there was marked inter-individual variation. It is very likely that exposure to other micro-organisms with cross-reacting hsp60/65 contribute to the measured titres as well. Indeed another interpretation of our data is that the antibiotics given to eradicate *H.pylori* may have eradicated or decreased the load of other chronic bacterial infections and the reduction of these infections may be responsible to a greater or lesser extent for the fall in anti-hsp65 titre. In support of this is our data in from the group who received antibiotics but subsequent breath tests indicated persistent *H. pylori* infection. In this cohort (n=15) there was a trend for anti-hsp65 titre to fall (from 10.3 AU/ml to 9.31 Au/ml). It can at least be concluded that giving antibiotics leads to a fall in anti-hsp65 titre thus indicating that bacterial infections are an important influence on anti-hsp65 titre. In this regard it would be very interesting to examine the influence of *Chlamydia pneumoniae* on anti-hsp65 titres as this organism has also be associated with CHD (reviewed in chapter 2 and see 74,77-80,82-84) and expresses cross-reacting hsp60/65 (76).

Thus, an alternative hypothesis to explain the association between *H.pylori* and ischaemic heart disease based on my data and the evidence of Xu et al is as follows; endogenous hsp60 expression is induced on normal arterial intima by stresses such as hypertension and smoking. Exposure to *H.pylori* and other micro-organisms induces an immune response to bacterial hsp60/62/65 and the antibodies produced cross-react with hsp60.

This could either initiate or contribute to the local inflammatory and autoimmune process in the arterial intima, leading to initiation or worsening of atherosclerotic lesion.

Our data links in very well with some interesting evidence emerging in the last three years since the conception of this thesis, which has implicated elevated levels of CRP as a risk factor for ischaemic heart disease (267) and for the progression of stable (90) and unstable (91) angina. CRP is the major acute phase reactant in humans and the level reflects the stimulation of hepatic production by circulating inflammatory mediators such as cytokines (268). In unchallenged subjects, concentrations are usually low, rising several hundred fold in acute illness (269). The causes of variations in CRP in otherwise normal people has received little attention (267). In the first of these papers in 1994 Liuzzo et al (91) reported a group of 32 patients with UA. Eighteen of 20 patients with CRP > 0.3 mg/dl had a further CHD event during their hospital stay. In contrast, only 2 out of 11 with CRP < 0.3 mg/dl had CHD events ($p<0.001$).

The second paper was reported in 1995 by Thomson et al (90) on behalf of the ECAT study group. They followed 3,043 patients with stable angina for 2 years who had undergone coronary angiography at baseline. The group who experienced further CHD events (n=106) had a mean baseline CRP of 0.22 mg/dl in comparison to those without events (n=2700) with mean baseline CRP 0.16 mg/dl ($p=0.05$). The values were adjusted for age,

sex and all other established CHD risk factors including baseline severity of coronary atherosclerosis and left ventricular ejection fraction.

Thirdly, Mendal et al in 1996 (267) showed in a population based cross-sectional study of 303 white men aged 50-69 years that the independent odds ratio per doubling of CRP for all prevalent coronary heart disease was 1.55 (95% CI, 1.25-1.92). Further, they examined factors which might influence CRP levels and showed that age, smoking, history of chronic bronchitis and seropositivity to *H.pylori* or *C.pneumoniae* were all individually related to increased CRP levels. This supports the idea that the acute phase response is a continuum and not an all or nothing phenomenon. In addition, they showed that triglycerides, cholesterol and fibrinogen were all positively associated with increased levels of CRP.

It is unlikely that CRP reflects underlying quantity or severity of atherosclerosis as we (data from chapters 6 and 11 not shown) and others (90) observed no correlation between CRP and the coronary angiographic extent of disease (Chapters 6 & 11). Elevated CRP is also unlikely to reflect more frequent episodes of ischaemia as we could find no evidence of a correlation between CRP and frequency of anginal episodes (Chapters 6 & 11). Similarly, Liuzzo et al (270) showed that there was no elevation of CRP in patients with variant angina (characterised by severe transmural myocardial ischaemia associated with transient ST segment elevation and accompanied by occlusive epicardial coronary artery spasm). Other more likely possibilities include that serum CRP could be related to the

pathogenesis of atherosclerosis via the effects of inflammation (induced by e.g. smoking, *H.pylori*, chronic bronchitis etc.) on conventional risk factors. Alternatively the raised CRP may result from inflammation in the arterial wall associated with the atherosclerosis itself. A final possibility is that the cytokine and cellular mediators of the acute phase response originating at a distance from the coronary arteries are directly involved in the pathogenesis of atherosclerosis. Potentially a combination of these might be the true explanation and certainly our data would fit very well with the latter two.

Thus, it has been suggested (268) that the biological (inflammatory) state of a coronary lesion might be a more important determinant of the clinical outcome than for example the degree of stenosis. Certainly mechanical testing of aortic fibrous caps indicates that increased foam cell infiltration weakens caps locally, reducing their tensile strength (96). This has been corroborated by immunohistochemical studies showing there are more macrophages at regions of plaque rupture than at unruptured segments (97). This might help to explain why most myocardial infarctions occur as a result of the formation of thrombi on lesions that are not highly stenotic (40) and why knowledge of the angiographic location of coronary stenosis does not allow one to predict the location of subsequent sites of acute occlusion (41). Conversely, elimination of the inflammatory response in the arterial wall may be associated with a decreased risk of subsequent acute events, even without substantial reduction in the degree of atherosclerosis. This may explain the recent findings from a number of large lipid-lowering studies for

the regression of coronary artery disease. In these studies there was only a minor angiographic regression of atherosclerosis, but a dramatic reduction in the rate of a cardiac events (268). Further, in studies of non-human primates, inflammatory cells in atherosclerotic lesions decreased in number after reversion from a high cholesterol to a normal cholesterol containing diet (277).

This emerging data which has changed since the original conception of this thesis indicates that the ideal prognostic assessment of coronary atherosclerosis would measure both the stenotic severity of the disease and, perhaps even more importantly, the biological (inflammatory) activity of the atherosclerosis (268).

In this regard, CRP is currently being assessed in further prospective studies (270). However, the search must continue for other reliable markers of the inflammatory activity of the disease which have prognostic significance (268).

Anti-hsp65 already has an advantage over CRP in that it correlates, albeit weakly, with the stenotic severity of disease. The second stage would be to show that elevated levels of anti-hsps65 have an adverse prognostic significance independently of the angiographic severity of disease, and certainly there is considerable theoretical evidence to suggest that this might be so. Although Chapter 5 indicates that there was no difference between anti-hsp65 titres between stable and unstable atherosclerosis (Figure 5.4), this was performed on small numbers, on a cross-sectional basis and without adjustment for severity of underlying

atherosclerosis (This data was not available for the acute coronary syndrome cohorts). Anti-hsp65 should be assessed prospectively in cohorts of patients with stable and unstable angina. Further, anti-huhsp65 should be assessed similarly as well as in cross-sectional studies. In addition, further basic work to identify which epitopes react with hsp60/65 antibodies and T cells is required. It is also not as yet clear where the sensitisation of hsp60/65 reactive lymphocytes occur and whether we are dealing with a cross-reaction between hsp65 and hsp60 induced by an exogenous antigen (e.g. following an infection) or with a bona fide autoimmune reaction against hsp60.

Thus, in conclusion, we have been unable to substantiate the original hypothesis that anti-hsp65 titres could be a useful clinical diagnostic marker (131) of atherosclerosis. However clearly they may be may be an important prognostic marker, and much further work is required.

12.3 CAROTID IMT IN THE ASSESSMENT OF CORONARY ATHEROSCLEROSIS

Over 40,000 patients have been recruited in more than 20 trials using carotid IMT measurements as a surrogate marker for CHD morbidity and mortality (186). This wide acceptance is remarkable in view of the fact that there has been only one prospective study of carotid IMT with CHD endpoints (220). The other grounds for the use of carotid IMT as a surrogate marker are the close autopsy correlations between carotid and coronary disease (192-195), the associations between carotid IMT and CHD risk factors

(reviewed in Chapter 9) (196-212) and the correlations between carotid IMT with the angiographic severity and extent of coronary atherosclerosis. There is reasonable evidence to support the former two, but insufficient data regarding the latter point. Therefore, in Chapter 8, the correlations between a detailed assessment by B-Mode ultrasound of the extent and severity of carotid atherosclerosis with a detailed angiographic assessment of severity and extent of coronary atherosclerosis were examined for the first time. The results were very disappointing for those who advocate the use of carotid IMT as a surrogate marker. At best the correlations between the severity of coronary atherosclerosis and carotid IMT score (CCA_{MEAN}) was 0.29 ($p=0.016$). In the last three years, two other studies (186,219) have examined correlations between continuous assessments of carotid IMT and coronary atherosclerosis in similar but less detailed manner than my study, and their results (correlations ranging from 0.23 to 0.29) were also very similar.

This data casts significant doubt on the validity of carotid IMT as a surrogate for CHD morbidity and mortality. However, further prospective studies of carotid IMT are underway and these should clarify this definitively.

However, it is likely that carotid IMT by itself will have a limited future as a surrogate marker for CHD events, not least because it poorly correlates with the stenotic severity of atherosclerosis. Further, it is difficult to see how a physical measurement of plaque and wall thickening will be able to reflect the biological, inflammatory activity of plaques.

Carotid IMT may continue to have a role to play as a surrogate in conjunction with a marker of biological activity. In addition, it is likely to continue to have a role in large scale epidemiological studies of pre-morbid atherosclerosis such as the ARIC study.

Also In Chapter 8, data is presented towards answering a number of other issues regarding the measurement of carotid IMT (72). Adjustment for body size or mass or carotid artery diameter does not importantly improve the correlations between the extent and severity of atherosclerosis in the two vascular beds. Also coronary atherosclerosis correlated better with CCA_{MEAN} IMT than with BIF_{MEAN} or more complex IMT scores, combining measurements from multiple coronary artery sites, suggesting that the former should be used. This is perhaps because the contribution of various CHD risk factors is different between the two carotid artery sites and that the risk factor profile of CCA_{IMT} is more similar to that of coronary atherosclerosis than that of BIF_{IMT} and we show some data to support this (Table 9.11). Certainly we and others (188) show that various risk factors differ in the relative importance between the two vascular beds and this further casts doubt on the validity of carotid IMT as a surrogate marker of CHD.

12.4 FIBRINOLYTIC FACTORS IN THE ASSESSMENT OF CORONARY ATHEROSCLEROSIS

One paper by Negri et al (150) in 1993 suggested that PAI-1 antigen might be a useful diagnostic marker of the severity of coronary atherosclerosis, demonstrating a correlation of 0.55. To my knowledge this is a higher

correlation than with any other peripheral blood marker. It has great potential as a marker as *in vitro* and animal studies had suggested that PAI-1 antigen might be involved in the pathogenesis of each of chronic atherosclerosis, plaque rupture and thrombosis.

Improvements to Negri et al's (150) methodology were made, to investigate whether the correlations were even greater than they reported. However we could demonstrate no evidence of a correlation between PAI-1 antigen (or the other fibrinolytic parameters, PAI-1 activity or t-PA antigen) and measures of the severity and extent of coronary atherosclerosis. The marked discrepancy between our results and those of Negri et al are difficult to explain, but may be due to the confounding influence of triglycerides.

Whilst none of the fibrinolytic parameters were useful in assessing the stenotic severity of coronary atherosclerosis, t-PA antigen has some promise as a prognostic marker in other studies (90,250 see Chapter 10). The data in Chapter 11 did produce some interesting insights into factors which regulate endogenous fibrinolysis. Previous findings that components of the insulin-resistance syndrome, hyperinsulinaemia, increased weight, increased central obesity, hypertension and triglyceridaemia are all related to fibrinolysis and that the most important relationship for PAI-1 antigen and activity is with insulin were confirmed. Levels of PAI-1 antigen was the most important determinant of t-PA antigen in our study and this helps to explain the apparently paradoxical finding that higher levels of t-PA antigen (90,250) carry a worst prognosis. This data certainly agrees with the consensus (239)

that the elevated t-PA antigen reflects increase in t-PA and PAI-1 complexes and that high measured t-PA is secondary to high PAI-1 antigen and overall reflects reduced fibrinolytic activity.

For the first time we showed that levels of γGT seem to be an important influence on PAI-1 antigen levels in a normo- triglyceridaemic population. The source of the majority of plasma PAI-1 is unclear (226) but our data suggests that hepatic production is important. γGT reflects increasing hepatic liver enzyme induction generally, and potentially increased PAI-1 antigen production. Alternatively, there may be reduced hepatic clearance of PAI-1 antigen.

12.5 CONCLUSIONS

The first objective of this thesis was to investigate techniques for non-invasively assessing atherosclerosis for four main potential uses. Unfortunately the main conclusion from this thesis is that the techniques we assessed are not very promising.

On the basis of data which has largely been published since the conception of this thesis, it has been suggested (268) that the biological (inflammatory) state of a coronary lesion might be a more important determinant of the clinical outcome than for example the degree of stenosis. It is likely therefore that the most accurate prognostic assessment of coronary atherosclerosis in the future will combine a physical measurement of

stenotic severity with a biological measurement of atherosclerotic inflammatory activity.

In my opinion the peripheral inflammatory blood markers, including anti-hsp65, hold most promise for the assessment of the biological activity of coronary atherosclerosis and require urgent prospective validation. These may be combined in the future with a non-invasive assessment of the physical severity of coronary lesions by MRI or by measuring a surrogate like carotid IMT. The work of Lees et al (274) using radiolabelled peptides derived from an apolipoprotein holds great promise. The radioisotopes are mostly taken up into unstable plaques. Thus, potentially this technique could give information on both the physical severity and biological activity of lesions. The thesis was more successful in achieving the second objective in examining pathogeneic mechanisms of atherosclerosis and athero-thrombosis. Most importantly elevated anti-hsp65 is shown to be a risk factor for coronary atherosclerosis. Intriguing data is presented suggesting that *H.pylori* and (possibly other micro-organisms) may be important influences on anti-hsp65 titre and thus be involved in the pathogenesis of atherosclerosis. This interesting observation requires urgent further investigation. Novel data is also presented suggesting that γGT maybe an important influence on endogenous fibrinolytic function.

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APPENDIX 1 PUBLICATIONS FROM THIS THESIS

Birnie D, Hood S, Holme E, Hillis WS. Anti-heat shock protein 65 titres in acute myocardial infarction. Lancet 1994; 344: 1443.

Birnie D, McKay IC, Veitch J, Whaley K, Hood S, Hillis WS, Holme ER. Antimycobacterial hsp65 and rheumatoid factor titres in a population of normal twins: evidence of genetic control of rheumatoid factor. Clin and Exp Immunol 1995; 101: 393-397.

Birnie D, Holme ER, McKay IC, Hood S, McColl KEL, Hillis WS. Association of anti-hsp65 antibodies with coronary atherosclerosis : possible role of *Helicobacter pylori* infection. (submitted to: European Heart Journal Feb 1996).

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Birnie D, Hood S, Booth N, Hillis WS. The relationship between endogenous fibrinolysis, angiographic severity and extent of coronary atherosclerosis and cardiovascular risk factors. (manuscript in preparation).

Birnie D, Hood S, Booth N, Hillis WS. The relationship between endogenous fibrinolysis and liver function. (manuscript in preparation).

APPENDIX 2 PRESENTATIONS FROM THIS THESIS

Birnie D, Veitch J, McKay IC, Whaley K, Hillis WS, Holme E. Antimycobacterial hsp65 and rheumatoid factor titres in a population of normal twins. Medical Research Society 13-14 April 1994. (Abstract: *Clin Sci* 1994; 87:9P-10P).

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