GENETIC POLYMORPHISMS AND THE RISK OF CORONARY ARTERY DISEASE.

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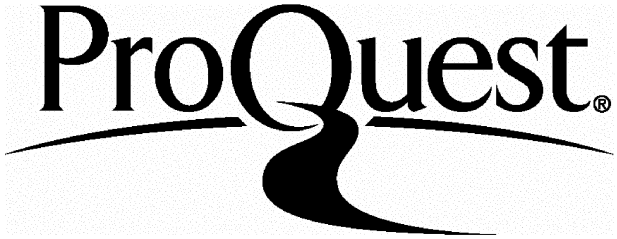

0.1. ABSTRACT

Background

Myocardial infarction involves the three processes of atheroma development, plaque rupture and formation of a thrombus. Proliferation of smooth muscle cells and vasoconstriction are important aspects of atheroma formation and are influenced by the renin-angiotensin system. Angiotensin converting enzyme (ACE) pivotal to this system has recently been shown to be influenced by an insertion/deletion (I/D) polymorphism in the ACE gene. Patients homozygous for the D allele have the highest circulating levels. Inhibition of fibrinolysis through raised levels of plasminogen activator Inhibitor (PAI-1) has also been shown to be under the control of a PAI-1 promoter single nucleotide insertion/deletion (4G/5G) polymorphism. Patients homozygous for the 4G allele have the highest levels of circulating PAI-1.

Aims

The aim of this thesis, was to investigate the role of both polymorphisms in relation to atherothrombosis in subjects with coronary artery disease (CAD).

Results

609 Caucasian patients (420 males 189 females) admitted for angiography for known or suspected coronary artery disease were recruited from two centres. Patients were classified as having no significant coronary artery disease (20%), single (21%), double (21%) and triple vessel disease (38%) on the basis of 50% stenosis. Both the ACE genotype and the PAI-1 genotype were associated with their respective circulating levels ($P= 0.0008$) and ($P = 0.0001$) respectively.

There was no relationship between the ACE genotype or levels with either the degree of coronary stenosis or a history of MI. In contrast, the 4G/4G genotype was significantly related to a history of myocardial infarction, an association which was stronger in the group with pre-existing significant atheroma.

Conclusions

These data suggests that the ACE genotype-activity does not influence the atherothrombotic process, whereas the PAI-1 promoter polymorphism influences the development of myocardial infarction through its effects on thrombus formation in patients with pre-existing atheroma.

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ABBREVIATIONS

A-I	Angiotensin-I
A-II	Angiotensin-II
ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of variance
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CAM	Cell Adhesion Molecules
CASS	Coronary Artery Survival Study
DD	Deletion Deletion
ECAT	European Concerted Action on Thrombosis and Disabilities
ELISA	Enzyme-linked Immunosorbant Assay
4G/4G	4 Guanine/4 Guanine
4G/5G	4 Guanine/5 Guanine
5G/5G	5 Guanine/5 Guanine
FVII	Factor VII
HDL	High Density Lipoprotein
HUVEC	Human Umbilical Vein endothelial Cells
IHD	Ischaemic Heart Disease
II	Insertion Insertion
ID	Insertion Deletion
LDL	Low Density Lipoprotein
MCP-1	Monocyte Chemotactic protein -1
MI	Myocardial Infarction
NIDDM	Non Insulin Dependent Diabetes Mellitus
PAI-1	Plasminogen Activator Inhibitor –1
PCR	Polymerase Chain Reaction
PCS	Point of Critical Stenosis
RAS	Renin Angiotensin System
TG	Triglyceride
t-PA	tissue Plasminogen Activator
VLDL	Very Low Density Lipoprotein
vWF	von Willebrand Factor

ATTRIBUTIONS

My personal contributions to this thesis include the following:

- The recruitment of all cases and control subjects. I devised and implemented the recruitment protocol for the cases and controls
- I obtained the necessary ethical permissions for the haemostasis arm of the study. I was responsible for consenting the subjects in this study
- I collected the patient and control clinical data, made all the anthropometric measurements and took the blood samples
- I verified all of the clinical data from hospital records, in particular the verification of cardiac events (myocardial infarction) met the WHO criteria.
- I identified all of the cases and made all the follow-up appointments
- I completed all of the data collection forms and entered them onto the database and was responsible for data protection
- I processed all the blood samples.
- I extracted and quantified the DNA from the samples on all subjects
- I genotyped the samples for the ACE I/D polymorphism and PAI-1 4G/5G polymorphism.
- I also undertook a number of the PAI antigen assays and with the assistance of Angela Carter undertook the fibrinogen and vWF assays. ACE levels and other biochemical tests were performed in the Chemical Pathology Department at the LGI.
- Coronary angiography were performed and reported by Consultant Cardiologists at the LGI and Pinderfields Hospital. I was involved both in the procedures and reporting in about half the patients.
- I wrote all the chapters in this thesis.
- The statistical methods were mainly performed with the help of a Statistician Daren Greenwood from the University of Leeds.
- This thesis was typed by Hilary Davies and printed by myself and I accept total responsibility for the accuracy of the contents.
- My thesis was supervised by Professor Grant with advice from Professor John Yudkin (London Advisor)

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1.0 PREFACE

In most industrialised countries Ischaemic heart disease (IHD) is the commonest cause of death. In England and Wales 30% and 22% of all deaths amongst men and women respectively are the results of IHD. In recent years, in addition to 156,000 deaths every year in England and Wales, there have been an average 115,000 hospital discharges with the diagnosis of IHD.[1]

Sixty percent of all myocardial infarction (MI) fatalities occur in the first hour after the episode. Hence most IHD deaths occur too rapidly for treatment to influence prognosis.[2] It is clear therefore, that prevention of MI is of major Public Health importance.

IHD emerged as the major cause of death in the twentieth century, this correlated with a decrease in infectious disease mortality on the one hand and an increase in age-specific risk of IHD on the other. In the late 1960's, this "epidemic" seemed to have reached its peak and started to decline in countries such as the USA and Finland. This decline over a relatively short period of time suggests that the causes of this disease are either modifiable and/or preventable.

The major manifestations of IHD are MI (fatal and non-fatal), sudden cardiac death, chronic angina pectoris and unstable angina. These ischaemic syndromes present themselves with different pathological backgrounds and risk factors.

In the search for the aetiology of IHD and its manifestations, there are 2 major lines of research which need to be pursued; 1) the pathophysiology of atherosclerosis and thrombosis and 2) epidemiological methods to define risk factors for IHD.

Both experimental animal studies and clinical evidence indicate that atherosclerosis is the basic requirement for the development of IHD. Furthermore, thrombosis within the coronary arteries plays a critical role in the initiation of the major IHD events - MI and sudden cardiac death.[3] Moreover, there is interplay between the atherosclerotic and the thrombotic processes in the development and progression of atherosclerotic plaques.[4] The balance between fibrinolytic activity and coagulation may have an important role in determining whether thrombi, once formed, persist and increase in size or undergo lysis.

The classical risk factors that have been associated with CAD (smoking, hypertension, hypercholesterolaemia, diabetes and family history) do not fully account for the risk of developing CAD. Attention has focused recently on the influence of genetic

polymorphisms on circulating proteins in the haemostatic and renin-angiotensin systems (RAS).

This thesis focuses on two circulating proteins; plasminogen activator inhibitor –1 (PAI-1) and angiotensin converting enzyme (ACE). ACE plays a key role in the modulation of vascular tone and smooth muscle cell proliferation, both of which are important aspects of the atherothrombotic process. PAI-1 is the main inhibitor of fibrinolysis, and thus high levels of PAI-1 lead to a pro-thrombotic state. Both ACE and PAI-1 levels in the plasma have been shown to be influenced by genetic polymorphisms in their respective genes. While the levels of these circulating proteins tend to vary (because of the acute phase reaction or the effects of other environmental factors) genes do not change. The study of the relationship between these genetic polymorphisms and CAD may therefore enhance in a significant way our understanding of the associations between the Fibrinolytic and the RAS system and CAD.

This thesis first reviews the processes leading to atheroma and thrombosis, with emphasis on the possible role of PAI-1 and ACE. Secondly, the risk factors for CAD are reviewed. Thirdly, a review of the literature is undertaken with regard to ACE and PAI-1, and their relation to CAD. Finally, gender differences in coagulation and fibrinolysis with respect to CAD are discussed.

2.0 INTRODUCTION

2.1 DEVELOPMENT AND MORPHOLOGY OF ATHEROMA

2.1.1 Atheroma

Atheroma is a focal, intimal disease of arteries. The development of an atheromatous plaque begins with the accumulation of lipid-filled macrophages at focal sites within the intima. .[5] These lesions seen as flat yellow dots or lines macroscopically on the intima are known as the fatty streak. Many such fatty streaks remain static or even vanish. They are common, for example, in young individuals from populations in whom CAD is virtually absent. .[5] Indeed such adults often have fewer fatty streaks than children do from the same population, suggesting that some fatty streaks must disappear. .[5] Progression from the fatty streak stage is associated with extracellular lipid within the intima, smooth muscle cell proliferation then develops with the formation of a layer of cells covering the extra cellular liquid, and separating it from the adaptive smooth muscle thickening in the intima. Collagen is then produced in larger and larger amounts by the smooth muscle cells. This sequence of events culminates in the formation of the "advanced plaque".

2.1.2 Forms Of Advanced Plaque

Most advanced plaques have a lipid core composed of extra cellular cholesterol and cholesterol esters, some of which is in crystalline form. The lipid core is soft and can be extruded by pressure. The lipid core occupies anything from 5% to more than 70% of overall volume of plaque. There are 3 types of advanced plaque; soft, hard and gelatinous. Lesions containing a lot of lipid are soft in contrast to hard lesions with a high collagen content. As we shall see, soft and hard lesions have important clinical implications in relation to the risk of subsequent coronary events. The final type, known as the gelatinous lesion is a raised soft semi-translucent brown plaque, which contains no foam cells and has an oedematous connective tissue stroma. [6]

Patients show every possible combination of plaque types in their coronary arteries. It is not known whether lipid rich plaques evolve spontaneously into purely fibrous plaques or vice-versa. The significance of gelatinous plaques is unknown.

The formation of much of the lipid core is thought to take place by cell necrosis (or by apoptosis), [7]other evidence suggests that dead macrophages release lipid into "the

core".[8] In addition, smooth muscle cell death and destruction of the plaque collagen by proteases creates the space within the connective tissue matrix, which becomes filled with lipid debris, producing the lipid core, which is highly thrombogenic. It contains lipid in a lamellar form on which coagulation is rapid, collagen with its ability to initiate platelet aggregation and tissue factor (produced by the macrophages) which has an important role in the coagulation system.[9]

The fibrous cap is a structure of considerable importance separating the highly thrombogenic core within the intima from the arterial lumen. The raised plaque may either grow slowly and encroach on the lumen or become unstable, rupture and undergo thrombosis to produce acute obstruction. These two processes however, are not mutually exclusive, and plaque growth often involves sub-clinical thrombosis. Several epidemiological studies have shown that in populations the proportion of the intima of coronary arteries occupied by raised plaques at necropsy mirrors the incidence of clinically expressed IHD in that population. [10] Furthermore, other studies have showed that populations with risk factors for IHD have more raised plaques than those without these risk factors. Three issues are worth noting from the relationship between atheroma formation and clinical events. Firstly, individuals may sustain an acute coronary syndrome from a single plaque in a strategically important vessel such as the left main stem rather than several plaques in, for example, a small distal right coronary artery. Secondly, the composition of the plaque is of greater importance clinically than the size or number of plaques. Furthermore, the advanced plaque may not always be seen angiographically.

2.1.3 Inflammatory And Immune Mechanism In Atherogenesis

Recent evidence suggests an increasing role for inflammatory and immune mechanisms in the development of atheroma. This is evident in the close association of macrophages and T lymphocytes, both of which are present in large numbers in the region of the plaque.[11]

These cells are able to release a large number of proteases, cytokines and growth factors. A key step is the adhesion of these cells to the intact endothelial surface. Activated endothelial cells express adhesion molecules on their receptors for which monocytes and T cells have specific ligands. These adhesion molecules include E-selectin and cell adhesion molecules (CAM).[12] Most endothelial adhesive molecules are

induced by stimuli such as cytokines. In addition modified LDL may also induce monocyte adhesion.

Adhesion is followed by migration of monocytes through endothelial cell junctions to enter the intima by chemotaxis. Monocyte Chemotactic protein (MCP-1) is the most powerful chemo-attractant and is released from endothelial cells, smooth muscle cells and monocytes after stimulation).[12]

Inhibitors of monocyte migration include nitric oxide, prostacyclin and possibly fish oils. The role of T lymphocyte in the atheromatous plaque remains uncertain. One function appears to be the modulation of the inflammatory process, in particular the monocyte. T-cells produce Interferon-γ, a potent growth inhibitor for both endothelial and smooth muscle cells. Both Interferon-γ and TNF-α produced by macrophages and lymphocytes down-regulate the scavenger-receptor on the macrophage, inhibiting foam cell formation. Adjacent to the plaque, in the adventitia of the artery, B-lymphocytes and plasma cells accumulate indicating an antibody response to antigens such as oxidised LDL in the plaque. These circulating antibodies have been used to predict the progression of clinical disease.[13] Oxidised LDL/antibody complexes can be taken up by the Fc receptor of monocytes and provide a further mechanism for intracellular accumulation of lipid.

The phenomenon of infiltration of the coronary arteries by inflammatory mononuclear cells (revealed in autopsy studies) raised the possibility that atherosclerosis has an important inflammatory component in its pathogenesis. This chronic inflammatory disease is characterised by foci of monocytes or macrophages and T lymphocytes in the arterial wall, as well as proliferation and migration of vascular smooth muscle cells, matrix formation and neo-vascularization.

2.1.4 Infective Agents and CAD

Evidence has emerged to implicate infective agents in the inflammatory process leading to the formation of atheroma. Specific antigenic components of *Chlamydia pneumoniae* have been demonstrated in atherosclerotic lesions but not elsewhere in the arterial wall. Furthermore, specific antibodies to this pathogen can be identified serologically in the blood of many victims of CAD or MI. *Helicobactor pylori (H. Pylori)* has also emerged as an important infective agent implicated in the pathogenesis of CAD.[14; 15] Indirect evidence includes studies suggesting that childhood deprivation (a condition with a high prevalence of *H. pylori* infection) may influence adult risk of CAD.[16] Chronic bacterial

infections have also been linked to the risk of CAD in adults. *H. pylori* infection is a chronic bacterial infection of the stomach that is usually acquired in childhood. It causes an active gastritis in all subjects infected with it, and is now known to be the causative agent in peptic ulcer disease, and probably gastric cancer.[17] These gastric conditions are more prevalent in patients with CAD. *H pylori* infection is acquired in childhood and related to poverty and may partly explain the link between childhood poverty and CAD. Other evidence comes from *H pylori*-infected patients who have been shown to have higher levels of TNF-α and interleukin-6 in their gastric antrum than others not affected. This has led to the suggestion that infection with *H pylori* can promote a low grade chronic inflammatory response, a process which is integral to atherosclerosis.[18]H. *pylori* has also been associated with heat shock proteins which have been implicated in the formation of CAD. Its relationship to circulating levels of IHD risk factors such as fibrinogen and factor VII:C are more controversial. .[18]

2.1.5 Angiotensin In Smooth Muscle Cell Proliferation

The Renin-angiotensin system (RAS) is involved in the regulation of the growth factors that control smooth muscle cell proliferation. Angiotensin-II (A-II) plays a pivotal role in this system (see section 4.2). A-II has been shown to promote growth of vascular smooth muscle cells in certain conditions. Because A-II is a powerful vasoconstrictor, and calcium is involved in the regulation of muscle growth, it has been proposed that by increasing the level of cytosolic calcium, prolonged stimulation of vascular smooth muscle may lead to myointimal proliferation.[19] Support for this hypothesis is the finding that A-II also activates the growth-stimulating proto-oncogenes including c-fos via protein kinase C.[20] Furthermore, there is evidence that A-II may stimulate growth of the vascular matrix and in particular collagen. . .[19] Because increased arteriolar growth leads to a decreased ratio between the lumen and the media of the arterioles, which increases the arteriolar vascular resistance, such a growth promoting potential of A-II can have an important effect on the systemic arteriolar resistance and hence on blood pressure and atheroma formation.

A-II receptors have now been identified in cardiac myocytes. A role for cardiac RAS as a growth regulator has been proposed. .[19] In addition to effects on proto-oncongenes, A-II appears to up-regulate the cardiac hypertrophic response by inducing the angiotensinogen gene and the gene for transforming growth factor. This has provided the link between A-II and left ventricular hypertrophy (LVH). In the Framingham

study,[21] LVH was a powerful risk factor for IHD. The physiological effects of A-II receptor stimulation could include inotropic and chronotropic changes but whether it is circulating or myocardial RAS that is involved is not clear. The production of A-II is catalysed by the angiotensin-converting enzyme (ACE). As discussed below, circulating levels of ACE have been shown to be partly determined genetically.[22] This implies that smooth muscle cell proliferation and atheroma formation could be influenced by possession of different ACE gene polymorphisms.

2.1.6 Vascular Control

Though atheroma is a focal disease, it is associated with abnormalities of vascular tone in affected arteries which favour vasoconstriction often at inappropriate times such as exercise. A dominant factor governing vascular smooth muscle tone is the release of noradrenaline from terminal neurones into the synaptic space, with stimulation of the post-synaptic vasoconstrictory $alpha_1$-and $alpha_2$-receptors. .[19] By altering the rate of noradrenaline release, a large number of hormones and autonomic signals can achieve indirect control of the degree of vasoconstriction. The RAS modulates vascular tone directly and indirectly by the actions of angiotensin-II (A-II). A-II acts to increase the rate of release of noradrenaline from the terminal neurone by its action on the pre-synaptic A-II receptor. This is independent of its direct vasoconstrictory effect on the vascular receptor. As we have intimated above, circulating levels of ACE are genetically influenced indicating another potential mechanism by which genetic polymorphism of ACE could be implicated in atherogenesis.

2.1.7 Fibrinogen And Plaque Growth In Atherogenesis

Fibrinogen crosses the normal endothelium to enter the intima. At the sites of potential plaque formation more fibrinogen enters and is retained for a longer period.[23] Within the plaque, thrombin is generated by macrophage activity leading to intra-intimal fibrin deposition. Fibrin generation and degradation by plasmin within the plaque are held in balance, with the former dominating in growing plaque.[24] Both thrombin and fibrin degradation products are potent stimulants of smooth muscle growth. This implies that the fibrinolytic system does have a key role in atherogenesis. Consequently, if genetic polymorphisms do influence circulating levels of the key components of the fibrinolytic system, they may be important risk factors in atheroma formation.

2.1.8 Vascular Remodelling And The Media In Atherosclerosis

The arterial media is a changeable structure, and has a considerable capacity to remodel. As atheroma develops, the vessel wall adapts to preserve the luminal dimensions for as long as possible. Consequently, the external diameter of the vessel increases. This adaptation is mainly achieved by rearrangement of medial smooth muscle. Glagov et al have shown that the intima has to be increased by more than 40% of the original cross

sectional area of the vessel before this capacity of the arterial wall to accommodate the plaque is overcome.[25]

Many advanced coronary plaques have a more fundamental local effect on the media immediately behind the lesion. The media undergoes thinning and atrophy with loss of medial smooth muscle cells. This results in plaque bulging outward rather than inward. The external outline of the vessel becomes eccentric although the lumen remains circular in shape.

The result of the two processes, medial rearrangement and destruction, is that coronary angiography will always underestimate the amount of intimal disease to a wide and unpredictable degree. [26] Many angiographically normal coronary arteries will contain advanced plaques. A new lesion developing on angiography between 2 successive examinations does not indicate that a new plaque has developed. It may simply be due to a previously existing advanced lesion that has undergone sufficient enlargement that the capacity of the vessel to remodel has been overcome.

In ectasia the increase in the external diameter of the artery is so excessive that even though atheroma is present within the intima, the lumen is enlarged rather than narrowed. Such ectatic segments may alternate with segments in which atherosclerotic narrowing has developed. Morphologically, two factors mark ectatic segments; 1) diffuse involvement of the intima 2) an extreme degree of medial smooth muscle loss. For these reasons any angiographic study has some limitations, and these are considered further in a later section.

2.1.9 The Progression Of Atheroma

Lipid accumulation and smooth muscle proliferation inherent in the primary process of atheroma formation are in themselves capable of slowly increasing plaque volume to a degree at which the lumen becomes compromised. Sudden increases in plaque volume that are responsible for angiographic progression are due to the incorporation of thrombus into the plaque.[27] This process can invoke florid smooth muscle proliferation as a repair process. A plaque may become unstable and be complicated by formation of a thrombus. The latter may itself encroach on or occlude the arterial lumen or break away and embolize and impact in smaller more distal vessels. . .[27]

Plaque formation begins in early life, and by the third decade advanced plaques are common in populations in the developed world. These advanced plaques do not often cause symptoms and may not be seen angiographically. The transition from

asymptomatic to the symptomatic phase is related to complications occurring in relation to the plaque, principally plaque rupture and thrombus formation.

Information about the progression of coronary disease is derived from angiography in subjects without acute events. Such studies are limited by the insensitivity of the techniques for non-stenosing plaques. However, in general, progress of an atheromatous plaque is slow and intermittent rather than a steady linear progression. Furthermore, progression is largely due to the appearance of new angiographic lesions rather than the growth of pre-existing lesions. [28] Moreover, high-grade and occluding lesions often develop in segments of the artery previously judged to be normal or to have minor disease.[28; 29]

2.1.10 Plaque Stability

A greater understanding of the factors that lead to plaque instability and rupture causing thrombosis is increasing rapidly. Unstable plaques have morphological characteristics that have given insight into the structural and cellular features of presently stable plaques. The risk of any individual with coronary atherosclerosis developing an acute ischaemic event depends on the number of such vulnerable plaques present in that individual rather than the number of plaques overall.[30]

Necropsy studies and tissues from atherectomy have compared stable and unstable plaques. These studies have shown the latter to have a large core of extracellular lipid, a high density of macrophages containing lipid, a reduced smooth muscle content, and a thin cap. [31; 32] In plaque rupture, the fibrous cap of a plaque tears, exposing the highly thrombogenic lipid core to blood in the lumen of the artery. [33; 34]The mechanical strength of the plaque cap is therefore a vital component of plaque stability and depends on the amount and organization of collagen and other connective tissue proteins. Smooth muscle cells exist in lacunae in the plaque cap, where they produce and maintain the connective tissue matrix on which the cap integrity depends. The production and degradation of the matrix are held in balance, and the cap tissue is therefore dyanamic.[35] Both sides of this equation are detrimentally altered by inflammatory processes within the plaque. A reduction in the smooth muscle cell density will inevitably lead to a decline in connective tissue synthesis. There is growing evidence that smooth muscle cell death by apoptosis occurs in plaques, perhaps related to a decline in growth factors

needed for their maintenance or to the activity of macrophages in the vicinity producing reactive oxygen species(ROS).[36] Interferon-γ production by lymphocytes depresses collagen synthesis by smooth muscle cells.[35] Enhancement of the catabolic side of the equation of connective tissue synthesis, however, is probably more important.

2.1.11 The Metalloproteinases

Connective tissue matrix proteins are degraded by a range of proteases, the most widely studied of which are the metalloproteinase family.[37] There are at least 12 members of this family, with a large range of molecular weights and with considerable individual variation in their affinity for different components of the connective tissue matrix. One form membrane-type matrix metalloproteinase (MT-MMP) is bound to cell membranes, and its activation plays a role in cell migration. Those with the ability to initiate or enhance the degradation of collagen include interstitial collagenase (matrix metalloproteinase-1 or MMP-1), gelatinase B (MMP-9), and stromelysin (MMP-3). Although several cell lines in the plaque, including smooth muscle cells and basophils, produce metalloproteinases, the major source is the macrophage. A feature common to all these metalloproteinases is that they are secreted into the extracellular milieu as an inactive precursor that is then converted to an active lower-molecular-weight enzyme. The same cell type, although not necessarily the same cell, also produces tissue inhibitors of metalloproteinases (TIMPS), which bind to and neutralize the active enzyme. Control of the catabolism of connective tissue is potentially exerted at three levels. The first is in the transcription and secretion of metalloproteinases by the macrophage, the second is at the activation point, and the third is at the level of inhibition because of binding of TIMPS to the active enzyme.

Observational studies on human plaque tissue have used either in situ hybridization to show metalloproteinase mRNA or immunohistochemistry to show the metalloproteinase itself.[38] The difficulty of such observational studies is that most of the antibodies used recognize both the active and inactive forms of the metalloproteinase and therefore give no indication of the balance of the active enzyme with its inhibitor. Nevertheless, these observational studies have shown large amounts of, in particular, MMP-9 (gelatinase B)[39] and MMP-3 (stromelysin) in macrophages in unstable plaques. A biological assay of dynamic enzyme activity within the plaque can be made by placing the tissue section on a gelatin sheet and observing where lysis occurs.[40] This approach has confirmed that

an excess of active enzyme over its inhibitor is present in unstable human plaques and is maximal at vulnerable areas in the cap.[41]

These data suggest that one potential way of inhibiting or preventing atherosclerotic plaque progression and clinical events is to reduce metalloproteinase production or activation. Although MMP-9 is emerging as a major member of the metalloproteinase family in the context of plaque events, it must be remembered that its proenzyme is constitutively expressed by monocytes and macrophages, for example in fatty streaks, long before any question of instability of the plaque arises. Mechanisms must therefore exist for the upregulation of expression, enhanced release of the proenzyme, or increased extracellular activation. Tumor necrosis factor-α and interleukin-1 are known to upregulate metalloproteinase activity by macrophages in culture[42] and are one way in which enhanced inflammatory activity in the plaque leads to a detrimental effect. The interaction of macrophages with lymphocytes using CD40 and its ligand also upregulates metalloproteinases.[43] Although the classic activation pathway for metalloproteinases in the tissues is by plasmin,[44] there is also now evidence that active metalloproteinases can further activate the proenzymes in the adjacent tissue, that MT-MMP will induce activation, that mast cells may play a role, and finally, that ROSs can lead to direct activation of the proenzyme.[45]

2.2 HAEMOSTASIS

The haemostatic process is often subdivided into five somewhat overlapping phenomena: vasoconstriction, platelet adhesion, formation of the platelet plug, coagulation and fibrinolysis. The first event in haemostasis in response to vascular injury is vasoconstriction. The majority of vessels are capillaries where smooth muscle cells are absent and haemostasis depends on direct sealing. Breaches in arterioles and veins on the other hand depend on vasoconstriction, followed by activation of platelets and coagulation components.

Vessel injury exposes subendothelial tissue with various elements to which platelets can adhere.[46] Collagen and fibronectin interact readily with platelet membrane glycoproteins. Several adhesive proteins such as von Willebrand factor (vWF) strengthen the initial bonds. Through the action of activators such as collagen and

eventually thrombin, the adhered platelets soon become activated, whereby platelet receptors are expressed and several mediators released. These mediators include, adenosine diphosphate (ADP), serotonin, thromboxaneA_2, platelet derived growth factor and vWF. These potent inducers of platelet aggregation are capable of recruiting additional circulating platelets, which in turn adhere and transform the initial monolayer of platelets into an aggregate. The platelet glycoproteins IIb-IIIa on the platelet membrane undergo a conformational change in the activation process, so that they can interact with plasma fibrinogen and other adhesive proteins such as fibronectin which serve to link platelets together into a tighter aggregate. As fibrinogen is required for platelet aggregation and is an integral part of the coagulation system, the two systems interrelate. Activated platelets exhibit procoagulant activity through platelet factor 3 (PF3) which presents a platelet surface for the activation of thrombin by factor Xa and also increases the activation of factor X to factorXa by factorXI.

2.2.1 The Coagulation System

Maintenance of haemostasis requires the formation of fibrin clot by the coagulation system. Activated platelets rearrange their surface lipoproteins so that phospholipids on which coagulation factors can concentrate are exposed to the bloodstream. Furthermore, they markedly accelerate the formation of thrombin, which occupies a central position in the coagulation process. Thrombin is formed as the end-result of a chain of reactions, which transform in sequence a number of coagulation factors present as precursors (zymogens) in plasma into activated factors. The reactions mainly occur on the membrane of activated platelets and other stimulated cells and tissue factor (a membrane protein that is exposed to the blood for example after trauma) on which these factors bind. Because of the low concentration of coagulation factors in plasma and the abundance of circulating inhibitors, the interaction of procoagulants and their subsequent activation would proceed only slowly unless they concentrate on phospholipids where they are protected from inhibitors. Coagulation factors are activated mainly through limited proteolysis.

The traditional scheme of the coagulation cascade distinguishes an intrinsic from an extrinsic activation pathway. The two pathways both result in the activation of factor X which converts prothrombin to thrombin a final common pathway to fibrin formation. The two pathways do not act in isolation, and there is in addition cross-activation of the

two pathways.

The extrinsic pathway is triggered when tissue factor present on non-vascular cells is exposed to blood. Factor VII (FVII) binds to tissue factor and is rapidly activated to FVIIa. FVIIa then activates factors IX and X leading to thrombin generation. That FVII is essential to ensure normal haemostasis is underlined by the bleeding condition of patients with severe congenital FVII deficiency.

The reaction sequence in the intrinsic pathway is initiated by contact of platelets and/or coagulation components with subendothelial tissue. The initial contact phase involves factor XII (Hageman factor), prekallikrien and high molecular weight kininogen. The activation of factor XII is facilitated by kallikrien. The latter is generated by small traces of FXIIa from prekallilrien. FXIIa now converts FXI to FXIa. Factor XIa activates FIX to IXa which then catalysis the activation of factor X in the presence of phospholipid and factor VIII. See fig 1.

In the common coagulation pathway factor Xa generates thrombin from prothrombin in the presence of phospholipid. Thrombin cleaves fibrinopeptides A and B from fibrinogen to produce fibrin. Thrombin also activates factor XIII which in the presence of ionised calcium stabilises fibrin in a cross-linked mesh. Thrombin also promotes the activation of factors XI, VIII, and V.

The cascading nature of the coagulation system requires effective inhibition to prevent consumption of coagulation factors, which generally circulate in low concentrations. Factor VII is inactivated by the formation of a complex involving a lipid-associated coagulation inhibitor (LACI), tissue factor, phospholipids and calcium ions. LACI is the same molecule as the extrinsic pathway inhibitor (EPI) and appears to be the only plasma inhibitor of the catalytic activity of factor VIIa-tissue factor complex.

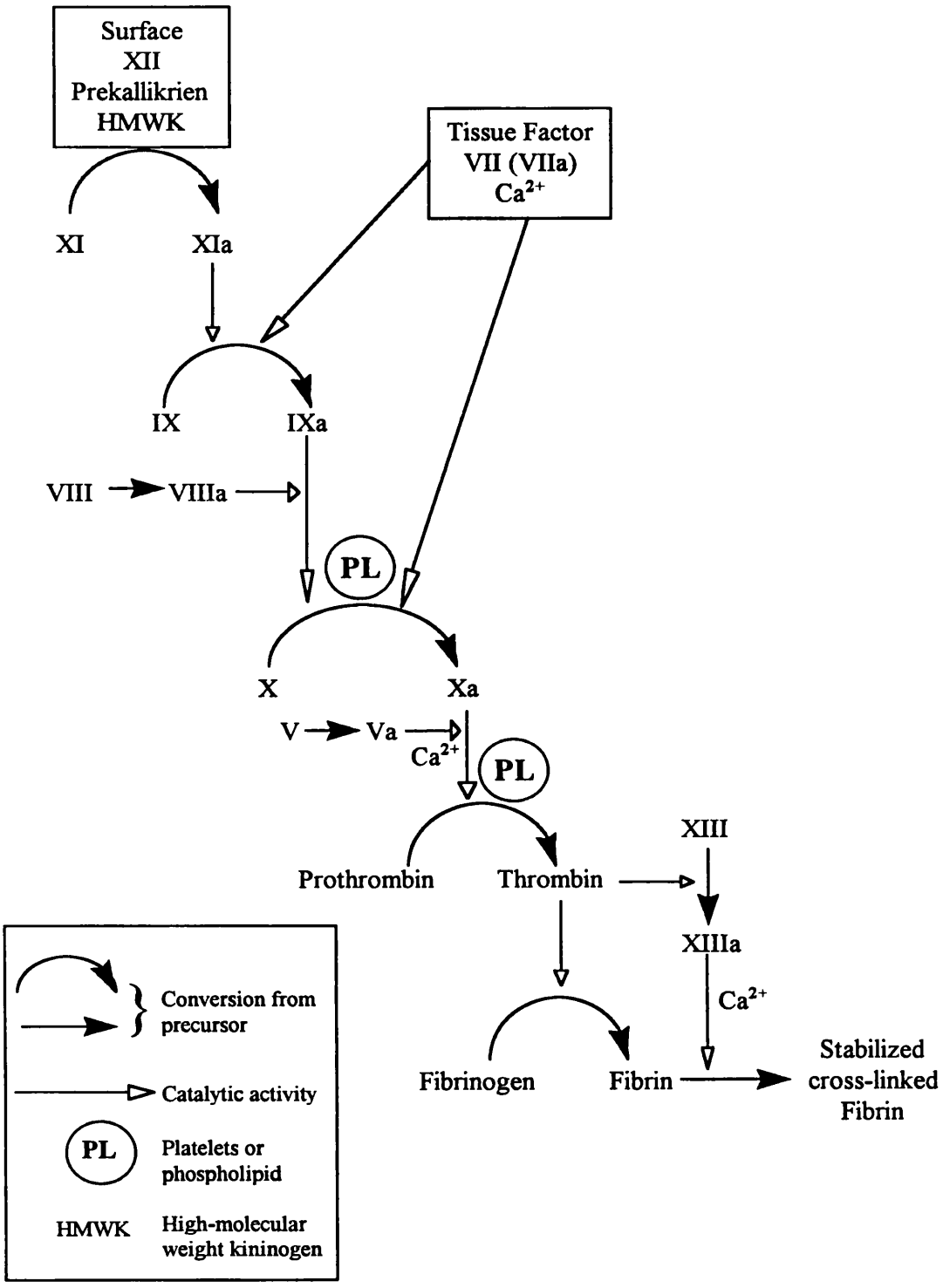
Several mechanisms help to prevent uncontrolled formation of fibrin in the circulation. First, coagulation remains a strictly localised process because it requires negatively charged surfaces which are in the first place provided by activated platelets. Platelet activation, in turn, is limited to sites of vessel injury. Furthermore, flowing blood will itself rapidly dilute any inadvertently activated clotting factor before it perpetuates the reaction sequence to form fibrin. Finally, a number of proteins circulate in the blood to inhibit the coagulation process at various stages of the cascade in addition to those mentioned above. Two of these appear particularly important to prevent thrombosis: antithrombin and protein C.

Antithrombin III inhibits thrombin and the activated forms of several coagulation factors, but inhibition of thrombin and of factor Xa are particularly important and clinically relevant. Protein C is a proenzyme that is activated by thrombin to become a serine protease that inhibits factor Va and VIIIa. Protein S is another vitamin K dependent protein that appears to function as a cofactor for activated protein C by facilitating its binding to membrane phospholipids.

In addition to being a powerful anticoagulant, activated protein C initiates fibrinolysis by releasing tissue plasminogen activator (t-PA) from the endothelium and neutralises plasminogen activator inhibitor.

Thrombin in association with intact endothelium induces the production and release from the vascular endothelial cells of two highly potent local anti-aggregatory vasodilators: prostacyclin and nitric oxide. These are thought to provide significant antithrombotic protection for microcirculatory beds adjacent to sites of thrombus formation.

Figure 1 The Coagulation cascade



2.2.2 Plaque Thrombosis

Two different mechanisms are responsible for thrombosis on plaques.[27] Each process can cause either microthrombi or at the other extreme thrombosis sufficient to occlude the lumen. The first process is superficial intimal injury, a process that involves denudation of the endothelial covering over the plaque. Subendothelial connective tissue matrix is exposed and platelet adhesion due to reactions with collagen occurs. The thrombus becomes adherent to the surface of the plaque. . .[27]

The second process is deep intimal injury; this process involves an advanced plaque with a lipid core. The plaque cap tears, allowing blood from the lumen into the lipid core which is a highly thrombogenic material. Thrombus thus forms within the plaque, expanding its volume and distorting its shape. Subsequently, thrombus may extend into the lumen, although this is not inevitable. This process of mechanical tearing of the plaque has been referred to variably as plaque fissuring, rupture and ulceration, and is clearly central to the processes that lead to acute coronary syndromes. .[27]

2.3 EVOLUTION OF CORONARY THROMBOSIS

Coronary thrombosis is a dynamic process, involving mechanisms within the vessel wall. Spontaneous lysis due to activation of plasminogen bound to fibrin is rapid and initiated by any tissue damage in the intima. If not removed by lysis, surface thrombi are rapidly organised and incorporated into the vessel wall. Thrombus invokes smooth muscle cell proliferation with these cells invading the thrombus and replacing it with collagen. The repair processes that follow deep intimal injury are more complex and there are a range of possible outcomes. Spontaneous fibrinolysis of thrombus within the lumen is responsible for transitions between occlusive to mural thrombus, and often to restoration of the lumen. If intraluminal thrombus is not removed by lysis, it is invaded by endothelial and smooth muscle cells. The thrombus is thus converted to a mass of collagenous tissue through which new vascular channels may develop. Studies of coronary arteries taken from control subjects who have coronary atheroma but no history of IHD and who have died of accidental causes, show that small episodes of plaque fissuring and resultant intraplaque thrombosis are not uncommon.[47] In subjects without diabetes or hypertension a small recent plaque fissure has been found in 8% of

subjects while in those with these risk factors the frequency rises to 16%. Such data indicate that plaque fissuring is an integral part of the progress of atheroma and not simply a cause of an acute coronary ischaemia.[48] A major determinant of whether plaque rupture leads to clinical symptoms must be whether intraluminal formation of thrombus occurs. Acute MI is associated with occlusive thrombus whereas unstable angina is associated with non-occlusive thrombus.

2.4 THE PROPENSITY TO THROMBOSIS

2.4.1 Fibrinolysis vs. Thrombosis

The propensity of the thrombus to cause an occlusion depends on the balance between systemic thrombotic potential and systemic fibrinolytic activity. Fibrin is primarily cleared from the circulation by the fibrinolytic system that acts as an antithrombotic defence mechanism. Plasmin is the effector enzyme of the system. It is formed from an inactive precursor, plasminogen, under the action of plasminogen activator. The most important physiological activator is tissue plasminogen activator (t-PA). [49]Controlling the release of t-PA represents a first way of regulating fibrinolysis. However, the most important regulation comes from the interaction of the interaction of t-PA with specific inhibitors of which plasminogen activator inhibitor-1 (PAI-1) is the most important.[50] Clinical studies support a role for an elevated plasma level of PAI-1 in the genesis of thrombotic events. Circulating levels of PAI-1 has been shown to be partly controlled genetically. Polymorphisms of PAI-1 may therefore influence the thrombotic process and acute coronary events. The fibrinolytic system is dealt with in more detail in section 4.5.

2.4.2 Summary Of Atherothrombosis

Occlusive thrombi, which consist of platelet and fibrin, frequently precipitate acute ischaemic events. Rapid progression of atherosclerotic plaque with rupture of the plaque surface, as provoked, for example by plaque haemorrhage, causes platelet adhesion and aggregation where subendothelial tissue has been exposed. High sheer stress as occur at critical arterial stenosis might also promote mechanical plaque ulceration and platelet activation, which is ultimately followed by fibrin deposition to stabilise the early platelet thrombus. The same basic mechanisms, which regulate the formation of a haemostatic plug, are responsible for the development of a pathological thrombus. The

degree of pre-existing stenosis, the severity of the intimal damage and the local balance between prothrombotic, antithrombotic and thrombolytic forces determine whether the platelet-fibrin thrombus remains limited to seal the fissure or evolves into an occlusive thrombus.

3.0 EPIDEMOLOGICAL METHODS

In the search for risk factors, it is important to be aware that there are differences in risk factor patterns between different countries. Factors may be of markedly different importance depending upon the prevalence of other factors in the Community. For example, among the Japanese, smoking seems to be of limited risk compared to its importance in the Western world. A probable explanation is the low cholesterol levels in Japan compared to many Western countries.

3.1 DEFINITION 0F A RISK FACTOR

A commonly used definition by epidemiologists of a risk factor is that its presence is associated with increased risk of morbidity and mortality, without necessarily imputing a direct causal relationship between the factor and the disease process. A risk factor can be inborn (genetic) or acquired (environmental) and should be present before the disease is manifest (clinically or sub-clinically). After detection of an association between risk factor and disease, further studies should be directed at investigating its role as an independent risk factor, a causal relationship and whether modification of the factor alters disease activity. However, it is not always necessary to completely define the factor as causally linked in order to recommend modifications of that factor.

In the setting of IHD, the search for risk factors traditionally starts with cross-sectional case-control studies that compare patients who have survived MI with randomly chosen control subjects from the same population. These studies are liable to bias however, as special characteristics of the non-survivors are missed. They do not provide quantitative measures of the absolute risk of IHD associated with the factor. In addition, the disease itself may have caused changes in the risk factor after the event. However, case-control studies are cost-effective and easier to conduct than large-scale prospective studies. They and are often performed as pilot studies to obtain information on what factors need to be studied prospectively.

Prospective studies of large population groups are often continued for long periods of time. If sufficiently large and therefore statistically valid they can provide valuable information on absolute, relative and independent risk factors. However, sophisticated multivariate analyses should not replace biological plausibility regarding cause and effect. Some risk factors may lose statistical weight after multivariate analyses due to

strong correlation with others, but may still represent important clinical endpoints. For example, obesity is associated with dyslipidaemia and hypertension, but disappears as an independent risk factor in many multivariate analyses, although it usefully serves as a surrogate and potentially modifiable risk factor in the early chain of events lending to IHD. As many as 246 risk factors have been linked to IHD many of which are closely related and may interact with each other. It is therefore important to determine which are of greatest (causal) importance for IHD, and moreover, which are modifiable.[51]
Serum cholesterol level, smoking and hypertension have emerged in several studies as the 3 major independent risk factors for IHD in the Western world. Together, they account for nearly half of the variation seen in the incidence of IHD between different populations.

It is worth emphasising that acquired risk factors can change with time and will have implications when observing a cohort of patients over a given period. Peto has shown that repeated measurements of a variable will give a considerably better prediction and a steeper 'dose-response' curve than a single measurement.[52] It is for this reason that genetic risk factors (which do not change with time in an individual) have entered the scientific arena in recent years for evaluating the aetiology of IHD.

3.2 RISK FACTORS FOR CAD

The observation in the 1950's of the striking differences in the frequency of IHD between different countries stimulated the search for various predictive factors for CAD. Risk factors can be divided into reversible (modifiable) and irreversible (unmodifiable) as shown below.

IRREVERSIBLE	**REVERSIBLE**
Age	Smoking
Male Gender	Hypertension
Race	Diabetes mellitus
Family History	Hyperlipidaemia
	Social class
	Haemostatic factors

3.3 IRREVERSIBLE RISK FACTORS

3.3.1 Age

The incidence and mortality of IHD increase with age. From age 35-44 to 55-64 there is a 15-fold increase in IHD amongst men and about 30-fold increase in women. Consequently the male/female ratio decreases with advancing age. CAD is the leading cause of death amongst males by the 4th decade of life and among females by the 6th decade. Advanced age is also a poor prognostic factor post-MI.[53]

3.3.2 Gender

The incidence of IHD is higher in men compared to women for most age groups. Between the ages of 50-59, it is about 5 times as common in men. The incidence in women after the menopause rises sharply, diminishing the gender difference. It seems likely that the gender difference may be partly explained by the hormonal milieu offering protection to the pre-menopausal female. Data relating IHD rates in women who have had premature ovarectomy (with a consequent increase in IHD prevalence) supports the hormonal hypothesis[54]

Environmental differences may also play a role. Until recently, smoking has been considerably less common amongst women. Differences in abdominal fat distribution have been reported as providing an explanation for the gender difference.[55] Such constitutional differences must, to a large extent, reflect genetic differences between the sexes.

The recognised risk factors for IHD in general tend to operate in both sexes, however, what is intriguing is that obesity and more importantly diabetes mellitus seem to have a much greater effect amongst women. Furthermore, women who have suffered a MI seem to lose their protection and have a worse prognosis than their male counterparts. These gender differences, and the possible mechanisms are discussed in more detail later in chapter five.

3.3.3 Race

IHD affects all racial groups. The genetic background that influences major risk factors has not been fully investigated although available evidence suggests that differences exist in the incidence of IHD between populations. Compelling data from migration studies on Japanese in Japan, Hawaii and USA demonstrate the strong influence of

environmental factors such as diet and smoking. [56] Asian immigrants in Britain have a higher incidence of IHD than the indigenous population.[57] Whilst cigarette smoking and blood pressure tend to be lower among Asians, the incidence of diabetes is much higher, and is associated with a higher population attributable risk to IHD than other populations.[58] Furthermore, South Asian migrants are characterised by features of the metabolic syndrome of which insulin resistance plays a central role.[59]. Raised levels of plasma plasminogen activator inhibitor-1 (PA1-1) have recently been included as a feature of the metabolic syndrome, and it has been postulated that the resulting prothrombotic state may contribute to the measured cardiovascular risk. In this respect, the genetics of PA1-1 presents us with a possible reason for the racial differences in the incidence of IHD. In a study of diabetic subjects, those of Afro-Caribbean race were characterised by an absence of the genotype 4G/4G which is associated with the highest circulating levels of PA1-1 and with a history of myocardial infarction.[60] .

The gene encoding angiotensin-converting enzyme (ACE) has important allelic differences in different racial groups. The significance of this remains to be elucidated.

3.3.4 Family History

Familial clustering of IHD has been known since the studies of Slack and Evans, which indicated that there was a-2.5 - 7 fold, increase in the risk of fatal MI in first degree relatives of patients with this disorder.[61] This may be due to factors that are environmental, genetic or both.

3.3.5 Environmental And Genetic factors

Environmental factors such as diet and smoking habits tend to be similar among family members. There is genetic predisposition to the established risk factors for IHD such as dyslipidaemia, diabetes mellitus, hypertension and obesity. In a prospective study of 4014 healthy subjects, men under 60 years with a positive family history of stroke had a higher mean blood pressure and plasma cholesterol concentrations compared to those with a negative history. In addition older men with a positive history of stroke had a higher incidence of diabetes mellitus.[62] Conversely, in a prospective study of 4050 men in Finland, no association was found between having a family history of premature CAD (under age 50) and risk of either stroke or MI when other factors were adjusted for.[53; 63] Other studies have also failed to show an association.[64] Clearly these studies highlight the uncertainties that still exist about the importance of

family history as an independent risk factor for CAD. There are difficulties inherent in these studies in which a primary risk factor must be distinguished from a genetic tendency to that risk factor, an increased susceptibility in some families to the effect of such a risk factor and the shared environmental influences that exist within families. The situation may be compounded further by the probability that two or more of these factors may be operative and by the fact that the family history status may change with the development of new cases of CAD in family members.

It has been argued, rather controversially, from these studies that a positive family history as an independent risk factor is not of the same magnitude as hypertension, smoking, diabetes and hyperlipidemia. [53] The importance of a particular risk factor must differ not just between populations, but within populations. The genetic background may also determine other, as yet unidentified risk factors. It is unlikely that a single gene is responsible for this effect since the different risk factors that influence IHD are in turn affected by several genes. The heterogeneity of familial hypercholestrolaemia and associated IHD events exemplifies this well. The role of genetic alterations that separately or in concert contribute to IHD is currently under intense investigation.

3.4 REVERSIBLE RISK FACTORS

3.4.1 Smoking

Several longitudinal studies have shown that smoking is associated with a higher incidence of IHD and risk of death.[65] A dose-response relationship to risk has also been shown in some studies. Cigarette smoking causes endothelial dysfunction, platelet aggregation and an increase in fibrinogen levels all of which are important aspects of atheroma and thrombosis although the precise mechanism is unknown. The demonstration of an association between smoking and IHD is not proof of causation, since it can be argued that smokers and non-smokers differ in aspects other than smoking, which may predispose to IHD. The evidence for a causal relationship however, comes from the following:

The consistency of the relationship demonstrated in many studies in many countries.

The strength of the risk, which is 3 fold greater amongst heavy smokers compared to non-smokers; Its independence to other factors; The lower risk of IHD in ex-smokers; the greater the number of years as an ex-smoker, the closer the mortality risk to that of a life-time non-smoker. A decrease in IHD amongst middle and upper class associated

with a decrease in smoking in this group, with no change in the lower social classes either in smoking habit or incidence of IHD.[66]

3.4.2 Blood Pressure

Risk of IHD is related to increasing levels of systolic and diastolic BP in a direct and a continuous manner with no clear cut-off point below which risk becomes negligible. In most epidemiological studies, systolic BP has been a better predictor than diastolic, but a combination of the two is more informative. Especially among the elderly, it has been shown that systolic BP elevation is associated with increased risk[67]. As intimated above, from an epidemiological point of view there is no natural level at which an individual can be defined as "hypertensive". The definition is based on a rather arbitrary level at which people are labelled as hypertensive in order to initiate treatment. The prevalence of hypertension may be closely related to shifts in the whole distribution of population values. Thus, in populations in which BP's are moved far to the left, there is no hypertension. Rose.[68] It is of note that in some rural and nomadic populations, BP does not increase with age.

Hypertension is closely related to other risk factors for IHD. It forms part of the metabolic syndrome. It has been shown to be related to physical inactivity, excessive intake of salt, excessive alcohol use and possibly to the balance of polyunsaturated to saturated fats. Psychological stress may also be important.

It has been postulated that hypertension is an aggravating factor for IHD provided sufficient atheroma is established. Evidence for this comes from studies from Japan in which elevated BP seem to be less important for IHD risk perhaps because of the low serum cholesterol levels[56]. Conversely in populations with relatively high levels of serum cholesterol, the increased risk associated with hypertension is similar to that of serum cholesterol. A recent meta-analysis of 14 randomised trials in which the diastolic BP was reduced by 5-6mm of mercury showed a good and predictive effect for stroke, but was less predictive of IHD.[67] Furthermore, studies among elderly patients have also indicated beneficial effects on total mortality when BP was reduced, but the risk reduction on IHD was not significant.[69]. It would appear then that BP reduction, which on the whole has not been very effective, does not lead to full reversibility of IHD risk. Elevation of BP is associated with several other risk factors, and it may well be that its risk on IHD is conveyed through other risk factors.

3.4.3 Diabetes Mellitus And Insulin Resistance

Diabetes mellitus occurs in an estimated 4% the UK population of which half is undiagnosed. Macrovascular disease accounts for 75% of all deaths, whilst cardiovascular disease accounts for up to 3 times the mortality of subjects without diabetes.[70]

Additionally, the incidence of asymptomatic IHD in diabetes is much higher than in non-diabetic subjects. Population studies indicate that age-adjusted mortality rates for IHD are 2 to 3 times and 7 times higher among men and women respectively with diabetes than in those without the disease[71]. Angiographic studies indicate that patients with diabetes tend to have a greater number of coronary arteries involved by disease.

Whilst most of the known risk factors for the development of CAD occur in excess in diabetes it has been estimated that they still only account for 25% of the excess risk. In the diabetic milieu 3 major abnormalities exist that could contribute to this state of affairs: hyperglycaemia, with associated protein glycosylation, changes in lipoprotein metabolism and insulin resistance. Some of the possible mechanisms involved in the pathogenesis of vascular disease in diabetes include abnormalities in the polyol pathway, platelet function, coagulation, fibrinolysis, anti-oxidant status and blood flow. In particular, patients with type 2 diabetes mellitus are characterised by suppression of fibrinolysis, which may contribute to the development of CAD.

Resistance to the action of insulin is a feature of type 1 and type 2 diabetes mellitus and of Reaven's syndrome (Syndrome X).[72] The prominent feature of this syndrome is a clustering of cardiovascular risk factors around insulin resistance. There is suppression of fibrinolysis through raised levels of PAI-1 and it has been postulated that it is this relationship which provides one of the links between insulin resistance and IHD.[73]

3.4.4 Coagulation And Fibrinolysis

Thrombus formation has an important role in the pathogenesis of myocardial infarction. The implication of this well established phenomenon is that the haemostatic system has an important role in the evolution of acute myocardial infarction and that tests of haemostasis may help to predict patients at risk.

3.4.5 Coagulation

Meade et al in 1980 reported fibrinogen level as a predictor of subsequent development of IHD in middle aged men.[74] This has gained support from other large prospective studies.[75] Circulating fibrinogen levels have a major genetic component, and raised

levels are associated with smoking, lower social class and increased stress. .[74].[75; 76] Patients with hypercholestrolaemia and hypertriglyceridaemia have been reported to have raised fibrinogen levels.[77] In the recent ECAT study, cholesterol was associated with a high risk of subsequent ischaemic event in as far as it was associated with raised fibrinogen levels.[78]

Evidence is emerging that increased levels of Factor VII may be an independent risk factor for IHD.[79] Its activity is influenced by dietary fat, and can be rapidly reduced by dietary methods. The traditional Eskimo diet of polyunsaturated fat produces a prolonged bleeding time. It is not clear whether this results in protection from CAD, but diet can be seen to have major effects on platelet function and prostaglandin metabolism.

Abnormalities of fibrinogen metabolism might be linked directly to lipoprotein abnormalities. Plasminogen and lipoprotein a (Lp(a)) have a similar molecular structure. Raised levels Lp(a)are associated with atherosclerosis,[80] and the kringle structures common to both proteins are responsible for binding of plasminogen to fibrin prior to activation to plasmin. It is possible that Lp(a) might bind in this way and block fibrinolysis either by preventing activation of plasminogen or by preventing initial access and binding of plasminogen.

3.4.6 Fibrinolysis

PAI-1 is the main inhibitor of fibrinolysis, and raised levels have been associated with the atherothrombotic process. This is discussed in more detail in chapter 4.

3.4.7 Platelets

The importance of platelets in the pathogenesis of CAD rests on 2 pieces of accepted wisdom; first, that platelets in association with subendothelial interactions have pivotal effects on haemostasis, and second, that the use of antiplatelet agents such as aspirin have beneficial effects on outcome.

Platelets do not only contribute to the late thromboembolic complications of obstructive arterial disease, they also play a critical role in the development of early atheroma. The available evidence indicates that flow disturbance in a vessel with damaged endothelium leads to attachment of platelets to the subendothelial layer. This interaction is promoted by vWF, a finding supported by the association between increased circulating levels of vWF and re-infarction. Attachment of platelets would lead to the release of growth

factors and platelet activation products thus further promoting the development of both atheroma and thrombosis. Other studies have demonstrated an association between platelet aggregation and myocardial infarction.[81]

3.4.8 Cholesterol

There are now several studies that point to raised total serum cholesterol as an independent risk factor for IHD.[82] The risk seems to be predominantly due to high levels of LDL cholesterol, and provides the background for acute coronary events. The available evidence suggests that raised serum cholesterol is a necessary factor for IHD.

In communities with low levels of total serum cholesterol, there is a low incidence of IHD, but even in these communities there is a gradual increase of risk with increasing levels of serum cholesterol. .[82]. Moreover, in these countries smoking does not appear to be a risk factor for IHD, pointing to the importance of serum cholesterol as a necessary factor for the development of IHD.

The total serum cholesterol level within a country does not vary as much as it does between countries. These differences are likely to be due to genetic as well as environment factors or more correctly to differences in gene-environment interactions. Within a country there is a continuous relationship between serum total cholesterol concentration and incidence of IHD as well as mortality. [82]

In the Multiple Risk factor Intervention Trial (MRFIT),[83], men in the lowest fifth of the cholesterol distribution were regarded as the baseline risk group. Above this level, risk for mortality from IHD increased with the serum levels. In men in the top quintile, the relative risk was 3.4 compared to men in the lowest fifth (However, even in the lower cholesterol levels, a risk gradient was still seen).

Non-fatal myocardial infarction has also been shown to be related to serum cholesterol level in a similar way, and the findings are consistent among different populations[53; 84]

Some previous studies suggested that there was an increase in total mortality at the lower end of the total serum cholesterol concentration. However, the recent 4S study, a cholesterol-lowering prospective study of 4444 patients with Simvastatin has helped to resolve some of the controversy in this area.[85] Patients with evidence of IHD who had their cholesterol lowered (from 5.5-8mmol/l), had a 30% risk reduction in total mortality whatever their cholesterol level. Furthermore, the West of Scotland Study has extended the findings of 4S to include primary prevention (i.e. of patients without evidence of IHD[86].

3.4.9 High Density Lipoprotein (HDL)

The concept of HDL playing an important role in mobilising cholesterol from the tissues (reverse transport) is now firmly established. The inverse relationship between plasma levels of HDL cholesterol and the incidence of IHD has been observed in prospective epidemiological studies from several countries[86; 87]. In the British Regional Heart Study, men in the lowest quintile of the HDL distribution had a two-fold risk of a major ischaemic event[53]. In the Framingham study, a beneficial effect of increased levels of HDL cholesterol on decreased risk of IHD was reported in both sexes[88].

Based on the evidence linking levels of lipids and lipoproteins to the incidence of IHD, the use of total serum cholesterol has been questioned, with advocates of LDL: HDL ratio or total cholesterol: HDL ratio, as a useful means of judging an individual's future risk of IHD. However, because high total serum cholesterol is rarely due to a high proportion of HDL cholesterol, measurement of the former as a good indicator of IHD risk is still widely used.

3.4.10 Triglycerides (TG)

Elevated fasting plasma TG has been implicated as a risk factor for IHD. Because of its strong positive correlation with serum total cholesterol, in some Scandinavian studies the significance has been lost in multivariate analysis. Consequently its importance as an independent risk factor has been questioned[89]. In the Evans County Study, elevated TG was associated with increased risk of IHD only among white women aged 50 years and older.[90] In the Framingham study, elevated TG was found to be an independent risk factor in men, especially in those with a high plasma LDL cholesterol/HDL cholesterol >5, but was a greater predictor in women.[91] Serum TG is significantly and positively correlated with serum total cholesterol, body mass index (BMI), and negatively with HDL. It is difficult, statistically, to separate the effects of high TG from that of low HDL, because the two are linked metabolically.

A prospective interventional study in which TG reduction is shown to reduce cardiovascular mortality seems to be the only way of resolving this.

3.4.11 Chronic Infections

Recent studies have reported an association between infections with *helicobacter pylori* [15] and *Chlamydia pneumoniae* with CAD.[92] It has been postulated that by promoting a low grade chronic inflammatory response with the release of interleukins and possibly fibrinogen,[18] these infections may contribute to the atherogenic process.[14]

3.5 SYNERGISTIC EFFECTS OF RISK FACTORS

It has been known for many years that the prediction of cases of major IHD can be improved by assessing the combined effects of several risk factors acting simultaneously. In a study of men born in 1913, it was found that the 3 major risk factors; smoking, hypertension and cholesterol acted in synergy to increase risk.[51; 86]. These factors usually act in an independent but additive fashion to create a high risk group. In the ECAT study, for example, levels of cholesterol, fibrinogen, vWF and tPA antigen were independent predictors of subsequent events in patients with angina pectoris.[78] Furthermore, in patients with high cholesterol levels, the risk of coronary events rose with increasing levels of fibrinogen. It is not only these "environmental factors" that act in concert. It would be expected that the genes that influence these factors might act in synergy with each other and perhaps with other "environmental factors". This concept was put to the test with the publication of the first study linking the angiotensin converting enzyme (ACE) genotype (DD) with the risk of myocardial infarction.[93] The overall increase in risk was modest, with an odds ratio of 1.34 in patients with the DD genotype. It is only when a subgroup analysis was carried out in patients who had sustained an infarct despite being at low risk, that the presence of the deletion genotype DD assumed much greater importance with an odds ratio of 3.2. The DD genotype was therefore acting independently and in a non-additive manner to other classical risk factors such as smoking, hyperlipidaemia and hypertension. This is unusual, because as indicated above the other risk factors interact cumulatively to create high-risk individuals. If the hypothesis proposed by Cambien et al is correct, we are left with an unusual problem, namely, identification of the DD genotype helps us to define a high risk group but only if they do not have other (more) classical risk factors. On the other hand it may provide us with some insight into the mechanisms of the atherothrombotic process. The group have argued that their finding does explain the phenomenon whereby patients with no risk factors present with an MI, this requires confirmation and is discussed further below.

4.0 GENETIC POLYMORPHISMS

4.1 INTRODUCTION

As the above account would make clear, atherogenesis is a multifactorial disease, an end product of many influences both environment and genetic, this may be expressed in terms of a Venn model (figure2). The sets define subgroups in the general population. A, defines those exposed to a highly atherogenic environment. B, represents a subset exposed to a higher than background risk due to a disorder known to predispose to atheroma such as hypertension and diabetes mellitus. C, represents those possessing genetic variants conferring a predisposition to develop atheroma. When this genetic liability coincides with other risk factors atheroma develops. However, prediction of CAD is ineffective due to difficulties in the relative weighting of these factors for any individual. Progress may be made by limiting early environmental risk factors in individuals with a specific genetic predisposition.

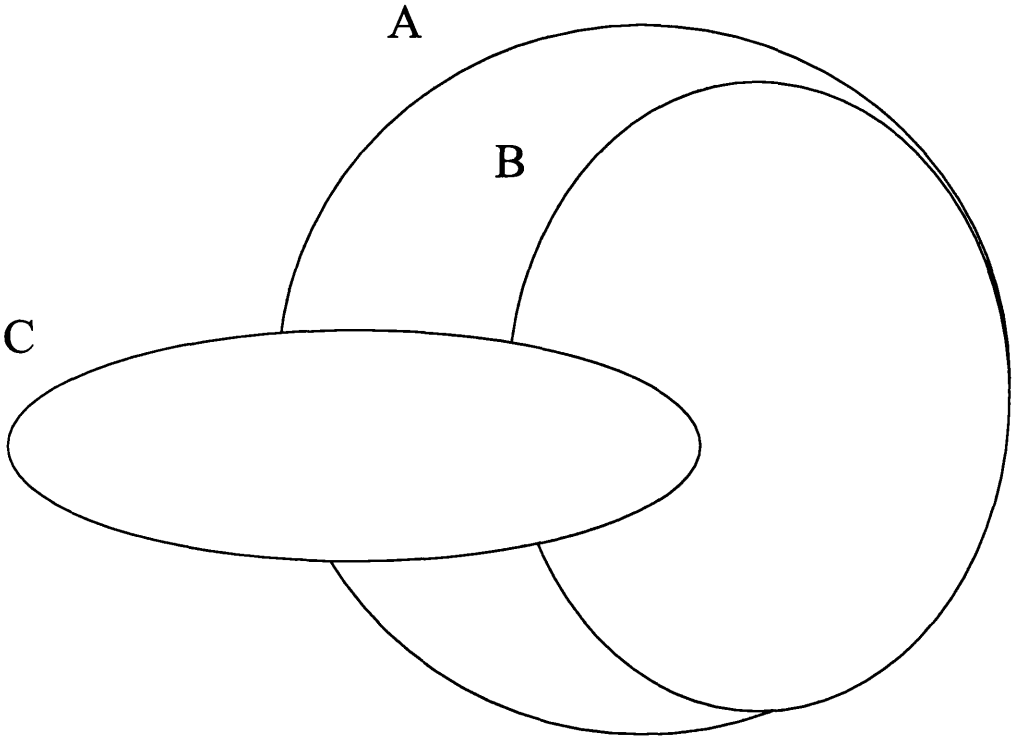


Figure 2. Venn diagram demonstrating factors that may interact to produce atherosclerosis. Subset A: members exposed to atherogenic environment; Subset B: individuals with disorder predisposing to atherosclerosis. Subset C: individuals with genetic predisposition.

The evidence for aggregation of premature CAD within families was briefly reviewed above. Slack and Evans (in family studies) analysed first degree relatives with CAD and showed the increased risk of death from CAD was 5 and 7 fold greater than in matched controls for males and females respectively.[61] Since then other studies have confirmed this finding.[94; 95] Further evidence comes from twin studies.[96] Berg found concordance rates for angina pectoris or MI in monozygotic twins to be 0.65 as compared with 0.25 in dizygotic twins. If twins with premature CAD appearing before the age of 60 were alone considered these figures were 0.83 and 0.22 respectively. The Venn model described above helps to explain some of the features of atherosclerosis. Firstly, the relatively high frequency of the disease in the Western world may be related to the fact that genes conferring susceptibility to atheroma would be at no selective disadvantage under favourable environmental conditions and thus be expected to be widespread in healthy subgroups. Secondly, in the face of such a complex model, the failure of classical genetic epidemiology to elucidate recognisable patterns of disease inheritance becomes easily explained.

DNA technology has made it possible to study 'CANDIDATE GENES'. This is defined as a gene whose protein product could plausibly be involved in the process of atheroma and/or thrombosis.

4.1.2 Principles For Identification Of Genetic Polymorphisms

To determine which of the 1.4 million potential genes in the human genome are likely to be involved in the pathogenesis of atherothrombosis, two complementary but different approaches have been used:

CANDIDATE GENE TARGETING

This assumes that those genes known to code for a protein (intermediate phenotype) suspected to be involved in the atherogenic process are the genes most worthy of study. For example a candidate gene approach may involve concentrating on a gene producing a protein known to be central to lipid metabolism, such as apolipoprotein B. By using various molecular genetic tools to identify polymorphisms in that gene, one could proceed to see if the relative frequencies are altered between patient and control groups in various racially different populations.

The identification of candidate genes uses at its source the wealth of data regarding proteins that have been identified, characterised and thought to be implicated in the onset or development of atherosclerosis as intimated above.

COMPLETE GENOMIC MAPPING

Early approaches were based on the production of complementary DNA and genomic DNA libraries, which allow the isolation of random unique DNA fragments with regular spacing along each and every chromosome and the subsequent use of gene fragments as hybridisation probes to detect Restriction Fragment Length Polymorphisms (RFLP). Pedigree studies are then pursued and should any association be found, the gene fragment can be mapped to the genome. Then by "walking along" the chromosomal segment with further probes one might expect to be able to pinpoint the aetiological mutation involved.[97]

A more recent is to make use of Microsatellites or short tandem repeats (STR). These have a large number of alleles for each locus and are spread throughout the genome and can be used as markers for disease traits. An example of this is the CA repeat region in the PAI-I gene as discussed in section 4.6.

An initial trawl is performed using 400 STR markers throughout the genome. If any markers show linkage to the disease of interest, a second screening is performed with closer markers in that region of the genome.

The genes in the defined region are identified, and functional studies are then performed.

Other workers have used the Single Nucleotide Polymorphism (SNIP) maps. These markers are at a higher density and can be used to pinpoint functional polymorphisms.

The candidate gene approach is only useful and a quick method when the assumptions made with regard to disease causality is correct. The Microsatellite and SNIP approaches are not based on such assumptions. The gene(s) involved in the disease can be identified without prior knowledge of its existence or involvement.

Our justification for the candidate approach was because the intermediate phenotypes (circulating levels of PAI-I and ACE) were implicated in the atherothrombotic process. By examining these candidate genes the relationship to disease could be studied. Although this is a quick method for identifying functional polymorphisms within these

genes, such an approach could miss important mutations in other genes that could alter the circulating levels of these intermediate phenotypes.

The Microsatellite approach could identify such un-linked genes that could directly or indirectly modulate the levels of these gene products. However, such an approach is more intensive, expensive and time-consuming.

These recent approaches were unavailable in our unit at the time the present work was conducted.

Large nuclear family studies

Many less complex genetic conditions have been effectively studied by segregation of family members into affected and unaffected categories. Subsequent analysis of the gene transmission patterns have then been performed to determine the presence or absence of linkage disequilibrium (inheritance of gene loci more or less frequently than expected based on known allele frequencies and mendelian inheritance patterns). Thereafter abnormal genes have been cloned and identified by direct sequencing or by use of restriction enzymes. This approach has not received much attention in the study of myocardial infarction because (a) it is considered to be genetically and otherwise more complex (b) because it presents late in life making identification of unaffected individuals difficult (c) it is often fatal resulting in loss of informative genetic information.

Nevertheless new statistical methods have been described that permit this approach in multiple small pedigrees. The transmission disequilibrium test (TDT) described by Spielman in 1993 originally required multiple trios (two parents and a single affect offspring).[98] Later modifications suggest that only a single parent is required (TDT-1). Furthermore the test remains valid in the presence of no parents based on larger collections of affected (A) and unaffected (U) siblings (S-TDT). The use of TDT requires both parents to be heterozygous making collection of subjects more difficult. Furthermore, it necessitates recruitment of much larger numbers.

Discordant-Sibship Test(STD) for disequilibrium and linkage

More recently, the discordant-alleles and discordant-sibling disequilibrium tests suggests that both pairs (AU) and preferably (AAU or AUU) can be informative both separately and in combination with other information that may be available from surviving parents and additional siblings.[99] Basically, this is a non-parametric sign test that compares within each family the number of alleles in affected siblings versus those in unaffected siblings. As well as being a valid test for gene-disease association it is also able to test for linkage between any disease-causing/modifying genetic polymorphisms and the genetic markers studied. In addition, false-positive results that may be caused by population stratification are avoided, statistical power is greatly increased and hence conclusions regarding diseases-causation/modifying are more valid.

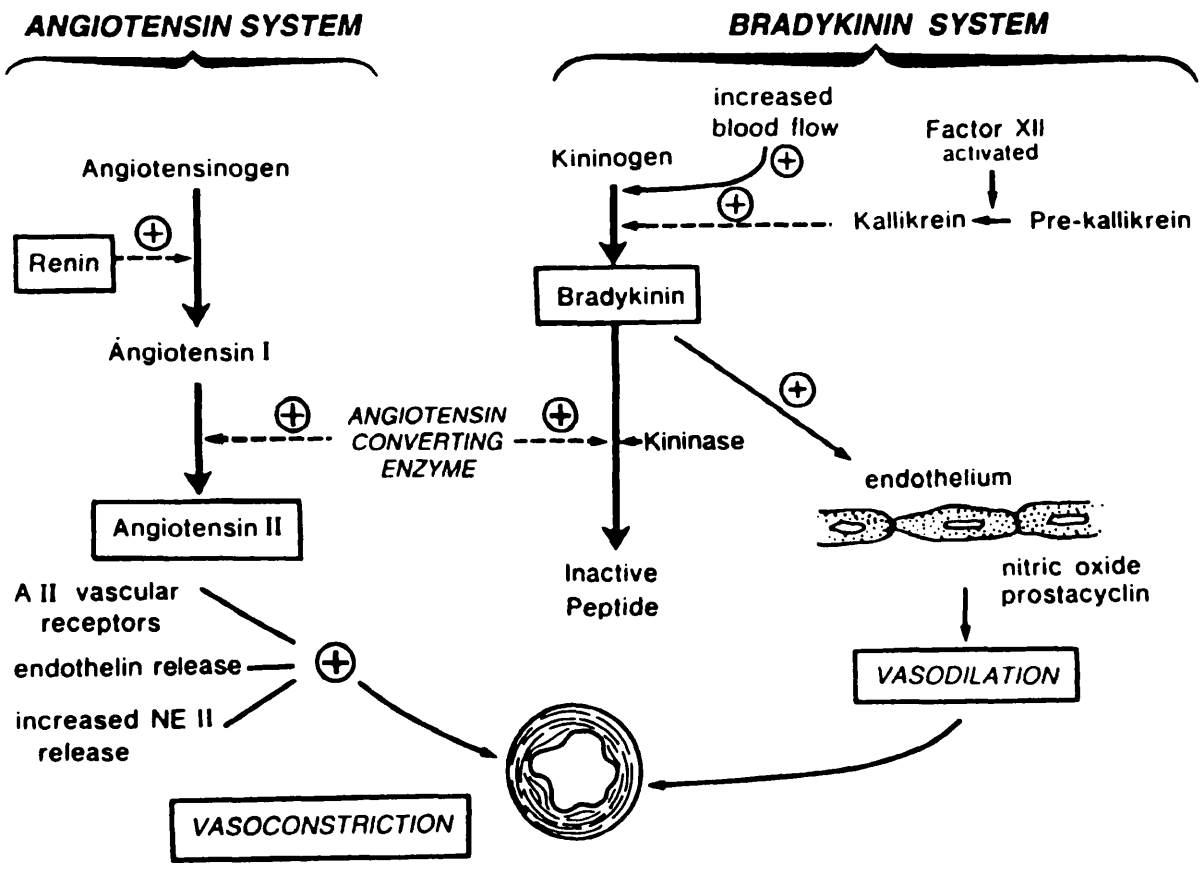
When analysing the genetic component as CAD, the major aim is to identify those common genetic variants that will alter expression of a single gene in a relatively small but significant way. These common variants will not precipitate disease in an individual alone, but on a population basis could be associated with an increased risk of disease. It will then be possible to build a risk factor profile for an increased risk of disease based not on levels of plasma proteins but also on genotypes for common functional variants. This may be a more reliable risk profile than single measurements of basal, fasting circulating levels of proteins since these do not take into account intra-individual variation. Furthermore, they do not allow for stress-induced acute phase responses, nor the response to other environmental factors. The identification of common functional, genetic variants, and the effect of such variants (through a single gene product) on the pathogenesis of atheroma and thrombosis is therefore of critical importance for a multifactorial, polygenic disease such CAD.

4.2 ANGIOTENSIN CONVERTING ENZYME

4.2.1 The Renin-Angiotensin And Bradykinin System

The components of the Renin-Angiotensin-bradykinin System (RABS) figure 3, has attractive candidates for cardiovascular disease. It's importance lies in its effects, which includes: 1) proliferation of vascular smooth muscle, 2) sodium and water homeostasis 3) modulation of vascular tone and 4) effects on fibrinolysis.[100]

Figure 3 The Renin-angiotensin and bradykinin System.



Renin is released from the juxta-glomerular apparatus of renal tubules in the renal cortex. It converts the plasma protein angiotensinogen to angiotensin 1. This is the rate limiting step in the system.

Angiotensin-converting enzyme (ACE) belongs to the serine protease family and is produced by several different tissues in the body including the vascular endothelium.[101] It catalyses the conversion of angiotensin I to angiotensin-II (AII). A-II stimulates smooth muscle cell proliferation and is a potent vasoconstrictor. In the adrenal cortex it stimulates glomerulosa cells to release aldosterone which acts on the distal convoluted tubules to reabsorb sodium.

Until recently the kininogen system was thought to be separate from the RAS, however, kininase is now known to be identical to ACE, and causes the breakdown of bradykinin. The latter releases nitric oxide from the vascular endothelium. Nitric oxide (NO), is not

only a potent vasodilator, but inhibits the proliferation of smooth muscle cells. An increase in ACE activity will have the dual effects of increasing the levels of A-II and reducing bradykinin levels. The effect of this may be the proliferation of smooth muscle cells and cause vasoconstriction, both of which are important aspects of the development of atheroma.

Recent studies suggest that A-II in addition stimulates the release of plasminogen activator Inhibitor-1 (PAI-1), the main inhibitor of the fibrinolysis (see below). Raised levels of PAI-1 may lead to a prothrombotic state[102; 103] A-II induces PAI-1 production by endothelial and smooth muscle cells in vitro [102] It has been shown to acutely elevate circulating PAI-1 levels in a dose-dependent way when infused in human subjects in vivo.[104] Finally, the lowering of circulating A-II levels by the administration of ACE inhibitors following myocardial infarction is associated with a reduction in circulating PAI-1 levels [105] In vitro studies indicate that specific antagonists to either of the recognised angiotensin receptor types (AT_1 or AT_2) do not abolish the induction of PAI-1 synthesis by A-II. However, a receptor to a six-peptide form of angiotensin (angiotensin IV) has been identified, and a specific antagonist for this receptor does block angiotensin-induction of PAI-1 in vitro [102]. These data suggest that angiotensin increases PAI-1 levels by a different receptor interaction to its vasopressor activity. Selective AT_1 and AT_2 receptor blockers have been introduced as novel therapies in the treatment of hypertension. As blockade of AT_1 receptors is associated with increased renin production it is possible that such agents may have an adverse influence on vascular risk by increasing circulating levels of PAI-1 via the angiotensin IV receptor. This is purely speculative, and there is no data to suggest that these newer agents may have an adverse vascular risk.

It is evident therefore that at least theoretically, an increase in ACE activity may not only promote the development of atheroma, but also thrombosis, the two main aspects that lead to myocardial infarction. Support for this is derived from the observation that ACE inhibitors reduce atherosclerosis in cholesterol-fed rabbits. Furthermore, in large prospective clinical trials such as the SAVE study, ACE-inhibitors were shown to reduce future acute coronary events.

4.2.2 Plasma ACE

ACE is a dipeptidyl carboxypeptidase and as noted above converts angiotensin-I to the potent vasoconstrictor angiotensin-II and inactivates the vasodilator bradykinin. ACE

has been purified from several sources, including lung, seminal fluid and plasma. ACE derived from different organs of the same species is immunologically similar, but those purified from different species are antigenically distinct. The molecular weight ranges from 140 - 160 Kd for the endothelial ACE to 90 - 100 Kd for the testicular form, depending on the carbohydrate content of the molecule.[106]

ACE acts by cleaving terminal dipeptides, and rates of hydrolysis of angiotensin-I are influenced by the presence of chloride anions, but the latter is less critical for activity on bradykinin. Chloride appears to enhance activation by inducing a conformational change in ACE that increases substrate-binding, an effect potentiated by sulphide.

4.2.3 ACE Distribution & Regulation

Recent studies have shown that ACE is present in nearly all mammalian tissues and body fluids. [101]The highest levels have been found in the kidney, ileum, duodenum and uterus, with lesser levels in the lung, prostate, jejunum, testis and adrenal. The availability of ACE enzyme mRNA has permitted more exact localisation of ACE transcripts to specific cell types. Thus, the pulmonary form of ACE appears to be restricted to epithelial and endothelial cells of specific tissues and to stimulated macrophages.[107]

Little is known about the regulation of ACE mRNA expression, however, regulation of the protein has been studies in vivo and in culture. Corticosteroids in cultured endothelial cells, alveolar macrophages, monocytes and lung can induce ACE. [101] Hyperthyroidism tends to elevate levels of circulating ACE. Both protein kinase C and cAMP dependent-mechanisms appear to increase ACE activity in cultured endothelial cells, but only activators of protein kinase C increase the release of ACE into the medium. To date only the testicular form of ACE has been shown to be under hormonal control; specifically, FSH/LH, human chorionic gonadotrophin and testosterone, these affect the depletion of testicular ACE activity induced by hypophysectomy.[108]

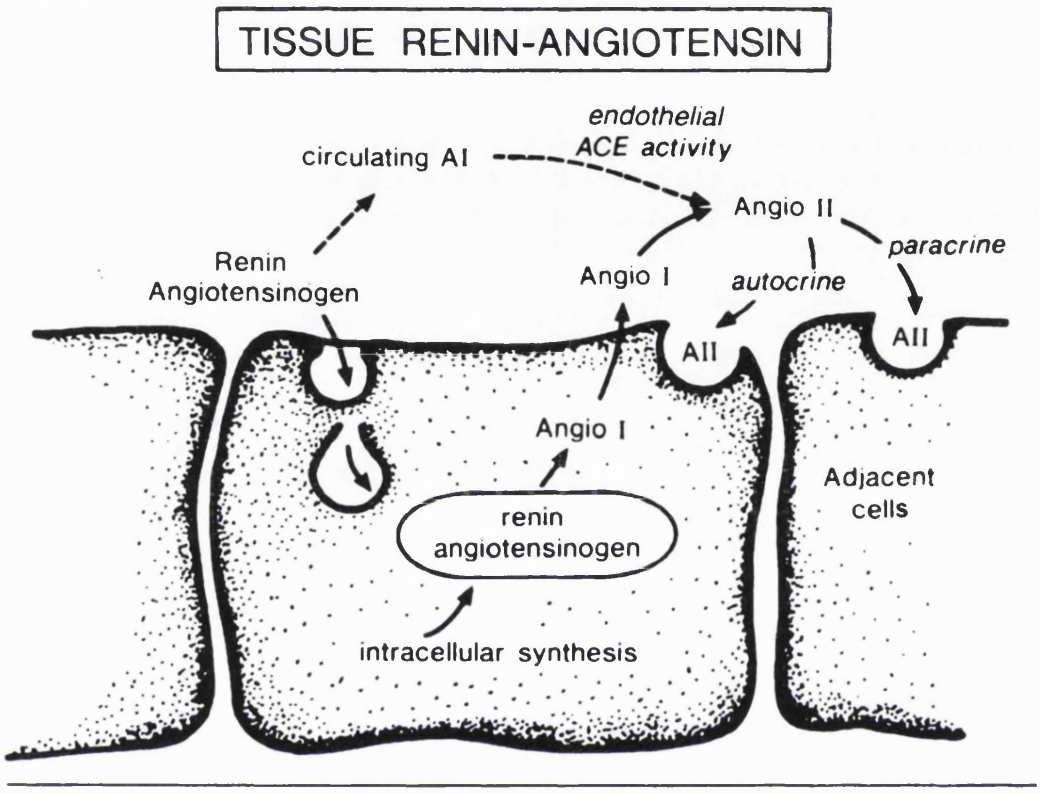
4.2.4 Tissue ACE Systems

Although the acute hypotensive effects of ACE inhibition can clearly be linked to a decrease in circulating levels of A-II, during chronic ACE inhibition there is a reactive hyperreninemia linked to re-emergence of circulating angiotensin-II.[109] Furthermore,

ACE inhibition can be effective even in low renin states.[110] There is also a discrepancy between haemodynamic effects and circulating levels of ACE inhibitors, possibly due to binding of ACE inhibitors to tissue sites.[111] An increasingly important question is whether angiotensin can be formed in crucial tissues (it can) and whether tissue ACE is potential or even major site of action for the ACE inhibitors. .[109]

All the components of the renin-angiotensin system are found in vascular tissue including the coronary vessels. In a number of vascular beds, local production is the major source of A-I and A-II found in the veins.[112] Tissue ACE activity is found in the lungs, myocardium, brain, kidneys, and testis. .[109]. An especially striking finding is the degree to which renal ACE activity is depressed to very low levels by ACE inhibitor therapy given to animals.[113] Furthermore, during chronic ACE inhibitor treatment when plasma ACE activity increases as a result of compensatory induction, there are still low levels of free tissue ACE in kidney, adrenals, and aorta of rats.[114] The implication of the discovery of these tissue components of renin-angiotensin system means that A-II can be locally formed in or around blood vessels with possible vasoconstrictory effects. ACE can be made in the endothelium, with the major part of the enzyme protruding into the vascular lumen. The result is that A-II is always generated at this site, and it does not matter whether the precursor A-I is locally made or derived from the circulation.[115] Local A-II formation accounts for the autocrine-paracrine effects of the RAS (autocrine, local action on the same cells that produce the A-II; paracrine, effects on neighbouring cells) figure 4.

Figure 4 Proposed role of Tissue RAS



Proposed role of Tissue RAS. Renin and angiotensinogen from the extracellular space may enter the cell by means of receptor endocytosis. Intracellular R-A can be derived from such endocytosis or local intracellular synthesis. For paracrine and autocrine effects see text.

An important observation now made in several pharmacokinetic studies in rats is that, when an ACE inhibitor is given to spontaneously hypertensive rates (SH rats), the fall in blood pressure correlates better with the inhibition of the tissue than the circulating RAS.[116] Hence, it is now increasingly believed that tissue systems are of (at least) some importance in the blood pressure lowering effects of ACE inhibitors, which would explain why antihypertensive effects can be maintained despite the later reactive increase of circulating A-II. .[109] Nonetheless, decisive studies to prove the significance of local tissue ACE inhibition in humans are still lacking.

Not all A-II is generated as a result of the activity of ACE. In the failing human heart, it appears that most A-II could be formed by a path sensitive to a membrane-bound serine proteinase and not to an ACE inhibitor.[117] Similar observations have been made in the dog heart after coronary ligation,[118] where both a serine and a cysteine protease are implicated. Other tissues in which A-I can be converted to A-II independently of the ACE activity are the renal arteries[119] and the lungs.[120] A major controversy is the extent to which these independent pathways are of pathophysiological importance.[121] Use of specific protease inhibitors suggests that the local formation of A-II by proteases does not regulate infarct size in dogs.[118]

4.2.5 Plasma ACE And Genetic Influence

In healthy men, plasma ACE can differ greatly among subjects by as much as 5.7 times, [106]but remain remarkably stable when measured repeatedly in a given individual.[122] A search for environmental or hormonal parameters accounting for this interindividual variability only yielded weak associations. .[109] Results of a family study showed that familial similarity in ACE level was compatible with the transmission of a major gene, explaining a large part of the interindividual variation in circulating ACE levels.[123] These facts strongly suggested a genetic component to be at least in part responsible for determining the level of plasma ACE.

4.3 ACE GENE POLYMORPHISM

The ACE gene was cloned in 1988 by Florent Soubrier in Paris and was found to comprise of 26 exons. [124] The presence of a polymorphism in the 16th intron (non-coding region) due to the presence (I) or absence (D) of a 287 base pairs was discovered. The region was amplified using the polymerase chain reaction (PCR) and

three genotypes were identified; the heterozygotes (ID), and the homozygotes for insertion (II) and deletion (DD). [125]

4.3.1 Relationship Between Polymorphism And Plasma ACE

The search for a relationship between the ACE polymorphism and the level of plasma ACE revealed a strong co-dominant association, with subjects homozygous for the D allele having roughly twice the level of those with the II genotype, and ID subjects having intermediate levels.[126] Furthermore, the level of ACE activity in T lymphocytes has been shown to be related to the polymorphism. This is a significant finding since circulating ACE may not necessarily reflect tissue ACE. [127] As discussed above, tissue ACE may be of greater importance and the above finding could suggest that the level of ACE activity in all cells in which it is expressed (in particular in the vascular wall and myocardium), could be related to the polymorphism. [127]

In order to establish whether the polymorphism (functionally expressed as variations in plasma ACE levels), which had been identified by segregation analysis in earlier family studies, was the same polymorphism as the ACE ID polymorphism, a further study was performed in a series of families.[128] By linkage segregation analysis, it was concluded that the ACE ID polymorphism was not functional, but a marker for an unknown functional variant probably located in the regulatory region of the gene. It was however, a fairly good marker with a sensitivity of 100% and specificity for the functional variant of about 70%. The functional variation could explain 44% of the variability of plasma ACE, whereas ACE ID polymorphism only explained 28%..[128]

Having shown that the polymorphism was related to the intermediate phenotype i.e. circulatory (and probably tissue) ACE, and that the latter could be related to vascular homeostasis, the question was raised whether this genetic polymorphism could be related to the atherothrombotic process.

4.3.2 ACE Polymorphism And Atherothrombosis

In 1988, a case control study designed to identify genetic factors that could predispose to myocardial infarction was set up.[93] It was a collaborative study between 3 centres in France and a centre in Belfast. The patients were selected from the WHO/MONICA registry, and control subjects selected randomly from the population. Cambien et al

published the landmark study in Nature 1992. Their results demonstrated a higher risk of MI associated with the DD genotype. Taking the risk associated with the II genotype level as 1, the relative risk associated with the heterozygote (ID) was 1.3, and with the DD genotype, 1.6. Furthermore, in a subgroup of subjects considered to be at low risk of MI (selected from patient and control groups on the basis of BMI, apoB levels and hypolipidemic treatment), the risk attributed to the DD genotype assumed greater significance. This finding was consistent in all the centres studied. Surprisingly, within the high risk group, although the presence of the DD genotype was still associated with a significant risk of MI, it was of much less importance. Among the higher risk population of Belfast where the risk of MI was 4 times higher than in the French population in the study, the DD genotype was associated with less risk of MI.

To explain these patterns seen, it was hypothesised that carriage of this particular genotype (DD) in a high risk population was associated with an increased risk of dying from an MI. In other words, the risk factors were acting in concert to produce a very high risk group who would have 'died out' and therefore not participated in the study. The DD genotype was thus serving as a useful marker for patients who are at low risk and will sustain an MI. Patients with the DD genotype and other risk factors have an increased risk of dying from an MI. The ECTIM study recruited their MI subjects 3 to 9 months after the event. Since 50% die of acute coronary syndromes even before reaching hospital, this explanation was plausible. Support for this hypothesis came from a further study from the same group.[129]. Tissue recovered at autopsy from patients who had died from MI and a control group showed that taking the relative risk of death from MI for the II genotype as 1, the risk for ID and DD were 1.8 and 2.2 respectively. There appeared therefore to be a relationship between death from CAD and the ID polymorphism. A further study from Belfast, which lent support to the hypothesis that the DD genotype was a risk factor for fatal events, came from a study in which family histories were investigated. Patients with a parental history of fatal MI had a higher prevalence of the DD genotype. Their results demonstrated a large difference in parental history of fatal MI in the Northern Irish (where the risk of MI is 4 times higher) and French populations. About 70% of subjects in Belfast had at least one parent who had died of an MI, compared to only 30% in France.[130]

To summarise this aspect of the studies from ECTIM, subjects in a high risk population with the DD genotype have an increased risk of fatal MI. However, those with low risk

factors defined by BMI, apoB and not on hypolipidaemic treatment who possess the DD genotype was an increased risk of a non-fatal MI.

These studies raised more questions than they answered. What were the risks of those with the DD genotype in a low risk population with and without the more classical risk factors; smoking, hypertension, diabetes mellitus and hypercholestrolaemia? Was there a gene-environment interaction? One of the main problems with the Cambien hypothesis has to do with the apparent distinction between fatal and non-fatal MI. All the risk factors act in concert to produce a high risk group, who are at risk of an MI (fatal or non-fatal). The proposed mechanism through which the DD genotype exerts its influence is through high circulating (and possibly tissue) ACE levels and hence AT-II levels, although the conversion of AT-I to AT-II is not the rate-limiting step. It would be expected that AT-II would act together with the other classical risk factors in the atherothrombotic process to produce these high risk groups. The second problem has to do with the choice of BMI, ApoB and patients not on hypolipidaemic treatment as classical risk factors. These risk factors are not particularly classical by criteria in general use. A third problem, is that the risk-association of the DD genotype was extremely modest until sub-group analyses were performed. Despite these problems, however, these studies generated a lot of interest and debate in the scientific community. Since the original report in Nature, there have been several conflicting reports on the role of the ID polymorphism and CAD. Mattu et al reported an association between the DD genotype and D allele with CAD in men apparently at low risk defined by blood pressure (150/100 and 140/90) and total cholesterol/HDL ratio (5.0 and 5.6). Interestingly, in subjects with classical risk factors in the CAD group (defined by the Rose questionnaire and ECG Minnesota coding) the DD genotype did not confer any risk.[131] Though this study was said to further strengthen Cambien's work, it raised several problems. If as Cambien's work suggested, those with classical risk factors and the DD genotype constituted a very high risk group at risk of fatal MI, Mattu's work certainly does not take us any further on that score. It could be argued however, that Cambien was looking at an association with the thrombotic process (i.e. MI) whereas Mattu only addressed atherogenesis, this point is discussed further below.

Bohn M et al. in a similar study to Cambien found no relationship between the ID polymorphism and survivors of MI.[132] Three studies looking at parental or grandparental history of premature MI have found an association with the DD genotype (Table1).[130; 133;

[134] All the studies hitherto mentioned have been retrospective studies. A prospective study would clearly be more helpful in clarifying the association or lack of it. Lindpaintner and colleagues in the New England Journal reported a lack of association among 3590 North American Physicians' (Table1) with respect to coronary events (angina, MI and revascularizations) in patients who had been followed up prospectively.[135]

Discrepancies in the frequency of the DD genotype especially in the control group, which ranged from 17 - 39% highlighted the need for a genetically appropriate control group. If the DD genotype was associated with the development of atheroma rather than thrombosis (although there is biological plausibility for both), it could explain the apparent discrepancies in the frequency of DD in the control group since an apparently normal subjects may have significant atheroma. Coronary angiography is one way of addressing this issue. Table 2 gives an overview of the coronary angiographic studies to date. In over 90% of the ECTIM cases in France who had coronary angiography, there was no relationship between the DD and degree of atheroma.[136] This negative finding was confirmed by both Ludwig[137] and Gardemann. [138] However, Nakai found an association with the degree of atheroma among Japanese subjects [139]and Arbustini among Italians.[140] In contrast to Lindpainter's work, the above angiographic studies found an association with MI in subgroups (Ludwig in the group with .60% stenosis), (Gardemann - subgroup with low TG and total cholesterol). Arbustini however, found the association in the whole group of MI subjects.

4.3.3 Associations With Other Cardiovascular Pathology

Table3 summarises the relationship between the ACE I/D polymorphism and various cardiovascular pathologies in which the proliferative effect of AT-II may be involved. It is striking that the results from these studies have been so conflicting. Restenosis injury after angioplasty is a very useful model to study since it is relatively easy to demonstrate the smooth muscle proliferation subsequent to angioplasty. Ohishi demonstrated an association between the DD genotype and restenosis.[141] The larger and more careful study be Samani et al, however, showed no relationship to the ACE polymorphism.[142]

Table 4, summarises the relationship between the ACE polymorphism and vascular disease in subjects with diabetes. Whilst it is clear that the majority of these studies do suggest an association with the DD genotype, these studies are of relatively small size. Furthermore, there is the problem of publication bias in favour of studies showing positive findings. The study by Schacter et al Table3, deserves closer attention, since it is a unique study.[143] The DD genotype, which has been associated with premature death in the original report and some subsequent reports, was found to be preponderant among some 300 French centenarians compared to a control group of 160 adults aged 20 - 70. The doubts created by this study are further strengthened by the largely negative studies reported for studies in hypertension. Since the RAS plays such an important part in the regulation of blood pressure, it is perhaps somewhat surprising that the ACE I/D polymorphism does not appear to play any role in human hypertension.[126; 144; 145]
The wide variety of biologically seemingly unrelated conditions that have been reported has raised suspicions in this whole area of molecular biological research.

4.3.4 Plasma ACE And Cardiovascular Disease

Since the circulating ACE activity is only partly explained by the ACE I/D polymorphism, the question was raised as to whether plasma ACE activity per se had an independent relation to the risk of myocardial infarction. The ECTIM investigators provided evidence for such as association but after subgroup analysis in young subjects (< 55 years old). [22] This was supported by a study in which carotid intima - medial wall thickness was measured by ultrasound and suggested that chronic exposure to high levels of plasma ACE could be involved in structural changes of the arterial wall.[146] In contrast to this however, Gardemann, who showed an association between the ID polymorphism and MI, found no relationship between circulating ACE and CAD.[138]

4.4 AIMS

This study was designed to look for a possible differential effect of the ACE gene polymorphism on atheroma on the one hand, and thrombosis on the other. Atheroma was defined by significant lesions at coronary angiography, and thrombosis by a history of myocardial infarction. The relationship between the ACE gene and the classical risk factors was also assessed. Furthermore, any independent association between circulating levels of ACE and atherothrombosis was ascertained.

TABLE 1 ACE AND CAD

AUTHOR	n CAD/ Control	Definition of CAD	Study Population	+ or -	RR (OR)
Cambien et al	610/733	MI 3-9 months (all f)	France/Belfast	+	1.34 (3.2 in low risk groups + (subgroup with low TC & TG)
Bohn et al	234/366	MI (up to 4yrs (f + m)	Norway	- (P= 0.002)	-
Bohn et al	229/366	premature parental MI	Norway	+ (P=0.03)	3.1
Tiret et al	1343	Parental history MI	France/Belfast	+	2.6
Mattu et al	404/822	ECG's & Rose questionnaire	Wales (Caerphilly)	+ (P<0.009)	-
Evans et al	213	fatal MI (autopsy)	Belfast Caucasians	+ (P<0.02)	2.2
Badenhop et al	404	CAD in grandparents	Australian Caucasians	+ P=0.01	2.8
Lindpaintner et al	1250/2340	MI, Angina, revascularisation *(Prospective)	North American Physicians	-	

TABLE 2 ACE AND ANGIOGRAPHIC STUDIES

AUTHOR	N Cases/ Control	DEFINITION CAD	ATHEROMA (ANGIO)	THROMBOSIS (MI)	STUDY POPULATION	+/-
Nakai et al	178/100	75% (MI and Angina)	no distinction	made	Japanese	+ (Multi > Single CAD > control)
Cambien (ECTIM)	368	?			France	-
Ludwig	697/203 159 had MI	>60% (n = 362) <10% disease free (n = 335) quantitative angio n = 27	-	+ (P = 0.005) in >60% - (< 10% group)	Utah (US whites)	+/-
Gardemann	920	> 50%	-	-	Germany	+/-
Talmud[147]	73	Progression/ Regression study	DD associated of	with progression of disease	UK Caucasians	+
Arbustini	255/133	MI/Angio	+	+	Italy	+

TABLE 3 ACE AND OTHER CARDIOVASCULAR PATHOLOGY

AUTHOR	CONDITION (Cardiovascular)	CASES/ CONTROL	ASSOCIATION WITH DD
Pinto et al[148]	Progressive Ventricular Dilation after anterior MI	96/0	+
Raynolds et al[149]	Ischaemic and Idiopathic dilated Cardiomyopathy	214/79	+
Montgomery et al[150]	Idiopathic dilated Cardiomyopathy	99/364	-
Castellano et al (Vobarno Study)[151]	Carotid artery wall thickness	189/0	+
Marian et al[152]	Hypertrophic Cardiomyopathy and Sudden death	100/106	+
Samani et al	Restenosis after Coronary angioplasty	233/0	-
Schunkert et al[153]	Left Ventricular hypertrophy (ECG)	290/1138	+
Iwai[154]	Left Ventricular hypertrophy (Echocardiogram)	268/-	+
Celermajer[155]	Endothelial dysfunction in subjects without other CAD risk factors	184/-	-
Kupari[156]	Left ventricular size, mass & function	86/-	-
Dessi-Fulgheri[157]	Carotid atheroma	240	-
Ohishi	Restenosis after Coronary angioplasty	82/102	+
Catto [158]	Stroke	467/231	-
Schacter F et al	Human Longevity (in centenarians)	338/160	+
Harden[159]	Progression of IgA nephropathy	100/100	+

TABLE 4 ACE AND DIABETES

AUTHOR	CONDITION	CONTROL	ASSOCIATION WITH DD
Keavney[160]	MI in NIDDM patients	173/297	+ (D allele)
Zingone[161]	Hyperglycaemia	174/-	+
Panahloo[162]	Insulin sensitivity in NIDDM Insulin Resistance	103/533	+ -
Ruiz[163]	CAD in NIDDM	132/184	+
Fujisawa[164]	MI in NIDDM Diabetic nephropathy & Retinopathy	61/136, 54/35, 191/-	+ -

4.5 PLASMINOGEN ACTIVATOR INHIBITOR-1

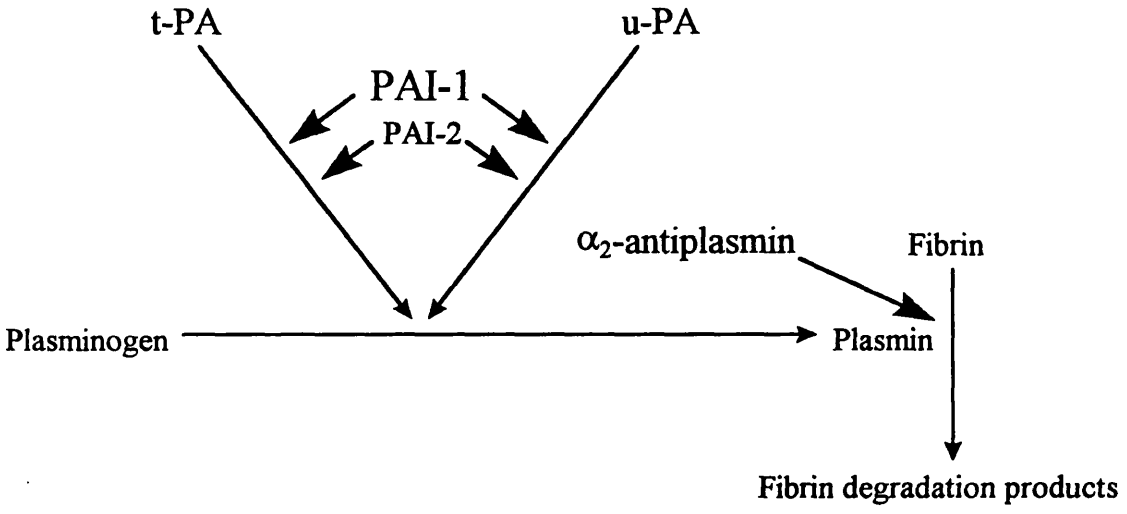
4.5.1 The Fibrinolytic System

The fibrinolytic system is responsible for the degradation of the solid-phase fibrin network that constitutes the major protein component of thrombus. [165] As a consequence, the fibrinolytic system facilitates the dissolution of blood clots after repair of a locally injured vessel wall and thus ensures unobstructed circulation. [165] Fibrinolysis is initiated by either one of the serine proteases urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (tPA). The activity of the latter is dependent on the presence of the obligatory cofactor fibrin. Binding of tPA to fibrin increases the affinity of tPA for the zymogen plasminogen, resulting in a significant acceleration of the conversion of plasminogen to the active enzyme plasmin. Plasmin converts insoluble intact fibrin polymers into a distinct series of fibrin degradation products. Urokinase also activates plasminogen but is chemically distinct from t-PA. [165]

PAI-1 and α_2-antiplasmin are the two major inhibitors of fibrinolysis. Physiologically PAI-1 is the more important inhibitor, and plasma levels of PAI-1 is attributed with being the principle determinant of fibrinolysis.

PAI-1 is a 50 KD glycoprotein that rapidly forms inactive 1:1 equimolar complexes with tPA (and u-PA). It is synthesised by endothelial cells, hepatocytes and is found in high concentrations in the alpha granules of platelets and probably vascular smooth muscle cells. The activity of plasmin is controlled by α_2-antiplasmin, a glycoprotein that is present in high concentrations in plasma. While α_2-antiplasmin is capable of inhibiting free plasmin, the latter generated on the surface of fibrin is protected from α_2-antiplasmin. The main components of the fibrinolytic system are shown in figure 5.

Figure 5 Components of the fibrinolytic system



t-PA	Tissue plasminogen
u-PA	Urokinase
PAI-1	Plasminogen activator inhibitor-
PAI-2	Plasminogen activator inhibitor-
↘	Inhibitor

PAI-2 is present in the trophoblastic epithelium in human placenta. It is a major inhibitor of fibrinolysis during pregnancy and with the exception of rare tumours, high levels of PAI-2 are rarely encountered. [165]

The fibrinolytic system acts as a counterbalance to the coagulation cascade, and in common with this system, control mechanisms exist at several levels to prevent abnormal activity. Under normal circumstances t-PA binds to fibrin to prevent inhibition by PAI-1. In the circulation t-PA activity is effectively quenched by circulating PAI-1 to prevent fibrinogenolysis and bleeding.

Evidence for the regulatory function of PAI-1 in vivo is derived from studies conducted by Erickson et al. Transgenic mice with stimulated expression of human PAI-1 cDNA were found to be born with thrombotic abnormalities in the tail and hind legs.[166] Secondly, Carmeliet and collaborators employed murine embryonic stem cells and the technique of homologous recombination to inactivate the PAI-1 gene. Subsequent crossing of heterozygotes yielded "double knock-out" mice (PAI-/-) that were fully viable. Challenging the fibrinolytic system by injecting fibrin in the lungs of these animals showed that the potency to dissolve fibrin was significantly higher in the PAI-/- mice than in the normal mice (PAI+/+), whereas an intermediate potency was observed in the heterozygotes (PAI-/+). The fibrinolytic capacity of PAI-/- mice is reduced to that of normal mice after injection of adenoviruses that enable functional expression of PAI-1.[167] Thirdly, Fay et al, examined an 11 year old with a haemorrhagic diathesis whose plasma was completely devoid of PAI-1 antigen and activity, due to a dinucleotide insertion in exon 4 of both PAI-1 alleles. Whether this patient synthesised a truncated PAI-1 protein that was subsequently degraded is unknown.[168] Furthermore, several other groups have reported (partial) PAI-1 deficiency in patients suffering from hyperfibrinolysis, manifested by bleeding tendencies. Elevated levels of PAI-1 are found in patients with thrombotic disorders such as deep vein thrombosis [169] and myocardial infarction [170]. Levels of PAI-1 rise during the second and third trimesters of pregnancy coinciding with the time of increased risk of thrombosis.[165] [150] These observations provide evidence that PAI-1 is an essential regulatory protein of the fibrinolytic system..

4.5.2 PAI-1 and Physiology

In 1963 Brakman and Astrup claimed the existence of an inhibitor of fibrinolysis that did not act directly upon plasmin [171] and in 1982 Kruithof et al demonstrated the presence of a

physiological inhibitor of tissue plasminogen activator in human plasma, [165]. It belongs to the group of protein inhibitors termed SERPINS (serine protease inhibitor super family). In human subjects PAI-1 is present in plasma at low concentrations of between 0 and 60 ng/ml

PAI-1 synthesis has been demonstrated in a number of differing cells lines including endothelial cells, fibrosarcoma cells, mesothelial cells and hepatoma cells.[172] PAI-1 production can be induced in other cells by a number of stimuli as listed in table5.

PAI-1 is present in plasma as a complex with vitronectin, which acts to stabilise it. PAI-1 is also found at high concentrations in the α-granules of circulating platelets.[172] With the exception of platelets, PAI-1 is not stored within cells and is secreted after synthesis, indicating that increases in circulating levels of PAI-1 seen in disease states may be due to increased synthesis rather than release of intracellular stores.[173]

PAI-1 adopts at least two different conformations. It is secreted in its active form and spontaneously converts to its latent form with a half-life of about 1 hour at 37°C in neutral or alkaline pH. The latent form has no inhibitory activity but there is limited evidence that it can be physiologically converted back to the active form. .[173] The majority of PAI-1 in fresh human plasma is in the active form whereas that in platelets is in the latent form. The physiological significance of the PAI-1 in platelets which accounts for 90% of total blood PAI-1is unclear although it may contribute to the resistance of platelet rich thrombus to thrombolysis. Alternatively it may act as a store of PAI-1 to be activated and released upon some stimulus. Vitronectin is present in platelets and it has some ability to activate latent PAI-1.

TABLE 5 STIMULANTS OF PAI-1 GENE EXPRESSION.

Stimulus	Cell type	Reference
lipopolysaccharide	bovine aortic endothelial cells human umbilical vein endothelial cells (but not hepatoma cells)	(Loskutoff 1991)
tumour necrosis factor and interleukin-1	bovine aortic endothelial cells human umbilical vein endothelial cells microvascular endothelial cells	(Loskutoff 1991)
transforming growth factor β	bovine aortic endothelial cells, fibroblasts, epithelial cells, hepatoma cells	(Loskutoff 1991)
Epidermal growth factor, platelet derived growth factor and basic fibroblast growth factor	various cell lines	(Loskutoff 1991)
corticosteroids	hepatocytes, mammary carcinoma, human and rabbit endothelial cells, human dermal fibroblasts	(Loskutoff 1991)
lipoprotein (a)	endothelial cells	(Loskutoff 1991)
phorbol-myristate acetate	variety of cells	(Loskutoff 1991)
thrombin	human umbilical vein endothelial cells	(Dichek 1989)
insulin	HepG2 (hepatic) cells, bovine aortic endothelial cells	(Kooistra 1989; Grant 1991a)
insulin-like growth factor	porcine aortic endothelial cells	(Schneider 1991)
proinsulin	porcine aortic endothelial cells	(Schneider 1992)
very low density lipoprotein	human umbilical vein endothelial cells and Hep G2 cells	(Mussoni 1992)
angiotensin II	murine astrocytes, bovine aortic endothelial cells, vascular smooth muscle cells	(Olson 1991; Feener 1995; Vaughan 1995)

Secretion of PAI-1 into the circulation is known to be derived from several sources including endothelial cells, vascular smooth muscle cells, adipose tissues and hepatic cells. It is possible that under different conditions these sources contribute to different extents. .[172] .[173]This uncertainty necessitates some caution in extrapolating the results from in vitro experiments on PAI-1 gene expression from any cell line to the in vivo regulation of PAI-1 levels.

4.5.3 Regulation Of PAI-1

There is intra and interindividual variation in PAI-1 levels over time. The source of this variation may be contributed to by different assay techniques. Circulating levels of PAI-1 follow a circadian pattern reaching its peak in the morning. This relative hypofibrinolytic state coincides with the increased incidence of coronary events at this time.[174]

PAI-1 is raised in response to acute phase reactions including stress, trauma, surgery, infection and inflammation.[165] This may be mediated through the action of cytokines which have been found to stimulate PAI-1 gene transcription in vitro.[175] Smoking has also been associated with raised plasma PAI-1 levels. [160]. Finally, evidence from population studies suggests that levels of PAI-1 relate to the metabolic changes associated with the insulin resistance syndrome (Syndrome X).

4.5.4 PAI-1 And Insulin Resistance Syndrome

Reaven [72]described a clustering of cardiovascular risk factors including hypertension, low HDL cholesterol and high VLDL triglyceride figure 6. Subsequently, male pattern obesity was added to his original description. Central to these metabolic abnormalities is Insulin resistance. Several studies have demonstrated a close association between PAI-1 and the features of the metabolic syndrome. Juhan-Vague *et al* showed a relation to BMI and systolic hypertension,[176] and Landin to waist to hip ratio and systolic hypertension.[105] Correlations between PAI-1 levels with Insulin in angina patients[177] and obese subjects with NIDDM have also been demonstrated. [177]

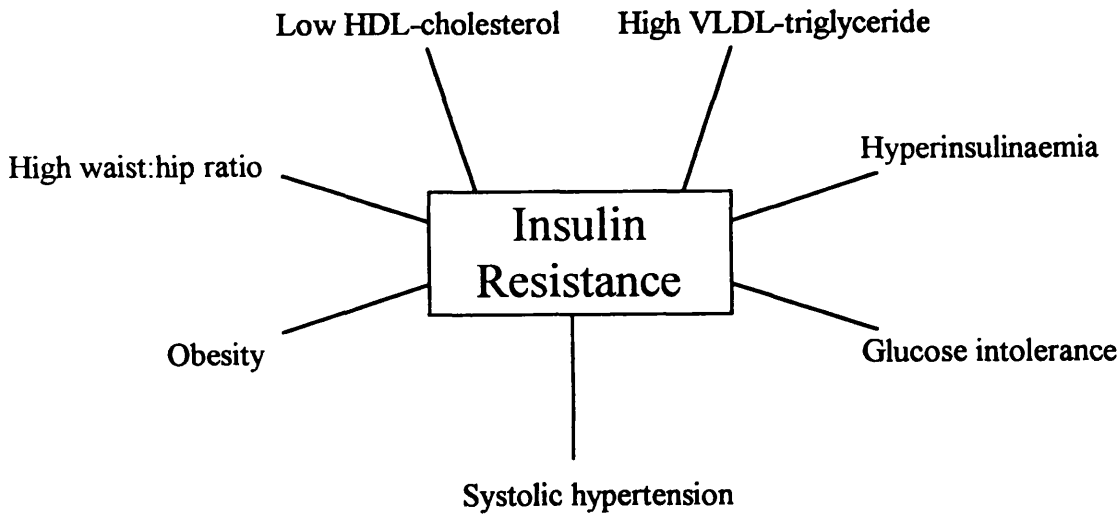
The strongest evidence for the association of PAI-1 and insulin resistance is from human studies in which insulin resistance, evaluated directly by hyperinsulinaemic euglycaemic clamping techniques, has been significantly correlated with PAI-1 in hypertensive subjects and obese subjects by Landin et al and with correlation coefficients as high as 0.98 in obese subjects and 0.87 in NIDDM subjects reported by Potter van Loon *et al.* [178]

When insulin resistance is reduced by starvation, weight loss, or physical exercise there is an associated normalisation of elevated PAI-1 levels. Metformin reduces insulin resistance and has been shown to increase fibrinolytic activity. Metformin therapy causes a reduction in PAI-1 levels in obese subjects and patients with NIDDM.[179]

The existence of a correlation between triglyceride and PAI-1 levels have been demonstrated in several studies in both normal subjects and NIDDM patients.[60] The reduction of triglyceride levels over the medium term by dietary intervention in non diabetic subjects with cardiovascular risk factors tends to normalise elevated PAI-1 levels.[180]

Both VLDL in a receptor dependent mechanism and LDL, independently of receptors specifically stimulate synthesis of PAI-1 in cultured endothelial cells and LDL also acts in this way on cultured HepG2 cells.[181] VLDL from patients with hypertriglyceridaemia has a greater stimulatory effect than that from normotriglyceridaemic subjects.[182] This fact may be of critical importance in our understanding of the relationship between PAI-1, its relation to insulin resistance and the association with CAD. Although, whether the association of triglyceride and PAI-1 levels accounts for the relationship between insulin resistance and hypofibrinolysis is not entirely clear.

Figure 6 The Insulin Resistance Syndrome.

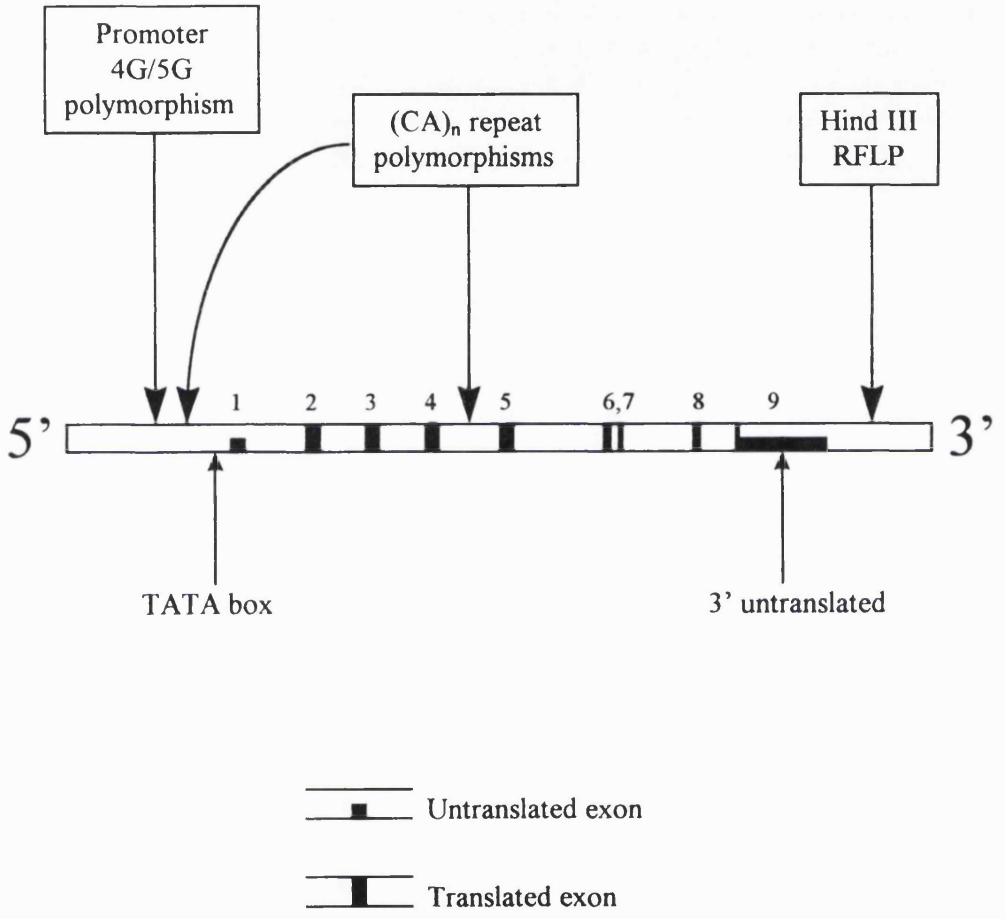


4.6 GENETIC POLYMORPHISMS OF PAI-1

The PAI-1 gene which contains 9 exons and 8 introns, distributed over approximately 12.3 kb of DNA has been cloned and localised to q21.3-q22 of chromosome 7. [183-185]Most of the regulation of PAI-1 is through activation or repression of transcription. A large number of physiological regulators affect PAI-1 expression and do so through direct changes in transcriptional activation and repression. The promoter is responsible for driving transcription of the gene and contains sites to which transcriptional activators and repressors can bind, figures7 and 8. Functional studies in which the promoter region of the PAI-1 gene was attached to a reporter gene have provided some reliable information on the ability of different sections of the gene to promote transcription in response to different stimuli in cell culture. Various experiments have localised specific regulatory elements to regions of the PAI-1 promoter including those responsive to tissue growth factor and dexamethasone. Further work to establish the transcription factors and mechanisms responsible this tissue specificity and regulation in vitro is one way in which it may be possible to gain more understanding of the very complex regulation of PAI-1 in vivo.

Similar to ACE, a large degree of inter- and intra-individual variation in PAI-1 levels has been observed.[186] It has been postulated that some of this variation is due to common genetic variants in the PAI-1 gene which affect gene expression. Different polymorphisms have been described in the PAI-1 gene, figure 7.[183; 187; 188] Klinger et al reported an allelic variation at a HindIII restriction fragment length polymorphism (RFLP). This has been associated with altered levels of PAI-1 in healthy individuals and young male survivors of an MI.[187] This association however, did not reach statistical significance possibly due to the small numbers in the study. Dawson *et al* proposed from this study that the correlation that exists between PAI-1 and VLDL and that between insulin response to an oral glucose load and plasma PAI-1 levels are affected by genotype at this RFLP.

Figure 7 Schematic representation of the human PAI-1 gene showing the relative location of 3 common polymorphisms.



In common with other dinucleotide repeat sequences, the cytosine-adenine (CA) repeat sequence in the 4th intron of the PAI-1 gene shows polymorphic variation with differing numbers of CA dinucleotides on different alleles. Dawson et al studied this CA repeat polymorphism in young male Swedish survivors of myocardial infarction and control subjects. .[187] They used polymerase chain reaction amplification of the CA repeat sequence and polyacrylamide electrophoresis to separate DNA bands and identified 8 alleles at this locus. According to a nomenclature suggested by Weber and May , they named the most frequent allele "z" and other alleles by their nucleotide difference from this allele. So in order of increasing size the alleles were named "z-2", "z" "z+2" and so on. The largest allele identified was "z+10". Some confusion has arisen about the location of this CA repeat polymorphism. Dawson et al described it as being in the third intron .[187] whereas the PAI-1 gene sequence published by Bosma *et al* clearly places it in the 4th intron.[185] The fact that the 1st exon is not translated may have led to this confusion. Dawson et al found that the intron CA repeat sequence was in linkage disequilibrium with the Hind III RFLP with allele 1 associating with the most frequent CA repeat allele "z" and the Hind III allele 2 associating with longer CA repeat alleles "z+4", "z+8" and "z+10". .[187]

Dawson et al used the Hind III RFLP and the intronic CA repeat polymorphism to examine the relationship of PAI-1 genotype with circulating levels of PAI-1 in a group of young Swedish males who had survived a myocardial infarction and 95 age matched healthy control subjects. They found a trend to higher PAI-1 levels in both patient and control groups, in subjects homozygous for the HIND III 1 allele. This did not reach statistical significance possibly due to the low numbers in the study. At the CA repeat polymorphism they found an association between high levels and shorter alleles, p=0.03, in the patient group, and a non-significant trend in the control group. They further demonstrated that a relationship existed between PAI-1 and triglyceride which was influenced by PAI-1 genotype

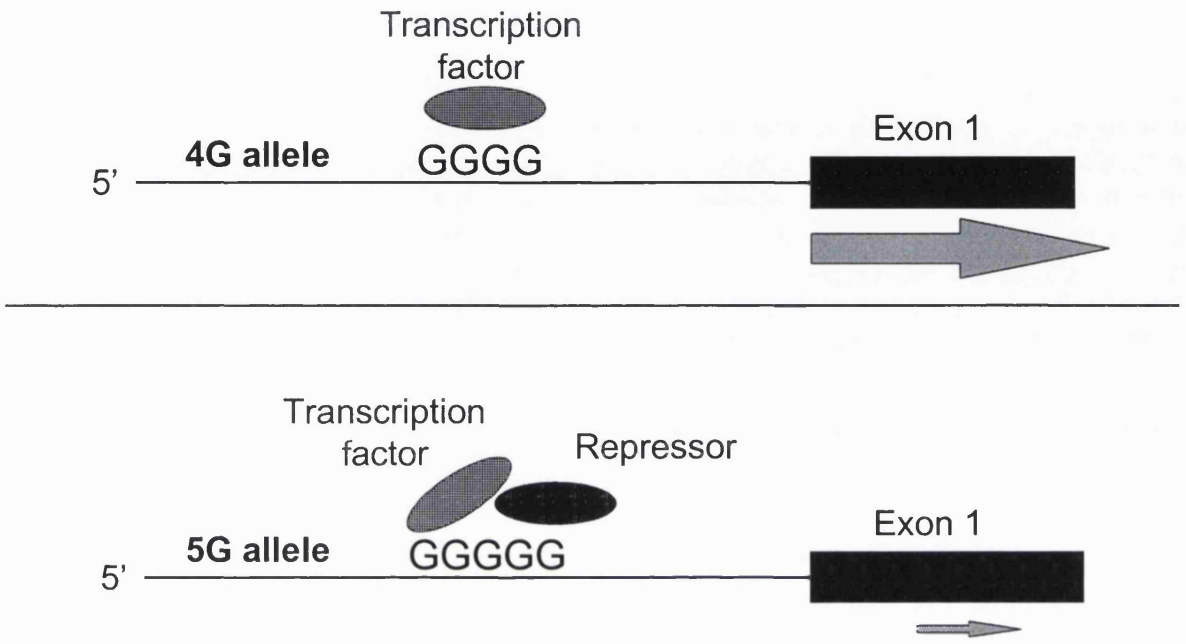
These data prompted the search for polymorphisms in the promoter region of the PAI-1 gene. Any sequence changes at these sites could be envisaged to alter either basal levels of PAI-1 or alter the response of PAI-1 to a specific regulator. It was postulated that PAI-1 responses in vivo, may be mediated by changes in the rate of gene transcription, and that sequence elements within the promoter region control this response.[189; 190]. Dawson and colleagues went on to further examine the promoter and 3' region of the PAI-1 gene in an effort to identify sequence chains responsible for the association between genotype and PAI-1 levels demonstrated by their earlier study. They identified a polymorphism in the promoter region

which was shown on sequencing to be insertion of a fifth guanine base in a run of four guanine bases at position –675 from start of transcription. .[189] (The relative locations of 3 common polymorphisms are depicted on figure 7).

They went on to show differential binding of crude hepatoma cell nuclear proteins to the alleles when present as double-stranded oligonucleotide sequences. They used an in vitro system whereby a length of the PAI-1 promoter included either 4G or 5G allele. This was placed upstream of a gene for a marker protein chloramphenicol acetyltransferase, (CAT) and transfected into cultured liver cell line (HepG cells). The influence of the polymorphism on gene transcription was then studied. There was no difference in basal production of CAT mRNA but when the cells were stimulated with interleukin-1, which is known to increase PAI-1 transcription in vitro they found significantly greater CAT mRNA production from the 4G than the 5G allele. They also re-examined the relationship between PAI-1 levels and genotype in the original patient groups finding increased PAI-1 levels in subjects with the 4G/4G genotype although this reached significance in only the patient and not the control sample. When PAI-1 levels were adjusted for triglycerides and BMI, this difference failed to reach standard levels of significance.

Eriksson et al have repeated much of this work confirming that the PAI-1 promoter shows allele-specific binding of nuclear proteins extracted from HepG2 cells and also from human umbilical vein cells (HUVEC) and smooth muscle cells.[190] Using a methylation interference assay the binding sites of two particular proteins have been localised. The first, named protein A binds equally to the 4G and 5G alleles at a site upstream and adjacent to the polymorphic site. The second, protein B, binds only to the site of the sequence of 5 guanine residues and is believed to be the allele-specific factor. In HepG2 cell and HUVEC transfection studies using PAI-1 promoter-driven CAT transcription, Eriksson's findings differ from those of Dawson et al. The 4G allele showed greater basal transcriptional activity than the 5G allele but there was no difference in the response of the two alleles to cytokine stimulation. The discrepancy in the results of these two studies is unresolved, although it may arise from the different lengths of the PAI-1 promoter that were transfected and from the use of differing HepG2 cell lines. In addition differences in culture media and treatment of the cells may also affect the relationship between promoter function and the rate of transcription of the reporter gene. However, in each case the 4G allele was found to be associated either with increased basal or stimulated gene transcription compared to the 5G allele, figure 8.

Figure 8 Schematic representation of transcription factors and effect on circulating PAI-1 levels.



Eriksson *et al* also examined PAI-1 genotype in relation to circulating levels in a group of young Swedish male survivors of myocardial infarction (MI) and control subjects. They found a significant association between higher PAI-1 levels and increasing numbers of 4G alleles in control subjects and a similar but non-significant trend in the MI patients. .[190]

4.7 PLASMA PAI-1 IN RELATION TO ATHEROTHROMBOSIS

The mechanisms responsible for the generation of atheromatous lesions have been outlined above. Fibrin deposition is an invariable feature of plaques. It could play a role in binding LDL and stimulating smooth muscle cell proliferation. The fact that transmural myocardial infarction is caused by the formation of an occlusive fibrin thrombus on a pre-existing plaque is established. Defective fibrinolysis through elevated PAI-1 may therefore determine vascular risk through the two separate but related events of atheroma and thrombosis.

4.7.1 Atheroma

Elevated expression of PAI-1 has been demonstrated around atherosclerotic lesions in diseased human arteries.[191] In the ECAT study PAI-1 levels were related to the presence of coronary stenosis in patients undergoing coronary angiography.[77] Sakata et al showed that patients with multivessel CAD had significantly greater PAI-1 levels than those with 1-vessel disease.[192]

These findings indicate an association between circulating levels of PAI-1 and coronary atheroma. However, they do not tell us whether the raised levels of PAI-1 are due to cause or effect or indeed both cause and effect.

4.7.2 Thrombosis

The circadian fluctuation in levels of PAI-1 coincides with the circadian pattern in the incidence of acute myocardial infarction with a daily peak at around 8.00a.m.[193] This pattern is preserved during episodes of myocardial ischaemia suggesting that this may not be purely a reactive phenomenon. A number of studies have reported on the association between suppressed fibrinolysis (through raised PAI-1) and angina or MI.[194]

Prospective studies have shown that reduced fibrinolysis predicts myocardial infarction and that high PAI-1 activity predicts recurrence of myocardial infarction in young Swedish men.[195] Since PAI-1 may be related to atheroma, showing that high levels of PAI-1 predict recurrence of MI does not establish a causal link with thrombosis. It could simply represent the fact that elevated PAI-1 levels reflect increased severity of coronary disease, in terms of risk of MI. However there are other lines of evidence supporting a causal link with thrombosis.

Thrombolysis resistance and reocclusion after thrombolytic therapy of patients suffering from acute MI is associated with the occurrence of platelet- rich thrombi. Different approaches have been used to support the hypothesis that PAI-1 released from thrombin-activated platelets and retained within the thrombus by binding to fibrin, is a major contributor to thrombolysis resistance and re-occlusion.

First, incorporation of active PAI-1 into preformed clots applied to an experimental animal model for pulmonary embolism, showed that the inhibitor suppressed endogenous fibrinolysis.[196] Furthermore, systemic administration of active PAI-1 to experimental animals substantially compromised t-PA-mediated fibrinolysis.[197] Conversely, the incorporation of anti-PAI-1 monoclonal antibodies enhanced endogenous t-PA-mediated thrombolysis in the rabbit jugular vein model and partially prevented thrombus extension.[198] Significantly, in a canine experimental model for coronary thrombosis that mimics reocclusion in man,[199] a comparison was made between systemic administration of an anti-PAI-1 monoclonal antibody together with t-PA versus t-PA alone. The antibody substantially reduced the time to reperfusion, prolonged the time to reocclusion and ultimately caused a reduction in the number of reocclusions.[200]

Ex-vivo experiments measuring t-PA-mediated clot lysis with purified platelets, demonstrated that a peptide derived from the amino-acid sequence of the mobile loop of PA1-

1, also greatly increased clot lysis presumably due to inactivation of PAI-1.[201] Possibly, this approach or one employing anti-PAI-1 monoclonal antibodies may lead to compounds that promote thrombolysis in vivo and may prevent reocclusion after thrombolysis in patients with an acute MI. These findings have gained support from the studies by Gray and Yudkin.[202] They compared the activity of PAI-1 in diabetic and non-diabetic patients admitted with acute MI and also determined whether PAI-1 activity influenced reperfusion after thrombolytic therapy. They found that both raised PAI-1 activity on admission and diabetes were associated with a reduced likelihood of reperfusion after a thrombolytic therapy. Support for this finding comes from the study by Barbash *et al* in which PAI-1 levels were correlated to patency of infarct-related arteries after thrombolytic therapy with recombinant t-PA. In 125 consecutive patients with an acute MI, pre-treatment levels of PAI-1 were significantly higher in patients with occluded arteries compared to those with patent arteries.[203]

While the above data indicate an association between PAI-1 levels and both atheroma and thrombosis, it is unknown whether these changes represent cause, effect, both cause and effect or a shared association with some other factor. PAI-1 is released as part of an acute phase response, and it is therefore possible that the elevated levels seen in previous studies may have resulted from rather than lead to atherogenesis and thrombosis. Furthermore, an underlying association between PAI-1 levels and CAD may be hidden by the confounding effect of the close correlation between levels of PAI-1 and other established cardiovascular risk factors. If genotype contributes to determination of PAI-1 levels, it may act as a marker for lifelong exposure to differing levels representing a means to test the hypothesis that elevated PAI-1 levels contribute to coronary atheroma and/or thrombosis.

4.8 PAI-1 GENOTYPE AND ATHEROTHROMBOSIS

Differences in PAI-1 genotype frequencies between affected and unaffected population groups may suggest that elevated PAI-1 levels are the cause rather than the effect of atherothrombotic disease. Dawson et al found no difference in PAI-1 Hind III RFLP and promoter polymorphism genotypes between young Swedish male long-term survivors of MI and control subjects from the same area.[187] However, Eriksson et al examined a similar group who had recently survived an MI and found an increased frequency of the 4G/4G genotype in the MI group.[190] Support for Eriksson's findings came from Mansfield et al, who found a preponderance of the 4G/4G genotype in non-insulin dependent diabetes subjects with a history of myocardial infarction.[204] Such studies suffer from a number of confounding factors

a. the small sample size

b. the phenomenon of allele drop-out - this is due to the death of subjects expressing the clinical phenotype that may lead to under representation of the deleterious genotype.

The ECTIM group reported their analysis of PAI-1 promoter genotype from their case control study of candidate genes in MI [205]. They confirmed the association between PAI-1 promoter genotype and circulating PAI-1 levels but found no evidence between the genotype and survivors of MI. Patients were recruited three to nine months after the event and hence this study could suffer from the possibility of allele drop-out. One may have expected any genotype-related drop-out through mortality to have disturbed the Hardy-Weinberg equilibrium. However, this was demonstrated in the MI subjects studied at all four centres in ECTIM. An important problem with ECTIM as well as most of the studies published to date in this field is that it fails to distinguish between a possible differential effect on atheroma on the one hand and thrombosis on the other. The importance of this lies in the fact that an apparently normal control group will have subjects with significant atheroma that is not yet apparent.

At the time of this project, no study had been published that addressed the separate issues of the relationship of PAI-1 levels and genotype to atheroma on the one hand and thrombosis on the other.

4.9 AIMS OF THESIS 2

The emerging evidence that circulating levels of PAI-1 relate to genotype at a common polymorphism in the promoter of the PAI-1 gene has opened the possibility of using PAI-1 genotype as a surrogate measure of pre-morbid PAI-1 levels to tease apart the cause and effect limbs of the PAI-1-CAD relationship.

As with the ACE gene the relationship between the PAI-1 promoter polymorphism and PAI-1 levels, and the presence of atheroma as assessed by coronary angiography, and thrombosis by a history of myocardial infarction was examined. Furthermore, the relationship between PAI-1 and other cardiovascular risk factors were assessed.

5.0 GENDER DIFFERENCES IN PATIENTS WITH CAD

The study of coagulation and fibrinolytic factors in relation to IHD afforded us the opportunity to look at differences that exist with respect to gender.

Introduction

Although the majority of studies investigating cardiovascular risk factors have been conducted in men, differences between men and women in the epidemiology, presentation, management and prognosis of coronary artery disease (CAD) have become apparent recently.[206; 207] While the major risk factors (cigarette smoking, hypertension, diabetes, cholesterol) are important in both sexes, gender differences with respect to other risk factors are being investigated to explain some of this dissimilarity.

The Framingham[208-210]and other studies[211] highlight several differences in men and women presenting with acute and chronic coronary syndromes. Women and men who at angiography are found to have a "normal" or minimally diseased coronary artery have a good prognosis.[212; 213] However, women who present with definite evidence of CAD, for example myocardial infarction, have a poorer prognosis than men who present similarly.[210] Furthermore, the cardiovascular influence of diabetes is more marked in female than male patients of a similar age[214] a finding that may relate to sex-specific differences in circulating levels of haemostatic factors.[215] Little information is available with regard to these haemostatic systems and their gender-specific influence on atherothrombotic disease in non-diabetics subjects. Four components of these systems have been related to the risk of future coronary events. Fibrinogen has emerged as a potent predictor of CAD and future coronary events as shown in a number of large prospective studies including Framingham, [216] Northwick Park Heart[74] PROCAM,[75] and the Speedwell/Caerphilly Studies.[217] FVII:C independently predicted fatal coronary events in the Northwick Park Study. [74] In the European Concerted Action on Thrombosis and Disabilities (ECAT) study, vWF as well as fibrinogen levels were identified as independent and significant predictors of MI or sudden death in patients who presented with angina.[218] Raised levels of PAI-1 resulting in suppression of fibrinolysis have been found in a number of studies to relate to the severity of CAD (assessed by angiography),[77] and to predict re-infarction in young male survivors of a myocardial infarction.[195]

Aim

In this study we have investigated the hypothesis that gender-specific differences in coagulation and fibrinolysis occur in relation to coronary atheroma which could explain some of the differences observed with regard to the presentation and respective prognoses in CAD.

6.0 MATERIAL AND METHODS

6.1 THE STUDY POPULATION

Six hundred and nine Caucasian patients were recruited from two centres in Leeds (409) and Wakefield(200). Patients were admitted for coronary angiography for investigation of chest pain, with suspected or known CAD. Our control population comprised of patients with no evidence of significant atheroma at coronary angiography. This group of patients usually had chest pain, and had one or more CAD risk factors. The majority of patients had inconclusive or positive exercise test. A positive exercise test was defined as > 1mm ST segment depression in 2 successive leads on a standard Bruce protocol. Patients with negative exercise tests who continued to have chest pain and were admitted for angiography were also included. Each patient gave informed consent, and the study was approved by the United Leeds Teaching Hospitals (NHS) Trust and Pinderfields Health Trust Research Ethics Committee.

6.2 ASSESSMENT OF RISK FACTORS FOR CAD

We used a standard questionnaire to assess risk factors for CAD as shown in appendix..

6.2.1 Smoking history

We distinguished groups of patients: currents smokers, patients who had stopped smoking but had been smokers in the previous 10 years, patients who had smoked consistently at any time previously, and those who had never smoked.

6.2.2 Family history of CAD

A history of angina or myocardial infarction in a first-degree relative < 60 years of age was taken from patients. A history of CAD in family members other than first-degree relatives was noted separately. We also ascertained other cardiovascular risk factors in any relative.

6.2.3 Diabetes

We recorded a history of diabetes mellitus, both insulin and non-insulin dependent types by WHO criteria (Report of a WHO Study Group Geneva Switzerland). This was ascertained by clinical history, and confirmed with reference to patients' case notes.

6.2.4 Hypertension

A past history of hypertension was ascertained from patients' medical records.

Blood pressure was measured (at the time of study) with patients in the supine position to the nearest 2mmHg using the Dynamap automated sphygmomanometer (Critikon, 1846 SX/P

Version 086). For patients on antihypertensive or antianginal medication we established from the medical records the status of the patient with regards to a past history of hypertension.

6.2.5 Hyperlipidaemia

Patients reported to have a history of hyperlipidaemia or those on dietary and/or antilipid medication were noted from medical records. Total cholesterol and triglyceride were measured on fasting blood samples using the Hitachi 747 automatic analyser (Boehringer Mannheim, Mannheim, Germany) in the biochemistry laboratory.

6.2.6 Body mass index

This was calculated from weight in kilograms divided by height in metres squared.

6.2.7 Angina and Myocardial infarction

Current angina was defined as any episode of typical cardiac pain in the three months preceding angiography. The diagnosis of myocardial infarction (MI) was ascertained from patients' hospital records using WHO criteria of at least 2 out of 3 from ST elevation of 1mm in two or more successive leads, typical chest pain longer than 20 minutes duration, and a creatinine kinase rise of more than twice the baseline value. Patients who were reported to have had a history of MI but did not meet the WHO criteria (n=16) were excluded from the analysis. The diagnosis was confirmed by the presence of hypo-and akinetic segments at angiography. The duration between the most recent episode of MI to the time of recruitment was noted in view of the effect of acute phase reaction.

6.3 CORONARY ANGIOGRAPHY

6.3.1 Technique

Two techniques , the Sones (brachial artery approach) and the Judkins (femoral artery approach), were used depending on the cardiologists preference. Patients were given a light premedication (diazepam 5-10 milligrams). Local anaesthesia was administered in the region of the femoral sheath. A haemostatic sheath was inserted into the femoral artery, and the preshaped catheters passed up the aorta. Left ventriculography was usually performed in conjunction with coronary angiography. A single (or double) view (s) of the ventricle was taken. A left ventriculogram is an important part of the study since CAD may cause abnormalities of left ventricular function. Regional wall abnormalities (hypokinetic or akinetic segments) are suggestive of prior coronary events. Other disease processes (such as the cardiomyopathies) may lead to more global abnormalities of ventricular function. Catheters

were then manipulated into the respective coronary ostia. Angiographic assessment was made in multiple projections as anatomically coronary arteries curve around the heart and eccentric lesions may be seen in some projections and not in others. This enabled the operator to assess the severity and extent of stenosed vessels.

6.3.2 The Grading System

Angiography was carried out and reported by Cardiologists who had no knowledge of biochemical, coagulation or fibrinolytic factors. Significant coronary atheroma was defined as 50% stenosis in each of the three major coronary arteries or their branches. Patients were then categorised into " normal", single, double or triple vessel disease.

The use of coronary angiography and the grading system is discussed in section....

6.4 SAMPLING METHODS

Each patient was studied between 0700h and 1030h (to minimise the influence of the circadian rhythm of PAI-1 samples) after an overnight fast of at least 8 hours. Free-flowing blood samples were taken using a 19 gauge butterfly needle from an antecubital vein without venous stasis. Blood was taken into 0.9% citrate (ph 8.8) on ice, in the ratio of 9 parts blood to 1 of citrate for PAI-1 assay. This was centrifuged at 2560×g and 4°C for 30 minutes. Platelet poor plasma was aliquatted from the spun samples and snap frozen prior to storage at -40°C. Blood was collected into 0.9% citrate at room temperature for analysis of Factor VII:C levels. The samples were centrifuged at 2560g at room temperature for 20 minutes, snap frozen and stored at -40° until assay. Blood was also collected into lithium heparin for analysis of plasma cholesterol and triglycerides and into EDTA tubes (sarsted) for DNA extraction.

6.5 DETECTION OF GENETIC POLYMORPHISMS

6.5.1 DNA Extraction

DNA was extraction by a salt detergent method adapted from that described by Gustincich *et al.*[219]

Step 1- Blood lysis/denaturation.

300ul of EDTA anticoagulated blood was mixed with 600 ul of blood lysis buffer ,8% dodecyltrimethylammonium bromide (DTAB), 1.5M NaCL, 100mM Tris-HCL, pH 8.6, and 50mM EDTA in a 2ml Eppendorf tube and incubated at 68(for 5 minutes.

Step 2- Deproteinization.

Chloroform (900ul) is then immediately added and after mixing by inversion, the sample is centrifuged at 10 000 rpm for 2 minutes in an Eppendorf table centrifuge. A solid plug is present at the layer between the organic and aqueous phase: this allows the pouring of the upper aqueous phase containing the genomic DNA into a new 2-ml Eppendorf tube.

Step3- DNA precipitation and resuspension

900 µl of molecular biology grade water and 100µl of cetyltrimethylammonium bromide (CTAB) from a 5% solution made up in 0.4M NaCL were added. The final CTAB concentration will thus be 0.3%. After gentle mixing by inversion at room temperature, samples are centrifuged at 10 000 rpm in an Eppendorf tube for 2 minutes: the DNA-CTAB pellets were resuspended in 300 ul of NaCL 1.2 M. After resuspension of the pellet, the DNA was recovered by ethanol precipitation: 750 ul of ethanol was added, and after mixing by inversion, the samples were centrifuged for 10 minutes at 10 000 rpm at room temperature. The supernatant was discarded by pouring, and the pellets rinsed with 70% ethanol/30% molecular biology grade water. After removing the 70% ethanol, the DNA was dissolved in 1ml of molecular biology grade water and stored at -4C°.

DNA concentration was assessed by spectrophotometry.

6.5.2 Polymerase Chain Reaction (PCR)

This technique developed by Mullis and coworkers in 1985, allows for the specific amplification of discrete DNA fragments present in very small (picogram) quantities. The basic premise of PCR is that in a test tube containing a single molecule of DNA as template, appropriate flanking primers to bind to either end of the portion of DNA of interest, nucleotide triphosphate building blocks, and DNA polymerase, one can amplify that portion of the DNA of interest to 100 billion similar molecules in a single day. An essential requirement is knowing the sequence of a portion of each end of the DNA fragment to be amplified, but one need not know the intervening sequences. These short sequences at either end , usually 10-20 base pairs, are used to make complimentary oligonuecleotides that serve as primers, and in the presence of the nucleotide building blocks a complimentary fragment of DNA is synthesized. This cycle is repeated in an exponential fashion such that in 20 to 30 cycles over 3 to 4 hours, more than 1 million copies are synthesized. The DNA polymerase lengthens the short oligonucleotide

primer by attaching additional oligonucleotide to its 3' end. The nucleotide that the polymerase attaches will be complimentary to the base in the corresponding position on the template strand. If the adjacent nucleotide is an A, the polymerase attaches a T base; if the template nucleotide is a G, the enzyme attaches to a C. Repetition of this process allows the polymerase to extend the 3' end of the primer all the way to the 5' end of the template.

The steps that make up the PCR cycles include DNA denaturation, extension primer annealing, and amplification (or extension).

DNA Denaturation

The double-stranded template DNA is denatured under high temperatures and the dissociated single strands remain free in solution.

Extension primer annealing

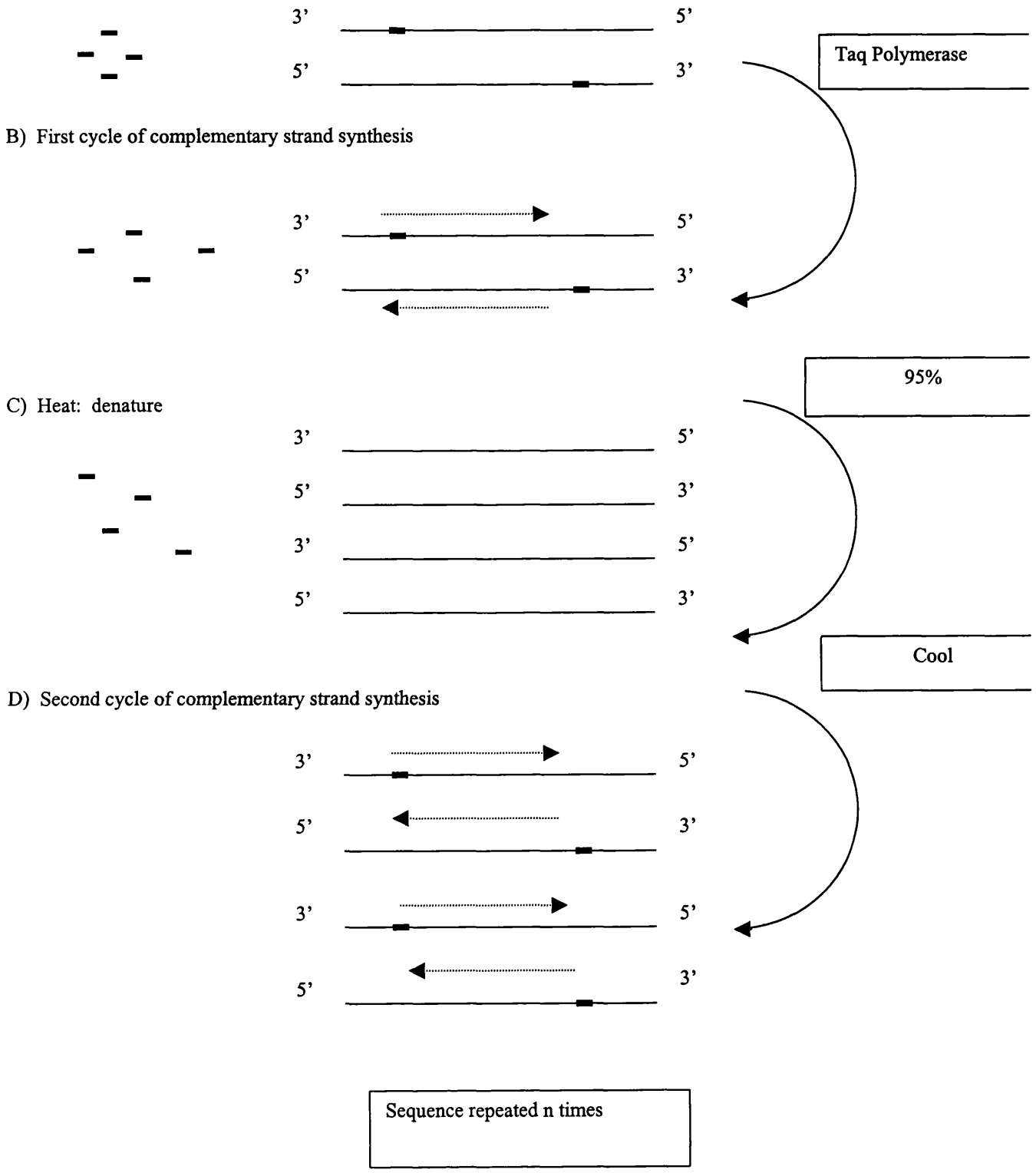
Two extension primers, which are selected by the sequence of the DNA at the boundaries of the region to be amplified, are utilised to anneal to one of the DNA strands. Each anneals to the opposite strand; generally they are different in their sequence and are not complimentary to each other. The primers are present in large excess over the DNA template, which favours the formation of primer-template complexes at the annealing sites, rather than reassociation of DNA strands when the temperature is lowered.

Amplification (extension)

The 5'(3' extension of the primer-template complex is mediated by DNA polymerase, and as a result, extension primers become incorporated into the amplification product. A thermostable DNA polymerase purified from Thermus aquaticus, Taq DNA polymerase, which catalyses the reaction at high temperatures, has gained wide usage and greatly simplified this process since fresh enzyme is no longer required after each denaturation step. Amplification mimics the natural DNA replication process of doubling the number of molecules after each cycle. The amplification product of interest ("short product") begins to accumulate after 3 cycles. The short product is the region between the 5' ends of the extension primers (synthetic oligonucleotides that anneal to the sites flanking the region to be amplified) that contains discrete ends corresponding to the sequence of these primers. As the cycle number increases, the short product becomes the predominant template which the extension primer anneals. Theoretically the amount of product doubles after each cycle, leading to exponential accumulation. Due to enzyme kinetics, however, the actual amount is lower. As the cycles proceed, other products also form, but the end of the amplification process, the short product is overwhelmingly more abundant. These stages are depicted in figure 9.

Figure 9

Schematic view of Polymerase Chain Reaction (PCR)



6.5.3 Determination Of Ace Genotype

The ACE I/D polymorphism was detected by the polymerase chain reaction (PCR), according to the presence or absence of an insertion in intron 16.[220] PCR products (490bp insertion and 190bp deletion) were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and viewed with UV light as described below. This procedure allows discrimination among the three ACE genotypes: II, ID and DD.

Mistyping ACE Heterozygotes

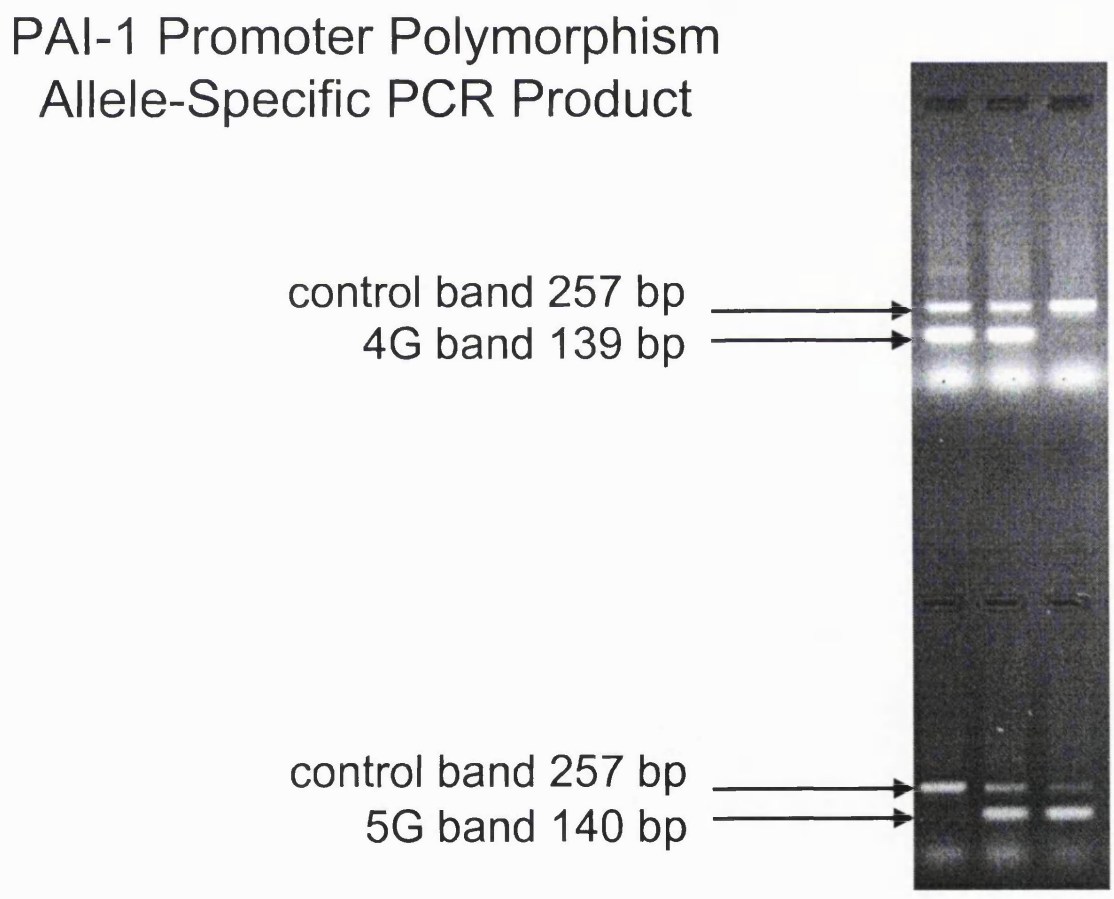
In genotyping a large pedigree in which one of the parents was an II homozygote Shanmugam *et al* were disturbed to encounter several DD genotypes among the off-spring.[221] On repeating the PCR amplification under slightly different conditions all of the DD genotypes amplified as ID. They were the first to point out the possibility of mistyping ID heterozygotes as DD. This mistyping is due to the preferential amplification of the smaller D allele. To overcome this, all samples typed as DD homozygotes were subjected to a further amplification by using the original antisense primer together with a primer specific for the insertion sequence as described by Shanmugam et al.[221] This identified 6 out of 121 subjects initially genotyped as DD. This 5% level of mistyping is consistent with the large American Physicians Health Study. Several studies have been published which have not taken into account this problem of mistyping and as such their results and conclusions have to be viewed with caution. This point is discussed further below.

6.5.4 Determination Of PAI-1 Gene

Genotyping was performed for the 4G/5G Promoter Polymorphism. This single allele insertion/deletion polymorphism is situated 675 base-pairs upstream from the start of transcription and gives rise to a sequence of either 4 or 5 guanine bases; (4G/5G). Genotype at this polymorphism was determined in all subjects by PCR amplification of genomic DNA using the allele specific primers: insertion 5G allele; 5'GTC TGG ACA CGT GGG GG-3', deletion 4G allele; 5'-GTC TGG ACA CGT GGG GA-3' each in a separate PCR reaction together with the common downstream primer 5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3' and a control upstream primer 5' -AGC CAG CCA CGT GAT TGT CTA G-3' to verify the occurrence of DNA amplification in the

absence of the allele on the genomic DNA. In addition to these primers each PCR reaction mix of 25 microlitre contained 100 ng genomic DNA with 1.5 mM $MgCL_2$, 3.2 mmol/l of dATP, dCTP, dGTP, dTTP, 50 pmol of the specific (4G or 5G) primer, 0.0025% W1, and 1 X Taq buffer. 1.25 U of dynazyme Taq polymerase was added to each reaction at 94° (the hot-start). DNA amplification was achieved through 30 cycles of the following steps: 60 seconds at 94°C denaturation step, 45 seconds at 65°C, annealing step and 75 seconds at 72°C extension step. A final extension of 5 minutes at 72°C was followed by cooling of the PCR reaction product to 4°C until fragment separation. The PCR amplification conditions were determined by running multiple reactions with differing concentrations of the primers or magnesium chloride and at different annealing temperatures.

Figure 10 PAI-1 Promoter Polymorphism Allele-specific PCR product



6.5.5 Agarose Gel Electrophoresis

The PCR products are loaded into a well of an agarose gel and subjected to electrophoresis. Since DNA fragments are negatively charged (each nucleotide

possesses a net negative charge), they migrate towards the anode at a rate that correlates with their length, the longer fragments migrating slowest, and remaining near the origin, namely, the well in which the sample was inserted. After running the gel for sufficient time to separate the fragments, the gel is stained with ethidium bromide, which intercalates within bases and fluoresces under ultraviolet illumination, from which photographs of DNA fragments are taken. To determine the size of the fragments of the unknown DNA, a standard DNA sequence, which is known to give fragments of specific lengths, is electrophoresed concomitantly. Electrophoreseis through agarose will separate double-stranded DNA fragments varying from 70 to 100 000 base pairs.

The amplified DNA fragments from PCR were separated by agarose gel electrophoresis and viewed under ultraviolet light after staining with ethidium bromide as described above. Each subject was classified by two independent observers into one of three possible genotype groups: 4G/4G, 4G/5G or 5G/5G , for PAI-1 figure 10, and II, ID and DD for ACE.

The PCR results for both PAI-1 and ACE genotypes were scored by two independent observers who had no prior knowledge of either cases or controls. No intraobserver variability was found on repeated readings of the same gel and the interobserver variability was 1%. All ambiguous samples were analysed a second time.

As an additional measure of quality control, validation of the genotyping by using control subjects of each genotype as shown by direct sequencing was carried out. These known controls were run with each batch of samples. Furthermore, samples already genotyped were repeated on different occasions in order to confirm that the previous genotyping was accurate.

6.6 MEASUREMENT OF CIRCULATING LEVELS

6.6.1 Measurement of plasma ACE activity

Plasma ACE activity (measured in our Chemical Pathology laboratory) was estimated using 5ml venous blood anticoagulated with lithium heparin. Samples for ACE activity were centrifuged at room-temperature, plasma separated into cryotubes, snap-frozen in liquid nitrogen and stored at -40°C. ACE activity was determined by an automated method, in which hydrolysis of a synthetic TRIS buffered substrate, furanacrylol-1-

phenylalanyl-glycylglycine by ACE produced a furanocrylol blocked amino acid and a dipeptide. The resultant decrease in absorbance at 340 nm is a measure of ACE activity.

6.6.2 Measurement of Plasma PAI-1 antigen

An enzyme-linked immunosorbant assay (ELISA) was used to measure the levels of PAI-1 antigen (Imulyse, Biopool, Umea, Sweden).

ELISA uses monoclonal antibody to the analyte which is conjugated to peroxidase. Peroxidase is an enzyme which catalyses a change in the substrate ortho-phenylenediamine di-hydrochloride (OPH) resulting in the development of a yellow colour in the reaction medium which can be measured by colorimetry. The intensity of the colour change is a function of the concentration of the analyte present in the sample.

The testing wells are coated with an anti-PAI-1 monoclonal antibody, raised in mouse, which binds all the PAI-1 present in the sample. A second anti-PAI-1 monoclonal antibody is added which binds to an exposed site of the PAI-1 molecule already bound to the first antibody. This second antibody is conjugated to peroxidase. The excess antibodies are then washed away and the peroxidase substrate OPH is added, resulting in colour change proportional to the concentration of PAI-1 antigen in the original sample.

Substances such as rheumatoid factor which bind to immunoglobulin may bind non-specifically to monoclonal anti-PAI-1 antiboby. This would result in increased peroxidase activity and a falsely high estimate of PAI-1 antigen concentration. To increase the specificity of the assay the Immunological Specificity and Accuracy Control (ISAC) principle is used. Ten micolitre of each sample to be measured is placed in two monoclonal antibody coated wells. To one well (the A-well) is added an excess of the coating monoclonal antibody to quench PAI-1 binding so that only non-specific binding occurs to the well coat. To the other well (N well) a non-reactive mouse monoclonal antibody is added so that both specific binding of PAI-1 and non-specific binding will occur to the well coat. PAI-1 concentration can then be measured specifically from the difference in colour change between A and N wells.

Method for 40 samples

250 µl of coating antibody solution was diluted to 25ml with 0.1M $NaHCO_3$ buffer and 200µl was then added to each well. The plate was covered and incubated at 25°C on a

plate shaker for 2 hours. The solution was then discarded by inverting and tapping the plate. The wells were then washed 3 times with PET buffer (PBS buffer substance with EDTA and Tween 20).

200µl of coating antibody solution was diluted to 10ml with PET buffer and mixed. 150 µl was added to each A well. 200µl of non-reactive mouse monoclonal antibody solution was diluted to 10ml with PET buffer and mixed. 150µl was added to each N well.

50µl of PAI-1 standard concentrations of 50,25, 12.5 and 0 ng/l were prepared by mixing a standard PAI-1 plasma of 50ng/ml with PAI-1 depleted plasma in the proportions 4:0, 2:2, 1:3 and 0:4. Plasma samples known to contain more than 50ng/ml PAI-1 were diluted with PET buffer. 10 µl of each standard and each sample were added to paired A and N wells. The samples were covered and incubated at 25°C on the plate shaker for 1 hour. 80µl of conjugated antibody solution was diluted with 6ml PET buffer and mixed and then 50µl of this solution was added to each well prior to further incubation at 25°C for 1 hour on a plate shaker. The contents of the plate were then discarded and the wells washed 4 times with PET-buffer.

The OPH substrate was dissolved in 3ml of water for 30 minutes before use and then diluted further to 24ml with water. Immediately before use 10µl of 30% hydrogen peroxide was added. 200µl of the substrate mix was added to each well before incubation for 15 minutes at 25°C on a plate shaker. The reaction was stopped by the addition of 50µl 4.5M sulphuric acid.

Absorbance at 492nm was measured using a micro-test plate spectrophotometer and the difference in absorbance between the N and the A well (ΔA) for each standard and sample calculated. ΔA was plotted against concentration for the standard samples to obtain a calibration curve from which the PAI-1 concentration of the other samples was read. Where samples had been diluted the concentration was multiplied by the dilution factor.

The coating anti-PAI-1 mouse monoclonal antibody (Biopool MAI-12) binds to all known forms of PAI-1 and the conjugated mouse monoclonal anti-PAI-1 antibody (MAI-11) binds to both latent and active PAI-1 and to a lesser extent complexed PAI-1. The Imulyse PAI-1 assay is therefore claimed to measure active and latent PAI-1 with poor detection of PAI-1 complexed with either t-PA or urokinase. The stated coefficients of variation (CVs) for this assay at 20ng/ml are within-assay 5% and

between-assays 9%. In the laboratory where these tests were performed the CVs were within-assay 8.4% and between-assays 10.5%. As this assay can measure PAI-1 antigen in the range 0.9-50ng/ml, samples with PAI-1 antigen concentrations greater than 40ng/ml were diluted 1:1 with PET-buffer, re-assayed and the resulting concentration multiplied by two.

6.6.3 Measurement of other haemostatic factors

Fibrinogen was measured by the Clauss method, vWF was measured by an ELISA (with antibodies from Dako, Sweden), FVII:C was assayed on the ACL 3000 (Instrumentation laboratory, Warrington, UK) using FVII:C deficient plasma rabbit thromboplastin as reagents.

7.0 STATISTICAL METHODS

In cases and controls, certain variables including body mass index, total cholesterol, triglyceride, PAI- 1 , showed a positively skewed distribution and these values were log transformed to a normal distribution.

Where data was log-transformed, the values presented in tables are reverse transformed means (i.e. geometric means) with the reverse transformed 95% confidence interval for the mean. For the sake of uniformity, those data that are not log-transformed are also presented as mean (95% confidence interval). Data analysed by non-parametric methods are presented as median (25th, 75th centile).

Mean values of normally distributed data were compared by unpaired Student t-test. The Mann-Whitney U-test compared median values of non-parametrically distributed data.

Deviation of genotype distribution from that predicted by Hardy-Weinberg Equilibrium was tested by comparing observed genotype frequencies with those expected from the observed allele frequencies using the chi-squared test with 1 degree of freedom.

Differences in categorical data (for example genotype) between groups were assessed using the chi-squared test. Bivariate correlation coefficients were calculated according to Pearson's method in both variates were normally distributed or Spearman's method if one or both variates did not conform to a normal distribution. Factorial analysis of variance (ANOVA) was used to compare mean values of normally distributed data between two or more groups allowing for differences between the groups in other covariates. Using this method, mean values using the Bonferroni adjustment, for the influence of the other covariates was estimated for the groups studied.

Multiple linear regression analysis was used to assess the significant and independent associations of normally distributed variables allowing for the influence of other covariates on the association. Stepwise linear regression analysis was used to investigate the most significant and importance of many covarying factors which associate with a target variable.

Logistic regression analysis was used to examine the significant and independent associations of dichotomous variables such as the presence or absence of a history of ischaemic heart disease.

With 600 genotyped patients, the primary comparison, extent of coronary disease according to genotype, would have 80% power to a statistically significant difference between II and ID or between ID and DD groups if one group had 15% more double and triple vessel disease that the other (baseline 50%) at a significance level of 5%. 300 patients with measured ACE levels would give the study over 80% power to detect a difference between mean levels of 20 IU/l between II and ID or between ID and DD.

All standard statistical techniques were performed using SPSS for Windows (SPSS Inc, Chicago) Versions 6.1 for Windows 95.

8.0 RESULTS

8.1 GENDER DIFFERENCE

Males vs females in total study population

In all subjects (420 men and 189 women) the proportions of non-significant, single, double and triple vessel disease were as follows: (20%, 21%, 21% and 38% for men) and (45%, 20%, 17% and 18% for women). Table 6 shows the clinical, biochemical and haemostatic characteristics by gender. Women were older than men, had a higher prevalence of hypertension, a lower prevalence of cigarette smoking and a trend towards lower BMI. As expected men had a higher prevalence of previous myocardial infarction (MI), triple vessel disease (38% vs 18%) and a lower incidence of angiographically non-significant coronary vessel disease (20% vs 45%). Female subjects (n=107)had higher plasma fibrinogen (3.4g/l vs 3.2g/l, p=0.01) and FVII:C (134% vs 117%, p<0.0001) levels than men (n=189), the differences remaining in a factorial ANOVA model after adjusting for age, smoking history, cholesterol, triglyceride, BMI and white cell count. Circulating levels of PAI-1 were not different between the sexes before and after adjusting for age, BMI, and triglyceride. Similarly, there was no difference between men and women in vWF levels in both univariate and multivariate analysis.

The median duration (25th and 75th centile) after MI to time of recruitment was 16 months (7-56 months). Of the 296 patients with haemostatic factors, only 8 subjects had sustained an MI within 3 months prior to recruitment. Of these subjects, none were female. Differences in haemostatic factors between the sexes could not therefore be explained by acute phase response secondary to an MI.

Males vs females with significant atheroma

All subjects with angiographically significant atheroma in at least one vessel were analysed separately (Table 7). The differences with respect to clinical features (age, smoking and hypertension history) that were seen in the total study population were also present in this selected group. With respect to haemostatic factors, the females in this selected group (n=50)had higher levels of fibrinogen (3.5g/l vs 3.3g/l, p=0.02), and factor VII:C (139% vs 117%, p=0.0001) than men (n=147)the differences remaining in a factorial ANOVA model; (3.7g/l vs 3.3 g/l, p=0.003 for fibrinogen), and

(139% vs 117%, p=0.0001 for FVII:C). PAI-1 levels were not different in univariate analysis (25.0ng/ml vs 20.2 ng/ml, p=0.1) but assumed statistical significance in multivariate analysis (26.2 ng/ml vs 19.7 ng/ml p=0.02). Levels of vWF were not different in this group in either univariate or multivariate analysis.

Males vs females (normal vs atheromatous vessels)

Men and women were separately analysed comparing subjects with significant coronary stenosis to those without significant disease (Table 8).

Subjects with significant atheroma were older in both sexes, and had increased plasma levels of cholesterol (6.8 mmol/l vs 6.0 mmol/l ,p<0.0001 for women, 6.2 mmol/l, vs 5.6 mmol/l, p<0.0001 for men) and triglycerides (2.0 mmol/l vs 1.5 mmol/l, p<0.0001 for women, 2.1 mmol/l, vs 1.6 mmol/l, p<0.0001 for men). A trend towards higher BMI (27.1 kg/m^2 vs 26.0, p=0.09) was only found in female subjects. A previous history of cigarette smoking was associated with significant atheroma in women (63% vs 40%, p=0.001) but not in men. In contrast there was a higher prevalence of diabetes in men with significant atheroma (10% vs 1% p=0.006) but not in women. Women with significant coronary stenosis (n=50) had higher circulating levels of PAI-1 (25.0ng/l vs 13.4 ng/l, p<0.0001) and vWF (1.2 IU/ml vs 1.0 IU/ml, p<0.03) than women without significant coronary disease (n=57) in univariate analysis, the differences remaining when adjusted for other covariates. FVII:C and fibrinogen levels tended to be higher in females with significant coronary disease, but these differences did not achieve statistical significance even in multivariate analysis. These differences were not seen in men with the exception of fibrinogen which was the only haemostatic parameter associated with significant atheroma (3.3g/l vs 3.0g/l, p=0.05).

Summary of results with respect to gender

In all subjects women have higher levels of fibrinogen and FVII:C than men irrespective of the atheroma status. PAI-1 levels are not different in the whole population but are different in the subgroup with atheroma, women having higher levels. vWF levels are not different irrespective of atheroma status.

When the sexes are studied separately, PAI-1 and vWF levels appear to reflect the presence of atheroma in women. Fibrinogen and FVII:C levels are not related to the presence of coronary atheroma in women. In men, only fibrinogen appears to reflect the presence of atheroma.

TABLE 6 CLINICAL, HAEMOSTATIC AND BIOCHEMICAL FEATURES OF 609 PATIENTS

Characteristic	**Men (n=420)**	**Women (n=189)**	**p**
Age, yr	58 (57-59)	61(60-62)	<0.0001
BMI, kg/m^2	27.2 (26.2-28.2)	26.6(24.6-28.5)	NS
Smokers, %	73	52	<0.0001
Hypertension, %	27	36	0.05
Diabetes, %	8	8	NS
History of MI, %	40	28	0.002
Measurement	**Men**	**Women**	**p**
Cholesterol, mmol/l	6.1 (6.0-6.2)	6.5 (6.3-6.7)	0.001
Triglyceride, mmol/l	1.9 (0.8-3.0)	1.8 (0.7-2.9)	NS
Fibrinogen, g/l	3.2 (2.2-4.2)	3.4 (2.4-4.4)	0.01
PAI-1 antigen, ng/ml	20.3 (19.2-21.4)	18.7 (17.5-19.9)	NS
FVII:C, %	117 (113-121)	134 (127-141)	<0.0001
vWF, IU/ml	1.17 (1.12-.1.23)	1.17 (1.09 -1.25)	NS

Values shown are mean and geometric mean (95% confidence interval) and antilogged where appropriate. Adjusted values are shown in the text. Values for fibrinogen, PAI-1,FVII:C, and vWF are shown for 189 males and 107 females. All other measures are reported in 420 males and 189 females respectively.

TABLE 7 CLINICAL FEATURES OF SUBJECTS 434 MEN AND WOMEN WITH SIGNIFICANT ATHEROMA AT ANGIOGRAPHY

Characteristic	**Men (n=339)**	**Women (n=101)**	**p**
Age, yr	59 (58-60)	62 (60-64)	0.001
BMI, kg/m^2	27.3 (26.3-28.3)	27.1 (26.1-28.1)	NS
Smokers, %	74	63	0.04
Hypertension, %	29	41	0.04
Diabetes, %	10	10	NS
History of MI, %	49	43	NS
Measurement	**Men**	**Women**	**p**
Cholesterol, mmol/l	6.2 (6.1-6.3)	6.8 (6.5-7.1)	<0.0001
Triglyceride, mmol/l	2.1 (1.0-3.2)	2.0 (0.9-3.1)	NS
Fibrinogen, g/l	3.3 (2.3-4.3) / 3.3*	3.5 (2.4-4.6) / 3.7*	0.02/ 0.003
PAI-1 antigen, ng/ml	20.2 (19.0-21.40) / 19.7*	25.0 (23.8-26.2)/ 26.2*	0.1/ 0.02
FVII:C, %	117 (112-122) / 117*	139 (130-148) / 139*	<0.0001
vWF, IU/ml	1.15 (1.01-1.29) / 1.13*	1.25(1.14-1.37) / 1.20*	NS

Values shown are mean and geometric mean (95% confidence interval) and antilogged where appropriate. *Figures shown are values after adjusting for age, BMI, cholesterol, triglyceride, white cell count where appropriate in the factorial ANOVA model. Values for fibrinogen, PAI-1, FVII:C, and vWF are shown for 147 males and 50 females. All other measures are reported in 339 males and 101 females respectively.

TABLE 8 CLINICAL, HAEMOSTATIC AND BIOCHEMICAL FEATURES IN MEN AND WOMEN WITH AND WITHOUT SIGNIFICANT ATHEROMA AT ANGIOGRAPHY

Characteristics	males			females		
	no atheroma (n=81)	atheroma (n=339)	p value	no atheroma (n=82)	atheroma (n =101)	p value
Age, yr	53 (51-55)	59 (58-60)	<0.0001	59 (57-61)	62 (60-64)	0.02
BMI, kg/m^2	27.1 (26.1-28.1)	27.3 (26.3-28.3)	NS	26.0 (25.0-27.0)	27.1(26.1-28.1)	0.09
Smokers %	70	74	NS	40	63	0.001
Hypertension %	20	29	NS	30	41	NS
Diabetes %	1	10	0.006	7	10	NS
History of MI %	8	49	<0.0001	10	43	<0.0001
Measurement						
Cholesterol, mmol/l	5.6 (5.3-5.9)	6.2 (6.1-6.3)	<0.0001	6.0 (5.7-6.3)	6.8 (6.5-7.1)	<0.0001
Triglyceride, mmol/l	1.6 (0.4-2.8)	2.1 (1.0-3.2)	<0.0001	1.5 (0.4-2.6)	2.0 (0.9-3.1)	<0.0001
Fibrinogen, g/l	3.0 (1.9-4.1)	3.3 (2.3-4.3)	0.05	3.3 (2.2-4.4)	3.5 (2.4-4.6)	NS
PAI-1 Ag ng/ml	20.6 (19.3-21.9)	20.2(19.0-21.40)	NS	13.4 (12.1-14.7)	25.0 (23.8-26.2)	<0.0001
FVII:C, %	115 (108-122)	117 (112-122)	NS	128 (117-139)	139 (130-148)	0.1
vWF, IU/ml	1.15(1.01-1.29)	1.18 (1.11-1.24)	NS	1.06 (0.95-1.18)	1.25 (1.14-1.37)	0.02

Values shown are mean and geometric mean (95% confidence interval) and antilogged where appropriate. For adjusted values see text.
Values for fibrinogen, PAI-1, FVII:C, vWF are shown for 147 males with and 42 males without atheroma, and in 50 females with and 57 females without atheroma. All other measures are reported in 339 and 81 males respectively and in 101 and 82 females respectively.

8.2 ACE Results

8.2.1 Relation of Established Risk Factors and of ACE Genotype to MI and CAD

Clinical Characteristics

A total of 609 consecutive patients were admitted for angiography and included in the study. The clinical and biochemical characteristics of these patients, by sex, are shown in Table 9. These characteristics are consistent with the sample being typical of patients admitted for angiography.

ACE genotype vs ACE activity

There were no differences between the ACE genotype subgroups and the established risk factors in the total population (data not shown).

In the total study population there was a strong relationship between ACE genotype and activity (mean plasma ACE levels were 61.8, 73.0 and 98.2 for II, ID and DD respectively; ANOVA, p=0.001). Adjusting for ACE inhibitor usage and other factors did not substantially alter these results.

ACE genotype and extent of CAD

Table 10 shows the number of subjects by extent of coronary disease and ACE genotype. There was no statistically significant association between these (median number of diseased vessels for II, ID and DD were 2, 2 and 1 with interquartile ranges of 0-3, 0-3 and 0-3 respectively; Kruskal-Wallis test p=0.527). Similarly there was no significant difference between genotype groups in terms of significant atheroma or not Table 6.

ACE genotype and MI

Table 11 shows the number of patients by history of MI and ACE genotype. There was no relationship between the ACE genotype and a history of MI (odds ratio for ID vs II = 1.10, 95% CI: 0.71 to 1.68; odds ratio for DD vs II = 1.10, 95% CI: 0.68 to 1.77, p=0.902). These results remained unchanged after adjusting for ACE inhibitor use and other factors.

ACE activity and extent of CAD

There was no significant relationship between plasma ACE levels and extent of disease odds ratio for increase in 10 IU/l = 0.97, 95% confidence interval: 0.93 to 1.02, p=0.202). This shows that the odds of having more diseased vessels are unrelated to plasma ACE levels (the non-significant trend was to have *fewer* diseased vessels with higher ACE levels). This result remained unchanged even when ACE inhibitor usage and other factors were taken into account (p=0.673).

ACE activity and MI

Subjects with higher plasma ACE levels were no more likely to have had an MI than those with lower ACE levels (odds ratio for increase of 10 IU/l = 0.96, 95% confidence interval 0.91 to 1.02, p=0.193). This remained unchanged after adjusting for ACE inhibitor usage and other factors.

Myocardial Infarction

Table 14 shows the relationship between risk factors and a history of myocardial infarction (MI). A past history of smoking and diabetes mellitus was related to a history of MI (p=0.007 and 0.04 respectively). MI patients were more likely to be on antianginal medication (p<0.001 for (-blocker and p=0.006 for Ca antagonists), and hence it is not surprising that a history of hypertension was not related to MI. There was a trend towards higher BMIs (p=0.06), cholesterol and triglycerides, but this was not statistically significant. There was a relationship between total white cell count and granulocyte count, and MI p=0.02. There was no relationship between the ACE genotype and a history of MI, either in the general population Table 12 or in any of the subgroups studied (in patients with one or more diseased vessels Table 13 , men and women separately, in patients with BMI < 26 kg/m2 and 25 kg/m2, patients who never smoked, without a history of hypertension and non-diabetics, either looked at separately or together).

Coronary Artery Disease

The relationship of risk factors according to extent of vessels disease is shown in Table 15. Patients with angiographic evidence of atheroma had higher BMIs ($p<0.02$), higher cholesterol ($p=0.0009$), and triglycerides (<0.0001), smoking history ($p=0.05$), and white cell count (0.02). There was, however, no clear trends with extent of disease apart from age and cholesterol. A history of MI was strongly related to atheroma $p<0.0001$, increasing in prevalence with an increase in number of vessels diseased (10%, 36%, 52%, 58% from non-significant to triple vessel disease respectively) (for increase of one vessel with >50% stenosis, OR = 2.04, 95% CI: 1.74 to 2.39, $p<0.001$).

Subjects with normal and single vessel disease had a higher prevalence of DD genotype (28% and 35% respectively), than patients with double (22%) and triple vessel (26%) disease but this was not statistically significant. In the subgroup of patients with significant atheroma there was no significant difference between genotype groups Table 13.

8.2.2 Relationship between ACE activity, CAD and MI

There was a trend towards higher ACE levels among subjects with normal (72.8IU/L) and single vessel (83.4IU/L) disease compared to multivessel disease groups, 62.8IU/L and 59.1IU/L for double and triple vessel disease groups respectively $p=0.08$. This trend became weaker after adjusting for ACE inhibitor treatment $p=0.1$ Table 14.

Patients with a past history of MI had lower levels of plasma ACE activity 68.1IU/L compared to those without an MI history 76.1IU/L $p=0.04$, a relationship which became weaker after adjusting for ACE inhibitor treatment, 70IU/L compared to 59IU/L $p=0.09$ Table15. In a multiple regression model after adjusting for other risk factors for MI, this negative relationship became stronger $p=0.01$. ACE activity was not age-dependent either in the total population studied or in any of the subgroups analysed (<55years, (55years, MI and no history of MI). In a linear regression model age remained unrelated to plasma ACE activity.

TABLE 9. PATIENT'S CLINICAL AND BIOCHEMICAL CHARACTERISTICS BY GENDER

	Male	Female	DIFFERENCE	95% CI
n=609	420	189		
Age	57.6 (9.4)	60.9 (8.6)	-3.3	-4.9 to -1.7
BMI(kg/m^2)	27.5 (4.0)	26.9 (4.2)	0.6	-0.1 to 1.4
Diastolic BP (mmHg)	82 (12)	77 (12)	4.8	2.6 to 7.1
Systolic BP (mmHg)	146 (22)	144 (26)	2.8	-1.6 to 7.2
Family history of CHD	234 (56%)	110 (58%)	-2.5%	-11% to 6%
History of hypertension	115 (27%)	67 (35%)	-8.1%	-15.9% to -0.2%
History of diabetes mellitus	35 (8%)	16 (9%)	-0.1%	-4.9% to 4.7%
Current smoker	81 (19%)	39 (21%)	-1.4%	-8.2% to 5.5%
Ever smoked in last 10 yrs	203 (49%)	72 (39%)	10.1%	1.5% to 18.6%
Ever smoked	307 (73%)	98 (52%)	21.0%	12.9% to 29.1%
History of myocardial infarction	168 (40%)	52 (28%)	12.9%	4.6% to 21.1%
Cholesterol(mmol/L)	6.1 (1.1)	6.5 (1.3)	-0.4	-0.6 to -0.1
Triglyceride (mmol/L)	2.3 (1.6)	2.1 (1.2)	1.09	0.98 to 1.21*
White cell count ($10^9/L$)	7.5 (2.1)	7.0 (2.1)	0.5	0.1 to 0.9
Antilipid	32 (8%)	21 (11%)	-3.5%	-8.3% to 1.3%
Aspirin	288 (69%)	107 (57%)	12.0%	3.8% to 20.2%
ß-blocker	199 (47%)	91 (48%)	-0.8%	-9.3% to 7.8%
Ca-antagonist	191 (46%)	106 (56%)	-10.6%	-19.2% to -2.0%

Values shown are mean (standard deviation), or number (percentage) as appropriate. Where data was missing, figures are based on patients for whom data was available.

* Triglyceride values were log-transformed before computing the t-test. Results quoted have been transformed back into mmol/l, where "difference" and 95% CI represent the ratio of the females' geometric mean to the males' geometric mean.

TABLE 10. RELATIONSHIP BETWEEN ACE GENOTYPE AND EXTENT OF CAD

		ACE Genotype			Alleles	
	N =	II	ID	DD	I	D
Normal	154	36 (24%)	74 (48%)	44 (29%)	0.47	0.53
Single	123	20 (16%)	60 (49%)	43 (35%)	0.41	0.59
Double	112	33 (29%)	54 (48%)	25 (22%)	0.54	0.46
Triple	185	46 (25%)	90 (49%)	49 (26%)	0.49	0.51
	574	135 (24%)	278 (48%)	161 (28%)	0.48	0.52

35 patients were either not genotyped or were not classified according to extent of disease.

TABLE 11 RELATIONSHIP BETWEEN CORONARY STENOSIS AND ACE GENOTYPE

		II	ID	DD
No atheroma	121	31 (26%)	56 (46%)	34 (28%)
Atheroma	314	66 (21%)	161 (51%)	87 (28%)

χ^2=1.3, df=2. p=0.5

10 patients had no angiograms, 14 patients could not be genotyped, and 5 patients had neither. These were excluded from the analysis.

TABLE 12 RELATIONSHIP BETWEEN THE ACE GENOTYPE AND HISTORY OF MI

		ACE Genotype			Alleles	
	N	II	ID	DD	I	D
MI	210	47 (22%)	103 (49%)	60 (29%)	0.47	0.53
No MI	366	88 (24%)	176 (48%)	102 (28%)	0.48	0.52
	576	135 (23%)	279 (49%)	162 (29%)	0.48	0.52

33 PATIENTS WERE EITHER NOT GENOTYPED OR WERE NOT CLASSIFIED ACCORDING TO HISTORY OF MI

TABLE 13 RELATIONSHIP BETWEEN ACE GENOTYPE AND MI IN PATIENTS WITH SIGNIFICANT ATHEROMA

	Patients with significant atheroma	II	ID	DD
MI	164	30 (19%)	84 (51%)	50 (31%)
No MI	156	36 (23%)	74 (47%)	46 (30%)

χ^2= 1.1, df=2, p=0.6

TABLE 14 CLINICAL AND BIOCHEMICAL CHARACTERISTICS BY MI

	No MI	MI	P
n= 455	282	173	
Age	57.9 (10)	59 (10)	NS
BMI kg/m^2	26.9 (4)	27.7 (4)	0.06
Diastolic BP(mmHg)	74 (25)	73 (25)	NS
Systolic BP (mmHg)	134 (45)	130 (45)	NS
Diabetes history	15 (5%)	18 (11%)	0.04
Hypertension history	90 (32%)	45 (26%)	NS
Family History	170 (60%)	104 (60%)	NS
Present smoker	57 (20%)	32 (19%)	NS
Smoked in previous 10 years	113 (63%)	92 (54%)	0.007
Ever smoked	178 (63%)	132 (77%)	0.003
ACE activity IU/L	70 (1.0)	59 (1.1)	0.04
ACE activity IU/L*	76.1 (1.0)	68.1(1.1)	0.09
Cholesterol(mmol/L)	6.0 (1.2)	6.2 (1.2)	NS
Triglygeride(mmol/L)	1.8 (1.7)	1.9 (1.7)	NS
Anti-lipid treatment	25 (9%)	14 (8%)	NS
ACE inhibitor	46 (16%)	35 (20%)	NS
Aspirin	158 (56%)	141 (81%)	<0.0001
ß-blocker	115 (41%)	98 (57%)	<0.0009
Ca-antagonist	131 (46%)	96 (56%)	0.06

Values shown are mean and standard error

*After excluding patients on ACE inhibitor treatment.
+ 9 patients who were reported to have had an MI, but did not meet the WHO criteria were excluded from the analysis.

TABLE 15 CLINICAL AND BIOCHEMICAL CHARACTERISTICS BY EXTENT OF CAD

	"Normal"	Single	Double	Triple	P
	125	92	87	145	
Age(years)	55 (10)	58 (10)	59 (7)	60 (11)	<0.001*
BMI(Kg/m^2)	26.4 (4)	27.6 (4)	27.9 (3.5)	27 (3.7)	<0.02*
Diastolic BP(mmHg)	73 (22)	78 (21)	76 (24)	74 (25)	NS
Systolic BP(mmHg)	132 (37)	138 (38)	136 (45)	136 (46)	NS
Diabetes History	4 (31%)	10 (11%)	7 (8%)	13 (9%)	NS
Hypertension History	27 (22%)	32 (35%)	30 (35%)	45 (31%)	NS
Family History	71 (57%)	56 (61%)	53 (61%)	91 (63%)	NS
Present smoker	25 (20%)	23 (25%)	22 (25%)	21 (14%)	NS
Ever smoked	75 (60%)	68 (74%)	63 (72%)	108 (75%)	0.05
Smoked in previous 10 years	48 (39%)	50 (55%)	47 (55%)	65 (45%)	0.05
Current angina	95 (76%)	81 (88%)	76 (87%)	126 (87%)	0.03
History of MI	12 (10%)	33 (36%)	44 (52%)	83 (58%)	<0.0001
Cholesterol(mmol/L)	5.8 (1.2)	6.1 (1.2)	6.3 (1.2)	6.3 (1.2)	0.0009
Triglyceride(mmol/L)	1.5 (1.7)	2.1 (1.8)	2.2 (1.7)	1.9 (1.7)	<0.0001
ACE activity(IU/L)	67 (1.3)	78 (1.8)	63 (1.9)	59 (2)	0.08
ACE activity(IU/L)*	73(1.1)	83(1.1)	66(1.1)	71(1.1)	0.1
Antilipid treatment	7 (6%)	10 (11%)	9 (10%)	14 (10%)	NS
Aspirin	61 (49%)	62 (67%)	66 (76%)	110 (76%)	0.0001
ß-blocker	40 (32%)	44 (48%)	50 (58%)	84 (60%)	0.0008
Ca antagonist	46 (37%)	45 (49%)	52 (66%)	83 (57%)	0.002
White cell count*10^9/L	6.3 (2.4)	7.5 (2.3)	6.8 (3)	7.1 (2.7)	0.02
Granulocyte count*10^9/L	4.3 (1.6)	4.9 (1.8)	4.8 (1.6)	5.0 (1.6)	0.04
Platelet count*10^9/L	240 (84)	253 (83)	218 (97)	226 (91)	NS

Values shown are mean and standard error
*Adjusted for ACE inhibitor treatment

8.3 PAI-1 RESULTS

The clinical and biochemical characteristics of the patients are shown in Table 6. Women were older than men (p=0.001), and had lower BMI (p=0.008). Male subjects had a higher diastolic BP (p=0.005), but there was no difference in systolic BP, although adjustment was not made for an influence of BP-lowering drug therapy. Despite the fact that more women were on lipid lowering treatments (12%vrs 7%, P>0.05), they had higher cholesterol levels. There were more smokers in the male population (p=0.01). There was a significant gender difference in the frequency of MI, with more men (42% vs29%) (p=0.02) having a history of MI. Male patients had a higher prevalence of multi-vessel disease, whilst, female subjects had a higher prevalence of normal coronary angiography (p<0.005).

Patients with coronary stenosis in one or more vessels were older (p<0.005), had a higher BMI (p=0.04), and higher diastolic and systolic BP (p=0.02, p=0.03, respectively). Smoking history was more frequent in subjects with one or more diseased vessels (p=0.04). Cholesterol levels increased with increasing vessel involvement (p=0.0009), and a history of myocardial infarction was related to the number of vessels disease (p<0.0001). There was no relationship between a family history of Ischaemic heart disease and presence of coronary stenosis Table 17.

8.3.1 PAI-1 levels in relation to CAD and MI

PAI-1 levels were weakly related to extent of disease although this did not reach the standard level of statistical significance (p=0.06). This association weakened after adjusting for TG, BMI, age and sex, factors that have been shown independently to relate to affect PAI-1 levels. The frequencies of the two alleles in the whole group were 0.54 for 4G and 0.46 for 5G. The genotype frequencies were in Hardy-Weinberg equilibrium.

PAI-1 levels were highest in subjects with the 4G/4G genotype (21.4ng/ml) lowest in the 5G/5G group (17.2ng/ml), with 4G/5G subjects having intermediate levels (20.3ng/ml). When values of PAI-1 were adjusted for differences in age, sex, BMI and TG, there was a significant difference in PAI-1 levels between genotype groups

(p=0.02) (Table16). Genotype frequencies differed between patients with and without a past history of MI, with a significantly greater frequency of the 4G/4G genotype in those with a history of MI (p=0.04) (Table16) and figure11. In a logistic regression model, possession of the 4G/4G genotype remained significantly related to MI (p<0.03, odds ratio=2.0, 95%CI=1.1-3.7), allowing for differences in age, sex, BMI, cholesterol and smoking history. The other independent significant factors were age (p=0.04), smoking history (p=0.05), BMI (p=0.01) and sex (p=0.0006). There was a trend towards higher levels of PAI-1 in subjects with a history of MI (22.2ng/ml vs 18.6ng/ml) (p=0.1) but this did not reach statistical significance even when adjusted for other factors.
Likelihood Chi-squared ratio did not reveal any differences in genotype frequency in the different angiographic groups.

8.3.2 TG and BMI in relation to PAI-1

The regression slope of PAI-1 levels on TG was steeper in those with the 4G/4G genotype than the other two genotypes. Similarly, the slope of PAI-1 on BMI was steeper in those with 5G/5G genotype. To assess the significance of these interactions, a factorial ANOVA model was designed with PAI-1 levels as the dependent variable. Genotype was expressed as two indicator variables. TG and BMI were expressed as covariates, with interaction terms of TG (genotype (4G/4G vs not 4G/4G), and BMI (genotype (5G/5G vs not 5G/5G). Both interaction terms remained significant with p-values of 0.004 for TG (genotype and 0.02 for BMI (genotype).
In order to assess whether the relationship between the promoter polymorphism and history of MI was gender specific, chi-squared analysis was performed in male and female subjects separately. In men, the genotype was related to MI (p=0.04),figure 13, but there was no relationship in females (p=0.57). In the multiple regression model, the relationship in men was stronger (p=0.007, OR=1.6, 95%CI=1.13-2.22), whilst there remained no relationship in women (p=0.14). In a subgroup including only subjects with atheroma (single and multi-vessel disease), figure12, the increased prevalence of the 4G/4G genotype in patients with a history of MI assumed greater significance (n=298, df=2, p=0.006) Table 18.

TABLE 16 PAI-1 LEVELS, MI AND EXTENT OF CAD BY GENOTYPE.

	4/4	4/5	5/5	P
Number	130(30%)	210(48%)	94(22%)	
PA1-1 Antigen(ng/ml)	21.4 (2.2)	20.3 (2.5)	17.2 (2.4)	NS
Adjusted * PA1-1(ng/ml)	22.5	21.5	15.8	0.02
Previous MI	60	80	27	0.02
No previous MI	68	124	67	
Vessels 0	37(31%)	65(54%)	18(15%)	
Vessels 1	25(29%)	36(41%)	26(30%)	NS
Vessels 2	66(30%)	107(48%)	49(22%)	

Values for PAI-1 antigen are geometric mean and antilogged standard deviation.
*Adjusted for sex, age, BMI and TG. 19 patients could not be genotyped, in 8 patients we could not confirm MI by the WHO criteria, in 4 patients we could not find the angiographic reports

TABLE 17 CLINICAL AND BIOCHEMICAL CHARACTERISTICS IN RELATION TO EXTENT OF ATHEROMA

	Normal Vessels **n=125**	**Single vessel disease** **n=92**	**Multi-Vessel disease** **n=232**	***p***
Age	55 (9.9) *55*	58 (9.0) *59*	60.6 (8.3) *62*	<0.0005 *<0.0005*
BMI (kg/m^2)	26.4 (4.2) *26.4*	27.7 (4.0) *27.5*	27.3 (3.6) *27.0*	0.04 *ns*
Cholesterol (mmol/l)	5.8 (1.2) *5.9*	6.2 (1.1) *6.3*	6.4 (1.1) *6.5*	<0.0001 *<0.0005*
Diastolic BP(mmHg)	78 (12) *78*	82 (13) *81*	81 (12) *80*	0.02 *ns*
Systolic BP (mmHg)	139 (21) *139*	145 (23) *145*	146 (25) *146*	<0.05
PA1-antigen (ng/ml)	16.4 (2.5) *16.4*	21.4 (2.2) *21.3*	21.3 (2.3) *21.1*	0.06 *0.086*
Triglyceride (mmol/l)	1.5 (1.7) *1.5*	2.1 (1.8) *2.1*	2.0 (1.7) *2.0*	<0.0001 *<0.0005*
Ever smoked	48(36%)	44 (47%)	107 (46%)	<0.05
Family History	78(59%)	57 (61%)	145 (62%)	NS
Previous MI	11(9%)	30 (34%)	121 (55%)	<0.0001

Values shown are mean (standard deviation), and antilogged where appropriate, except for PAI-1and TG where geometric mean (anti-logged standard deviation) are given. Values shown underneath in italics have been adjusted for sex, calculated using ANOVA.

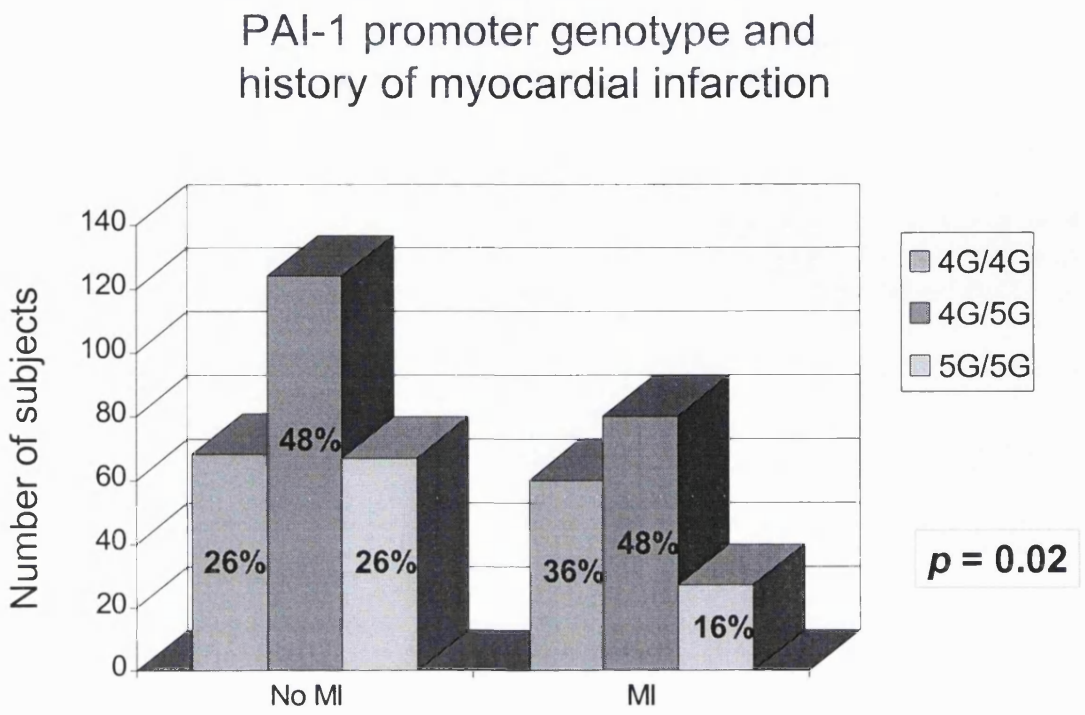


Figure 11 PAI-1 genotype and History of MI

Table 18 RELATIONSHIP BETWEEN GENOTYPE AND MI IN PATIENTS WITH SINGLE OR MULTI-VESSEL DISEASE

GENOTYPE	No MI	MI
4G/4G	36(24%)	52(36%)
4G/5G	66(43%)	68(46%)
5G/5G	50(33%)	26(18%)

Number of patients with percentage frequencies in brackets
χ^2=10.4, df=2, p=0.006

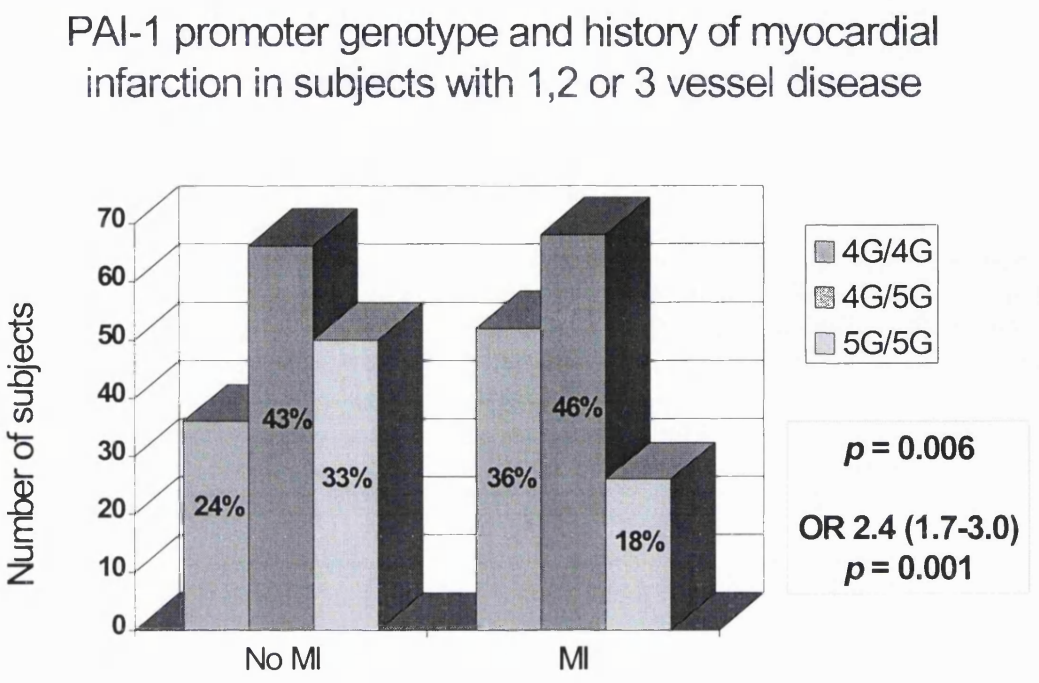


Figure 12 PAI-1 genotype and a history of MI in subjects with significant atheroma

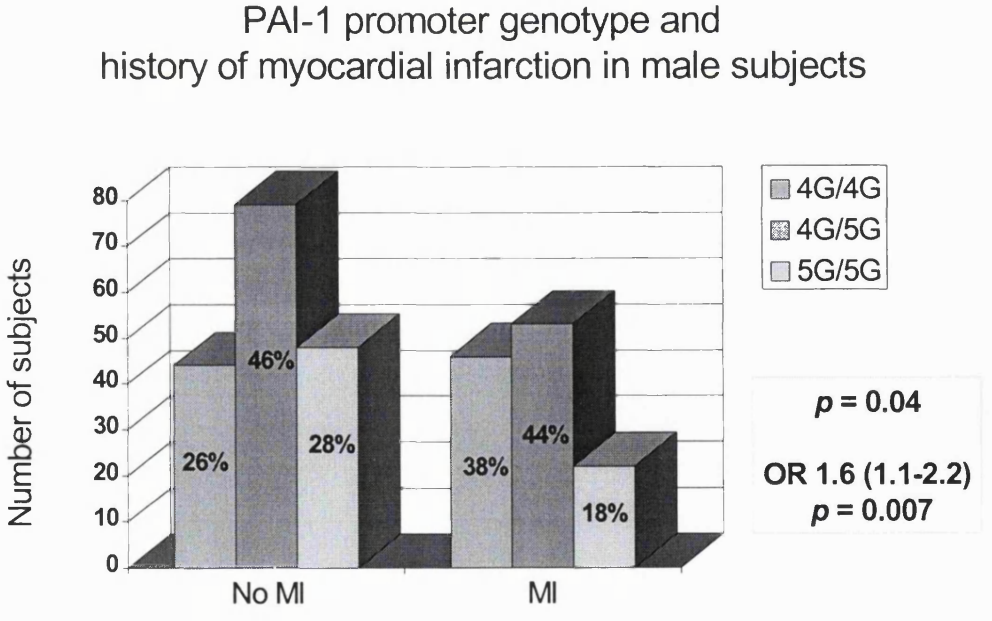


Figure 13 PAI-1 genotype and a history of MI in male subjects

9.0 GENERAL DISCUSSION

9.1 THE STUDY POPULATION

Patients were admitted for coronary angiography for investigation of chest pain, with suspected or known CAD. Since the aim of our study was to look at the relationship between genotype and atheroma, it was essential that both cases and controls should have a coronary angiogram. Our control population was therefore patients with no evidence of significant atheroma at coronary angiography. This group of patients usually had chest pain, and had one or more CAD risk factors. Furthermore, a number of these had positive exercise tests. Hence, it is likely that our control subjects were not normal controls. However, the control groups that have been used in studies of this kind have major difficulties. Some investigators have used the Rose questionnaire as a means of selecting patients likely to have CAD. The validity of this tool has been the subject of considerable scrutiny , with a sensitivity ranging from 25 to 83%, and a specificity from 48 to 98%. Its reliability has been shown to be particularly low in females. Other investigators have used the presence of electrocardiographic abnormalities using various coding systems such as the Minnesota coding system to select their CAD subjects. These again have a wide range of specificity and sensitivity. The use of exercise stress testing though more sensitive and specific than the above methods poorly correlates with the presence or absence of coronary atheroma. Since coronary angiography can only be performed in patients who have a clear indication for such an invasive procedure, the "best" control group were those who were found to have " normal" coronary arteries. I have discussed in a later section the use of coronary angiograms in assessing atheroma.

9.2 CORONARY ANGIOGRAPHY

9.2.1 Introduction.

The assessment of ischaemic burden of patients from history, examination and exercise testing has some limitations. The use of coronary angiography to assess the extent and severity of CAD has greatly improved our evaluation of such patients. It provides important anatomical information that, used in conjunction with the physiological information obtained from exercise testing allows detailed evaluation of the patient.

9.2.2 Coronary Stenosis And Haemodynamics.

Under resting conditions, progressive narrowing of a major artery does not cause a corresponding reduction in flow. Instead flow remains relatively constant until the luminal area has been markedly reduced, at which time an abrupt decrease occurs. Under hyperaemic conditions in the same artery, a considerably minor degree of stenosis will reduce peak blood flow. In normal human coronary arteries, it has been noted that hyperaemic or peak coronary blood flow (such as can occur during exercise) is about six times the normal resting blood flow. The hyperaemic state is mediated by dilatation of the distal arteriolar bed often referred to as the coronary flow reserve. The point at which reduction in flow begins to occur can be called the point of critical stenosis (PCS). In a major artery at resting conditions the PCS is not reached until severe stenosis (about 90% of the luminal diameter) exists. During exercise the PCS occurs at about 50% of arterial narrowing. It has been postulated that the maintenance of resting blood flow in the face of severe proximal stenosis results from compensatory vasodilation of the arteriolar bed distal to the lesion. Presumably, when the arterial luminal area is about 90% narrowed, the maximum vasodilatory capacity of the small arterioles is reached and they cannot compensate any further. Additional narrowing of the artery beyond this PCS then leads to rapid fall off in flow. It is further assumed that during periods of hyperaemia, some of the coronary flow reserve has already been encroached on to maintain resting flow, so that the maximum vasodilatory capacity of the arteriolar bed is reached with lesser degrees of arterial stenosis (about 50%).

Other authors have provided alternative explanations for the above relationship. Studies have been performed to show that measurements of arterial pressure distal to progressive stenoses failed to show a pressure drop as the degree of stenosis increased. Instead both pressure and flow remained constant until the PCS was reached. This meant that peripheral resistance was being maintained as stenosis increased. It further suggested that compensatory arteriolar dilatation is not the explanation for the maintenance of resting blood flow.

Logan has postulated another explanation for the phenomenon. The rate of flow through a vessel equals the driving pressure divided by the total resistance in its vascular bed. Total resistance (Rt) is the sum of stenosis resistance (Rs) in the proximal artery and peripheral resistance in the arteriolar bed (Rp). Under resting conditions when flow is relatively low and Rp is high, Rt is high but consists almost entirely of Rp. As major

arterial stenosis increases, Rs begins to increase, but is initially of very small magnitude compared with Rp and therefore has little overall effect on the magnitude of Rt of the entire system. Only when proximal arterial stenosis becomes severe does Rs begin to approach Rp. At this point further increase in Rs significantly elevates Rt, which in turn causes flow through the entire system to drop off rapidly. During hyperaemic states induced by exercise or other vasodilator stimuli, Rp and therefore Rt are much lower to begin with. A given degree of arterial stensosis will create a level or Rs that is more significant relative to Rp. Any increase in the severity of that stenosis will begin to raise Rt by significant increments at an earlier stage, and the PCS accordingly is reached at an earlier stage.

These concepts explain why resting blood flow may be entirely normal even when a significant proximal stenosis is present and why ischaemic symptoms develop during exercise when they are not present at rest. They also explain why a minor increase in degree of an already existing stenosis can cause a profound decrease in blood flow and lead to abrupt onset of severe ischaemia.

Other important aspects of the flow- stenosis relationship relate to the effect of sequential arterial stenosis and length of the lesion. Gould showed that the resistance of coronary stenoses in series are additive. The effects of such sequential lesions are not determined solely by the most severe lesion. Haemodynamic effects of coronary lesions have also been shown to increase significantly as their length increased.

White et al, has called into question the reliability of coronary angiography in predicting the haemodynamic significance of a coronary stenosis. They compared the degree of coronary stenosis measured by arteriography with the reactive hyperaemic response measured during coronary bypass surgery by a doppler probe after 20 seconds of arterial occlusion. In each case the ratio of peak resting flow velocity (coronary flow reserve) was measured. It would be expected that if angiography is an accurate way of assessing the haemodynamic significance of stenoses, the ratio of peak to resting flow would consistently be lower for severe than for minor stenoses. However, the study revealed a lack of correlation between this ratio and the degree of stenoses. They suggested that the lack of correlation could be explained by a variety of factors, including inter-observer and intra-observer variability, technical problems relating to radiographic magnification

and distortion, and the diffuse nature of coronary arteriosclerosis (which makes it difficult to establish an accurate denominator for the expression of percentage stenosis.)

9.2.3 The Grading System

The simplest and the most widespread grading system has categorised patients according to whether one, two or three of the major coronary arteries are involved. Several major studies describe the prognostic significance of coronary artery obstruction whereby the greater the extent of coronary artery narrowing, the worse the outcome. In particular, the CASS registry reported in more than 20 000 treated patients. [222] Survival was related to number, site (proximal or distal), and vessels stenosed. Mortality increased significantly as the extent of coronary disease increased as well as to left ventricular impairment. In fact ejection fraction was of more prognostic importance than the extent of coronary stenosis.

Angiographic studies have shown that patients whose pain is predictable and provoked by a constant level of cardiac work have segments of high grade stenosis in one or more epicardial coronary arteries. In this context, significant stenosis means a reduction of more than 50% in the diameter of the vessel lumen when compared with an adjacent reference segment of 'normal' artery. This corresponds to 75% reduction in cross-sectional area of the lumen. The evidence for regarding this degree of stenosis as significant is derived from the practical clinical experience of symptomatic, as compared with asymptomatic patients, and the theory of flow in tubes. At a standard perfusion pressure, flow begins to fall sharply when the lumen is narrowed by 75% in cross sectional area and there is a significant drop of pressure across the stenosis. This has been the justification for the use of 50% stenosis as significant CAD disease in this and many angiographic studies such as in the CASS study.

9.2.4 Problems With The Grading System.

Firstly, visual interpretation of the of coronary arteriograms by individuals are highly variable.[223-228] and poorly correlate to necropsy findings[229; 230] Reported visual intraobserver variability ± 1 standard deviation) ranges from 7% to 18% depending on technique. The former value was obtained from analyses of individual frames rather than cine projections.[228] Variability is lessened by the use of manual or electronic callipers or a calibrated magnifying glass.[227] Using maximal stenosis severity criteria, Zir *et al.* [224] found 35% disagreement in the assessment of lesions ≥ 50%, and DeRouen

et al. found 31% using a 70% as standard. Interobserver variability may be reduced by using the mean value of estimations in multiple projections.[227] Factors that increase observer variability include lesion location, such as the left main coronary artery,[226] distal location and poor quality cineangiograms and postangioplasty status. Two studies[223; 228] have suggested that observer variability improves with prior angiographic experience and frequency of reading. The superiority of mean observer values or consensus panel analysis has not been consistently reported. Whereas Sanmarco *et al.*[231] reported 95% agreement regarding lesion severity determined by consensus interpretations (panel of 4 angiographers), the standard deviation was found to be 14%. Galbraith *et al.*[229] found no improvement in correlation with necropsy findings between three individual observers and a three member panel.

Less data is available on percent stenosis observer accuracy. Whereas Scoblionko *et al* [228] reported overestimation of severe quantitatively assessed stenosis by visual analysis, Arnett *et al.*[230] found that visual interpretation underestimates the necropsy findings of lesions more than 50%. In a recent study, Beauman *et al.* [232] confirmed earlier findings that individual visual interpretation of coronary percent stenosis is limited by an approximate 15% variability ± 1 SD. Confidence limits (95%) for visually interpreted stenosis severity appear to be 30%. In clinical practice, a 50% stenosis implies that a specific lesion could range from 20% to 80%. In this study this imprecision was not lessened with angiographic experience but by a large panel and quantitative analysis. Although the severity of symptoms and exercise stress test data are employed in clinical interventional decisions, greater importance is given to angiographic assessment of stenosis severity. Assessment of severity in individual coronary stenosis is not critical for patients being considered for coronary artery bypass because of the usual presence of multivessel disease, impaired left ventricular function and severe chest pain syndromes. Angioplasty decisions require greater accuracy because they depend on analysis of individual coronary arteries. For this purpose, this study[232] concluded that visual interpretation was inadequate to determine the need for individual vessel intervention. Neither extensive experience nor analysis by a small panel of observers offered significant improvements in accuracy. A large panel and quantitative angiography did offer some improvements.

Secondly, although the simple division into one, two and three vessel disease has provided a convenient scheme for classifying patients, it may underestimate the

potential importance of coronary anatomy as a prognostic factor, and the *total "ischaemic burden"* of the patient[233] Patients with left main stem disease had a particularly bad prognosis in the CASS registry[222], a finding confirmed in other studies. The 5 year survival in such patients was only 48%. Patients with left main stem equivalent (combined proximal circumflex and left anterior descending stenoses) were also associated with poor prognosis (5 year survival 54%) , as have those with LAD stenosis proximal to its first septal branch. The total ischaemic burden or the amount of myocardium in "jeopardy", can be defined as the sum of the amount of myocardium distal to each lesion in the coronary artery tree.[234] Several coronary artery jeopardy scores have been used[235] These have been shown to predict the degree of left ventricular dysfunction resulting from spontaneous or pacing-induced ischaemia in patients with CAD.[236] In a study of 462 patients, Califf *et al* [234]showed that one such jeopardy score was superior to the number of diseased vessels as an indicator of survival in patients with CAD. The improvement in prognostic stratification was thought to result from the weighting of the jeopardy score so that proximal lesions were given more weight than distal lesions. A central theme of the jeopardy score is that the amount of myocardium that is damaged in acute myocardial infarct is related to the amount in jeopardy as estimated from the coronary anatomy. Califf *et al.* confirmed that in patients with prior myocardial infarction the left ventricular ejection fraction was related to the jeopardy score.[234] A human autopsy study[237] recently found that the amount of infarcted myocardium was directly proportional to the amount of myocardium distal to the vascular tree of the infarct-related vessel. Other factors such as the presence of serial lesions, the length of the lesions and the presence of collateral vessels, may be important in the relationship between coronary anatomy and mortality. However, with the addition of each new factor the calculation of the risk based on coronary anatomy becomes more cumbersome. As the difficulty increases, the likelihood the score will achieve wider clinical usage decreases.

Symptomatic patients with known coronary disease often die from myocardial infarction or die suddenly because of the acute progression of a lesion to coronary occlusion. Unfortunately, the mere fact that symptoms were present tells us little about the subsequent acute event as symptoms are not necessarily related to the plaque that progressed and was responsible for the acute event.[29] Even the presence of ischaemia on non-invasive testing may not help in risk stratification for future acute coronary events.

On follow up of a stable group of patients with a positive exercise stress test or evidence of ischaemia on ambulatory monitoring, Mulcahay et al reported that ischaemia could not predict the future occurrence of an acute coronary syndrome[226]

If we presume that progression to an acute syndrome is not related to the prior severity of a coronary stenosis, it is reasonable that in many patients an acute presentation might be the first manifestation of coronary disease. In fact, an acute syndrome particularly in men is the first symptomatic clinical manifestation of coronary disease in 50-60% of cases.[208] In most of these, the first manifestation is an acute myocardial infarction but sudden death may occur in 10-25% of cases without prior symptoms.

At least one mechanism for this inability to predict future acute events in patients with coronary artery disease is that such events are unrelated to prior severity of a coronary stenosis. Acute coronary events, which include the diagnoses of unstable angina, acute myocardial infarction, and some cases of sudden death, are related to a sudden disruption, fissure or erosion of an atherosclerotic plaque with the formation of an intracoronary thrombus that partially occludes (a majority of patients with unstable angina) or totally occludes (most patients with myocardial infarction or sudden death) the coronary artery. These so called vulnerable plaques, prone to thrombosis, usually contain a large lipid core and a thin fibrous cap with an abundance of inflammatory cells.[238; 239] These inflammatory cells are generally macrophages capable of degrading the fibrous cap and expressing tissue factor, which contributes to the formation of thrombus. Several angiographic studies in which more than one angiogram was performed on patients who subsequently developed acute syndromes have shown that a majority of these syndromes appears to develop from lesions that on the first angiogram were classified as non-significant stenosis.[240] Ambrose et al. found less than 50% narrowing in 48% of subsequent infarctions on serial angiography,[29] while in a study by Little et al the incidence was 66%.[240] A recent angiographic study found progression of coronary disease on a second angiogram in 89% of patients with an acute syndrome and 74% of the lesions caused less than 50% stenosis on the first angiogram.[226] Although these studies have selected out a population of patients in whom serial angiography was performed, the results differ significantly from patients who had been restudied and found to have a new total occlusion but without an intervening infarction. In this latter group, a severe lesion with greater than 70% narrowing is found in the majority of

patients on the first angiogram. In addition to these retrospective studies, angiographic studies performed after successful thrombolysis often show moderate coronary stenoses of the infarct related artery. Brown et al found less than 50% or 60% stenosis in 66% of lesions after myocardial infarction, similar to the incidence reported by other investigators.[241] In a recent post-mortem study in which Mann and Davies performed angiography post-mortem to assess the stenoses of so called lipid rich vulnerable plaques, there was no correlation between percentage diameter stenosis and the presence of these lesions.[230] These pathological data support the concept that stenosis severity is not an important determinant of future acute coronary events. Angiographic studies, because of the nature of angiography, will underestimate the degree of atherosclerosis in comparison to either pathological studies or the use of newer techniques like intravascular ultrasound. The concept that certain high grade stenotic lesions remain stable over long periods of time is a valid concept, while lesions progressing to acute coronary syndromes are often not severely obstructed before disruption. It is these less than severely stenotic lesions that lead to infarction when an occlusive thrombus forms as collateral vessels cannot be acutely recruited to prevent or limit the extent of myocardial necrosis. These minor or silent plaques are also more frequently seen in patients with multivessel disease and may partially explain why long term mortality is higher with multivessel rather than single vessel disease.[242] The more plaques present, the higher is the chance that one or more might be sites for future acute coronary events. While angiography is limited in it's ability to help predict the site or future development of acute coronary events, new invasive and non invasive techniques are available to detect the different characteristics of coronary lesions that may further add to our ability to predict future events. Angiography the traditional gold standard, can only detect advanced lesions and provide a measure of the degree of stenosis, which has limited prognostic value. Other techniques like intravascular ultrasound or angioscopy , can identify the presence of thrombotic lesions or even lipid rich , vulnerable plaques. However the ability to predict subsequent acute events has not been fully evaluated. Ultra-fast computerised tomography is useful in measuring the calcium content of coronary arteries. This non-invasive test may be useful for predicting the presence of CAD but clinical studies have yet to confirm that there is good correlation between calcium content and plaque vulnerablility. Furthermore, on the basis of intravascular ultrasound data, unstable (acute) lesions are less likely to be calcified than stable

coronary lesions. Magnetic Resonance Imaging techniques in the future may be able to image vulnerable plaques by characterising the various components of the plaque in terms of lipid, fibrous, and thrombotic material.[243]

Fourthly, I have discussed above the 2 processes of medial rearrangement and destruction, which will result in coronary angiography tending to underestimate the amount of intimal disease to a wide and unpredictable degree. Consequently, many angiographically normal coronary arteries will contain advanced plaques. Despite this, The CASS Registry Study showed that the seven year survival of patients with normal or near normal (<50% stenosis) coronary angiograms were 96% and 92% respectively.[244] This result from the CASS registry is extremely important and has formed part of the reasoning for grouping together patients with "clean" coronary arteries and those with minor disease as prognostically they fall into the same category of being "extremely good". Erikssen et al. reported a 7 year follow-up study of 36 apparently healthy middle-aged men with a positive stress test and normal coronary angiograms. Of the 36, 3 had a sudden cardiac death and 4 were diagnosed as having developed a cardiomyopathy within the 7 years of follow-up. The investigators concluded that patients with a positive stress test could not be assured of a good prognosis on the basis of a normal coronary angiogram. Clearly, the data from the large CASS registry of 20 000 (in contrast to 36) does not support that conclusion. There were a total of 843 who had an exercise stress test. Among the patients with a strongly positive response who were observed over 7 years, there were only 3 deaths. The ECG response to exercise was not predictive of survival, no matter how strong the response. This was true in subgroup analysis of those with and without minimal disease. Of note was that a history of hypertension recorded at study entry and a history of smoking were associated with increased mortality over the 7 years.

9.2.5 Quantitative Angiography

Using edge detection methods, a variety of algorithms have been created for detecting and tracing the vessel wall and calculating the percentage of stenosis by comparing the maximum or "normal" vessel diameter with the minimum diameter of a lesion.[245] Absolute measurements are possible with this method by using the known diameter of the angiographic catheter as a scaling device. For the most accurate calculations possible, the arterial segment of interest is magnified and the distortions related to

image acquisition are corrected. Measurements of experimental and clinical stenoses using this method have been accurate and precise.[246] Such measurements are not completely operator-independent however, and therefore may not be entirely objective. For instance, frame-to-frame variability in the measured vessel diameter can be demonstrated, apparently related to the degree of opacification and projection of the lesion in a rapidly moving artery. Other pitfalls in this method include a normal biological variability in vessel diameter, crossing vessels or arterial branches, eccentric lesions, and diffusely diseased arteries. The selection of the arterial segment considered normal by the operator or computer program significantly influences the calculated percentage of stenosis.[247]

Quantitative angiography is an improvement on visual assessments, however, in many centres this remains a research tool, and visual methods remain the cornerstone of making clinical judgements about patients with CAD.

Our reason for using the visual method as well as the more basic angiographic grading of normal, single, double or triple vessel disease is as follows. Firstly, it is still clinically the most widely used method upon which clinical judgements are based. Secondly, as we have shown above the largest mortality data (from the CASS registry) was based on these simple visual assessments and grading system. Consequently, most of the research performed in this area has been based on similar assessments.

10.0 DISCUSSION 2-GENDER DIFFERENCES

The "classical" cardiovascular risk factors (cholesterol, hypertension, diabetes, smoking and family history) do not fully account for CAD risk. Furthermore, they do not account for the gender differences in prognosis in those with proven CAD.[210]. Women who present with MI have been shown to have a poorer prognosis than men who present similarly. .[210] Fibrinogen, FVII:C, PAI-1 and vWF have been shown in large studies to predict acute coronary events.[74; 75; 77; 78; 195; 217] The question arises as to whether sex differences between these haemostatic parameters could explain the differences in disease presentation and prognosis.

As in the CASS study[248], we found that nearly half of the female patients undergoing coronary angiography had insignificant coronary disease or apparently normal coronary arteries compared to only 20% in our male subjects. This indicates the need for other non-invasive markers that may better predict the presence of significant coronary atheroma

As expected, women presenting for angiography were older and had higher plasma cholesterol levels than men. They were also more likely to be hypertensive,[210; 249] and less likely to smoke cigarettes. In the whole study group and in those subjects with atheroma, females had higher levels of both fibrinogen and factor VII with no differences in vWF concentrations. PAI-1 concentrations were elevated in females with atheroma compared to both men with atheroma and to females with normal coronary angiography. vWF was also increased in females with atheroma compared to those without, whilst factor VII did not appear to be related to the presence of atheroma. These results indicate that elevated factor VII and fibrinogen in females is gender rather than disease related, whilst PAI-1 and vWF appear to be associated with underlying atheroma rather than gender.

Higher levels of fibrinogen in women than men have been described in the healthy general population notably in the Scottish Heart Health Study of 8824 subjects, [249] and our findings in this study would support the view that in women, fibrinogen reflects gender rather than the presence of atheroma. By contrast, in men, fibrinogen appears to be related to the presence of coronary disease. The ECAT study[21863] reported no sex difference in fibrinogen levels in subjects who had been investigated for CAD by angiography. Fibrinogen is an acute phase protein, and circulating levels are affected by

a number factors including age, smoking, BMI, and cholesterol [249] It is unclear from the ECAT study whether adjustments were made for these possible confounders nor did the study look at men and women separately. In our study after adjusting for a large number of possible confounders the sex differences in fibrinogen remained. The reason for these sex differences is not immediately apparent, although it is possible that sex hormones play a role. If fibrinogen is not associated with the presence of atheroma in women, it may not influence the differences in prognosis between the sexes in subjects with evidence of CAD.

Previous studies that have investigated sex differences in the fibrinolytic system in healthy populations have yielded conflicting results.[250; 251] Large cross-sectional studies suggest that PAI-1 levels are similar in men and women, [252] and our study finding in the total population is in agreement with this. As in the ECAT study we have found higher PAI-1 levels in women with significant coronary stenosis than men. A recent study reported higher PAI-1 levels in diabetic women compared to diabetic men.[215] Suppression of fibrinolysis through raised PAI-1 is related to the syndrome of insulin resistance[178] and it has been suggested that sex-specific interference of the haemostatic system in association with insulin resistance may be the explanation for this finding.

Insulin resistance without clinical diabetes appears to be a factor in coronary artery disease, although our results imply that increased PAI-1 in females is related to atheroma, either causally or secondary to the underlying disease.

In the Framingham Offspring Study,[251] postmenopausal women and men of similar age did not differ significantly in their PAI-1 levels. However, healthy women with higher oestrogen levels, or those on hormone replacement therapy (HRT) had lower PAI-1 levels than other women not on HRT. In the present study, we made no assessment of the sex hormone levels, nor did we assess the use of HRT in our female population. However, examination of the age group in the female population suggests that the majority of the women would be postmenopausal. Furthermore, the use of HRT in this population at the time of recruitment was 3%. Despite this there was a substantial difference in PAI-1 levels to indicate that hormonal status does not fully account for the observed gender difference.

The majority of studies of vWF in healthy white subjects, and those with angina,[253] show no sex differences, and this was our finding in the total study population

and in the subgroup with significant coronary stenosis. However, vWF levels were related to the presence of CAD in women but not in men. Hence, despite the similarity in the levels of vWF between the sexes, vWF levels may contribute to the differences in prognosis.

Higher levels in FVII:C in women than men have also been described in the general population,[254] and as with fibrinogen, our finding in the total study population and in the subgroup with significant atheroma would not therefore be unexpected. Raised FVII:C levels have been described in women but not men in a small study of subjects with unstable angina compared to a normal control population.[245] The ECAT study also found higher circulating levels of FVII:C in women compared to men with significant coronary stenosis[255]. These findings with our observation that FVII:C levels were similar in women regardless of the presence of atheroma indicates that the increased levels reflect gender rather than atheroma . In a recent report among diabetic subjects women had higher levels of FVII:C than men.[215] A gender-specific influence of insulin resistance on the coagulation system was suggested. As with circulating PAI-1, a contribution of insulin resistance on the gender difference in circulating FVII:C cannot be excluded. FVII:C levels may therefore not contribute to the differences in prognosis between the sexes.

The importance of haemostatic factors on the pathogenesis of CAD in women has gained further support by the recent finding that Factor V Leiden increases the risk of myocardial infarction in young women.**(quote246)** However, the authors make it clear that the finding is unlikely to be applicable to populations of older women (as in our study population) in whom the overall baseline risk is higher. We have not measured Factor V Leiden in our study and are therefore unable to comment further on this finding.

In conclusion, we have shown in this study that in patients undergoing angiography for investigation of chest pain, women have a worse classical risk factor profile, and increased levels of fibrinogen, FVII:C and PAI-1 compared to men. However, in women with atheroma only PAI-1 and vWF levels were related to the presence of CAD which may be part of the explanation for the poorer prognosis in women than their male counterparts.

In the search for non-invasive markers of CAD, large prospective trials are needed to evaluate the role of circulating levels of these coagulation and fibrinolytic

factors and whether they may provide an additional means of predicting the likelihood for the presence of significant atheroma, particularly in women. This may be of use in selecting female patients who require a coronary angiogram. It may help reduce the number of female patients who present with chest pain and are subsequently found to have a normal coronary angiogram. The evidence for an association of PAI-1 with the presence of significant disease is growing, and the striking differences of PAI-1 levels in our study between females with and without significant stenosis raises the possibility that PAI-1 may fulfil such a role as a non-invasive marker at least in women. A prospective study is now underway to examine this.

The present study has some limitations. Firstly, it is retrospective, and therefore unable to yield sensitivity, specificity and predictive values for the possible non-invasive markers. Secondly, we have not measured insulin resistance which may be the explanation for some of the differences seen. Thirdly, the lack of data on the use of HRT presents a further limitation, as HRT use has been shown to influence fibrinolytic factors. Further studies are underway to address this. This study supports the growing body of evidence that the coagulation and fibrinolytic system needs to be considered in the assessment of risk factor profiles in patients (especially in women) who present for the assessment of chest pain.

11.0 DISCUSSION 3-ACE

The angiotensin converting enzyme I/D polymorphism has been implicated in the pathogenesis of ischaemic heart disease after the original description by Cambien et al relating a higher prevalence of the DD genotype in low risk patients with myocardial infarction.[93] This has however, been called into question by recent studies, notably the large prospective American Physician's Health Study[256] which showed no preponderance of the DD genotype in patients with coronary events. These studies were not however, designed to evaluate the presence or extent of coronary atheroma by angiography or otherwise. Hence the degree of atheroma in both cases and controls is unknown. If the ACE genotype were related to the degree of atheroma rather than thrombosis, this could explain the wide variation in the control population for whom the degree of atheroma is unknown. Two studies have attempted to distinguish whether the ACE gene is related to coronary atheroma or thrombosis,[137; 138] these have also yielded conflicting results. Our study investigated the prevalence of the DD genotype and its relation to coronary atheroma and thrombosis.

11.1.1 ACE and MI

We found no association between the ACE genotype and a history of MI either in the total study population, or in any of the subgroups who were at low risk of an MI by classical risk factors (smoking, hypertension, diabetes mellitus, hypercholestrolaemia, family history) and low BMI. This is in keeping with the American Physician's Health Study and other retrospective studies[132]. In contrast however, recent work by Gardemann et al[138] in support of the original report by Cambien, found an association between the ACE genotype and MI, but in common with Cambien, only after sub-group analysis. Lindpainter, has pointed out that such sub-group analysis has to be viewed with great caution[257] hence throwing into question the associations that have been made. Furthermore, no adequate explanation has been given for the phenomenon whereby, gene-environment interaction should act independently and in a non-additive manner to other known and classic risk factors[258]. Gardemann points out that it is a common observation that there is a group of patients who develop MI without classical risk factors. However, the definition for low risk that have been used in these studies include; low BMI<25kg/m2or 26kg/m2 (Gardemann and Cambien respectively), low apoB<1.25g/l, low cigarette consumption(less than 5 pack years, and non-hypertensives

(a difficult group to evaluate because of antianginal medication). These definitions are rather arbitrary, and not particularly classical (as usually defined). Our results suggest that the DD genotype does not confer an increased risk on MI and hence on thrombosis.

11.1.2 ACE and Coronary Atheroma

We found no association between the ACE genotype and either the presence or extent of coronary atheroma. This is in keeping with previous angiographic studies[137; 138] except a small (n=245) Japanese study which found an increase in the prevalence of the DD genotype with an increase in the number of vessels diseased.[139] Foy et al have reported allele frequencies of 0.3 and 0.7 for the deletion and insertion alleles respectively in Pima Indians, similarly, Lee et al reported identical allele frequencies in a Chinese population.[259] This is very different from that reported in Caucasian populations with allele frequencies of 0.53 and 0.47 for deletion and insertion alleles respectively,[128; 149] allele frequencies not dissimilar to that in our study (Table 4). These differences suggests that ethnicity may play an important role. Further support for ethnic differences comes from the study in Black Americans in which the ACE genotype was not even related to plasma levels.[260] There is no evidence from ours and other studies that in Caucasians the ACE I/D polymorphism plays an important role in the formation of atheroma.

11.1.3 ACE Genotype, Coronary Atheroma And Coronary Thrombosis

The formation of atheroma and thrombosis are different pathological processes, and it is therefore possible for the ACE genotype to have differential effects on the two processes. A hypothesis whereby pre-existing atheroma may be necessary for the effect of the ACE genotype on coronary thrombosis to become evident was put forward by Ludwig et al in a study of a US white population.[137] They found no relationship between the ACE gene and MI in the total study population, but in a sub-group of patients with >60% stenosis in any major vessel the DD genotype became significantly related to MI. In our study although there was a strong relationship between the presence and extent of atheroma with MI, we found no relationship between the ACE genotype in the subgroup with atheroma. If Ludwig's hypothesis was correct, it would be expected that the prevalence of the DD genotype in MI patients would increase with an increase in the number of vessels diseased, but this was not the case either in our study or in Ludwigs.

Furthermore, there is increasing evidence that infarct-related arteries tend to have stenosis of less than 50%, these smaller plaques having a higher lipid content and therefore more likely to rupture leading to coronary thrombosis[29]

In a recent meta-analysis using data on over 8500 subjects, Samani *et al* found an association between the ACE D allele and MI risk.[261] They suggested from their analysis that the increased risk associated with the D allele was unlikely to make a useful contribution to the risk stratification of an individual; it's main value was in elucidating the mechanism of the association. The authors were careful to point out several limitations of such a meta-analysis. The most important in this author's view was selection bias in favour of positive studies. This was only partially addressed by the use of the funnel plot analysis. Samani and his colleagues' main contribution with this analysis was to stop this area of molecular biological research from sudden death after the publication of the American Physician's Health Study, and that the later was not the last word on the relationship between the ACE gene polymorphism and CAD.

11.1.4 ACE Activity And Atherothrombosis

In keeping with most of the previous studies ACE activity was strongly related to genotype.[126; 128] There was no relationship however between ACE activity and coronary atheroma. Indeed there was a trend towards lower ACE levels in subjects with evidence of more extensive coronary atheroma. Support for this finding comes from a study by Bonithon-Kopp[262] in which significantly higher ACE activity was found in subjects with evidence of carotid wall thickening but with no evidence of plaque[146] Conversely, subjects with plaque tended to show lower ACE levels. In the report by Gardemann, plasma ACE activity was not related to atheroma. In the other two angiographic studies,[137; 139] no data was given on the relationship between plasma levels and coronary atheroma. A conclusion that may be drawn from ours and the other studies is that one cannot exclude the possibility that circulating ACE activity may be related to early structural changes to the arterial wall but is not related to coronary atheroma.

11.1.5 ACE Activity And MI

In view of the strong relationship between the presence of coronary atheroma and MI, and the fact that both share similar risk factors, it is perhaps not surprising (from the above discussion) that a non-significantly negative relationship was found between ACE and MI independent of age. It is seductive to speculate from these non-significant findings that plasma ACE activity may be of some pathological importance in the early phase of the atherothrombotic process, but declines in established disease. Gardemann found no relationship between ACE and MI, regardless of age. This is at variance with the report from Cambien in which ACE activity was an independent risk factor for MI only in the subgroup younger than 55 years.[22] It is difficult to reconcile these findings, one possible explanation may be that the cases in Cambiens under 55 age group had a preponderance of single vessel disease or non-significant coronary atheroma, the subjects with the highest ACE activity in our group.

Other ACE polymorphisms

One of the candidate regions for an active site influencing ACE levels is the ACE gene promoter. The promoter region has previously been characterised and found to contain sequences obligatory for transcriptional activity in the 132-bp region upstream of the transcriptional site and negative regulatory elements further upstream.[263] Ten novel polymorphisms have been identified in the ACE gene[264] including six in the 5' region, three in the coding sequence and one in the 3'untranscribed region. None have as yet been identified as being functional. In our unit, Foy C *et al* used single strand conformational polymorphism (SSCP) analysis QUOTE "{Orita, Suzuki, et al. 1989 ID: 1836}" as a means of detecting point mutations in the 5'region of the transcription start site. Two common two-allele polymorphisms were identified and characterised by direct sequencing. These were later found to correspond to two of the recently identified ACE gene polymorphisms (A-240T and T-93C). A commingling analysis between the T-93C and the I/D polymorphisms have provided further evidence in support of the I/D polymorphism either being in linkage disequilibrium with an active site elsewhere or alternatively having an effect together with a further functional site[265]. The six polymorphisms studied included the following: T-5491C, A-240T, T-93C, T1237C, I/D and $4656(CT)_{2/3}$. These were related to circulating ACE levels and to MI .

All the polymorphisms were associated with ACE levels when analysed individually by one way ANOVA.. A-240T accounted for the greatest variability in ACE levels with an

R^2 of 14%. After adjusting for the effects of the A-240T polymorphism, none of the other polymorphisms assumed significance.

An association of two of the promoter region polymorphisms with a history of MI was found. The TT genotype of the A-240T polymorphism appeared to be protective against MI. This finding, although supported by the work of Bohn *et al* (see Table 1) is at variance with Samani's meta-analysis.

The wide variation in circulating ACE concentrations between individuals appears to be universal across populations. The genetic variants responsible for such a range may be maintained in populations because of an unidentified protective effect of higher ACE levels. This possibility is suggested by the study of Schachter as discussed previously.

Alternatively, rather than being protective against MI, the haplotype containing the T and D alleles may be associated with fatal MI and therefore presents at a reduced frequency in survivors of MI. This hypothesis is supported by Evans' study alluded to above (Table1). The fact that all genotypes remained in Hardy-Weinberg equilibrium however, (when split according to a previous history of MI), militates against this possibility.

These findings may of course reflect the play of chance statistical associations either due to the small patient groups or to multiple testing (despite the use of bonferonni correction). Clearly much larger prospective studies are needed to reduce these possibilities.

11.1.6 Study Limitations

A major limitation in our study as with all retrospective study of this nature is that many of the cases were recruited some months after their MI, raising the possibility of selection by mortality as discussed. I have discussed the problems associated with our control group and the use of the visual method in assessing coronary angiography on pages 103-112. Furthermore, any conclusions drawn from such a relatively small study have to be viewed with caution.

In conclusion, the results of this study suggests that neither the ACE I/D polymorphism nor ACE activity are independent risk factors for atheroma or thrombosis. The possibility that ACE activity may be involved in early structural changes declining after the atherothrombotic process is established remains speculative. Furthermore, other ACE polymorphisms (in particular the A-240T) may have a protective role for future

coronary events. Other large prospective studies with greater numbers of coronary events are needed to help clarify this controversial area of cardiovascular research.

12.0 DISCUSSION 4 PAI-1

Circulating levels of PAI-1 may determine vascular risk through two separate though related mechanisms. Fibrin deposition is an invariable feature of atherosclerotic plaques[266] and high local levels of PAI-1 may theoretically result in increased fibrin deposition and encourage plaque formation or growth. Data showing increased circulating PAI-1 levels in subjects with coronary atheroma supports this possibility.[77] Distinct from this, acute myocardial infarction is usually associated with thrombosis at the site of a ruptured atherosclerotic plaque.[267] By inhibiting fibrinolysis, elevated levels of PAI-1 may contribute to a prothrombotic state increasing the likelihood of coronary thrombosis and occlusion. The work of Hamsten and others supports a role for elevated PAI-1 levels in coronary thrombosis[195; 268] The possible influence of PAI-1 levels on atherogenesis and occlusive thrombosis should be distinguished. However, as there is some evidence that PAI-1 release is increased from atherosclerotic lesions[191] and as a part of the acute phase response [165150] it is possible that the elevated levels of PAI-1 seen in previous studies may result from, rather than lead to atherogenesis or thrombosis. Furthermore an underlying association between PAI-1 levels and coronary disease may be hidden by the confounding effect of the close correlation between levels of PAI-1 and other established cardiovascular risk factors, particularly those that occur in association with insulin resistance.[73; 176]

The emerging evidence that circulating levels of PAI-1 relate to genotype at a common polymorphism in the promoter of the PAI-1 gene[60; 189; 190; 205] has opened the possibility of using PAI-1 genotype as a surrogate measure of pre-morbid PAI-1 levels to tease apart the cause and effect limbs of the PAI-1- coronary disease relationship. Previous studies examining the association between genotype and coronary disease have not distinguished between atherogenesis and occlusive coronary thrombosis. In this study we have examined the relationship between both PAI-1 genotype and PAI-1 levels with both coronary atheroma as visualised by angiography and with previous coronary thrombosis as assessed by WHO criteria.

12.1.1 PAI-1 genotype and PAI-1 levels.

In keeping with previous studies we found an association between PAI-1 promoter (4G/5G) genotype and PAI-1 levels after adjusting for BMI and triglyceride with the highest circulating levels in 4G/4G subjects, intermediate levels in 4G/5G heterozygotes and lowest levels in 5G/5G subjects suggesting a codominant gene effect [60; 189; 190; 205] We have also repeated the finding of others that the relationship between fasting PAI-1 and triglyceride levels is influenced by PAI-1 promoter genotype with a steeper regression slope in the 4G/4G group[60; 269] Additionally we found a similar influence of genotype on the regression slope of PAI-1 on BMI although in this case the slope was steeper in the 5G/5G group. These interactions of both TG and BMI with genotype remained both independent and significant in a regression model. While the interaction of triglyceride with genotype fits with the finding of higher levels of PAI-1 in the 4G/4G group it is unclear exactly what the statistical interaction between BMI and the 5G/5G genotype tells us about the regulation of PAI-1. In a similar study in Pima Indians interaction between PAI-1 genotype and BMI was observed but with a steeper regression slope in the 4G/4G group.[270] Clearly ethnic differences or chance may account for these findings and further studies will be required to confirm significant interaction between BMI and genotype in relationship to PAI-1 levels.

12.1.2 Associations with coronary atheroma

The proportions in the various angiographic groups were similar to that reported in the ECAT study,[77] and identical to data from the CASS registry [244]

This study found the expected relationship between the presence of coronary stenosis with age, BMI, hypertension, cigarette smoking and cholesterol levels. However, the relationship between PAI-1 levels and the presence of stenosis in one or more coronary arteries was weak falling just above standard levels of significance and there was no trend in PAI-1 levels in relation to the extent of coronary disease. In this latter respect our results are in agreement with those of the larger ECAT study,[77] To our knowledge this is the first study to investigate the relationship between the extent of coronary atheroma and PAI-1 promoter genotype and we found no evidence of an association

with the frequency of the potentially deleterious 4G/4G genotype, being similar in each angiographic class. This suggests that genotype and thus pre-morbid PAI-1 levels do not influence the generation of coronary atheroma. If this is the case, then the relationship between PAI-1 levels and the presence of coronary atheroma that others have shown may have resulted from production of PAI-1 in response to or triggered by the presence of atheroma. An alternative explanation for the results is that within those patients without significant coronary stenosis a number may have had microvascular coronary disease (25) and that PAI-1 genotype may influence the development of microvascular disease in the same way as macrovascular disease. However, there are no previous data to support or refute this and the majority of the patients with 'clean' coronary arteries are likely to have suffered from either coronary vasospasm or non-cardiac chest pain.

12.1.3 Associations with coronary thrombosis

As would be expected a history of myocardial infarction was more frequent with male sex, cigarette smoking and increased number of stenosed coronary arteries as well as being related to cholesterol levels, BMI and age. While there was only a trend to higher levels of PAI-1 in the MI group, we found a significant association between PAI-1 genotype and history of MI which remained after adjusting for the other risk factors. A single measurement of PAI-1 levels is unlikely to reflect lifelong levels since it is an acute phase reactant. Genotype which does not change may act as an effective marker for lifelong exposure to differing levels. This could explain why we have been able to demonstrate a relationship between genotype and a history of MI, while showing only a trend between PAI-1 levels and a history of MI.

Our findings concur with the results of two small and restricted studies which found the 4G/4G genotype to be more frequent in young male survivors of MI[190] and in NIDDM patients with a history of ischaemic heart disease[204] On the other hand our results differ from those of the large four centre ECTIM study in which no association between PAI-1 genotype and MI was found[205] The differences between our study and ECTIM are important for while they may reflect the play of chance they may also suggest the way in which PAI-1 genotype affects the processes causing coronary disease.

12.1.4 PAI-1 genotype, coronary atheroma and coronary thrombosis

In ECTIM, subjects who had survived a myocardial infarction were compared to control subjects recruited from the general population in the three French centres and Belfast[205] While these subjects were apparently healthy the frequency of significant though asymptomatic coronary disease in this control group is not known. The ECTIM study was not designed to be able to distinguish the influence of genotype on coronary thrombosis from that on atherogenesis. In contrast to this our study examined both coronary atheroma and thrombosis finding no relation on PAI-1 genotype to extent of atheroma but finding an association with previous coronary thrombosis. A hypothesis whereby pre-existing coronary atheroma may be necessary for the effect of PAI-1 genotype on coronary thrombosis to become evident, presumably through altered PAI-1 levels, is supported by the finding that the relationship between genotype and MI strengthened when only those patients with significant coronary artery stenosis were examined. Furthermore this influence of PAI-1 genotype on PAI-1 levels leading to a prothrombotic state could all take place at the local level. PAI-1 production is increased at and around atherosclerotic plaques[191] so the rate of both basal and stimulated PAI-1 gene transcription may be influenced by PAI-1 promoter genotype through molecular mechanisms being elucidated. This would account for the weak association that we found between history of myocardial infarction and levels of PAI-1 in the systemic circulation.

In conclusion, our findings suggest that genotype at the PAI-1 promoter polymorphism is an independent risk factor for myocardial infarction in patients being investigated for chest pain or suspected coronary disease and particularly in subjects known to have significant coronary artery stenosis. The results indicate that this effect is mediated by involvement of the PAI-1 gene in the pathogenesis of coronary thrombosis with greatest risk in those subjects possessing the 4G/4G genotype. Our findings also support the hypothesis that elevated levels of PAI-1 may predate the development of disease and indicate that PAI-1 genotype may serve as a useful marker of future risk. Prospective longitudinal studies are warranted to confirm these findings.

13.0 SUMMARY

In most industrialised countries IHD is the commonest cause of death. 60% of all fatal MI occurs in the first hour of the attack. Hence intervention as well as prevention of MI is of major public health importance. In order for this to happen we need to understand the pathophysological processes that result in atheroma and thrombosis. The atherothrombotic process results from a complex interaction between various circulating proteins (intermediate phenotype) and environmental risk factors

One major problem in the study of these circulating proteins revolves around differences in their levels in subjects studied. This is due to the fact that they are affected by acute phase reactions, and possibly by the atherothrombotic process itself. Consequently, whether the finding of raised levels of circulating proteins associated with CAD are due to cause or effect is unknown. The candidate gene approach is one way of dealing with this problem. These are genes that are thought to influence (or regulate) circulating proteins involved in the atherothrombotic process. Because these genes do not change, their study will go some way to answer whether an intermediate phenotype causes the disease or results from it figure 10. This is important, as it may be possible to intervene both at the level of the gene (by altering the regulation of transcription) and it's protein product (intermediate phenotype). Furthermore, if risk factors act in concert to produce a high risk group; the study of such interactions will be highly informative in our selection of groups worthy of close attention.

In all these respects plasminogen activator inhibitor-1 (PAI-1) presents itself as an intermediate phenotype of some importance. It plays a key role in fibrinolysis as its main inhibitor. Raised levels of PAI-1 therefore, could lead to a prothrombotic state. Furthermore, PAI-1 has a close relationship with features of the insulin resistance syndrome, all of which have been associated with the risk of IHD. Several studies linked raised levels of PAI-1 to the atherothrombotic process but failed to tease apart the cause and effect limbs, especially as PAI-1 is an acute phase protein. The discovery of the PAI-1 4G/5G polymorphism in the promoter region of the gene, and the finding that it influenced circulating PAI-1 levels paved the way for a study of the relationship of the PAI-1 genotype with the clinical phenotype – coronary stenosis and myocardial infarction.

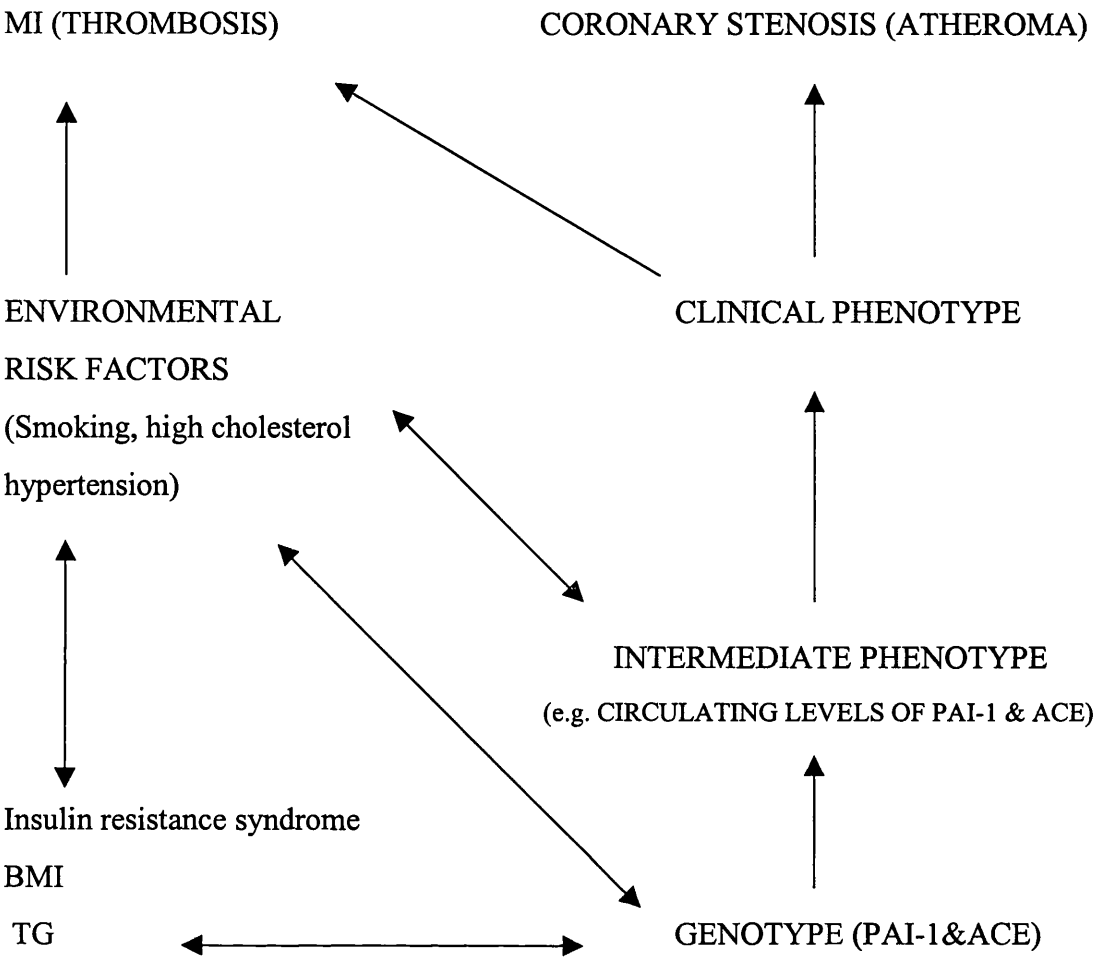


Figure 14 Proposed mechanism for gene /environment interaction and atherothrombosis

At the time this study was conducted, two small and restricted studies had shown an association between the 4G/4G genotype and MI. The ECTIM Study had reported a negative finding but confirmed the relationship between the 4G/4G genotype and the highest levels of circulating PAI-1. These studies however, did not attempt to tease apart the influence PAI-1 genotype and atheroma on the one hand and thrombosis on the other. A further major limitation had to do with the choice of an apparent normal control group who may well have had atheroma not as yet clinically apparent.

Although a study using coronary angiography has some limitations it is currently the gold standard for investigating subjects with known or suspected CAD. Subjects with normal or near normal coronary angiograms are prognostically distinct from those with

evidence of significant coronary atheroma. Although the emerging evidence suggests that it is small (non-significant) plaques which (because of their nature rather than size) rupture to cause acute coronary syndromes, subjects with multiple and significant lesions tend to have more such plaques. This explains why long term mortality studies shows that subjects with multi-vessel disease have a higher long term mortality than those with single- vessel disease, and much higher than those with "normal" coronary arteries.

This study confirmed the relationship between genotype and levels. Of greater importance was the finding of a relationship between the 4G/5G polymorphism and MI, a relationship which was stronger in subjects with pre-existing significant atheroma. This suggests that the presence of atheroma may be necessary for PAI-1 to exert its influence on the atherothrombotic process and has biological plausibility. It may also explain the difference between ECTIM and this study.

The review of atherothrombosis shows the complex interaction of various proteins, and highlights the possibility of gene-environment interactions. Clinical studies have reported a genotype-specific response of PAI-1 to triglyceride. Recent work suggests that a triglyceride-responsive motif exists in close proximity to the 4G/5G site that interferes with the binding of transcription factors. This study confirmed the genotype-specific response of PAI-1 to triglyceride. The emerging evidence indicates that the effects of the PAI-1 gene may be mediated through interactions with environmental risk factors of the insulin resistance syndrome. This gene/environment interaction may be critical to our understanding of the differences between past, present and future studies. For example, since this study was performed, the American Physicians Health study has reported their findings. They found no relationship between the 4G/5G genotype and 374 subjects with first MI and 121 with venous thromboembolism compared to control subjects. The subjects under study are a notoriously health conscious population with low rates of smoking, relatively low BMI and lipid levels. The study does not report on triglyceride levels, but the low levels of other features of the insulin resistance syndrome suggests that these subjects may have had low levels of TG. If this is the case the crucial gene/environment interaction between PAI-1 and TG may not have been evident as a causative factor in atherothrombotic risk.

These gene/environment interactions are likely to be the explanation (at least in part) for the stark differences between individuals and populations with regard to the importance

of different risk factors.

The interaction between BMI and PAI-1 genotype found in this study highlights the imperfections in our understanding in this area. Its close association with TG would lead us to expect results different from what was seen. Other studies are awaited to either confirm or refute this finding.

One of the striking features of IHD is the difference between the sexes with regard to epidemiology, presentation and prognosis. Whether differences in the haemostatic systems are gender-specific and could account for some of these differences is unknown. This study indicates that levels of PAI-1 may explain some of those gender differences. In particular, the difference in PAI-1 between women with and without coronary stenosis suggests a possible interaction between the female hormonal milieu and PAI-1 in the chain of events leading to atherothrombosis. This needs further confirmation. Of particular clinical interest is the seductive suggestion that PAI-1 may act as a non-invasive marker of significant coronary atheroma. The cost-savings, as well as the risks associated with coronary angiography make PAI-1 even more compelling for future study.

The RAS presents attractive candidates for cardiovascular pathology. Its role in vasoconstriction, smooth muscle cell proliferation, sodium and water homeostasis and finally in stimulating the release of PAI-1 is clear. Circulating and tissue ACE are under genetic influence.

Whilst there is biological plausibility for a relationship between the ACE ID polymorphism and atherothrombosis, several of the studies seem to have generated more heat than light. This study demonstrated the expected relationship between the ACE polymorphism and circulating levels but did not find any relationship between the genotype and clinical phenotypes. Indeed such trends as existed was in the other direction, such that subjects with minimal disease had a greater preponderance of the supposed deleterious deletion as well as the higher levels of ACE.

This could merely be the play of chance. It could highlight a major limitation in all such retrospective data namely the phenomenon of allele drop-out. On the other hand these results could be telling us something of the pathogenesis of early lesions. Perhaps ACE is involved in the early intimal – medial wall changes through its effect on smooth muscle proliferation, as suggested in one study. However this is purely speculative. The temptation to confine the ACE gene polymorphism to the "archives of medicine",

however, has been tamed by the recent meta-analysis from Samani *et al.* While this study suffers from publication bias as well as other problems common to meta-analysis its main contribution has been to give validity to future work in this area.

13.1 SUMMARY OF FUTURE WORK

IHD is largely thought to be a disease of the 20th Century. It is likely that this is the result of significant environmental changes or more correctly changes in gene environment reactions. The genetics of PAI-1 and its regulation by triglyceride presents us with an example of how genes may interact with the environment to enhance the atherothrombotic process.

It has been postulated that the PAI-1 4G/5G promoter polymorphism may be important only in the context of high triglycerides and insulin resistant states. Future work in this area would have to include a large prospective study involving several thousand patients with and without the insulin resistant syndrome. The relationship between PAI-1 and the atherothrombotic process in each group could be elucidated. If the above hypothesis is correct, there would be a strong relationship between PAI-1 and coronary events in subjects with insulin resistant syndrome compared to the general population. This study is now underway.

Further studies are needed to increase our understanding of the regulation of PAI-1 and the role of TG and BMI. The possibility that PAI-1 may serve as a non-invasive marker of significant coronary atheroma (especially in women) requires a large prospective study which is currently being undertaken. Furthermore, whether the different PAI-1 genotypes respond differently to thrombolytic therapy will be of some clinical interest.

Since this study was undertaken, other ACE gene polymorphisms have been identified which are thought to be of greater importance than the ID polymorphism. Further work to elucidate how these interact are underway. Angiotensin-II receptor polymorphisms and their role in the atherothrombotic process are under intense scrutiny.

In such a polygenic disease as CAD various genes are probably regulated by other genes, just as the various environmental factors that have been shown to contribute to CAD influence each other. This concept of gene/gene, gene/environment and environment/environment interactions are of great importance to our understanding of

the pathogenesis of CAD and need further study.

13.1.1 Clinical Inplications

It is unlikely that any single polymorphism will have great predictive value for complex, multi-factorial disorders such as CAD. However, there is emerging evidence to suggest that knowledge of genetic polymorphisms may improve our understanding of mechanisms of disease. For example, the recently discovered Factor XIII Val 34 leu[271] is associated with the formation of weaker fibrin clot and therefore protection from myocardial infarction.

Knowledge of genotype may also provide insight into appropriate pharmacological therapies. An example of this is the use of ACE inhibitors in coronary artery restenosis depending on ACE genotype.[272]

The predictive value of a single case control study is limited. However, attempts are currently underway to harness the findings of the Human Genome Mapping Project. It is likely that large patient cohorts as proposed by the Medical Research Council will overcome some of the constraints associated with case control studies. Ethical concerns have been raised by the media which may impede the rapid progress that could be achieved by such studies. However, these studies may more reliably associate genotype (and environmental factors) with the subsequent development of thrombotic vascular disorders.

14.0 REFERENCE LIST

1. de Bono DP, Hopkins A: The management of acute myocardial infarction: guidelines and audit standards. Report of a workshop of the Joint Audit Committee of the British Cardiac Society and the Royal College of Physicians. *J R Coll Physicians Lond* 1994;28:312-317

2. Demirovic J, Myerberg RJ: Epidemiology of sudden cardiac death: an overview. *Prog Cardiovas Dis* 1996;XXXVII:39-48

3. Davies MJ, Thomas A: Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. *N Engl J Med* 1984;310:1137-1140

4. Rokitansky C. A manual of Pathological Anatomy. 4(271). 1852. London, Sydenham Society.

5. Restrepo C, Tracy RE: Variations in human aortic fatty streaks among geographic locations. *Atherosclerosis* 1975;21:179-193

6. Smith E. Atherosclerotic lesions - an overview. (eds G Crepaldi, A Gotto and E Manzato. Atherosclerosis VIII , 13-19. 1989. Amsterdam, Excerpta Medica.

7. McGill HC. (eds S.Glagov, WP Mewman and SA Schaffer. Pathology of the Human Atherosclerotic Plaque , 1-11. 1990. New York, Springer-Verlag.

8. Guyton JR, Klemp KF, Black BL, Bocan TM: Extracellular lipid deposition in atherosclerosis. *Eur Heart J* 1990;11 Suppl E:20-28

9. Wilcox JN, Rodriguez J, Subramanian R, Ollerenshaw J, Zhong C, Hayzer DJ, Horaist C, Hanson SR, Lumsden A, Salam TA, et al: Characterization of thrombin receptor expression during vascular lesion formation. *Circ Res* 1994;75:1029-1038

10. Tejada C, Strong JP, Montenegro MR, Restrepo C, Solberg LA: Distribution of coronary and aortic atherosclerosis by geographic location, race, and sex. *Lab Invest* 1968;18:509-526

11. Hansson GK: Immune and inflammatory mechanisms in the development of atherosclerosis. *Br Heart J* 1993;69:38-41

12. Faruqi RM, DiCorleto PE: Mechanisms of monocyte recruitment and accumulation. *Br Heart J* 1993;69:19-29

13. Salonen JT, Yla-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssonen K, Palinski W, Witztum JL: Autoantibody against oxidised LDL and progression of carotid atherosclerosis [see comments]. *Lancet* 1992;339:883-887

14. Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ, Northfield TC: Relation of Helicobacter pylori infection and coronary heart disease. *Br Heart J* 1994;71:437-439

15. Ossei-Gerning N, Moayyedi P, Smith S, Braunholtz D, Wilson JI, Axon ATR, Grant PJ: Helicobacter pylori infection is related to atheroma in patients undergoing coronary angiography. *Cardiovasc Res* 1997;35:120-124

16. Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Northfield TC: Childhood living conditions and *Helicobacter pylori* seropositivity in adult life. *Lancet* 1992;339:896-897

17. Goodwin CS, Mendall MM, Northfield TC: *Helicobacter pylori* infection. *Lancet* 1997;349:265-269

18. Patel P, Carrington D, Strachan DP, Leatham E, Goggin P, Northfield, TC, Mendall MA: Fibrinogen: a link between chronic infection and coronary heart disease. *Lancet* 1994;343:1634-1635

19. Opie L.H. Angiotensin Converting Enzyme Inhibitors. 1994. New York, Wiley-Liss.

20. Lyall F, Dornan ES, McQueen J, Boswell F, Kelly M: Angiotensin II increases proto-oncogene expression and phosphoinositide turnover in vascular smooth muscle cells via the angiotensin II AT1 receptor. *J Hypertens* 1992;10:1463-1469

21. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP: Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* 1990;322:1561-1566

22. Cambien F, Costerousse O, Tiret L, Poirier O, Lecerf L, Gonzales MF, Evans A, Arveiler D, Cambou JP, Luc G, Rakotovao R, Ducimetiere P, Soubrier F, Alhenc-Gelas F: Plasma level and gene polymorphism of angiotensin-converting enzyme in relation to myocardial infarction. *Circulation* 1994;90:669-676

23. Kao V, Wissler R. A study of the immunohistochemical localisation of serum lipoproteins and other plasma proteins in human atherosclerotic lesions. Exp Molec Pathol 4, 465-479. 1965.

24. Bini A, Fenoglio JJ Jr, Mesa-Tejada R, Kudryk B, Kaplan KL: Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. Use of monoclonal antibodies. *Arteriosclerosis* 1989;9:109-121

25. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ: Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 1987;316:1371-1375

26. de Feyter PJ, Serruys PW, Davies MJ, Richardson P, Lubsen J, Oliver MF: Quantitative coronary angiography to measure progression and regression of coronary atherosclerosis. Value, limitations, and implications for clinical trials. *Circulation* 1991;84:412-423

27. Davies MJ: A macro and micro view of coronary vascular insult in ischemic heart disease. *Circulation* 1990;82:38-46

28. Ambrose JA, Winter SL, Arora RR, Eng A, Riccio A, Gorlin R, Fuster V: Angiographic evolution of coronary artery morphology in unstable angina. *J Am Coll Cardiol* 1986;7:472-478

29. Ambrose JA, Tannenbaum MA, Gorlin R, et al: Angiographic progression of coronary artery disease and the development of myocardial infarction. *J Am Coll Cardiol* 1988;12:56-62

30. Davies MJ: Reactive oxygen species, metalloproteinases, and plaque stability [editorial; comment]. *Circulation* 1998;97:2382-2383

31. Davies MJ: Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. *Circulation* 1996;94:2013-2020

32. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT: Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 1994;90:775-778

33. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R: Coronary risk factors and plaque morphology in men with coronary disease who died suddenly [see comments]. *N Engl J Med* 1997;336:1276-1282

34. Davies MJ: The composition of coronary-artery plaques [editorial; comment]. *N Engl J Med* 1997;336:1312-1314

35. Libby P: Molecular basis of the acute coronary syndromes. *Circulation* 1995;91:2844-2850

36. Isner JM, Kearney M, Bortman S, Passeri J: Apoptosis in human atherosclerosis and restenosis [see comments]. *Circulation* 1995;91:2703-2711

37. Dollery CM, McEwan JR, Henney AM: Matrix metalloproteinases and cardiovascular disease. *Circ Res* 1995;77:863-868

38. Henney AM, Wakeley PR, Davies MJ, Foster K, Hembry R, Murphy G, Humphries S: Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc Natl Acad Sci U S A* 1991;88:8154-8158

39. Brown DL, Hibbs MS, Kearney M, Loushin C, Isner JM: Identification of 92-kD gelatinase in human coronary atherosclerotic lesions. Association of active enzyme synthesis with unstable angina. *Circulation* 1995;91:2125-2131

40. Galis ZS, Sukhova GK, Lark MW, Libby P: Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994;94:2493-2503

41. Galis ZS, Sukhova GK, Libby P: Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J* 1995;9:974-980

42. Saren P, Welgus HG, Kovanen PT: TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol* 1996;157:4159-4165

43. Schonbeck U, Mach F, Sukhova GK, Murphy C, Bonnefoy JY, Fabunmi RP, Libby P: Regulation of matrix metalloproteinase expression in human vascular smooth muscle cells by T lymphocytes: a role for CD40 signaling in plaque rupture? *Circ Res* 1997;81:448-454

44. Murphy G, Willenbrock F, Crabbe T, O'Shea M, Ward R, Atkinson S, O'Connell J, Docherty A: Regulation of matrix metalloproteinase activity. *Ann N Y Acad Sci* 1994;732:31-41

45. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS: Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996;98:2572-2579

46. Harker LA, Hanson SR: Platelet factors predisposing to arterial thrombosis. *Baillieres Clin Haematol* 1994;7:499-522

47. Davies MJ, Bland JM, Hangartner JR, Angelini A, Thomas AC: Factors influencing the presence or absence of acute coronary artery thrombi in sudden ischaemic death. *Eur Heart J* 1989;10:203-208

48. Duguid J.K. Thrombosis as a factor in the pathogenesis of coronary atherosclerosis. J.Pathol Bacteriol 58, 207-212. 1946

49. Nicoloso G, Hauert J, Kruithof EKO, Van Melle G, Bachman F: Fibrinolysis in normal subjects: comparison between plasminogen activator inhibitor and other components of the fibrinolytic system. *Thromb Haemost* 1988;59:299-303

50. Sprengers ED, Kluft C: Plasminogen activator inhibitors. *Blood* 1987;69:381-387

51. Wilhelmsen L, Wedel H, Tibblin G: Multivariate analysis of risk factors for coronary heart disease. *Circulation* 1973;48:950-958

52. Peto R. Two properties of multiple regression analysis; and regression to the mean (and regression from the mean). Fletcher CN, Peto R Tinker CM Speizer FE. In The Natural History of chronic Bronchitis and Emphysema. An 8-Year Study of Early chronic Obstructive Lung Disease in Working Men in London. 218-223. 1976. Oxford, Oxford University Press.

53. Shaper AG, Pocock SJ, Walker M, Phillips AN, Whitehead TP, Macfarlane PW: Risk factors for ischaemic heart disease: the prospective phase of the British Regional Heart Study. *J Epidemiol Community Health* 1985;39:197-209

54. Colditz GA, Willett WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH: Menopause and the risk of coronary heart disease in women. *N.Engl.J.Med.* 1987;316:1105-1110

55. Larsson B, Bengtsson C, Bjorntorp P, Lapidus L, Sjostrom L, Svardsudd K, Tibblin G, Wedel H, Welin L, Wilhelmsen L: Is abdominal body fat distribution a major explanation for the sex difference in the incidence of myocardial infarction? The study of men born in 1913 and the study of women, Goteborg, Sweden [see comments]. *Am J Epidemiol* 1992;135:266-273

56. Marmot MG, Syme SL, Kagan A, Kato H, Cohen JB, Belsky J: Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: prevalence of coronary and hypertensive heart disease and associated risk factors. *Am J Epidemiol* 1975;102:514-525

57. McKeigue PM, Miller GJ, Marmot MG: Coronary heart disease in south Asians overseas: a review. *J Clin Epidemiol* 1989;42:597-609

58. Woods KL, Samanta A, Burden AC: Diabetes mellitus as a risk factor for acute myocardial infarction in Asians and Europeans. *Br Heart J* 1989;62:118-122

59. McKeigue PM, Shah B, Marmot MG: Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians [see comments]. *Lancet* 1991;337:382-386

60. Panahloo A, Mohamed-Ali V, Lane A, Green F, Humphries SE, Yudkin JS: Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. *Diabetes* 1995;44:37-42

61. Slack J, Evans KA. The increased risk of death from ischaemic heart disease in first-degree relatives of 121 men and 96 women with ischaemic heart disease. Journal of Medical Genetics 3, 239. 1966 62. Khaw KT, Barrett-Connor E: Family history of stroke as an independent predictor of ischemic heart disease in men and stroke in women. *Am J Epidemiol* 1986;123:59-66

63. Pekkanen J, Nissinen A, Puska P, Punsar S, Karvonen MJ: Risk factors and 25 year risk of coronary heart disease in a male population with a high incidence of the disease: the Finnish cohorts of the seven countries study. *BMJ* 1989;299:81-85

64. Conroy RM, Mulcahy R, Hickey N, Daly L: Is a family history of coronary heart disease an independent coronary risk factor? *Br Heart J* 1985;53:378-381

65. Hammond EC, Horn D. Smoking and death rates - Report on 44 months of folow-up of 187783 men. 11 Death rates by cause. 166, 1294-1308. 1958.

66. Holbrook JH, Grundy SM, Hennekens CH, Kannel WB, Strong JP: Cigarette smoking and cardiovascular diseases. A statement for health professionals by a task force appointed by the steering committee of the American Heart Association. *Circulation* 1984;70:1114-1117

67. Collins R, Peto R, MacMahon S, Hebert P, Fiebach NH, Eberlein KA, Godwin J, Qizilbash N, Taylor JO, Hennekens CH: Blood pressure, stroke, and coronary heart disease. Part 2, Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context [see comments]. *Lancet* 1990;335:827-838

68. Rose G. The strategy of preventive medicine. 1-138. 1992. Oxford, Oxford University Press.

69. SHEP Cooperative Research Group: Prevention of stroke by antihypertensive drug treatment in older persons with isolated systolic hypertension: final results of the Systolic Hypertension in the Elderly Program. *JAMA* 1991;265:3255-3264

70. Davies M, Rayman G, Day J: Increased incidence of coronary disease in people with impaired glucose tolerance: link with increased lipoprotein(a) concentrations? *BMJ* 1992;304:1610-1611

71. Pan WH, Cedres LB, Liu K, Dyer A, Schoenberger JA, Shekelle RB, Stamler R, Smith D, Collette P, Stamler J: Relationship of clinical diabetes and asymptomatic hyperglycemia to risk of coronary heart disease mortality in men and women. *Am J Epidemiol* 1986;123:504-516

72. Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988;37:1595-1607

73. Juhan-Vague I, Alessi MC, Vague P: Increased plasma plasminogen activator inhibitor-1 levels. A possible link between insulin resistance and atherothrombosis. *Diabetologia* 1991;34:457-462

74. Meade TW, Mellows S, Brozovic M, Miller GJ, Chakrabarti RR, North WR, Haines AP, Stirling Y, Imeson JD, Thompson SG: Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. *Lancet* 1986;2:533-537

75. Heinrich J, Balleisen L, Schulte H, Assmann G, van de Loo J: Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. *Arter Thromb* 1994;14:54-59

76. Meade TW, Chakrabarti R, Haines AP, North WR, Stirling Y: Characteristics affecting fibrinolytic activity and plasma fibrinogen concentrations. *British Medical Journal* 1979;1:153-156

77. Anonymous: ECAT angina pectoris study: baseline associations of haemostatic factors with extent of coronary arteriosclerosis and other coronary risk factors in 3000 patients with angina pectoris undergoing coronary angiography. *Eur Heart J* 1993;14:8-17

78. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC: Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group [see comments]. *N Engl J Med* 1995;332:635-641

79. Broadhurst P, Kelleher C, Hughes L, Imeson JD, Raftery EB: Fibrinogen, factor VII clotting activity and coronary artery disease severity. *Atherosclerosis* 1990;85:169-173

80. Berg K: Confounding results of Lp(a) lipoprotein measurements with some test kits. *Clin Genet* 1994;46:57-62

81. Elwood PC, Renaud S, Sharp DS, Beswick AD, O'Brien JR, Yarnell JW: Ischemic heart disease and platelet aggregation. The Caerphilly Collaborative Heart Disease Study. *Circulation* 1991;83:38-44

82. Chen Z, Peto R, Collins R, MacMahon S, Lu J, Li W: Serum cholesterol concentration and coronary heart disease in population with low cholesterol concentrations [see comments]. *BMJ* 1991;303:276-282

83. Martin MJ, Hulley SB, Browner WS, Kuller LH, Wentworth D: Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361 662 men. *Lancet* 1986;2:933-936

84. Rose G, Shipley M: Plasma cholesterol concentration and death from coronary heart disease: 10 year results of the Whitehall study. *Br Med J (Clin Res Ed)* 1986;293:306-307

85. AnonymousRandomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S) [see comments]. *Lancet* 1994;344:1383-1389

86. AnonymousWest of Scotland Coronary Prevention Study: identification of high-risk groups and comparison with other cardiovascular intervention trials. *Lancet* 1996;348:1339-1342

87. Shaper AG, Pocock SJ, Walker M, Phillips AN, Whitehead TP, Macfarlane PW: Risk factors for ischaemic heart disease: the prospective phase of the British Regional Heart Study. *J Epidemiol Community Health* 1985;39:197-209

88. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR Jr, Bangdiwala S, Tyroler HA: High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation.* 1989;79:8-15

89. Carlson LA, Bottiger LE: Ischaemic heart-disease in relation to fasting values of plasma triglycerides and cholesterol. Stockholm prospective study. *Lancet* 1972;1:865-868

90. Heyden S, Heiss G, Hames CG, Bartel AG: Fasting triglycerides as predictors of total and CHD mortality in Evans County, Georgia. *J Chronic Dis* 1980;33:275-282

91. Castelli WP: The triglyceride issue: a view from Framingham. *Am Heart J* 1986;112:432-437

92. Patel P, Mendall MA, Carrington D, Strachan DP, Leatham E, Molineaux N, Levy J, Blakeston C, Seymour CA, Camm AJ, et al: Association of *Helicobacter pylori* and *Chlamydia pneumoniae* infections with coronary heart disease and cardiovascular risk factors. *BMJ* 1995;311:711-714

93. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, Luc G, Bard JM, Bara L, Ricard S, Tiret L, Amouyel P, Alhenc-Gelas F, Soubrier F: Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992;359:641-644

94. Rissanen AM. Familial aggregation of coronary heart disease in a high incidence area (North Karelia, Finalnd). British Hearth Journal 42, 294. 1979.

95. Nora JJ, Lortscher RM Spangler RD Kimberling WJ. Genetic epidemiology study of early onset ischaemic heart disease. Circulation 61, 503. 1980.

96. Berg K. Twin studies of coronary heart disease and its risk factors. Acta Genet Med Gemell 33, 349. 1984.

97. Weissman SM: Molecular genetic techniques for mapping the human genome. *Mol Biol Med* 1987;4:133-143

98. Spielman RS, McGinnis RE, Ewens RJ: Transition test for linkage disequilibrium: the insulin gene region and insulin dependent diabetes mellitus. *Am J Hum Genet* 1993;52:506-516

99. Horvath S, Laird NM: A discordant-sibship test for disequilibrium and linkage: no need for parental data. *Am J Hum Genet* 1998;63:1886-1897

100. Erdos EG, Boggs LE: Hydrolysis of paraoxon in mammalian blood. *Nature* 1961;190:716-717

101. Ehlers MRW, Riordan JF: Angiotensin-converting enzyme: New concepts concerning its biological role. *Biochemistry* 1989;28:5311-5316

102. Kerins DM, Hao Q, Vaughan DE: Angiotensin induction of PAI-1 expression in endothelial cells is mediated by the hexapeptide angiotensin IV. *J Clin Invest* 1995;96:2515-2520

103. Vaughan NJ, Vaughan DE: The renin-angiotensin and fibrinolytic systems. Co-Conspirators in the pathogenesis of ischemic cardiovascular disease. *Trends in Cardiovascular Medicine* 1996;6:239-243

104. Ridker PM, Gaboury CL, Conlin PR, Seely EW, Williams GH, Vaughan DE: Stimulation of plasminogen activator inhibitor in vivo by infusion of angiotensin II. Evidence of a potential interaction between the renin-angiotensin system and fibrinolytic function. *Circulation* 1993;87:1969-1973

105. Ossei-Gerning N, Mansfield MW, Stickland MH, Wilson IJ, Grant PJ: Plasminogen activator inhibitor-1 (PAI-1) promoter 4G/5G genotype and levels in relation to a history of myocardial infarction in patients characterised by coronary angiography. *Arterioscler Thromb Vasc Biol* 1997;In press:

106. Alhenc-Gelas F, Richard J, Courbon D, Warnet JM, Corvol P: Distribution of plasma angiotensin I-converting enzyme levels in healthy men: relationship to environmental and hormonal parameters. *J Lab Clin Med* 1991;117:33-39

107. Kumar RS, Thekkumara TJ, Sen GC: The mRNAs encoding the two angiotensin-converting isozymes are transcribed from the same gene by a tissue-specific choice of alternative transcription initiation sites. *J Biol Chem* 1991;266:3854-3862

108. Griendling KK, Murphy TJ, Alexander RW: Molecular biology of the renin-angiotensin system. *Circulation* 1993;87:1816-1828

109. MacFadyen RJ, Lees KR, Reid JL: Tissue and plasma angiotensin converting enzyme and the response to ACE inhibitor drugs. *Br J Clin Pharmacol* 1991;31:1-13

110. Chatterjee K, Opie LH: Angiotensin inhibitors and other vasodilators with special reference to congestive heart failure. *Cardiovasc Drugs Ther* 1987;1:1-8

111. Sakaguchi K, Chai SY, Jackson B, Johnston CI, Mendelsohn FA: Inhibition of tissue angiotensin converting enzyme. Quantitation by autoradiography. *Hypertension* 1988;11:230-238

112. Campbell DJ: Circulating and tissue angiotensin systems. *J Clin Invest* 1987;79:1-6

113. Veltmar A, Gohlke P, Unger T: From tissue angiotensin converting enzyme inhibition to antihypertensive effect. *Am J Hypertens* 1991;4:263-269

114. Kohzuki M, Johnston CI, Chai SY, Jackson B, Perich R, Paxton D, Mendelsohn FA: Measurement of angiotensin converting enzyme induction and inhibition using quantitative in vitro autoradiography: tissue selective induction after chronic lisinopril treatment. *J Hypertens* 1991;9:579-587

115. Gohlke P, Bunning P, Unger T: Distribution and metabolism of angiotensin I and II in the blood vessel wall. *Hypertension* 1992;20:151-157

116. Cohen ML, Kurz KD: Angiotensin converting enzyme inhibition in tissues from spontaneously hypertensive rats after treatment with captopril or MK-421. *J Pharmacol Exp Ther* 1982;220:63-69

117. Urata H, Healy B, Stewart RW, Bumpus FM, Husain A: Angiotensin II-forming pathways in normal and failing human hearts. *Circ Res* 1990;66:883-890

118. Noda K, Sasaguri M, Ideishi M, Ikeda M, Arakawa K: Role of locally formed angiotensin II and bradykinin in the reduction of myocardial infarct size in dogs. *Cardiovasc Res* 1993;27:334-340

119. Okamura T, Okunishi H, Ayajiki K, Toda N: Conversion of angiotensin I to angiotensin II in dog isolated renal artery: role of two different angiotensin II-generating enzymes. *J Cardiovasc Pharmacol* 1990;15:353-359

120. Mento PF, Wilkes BM: Plasma angiotensins and blood pressure during converting enzyme inhibition. *Hypertension* 1987;9:42-48

121. Johnston CI: Angiotensin receptor antagonists: focus on losartan. *Lancet* 1995;346:1403-1407

122. Sassano P, Chatellier G, Billaud E, Alhenc-Gelas F, Corvol P, Menard J: Treatment of mild to moderate hypertension with or without the converting enzyme inhibitor Enalapril. *Am J Med* 1988;83:227-235

123. Cambien F, Alhenc-Gelas F, Herbeth B, Andre JL, Rakotovao R, et al.: Familial resemblance of plasma angiotensin-converting enzyme level: the Nancy Study. *Am J Hum Genet* 1988;43:774-780

124. Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Tregear G, et al.: Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Nat Acad Sci USA* 1988;85:9386-9390

125. Soubrier F, Hubert C, Testut P, Nadaud S, Alhenc-Gelas F, Corvol P: Molecular biology of the angiotensin I converting enzyme: I. Biochemistry and structure of the gene. *J Hypertens* 1993;11:471-476

126. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in the angiotensin I- converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990;86:1343-1346

127. Costerousse O, Allegrini J, Lopez M, Alhenc-Gelas F: Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J* 1993;290:33-40

128. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F: Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet* 1992;51:197-205

129. Evans AE, Poirier O, Kee F, Lecerf L, McCrum E, Falconer T, Crane J, O'Rourke DF, Cambien F: Polymorphisms of the angiotensin-converting-enzyme gene in subjects who die from coronary heart disease. *QJM* 1994;87:211-214

130. Tiret L, Kee F, Poirier O, Nicaud V, Lecerf L, Evans A, Cambou JP, Arveiler D, Luc G, Amouyel P, et al: Deletion polymorphism in angiotensin-converting enzyme gene associated with parental history of myocardial infarction. *Lancet* 1993;341:991-992

131. Mattu RK, Needham EWA, Galton DJ, Frangos E, Clark AJL, Caulfield M: A DNA variant at the angiotensin-converting enzyme gene locus associates with coronary heart disease in the Caerphilly Heart Study. *Circulation* 1995;91:270-274

132. Bohn M, Berge KE, Bakken A, Erikssen J, Berg K: Insertion/deletion (I/D) polymorphism at the locus for angiotensin I-converting enzyme and myocardial infarction. *Clin Genet* 1993;44:292-297

133. Bohn M, Berge KE, Bakken A, Erikssen J, Berg K: Insertion/deletion (I/D) polymorphism at the locus for angiotensin I-converting enzyme and parental history of myocardial infarction. *Clin Genet* 1993;44:298-301

134. Badenhop RF, Wang XL, Wilcken DEL: Angiotensin-converting enzyme genotype in children and coronary events in their grandparents. *Circulation* 1995;91:1655-1658

135. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, Buring J, Hennekens CH: A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischaemic heart disease. *N Engl J Med* 1995;332:706-711

136. Cambien F. ACE polymorphism as a risk factor for myocardial infarction. British Journal of Cardioloy Supplement1, S4-S6. 1995.

137. Ludwig E, Corneli PS, Anderson JL, Marshall HW, Lalouel J-M: Angiotensin converting enzyme gene polymorphism is associated with myocardial infarction but not with development of coronary stenosis. *Circulation* 1995;2120-2124

138. Gardemann A, Weiβ T, Schwartz O, Eberbach A, Katz N, Hehrlein FW, Tillmanns H, Waas W, Haberbosch W: Gene polymorphism but not catalytic activity of angiotensin I-converting enzyme is associated with coronary artery disease and myocardial infarction in low-risk patients. *Circulation* 1995;92:2796-2799

139. Nakai K, Itoh C, Miura Y, Hotta K, Musha T, Itoh T, Miyakawa T, Iwasaki R, Hiramori K: Deletion polymorphism of the angiotensin I-converting enzyme gene is associated with serum ACE concentration and increased risk for CAD in the Japanese. *Circulation* 1994;90:2199-2202

140. Arbustini E, Grasso M, Fasani R, Klersy C, Diegoli M, Porcu E, Banchieri N, Fortina P, Danesino C, Specchia G: Angiotensin converting enzyme gene deletion allele is independently and strongly associated with coronary atherosclerosis and myocardial infarction. *Br Heart J* 1995;74:584-591

141. Ohishi M, Fujii K, Minamino T, Higaki J, Kamitani A, Rakugi H, et al: A potent genetic risk factor for restenosis. *Nat Genet* 1993;5:324-325

142. Samani NJ, Martin DS, Brack M, Cullen J, Chauhan A, Lodwick D, et al: Insertion/deletion polymorphism in the angiotensin-converting enzyme gene and risk of restenosis after angioplasty. *Lancet* 1995;345:1013-1016

143. Schachter F, Faure-Delanef L, Guenot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D: Genetic associations with human longevity at the APOE and ACE loci. *Nat Genet* 1994;6:29-32

144. Berge KE, Berg K: No effect of insertion/deletion polymorphism at the ACE locus on normal blood pressure level or variability. *Clin Genet* 1994;45:169-174

145. Bonnardeaux A, Davies E, Jeunemaitre X, Fery I, Charru A, Clauser E, Tiret L, Cambien F, Corvol P, Soubrier F: Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension* 1994;24:63-69

146. Bonithon-Kopp C, Ducimetiere P, Touboul PJ, Feve JM, Billaud E, Courbon D, Heraud V: Plasma angiotensin-converting enzyme activity and carotid wall thickening. *Circulation* 1994;89:952-954

147. Talmud PJ, Watts GF, McBride S, Mandalia S, Brunt JNH, Coltart DJ, Lewis B, Humphries SE: Angiotensin converting enzyme gene polymorphism and the course of angiographically defined coronary artery disease. *Atherosclerosis* 1995;114:133-135

148. Pinto YM, van Gilst WH, Kingma JH, Schunkert H: Deletion-type allele of the angiotensin-converting enzyme gene is associated with progressive ventricular dilation after anterior myocardial infarction. *J Am Coll Cardiol* 1995;25:1622-1626

149. Raynolds MV, Bristow MR, Bush EW, Abraham WT, Lowes BD, Zisman LS, Taft CS, Perryman MB: Angiotensin-converting enzyme DD genotype in patients with ischaemic or idiopathic dilated cardiomyopathy. *Lancet* 1993;342:1073-1075

150. Montgomery HE, Keeling PJ, Goldman JH, Humphries SE, Talmud PJ, McKenna WJ: Lack of association between the insertion/deletion polymorphism of the angiotensin-converting enzyme gene and idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 1995;25:1627-1631

151. Castellano M, Muiesan ML, Rizzoni D, Beschi M, Pasini G, Cinelli A, Salvetti M, Porteri E, Bettoni G, Kreutz R, Lindpaintner K, Rosei EA: Angiotensin-converting enzyme I/D polymorphism and arterial wall thickness in a general population. The Vobarno Study. *Circulation* 1995;91:2721-2724

152. Marian AJ, Yu QT, Workman R, Greve G, Roberts R: Angiotensin-converting enzyme polymorphism in hypertrophic cardiomyopathy and sudden cardiac death. *Lancet* 1993;342:1085-1086

153. Schunkert H, Hense HW, Holmer SR, Stender M, Perz S, Keil U, Lorell BH, Riegger GA: Association between a deletion polymorphism of the angiotensin- converting-enzyme gene and left ventricular hypertrophy. *N Engl J Med* 1994;330:1634-1638

154. Iwai N, Ohmichi N, Nakamura Y, Kinoshita M: DD genotype of the angiotensin-converting enzyme gene is a risk factor for left ventricular hypertrophy. *Circulation* 1994;90:2622-2628

155. Celermajer DS, Sorensen KE, Barley J, Jeffery S, Carter N, Deanfield J: Angiotensin-converting enzyme genotype is not associated with endothelial dysfunction in subjects without other coronary risk factors. *Atherosclerosis* 1994;111:121-126

156. Kupari M, Perola M, Koskinen P, Virolainen J, Karhunen PJ: Left ventricular size, mass, and function in relation to angiotensin-converting enzyme gene polymorphism in humans. *Am J Physiol* 1994;267:H1107-1111

157. Dessì-Fulgheri P, Catalini R, Sarzani R, Sturbini S, Siragusa N, Guazzarotti F, Offidani M, Tamburrini P, Zingaretti O, Rappelli A: Angiotensin converting enzyme gene polymorphism and carotid atherosclerosis in a low- risk population. *J Hypertens* 1995;13:1593-1596

158. Catto AJ, Carter AM, Barrett J, Stickland MH, Bamford JM, Davies JA, Grant PJ: Angiotensin converting enzyme insertion/deletion polymorphism and cerebrovascular disease. *Stroke* 1996;27:435-440

159. Harden PN, Geddes C, Rowe PA, McIlroy JH, Boulton-Jones M, Rodger RSC, Junor BJR, Briggs JD, Connell JMC, Jardine AG: Polymorphisms in angiotensin-converting-enzyme gene and progression of IgA nephropathy. *Lancet* 1995;345:1540-1542

160. Keavney B, McKenzie C: Coronary atherosclerosis and the angiotensinogen gene. *Circulation* 1995;92:2356-2357

161. Zingone A, Dominijanni A, Mele E, Marasco O, Melina F, Minchella P, Quaresima B, Tiano MT, Gnasso A, Pujia A, et al: Deletion polymorphism in the gene for angiotensin converting enzyme is associated with elevated fasting blood glucose levels. *Hum Genet* 1994;94:207-209

162. Panahloo A, Andrès C, Mohamed-Ali V, Gould MM, Talmud PJ, Humphries SE, Yudkin JS: The insertion allele of the ACE gene I/D polymorphism. A candidate gene for insulin resistance? *Circulation* 1995;92:3390-3393

163. Ruiz J, Blanche H, Cohen N, Velho G, Cambien F, Cohen D, Passa P, Froguel P: Insertion/deletion polymorphism of the angiotensin-converting enzyme gene is strongly associated with coronary heart disease in non-insulin-dependent diabetes mellitus. *Proc Nat Acad Sci USA* 1994;91:3662-3665

164. Fujisawa T, Ikegami H, Yamato E, Takekawa K, Nakagawa Y, Hamada Y, Ueda H, Rakugi H, Higaki J, Ohishi M, Fuji K, Fukuda M, Ogihara T: Angiotensin 1-converting enzyme gene polymorphism is associated with myocardial infarction, but not with retinopathy or nephropathy, in NIDDM. *Diabetes Care* 1995;18:983-985

165. Kruithof E: Plasminogen activators a review. *Enzyme* 1988;40:113-121

166. Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR: Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. *Nature* 1990;346:74-76

167. Carmeliet P, Collen D: Role of the plasminogen/plasmin system in thrombosis, haemostasis, restenosis and atherosclerosis. Evaluation in transgenic animals. *Trends in Cardiovascular Medicine* 1995;5:117-122

168. Fay WP, Shapiro AD, Shih JL, Schleef RR, Ginsburg D: Brief report: complete deficiency of plasminogen-activator inhibitor type 1 due to a frame-shift mutation. *N Engl J Med* 1992;327:1729-1733

169. Juhan-Vague I, Aillaud MF, De Cock FP-JC, Arnaud C, Serradimigni A, Collen D: The fast-acting inhibitor of tissue-type plasminogen activator is an acute phase reactant, in Davidson JF, Donati MB, Coccheri S (eds): *Progress in fibrinolysis*. Edinburgh, Churchill Livingstone, 1985, pp 146-149

170. Hamsten A, Blomback M, Wiman B, Svensson J, Szamosi A, De Faire U, Mettinger L: Haemostatic function in myocardial infarction. *Br Heart J* 1986;55:58-66

171. Brakman P, Mohler ERJ, Astrup T: A group of patients with impaired plasma fibrinolytic system and selective inhibition of tissue activator-induced fibrinolysis. *Scand J Haematol* 1966;3:389-398

172. Tuddenham EGD, DN Cooper: *The molecular genetics of haemostasis and its inherited disorders*. Oxford, Oxford University Press, 1994,

173. Loskutoff DJ: Carboxypeptidases: new regulators of plasminogen activation in vivo? *J Clin Invest* 1995;96:2104-2105

174. Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, Loskutoff DJ: Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proc Nat Acad Sci USA* 1992;89:6998-7002

175. Loskutoff DJ: Regulation of PAI-1 gene expression. *Fibrinolysis* 1991;5:197-206

176. Juhan-Vague I, Thompson SG, Jespersen J: Involvement of the hemostatic system in the insulin resistance syndrome. A study of 1500 patients with angina pectoris. The ECAT Angina Pectoris Study Group. *Arter Thromb* 1993;13:1865-1873

177. Juhan-Vague I, Alessi MC, Joly P, Thirion X, Vague P, Declerck PJ, Serradimigni A, Collen D: Plasma plasminogen activator inhibitor-1 in angina pectoris. Influence of plasma insulin and acute-phase response. *Arteriosclerosis* 1988;9:362-367

178. Potter van Loon, Kluft C, Radder JK, Blankenstein MA, Meinders AE: The cardiovascular risk factor plasminogen activator inhibitor type 1 is related to insulin resistance. *Metabolism* 1993;42:945-949

179. Grant PJ: The effect of high- and medium-dose metformin therapy on cardiovascular risk factors in patients with type II diabetes. *Diabetes Care* 1996;19:64-66

180. Andersen P: Hypercoagulability and reduced fibrinolysis in hyperlipidemia: relationship to the metabolic cardiovascular syndrome. *J Cardiovasc Pharmacol* 1992;20:Suppl 8:S29-31

181. Mussoni L, Mannucci L, Sirtori M, Camera M, Maderna P, Sironi L, Tremoli E: Hypertriglyceridemia and regulation of fibrinolytic activity. *Arter Thromb* 1992;12:19-27

182. Stiko-Rahm A, Wiman B, Hamsten A, Nilsson J: Secretion of plasminogen activator inhibitor-1 from cultured human umbilical vein endothelial cells is induced by very low density lipoprotein. *Arteriosclerosis* 1990;10:1067-1073

183. Klinger KW, Winqvist R, Riccio A, Andreasen PA, Sartorio R, Nielsen LS, Stuart N, Stanislovitis P, Watkins P, Douglas R, et a: Plasminogen activator inhibitor type 1 gene is located at region q21.3-q22 of chromosome 7 and genetically linked with cystic fibrosis. *Proc Natl Acad Sci U S A* 1987;84:8548-8552

184. Follo M, Ginsburg D: Structure and expression of the human gene encoding plasminogen activator inhibitor, PAI-1. *Gene* 1989;84:447-453

185. Bosma PJ, van den Berg EA, Kooistra T, Siemieniak DR, Slightom JL: Human plasminogen activator inhibitor-1 gene. Promoter and structural gene nucleotide sequences. *J Biol Chem* 1988;263:9129-9141

186. Dawson S, Henney A: The status of PAI-1 as a risk factor for arterial and thrombotic disease: a review. *Atherosclerosis* 1992;95:105-117

187. Dawson S, Hamsten A, Wiman B, Henney A, Humphries S: Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arter Thromb* 1991;11:183-190

188. Humphries SE, Lane A, Dawson S, Green FR: The study of gene-environment interactions that influence thrombosis and fibrinolysis. Genetic variation at the loci for factor VII and plasminogen activator inhibitor-1. *Arch Pathol Lab Med* 1992;116:1322-1329

189. Dawson SJ, Wiman B, Hamsten A, Green F, Humphries S, Henney AM: The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in Hep G2 cells. *J Biol Chem* 1993;268:10739-10745

190. Eriksson P, Kallin B, Van'T Hooft FM, Bavenholm P, Hamsten A: Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Nat Acad Sci USA* 1995;92:1851-1855

191. Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, Loskutoff DJ: Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proc Nat Acad Sci USA* 1992;89:6998-7002

192. Sakata K, Kurata C, Taguchi T, Suzuki S, Kobayashi A, Yamazaki N, Rydzewski A, Takada Y, Takada A: Clinical significance of plasminogen activator inhibitor activity in patients with exercise-induced ischemia. *Am Heart J* 1990;120:831-838

193. Andreotti F, Davies GJ, Hackett DR, Khan MI, De Bart ACW, Aber VR, Maseri A, Kluft C: Major circadian fluctuations in fibrinolytic factors and possible relevance to time of onset of myocardial infarction, sudden cardiac death and stroke. *Am J Cardiol* 1988;62:635-637

194. Meade TW, Ruddock V, Stirling Y, Chakrabarti R, Miller GJ: Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. *Lancet* 1993;342:1076-1079

195. Hamsten A, Walldius G, De Faire U, Dahlen G, Szamosi A, Landou C, Blomback M, Wiman B: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. *Lancet* 1987;2:3-9

196. Reilly CF, Fujita T, Mayer EJ, Siegfried ME: Both circulating and clot-bound plasminogen activator inhibitor-1 inhibit endogenous fibrinolysis in the rat. *Arterioscler Thromb* 1991;11:1276-1286

197. Vaughan DE, Declerck PJ, Van Houtte E, De Mol M, Collen D: Reactivated recombinant plasminogen activator inhibitor-1 (rPAI-1) effectively prevents thrombolysis in vivo. *Thromb Haemost* 1992;68:60-63

198. Levi M, Biemond BJ, van Zonneveld AJ, ten Cate JW, Pannekoek H: Inhibition of plasminogen activator inhibitor-1 activity results in promotion of endogenous thrombolysis and inhibition of thrombus extension in models of experimental thrombosis [see comments]. *Circulation* 1992;85:305-312

199. Coller BS: Platelets and thrombolytic therapy [see comments]. *N Engl J Med* 1990;322:33-42

200. Biemond BJ, Levi M, Coronel R, Janse MJ, ten Cate JW, Pannekoek H: Thrombolysis and reocclusion in experimental jugular vein and coronary artery thrombosis. Effects of a plasminogen activator inhibitor type 1-neutralizing monoclonal antibody. *Circulation* 1995;91:1175-1181

201. Eitzman DT, Fay WP, Lawrence DA, Francis-Chmura AM, Shore JD, Olson ST, Ginsburg D: Peptide-mediated inactivation of recombinant and platelet plasminogen activator inhibitor-1 in vitro. *J Clin Invest* 1995;95:2416-2420

202. Gray RP, Yudkin JS, Patterson DL: Enzymatic evidence of impaired reperfusion in diabetic patients after thrombolytic therapy for acute myocardial infarction: a role for plasminogen activator inhibitor? *Br Heart J* 1993;70:530-536

203. Barbash GI, Hod H, Roth A, Miller HI, Rath S, Zahav YH, Modan M, Zivelin A, Laniado S, Seligsohn U: Correlation of baseline plasminogen activator inhibitor activity with patency of the infarct artery after thrombolytic therapy in acute myocardial infarction. *Am J Cardiol* 1989;64:1231-1235

204. Mansfield MW, Stickland MH, Grant PJ: Plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and coronary artery disease in Non-Insulin-Dependent Diabetes. *Thromb Haemost* 1995;74:1032-1034

205. Ye S, Green FR, Scarabin PY, Nicaud V, Bara L, Dawson SJ, Humphries SE, Evans A, Luc G, Cambou JP, Arveiler D, Henney AM, Cambien F: The 4G/5G genetic polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene is associated with differences in plasma PAI-1 activity but not with risk of myocardial infarction in the ECTIM study. *Thromb Haemost* 1995;74:837-841

206. Ayanian J Z. Differences in the use of procedures between women and men hospitalized for coronary heart disease. Epstein A M. New England Journal of Medicine 325, 221-225. 1991. 1991.

207. Healy B. The Yentl Syndrome. New England Journal of Medicine 325, 274-276. 1991. 1991.

208. Kannel WB, Feinleib M: Natural history of angina pectoris in the Framingham study. Prognosis and survival.

209. Lerner D J. Patterns of coronary heart disease mobidity and mortality in the sexes; a 26 year follow up of the Framingham population. Kannel W B. American Heart Journal 111, 383-390. 1986. 1986.

210. Murabito J M. Prognosis after the onset of coronary heart disease. An investigation of differences in outcome between the sexes according to initial coronary disease presentation. Evans J C. Circulation , 2548-2555. 1993. Larson M G, Levy D.

211. Orencia A. Effect Effect of gender on long term outcome of angina pectoris and myocardial infarction/sudden unexpected death. Bailey K. JAMA 269, 2392-2397. 11993. Yawn B P, Kottke T E.

212. Bemiller C R. Long term observations in patients with angina and normal coronary arteriograms. Pepine C. Circulation 47, 36-43. 1973. Rogers A K.

213. Kemp HG, Kronmal RA, Vlietstra RE, Frye RL: Seven year survival of patients with normal or near normal coronary arteriograms. A CASS registry study. *J Am Coll Cardiol* 1986;7:479-483

214. Kannel WB, McGee DL: Diabetes and cardiovascular risk factors: the Framingham study. *Circulation* 1979;59:8-13

215. Mansfield MW, Heywood DM, Grant PJ: Sex differences in coagulation and fibrinolysis in white subjects with non-insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* 1996;16:160-164

216. Kannel WB, Wolf PA, Castelli WP, D'Agostino RP: Fibrinogen and risk of cardiovascular disease. *Journal of the American Medical Association* 1987;258:1183-1186

217. Yarnell JW, Baker IA, Sweetnam PM, Bainton D, O'Brien JR, Whitehead PJ, et al.: Fibrinogen, viscosity, and white cell count are major risk factors for ischaemic heart disease. The Caerphilly and Speedwell collaborative heart disease studies. *Circulation* 1991;83:863-844

218. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC: Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group [see comments]. *N Engl J Med* 1995;332:635-641

219. Gustincich S, Manfioletti G, Del Sal G, Scheinder C: A fast method for high-quality genomic DNA extraction from whole human blood. *BioTechniques* 1991;11:298-301

220. Rigat B, Hubert C, Corvol P, Soubrier F, et al: PCR detection of the insertion/deletion polymorphism of the human ACE gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res* 1992;20:1433

221. Shanmugam V, Sell KW, Saha BK: Mistyping ACE heterozygotes. *PCR Methods Appl* 1993;3:120-121

222. Caracciolo EA, Davis KB, Sopko G, Kaiser GC, Corley SD, Schaff H, Taylor HA, Chaitman BR: Comparison of surgical and medical group survival in patients with left main coronary artery disease. Long-term CASS experience. *Circulation* 1995;91:2325-2334

223. Detre KM, Wright E, Murphy ML, Takaro T: Observer agreement in evaluating coronary angiograms. *Circulation* 1975;52:979-986

224. Zir LM, Miller SW, Dinsmore RE, Gilbert JP, Harthorne JW: Interobserver variability in coronary angiography. *Circulation* 1976;53:627-632

225. Sanmarco ME, Brooks SH, Blankenhorn DH: Reproducibility of a consensus panel in the interpretation of coronary angiograms.

226. Fisher LD, Judkins MP, Lesperance J, Cameron A, Swaye P, Ryan T, Maynard C, Bourassa M, Kennedy JW, Gosselin A, Kemp H, Faxon D, Wexler L, Davis KB: Reproducibility of coronary arteriographic reading in the coronary artery surgery study (CASS). *Cathet Cardiovasc Diagn* 1982;8:565-575

227. Meier B, Gruentzig AR, Goebel N, Pyle R, von Gosslar W, Schlumpf M: Assessment of stenoses in coronary angioplasty. Inter- and intraobserver variability. *Int J Cardiol* 1983;3:159-169

228. Scoblionko DP, Brown BG, Mitten S, Caldwell JH, Kennedy JW, Bolson EL, Dodge HT: A new digital electronic caliper for measurement of coronary arterial stenosis: comparison with visual estimates and computer-assisted measurements. *Am J Cardiol* 1984;53:689-693

229. Galbraith JE, Murphy ML, de Soyza N: Coronary angiogram interpretation. Interobserver variability. *JAMA* 1978;240:2053-2056

230. Arnett EN, Isner JM, Redwood DR, Kent KM, Baker WP, Ackerstein H, Roberts WC: Coronary artery narrowing in coronary heart disease: comparison of cineangiographic and necropsy findings. *Ann Intern Med* 1979;91:350-356

231. Sanmarco ME, Brooks SH, Blankenhorn DH: Reproducibility of a consensus panel in the interpretation of coronary angiograms. *Am Heart J* 1978;96:430-437

232. Beauman GJ, Vogel RA: Accuracy of individual and panel visual interpretations of coronary arteriograms: implications for clinical decisions [see comments]. *J Am Coll Cardiol* 1990;16:108-113

233. Hutter AMJ: Is there a left main equivalent? *Circulation* 1980;62:207-211

234. Califf RM, Phillips HR, Hindman MC, Mark DB, Lee KL, Behar VS, Johnson RA, Pryor DB, Rosati RA, Wagner GS: Prognostic value of a coronary artery jeopardy score. *J Am Coll Cardiol* 1985;5:1055-1063

235. Dash H, Johnson RA, Dinsmore RE, Harthorne JW: Cardiomyopathic syndrome due to coronary artery disease. I: Relation to angiographic extent of coronary disease and to remote myocardial infarction. *Br Heart J* 1977;39:733-739

236. Johnson RA, Zir LM, Harper RW, Leinbach RC, Hutter AMJ, Pohost GM, Block PC, Gold HK: Patterns of haemodynamic alteration during left ventricular ischaemia in man. Relation to angiographic extent of coronary artery disease. *Br Heart J* 1979;41:441-451

237. Lee JT, Ideker RE, Reimer KA: Myocardial infarct size and location in relation to the coronary vascular bed at risk in man. *Circulation* 1981;64:526-534

238. Falk E, Shah PK, Fuster V: Coronary plaque disruption. *Circulation* 1995;92:657-671

239. Libby P: Molecular bases of the acute coronary syndromes. *Circulation* 1995;91:2844-2850

240. Little WC, Constantinescu M, Applegate RJ, Kutcher MA, Burrows MT, Kahl FR, Santamore WP: Can coronary angiography predict the site of a subsequent myocardial infarction in patients with mild-to-moderate coronary artery disease? *Circulation* 1988;78:1157-1166

241. Brown BG, Gallery CA, Badger RS, Kennedy JW, Mathey D, Bolson EL, Dodge HT: Incomplete lysis of thrombus in the moderate underlying atherosclerotic lesion during intracoronary infusion of streptokinase for acute myocardial infarction: quantitative angiographic observations. *Circulation* 1986;73:653-661

242. Nakagomi A, Celermajer DS, Lumley T, Freedman SB: Angiographic severity of coronary narrowing is a surrogate marker for the extent of coronary atherosclerosis. *Am J Cardiol* 1996;78:516-519

243. Toussaint JF, LaMuraglia GM, Southern JF, Fuster V, Kantor HL: Magnetic resonance images lipid, fibrous, calcified, hemorrhagic, and thrombotic components of human atherosclerosis in vivo. *Circulation* 1996;94:932-938

244. Kemp H G. Seven year survival of patients with normal or near normal coronary arteriograms; a CASS registry study. Kronmal R A. Journal of The American College of Cardiology 7, 479-483. 1996. Vliestra R E, Frye R L.

245. Ross AM: Interpretation of coronary angiograms [editorial; comment]. *J Am Coll Cardiol* 1990;16:114-114

246. Boller A, Lesperance J, Revel D, Marchand JL, Amiel M: [Automatic quantitative analysis of vascular stenosis. Experimental study of a digital angiography system applied to cardiology]. *Arch Mal Coeur Vaiss* 1989;82:381-390

247. Stadius ML, Alderman EL: Coronary artery revascularization. Critical need for, and consequences of, objective angiographic assessment of lesion severity [editorial]. *Circulation* 1990;82:2231-2234

248. Kennedy JW, Killip T, Fisher LD, Alderman EL, Gillespie MJ, Mock MB: The clinical spectrum of coronary artery disease and its surgical and medical management, 1974-1979. The Coronary Artery Surgery study. *Circulation* 1982;66 Suppl3):16-23

249. Lee AJ, Smith WC, Lowe GD, Tunstall-Pedoe H: Plasma fibrinogen and coronary risk factors: the Scottish Heart Health Study. *J Clin Epidemiol* 1990;43:913-919

250. Eliasson M, Evrin P-E, Lundblad D, Asplund K, Ranby M: Influence of gender, age and sampling time on plasma fibrinolytic variables. A population study. *Fibrinolysis* 1993;7:316-323

251. Gebara OC, Mittleman MA, Sutherland P, Lipinska I, Matheney T, Xu P, Welty FK, Wilson PW, Levy D, Muller JE, et al: Association between increased estrogen status and increased fibrinolytic potential in the Framingham Offspring Study. *Circulation* 1995;91:1952-1958

252. Ranby M. Activity of plasminogen activator inhibitor-1 (PAI-1) in a population in Northern Sweden. Sundell I B. 4 (supplement 2), 54-55. 1990. Fibrinolysis. Johnson O, Lendin M C, and Dahlen G.

253. Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, Wu KK: Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb Haemost* 1993;70:380-385

254. Balleisen L, Bailey J, Epping P-H, Schulte H, van de Loo JC: Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: I. Baseline data on the relation to age, gender, body weight, smoking, alcohol, pill-using, and menopause. *Thromb Haemost* 1985;54:475-479

255. Haverkate F, Thompson SG, Duckert F: Haemostasis factors in angina pectoris; relation to gender, age and acute-phase reaction. *Thromb Haemost* 1995;73:561-567

256. Lindpaintner K, Lee M, Larson MG, Rao VS, Pfeffer MA, Ordovas JM, Schaefer EJ, Wilson AF, Wilson PWF, Vasan RS, Myers RH, Levy D: Absence of association or genetic linkage between the angiotensin-converting-enzyme gene and left ventricular mass. *N Engl J Med* 1996;334:1023-1028

257. Lindpaintner K, Pfeffer MA: Molecular genetics crying wolf? The case of the angiotensin-converting enzyme gene and cardiovascular disease. *J Am Coll Cardiol* 1995;25:1632-1633

258. Swales JD: ACE gene: the plot thickens. *Lancet* 1993;342:1065-1066

259. Lee EJ: Population genetics of the angiotensin-converting enzyme in Chinese. *Br J Clin Pharmacol* 1994;37:212-214

260. Bloem LJ, Manatunga AK, Pratt JH: Racial difference in the relationship of an angiotensin I-converting enzyme gene polymorphism to serum angiotensin I-converting enzyme activity. *Hypertension* 1996;27:62-66

261. Samani NJ, Thompson JR, O'Toole L, Channer K, Woods KL: A meta-analysis of the association of the deletion allele of the angiotensin-converting enzyme gene with myocardial infarction. *Circulation* 1996;94:708-712

262. Bonithon-Kopp C, Ducimetiere P, Touboul PJ, Feve JM, Billaud E, Courbon D, Heraud V: Plasma angiotensin-converting enzyme activity and carotid wall thickening. *Circulation* 1994;89:952-954

263. Testut P, Soubrier F, Corvol P, Hubert C: Functional analysis of the human somatic angiotensin I- converting enzyme gene promoter. *Biochem J* 1993;293:843-848

264. Kruithof E: Plasminogen activators a review. *Enzyme* 1988;40:113-121

265. Aronson DC, Ruys T, van Bockel JH, et al: A prospective study of risk factors in young adults with arterial occlusive disease. *Eur J Vasc Surg* 1989;3:227-232

266. Smith EB: Fibrinogen, fibrin and the arterial wall. *Eur Heart J* 1995;16:11-15

267. de Wood MA, Spores J, Notske R, et al: Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N Engl J Med* 1980;303:897-902

268. Hamsten A, Wiman B, De Faire U, Blombäck M: Increased plasma levels of rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. *N Engl J Med* 1985;313:1558-1563

269. Mansfield MW, Stickland MH, Grant PJ: Environmental and genetic factors in relation to elevated circulating levels of plasminogen activator inhibitor-1 in caucasian patients with non-insulin-dependent diabetes mellitus. *Thromb Haemost* 1995;74:842-847

270. McCormack LJ, Nagi DK, Stickland MH, Mansfield MW, Mohamed-Ali V, Yudkin JS, Knowler WC, Grant PJ: Promoter (4G/5G) plasminogen activator inhibitor-1 genotype in Pima Indians: relationship to plasminogen activator inhibitor-1 levels and features of the insulin resistance syndrome. *Diabetologia* 1996;39:1512-1518

271. Kohler HP, Stickland MH, Ossei-Gerning N, Carter A, Mikkola H, Grant PJ: Association of a common polymorphism in the factor XIII gene with myocardial infarction. *Thromb Haemost* 1998;79:8-13

272. Okamura A. Pharmacogenetic analysis of the effect of angiotensin-converting enzyme inhibitor on restenosis after percutaneous transluminal coronary angioplasty. Ohishi M. Angiology 50(10), 811-822. 1999. United States. Rakugi H.

15.0 PUBLICATIONS

Sex differences in coagulation and fibrinolysis in subjects with coronary artery disease. Ossei-Gerning N, Wilson IJ, Grant PJ.
Thromb Haemost 1998 Apr 79:4 736-40

Helicobacter pylori infection is related to atheroma in patients undergoing coronary angiography. Ossei-Gerning N, Moayyedi P, Smith S, Braunholtz D, Wilson JI, Axon AT, Grant PJ
Cardiovasc Res 1997 Jul 35:1 120-4

Angiotensin-converting enzyme (ACE) gene polymorphisms in patients characterised by coronary angiography. Foy CA, Rice GI, Ossei-Gerning N, Mansfield MW, Grant PJ
Hum Genet 1997 Sep 100:3-4 420-5

Plasminogen activator inhibitor-1 promoter 4G/5G genotype and plasma levels in relation to a history of myocardial infarction in patients characterized by coronary angiography. Ossei-Gerning N, Mansfield MW, Stickland MH, Wilson IJ, Grant PJ
Arterioscler Thromb Vasc Biol 1997 Jan 17:1 33-7

16.0 PRESENTATIONS AT MEETINGS AND ABSTRACTS

ORAL PRESENTATIONS& ABSTRACTS

Ossei-Gerning N, Mansfield MW, Stickland MH, Wilson JI, Grant PJ.
Plasminogen activator inhibitor-1 genotype and levels in relation to myocardial infarction and extent of coronary atheroma.
Fibrinolysis 1996; 10 Suppl 3:3.
XIIIth International Congress on Fibrinolysis and Thrombolysis, Barcelona, Spain, 24th-28th June 1996.

Ossei-Gerning N, Mansfield M, Stickland M, Wilson JI, Grant PJ.
Plasminogen activator inhibitor-1 promoter 4G/5G promoter polymorphism and plasma levels in relation to myocardial infarction in patients undergoing coronary angiography.
European Heart Journal 1996.
European Society of Cardiology, Birmingham August 25-29 1996.

Ossei-Gerning N, Moayyedi P, Wilson JI, Grant PJ.
Helicobacter pylori in relation to coronary artery disease.
European Heart Journal 1996.
European Society of Cardiology, Birmingham August 25-29 1996.
(This presentation was selected for a **press release** at the conference)

Ossei-Gerning N., Foy C., Wilson J.I., Grant P.J. Angiotensin converting enzyme in relation to the extent of coronary artery disease. British Cardiac Society, Harrogate 23-25 May 1995. **Judges Choice 1.** *Br. Heart. J.* 1995; **73:** 22(supplement)

Ossei-Gerning N., Foy C., Wilson J.I., Grant P.J. Angiotensin converting enzyme in relation to the extent of coronary artery disease. **(Oral & Poster)**. Israel, 11-16 June 1995. *Journal of Int. Soc. Thr. & Haem.*1995; **73**: 868

Ossei-Gerning N., Foy C., Wilson J.I., Grant P.J. Angiotensin converting enzyme gene polymorphism in relation to the extent of coronary artery disease. The European Society for Clinical Investigation and the Medical Research Society, Cambridge 2-5 April 1995 **Plenary session** *Eur J Cli Investigation & M.R.S.* 1995; Communications: M7

Ossei-Gerning N., Stickland M., Wilson J.I., Grant P.J. Plasminogen activator inhibitor-1 polymorphism and levels in relation to the extent of coronary artery disease. British Society for Haemostasis and Thrombosis, London 8 September 1995. *Blood Coag. Fibrinolysis.*1995; **6**: 600

Foy C.A., **Ossei-Gerning N.**, Barrett J.H., Grant P.J. Detection of a common polymorphism in the angiotensin converting enzyme gene promoter. British Society for Haemostasis and Thrombosis London 8 September 1995. *Blood Coag. Fibrinolysis* 1995;**6**:590

Ossei-Gerning N., Mansfield M., Stickland M., Wilson J.I.,Grant P.J. Plasminogen activator inhibitor-1 promoter 4G/5G promoter polymorphism and plasma levels in relation to Myocardial Infarction in patients undergoing coronary angiography. Northern Cardiology Workshop Presentation Prize. Grange, October 13-15 1995.

Ossei-Gerning N., Mansfield M., Stickland M., Wilson J.I.,Grant P.J. Plasminogen activator inhibitor-1 promoter 4G/5G promoter polymorphism and levels in relation to Myocardial Infarction in patients undergoing coronary angiography. Northern Junior Cardiac Club, Sheffield, December 6 1996.

Ossei-Gerning N. , Moayedi P. , Wilson J,I. , Grant P. J. Helicobacter pylori in relation to coronary artery disease. Northern Cardiology Conference, Shrigley Hall 12-13 April 1996.

Ossei-Gerning N, Moayyedi P, Wilson JI, **Grant PJ.**
Helicobacter pylori in relation to coronary artery disease.
Gut 1996 (supplement): W2.
British Society of Gastroenterology, Brighton, 20th March 1996.

Ossei-Gerning N, Moayyedi P, Wilson JI, Grant PJ.
Helicobacter pylori in relation to coronary artery disease.
Japanese Research Society for Helicobacter Pylori Related Gastroduodenal Diseases, Tokyo, 16th April 1996.

Ossei-Gerning N, Moayyedi P , Wilson JI, Grant PJ.
Helicobacter pylori in relation to coronary artery disease.
Postgraduate Day, Leeds General Infirmary, 23rd April 1996.

POSTER PRESENTATIONS

Ossei-Gerning N., Foy C., Wilson J.I., Grant P.J. Angiotensin converting enzyme gene polymorphism and plasma levels in relation to the extent of coronary artery disease. European Cardiac Society, Amsterdam, 20-24 August 1995. *Eur. Heart. J.* 1995; **16:** 472(supplement)

Ossei-Gerning N, Wilson IJ, **Grant PJ**.
Sex differences in coagulation and fibrinolysis in subjects with coronary artery disease. ***Thrombosis & Haemostasis*** 1997; suppl; p524.
International Society on Thrombosis and Haemostasis, Florence, June 6th-12th 1997.

Ossei-Gerning N, Wilson IJ, **Grant PJ.**
Sex differences in coagulation and fibrinolysis in subjects with coronary artery disease. *European Heart Journal* 1997; 18 (abstr suppl): p333.
XIXth Congress of the European Society of Cardiology, Stockholm, Sweden, August 24th-28th, 1997.

Ossei-Gerning N, Mansfield M, Stickland M, Wilson JI, **Grant PJ.**

Plasminogen activator inhibitor-1 promoter 4G/5G promoter polymorphism and plasma levels in relation to Myocardial Infarction in patients undergoing coronary angiography. *British Heart Journal* 1996. **(Moderated Poster)**
British Cardiac Society, Glasgow 7th-9th May 1996.

Ossei-Gerning N, Mansfield M, Stickland M, Wilson JI, **Grant PJ.**
Plasminogen activator inhibitor-1 promoter 4G/5G promoter polymorphism and plasma levels in relation to Myocardial Infarction in patients undergoing coronary angiography.
Postgraduate Day, Leeds General Infirmary, 23rd April 1996.

Ossei-Gerning N, Foy C, Wilson JI, **Grant PJ**.
Angiotensin converting enzyme gene polymorphism and plasma levels in relation to the extent of coronary artery disease.
Postgraduate Day, Leeds General Infirmary, 23rd April 1996.