FIBRINOLYTIC AND ENDOTHELIAL MARKERS IN CARDIOVASCULAR DISEASE AND DIABETES MELLITUS

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This thesis is dedicated to Alan,
Esme, Joe and Chris.

SUMMARY

Plasminogen activator inhibitor (PAI) activity, tissue plasminogen activator (tPA) antigen and von Willebrand factor (VWF) antigen are endothelial products with roles in fibrinolysis and platelet function respectively. Fibrin D-dimer antigen (D-dimer) is a marker of cross-linked fibrin formation and lysis.

Plasma levels of these four haemostatic variables (as well as the acute phase reactants, fibrinogen and red cell aggregation) were studied with regard to variability, associations with ischaemic heart disease (IHD) and its risk factors, and associations with diabetes mellitus (including microalbuminuria and response to insulin and exercise). Laboratory, biological and total variability were defined and appeared satisfactory for clinical studies.

In a population sample (North Glasgow MONICA study) distributions and independent associations were defined. PAI was related to time of day, serum triglyceride, alcohol intake, diabetes and interleukin-6. tPA was related to PAI, age, male sex, time of day, cigarette smoking, triglyceride and alcohol use. VWF was related to age, smoking and diabetes. D-dimer was related to age, VWF, fibrinogen and red cell aggregation.

In a case control study, survivors of premature myocardial infarction had higher levels of PAI, VWF, D-dimer, fibrinogen and red cell aggregation (which were independent of standard risk factors);

and of tPA (which was not independent). In a prospective population study (Caerphilly Heart Study), D-dimer and VWF were independent predictors of IHD events, while PAI and tPA were not. In a prospective study of patients with peripheral arterial disease (Edinburgh Claudication Study), D-dimer was an independent predictor of IHD events and progression of arterial disease; while fibrinogen was an independent predictor of mortality.

Non-insulin-dependent diabetic patients with microalbuminuria (a predictor of IHD) had higher levels of tPA and VWF than diabetic or non-diabetic controls without microalbuminuria. Compared to non-diabetic controls, insulin-dependent diabetic patients had lower increments in tPA in response to insulin induced hypoglycaemia or exercise, suggesting an endothelial defect.

The results of these studies are consistent with potential roles of disturbed fibrinolysis, endothelial and platelet function, and fibrin turnover in the pathogenesis of ischaemic heart disease and of diabetic vascular complications.

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Thanks are also due to Mr. Christopher Rumley for the figures and tables.

Statement

I declare that I am the author of this thesis; that no part of the work reported in this thesis has formed part of any other thesis; and that I personally contributed to the work reported as follows:

Chapter 2 of this thesis reports studies of measurement and biolgoical variability in laboratory tests. These were performed by myself, using data collected in collaborative studies.

Chapters 3-6 of this thesis report studies of PAI, tPA, VWF, D-dimer, fibrinogen, red cell aggregation, haematocrit and white cell count in large epidemiological studies, which by their very nature involved collaboration with teams of epidemiologists and statisticians. My role in these studies was as follows:-

1)Discussions concerning the initial proposals to epidemiologists to measure these haematological factors in these studies, relating them to cardiovascular disease and its standard risk factors;

2) Discussion with the survey teams prior to laboratory analyses concerning standardisation of important pre-analytical variables including subject rest periods; and blood sampling, processing, transport and storage.

3) Performance of the majority of laboratory analyses. A minority of these analyses were performed by technicians in the Haemostasis and Thrombosis Laboratory, under my direct supervision. Von Willebrand factor antigen measurements were performed by Mrs. M Orr. IL-6 analyses were performed by Ms Anne

Crilly in the Department of Medicine. Monitoring of internal quality control, and participation in external quality control. Transcription of laboratory analyses onto computer databases and checking of accuracy prior to transfer of databases to statistician colleagues.

4) Discussion with statisticians during statistical analyses of the data.

5) Writing discussions of the biological significance of the data and its statistical analyses.

Chapters 7-9 of this thesis report studies of PAI, tPA, VWF, D-dimer, fibrinogen and haematocrit in diabetic subjects and non-diabetic controls. These were performed in collaboration with Drs. M. Fisher and A. Collier, Senior Registrars in Diabetic Medicine, Glasgow Royal Infirmary. My roles in these studies were as follows:-

1) Collaboration in study design;

2) Participation in sample collection, processing, transport and storage;

3) Performance of laboratory analyses; quality control; participation in statistical analyses;

4) Writing discussions of the data.

LIST OF PRESENTATIONS AND PUBLICATIONS

Chapter 3

Rumley A, Lowe GDO, Smith WCS, Tunstall-Pedoe HD. An epidemiological study of PAI-1 activity, fibrinogen and white cell count. Fibrinolysis 1990; 4, Supplement 3: 152. (Presented at 10th International Congress on Fibrinolysis, Indianapolis 1990).

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Chapter 6

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Fowkes FGR, Lowe GDO, Housley E, Rattray A, Rumley A, Elton RA, MacGregor IR, Dawes J. Cross-linked fibrin degradation products, progression of peripheral arterial disease, and risk of coronary heart disease. Lancet 1993; 342: 84-86.

Chapter 7

Rumley A, Collier A, Rumley AG, Leach JP, Small M, Lowe GDO. Abnormalities of the endothelium, fibrinolysis and lipid peroxides in type 2 diabetes with and without microalbuminuria. Thrombosis and Haemostasis 1991; 65: 988. (presented at the XII Congress of International Society of Thrombosis and Haemostasis, Amsterdam, 1991).

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Chapter 8

Fisher BM, Quinn JD, Rumley A, Lennie SE, Small M, MacCuish AC, Lowe GDO. Effects of acute insulin-induced hypoglycaemia on haemostasis, fibrinolysis and haemorheology in insulin-dependent diabetic patients and control subjects. Clinical Science 1991; 80: 525-531.

CHAPTER 1

REVIEW OF HÆMOSTASIS, FIBRINOLYSIS AND VON WILLEBRAND FACTOR IN CARDIOVASCULAR DISEASE AND DIABETES MELLITUS

INTRODUCTION

Cardiovascular disease, especially ischaemic heart disease (IHD), is the most important cause of death in Western countries. Ischaemic heart disease is due to the progressive development of atherosclerotic plaques in the coronary arteries during adult life, and to the formation of acute platelet-fibrin thrombi upon ruptured plaques (Davies 1994). These acute thrombi may cause sudden coronary death, myocardial infarction, or unstable angina (chest pain without evidence of infarction). Incorporation of thrombi into the ruptured plaque may cause an increase in chronic stenosis and stable angina (Davies 1994) (Figure 1.1).

The recent epidemic of IHD in Western countries started in the 1950's. Prospective studies such as the Framingham study established that certain "risk factors" predicted IHD and other cardiovascular diseases such as stroke and peripheral arterial disease (Kannel et al 1961). These risk factors include family history of cardiovascular disease, male sex, increasing age, cigarette-smoking, high blood pressure, abnormal blood lipids (high total and low density lipoprotein (LDL) cholesterol); high triglyceride and very low density

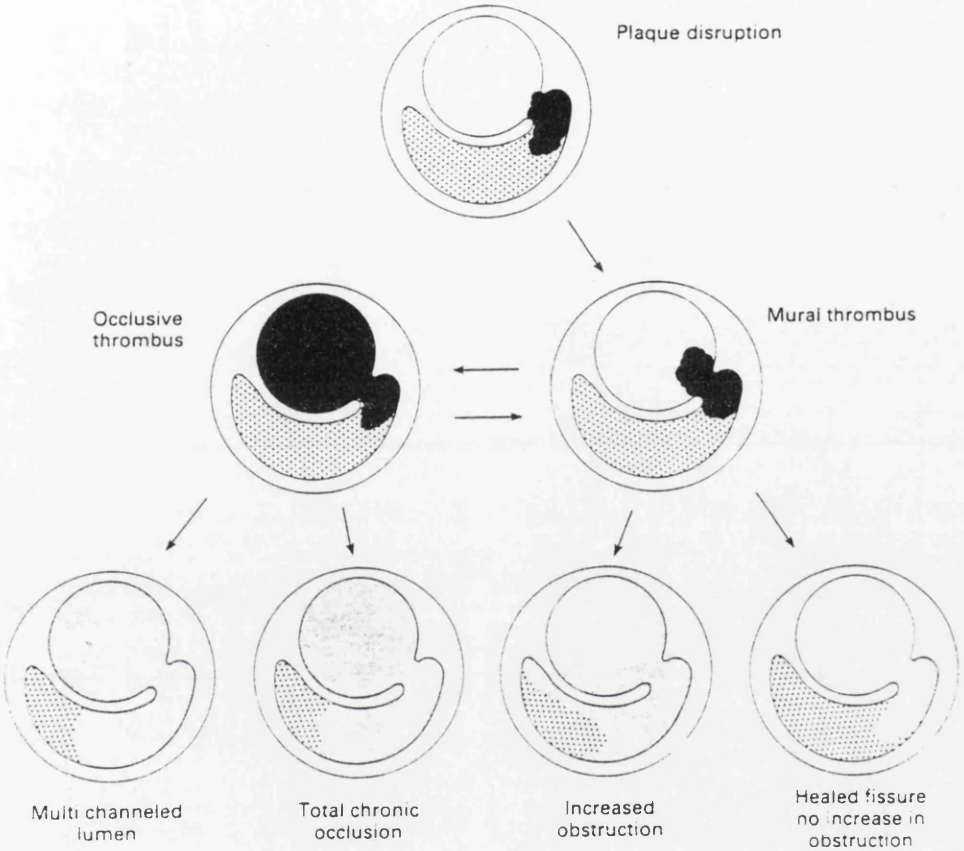

Figure 1.1 Diagramatic representation of thrombus formation (black) upon a ruptured atherosclerotic plaque (stippled). The thrombus may totally occlude the artery causing myocardial infarction; increase obstruction causing angina (unstable or stable); or heal with no increase in obstruction. Thrombus is either lysed by the fibrinolytic system, or replaced by new connective tissue (grey). From Davies (1994).

(VLDL) cholesterol; low high density lipoprotein (HDL) cholesterol; obesity; impaired glucose tolerance and diabetes mellitus. More recently, high blood pressure, abnormal blood lipids, obesity, glucose intolerance and high plasma insulin levels have been grouped and recognised as the "insulin resistance syndrome" (Reaven 1988). Prevention of cardiovascular disease has concentrated on lifestyle advice (reduce smoking and dietary salt and saturated fat; increase exercise and dietary unsaturated fat; avoid obesity), and drug treatment of hypertension, dyslipidaemia and diabetes.

Interest in the contribution of thrombosis to cardiovascular disease is relatively recent (Davies and Thomas 1984; Davies 1994). Angiographic studies (De Wood et al 1980) as well as post mortem studies (Davies 1994) have shown that coronary thrombosis is present in most patients with acute myocardial infarction or unstable angina. The ISIS-2 Trial (1988) showed that both aspirin and thrombolysis with streptokinase reduced mortality in acute myocardial infarction, suggesting that both platelets and fibrin are important components of the acute thrombus (Figure 1.1). Aspirin has also been shown to reduce the risk of myocardial infarction and stroke in persons with chronic arterial disease (Antiplatelet Triallists Collaboration 1994).

In 1980, Meade and colleagues in the Northwick Park Heart Study showed that fibrinogen (the precursor of fibrin) and clotting factors VII and VIII were predictors of fatal IHD (Meade et al 1980), and later of non-fatal IHD (Meade et al 1986). These coagulation factors were as strongly predictive as serum cholesterol. These findings have been confirmed by other groups (Ernst and Resch 1993; Heinrich et al 1994; Lowe et al 1995), and suggest that trials of

fibrinogen lowering drugs (e.g. bezafibrate) and oral anticoagulants which lower factor VII (e.g.warfarin) should be performed. Such studies are in progress (Meade 1995).

Platelets and clotting factors are called "haemostatic variables" because they contribute to the formation of the haemostatic plug which prevents excessive bleeding after injury to blood vessels. Low levels of platelets, of von Willebrand factor (VWF) which is required for platelet adhesion and aggregation, or of clotting factors such as factor VIII (anti-haemophiliac factor) result in bleeding disorders.

Haemostatic plugs require removal by the fibrinolytic system, which lyses fibrin deposits. Impaired fibrinolysis may promote thrombosis (Astrup and Permin 1947). Until recently, only global tests of the fibrinolytic system (such as clot lysis times or fibrin plate lysis area) were available for study of this system (Lowe and Prentice 1980). In the 1980's the identification of tissue plasminogen activator (tPA) and of its major inhibitor, plasminogen activator inhibitor type 1 (PAI-1) allowed the development of specific assays of these major components of the fibrinolytic system (Kluft 1988). Activity assays were facilitated by chromogenic substrates (Lijnen et al 1980).

The development of enzyme-linked immunosorbent assays (ELISA's) in the 1980's allowed the specific assays of not only PAI-1 and tPA, but also of fibrin degradation products resulting from fibrin formation and lysis in vivo such as fibrin D-dimer (Gaffney and Brasher 1973; Elms et al 1983). In the late 1980's, ELISA's became available for accurate assay of von Willebrand factor (VWF) which like tPA and PAI-1 is released from endothelial cells and may be a marker

of endothelial disturbance (Pearson 1994). Endothelial disturbance may be an important trigger of both atherogenesis (Ross 1993) and thrombogenesis (Pearson 1994).

The aims of the studies reported in this thesis were to relate these recently available assays of PAI activity, tPA antigen, fibrin D-dimer antigen, and von Willebrand factor antigen to ischaemic heart disease and to diabetes mellitus. This chapter reviews haemostasis, the fibrinolytic system, degradation of fibrinogen and fibrin to degradation products, von Willebrand factor, and knowledge of the relationship of these variables to cardiovascular disease and diabetes mellitus when these studies were started.

HAEMOSTASIS

Blood has a variety of essential functions including the maintenance of pH, transport of oxygen and carbon dioxide, the delivery of nutrients to cells, and the removal of waste products from them. The circulation of the blood therefore is essential to life, and this circulation occurs around a specialised type of closed system in which the volume is kept fairly constant. Like any plumbing system, there is the possibility of leaks, and these must be repaired to maintain the integrity of the system. The emergency mechanism for stopping blood loss is the formation of a haemostatic plug - haemostasis.

After injury to the endothelial lining of a blood vessel, subendothelial collagen fibres become exposed, and this initiates the

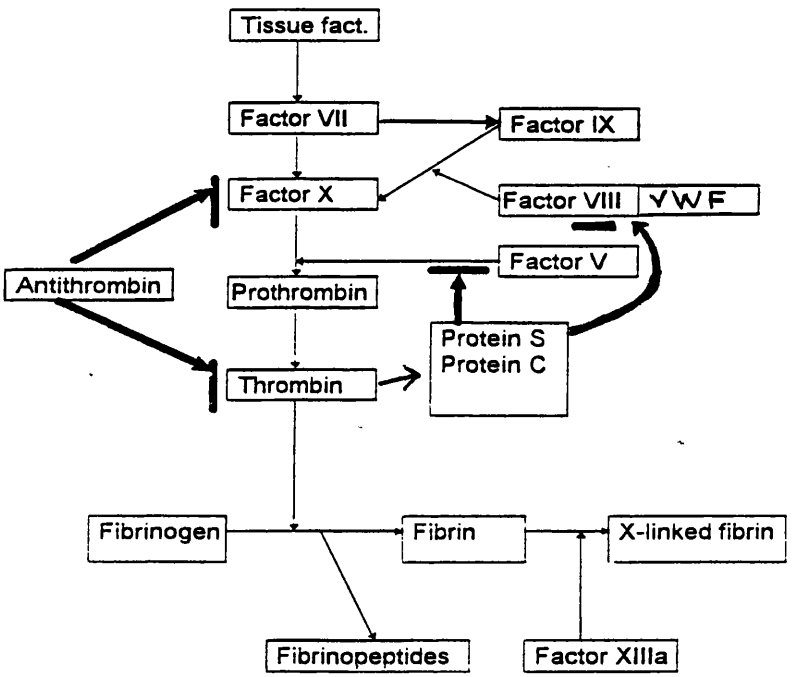

Figure 1.2 Scheme of the coagulation pathway (modified from Eliasson 1995). Tissue fact = tissue factor; VWF = von Willebrand factor. Heavy lines indicate inhibition.

platelet component of haemostasis. Adhesion of platelets is followed by morphological changes (shape change, degranulation) and the release of platelet products. These include thromboxane A_2 and serotonin (which cause vasoconstriction, reducing blood flow, and which promote platelet activation), growth factors (which stimulate tissue repair), platelet factor 4 (a heparin binding protein which prevents heparin-antithrombin complexes from inhibiting clotting factors, and is also chemotactic), factor V (a protein cofactor in the clotting process), von Willebrand factor, and a subunit of factor XIII (the transglutamidase required to cross link fibrin). The most important glycoprotein which facilitates platelet adhesion is von Willebrand factor, which is also the carrier protein for factor VIII (Bloom et al 1994).

Following platelet adhesion, inter-platelet aggregation occurs to form the initial platelet plug. Von Willebrand factor and fibrinogen link platelet membrane receptors (Gp Ib; Gp IIb/IIIa) specific for platelet aggregation, which is stimulated by exposure to collagen, thrombin, adenosine diphosphate (ADP), thromboxane and catecholamines. The platelet plug is subsequently stabilised by fibrin, formed through the coagulation pathways (extrinsic and intrinsic) (Bloom et al 1994)(Figure 1.2)(Eliasson 1995).

When the endothelium is injured, tissue factor (factor III), a membrane bound protein, is exposed. Coagulation factor VII then binds to tissue factor in the presence of Ca^{2+} ions. The resulting tissue factor-VII-Ca^{2+} complex is a catalytically active species (activated VII or VII_a), and has a high affinity for factor X. This complex activates factor X, which is attached to the endothelial or

platelet surfaces. X_a is the endopeptidase which activates prothrombin (factor II) to thrombin. Factor V_a is an essential co-factor for this activation. Factor V (which is present in platelets as well as plasma), binds to the tissue factor VII-Ca^{2+}-Xa complex and is activated by thrombin or X_a to give the activated form of the protein which is able to act as a co-factor for the activation of prothrombin. Thrombin converts soluble fibrinogen into an insoluble fibrin network at the site of injury, which stabilises the initial platelet plug. Thrombin also activates platelets and factors V and VIII; and activates factor XIII which cross-links fibrin. (Figure 1.2). This tissue factor (extrinsic) pathway of blood coagulation is currently thought to be the main pathway for initiation of thrombin and fibrin formation (Nemerson 1992).

The contact activation system which initiates the intrinsic pathway of blood coagulation involves the action of factor XII (Hageman factor), prekallikrein and high molecular weight kininogen (HMWK). The activated form of factor XII which is generated (factor XII_a) then activates factor XI. The precise mechanism for the initiation of contact activation has not yet been established. Factor XI_a in turn activates factor IX, which can also be activated by the factor VII_a-tissue factor complex (Figure 1.2). Factor X is then activated by the enzyme complex of IX_a, thrombin modified factor VIII, negatively charged phospholipid and Ca^{2+}. Factor VIII which acts as a cofactor in this activation must first be cleaved by thrombin or by factor X_a, to form factor $VIII_a$. In the presence of Ca^{2+} ions, negatively charged phospholipid and modified factor VIII, the rate of factor X activation is greatly enhanced. The intrinsic system of coagulation is currently thought to maintain thrombin and fibrin formation (Figure 1.2).

Thrombin can be directly inactivated by a number of coagulation inhibitors, the most important of which is antithrombin (antithrombin III). The activity of this protein is markedly enhanced by heparin. Heparin-antithrombin also inhibits free factor X_a (but not X_a when it is bound to V_a) and also IX_a (Figure 1.2). Other thrombin inhibitors include heparin cofactor II, α_2 macroglobulin and $\alpha_2$antitrypsin.

Protein C, which is activated when thrombin binds to thrombomodulin on the endothelial cell surface, is a vitamin K dependent serine protease which deactivates factor V_a and factor $VIII_a$, thus preventing further thrombin generation. This effect of protein C is dependent on its cofactor, protein S, another vitamin K dependent protein which binds protein C to the platelet surface (Figure 1.2). Just as coagulation deficiencies (haemophilias) are associated with bleeding, congenital deficiencies in antithrombin, protein C and protein S are associated with increased risk of venous thrombosis (thrombophilias). Recently, activated protein C resistance, due to a mutation in the gene for factor V, resulting in resistance to inactivation by activated protein C, has also been associated with increased risk of venous thrombosis (Bertina et al 1995, Dahlback 1995).

FIBRINOLYSIS

There is a balance between the formation of a platelet-fibrin clot (haemostasis), and its dissolution - fibrinolysis. While coagulation is essential to prevent blood loss from injured vessels, the process must be carefully controlled and be reversible. Both haemostatic and

fibrinolytic systems are internally balanced between active and inhibitory elements.

In Figure 1.3 the principal elements of the fibrinolytic system are shown. Essentially, plasmin attacks the insoluble fibrin enzymatically to produce soluble fibrin degradation products (FDP); it can also digest soluble fibrinogen and fibrin. Plasmin is formed from its inactive precursor, plasminogen, when activated by one of its activators, of which two exist in humans. These are tissue plasminogen activator (tPA) and urokinase (or urokinase type plasminogen activator) (uPA). These proteolytic enzymes can be inhibited by specific inhibitors, the plasminogen activator inhibitors PAI-1 and PAI-2 (Sprengers and Kluft 1987). Plasmin is inhibited by antiplasmins, principally by α_2 antiplasmin, but also by α_2 macroglobulin (Figure 1.3).

Serine proteases of the fibrinolytic system

The enzymes of the fibrinolytic system are all serine proteases (Table 1.1). These are so called because they have at their active site an unusually reactive serine residue. X-ray crystallographic studies show that the $-CH_2OH$ group of serine which is usually quite unreactive under physiological conditions is made strongly nucleophilic by the interaction with an aspartate residue and a histidine residue. Although not close to each other in the primary sequence of the protein, molecular folding enables these residues to set up a charge relay network. Studies have indicated that the rate of catalysis is enhanced by a factor of the order 10^3 by this charge relay

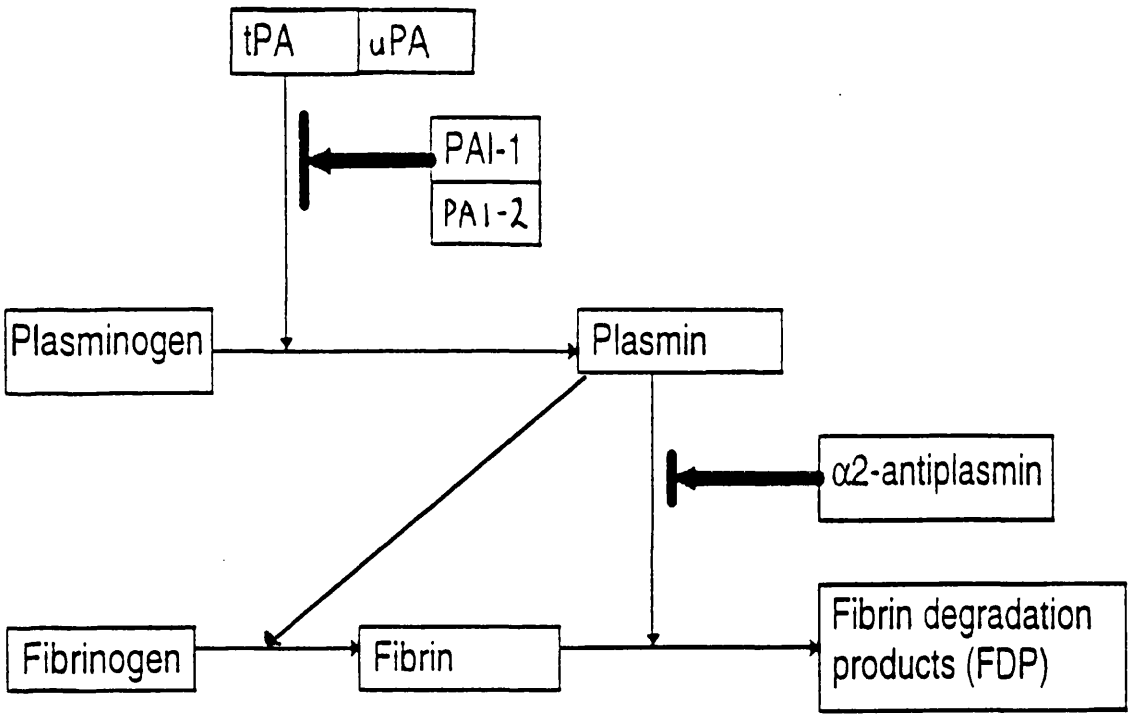

Figure 1.3 Scheme of the fibrinolytic system (modified from Eliasson 1995). tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; PAI = plasminogen activator inhibitors. Heavy lines indicate inhibition

network (Stryer 1988). The enzymes of the fibrinolytic system, plasmin, and the plasminogen activators tPA and uPA all belong to this family of serine proteases, as do thrombin, elastase, trypsin and chymotrypsin. The high degree of homology in these proteins denotes a common ancestor, and mutations of the ancestral gene are thought to have given rise to the present day proteins (Patthy 1985). Another common feature of these proteins is that the active enzyme has two chains held together by disulphide bonds, while the inactive zymogen exists as single chain molecules. All serine proteases have the active site of the enzyme located on the B (light) chain which comes from the C-terminal part of the zymogen. The A (heavy) chain which corresponds to the N-terminal of the inactive protein contains structural domains which are involved in the binding properties of the enzyme. Different exons are responsible for the coding of these domains. Table 1.1 summarises the general features of the three principal fibrinolytic enzymes.

Plasminogen

This 92kDa single chain glycoprotein has a relatively high concentration in the circulation (0.2 mg/ml, 2 M) and has a half life of about 2 days. This inactive species is specifically cleaved at the Arg 560 - Val 561 bond by tPA and uPA to form a two chain molecule bound by disulphide bridges.

While the active site is located on the B chain, the A chain has five homologous regions, known as kringles, which are involved in binding to lysine and its analogues, including tranexamic acid. Kringle 1, nearest the N-terminal end of the molecule, has the highest affinity

Table 1.1

Serine proteases of the fibrinolytic system (after Booth 1991)

Zymogen	Protease	Activation bond	A-chain domains	B-chain catalytic triad		
				His	Asp	Ser
plasminogen	plasmin	Arg560-Val561	5 kringles	602	645	740
scu-Pa	tcu-Pa	Lys158-Ile159	Growth factor 1 kringle	204	255	356
sct-Pa[a]	tct-Pa	Arg275-Ile276	Finger Growth factor 2 kringles	322	371	478

[a] The single chain form of t-Pa has significant activity.

scu-Pa, single chain urokinase; tcu-Pa, two chain urokinase; sct-Pa,single-chain tissue plasminogen activator; tct-Pa, two-chain tissue plasminogen activator.

site (K_d 5 M) for lysine while kringles 2-5 exhibit lower affinity binding (K_d 5mM). The high affinity of plasminogen for lysine is used in the purification of the protein by affinity chromatography. This ability to bind to lysine is responsible for the interaction of both plasminogen and plasmin with fibrin, and thus is immensely important in the regulation of fibrinolysis. These lysine-binding regions are also involved in the binding of the inhibitor $\alpha_2$antiplasmin to plasmin. Proteolysis of plasminogen, with the removal of a small N-terminal peptide, converts the native Glu-plasminogen to Lys-plasminogen. Addition of inhibitors such as aprotinin prevents this proteolysis during the purification of the protein. Activation by tPA is different in these two forms of plasminogen, and it has been shown that it is Lys-plasminogen that is more like fibrin bound plasminogen in its properties (Hoylaerts et al 1982). Some heterogeneity in plasminogen has been found and is attributed to differences in carbohydrate composition (Collen and DeMaeyer 1975).

Lysine binding sites are also involved in the binding of plasminogen to histidine rich glycoprotein. This reduces the plasminogen available for activation by about 50% (1 M) (Booth 1991).

Tissue plasminogen activator (tPA)

This activator of plasminogen was originally purified from various tissues including uterine tissue. It is present in the plasma at low concentrations of about 5-10 ng/ml (Ranby et al 1982b, 1986) and only about 15% of this is active. The rest is complexed to its main inhibitor PAI-1, and to a lesser degree to $\alpha_2$antiplasmin (Figure 1.3). A marked and rapid increase in plasma tPA levels is seen with various

stimuli, such as exercise, venous occlusion and catecholamines (Prowse and MacGregor 1988). tPA is a glycoprotein of about 65 kDa, and in contrast to most other serine proteases is active in the single chain form (Ranby et al 1982a). The two chain form occurs after cleavage of the Arg 275 - Ile 276 bond, and its active site is homologous to other serine proteases with the charge relay network involving His 322, Asp 371 and Ser 478. The A chain of tPA is the part of the molecule involved with substrate binding and a number of different domains on this chain show high homology with other similar proteins. The finger region is homologous to that in fibronectin, the growth factor domain is homologous to epidermal growth factor, and two kringles are homologous to plasminogen. The two domains most involved with substrate binding are the finger region and the second kringle (van Zonneveld et al 1986). In the absence of fibrin, the affinity of tPA for plasminogen is relatively low, with a K_m of 65 M. In the presence of fibrin, however, this affinity is increased with a K_m of 0.15 M (Hoylaerts et al 1982). tPA has both fibrin affinity and fibrin specificity, making it a key molecule in the regulation of fibrinolysis. These properties have also led to its development as a therapeutic agent for lysis of thrombi (e.g. coronary artery thrombi in acute myocardial infarction).

Urokinase (urokinase type plasminogen activator, uPA)

This protein is so named because it was originally purified from urine. It cleaves the same specific bond on plasminogen as tPA, but unlike tPA can act efficiently on its substrate in the absence of fibrin. uPA was thought for many years to have no role in hæmostasis, but more recently it has become evident that it occurs in the circulation at

levels of about 2 ng/ml, predominantly in the single chain form. Studies are continuing on the role of uPA in hæmostasis and thrombosis, but as yet this is not well defined (Bachman 1994), and will not be considered further.

Inhibitors of Fibrinolysis

Inhibitors in the fibrinolytic system are members of the SERPIN (SERine Protease INhibitors) family and like the serine proteases are closely related. The action of these inhibitors is the most important mechanism for regulation and control of fibrinolysis. The enzyme inhibitor forms a stable stoichiometric 1:1 complex with the enzyme, thereby rendering it inactive. Covalent bonds are formed and a fragment is released from the C-terminal end of the inhibitor. This comes about as the enzyme recognises a region on the inhibitor which is similar to the enzyme's natural substrate. The Arg-X bond which is usually recognised at the site when the plasminogen activators cleave their substrate is also present at the reactive centre of the inhibitor molecule, conferring specificity of the inhibitor for its enzyme.

The glycoprotein $\alpha_2$antiplasmin is the major inhibitor of plasmin, and is present in the plasma at a relatively high concentration (1 M). The rate constant for the formation of the plasmin-antiplasmin complex is very high, $2x10^7$/M s (Table 1.2). The high plasma concentration of this inhibitor, coupled with the rapid reaction with the enzyme makes it an important regulator of the fibrinolytic system.

Table 1.2

Inhibitors of fibrinolysis and rate constants (/M s) (after Booth 1991)

	α_2-Antiplasmin	PAI-1	PAI-2	PAI-3
sct-Pa		10^7	$9x10^{12}$	$<10^3$
tct-Pa		$>10^7$	$2x10^5$	$<10^3$
tcu-Pa		$>10^7$	10^6	$8x10^3$
Plasmin	$3x10^7$			10^2
Plasma concentration	70 (μg/ml) (1 μM)	20 ng/ml (0.4 nM)	0[a]	2 μg/ml (40 nM)

[a] Late pregnancy concentration is 250ng/ml (4nm).

Plasminogen Activator Inhibitors

The existence of specific inhibitors of plasminogen activator was disputed for many years. Most assays for plasminogen activators depend on the generation of plasmin, which is detected by its action on fibrin. This made it difficult however, to differentiate between inhibition of plasmin and inhibition of plasminogen activators. The occurrence of a specific inhibitor of the latter in plasma was confirmed using chromogenic assays by Chmielewska et al (1983), by Kruithof et al (1983) and later by other workers. Studies using immunohistochemical techniques then demonstrated the presence of the major plasminogen activator inhibitor (PAI), which is now known as PAI-1, in many tissues and in endothelial cells. PAI-1, the sequence of which has been deduced from its c-DNA structure, is a glycoprotein of 48kDa, and is a potent inhibitor of both tPA and uPA with a high rate constant (10^7/M s) similar to that for plasmin-antiplasmin (Table 1.2). Unlike $\alpha_2$antiplasmin, however plasma levels of the inhibitor are low at about 20 ng/ml (Booth et al 1988, Declerck et al 1988, Kruithof et al 1988). Almost 90% of PAI-1 in the blood is contained in platelets, but these are not the source of plasma PAI-1. Endothelium and the liver have been implicated as sources of PAI-1 (Sprengers and Kluft 1987).

Activity in the two pools of PAI-1 differs widely. While only 3 to 5% of platelet PAI-1 has been shown to be active (Booth et al 1990), almost all plasma PAI-1 is active. Activation of the latent platelet PAI-1 can occur under denaturing conditions , but it is not known whether this activation can occur under any physiological conditions.

A second inhibitor of plasminogen activator has been described (PAI-2) which is related to PAI-1 but quite distinct from it (Kruithof et al 1986). It is thought to be mainly placental in origin as it is found in maternal plasma during pregnancy; however there is also evidence for its presence in monocytes (Golder and Stephens 1983), so it may have a more general role in non-pregnant individuals. The specificity of PAI-1 and PAI-2 is similar, but the affinity of PAI-2 for tPA and uPA is much lower than that of PAI-1 (Table 1.2).

Regulation of Fibrinolysis (Figure 1.4)

The target molecule for the fibrinolytic system is fibrin, which therefore plays an important role in the regulation of the system. Lysine-binding sites on the kringles of plasminogen bind to fibrin, as does its major activator tPA (Figure 1.4). The zymogen and the activator are then in close proximity, and local production of plasmin at the site of fibrin formation is achieved. This activation occurs much more readily in the presence of fibrin than in its absence. The widely differing K_m values for these two situations (65 M in the absence of fibrin, 0.15 M in its presence) mean that plasminogen is not normally activated in the circulation unless fibrin formation has occurred (Hoylaerts et al 1982). Fibrinolysis is further regulated by the effect of $\alpha_2$antiplasmin, the main plasma inhibitor. When plasmin is formed, the avid binding of $\alpha_2$antiplasmin (to form $\alpha_2$antiplasmin-plasmin compexes) means that the half-life of the enzyme is very short, about 100 ms (Wiman and Collen 1978). The plasmin formed on the fibrin surface however is less susceptible to this binding since its active site is already bound to its substrate. Its lysine binding sites are also

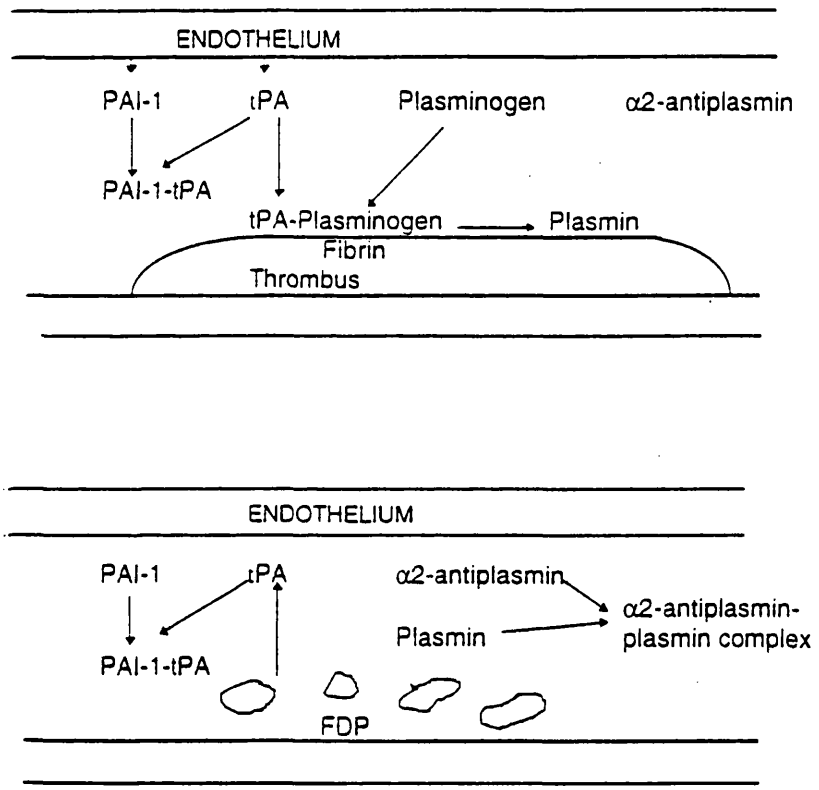

Figure 1.4 Regulation of fibrinolysis (modified from Eliasson 1995). For description see text.

bound to fibrin (Figure 1.4). These regulatory mechanisms mean that plasmin generation is limited to sites of fibrin formation and does not usually occur in the circulation.

Both plasminogen and $\alpha_2$antiplasmin are present in relatively high concentration (Table 1.3) and have half lives in the order of days, so these are the more stable part of the system. In contrast, tPA, uPA and PAI-1 are present in much lower concentration and have very short half lives due to rapid clearance (Table 1.3). Synthesis and release of these proteins must be continuous and fine control must exist to maintain the balance between them. Although plasma levels of tPA and its inhibitor are low (Table 1.3), it is likely that there is significant complex formation, due to the high affinity of the molecules for each other, and most of the tPA in plasma appears to be complexed to PAI-1 (Thorsen and Philips 1984) (Figures 1.4 and 1.5). There may be a higher proportion of free tPA in vivo than in in vitro samples since there is rapid clearance from the blood of both enzyme and inhibitor.

Various stimuli, such as exercise, venous occlusion and administration of vasopressin or its analogue DDAVP result in release of tPA from the endothelium. These responses have been used as a test for fibrinolytic potential in individuals by measuring plasma tPA levels before and after the stimulus. Only a small increase in tPA is required to completely overwhelm the low levels of PAI-1 normally present (Prowse and MacGregor 1988). It has also been shown that some people termed "non-responders" who were thought to have failed to release tPA after such stimuli had high levels of PAI-1 activity (Nilsson et al 1985). This would result in the inhibition of any released tPA. PAI-1 behaves as an acute phase reactant (Juhan-Vague et al

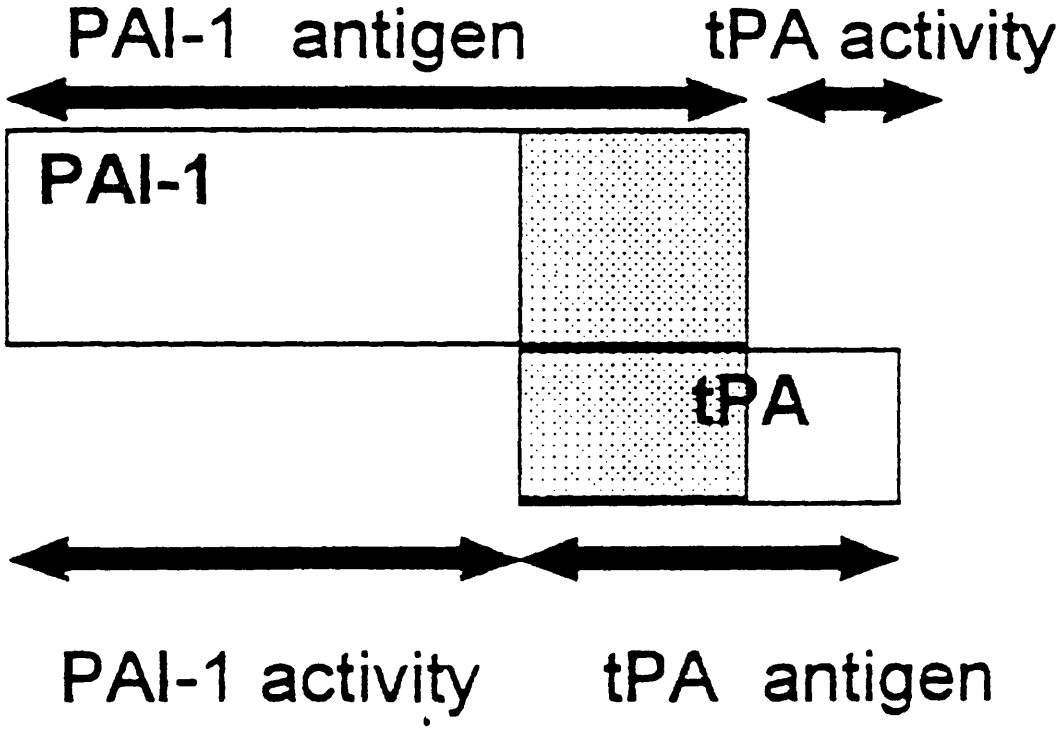

Figure 1.5 Binding of tissue plasminogen activator (tPA) to plasminogen activator inhibitor type 1 (PAI-1). The arrows indicate fractions measured by functional and antigenic assays of PAI and tPA (modified from Eliasson 1995).

Table 1.3

Constituents of the fibrinolytic system in plasma (after Booth 1991)

	Plasma concentration	Half-life in plasma
t-PA	80pM	5 min
scu-PA	40 pM	8 min
PAI-1	400 pM	7 min
Plasminogen	2 μM[a]	2.2 days
α_2-Antiplasmin	1 μM	3.3 days

[a] Effective concentration reduced to 1 μM by binding to histidine- rich glycoprotein.

1985, Kluft et al 1985) and has been found to be raised in a number of pathological conditions (Juhan-Vague et al 1984, 1988).

Laboratory investigations of the fibrinolytic system.

The measurement of fibrinolytic system components in recent years has become more reliable due to various technological developments. Many of the original assays were global measurements and these are still used. These include clot lysis times (whole blood and plasma), and lysis of preformed fibrin by the plasma euglobulin fraction (Lowe and Prentice 1980; Booth 1991). These assays are useful as a background for deciding whether more specific assays may be appropriate. Clots prepared using diluted whole blood are observed to determine the dilute whole blood clot lysis time, but this is very slow, therefore imprecise and impractical. The preparation of the euglobulin fraction of the plasma by acidification precipitates out almost all the tPA and plasminogen present. This also includes the PAI-1 that is complexed to tPA, but not all the free PAI-1 remains in the supernatent as is the case with other inhibitors. The clot formed with the euglobulin fraction will lyse in a few hours in a normal individual, and a shortening of this euglobulin clot lysis time implies increased fibrinolysis.

The addition of the euglobulin fraction to a plate of preformed fibrin made with a standard concentration of fibrin, and measurement of the fibrin plate lysis area is a more reliable estimate of total fibrinolysis.

More specific assays available for the components of the fibrinolytic system are now available, some of which are functional assays and some immunological. The development of chromogenic substrates some 15 years ago (Lijnen et al 1980) has made functional measurement of many of the fibrinolytic enzymes and inhibitors more precise and reliable. These substrates are short peptides of 3 or 4 amino acids which mimic the cleavage site of the enzyme. At the C-terminal end of the peptide, there is a molecule of *p*-nitroanaline. When this is split from the peptide by the action of the enzyme, a yellow colour develops which can be measured spectrophotometrically. These substrates are commercially available and are specific for the various proteases under investigation.

Plasminogen can be assayed by measuring the plasmin produced after its activation using a plasmin specific chromogenic substrate. Similarly, $\alpha_2$antiplasmin can be determined with this substrate by adding an excess of plasmin and measuring the residual enzyme after $\alpha_2$antiplasmin-plasmin complexes have been formed. tPA activity cannot be directly measured chromogenically as no substrate has yet been developed, but assays can use the chromogenic substrate for plasmin to determine tPA activity. Plasminogen added to a test plasma is cleaved by the endogenous tPA, and the resulting plasmin can be measured chromogenically. Fragments of fibrin are used as a stimulator in this method. Since PAI is present in about a five-fold excess compared to tPA in the plasma, after withdrawal of blood any free tPA will rapidly form complexes with PAI. To avoid this in vitro artefact, the sample must be collected into an anticoagulant which is either acidified (Chmieleweska and Wiman 1986) or which contains a specific reagent which will block the active

site of tPA. Because of this sampling requirement, stored citrated plasmas are unsuitable for tPA activity measurement, and such measurements in large epidemiological studies are made more difficult by the necessity for an additional sample.

PAI-1 activity measurement also makes use of this ability of plasmin to split its artificial substrate. An excess of tPA added to a test plasma results in complex formation. The excess tPA in the presence of fibrin fragments then activates plasminogen added to the system. The plasmin generated in this way can be measured when it acts on the chromogenic substrate; this concentration of plasmin is proportional to the remaining tPA and thus to the PAI activity in the test plasma (Chmieleweska and Wiman 1986).

These functional assays have improved the reliability of many of these enzyme assays, but they are not always completely specific. Immunological assays are highly specific and recent developments make such assays quick and easy to perform. Rocket immunoelectrophoresis has been used for a number of proteins which have relatively high concentrations in the plasma. This involves the addition of antiserum to an agarose gel. The test plasma is then applied to the gel, and during electrophoresis the antigen moves through the gel until maximal binding to the antibody occurs. After appropriate staining, the concentration in the test plasma can then be estimated from the height of the peak.

This technique has largely been superseded by the use of Enzyme Linked ImmunoSorbent Assays (ELISA), which are much more sensitive and can be used for proteins with very low

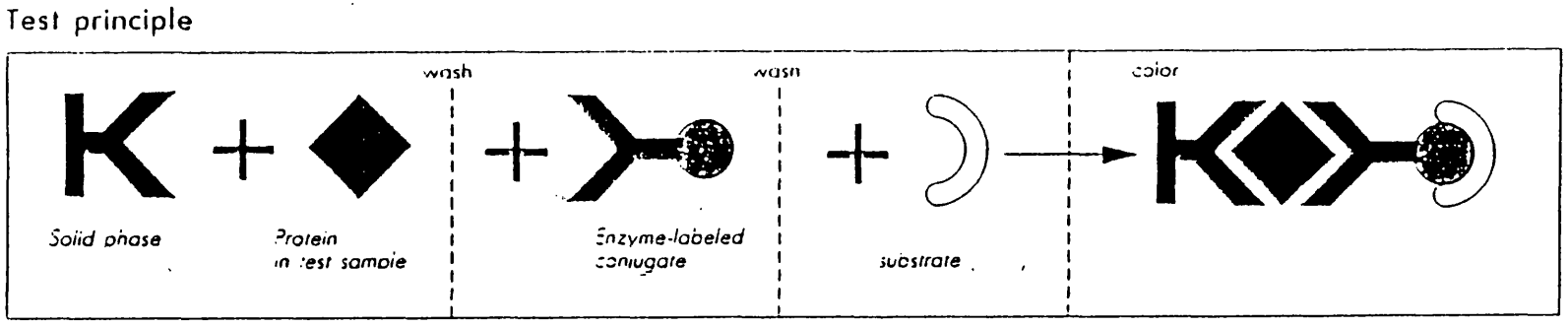

Figure 1.6 Principle of enzyme-linked immunosorbent assays (ELISA). For description see text.

concentrations in the plasma (e.g. tPA, VWF, FDP). They allow a more rapid estimation of the protein levels and a much larger throughput of samples. This is useful, for example, in epidemiological studies where sample numbers are high. The principle of the method is illustrated in figure 1.6. Typically, a monoclonal antibody to the protein under investigation is coated to a solid support. This can be a plastic tube, or more commonly a microtitre plate. The addition of standards and test plasmas to each well allows binding of the protein to the antibody during a suitable incubation period. The plate is then washed and a second antibody to the protein is added. Binding occurs during a second incubation period. This so-called second, or tag antibody is linked to an enzyme, often horseradish peroxidase. After further washing, the addition of substrate to the well results in a coloured product after the cleavage of the substrate by the enzyme. The absorbences in each well can be read using a microtitre plate reader which can be programmed to construct the standard curve and read the unknown values from the curve.

DEGRADATION OF FIBRINOGEN AND FIBRIN

Fibrin formation

Fibrinogen (Doolittle 1981) is a large elongated molecule (length 460Å) with a molecular weight of 340 kDa. It consists of six polypeptide chains and there are three kinds of these: Aα, Bβ, and γ. These chains have many disulphide bonds. Electron microscopy shows that the fibrinogen molecule is made up of 3 nodules connected by 2 rods. Biochemical studies indicate that the central nodule comprises the amino terminal ends of all six polypeptide chains.

Thrombin cleaves arginine-glycine bonds on each of the Aα chains to give a 16 residue peptide, fibrinopeptide A (FpA), and fibrin I or desAA fibrin. The new amino terminal end of the α chain formed by this cleavage is the site where the newly formed fibrin I can bind to a pre-existing site on the fibrinogen. At low concentrations of fibrin, the complexing to fibrinogen allows the fibrin to be kept in solution. This is known as soluble fibrin (Brass et al 1976, Graeff et al 1979). At a certain critical concentration, the fibrin I molecule spontaneously associates in a staggered array to form long fibrin I polymers (Figure 1.7). This is due to the change in the surface charge pattern when a number of negatively charged residues are released by the action of thrombin. The initial clot formed by the spontaneous aggregation of fibrin monomers is quite fragile, and is subsequently stabilised by the formation of cross links between the side chains of different molecules in the fibre. Peptide bonds are formed between specific glutamine and lysine side chains in a transamidation reaction involving Factor XIIIa (Doolittle 1981).

Fibrinopeptide B, a 14 residue peptide is released from the Bß chain simultaneously with FpA, but at a much slower rate. The fibrinogen molecule from which both FpA and FpB have been cleaved is known as fibrin II or desAABB (Figure 1.7). This will also aggregate and become cross linked (Hermans and McDonagh 1982).

Increased coagulation activity is indicated by increased plasma levels of soluble fibrin. If the fibrinolytic system does not adequately compensate for this, a thrombotic event may result.

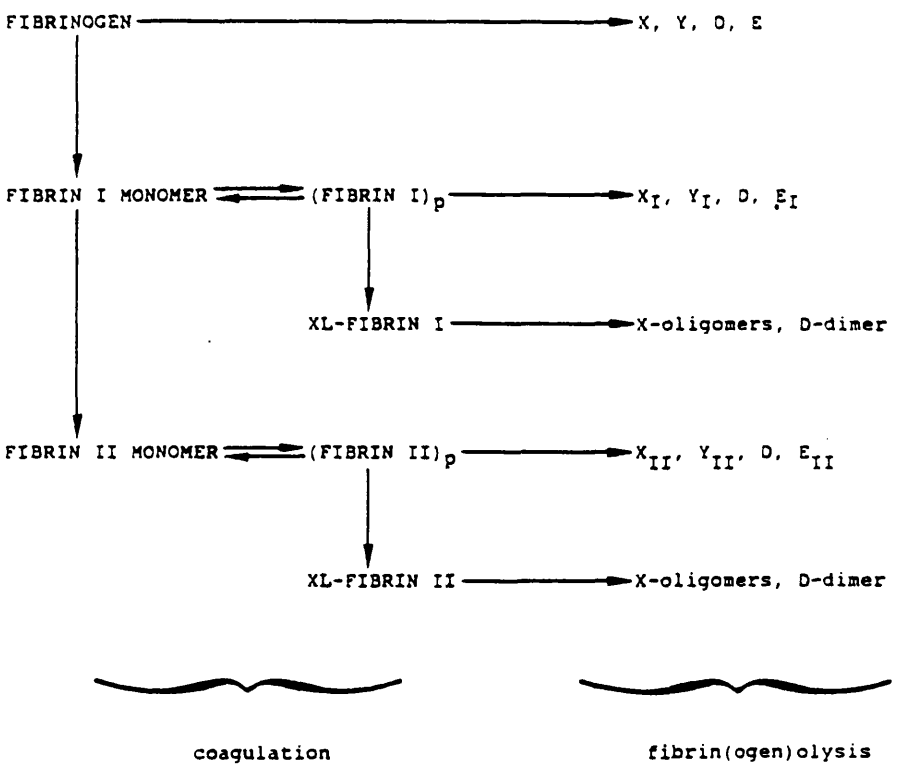

Figure 1.7 Formation of fibrin and degradation of fibrin by plasmin (from Nieuwenhuizen 1991). For description see text. p = polymer.

Molecular forms of fibrinogen

The enzyme plasmin which occurs when fibrinolysis is activated is not specific for fibrin. It will also attack fibrinogen under certain conditions. Three main forms of fibrinogen occur in the circulation (Holm et al 1985; Nieuwenhuizen 1991). These are:

(1) The high molecular weight form (HMW) in which both alpha chains are intact,
(2) The low molecular weight form (LMW) in which one of the alpha chains has been cleaved, and
(3) the lowest molecular weight form (LMW') which has both alpha chains shortened at the carboxy terminal end.

The relative amounts of these forms in normal plasma are 70%, 26%, and 4% respectively. It is possible that even small increases in proteolytic activity may result in a change in the ratio of LMW' to HMW fibrinogen. With further digestion of LMW', a product called fragment X is formed which is a form of fibrinogen with only about 35% of the alpha chains remaining. This is still clottable fibrinogen, but will clot only slowly. Since the amino terminal end of the alpha chains is still at least partly intact the FpA fragment is still present on the molecule (Nieuwenhuizen 1991).

Formation of fibrinogen degradation products

Fragment X is cleaved by plasmin symmetrically into fragments Y and D (see Figure 1.7). Fragment Y is the central nodule connected

to one of the outer nodules, and fragment D consists of the other outer nodule. Finally the remaining bond between fragment D and the central nodule in Y is cleaved. The central nodule is designated fragment E and still contains some FpA.

Conformational changes take place in fragments X, Y and E, exposing regions of the beta chain which are normally buried in the fibrinogen molecule.

Formation of fibrin degradation products

Both Fibrin I and Fibrin II (cross-linked and non-crosslinked) are digested by plasmin via a series of intermediate products, comparable to those formed in the lysis of fibrinogen. Plasmin seems to follow the interdomainal cleavage pattern in fibrin as in fibrinogen. The degradation of non-cross linked fibrin I (des AA fibrin) will sequentially yield fragments X_I, Y_I, D and E_I; and non-cross linked fibrin II (des AABB fibrin), X_{II}, Y_{II}, D and E_{II}, where the subscript I denotes the absence of FpA and the subscript II denotes the absence of both FpA and FpB (Figure 1.7).

Cross linked fibrins I and II are composed of long polymers of fibrin I and II whose α and γ chains are cross linked by isopeptide bonds. These subunits are susceptible to attack by plasmin and this occurs in random order. Soluble fragments of the original polymer with varying molecular weights are the result. These fragments are known as X-oligomers (Graeff and Hafter 1982, Gaffney and Perry 1985, Gaffney et al 1986). Further degradation of these X-oligomers yields fragments E_I (or E_{II}) and fragment D-dimer (Doolittle 1981,

Graeff and Hafter 1982). This D-dimer fragment is formed from two fragments covalently cross linked by peptide bonds on the gamma chain of each fragment (Figure 1.7).

Table 1.4 describes some of the molecular characteristics of fibrinogen and some of its breakdown products.

Measurement of fibrin(ogen) degradation products

It has been realised that the in vivo activity of the fibrinolytic system can be assessed from plasma levels of fibrin degradation products, and that these measurements give information about the turnover of fibrin in an individual. Previous assays were based on serum samples which were a notorious source of artefacts, since they depend on the effective removal of fibrinogen in vitro. This removal was sometimes incomplete. In addition, a serum sample is unsuitable for the measurement of any parameters of coagulation, precluding any measurements of fibrinolytic and coagulation parameters in the same sample. The fibrinolytic system is not absolutely specific for fibrin, and under some conditions may attack fibrinogen. This has only been shown conclusively by the advent of a plasma assay specific for fibrin*ogen* degradation products (Nieuwenhuizen 1991).

Reliable ELISA techniques now exist for the quantitative measurement of a number of fibrin(ogen) degradation products in plasma including D-dimer, X-oligomers and soluble fibrin (Nieuwenhuizen 1991). Latex agglutination assays are also used, where the monoclonal antibody to a specific fragment is bound onto latex particles. This binds to only one type of epitope and agglutination

Table 1.4

Molecular characteristics of fibrinogen and some of its derivatives (from Nieuwenhuizen 1991)

Fibrinogen or Fibrin(ogen) derivative	Molecular features
Intact fibrinogen	FpA present; carboxyl terminal end(s) of Aα-chain(s) intact
Soluble fibrin	FpA absent; new amino terminal ends on α-chains (and possibly β-chains) are neo-epitopes. Also neo-epitopes in Aα-[148-160]. No cross-links present
Cross-linked fibrin	Same as soluble fibrin plus the presence of cross-links
Fibrin*ogen* degradation products (FgDP)	FpA present; no cross-links; neo-epitopes amongst others in Bβ-[58-114]
X-oligomers	FpA absent; high molecular weight; cross-links present
Fibrin degradation products (FbDP)	FpA absent; neo-epitopes in Bβ-[58-114]; cross-links present in cross-linked FbDP
D-dimer	No FpA; cross-linked γ-chains

can only occur if there are multiple epitopes on the fragment under test. Virtually all fibrin(ogen) derivatives have at least two since they are symmetrical or polymeric. This makes them suitable for such agglutination tests, which have the practical advantage of a rapid result, but these assays are only semi-quantitative and inter-operator variability may occur when reading the test.

High levels of fibrin degradation products, including D-dimer, have been found in certain clinical situations, such as disseminated intravascular coagulation (Wilde et al 1989), and pulmonary embolism and deep venous thrombosis (Kroneman et al 1990). D-dimer is also elevated in acute coronary syndromes and during thrombolytic therapy of acute myocardial infarction (Seifreid and Tanswell 1987; DeClerck et al 1987), and raised levels have been reported in some patients with pre-eclampsia (Trofatter et al 1989).

VON WILLEBRAND FACTOR

This factor is deficient in the plasma of patients with the bleeding disorder, von Willebrand's disease. Von Willebrand factor (VWF) was first purified in the mid-seventies and found to be a multimeric protein. It is present in endothelial cells, megakaryocytes, platelets and plasma. The basic subunit has a molecular weight of 22 kDa and dimers are held together by disulphide bridges at the carboxyterminal end. Inter dimer disulphide bridges form multimers. VWF is complexed in the plasma to Factor VIII by non-covalent binding to the light chain of this clotting factor. The multimeric nature

of this protein has been demonstrated by SDS-PAGE (Meyer and Girma 1993).

This protein is synthesised in the endothelial cells and most of it is secreted constitutively into the plasma, although about 5% is stored by the cells in Weibel Palade bodies (Wagner et al 1982). VWF has been demonstrated immunohistologically in subendothelial tissues.

The primary importance of VWF in haemostasis is because of its interactions with platelets, endothelial cells and subendothelial structures. It mediates platelet adhesion to subendothelium when a platelet plug begins to form, binding to the GPIb membrane receptor. VWF also mediates platelet aggregation, as does fibrinogen which also bridges between platelets. In addition, VWF induces expression of the fibrinogen receptor on the platelet surface. These platelet-platelet interactions are essential for the formation of a haemostatic plug, and VWF therefore plays a key role in primary haemostasis. In the coagulation cascade, FVIII is an essential cofactor for the generation of thrombin. VWF acts as a carrier protein for FVIII, protects it from proteolysis, and also delivers it to the point of vascular injury (Meyer and Girma 1993).

The secretion of VWF is stimulated by thrombin (Loesberg et al 1983) and also by fibrin (Ribs et al 1987). The plasma concentration is about 10mg/ml but there is a wide range in normal individuals. There is also a tendency for plasma levels to increase with age.

Experimental animal studies suggest that VWF may contribute to atherogenesis and arterial thrombosis (Badimon et al 1993). Few

epidemiological or case control studies have looked at the association of VWF with cardiovascular disease in man. Since a reliable ELISA technique had recently become easily available, it seemed useful to look at VWF levels in parallel with the fibrinolytic variables, PAI and tPA. In particular, if plasma tPA antigen levels partly reflect endothelial disturbance, it would be useful to compare the associations of the two endothelial release products, VWF and tPA. Both are released from endothelial cells by adrenaline and vasopressin (Prowse and Macgregor 1988) and failure to release tPA has been reported in some cases of von Willebrand's disease (Ludlam et al 1980).

POTENTIAL ROLE OF IMPAIRED FIBRINOLYSIS AND OF ENDOTHELIAL DISTURBANCE IN CARDIOVASCULAR DISEASE AND DIABETES MELLITUS

The major cardiovascular diseases (ischaemic heart disease, ischaemic stroke and peripheral arterial disease) are due to the progressive development of atherosclerotic plaques and subsequent thrombus formation (Davies 1994). Progressive incorporation of fibrin into the vessel wall may play a part in the development of atherosclerosis: the Rokitansky-Duguid hypothesis (Rokitansky 1852; Duguid 1946; Schwartz et al 1988; Thompson and Smith 1989). Fibrin is a consistent component of atherosclerotic plaques (Smith 1986; Bini et al 1989; Thompson and Smith 1989), and it may contribute to plaque growth by stimulation of smooth muscle cell proliferation (Naito et al 1992) and by binding and accumulating with

low density (LDL) lipoprotein; especially lipoprotein (a) (Lp(a)) (Smith et al 1976, 1990).

It is therefore possible that underactive fibrinolysis could lead to decreased removal of arterial fibrin deposits and thus to promotion of atherosclerosis (Astrup and Permin 1947). Experimental testing of this hypothesis has been difficult until recently because only global assays of fibrinolytic activity were available. However, the purification of tPA in the 1980's and the development of specific assays for tPA and for its major inhibitor PAI-1, have allowed more specific testing of the hypothesis that increased levels of PAI activity (usually due to PAI-1) may promote atherosclerosis and thrombosis.

Recent experimental studies have shown that increased PAI-1 levels have prothrombotic effects (Krisnamurti et al 1987; Vaughan et al 1992). Quenching of PAI-1 activity with a specific antibody stimulates endogenous thrombolysis (Levi et al 1992). Transgenic mice with over-expression of the human PAI-1 gene developed peripheral venous thrombosis (Erickson et al 1990).

In man, congenital defficiency of PAI-1 causes a bleeding disorder (Fay et al 1992). Administration of certain anabolic steroids such as stanozolol in man lowers plasma PAI-1 levels and increases tPA activity and systemic plasmin activity as measured by plasma levels of the Bß $_{15-42}$ fragment (Lowe and Small 1988). However, to date clinical trials of anabolic steroids in prevention of thrombosis have been inconclusive, and adverse effects make them unsuitable for long-term prevention of thrombosis (Lowe and Small 1988). On the

other hand, recombinant tPA is a highly effective thrombolytic agent in acute myocardial infarction (Sobel 1988).

Locally, atherosclerotic lesions contain higher concentrations of PAI-1 and its messenger RNA than do normal arterial walls (Schneiderman et al 1992). Furthermore, the high concentration of platelets in arterial thrombi contains a large pool of latent PAI-1; release and activation of this pool may locally inhibit thrombolysis (Booth 1991).

As well as impaired fibrinolytic activity, there is increasing evidence for endothelial disturbance in cardiovascular disease. Endothelial disturbance may be one common mechanism through which several cardiovascular risk factors (e.g. smoking, infections) may promote atherogenesis. It may be followed by arterial wall cell proliferation in response to endothelial injury: proliferating macrophages and smooth muscle cells accumulate cholesterol, resulting in typical atherosclerotic lesions (Ross 1993).

Endothelial cells have several prothrombotic and anti-thrombotic mechanisms, the balance of which may change during both haemostasis and thrombosis (Pearson 1994).

Release of both PAI-1 and tPA from endothelial cells may reflect endothelial disturbance, and alterations in the balance of PAI and tPA activities may promote thrombogenesis. Release of VWF from endothelial cells may also promote thrombogenesis, by increasing platelet adhesion and aggregation; and by localising factor VIII at the sites of vessel injury (Badimon et al 1993).

The increased risk of cardiovascular disease in patients with diabetes mellitus may in part be due to impaired fibrinolysis and endothelial disturbance (Osterman and van de Loo 1986; Grant and Medcalf 1990).

The literature on the association of fibrinolytic variables and VWF with cardiovascular disease, which was available when the studies reported in this thesis were started, is now reviewed. Case-control studies of prevalent disease are considered first; followed by prospective studies of incident cardiovascular disease in non-diabetics and diabetics.

CASE CONTROL STUDIES OF FIBRINOLYSIS AND VWF IN CARDIOVASCULAR DISEASE AND DIABETES MELLITUS

Deep vein thrombosis

Almost all of the early case control studies have shown patients with deep vein thrombosis to have depressed fibrinolytic activity (Juhan-Vague et al 1988; Lowe and Small 1988). Lower levels of tPA activity observed after venous occlusion in some patients are now known to be the result not usually of defective tPA release, but of high PAI activity (Nilsson et al 1985). This causes the inactivation of tPA giving lower activity, but when measured by an antigenic method which measures both free and bound tPA these levels were found to be normal or increased (Bergsdorf et al 1983; Nilsson 1989).

Ischaemic heart disease

The relationship between ischaemic heart disease and fibrinolysis is summarised in Table 1.5. Most studies have observed raised levels of tPA antigen and PAI activity, and decreased levels of tPA activity. In patients who had restenosis after percutaneous transluminal coronary angioplasty, there were higher levels of tPA antigen and lower tPA release after venous occlusion when compared to an age matched group without restenosis (Kirschstein et al 1989). PAI activity was higher in the restenosis group in this study but the difference was not statistically significant. In a study comparing patients whose arteries occluded with those who had patent arteries after thrombolytic therapy with recombinant tPA, pretreatment levels of PAI activity and tPA antigen were significantly higher in the group who occluded (Barbash et al 1989). When compared to healthy controls, the patients whose arteries remained patent had significantly higher levels of tPA antigen but not PAI activity.

There are fewer studies which have looked at VWF in patients with ischaemic heart disease (Table 1.5). Higher levels of VWF were found in patients with angina pectoris who had myocardial infarcts than in such patients who had no history of myocardial infarction (Schmitz-Huebner et al 1988). One study showed a higher plasma VWF level in women who survived a myocardial infarct when compared to controls (Hamsten et al 1985), but the male groups had similar levels of VWF. Other reported associations of VWF with cardiovascular disease are an association with clinical severity of angina pectoris (Moisseev 1988), and ischaemic cerebrovascular

Table 1.5 Case control studies on VWF, tPA, PAI and ischæmic heart disease

Author	n	VWF	tPA	PAI	tPAac
Hamsten et al 1985	71	↑ women	↑	↑	↓
Nilsson & Johnson 1987	124		=	↑	↓
Verheugt et al 1987 (AMI)	18			↑	↓
Juhan Vague et al 1989 (AMI)	67			↑	
Barbash et al 1989 (AMI)	125		↑	=	
Paramo et al 1985	118		↑	↑	
Aznar et al 1988	92		↑	↑	
Francis et al 1988	99			↑	↓
Schmitz-Heubner et al 1988	225	↑			
Olofsson et al 1989	213		↑	↑	
Mehta et al 1987	75		=	=	
Vanderkerckhove et al 1988	71		=		=
Oseroff et al 1989	65			=	=

tPA = tPA antigen, PAI = PAI-1 activity, tPAac = tPA activity,

AMI = studies on patients with acute myocardial infarction.

Notice that only the most recent studies have been listed here.

disease (Wahlberg et al 1980, Mettinger 1982). These case control studies indicate that high levels of VWF may be a marker of ongoing atherosclerotic changes and risk of thrombotic events, possibly due to its release from disturbed arterial endothelium.

Diabetes mellitus

Fibrinolytic activity is impaired in patients with diabetes mellitus (Fuller et al 1979; Osterman and van de Loo 1985; Grant and Medcalf 1990) which may be relevant to their increased risk of arterial disease. Improved diabetic control with insulin shows improved fibrinolytic activity in both insulin dependent diabetes mellitus (IDDM) (Greaves et al 1983) and non- insulin dependent diabetes mellitus (NIDDM) (Small et al 1987). Both high PAI activity and high tPA antigen are associated with plasma insulin, obesity, triglyceride and and blood pressure: the insulin resistance syndrome (Juhan-Vague et al 1988).

Several studies have consistently shown that plasma VWF levels are elevated in insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) (Ostermann and van de Loo 1985). This may reflect both microvascular and macrovascular endothelial disturbance in this disease.

PROSPECTIVE STUDIES ON FIBRINOLYSIS AND VWF IN ISCHÆMIC HEART DISEASE AND DIABETES MELLITUS

Some recent prospective studies looking at associations of fibrinolysis and cardiovascular events are summarised in table 1.6. In a large prospective study in healthy men carried out at Northwick Park Hospital, 109 out of 1511 men developed ischæmic heart disease during the 7-year follow up (Meade et al 1986). In this study, global fibrinolytic activity was measured by the dilute whole blood clot lysis time, because assays of PAI and tPA were not yet available. Fibrinolytic activity was significantly lower in those who went on to develop IHD when compared to those who did not. On the other hand, euglobulin clot lysis time was not predictive of IHD or stroke in the Goteborg Study (Wilhelmsen et al 1984).

PAI activity was an independent risk factor for predicting reinfarction and cardiac death among survivors of premature myocardial infarction (under 45 years) (Hamsten et al 1987). However, neither PAI activity nor tPA antigen were predictive of recurrent myocardial infarction in older men (Jansson et al 1991b). In angina pectoris, tPA antigen, but not PAI activity was predictive of myocardial infarction (Jansson et al 1991b, 1993).

As already described, VWF is also a marker of endothelial disturbance and might therefore be expected to mirror altered levels of fibrinolytic proteins of endothelial origin (tPA, PAI-1). Very few prospective studies have assessed the relationship of VWF to ischæmic heart disease. The Northwick Park Heart Study showed that

Table 1.6 Relation between fibrinolytic variables and cardiovascular events in prospective studies

Author	n	events	FA	tPA	PAI	tPAac
Meade et al 1986	1511	109	↓			
Hamsten et al 1987	109	17	↓		↑	
Gram et al 1987	29	9	↓	↑		
Munkvald et al 1990	20	8	↓	↑		
Jansson et al 1991 b	123	45		=	=	=
Jansson et al 1991 a	213	47		↑	=	
Dotevall et al 1990	776	26			=	

FA = fibrinolytic activity, tPA = tissue plasminogen activator antigen, PAI = plasminogen activator inhibitor activity, tPAac = tissue plasminogen activator activity

higher incidence of IHD was associated with increased factor VIII levels, and factor VIII is known to correlate well with VWF concentrations, but this association was not statistically significant (Meade et al 1986). Haines et al (1983) showed that higher VWF levels occurred in patients with acute myocardial infarction who died within one year. Patients were sampled soon after admission and the results may therefore be affected by acute phase reactions. Breddin et al (1986) reported that VWF levels predict cardiovascular events in a group of diabetic patients.

AIMS OF STUDIES REPORTED IN THIS THESIS

The above literature review suggested that abnormalities in specific components of the fibrinolytic system (PAI, tPA, fibrin D-dimer), as well as in VWF (which like PAI-1 and tPA is a marker of endothelial disturbance), may be associated with cardiovascular disease especially ischaemic heart disease; and with diabetes mellitus in which the risk of cardiovascular disease including ischaemic heart disease is increased.

The recent availability of chromogenic assays for PAI activity, as well as specific ELISA's for tPA, D-dimer and VWF antigens, made it possible to perform further clinical and epidemiological studies of these variables in cardiovascular disease and diabetes mellitus. As well as their biolgical associations, their potential utility as predictors of ischaemic heart disease events was also addressed.

It was decided to assay plasma PAI activity (which is largely PAI-1 activity in non-pregnant persons) rather than antigen, on the premise that it is PAI activity which is the main potential determinant of fibrinolytic activity in vivo (evidence for this is summarised above). Furthermore, plasma PAI-1 antigen is partly inactive complexes with tPA (Figure 1.5); and can be artefactually further elevated by release of inactive PAI-1 from blood platelets during venepuncture (Booth 1991), which may be a problem especially in epidemiological studies under "field conditions".

It was also decided to assay plasma tPA antigen rather than tPA activity, on the basis that sampling conditions and anticoagulant to prevent complexing of active tPA with PAI-1 during sample handling (which would result in false low levels of tPA activity) were not well standardised at the time these studies were initiated. Between them, PAI activity and tPA antigen represent the total amount of circulating PAI-1, tPA and PAI-1-tPA complexes (Figure 1.5).

The aims of this study were as follows:-

(a) to assess the laboratory variability, biological (longitudinal intra-individual) variability, and total (laboratory and biological) variability in PAI activity, tPA antigen, fibrin D-dimer antigen and VWF antigen (Chapter 2);

(b) to assess the inter-individual variability of PAI activity, tPA antigen, fibrin D-dimer antigen and VWF antigen in a large random sample of the general population - the Second Glasgow WHO-MONICA Survey; within this sample to determine the relationships of these

variables to time of sample, demographic and life style variables, cardiovascular risk factors and prevalent cardiovascular disease; and within this sample to establish the major independent determinants of these variables in the population by multivariate analysis (Chapter 3);

(c) to assess whether some of these associations of PAI activity may arise because of its "acute phase protein" reactions (Juhan-Vague et al 1985; Kluft et al 1985) (possibly mediated through the cytokine interleukin-6,IL-6) (Chapter 3);

(d) to determine the association of PAI activity, tPA antigen, fibrin D-dimer antigen, VWF antigen, fibrinogen and red cell aggregation with prevalent ischaemic heart disease (IHD) in a population controlled case-control study of premature myocardial infarction (Chapter 4);

(e) to assess the predictive value of PAI activity, tPA antigen, fibrin D-dimer antigen and VWF antigen for incident IHD, in the prospective Caerphilly Heart Study (Chapter 5);

(f) to assess the predictive value of fibrin D-dimer antigen, VWF antigen and fibrinogen for incident IHD, in the prospective Edinburgh Claudication Study (Chapter 6);

(g) to compare PAI activity, tPA antigen, VWF antigen and fibrinogen in matched patients with non-insulin-dependent diabetes mellitus (NIDDM) with and without microalbuminuria (a risk factor for IHD), and in matched non-diabetic controls; and to correlate levels of

these variables with two measures of free radical formation which is one potential cause of endothelial disturbance (Chapter 7);

(h) to compare the responses of PAI activity, tPA antigen, fibrin D-dimer, VWF antigen and fibrinogen to acute insulin-induced hypoglycaemia in patients with insulin-dependent diabetes mellitus (IDDM) and matched non-diabetic controls (Chapter 8). This stress was used both to assess dynamic endothelial response as a test of endothelial function, and also to assess the possible contribution of hypoglycaemic episodes to elevation of these variables in IDDM;

(i) to compare the response of PAI activity, tPA antigen, VWF antigen and fibrinogen to acute submaximal exercise in patients with IDDM and matched non-diabetic controls (Chapter 9). This stress test was again used as a test of dynamic endothelial function.

The methods used in these studies are described in Chapter 2.

CHAPTER 2

SUBJECTS AND METHODS

SUBJECTS

The subjects who participated in each study are described in the relevant chapters of this thesis.

LABORATORY METHODS

Sample handling and storage

Careful blood collection, sample handling and storage are essential for the accurate measurement of the variables reported in these studies. The reliability of these results ultimately reflects the care taken with these procedures. Withdrawal of blood results in immediate changes in the components of haemostasis (Thomson, 1992), activating some factors (such as tissue factor and platelet release factors), while other labile factors such as tPA and Factor VIII may quickly deteriorate. To reduce the possibility of the pre-analytical phase affecting the results, adherence to recommended protocols must be strictly observed. This is particularly true when samples are being handled outside the laboratory, e.g. in population studies. Sampling procedures in such studies are discussed in the relevant chapters of this thesis.

In all studies where blood samples were brought to the laboratory for handling, the protocol for blood collection and storage designed by the

European Concerted Action on Thrombosis (ECAT) was followed (Thomson, 1992).

Minimal stasis was applied to the upper arm prior to venepuncture and blood was added to tubes containing 0.109 mM trisodium citrate with a final ratio of 9 volumes of blood to 1 volume of citrate. These tubes were pre-cooled on melting ice so that the blood sample was cooled rapidly, thus preserving those components that deteriorate at higher temperatures. The tubes were then put on melting ice, and were centrifuged within one hour at 2000g for 20 minutes in a refrigerated centrifuge at $+4^{o}C$ to give platelet poor plasma. After the careful removal from the cells with a disposable Pasteur pipette the middle layer of the plasma was aliquoted and snap frozen at $-70^{o}C$ until assay.

Plasminogen Activator Inhibitor (PAI) Activity

Plasma plasminogen activator inhibitor activity levels were determined by a commercially available chromogenic substrate assay (Coatest PAI; Chromogenix, Epsom, U.K.). The assay involves the addition of a fixed amount of single-chain tissue plasminogen activator (tPA) in excess to undiluted plasma, where most of it rapidly forms an inactive complex with the fast inhibitor PAI-1. (Single-chain tPA is used in the assay because PAI-2, another inhibitor of tPA found in plasma, inhibits this single-chain form very poorly). The residual tPA then activates plasminogen to plasmin in the presence of a stimulator. The amount of plasmin formed is directly proportional to the PAI activity in the plasma sample (which under most circumstances is mostly PAI-1). Plasmin levels are then determined by

measuring the amidolytic activity of plasmin on a chromogenic substrate (S-2403), which releases *p*-nitroaniline, levels of which are determined using an automatic microplate reader (MR700 from Dynatech, West Sussex, UK), at 405nm. The absorbance of the sample is then compared with the standard curve generated for each test run, and a value for the level of PAI activity obtained. This is expressed in arbitrary units (AU), one AU being defined as the amount which inhibits one IU of tPA/ml under the test conditions (Chmeilewska et al 1986, Gram et al 1993). A sample standard curve is shown in Figure 2.1.

At the time this work was undertaken, only two PAI activity kits were available commercially. These were the kit from Chromogenix (then known as Kabi) and a kit from Biopool AB, Umea, Sweden.The kits were compared and found to correlate well. It was decided on terms of cost to use the Chromogenix kit (Chmielewska et al. 1986). This kit has a reported sensitivity of 5 arbitrary units/ml, and is not affected by normal levels of alpha-2-antiplasmin. During the course of the MONICA 2 study (reported in Chapter 3), which was the first study in which PAI activity was measured, there were changes made to the kit and a new substrate was introduced. This resulted in higher values for PAI activity: the normal pooled plasma which was run with each assay for quality control was consistently higher with the new kits. (This control plasma was produced by pooling normal plasma from 20 healthy volunters, 10 men and 10 women, aged between 25 and 45 years. This was then aliquoted and rapidly frozen at -70^{o}C). It was therefore decided to express the results as a percentage of the normal pool rather than as arbitrary units/ml. The value obtained for the pool on each plate was used to calculate the PAI activity values for

CURVE FITTING

LINEAR CURVE FIT
SLOPE = -0.0272
Y-INT = 1.148
R-SQR = 0.992

Trial #1

STANDARDS

#	LABEL	LOCATION	O.D.#1	O.D.#2	AVG.	CONC.	PRED.CONC.	%ERROR
1	_____	A1 ,A2	1.150	1.172	1.161	0.000	*******	*****
2	_____	B1 ,B2	0.939	0.868	0.903	10.000	8.988	10.115
3	_____	C1 ,C2	0.567	0.522	0.545	20.000	22.176	10.879
4	_____	D1 ,D2	0.322	0.309	0.315	30.000	30.600	2.001
5	_____	E1 ,E2	0.095	0.094	0.095	40.000	38.715	3.212

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? INDICATES INVALID TERM
D INDICATES DELETED TERM

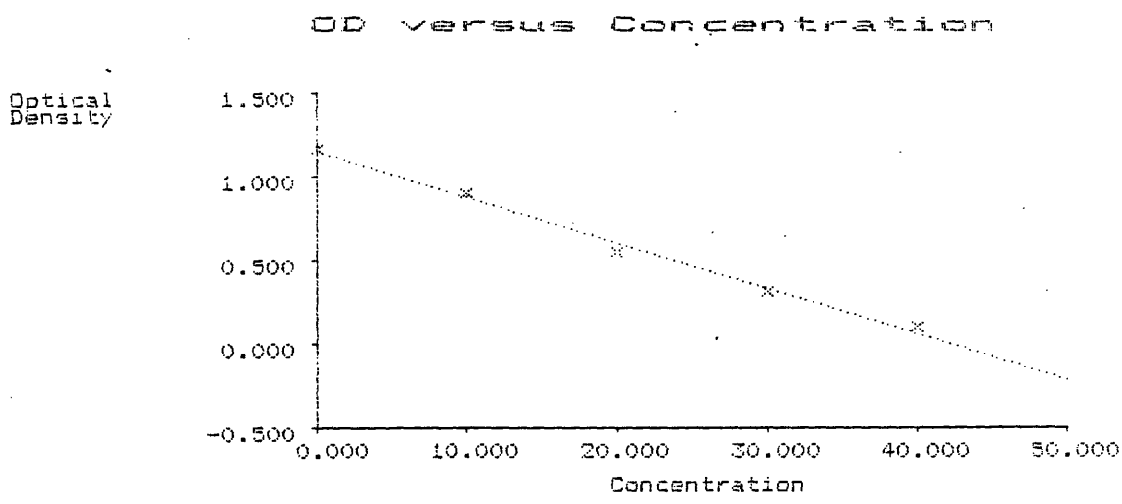


Figure 2.1 Sample curve for PAI activity.

LINEAR CURVE FIT
SLOPE = -0.0272
Y-INT = 1.148

the samples on that plate. This method of recording the results was then used in all subsequent studies. Successive plasma pools were calibrated against each other.

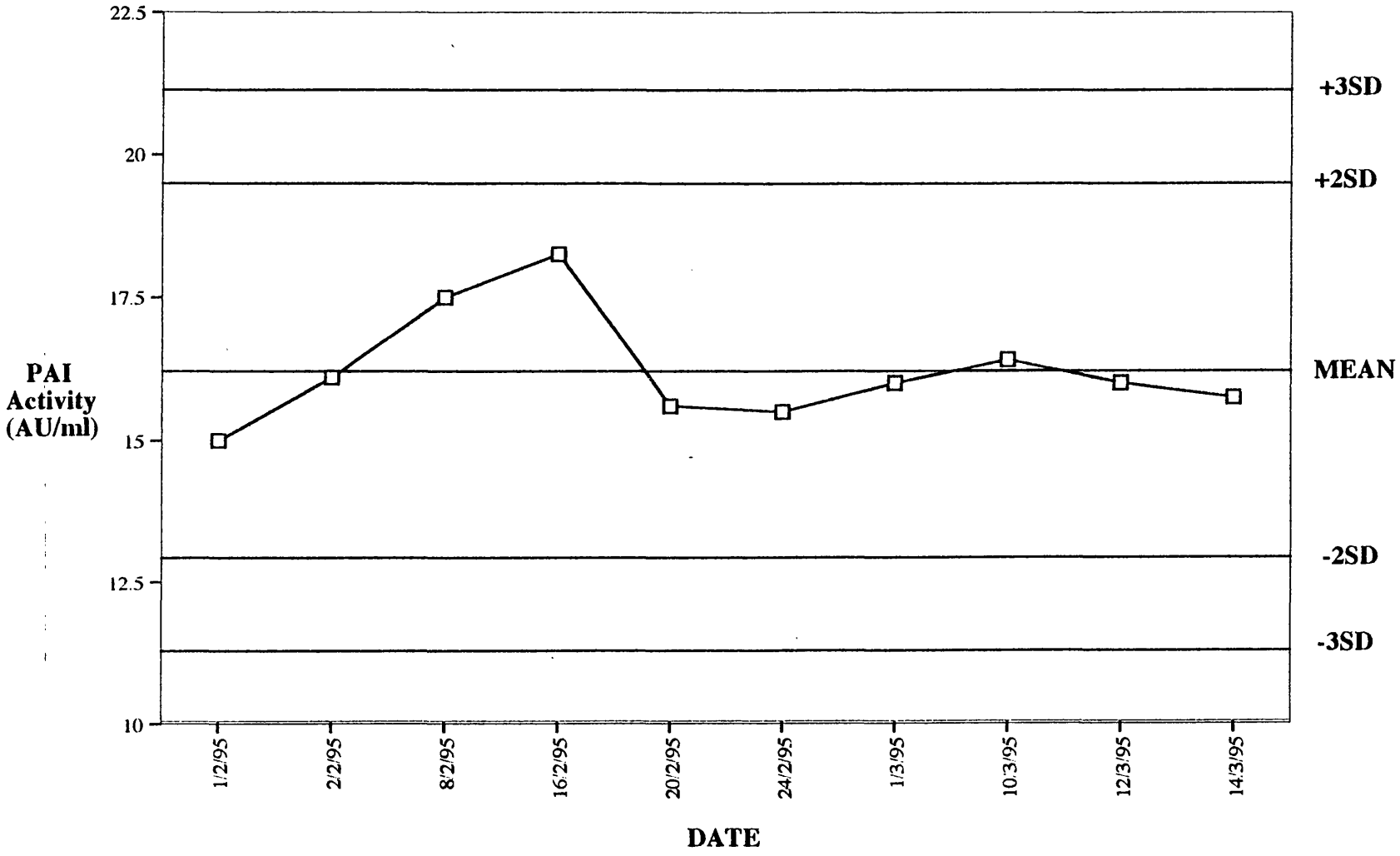
Assay of the normal pool in each set of assays also gave information about the coefficient of variation (CV) of the assay. The inter assay CV performed on a normal pool on 10 occasions was found to be 8.1%. The intra assay CV calculated on 5 single assays of a normal pool was 7.9%. The mean PAI activity level was plotted out daily on a Levey-Jennings plot (Levey and Jennings 1950). An example is shown in Figure 2.2.

At the time this work was undertaken, no standard reference method or standard for PAI was available, although one plasma standard is currently under study (Gaffney 1993). During the studies reported in this thesis, the Department of Medicine was asked to participate in an international comparison of PAI estimation. This was a study set up to assess the analytical performance of different commercial kits using eight "blind" split samples sent to 11 different haemostasis laboratories (Gram et al 1993). This study showed wide inter-laboratory and inter-kit variability, which might be reduced by expressing the results as a percentage of normal plasma pool.

Tissue Plasminogen Activator (tPA) Antigen

Plasma levels of tPA were measured with a commercially available enzyme linked immunosorbent assay (ELISA) from Biopool AB, Umea, Sweden (Tintelize #101120). The assay quantifies human single chain and two chain tPA antigen. No cross reaction with

FIGURE 2.2 A sample Levey-Jennings plot for PAI activity



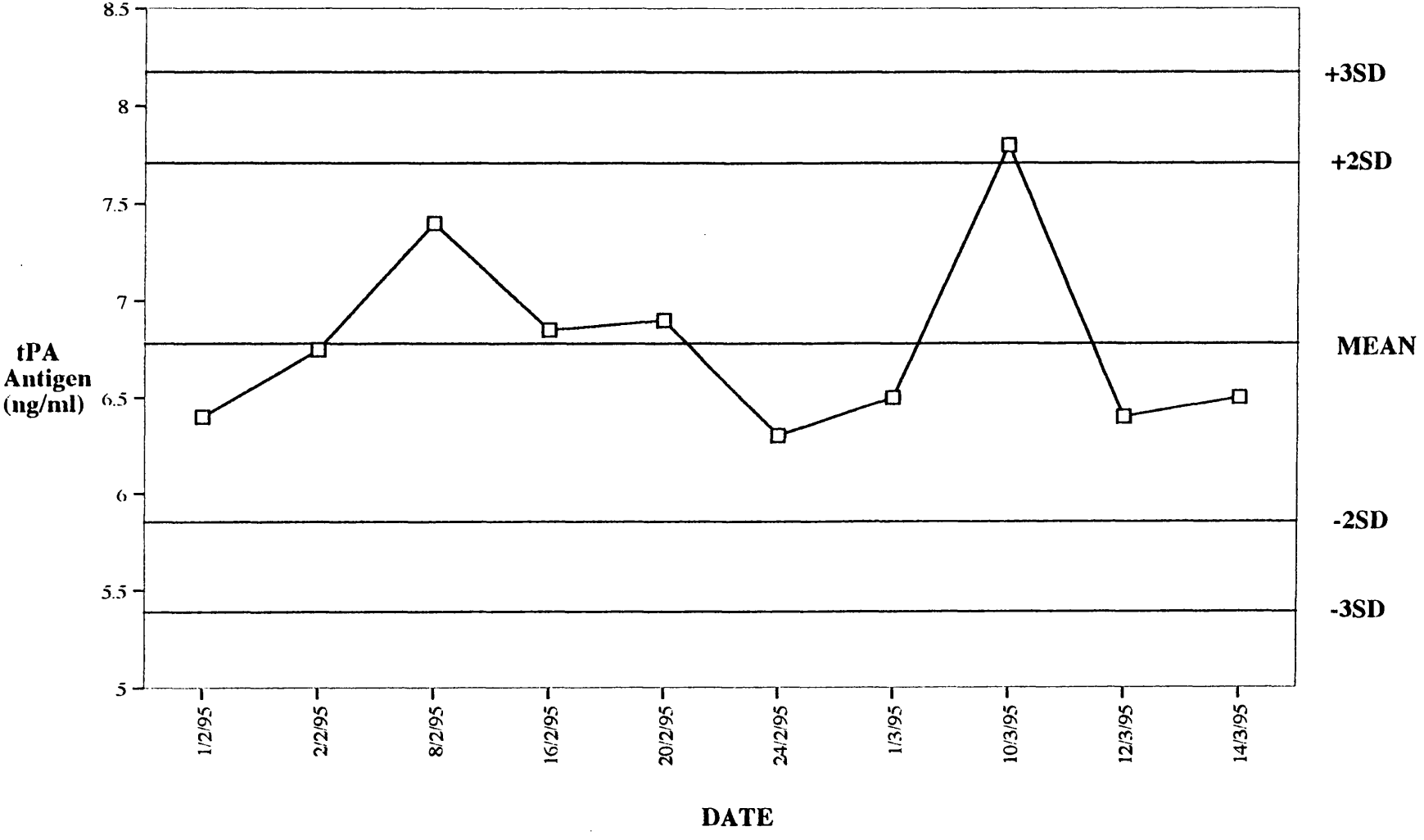
urokinase is observed. Maximal sensitivity of the assay is reported by the manufacturer as 1.5 ng/ml.

In this assay some interference may occur from plasma levels of other antibodies such as anti-goat antibodies and rheumatoid factor. To exclude false positives, each sample is applied to two wells, one containing normal goat IgG, and the other containing goat anti-human tPA IgG. The difference in assay response between these two wells is highly tPA specific. After initial binding to the pre-coated well, the second antibody which is conjugated to horseradish peroxidase is added to the wells. This will bind to free antigenic determinants on the tPA molecules present. Unbound conjugate is washed away after a further incubation period, and the remaining peroxidase is then measured by the addition of its substrate orthophenylenediamine dihydrochloride. The colour development is proportional to the amount of tPA bound to the well. The absorbancies were read at 492 nm on an MR 700 plate reader (Dynatech, West Sussex,UK). Because of the blank reading for each test and standard, the curve had to be plotted manually and the results from each test read from the curve.

Inter assay CV was determined as before using a normal pool measured on 10 occasions and found to be 9.8%. Intra assay CV calculated on 5 single assays was 9.0%. When 6 patients' plasmas with a wide range of tPA levels were used to calculate the CVs they were found to be very similar.

A Levey Jennings plot of the mean tPA antigen level of a normal pool is shown in Fig 2.3.

FIGURE 2.3 A sample Levey-Jennings plot for tPA antigen



An International Standard for tPA has recently been developed by Dr. P Gaffney, NIBSC, South Mimms, UK (Gaffney et al 1993), and in 1995, the laboratory in the Department of Medicine was asked to participate in its evaluation in an international collaborative study. The value assigned to the standard by this laboratory (24.7 ng/ml) was very similar to the consensus mean. This standard has now been submitted to the World Health Organisation (WHO).

Von Willebrand Factor (VWF) Antigen

Plasma von Willebrand factor (VWF) antigen levels were measured using an in-house enzyme linked immunosorbent assay (ELISA), employing rabbit anti-human polyclonal antibodies obtained from DAKO plc, High Wycombe, UK (Short et al 1982).

The standard curve was constructed using a normal pooled plasma from 20 volunteers as previously described, and the 5th British Standard (obtained from National Institute for Biological Standards and Control, South Mimms, UK) was used for pool calibration. This meant that the results could be reported as international units/decilitre (iu/dl), instead of as a percentage of the pool.

The capture antibody is coated onto the wells of a 96 well microtitre plate (Dynatech, West Sussex, UK). Diluted plasma samples and standards are then added and incubated for 30 minutes. After washing with buffer, the antibody, which is conjugated to horseradish peroxidase, is added. After this has incubated for a further 30 minutes, another washing procedure takes place. The peroxidase substrate, orthophenylenediamine which has been activated by

addition of hydrogen peroxide is added and after a rapid colour development, the reaction is stopped by the addition of 1.5M sulphuric acid. The absorbance in the wells is then read using the microplate reader (MR700 from Dynatech, West Sussex, UK) at 492 nm and the results of the test samples are read against the standard curve plotted for the assay. A sample curve is shown in Figure 2.4.

Using the 5th British Standard in 10 assays, the CV for the inter-assay variability was 7.7%. The intra-assay variability was 6.0%, being the CV of 5 aliquots of the standard in a single assay. As participants in the UK National External Quality Assesment Scheme (NEQAS) for Blood Coagulation, we regularly received samples during the studies for assay of von Willebrand factor antigen levels. Reports on the results of these surveys during the period of the studies gave us a satisfactory grading.

Since the normal pool was used to construct the standard curve in this assay, it could not be used as a quality control plasma. The 5th British Standard was therefore assayed on each plate and the results plotted out on a Levey Jennings plot (not shown).

Fibrin D-dimer antigen

The measurement of plasma fibrin D-dimer, which is present in several cross-linked fibrin degradation products, was carried out using the `Dimertest' enzyme linked immunosorbent assay (ELISA) kit from Agen Biomedical Limited (Parsippany, New Jersey, U.S.A.). The cleavage of both fibrinogen and fibrin by the fibrinolytic enzyme plasmin yields fibrin(ogen) degradation products (FDPs). However,

LOG CURVE FIT
E = 0.7811
NT = -1.806
R = 0.997

al £1

STANDARDS

LABEL	LOCATION	O.D.£1	O.D.£2	AVG.	CONC.	PRED.CONC.	%ERROR
____	A1 ,A2	1.303	1.306	1.304	300.000	288.025	3.992
____	B1 ,B2	1.008	0.968	0.988	200.000	201.709	0.854
____	C1 ,C2	0.807	0.804	0.806	150.000	155.389	3.593
____	D1 ,D2	0.611	0.560	0.586	100.000	103.290	3.290
____	E1 ,E2	0.330	0.316	0.323	50.000	48.259	3.481

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D INDICATES DELETED TERM

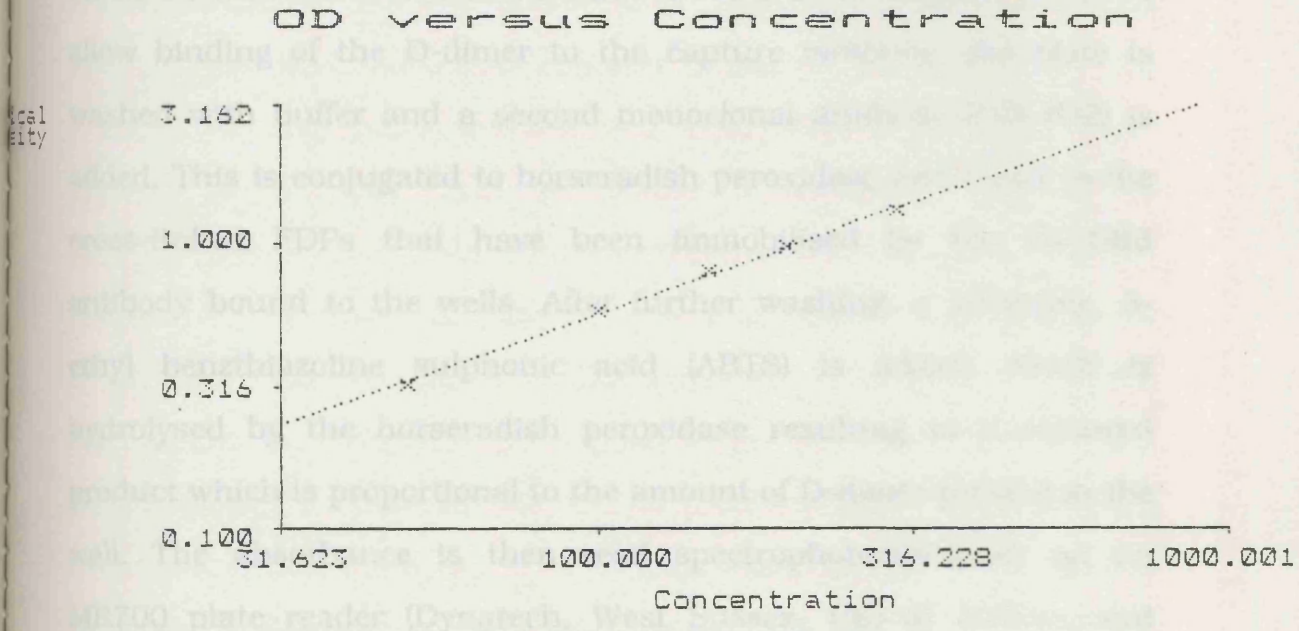


Figure 2.4 Sample curve for VWF antigen.

LOG CURVE FIT
E = 0.7811
NT = -1.806

only those products from the degradation of cross-linked fibrin contain D-dimer. Elevated levels of cross-linked FDPs detected by this method indicate increased formation and lysis of cross linked fibrin, and imply an increase in the turnover of cross-linked fibrin.

The principle of the test involves the use of the monoclonal antibody DD-3B6, which recognises D-dimer and FDPs containing it. The DD-3B6 is bound to microtitre plates to which either plasma or standard dilutions of D-dimer are added. After an incubation period to allow binding of the D-dimer to the capture antibody, the plate is washed with buffer and a second monoclonal antibody (DD-4D2) is added. This is conjugated to horseradish peroxidase and binds to the cross-linked FDPs that have been immobilised by the DD-3B6 antibody bound to the wells. After further washing, a substrate, 3-ethyl benzthiazoline sulphonic acid (ABTS) is added, which is hydrolysed by the horseradish peroxidase resulting in a coloured product which is proportional to the amount of D-dimer present in the well. The absorbance is then read spectrophotometrically on an MR700 plate reader (Dynatech, West Sussex, UK) at 405nm, and compared with the standard curve.

Quality control of this assay proved rather difficult. There is no international standard for D-dimer as yet (Gaffney 1993), and since the kit is designed to look at pathologically high levels of D-dimer, the use of a normal pool is not entirely suitable. Nevertheless, quality control as assessed by a Levey Jennings plot was satisfactory during these studies. The CVs for this assay were higher than for other assays, with an inter assay variation of 15.8% calculated by measuring the D-dimer level of the pool in 10 different assays. The

intra assay CV calculated from 5 aliquots of pool run on the same asssay was 14.2%.

Fibrin plate lysis area.

The fibrin plate lysis area of the plasma euglobulin fraction was performed by the method of Kluft et al (1976). This method involves the precipitation of the euglobulin fraction of the plasma by dilution and acidification. This fraction contains most of the plasma fibrinogen and plasminogen, and also contains plasminogen activators. The precipitated euglobulin fraction was resuspended in buffer, and applied to a petri dish in which a standard solution of fibrinogen/buffer had been clotted with thrombin. The test euglobulin precipitate was tested in parallel with a standard control plasma which had been precipitated in the same way. One control and the test plasma in triplicate were applied to the plate, which was then incubated at 37^{0}C for 18 hours. Two perpendicular diameters were measured to calculate the area of lysis. This area of lysis gives a measure of global fibrinolysis since lysis is affected by plasminogen activator activity, as well as the activity of plasmin and other proteases.

Fibrinogen

Clottable fibrinogen was measured by the dilute thrombin clotting time method of Clauss (1957). This was done on a Coag-A-Mate X2 automated coagulometer (Organon Teknika, Cambridge, UK) using reagents and standards from the manufacturer. The International Fibrinogen Standard (Gaffney and Wong 1992) was also

used to check the manufacturers standard; this was satisfactory. An in-house plasma pool from 20 healthy donors was used as the internal quality control; this was aliquotted and stored at -70^{o}C and used in each assay. Using fibrinogen levels of the normal pool over 10 assays the CV for inter assay variability was found to be 5.1%. The intra assay variability was 3.2%, calculated from fibrinogen levels measured on the pooled plasma run 5 times in the same assay. External quality control was provided as before by participation in the UK NEQAS scheme. Again, laboratory results were satisfactory during the studies reported in this thesis.

Red Cell Aggregation

Measurement of red cell aggregation was performed in whole blood anticoagulated with dipotassium EDTA (1.5 mg/ml). A photometric technique using an automated MA1 aggregometer (Myrenne GMBH, Roetgen, Germany) was employed (Schmid-Shonbein et al 1982; Stoltz et al 1984).

25µl of well mixed whole blood is placed on the aggregometer cone which contacts a glass slide on closing the machine. The sample is subjected to a 10 second period of high shear (around 600s^{-1}) to disrupt any red cell aggregates. The change in light transmission over a 5 second period of stasis is then measured. The greater the red cell aggregation, the greater the transmission of light through the sample, and the higher the numerical value obtained in arbitrary units from the aggregometer.

These measurements were carried out at ambient temperature (20-25^{o}C) and at native hæmatocrit. Standardising for hæmatocrit appears to have no effect on red cell aggregation in most clinical situations (MacRury and Lowe 1990). The mean of duplicate readings was taken and the procedure was repeated if there was a difference of more than 0.3 units between the readings, until consistent results were achieved.

Haematocrit

Haematocrit was measured by the Hawksley microhaematocrit method (Hawksley and Sons, Lancing, Sussex, UK) in accordance with International Committee for Standardisation in Haematology (ICSH) recommendations (1986).

Whole blood anticoagulated with dipotassium EDTA was well mixed, and duplicate samples of this were drawn up into glass capillary tubes of 1mm in diameter which were then sealed at one end. The tubes were then placed in the Hawksley microhaematocrit centrifuge and spun at 13,000g for 5 minutes. The hamatocrit was then read as a percentage of the sample which was packed cells, without correction for plasma trapping. An average reading of the two samples was taken.

Other Assays

In certain studies, IL-6, adrenaline, noradrenaline, cortisol and free radicals were assayed in other laboratories. Details of these measurements are given in the appropriate chapters.

STUDY OF TOTAL VARIABILITY IN PAI, TPA, VWF, D-DIMER AND FIBRINOGEN.

Having established the variability in laboratory measurement, I then proceeded to study total variability of PAI activity, tPA antigen, VWF antigen, D-dimer and fibrinogen. Total variability is the sum of biological variability and laboratory measurement variability (usually inter-assay) and is estimated by repeated measurements in a group of individuals over a period of time (usually several weeks) (Ernst et al 1985). While diurnal variation in plasma levels of PAI and tPA is well established (Andreotti et al 1988; Angleton et al 1989), there is little published information on longitudinal biological variation in PAI, tPA, VWF, D-dimer or fibrinogen. Thompson et al (1987) studied such variation over a 3 year period in several haemostatic variables, including VWF and fibrinogen. They concluded that biological variability within individuals was much greater than laboratory measurement variability. This is not surprising as fibrinogen is a "reactant " protein, and because plasma VWF levels also rise in response to endothelial stimulation (e.g. hormonal) or injury (see Chapter 1). De Bart et al (1992) compared variability in fibrinogen and PAI activity, and found that both laboratory and (especially) biological variability of PAI was higher than for fibrinogen (see Table 2.1).

For the study of total variability, 30 male patients aged 60-80 years with stable peripheral arterial disease (intermittent claudication) were recruited from the Peripheral Vascular Clinic, Edinburgh Royal Infirmary, or from the Edinburgh Artery Study (Smith et al 1993). Our previous studies in this laboratory have shown that such patients

Table 2.1

Results of laboratory (inter-assay), total and biological variabilities (mean CV, %) in hæmostatic and fibrinolytic variables.

	Current Study	Other Studies
Fibrinogen		
(Clauss)	(range 2.12-6.72g/l)	3-5% (de Bart 1992)
-inter assay	5.1%	5.8% (Rosenson 1994)
-total (n=30)	10.4%	11±5% (d Bart1992)
		17.8% (Rosenson 1994)
-biological	5.68%	
PAI activity	(range 30-196%)	
-inter assay	8.1%	10-15% (de Bart 1992)
-total (n=30)	14.6%	55±34% (de Bart 1992)
-biological	6.5%	
tPA antigen	(range 4.4-29.4ng/ml)	
-inter assay	9.8%	14.5% (Nguyen et al 1995)
-total (n=30)	14.0%	18.2% (Nguyen et al 1995)
-biological	4.7%	
vWF antigen	(range 65-276iu/dl)	
-inter assay	7.7%	12.7% (Nguyen et al 1995)
-total (n=30)	8.6%	21.6% (Nguyen et al 1995)
-biological	3.9%	
D-dimer antigen	(range 27-489ng/ml)	
-inter assay	15.8%	
-total (n=30)	16.3%	
-biological	0.59%	

have significantly higher mean levels and a wider range of these variables than controls (Reid 1991; Smith et al 1993). Therefore their plasma levels of PAI, tPA, VWF, D-dimer and fibrinogen cover a broader range than in healthy controls, allowing assessment of variability at both normal and moderately elevated levels in in older persons. This is appropriate when considering the associations of these variables with risk factors for cardiovascular disease, with cardiovascular disease, and with diabetes mellitus, which were investigated in the studies reported in this thesis.

The main purpose of this study was to assess variability in cytokine levels in peripheral arterial disease and their relationships to haemostatic variables; however it also gave me an opportunity to assess variability in PAI, tPA, VWF, D-dimer and fibrinogen. Subjects gave informed consent to the study, which was approved by the hospital Ethical Committee. Venous blood samples were taken at weekly intervals for 4 weeks, at approximately the same time of day to minimise diurnal variation. Samples were taken by nursing staff trained by myself in sample handling, processing, and storage which was performed as described above. PAI, tPA, VWF, D-dimer and fibrinogen were assayed as described above within 4 weeks of collection. Total variation in each variable was assessed for each subject by calculation of the coefficient of variation over the 4 samples. Biological variation was calculated according to the formula of Ernst et al (1985):

$$\text{Biological variability} = \frac{\text{Total - methological variability}}{\text{1- methodological variability}}$$

Table 2.1 shows the results for each variable, presented as the range of individual mean measurements across the 30 subjects; the inter-assay laboratory measurement variability (mean CV; as reported above); the total variability (mean CV); and the biological variability as calculated above. The results are compared to those of de Bart et al (1992) for fibrinogen and PAI activity; Rosenson et al (1994) for fibrinogen; Kluft (1994) for tPA antigen; and Nguyen et al (1995) for tPA antigen and D-dimer antigen (which were reported after performance of the present study).

For fibrinogen (measured by the Clauss assay), the inter-assay CV (5.1%) was similar to that of de Bart et al (1992) (3-5%) and Rosenson et al (1994) (5.8%). The total variability (10.4%) was similar to that of de Bart et al (1992) (11±5%) and rather lower than that of Rosenson et al (1994) (17.8%).

For PAI activity, the inter-assay CV (8.1%) was rather lower than that of de Bart et al (1992) (10-15%), possibly because the commercial kit used in the present study carries less risk of measurement error than the method of Verheijen et al (1984) used in the study of de Bart et al (1992). This difference was apparent when our laboratory changed from the method of Verheijen et al (1984) to the commercial Chromogenix/Kabi assay in 1988. The total variability (14.6%) was much lower than that reported by de Bart et al (1992) (55±34%). Possible reasons for this wide biological variability reported by de Bart et al (1992) include the performance of their study over a 6 month period (which might therefore include a seasonal variation in PAI levels); and the inclusion of young women (which therefore

includes menstrual variation). The latter group would not usually be assessed for their cardiovascular risk, which is very low.

For tPA antigen, the inter-assay CV (9.8%) was rather lower than that of Nguyen et al (1995) (14.5%); while the total variability (14.0%) was similar to that of Kluft (1994) (14%) and Nguyen et al (1995) (18.2%).

For VWF antigen, the inter-assay CV was 7.7% and the total variability was 8.6%, indicating that longitudinal variation was low. These results are similar to those of Nguyen et al (1995) (12.7% and 21.6% respectively).

For D-dimer antigen, the inter-assay CV was 15.8% and the total variability was 16.3%.

In conclusion, the results of this study are similar to those of the few recently reported studies of laboratory and biological variability in PAI, tPA, VWF, D-dimer and fibrinogen. Laboratory measurement (inter-assay) variability is lowest for fibrinogen (5%); higher for VWF, PAI and tPA (8-10%); and highest for D-dimer (16%). Total variability (laboratory measurement variability plus intra-individual biological variability over 4 weeks) is lowest for fibrinogen and VWF (9-10%); and highest for PAI, tPA and D-dimer (14-16%). These variabilities should be considered when designing studies of the associations of these variables with cardiovascular risk factors, cardiovascular disease, and diabetes mellitus. They must also be considered in calculating the number of samples required to obtain an

accurate estimate of a person's "average" plasma levels. This is important if these variables are to be measured in the future as "cardiovascular risk factors" in individual persons. For example, Rosenson et al (1994) calculated that the average of 4 plasma fibrinogen measurments over 6 weeks is required to reduce misclassification error to less than 10%.

STUDY DESIGN AND STATISTICAL ANALYSIS

The laboratory and biological variations in PAI, tPA, VWF, D-dimer and fibrinogen which were apparent in the literature, and from my own study reported in the previous section of this chapter, suggest that large studies are required to be sufficiently powerful to investigate their relationships to cardiovascular risk factors, and cardiovascular disease. Epidemiological studies using hundreds of subjects were therefore chosen for this purpose(Chapters 3, 5 and 6). In the case-control study of premature myocardial infarction (Chapter 4), it was calculated that 100 survivors of infarction should be studied to have a 90% power to detect biologically significant differences in fibrinogen (Meade et al 1986) or PAI activity (Hamsten et al 1988).

In the studies of diabetic microalbuminuria, insulin induced hypoglycaemia, and exercise (Chapters 7-9), smaller numbers were accepted, based on matching subjects for age and sex, and on the limited number of volunteer subjects available for the stresses of insulin and exercise.

Distributions of PAI, tPA, VWF, D-dimer and (in some studies) fibrinogen tended to be positively skewed, as found by other authors

(e.g. Eliasson 1995). The results are therefore usually presented as medians and interquartile ranges (IQR), or ranges or SEM for smaller studies. Statistical analyses were those recommended by the relevant statistician in each study after inspection of the data, and are described in each of the following chapters.

CHAPTER 3

DISTRIBUTIONS AND ASSOCIATIONS WITH CARDIOVASCULAR RISK FACTORS OF PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN ACTIVATOR ANTIGEN, VON WILLEBRAND FACTOR ANTIGEN, AND FIBRIN D-DIMER ANTIGEN IN A RANDOM POPULATION SAMPLE - NORTH GLASGOW MONICA STUDY

INTRODUCTION

In Chapter 1, it was noted that plasma levels of plasminogen activator inhibitor (PAI) activity, tissue plasminogen activator (tPA) antigen, von Willebrand Factor (VWF) antigen, and fibrin D-dimer antigen were recently introduced assays of fibrinolysis, endothelial disturbance, and fibrin turnover which showed potential in studies of cardiovascular disease and diabetes. In Chapter 2, the laboratory and biological variability of these assays was evaluated, and found to be sufficiently acceptable for such studies. In Chapter 1, it was noted that there were few reported studies of the distributions of these variables in random samples of the population, and their associations in such samples with demographic factors and cardiovascular risk factors (e.g. age, sex, hormonal status, smoking, alcohol use, diet, social class, diabetes, history of cardiovascular disease, obesity, blood pressure, cholesterol and triglyceride). It is important to perform such studies, to allow interpretation of the relationships of these plasma factors to cardiovascular disease or diabetes.

The opportunity to perform such a study occurred in 1989, when a random sample of men and women aged 25-64 years was performed in North Glasgow: the Second W.H.O. MONICA Survey of cardiovascular risk factors. In 1985, the World Health Organisation (W.H.O.) set up the multinational MONICA study (**MONI**toring of trends and determinants of **CA**rdiovascular disease) to study in a standardised way the incidence of cardiovascular events (myocardial infarction and stroke) over a 10 year period (W.H.O. MONICA Project 1988; Tunstall-Pedoe et al 1994). The study involved 38 populations in 21 different countries; North Glasgow was selected as the study population in the United Kingdom because it has one of the world's highest risks of ischaemic heart disease in both men and women (Tunstall-Pedoe et al 1994). At the start, middle and end of the 10 year study period, a representative population sample of men and women aged 25-64 years was performed by each study centre, in order to assess the prevalence of known cardiovascular risk factors such as smoking, hypertension, and hyperlipidaemia. Trends in risk factors will be compared at the end of the study period to changes in incidence of cardiovascular events, in order to clarify to what degree changes in event rates can be "explained" by changes in risk factors.

The Haemostasis and Thrombosis laboratory in the University Department of Medicine, Glasgow Royal Infirmary, established a collaboration at the start of the W.H.O. MONICA study with its leader Professor Hugh Tunstall-Pedoe, Cardiovascular Epidemiology Unit, University of Dundee. Plasma fibrinogen and blood viscosity were measured in the first MONICA survey in North Glasgow in 1984; and fibrinogen was measured in the Scottish Heart Health Study which shared its protocol (Lee et al 1990). For the Second MONICA survey in

North Glasgow in 1989; a grant was obtained to measure fibrinogen, PAI activity, haematocrit, white blood cell count and red cell aggregation (a marker of acute-phase protein reactions) in order to study their distributions and associations in this population survey. A subsequent grant was obtained to measure tPA, vWF and D-dimer antigens in the residual stored plasma samples, again to study their distributions and associations in this population sample.

AIMS OF STUDY

1) What are the distributions of plasma PAI activity and tPA, VWF and D-dimer antigens in a random sample of adult men and women?

2) What are their associations with standard demographic and cardiovascular risk factors, and with prevalent cardiovascular disease or diabetes mellitus?

3) What are the independent associations of such factors with each of PAI, tPA, VWF and D-dimer in multiple regression models? What percentage of variation in PAI, tPA, VWF and D-dimer can be "explained" by such factors in these models?

4) In further multiple regression models, what percentage of variation in plasma levels of PAI, tPA, VWF and D-dimer can be "explained" not only by such factors, but also by biological mechanisms such as haemoconcentration (haematocrit), increases in "reactant" plasma proteins (red

cell aggregation), and "reactant" increases in the white cell count?

5) Plasma levels of the cytokine interleukin-6 (IL-6) were also measured in a subsample of stored plasmas, to assess the contributions of this major mediator of "acute phase reactant" plasma proteins to PAI-1 levels.

SUBJECTS AND METHODS

The Second WHO-MONICA survey of cardiovascular risk factors in a random sample of the population of North Glasgow was performed between January and June, 1989. The MONICA study methods have been described in detail (WHO-MONICA Project 1988). Men and women aged 25-64 years were randomly selected from the registers of general practitioners in North Glasgow, balanced evenly between the four 10-year age groups, and invited to attend a local health screening clinic for examinations in the non-fasting state, between 08.00 and 16.00 hours. If they did not attend, a reminder with a new appointment was sent. Participants were asked to complete a standard questionnaire concerning their medical history, habits and social background; and underwent a standard examination including height, weight, calculation of body mass index (BMI=weight in kg/height^2 in m) as a measure of overweight, blood pressure, and measurement of serum cholesterol, total and high density lipoprotein (HDL) cholesterol, triglyceride, gamma glutamyl transpeptidase (GGT; a measure of alcohol intake) and thiocyanate (a marker of smoking), as well as blood glucose (WHO MONICA Project, 1988).

Prior to commencing the study, I held detailed discussions with the survey team concerning the collection and handling of extra blood samples for assay of the haematological variables, and the nurses were trained in blood sampling and processing by myself in the Haemostasis and Thrombosis Laboratory. Subjects rested for at least 20 minutes before blood sampling from a forearm vein, using minimum venous stasis to minimise local release of tPA and PAI-1 from forearm veins and to minimise haemoconcentration. Sample tubes for the "core MONICA protocol" biochemical analyses (listed in the previous paragraph), which were performed by Dr. R Tavendale in the Cardiovascular Epidemiology Unit, University of Dundee, were required to be filled first. Thereafter, further venous blood was anticoagulated with 0.109M trisodium citrate (9:1 v:v) in plastic sample tubes freshly prepared in the laboratory. After careful mixing, these samples were immediately centrifuged at 2000g for 20 minutes at room temperature. The middle layer of plasma was collected (to avoid platelet or leucocyte contamination) with a disposable plastic Pasteur pipette; snap frozen in aliquots and stored at -70^{o}C until assay of fibrinogen, PAI, tPA, VWF, D-dimer and IL-6. Apart from fasting, (which could not be demanded in this random population survey), these conditions accord with the Leiden Fibrinolysis Protocol for standardisation of PAI activity and tPA antigen assays (Kluft and Verheijen 1990). Further venous blood was anticoagulated with dipotassium edetate (1.5 mg/ml) for measurement of red cell aggregation, haematocrit and white cell count.

Haematological variables were measured as described in Chapter 2. Red cell aggregation, haematocrit and white cell count were measured within 24 hours of sample collection. Fibrinogen and PAI

activity were measured within 6 months of sample collection, and tPA, VWF and D-dimer antigens and IL-6 were measured within 18 months of sample collection (after obtaining a further grant for their measurement).

Statistical analysis was performed by Dr. A J Lee, Cardiovascular Epidemiology Unit, University of Dundee, with input from myself and Professor GDO Lowe, using the Statistical Package for the Social Sciences (SPSS) on the University main frame computer. Because of sex differences in both haematological variables and risk factor variables, men and women were analysed separately. Due to positively skewed distributions of PAI, tPA, VWF and D-dimer, data are presented as medians and inter-quartile ranges (IQR). Fibrinogen and red cell aggregation showed normal distributions and are presented as means and standard deviation (SD). Differences between groups were compared by the Mann-Whitney U test for two groups, and by the Kruskal-Wallis test for multiple groups. Spearman rank correlations were used to examine associations between the haematological factors and risk factors.

For multivariate analyses, square root transformations were performed for PAI, tPA and VWF; and natural log transformations for D-dimer, triglycerides, and GGT were performed. Stepwise multiple regression models were used to find the percentage of variation in each of PAI, tPA, VWF and D-dimer which could be explained by the following variables: age and time of day (both forced entry); smoking status (current, ex- or never); consumption of alcohol (units/week); white fish and oily fish (eaten rarely; once a week; twice or thrice weekly); social class; past history of angina, myocardial infarction,

high blood pressure, stroke or diabetes; body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, GGT, thiocyanate, glucose; and (in women) oral contraceptive use and menopausal status.

In a second multivariate analysis for each of PAI, tPA, VWF and D-dimer; haematocrit, white cell count and red cell aggregation were also included, to reflect the contributions of haemoconcentration, inflammation and acute-phase protein reactions respectively. A further model was also produced for D-dimer, including fibrinogen, PAI, tPA and VWF as well as the above variables, to examine the combined effect of all these variables on fibrin turnover.

In a random subsample of 139 subjects, plasma interleukin-6 (IL-6) was measured by Ms A Crilly in the Department of Medicine, using a sensitive bioassay (Aarden et al 1987) and correlated with plasma levels of haematological variables.

RESULTS

Of 2000 men and women invited to attend the Second WHO-MONICA Survey in North Glasgow, a total of 653 men and 744 women attended for examination(total 1397; response rate 70%). A citrated plasma sample was obtained for measurement of fibrinogen and PAI in 409 men and 386 women (total 795), due to either refusal of venepuncture, failed venepuncture, or inadequate blood to fill the citrate tube following filling of "core protocol" sample tubes for biochemistry. Following storage of citrated plasma samples pending obtaining of funding for assays of VWF, D-dimer and tPA antigen,

these assays were performed in that order on 300 men and 303 women (total 603), 284 men and 284 women (total 568), and 283 men and 275 women (total 558) respectively. The decreasing numbers of available samples for these assays reflect decreasing availability of plasma aliquots. Inspection of demographic variables in subjects in whom all assays were, and were not, performed did not suggest any significant bias; and at least 47 subjects were available in each 10 year age group for both men and women (Table 3.1).

Age and Sex (Table 3.1); Menopause (Table 3.2) and Oral Contraceptive Use (Table 3.3)

Overall there was no sex difference in plasma levels of PAI activity; however there was a marked sex difference in the relationship of PAI to age. There was no significant change in PAI between age 25-64 years in men ($p=0.93$); however there was a significant increase of PAI over this age range in women (from 80 to 95%; $p=0.02$). Premenopausal women had lower levels of PAI than post-menopausal women (Table 3.3; $p=0.006$). However, low PAI levels in young women appeared to relate to current use of oral contraceptives (Table 3.3; $p=0.07$).

Plasma tPA antigen levels were significantly higher overall in men than in women (6.5 vs. 4.9 ng/ml; $p< 0.001$), and this sex difference was apparent at all ages (Table 3.1). Plasma tPA increased markedly with age in both men and women, the increase being approximately two-fold over the range 25-64 years (Table 3.1). Premenopausal women had lower tPA levels than postmenopausal women (Table 3.2).

Plasma VWF antigen levels also showed an overall trend to be higher in men than women (93 vs 89 iu/dl; p=0.08); and showed a greater trend to increase with age. Plasma VWF levels did not increase after the menopause (Table 3.2); and oral contraceptive use had no effect (Table 3.3).

Plasma D-dimer antigen levels, in contrast, were higher in women (60 vs. 53 ng/ml; p=0.03); this sex difference disappeared after the menopause (Table 3.1). D-dimer levels increased with age in both men and women; and increased after the menopause (Table 3.2; p=0.001). Oral contraceptive users had lower levels (Table 3.3).

Time of sample (Table 3.4)

Significant decreases in plasma PAI activity and tPA antigen between 08.00 and 16.00 hours were observed in both sexes; and in VWF and D-dimer, in men only.

Cigarette smoking (Tables 3.5 and 3.6).

No effect of cigarette smoking on PAI activity in men was observed (p=0.42), although a trend to higher levels was observed in female current and ex-smokers (p=0.07).

Current cigarette smokers had higher tPA antigen levels than never-smokers in both sexes. Ex-smokers had intermediate levels in women; however ex-smokers had higher levels than current smokers in men.

Plasma VWF and D-dimer levels showed reversible increases in smokers among men, but not among women. The effect of smoking in men on D-dimer levels was not statistically significant(p=0.06). No significant relationships of PAI, tPA, VWF or D-dimer to reported daily cigarette consumption among smokers were observed.

Alcohol consumption

On univariate analysis, reported alcohol consumption showed only a weak association with tPA (r=0.11, p=<0.05) in men, and a negative association with both VWF (r=-0.16; p<0.01) and D-dimer (r=-0.11, p<0.05) in women (data not shown).

Fish consumption

Reported white fish consumption showed weak positive correlations with both VWF and D-dimer in men (r=0.10 and 0.12, p<0.05) and with D-dimer in women(r=0.10, p<0.05). Oily fish consumption showed no significant correlation with haematological variables (data not shown).

Social class

Both PAI activity and tPA antigen showed increases with decreasing social class in women; a similar trend was observed for tPA antigen and VWF antigen in men (data not shown).

Diabetes Mellitus (Table 3.7).

Although as expected the prevalence of diabetes was low in this age group (1.5%), diabetic subjects had significantly higher levels of PAI activity than non-diabetics ($p=<0.01$), and also tended to have higher levels of tPA and VWF antigens. Fibrinogen and red cell aggregation were also higher in diabetic subjects.

Hypertension (Table 3.8)

Subjects with a history of hypertension had higher levels of tPA antigen than persons with no such history (men $p<0.001$; women $p<0.05$) as well as higher levels of fibrinogen and (in women) red cell aggregation.

Angina or myocardial infarction (Tables 3.9 and 3.10)

Men with a history of angina or myocardial infarction had higher levels of fibrinogen, tPA, VWF and D-dimer antigens, but not PAI activity, than men with no such history. Women with such a history also had higher tPA levels.

Other cardiovascular risk factors (Table 3.11).

Plasma PAI activity correlated with body mass index, blood pressure, HDL cholesterol (inversely), triglyceride (Figure 3.1), and GGT ; and with total cholesterol and glucose in women.

Plasma tPA antigen correlated with body mass index, blood pressure, total cholesterol, triglyceride and GGT in both sexes; and with glucose, thiocyanate and HDL cholesterol (negative) in women (Figures 3.1-3.3).

Plasma VWF antigen correlated with blood pressure and GGT (positive) and triglyceride (negative) in men; and with total cholesterol, glucose and GGT (positive) and BMI, triglyceride and HDL cholesterol (negative) in women.

Plasma D-dimer antigen correlated with blood pressure, glucose and thiocyanate (positive) and triglyceride, body mass index (negative) in men; and with blood pressure and cholesterol in women.

Inter-relationships of haematological variables (Table 3.12)

The strongest correlation observed was between PAI activity and tPA antigen (r=0.31 for men, r=0.38 for women). PAI activity also correlated with fibrinogen (r=0.18) and red cell aggregation (r=0.14) in women. tPA antigen also correlated with haematocrit, red cell aggregation, fibrinogen, VWF and D-dimer. VWF antigen also correlated with red cell aggregation, fibrinogen and D-dimer.

Multivariate analysis: PAI activity

The significant independent associations of PAI activity in men were time of day, serum triglyceride, serum GGT, and history of diabetes (total variance explained=9.6%). The addition of haematocrit, white cell count and red cell aggregation had little effect on the model.

The significant independent associations of PAI activity in women were time of day and serum triglyceride (Fig 3.1) (total variance explained =16.5%). The addition of haematocrit, white cell count and red cell aggregation had little effect on the model.

Multivariate analysis: tPA antigen

The significant independent associations of tPA antigen in men were age (Table 3.1), serum GGT (Fig 3.3), and triglyceride (Fig 3.3); (total variance explained = 37.9%). The addition of haematocrit, white cell count and red cell aggregation resulted in replacement of triglyceride by haematocrit, red cell aggregation and smoking (total variance explained =44.5%).

The significant independent associations of tPA antigen in women were age (Table 3.1), triglyceride (Fig 3.1), serum GGT (Fig 3.2), time of day, reported alcohol consumption and social class (total variance explained =40.6%). The addition of haematocrit, white cell count and red cell aggregation resulted in replacement of social class by BMI (Fig 3.3) (total variance explained =41.4%).

Multivariate analysis: VWF antigen

The significant independent associations of VWF antigen in men were age, triglyceride (negative), serum GGT, thiocyanate and HDL cholesterol (negative) (total variance explained = 30.2%). The addition of haematocrit, white cell count and red cell aggregation resulted in the replacement of HDL cholesterol by red cell aggregation (total variance explained =28.7%).

The significant independent associations of VWF antigen in women were age, BMI and alcohol (negative) (total variance explained = 8.5%). The addition of haematocrit, white cell count and red cell aggregation to the model resulted in entry of BMI and alcohol (total variance explained = 8.8%).

Multivariate analysis: D-dimer antigen

The significant independent associations of D-dimer antigen in men were age, angina and triglyceride (total variation explained = 15.9%). Addition of haematocrit, white cell count and red cell aggregation to the model resulted in loss of angina and triglyceride (total variation explained = 12.8%). Further addition of fibrinogen, PAI, VWF and tPA into the model resulted in the addition of VWF (total variance explained = 16.9%).

The significant independent associations of D-dimer antigen in women were age and history of stroke (total variance explained = 8.5%). The addition of haematocrit, white cell count and red cell aggregation to the model resulted in the addition of red cell aggregation (total variance explained = 13.1%). Further addition of fibrinogen, PAI, VWF and tPA in the model resulted in the replacement of age by fibrinogen (total variance explained = 20.3%).

Relationship of IL-6 to haematological variables

Plasma IL-6 was assayed in a random sample of 139 samples. IL-6 showed significant correlations with red cell aggregation (r= 0.37),

Table 3.1

Distributions of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by age and sex

	Male			Female		
Age (yrs)	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)	*p=NS*			*p=0.02*		
Total	93	74, 118	409	90	67,113	386
25-34	91	73, 108	72	80	61, 99	75
35-44	93.5	72, 125	98	87	69, 106	91
45-54	94	73, 126	113	92.5	65, 113	112
55-64	92	76, 108	126	95	73, 128	108
tPA (ng/ml)	*p≤0.001*			*p≤0.001*		
Total	6.5	5, 9	282	4.9	3, 7	275
25-34	4.4	3, 6	52	3.0	2, 5	47
35-44	5.7	4.5, 8	66	3.5	2, 6	63
45-54	7.0	5, 10	74	4.9	4, 7	88
55-64	8.5	5, 12	90	7.0	5, 10	77
VWF (iu/dl)	*p≤0.001*			*p=0.01*		
Total	93	72, 124	300	89	70, 114	303
25-34	73	62, 98	53	80.5	68, 107	58
35-44	89	66, 107	71	84.5	66, 112	74
45-54	93	72, 124	82	89	72, 110	92
55-64	113	85, 154	94	100	75, 132	79

Table 3.1 (continued)
Distributions of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by age and sex

	Male			Female		
Age (yrs)	Median	I.Q.R.	n	Median	I.Q.R.	n
D-dimer (ng/ml)	*p≤0.001*			*p≤0.001*		
Total	53	35, 78	284	60	40, 82	284
25-34	42	24, 61	53	51	30, 78	52
35-44	45	30, 65	68	49	35, 68	68
45-54	50	34, 68	75	62	43, 83	93
55-64	77	52, 107	88	75	57, 94	71
	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)	*p≤0.001*			*p≤0.001*		
Total	2.33	0.61	487	2.42	0.56	462
25-34	1.92	0.34	85	2.31	0.59	90
35-44	2.14	0.58	114	2.21	0.41	107
45-54	2.41	0.59	130	2.46	0.56	134
55-64	2.61	0.60	158	2.63	0.57	131
Red Cell Aggregation (units)	*p≤0.001*			*p≤0.001*		
Total	3.54	1.19	432	3.71	1.22	422
25-34	3.06	1.04	75	3.41	1.19	83
35-44	3.47	1.06	109	3.39	1.17	92
45-54	3.62	1.27	118	3.86	1.18	128
55-64	3.82	1.21	130	4.02	1.22	119

Table 3.2

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by Menopause

	Pre			Post		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)	83.5	64, 106	210	94	73, 121	173
						p=0.006
tPA (ng/ml)	3.5	2.5, 5.5	142	6.25	4.5, 8.6	130
						p≤0.001
VWF (iu/dl)	89	69, 110	167	89	71, 121	133
						p=NS
D-dimer (ng/ml)	52	35, 78	156	71	48, 93	125
						p≤0.001
	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)	2.29	0.50	244	2.57	0.59	214
						p≤0.001
Red Cell Aggregation (units)	3.47	1.21	220	3.97	1.18	201
						p≤0.001

Table 3.3

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by use of oral contraception in 25 to 64 year old women

	Yes			No		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)	86	65, 109	213	92	72,118	170
						p=0.07
tPA (ng/ml)	4	2.5, 6.5	142	5.5	4, 8	131
						p≤0.001
VWF (iu/dl)	82.5	66.5, 110	162	95.5	72, 122.5	138
						p=0.003
D-dimer (ng/ml)	53	36, 78	153	70	47.5, 92.5	129
						p=0.001
	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)	2.35	0.55	256	2.52	0.57	203
						p≤0.001
Red Cell Aggregation (units)	3.54	1.18	234	3.93	1.24	184
						p≤0.002

Table 3.4
Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by time of sample

	Men			Women		
Sampling time	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI(%)	*p=0.007*			*p≤0.001*		
08.00-10.00	98.5	80, 137	118	95	75, 129	121
11.00-12.00	95	76, 121	109	96	73, 124	102
13.00-14.00	87	70, 104	108	79.5	63, 103	82
15.00-16.00	84	65, 110	73	78	64, 95	81
tPA(ng/ml)	*p≤0.001*			*p≤0.001*		
08.00-10.00	7	4.5, 11	79	6.3	4, 9	93
11.00-12.00	8	6, 11	67	5.5	4, 8	74
13.00-14.00	5.8	5, 9	75	4.1	3, 7	55
15.00-16.00	5.4	4, 8	61	3	2, 4.3	53
VWF(iu/dl)	*p=0.002*			*p=NS*		
08.00-10.00	107	76, 142	86	89	69, 119	99
11.00-12.00	98	73, 128	72	89.5	71, 115	84
13.00-14.00	87	69, 107	77	89	71, 107	65
15.00-16.00	81	70, 113	65	90	69, 114	55
D-dimer(ng/ml)	*p=0.001*			*p=NS*		
8.00-10.00	63	40, 82	79	59	36, 81	90
11.00-12.00	61	41, 93	68	65	40, 82	79
13.00-14.00	44.5	28, 61	74	59	42, 82	61
15.00-16.00	48	31, 71	63	60	40, 89	54

Table 3.4 (continued)

Sampling Time	Men			Women		
	Mean	SD	n	Mean	SD	n
Fibrinogen(g/l)	*p≤0.001*			*p=0.02*		
8.00-10.00	2.39	0.60	132	2.51	0.60	148
11.00-12.00	2.43	0.59	124	2.47	0.59	121
13.00-14.00	2.32	0.67	128	2.37	0.49	91
15.00-16.00	2.13	0.54	101	2.29	0.49	102
Red Cell Aggregation (units)	*p=NS*			*p=0.07*		
8.00-10.00	3.66	1.05	116	3.77	1.20	137
11.00-12.00	3.53	1.18	116	3.85	1.23	110
13.00-14.00	3.53	1.28	108	3.78	1.18	80
15.00-16.00	3.43	1.26	91	3.42	1.24	95

Table 3.5

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by smoking habit

	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
Current	94	75, 120	163	92	73, 121	177
Ex	94	74, 133	128	92	64, 108	75
Never	90	70, 108	116	83	62, 110	132
			p=NS			*p=0.07*
tPA (ng/ml)						
Current	7.0	4.5, 10	112	5.5	3.5, 8	122
Ex	7.5	4.5, 11	94	5.0	3, 6.6	60
Never	5.5	4, 7	75	4.5	2.9, 6.9	92
			p=0.002			*p=0.05*
VWF (iu/dl)						
Current	100	75, 144	122	89	70, 118	143
Ex	93	74, 118	98	90	75, 111	60
Never	86	69, 104	79	89	67,111	98
			p=0.009			*p=NS*
D-dimer (ng/ml)						
Current	61	41, 78	117	57	36, 78	131
Ex	53.5	34, 78	92	71	39, 91	60
Never	44.5	27, 78	74	65	44, 81	92
			p=0.06			*p=NS*

Table 3.5 (continued)
Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by smoking habit

	Men			Women		
	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)			*p≤0.001*			*p=0.01*
Current	2.46	0.53	196	2.51	0.62	209
Ex	2.40	0.74	155	2.36	0.51	91
Never	2.05	0.46	134	2.34	0.50	160
Red Cell Aggregation (units)			*p=0.001*			*p<0.001*
Current	3.18	0.99	163	3.44	1.18	197
Ex	3.85	1.36	140	4.02	1.25	80
Never	3.66	1.12	127	3.90	1.19	144

Table 3.6

Distribution of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by daily cigarette consumption among smokers

	Male			Female		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
1-9	86.5	66, 105	24	96	64, 120	17
10-19	92	74, 114	43	92	71, 127	68
20-29	97.5	76, 124	60	94	73, 121	71
30-39	105	86, 134	21	89	76, 124	13
40+	82	70, 128	15	90	60, 107	8
			p=NS			*p=NS*
tPA (ng/ml)						
1-9	5.3	4.5, 7.2	14	4.5	3.5, 9.5	11
10-19	7.5	4.9, 10.7	34	5.4	4, 7.6	46
20-29	7	5, 10.1	43	5.5	3, 7.5	51
30-39	5.4	3.5, 11.5	14	5.8	4.1, 8.6	10
40+	6.7	6, 11	7	6.4	3, 8.8	4
			p=NS			*p=NS*
VWF (iu/dl)						
1-9	108	66, 145	17	92	73, 127	13
10-19	98	75, 155	38	91	71, 116	53
20-29	100	79, 151	43	82	66, 111	60
30-39	94	70, 125	16	115	48, 150	13
40+	104	77, 157	8	123	75, 154	4
			p=NS			*p=NS*

Table 3.6 (continued)
Distribution of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by daily cigarette consumption among smokers

	Male			Female		
	Median	I.Q.R.	n	Median	I.Q.R.	n
D-dimer (ng/ml)						
1-9	56	30, 71	15	42	29, 61	11
10-19	65	45, 84	37	60	41, 80	46
20-29	56	42, 78	43	57	34, 74	57
30-39	59	32, 74	15	76	43, 134	13
40+	70	46, 108	7	47	42, 86	4
			p=NS			*p=NS*
	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)						
1-9	2.34	0.56	26	2.29	0.51	21
10-19	2.45	0.60	54	2.53	0.52	81
20-29	2.51	0.44	74	2.52	0.70	84
30-39	2.44	0.51	25	2.68	0.73	15
40+	2.45	0.61	17	2.56	0.60	8
			p=NS			*p=NS*
Red Cell Aggregation (units)						
1-9	3.25	1.07	24	3.68	1.24	21
10-19	3.28	1.09	44	3.53	1.20	74
20-29	3.03	0.90	62	3.32	1.18	79
30-39	3.25	1.06	20	3.31	1.07	14
40+	3.38	0.87	13	3.43	1.09	9
			p=NS			*p=NS*

ble 3.7

vels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell
gregation by Diabetes

	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
AI (%)						
Yes	121	97, 174	11	118	96, 141	12
No	92	74, 114	379	90	67, 111	360
			p=0.009			*p=0.01*
PA (ng/ml)						
Yes	8.5	4.7, 10.8	8	6	3.6, 10.6	8
No	6.5	4.5, 9.2	262	4.7	3, 7	259
			p=NS			*p=NS*
WF (iu/dl)						
Yes	105	84, 172	7	117.5	84, 161	8
No	93	72, 124	279	89	70, 113	288
			p=NS			*p=0.03*
-dimer (ng/ml)						
Yes	71	46, 110	8	46	31, 67	7
No	53	35, 78	264	60	40, 81	270
			p=NS			*p=NS*

ble 3.7 (continued)
vels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell
gregation by Diabetes

	Mean	SD	n	Mean	SD	n
rinogen (g/l)						
Yes	2.55	0.42	16	2.87	0.63	11
No	2.32	0.62	449	2.40	0.55	433
			p=0.02			*p=0.01*
d Cell						
gregation (units)						
Yes	4.31	1.24	11	4.16	0.78	8
No	3.53	1.19	402	3.68	1.22	397
			p=0.05			*p=NS*

Table 3.8

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by High Blood Pressure

	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
Yes	90	74, 118	77	91	71, 122	87
No	93	73, 117	322	90	65, 111	295
			p=NS			*p=NS*
tPA (ng/ml)						
Yes	8	6, 11	52	6	3.5, 8	63
No	6	4, 9	225	4.5	3, 7	209
			p≤0.001			*p=0.02*
VWF (iu/dl)						
Yes	98	81, 132	61	91	72, 116	69
No	91	72, 122	233	87	70, 113	231
			p=NS			*p=NS*
D-dimer (ng/ml)						
Yes	56	41, 94	55	63	42, 90	63
No	53	32, 78	223	59	39, 81	218
			p=NS			*p=NS*

Table 3.8 (continued)

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by High Blood Pressure

	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)						
Yes	2.46	0.57	90	2.55	0.53	104
No	2.30	0.62	385	2.38	0.57	353
			p=0.003			*p=0.003*
Red Cell Aggregation (units)						
Yes	3.67	1.38	88	4.02	1.24	98
No	3.51	1.14	334	3.61	1.20	321
			p=NS			*p=0.003*

Table 3.9 Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by Angina

	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
Yes	97	70, 121	28	93	71, 115	17
No	92	74, 117	368	90	67, 114	356
			p=NS			*p=NS*
tPA (ng/ml)						
Yes	10	7.5, 12	19	7	5.7, 8	12
No	6	4.5, 9	254	4.5	3, 7	254
			p≤0.001			*p=0.04*
VWF (iu/dl)						
Yes	112.5	91, 148	22	98	76, 110	13
No	91	71, 121	268	89	70, 114	283
			p=0.006			*p=NS*
D-dimer (ng/ml)						
Yes	69	55, 106	22	77	39, 124	12
No	52	33, 78	254	59	39, 81	264
			p=0.002			*p=NS*

Table 3.9 (continued)
Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by Angina

	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)						
Yes	2.64	0.60	32	2.58	0.49	20
No	2.30	0.61	441	2.40	0.56	427
			p≤0.001			*p=NS*
Red Cell Aggregation (units)						
Yes	3.53	1.23	30	3.99	1.50	19
No	3.55	1.19	390	3.68	1.20	390
			p=NS			*p=NS*

Table 3.10 Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by Myocardial Infarction

	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
Yes	90.5	81, 130	18	93	85, 139	11
No	92	73, 117	374	90	67, 114	358
			p=NS			*p=NS*
tPA (ng/ml)						
Yes	8.5	5.7, 12	16	7	5.7, 9.5	8
No	6.2	5, 9	257	4.5	3, 7	256
			p=0.07			*p=0.09*
VWF (iu/dl)						
Yes	115	84, 160	17	99	76, 109	8
No	92	72, 122	273	89	70, 114	286
			p=0.05			*p=NS*
D-dimer (ng/ml)						
Yes	78.5	58, 141	16	78	42, 89	7
No	52	35, 78	259	59	39, 81	267
			p=0.004			*p=NS*

Table 3.10 (continued)
Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by Myocardial Infarction

	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)						
Yes	2.69	0.61	24	2.66	0.44	12
No	2.31	0.61	444	2.40	0.56	431
			p≤0.001			*p=0.05*
Red Cell Aggregation (units)						
Yes	3.57	0.97	21	4.28	1.25	12
No	3.54	1.20	395	3.68	1.20	390
			p=NS			*p=0.07*

Table 3.11

Spearman rank correlation matrix of PAI, tPA, D-dimer and VWF with cardiovascular risk factors which are continuous variables.

BMI = body mass index; GGT = gamma glutamyl transpeptidase.

***p<0.001, **p<0.01, p<0.005.

	PAI	tPA	D-dimer	vWF
Men				
BMI	0.12 *	0.26 ***	-0.13 *	-0.07
Systolic BP	0.14 *	0.24 ***	0.15 **	0.14 **
Diastolic BP	0.10 *	0.30 ***	0.17 **	0.12 *
Total cholesterol	0.05	0.28 ***	0.01	0.06
HDL cholesterol	-0.11 *	-0.06	0.09	0.06
Triglyceride	0.11 *	0.30 ***	-0.014 ***	-0.11 *
Glucose	0.05	0.08	-0.13 *	0.01
Thiocyanate	0.01	0.02	0.12 *	0.09
GGT	0.19 ***	0.48 ***	0.01	0.19 ***
Women				
BMI	0.20 ***	0.38 ***	0.08	-0.14 **
Systolic BP	0.17 **	0.34 ***	0.12 *	0.10 *
Diastolic BP	0.12 *	0.23 ***	0.09	0.03
Total cholesterol	0.10 *	0.36 ***	0.20 ***	0.36 ***
HDL cholesterol	-0.15 **	-0.15 **	0.01	-0.12 *
Triglyceride	0.31 ***	0.46 ***	0.08	-0.10 *
Glucose	0.18 **	0.13 **	0.18 **	0.09
Thiocyanate	0.08	0.11 *	-0.08	0.00
GGT	0.24 ***	0.44 ***	−0.03	0.15 **

Table 3.12
Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by High Blood Pressure

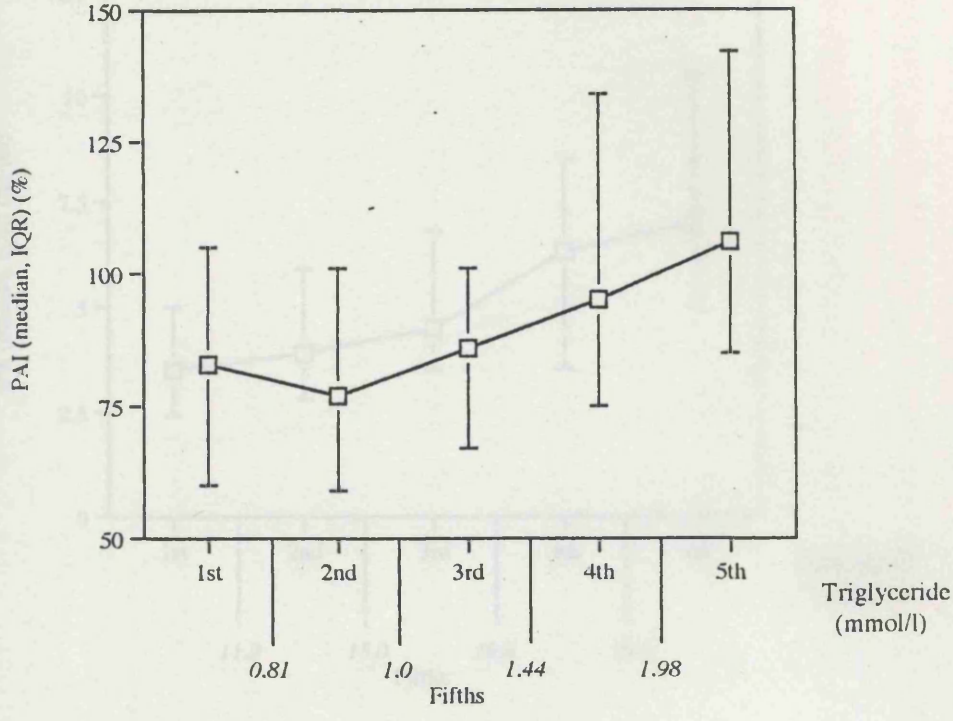
	Men			Women		
	Median	I.Q.R.	n	Median	I.Q.R.	n
PAI (%)						
Yes	90	74, 118	77	91	71, 122	87
No	93	73, 117	322	90	65, 111	295
			p=NS			*p=NS*
tPA (ng/ml)						
Yes	8	6, 11	52	6	3.5, 8	63
No	6	4, 9	225	4.5	3, 7	209
			p≤0.001			*p=0.02*
VWF (iu/dl)						
Yes	98	81, 132	61	91	72, 116	69
No	91	72, 122	233	87	70, 113	231
			p=NS			*p=NS*
D-dimer (ng/ml)						
Yes	56	41, 94	55	63	42, 90	63
No	53	32, 78	223	59	39, 81	218
			p=NS			*p=NS*

Table 3.12 (continued)

Levels of PAI, tPA, VWF, D-dimer, Fibrinogen and Red Cell Aggregation by High Blood Pressure

	Mean	SD	n	Mean	SD	n
Fibrinogen (g/l)						
Yes	2.46	0.57	90	2.55	0.53	104
No	2.30	0.62	385	2.38	0.57	353
			p=0.003			*p=0.003*
Red Cell Aggregation (units)						
Yes	3.67	1.38	88	4.02	1.24	98
No	3.51	1.14	334	3.61	1.20	321
			p=NS			*p=0.003*

PAI levels in women by fifths of Triglyceride



tPA levels in women by fifths of Triglyceride

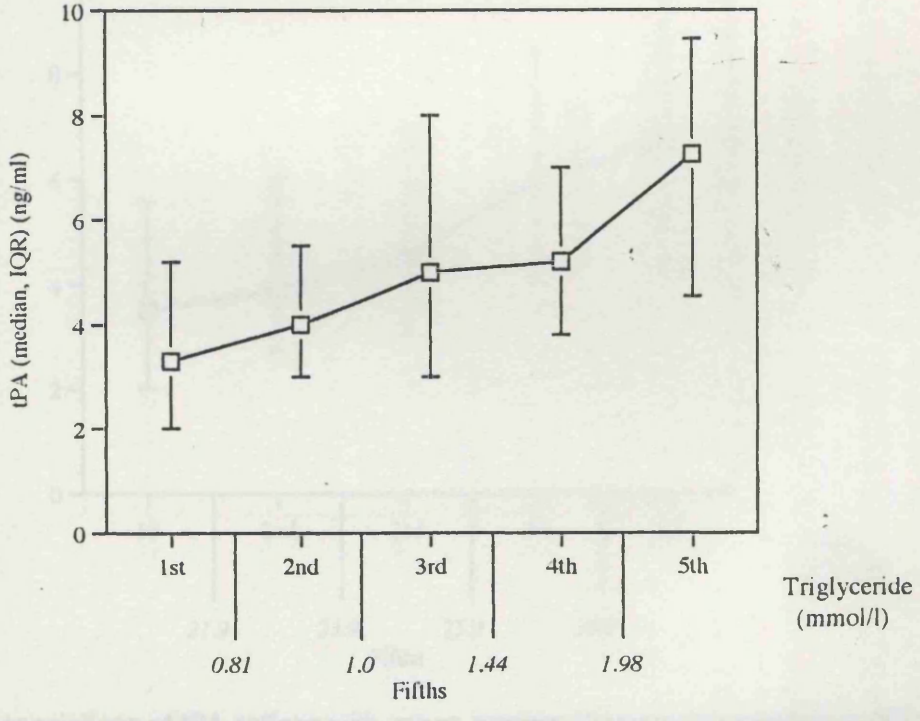
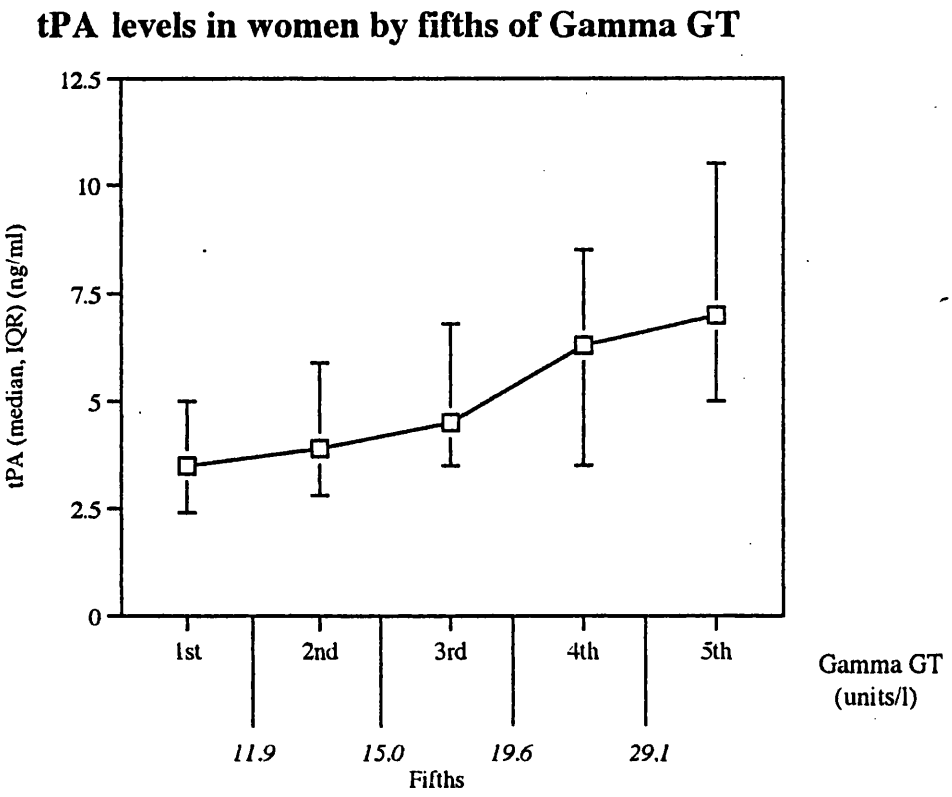


Figure 3.1 Associations of PAI activity and tPA antigen with serum triglyceride in women. Data shown are median and IQR by fifths of serum triglyceride.



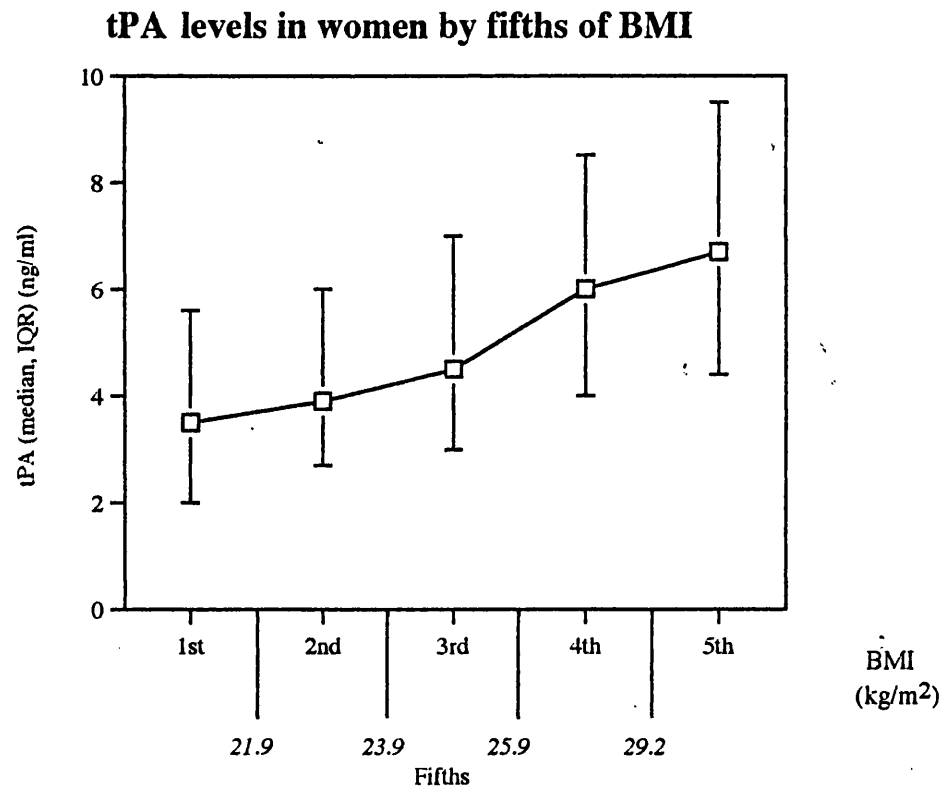
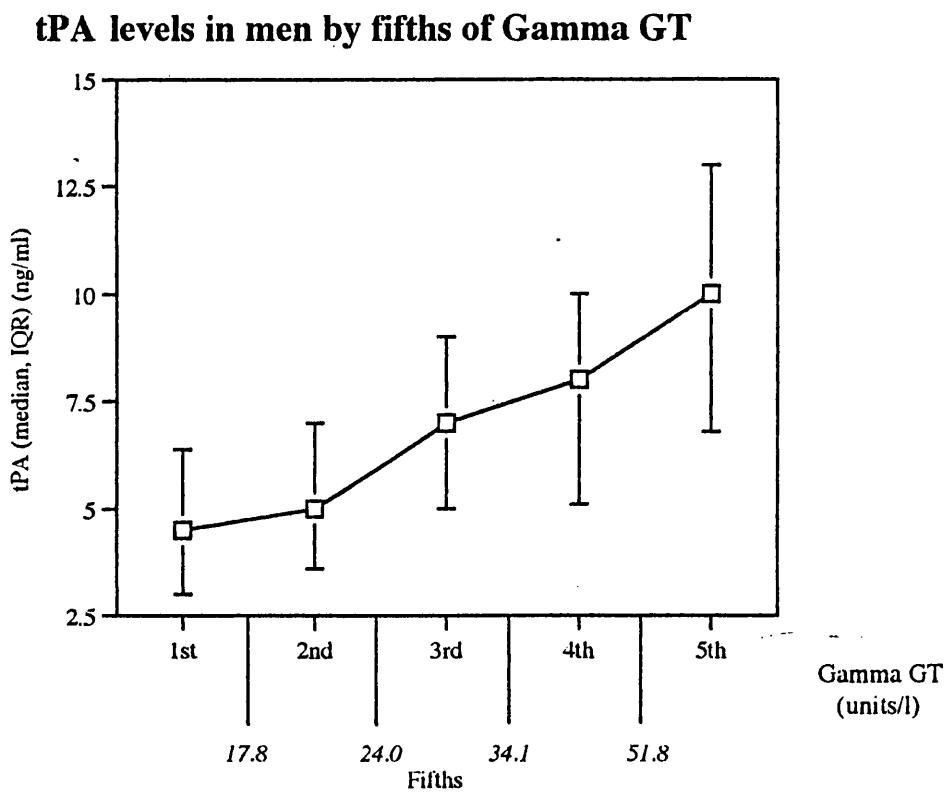


Figure 3.2 Associations of tPA antigen with serum gamma glutamyl transpeptidase (GT) and body mass index (BMI) in women. Data shown are median and IQR by fifths of serum gamma GT or BMI.



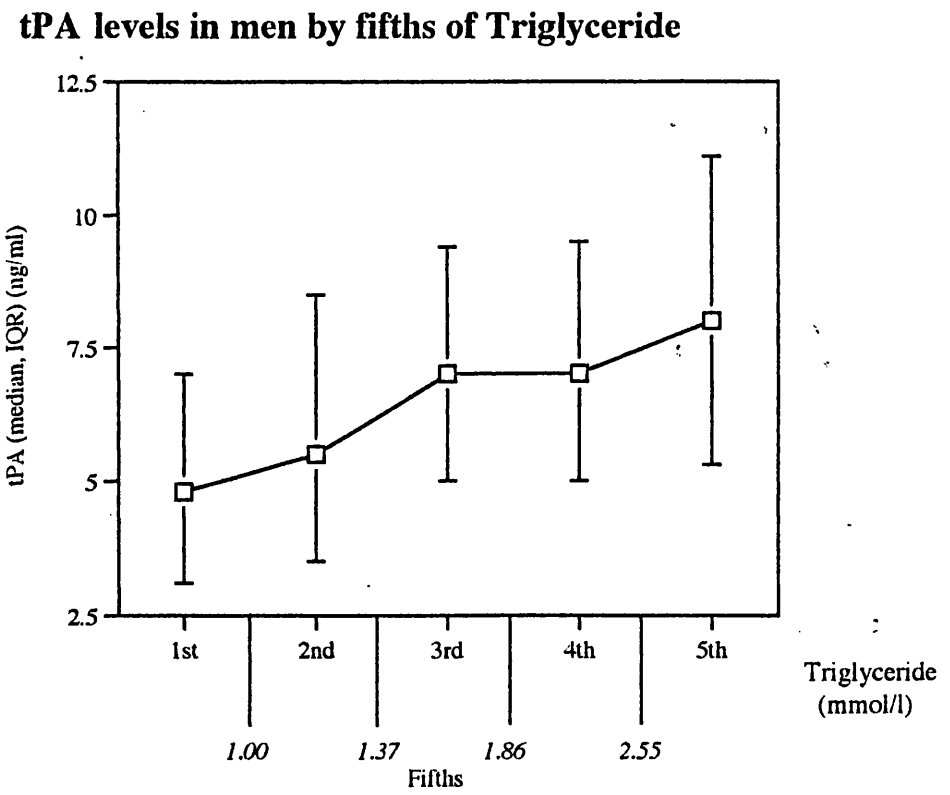


Figure 3.3 Association of tPA antigen with serum gamma glutamyl transpeptidase (GT) and triglyceride in men. Data shown are median and IQR by fifths of serum gamma GT or ttriglyceride.

fibrinogen (r=0.23), and PAI (r=0.24) (all $p<0.005$), but not with age, sex, smoking, white cell count, or other variables.

DISCUSSION

The distributions and associations of PAI activity, tPA antigen, VWF antigen and D-dimer antigen in this study will be discussed in turn, comparing the findings to those in the literature reported prior to the study, as well as that reported since this study was performed. The most directly comparable study of PAI activity and tPA antigen is the Second MONICA Survey in Northern Sweden, recently reported by Eliasson and colleagues (Eliasson et al 1993;1994 a, b; Eliasson 1995). This survey used similar methodology to the present study, but was performed on fasting subjects, sampled earlier in the day (07.00-15.00), who were advised not to smoke and who were not rested. This study obtained a higher response rate (79%) and a higher percentage of samples obtained for haematological studies. These included PAI activity and tPA antigen assays, performed by different methods (Biopool AG, Umea, Sweden) from the current study. Laboratory measurement variability appeared higher than in the present study, and surprisingly PAI activity was zero in 13% of samples (Eliasson 1995), which was never observed in the present study.

PAI activity

A wide distribution of plasma PAI activity was observed in the present study, in both men (range 10-300% pool) and women (range 10-302% pool). This is in agreement with many previous studies (Kruithof et al 1988; Eliasson 1995) and as noted above Eliasson

(1995) found 13% of the people in the Second MONICA survey in Northern Sweden to have undetectable levels of PAI activity. Despite this wide variation, the large number of subjects in the present study showed significant associations of PAI activity with several cardiovascular risk factors in the general population, including diabetes mellitus. On multivariate analysis, the independent determinants of PAI activity were time of day, serum triglyceride, and (in men) serum gamma-glutamyl transpeptidase (a marker of alcohol intake) and a history of diabetes mellitus.

While overall there was no sex difference in PAI levels, there was a sex-dependent change with age (Table 3.1). Median PAI level did not vary significantly wth age in men; however levels in premenopausal women were significantly lower than in men or in postmenopausal women (Tables 3.1 and 3.2). These results are very similar to those of Eliasson (Eliasson et al 1993; Eliasson 1995) in the Swedish MONICA Study. The lower PAI activity in premenopausal women appeared partly due to use of oral contraceptives (Table 3.3) which are known to increase fibrinolytic activity and to decrease PAI (Juhan-Vague et al 1988); this finding was also observed in the Swedish MONICA Study (Eliasson 1995). These results suggest that reference ranges for PAI activity should be age, sex and hormone-specific.

PAI activity showed a significant diurnal variation in both men and women, levels falling between 08.00 and 16.00 h (Table 3.4). Similar diurnal variation was observed in the Swedish MONICA Study (Eliasson et al 1993; Eliasson 1995). These results from cross-sectional population samples confirm the results of small longitudinal studies which frequently sampled healthy volunteers over a 24 h

period (Andreotti et al 1988; Angleton et al 1989). On multivariate analysis, time of day remained a significant determinant of PAI activity in the present study. Hence reference ranges for PAI activity should be time-specific (e.g. morning or afternoon ranges); and the diurnal variation in PAI should be considered in the design of studies of PAI activity. Andreotti et al (1988) have suggested that high PAI activity in the early morning may be relevant to the high incidence of myocardial infarction, coronary death and stroke at this time of day; by reducing endogenous lysis of arterial thrombi formed at this time.

Cigarette smoking was not significantly associated with PAI activity in the present study, although PAI activity tended to be lower in female non-smokers ($p=0.07$; Tables 3.5 and 3.6). Similar findings were reported in the Swedish MONICA Study (Eliasson 1995). Global fibrinolytic activity may be reduced in chronic smokers (Meade et al 1979; Allen et al 1984, 1985; Juhan-Vague et al 1988), but may reflect decreased release of tPA from endothelial cells (e.g. after desmopressin infusion) rather than an increase in PAI (Allen et al 1984, 1985). This decreased release of tPA is accompanied by decreased release of VWF in chronic smokers (Allen et al 1985); and may reflect exhaustion of tPA or VWF stores by the repeated release of tPA and VWF during acute smoking (Allen et al 1984), perhaps due to release of adrenaline and vasopressin (Juhan-Vague et al 1988).

Diabetic subjects had significant elevations of PAI activity compared to non-diabetic subjects (Table 3.7). PAI activity also correlated well with other variables associated with the "insulin resistance syndrome" (Reaven 1988): body mass index, blood pressure, triglyceride (Figure 3.1), glucose (in women), and low HDL

cholesterol (Table 3.11). These findings are consistent with the Swedish MONICA Study (Eliasson et al 1994a,b; Eliasson 1995); and with the growing literature which suggests that high PAI activity is consistently correlated with diabetes and insulin resistance (Juhan-Vague et al 1988; Gough and Grant 1991; Juhan-Vague and Alessi 1993). On multivariate analysis, history of diabetes mellitus remained a significant determinant of PAI in men in the present study. Insulin levels were not measured in the present study; however in the Swedish MONICA Study (Eliasson et al 1994a, b; Eliasson 1995) and in other large studies (Juhan-Vague and Alessi 1993) fasting insulin levels appeared to be the strongest predictor of PAI activity in both men and women (r=0.5 for each sex in the Swedish MONICA Study: (Eliasson 1995). In women, these correlations were stronger in the postmenopausal, the obese, and those with high triglyceride levels (Eliasson 1995). However, the strong inter-relationships between the components of the insulin resistance syndrome make it difficult to separate their associations in statistical analysis. Whether or not insulin stimulates PAI-1 release from endothelial cells or from hepatocytes is controversial (Juhan-Vague and Alessi 1993).

Neither history of hypertension (Table 3.8) or ischaemic heart disease (angina or myocardial infarction; Tables 3.9 and 3.10) were significantly associated with PAI activity. However the numbers of subjects with ischaemic heart disease were small; the association of PAI activity with myocardial infarction was subsequently studied in a larger, case-control study (Chapter 4).

Other cardiovascular risk factors (Table 3.11) associated with PAI activity on univariate analysis were (as noted above) components

of the insulin resistance syndrome: body mass index, blood pressure, triglyceride (Figure 3.1), glucose (in women), and low HDL cholesterol. PAI activity also correlated with serum gamma-glutamyl transpeptidase (GGT: a marker of alcohol intake), and total cholesterol (in women). On multivariate analysis, serum triglyceride remained a significant independent determinant of PAI activity in both men and women; while serum GGT remained a significant independent determinant of PAI in men.

These findings on univariate analysis are similar to those of the Swedish MONICA Study (Eliasson et al 1994a,b; Eliasson 1995) which found that in both men and women the strongest associations of PAI activity on univariate analysis were triglyceride (r=0.44), body mass index (r=0.40), waist-hip ratio (r=0.36), HDL cholesterol (r= -0.24), total cholesterol in women (r=0.24) and blood pressures (r=0.09-0.22). In the Swedish study, subjects were fasted, insulin and waist-hip ratio was measured, and alcohol consumption was not measured; while in the present study, subjects were not fasted, insulin and waist-hip ratio were not measured, and alcohol consumption was measured. These differences between the Glasgow and Swedish MONICA studies may explain different findings on multivariate analysis. The Swedish study found that independent predictors of PAI activity did not include triglyceride , but did include age, waist-hip ratio, low height and low HDL cholesterol in men; and body mass index, cholesterol, systolic blood pressure, smoking and age in women (Eliasson et al 1994a,b; Eliasson 1995).

Recent studies suggest that the findings in the present study, that serum triglyceride and alcohol consumption (serum GGT) were

independent determinants of plasma PAI activity are probably direct effects. Triglyceride-containing very low density lipoproteins (VLDL) increase endothelial cell production of PAI-1 (Stiko-Rahm et al 1990; Mussoni et al 1990), as does oxidised low density lipoprotein (LDL) (Latron et al 1991; Chautan et al 1993). Hendriks et (1994) observed in a controlled study that consumption of wine caused an acute increase in plasma PAI activity.

Plasma PAI activity levels are major determinants of tPA activity, which was not measured in the present study. In the Swedish survey, PAI activity was a strong, inverse determinant of tPA activity (r=-0.73 in men and -0.59 in women), which therefore showed relationships inverse to those of PAI with cardiovascular risk factors (Eliasson 1995).

tPA antigen

Plasma levels of tPA antigen in the present study were very similar to those reported in the literature (Kluft 1988); and to those recently reported in the Swedish MONICA Survey (Eliasson et al 1993; Eliasson 1995). As with PAI activity, tPA antigen showed significant associations with several cardiovascular risk factors in the general population. On multivariate analysis, the independent determinants of tPA antigen were age, serum triglyceride, and serum GGT; and (in women) time of day, reported alcohol consumption, and social class. These factors "explained" about 40% of the total variance in plasma levels.

The present study confirmed a strong association between tPA antigen and PAI activity levels (r=0.31 for men, r=0.38 for women; Table 3.12). This association may be partly due to circulating tPA-PAI-1 complexes; the normal excess of PAI-1 over tPA resulting in increased complex formation with increasing PAI-1 levels (Figure 1.3). Similar correlations were observed in the Swedish MONICA Survey (r=0.40 for men, r=0.37 for women) (Eliasson 1995). The association of tPA antigen with serum triglyceride and serum GGT may therefore partly reflect the associations of PAI activity with these variables. However, the relationships of tPA antigen to other cardiovascular risk factors were different from PAI activity. This suggests influences on plasma tPA antigen levels other than merely plasma PAI levels.

There was a significant sex difference in tPA antigen levels, men having higher levels than women at all ages. In addition tPA levels rose significantly with age in both men and women, doubling between ages 25 and 64 years (Table 3.1), and doubling after the menopause (Table 3.2). These results are almost identical to those from the Swedish MONICA Study (Eliasson et al 1993; Eliasson 1995), and are consistent with previous literature (Juhan-Vague et al 1988). Age remained the most important determinant of tPA levels on multivariate analysis, in both men and women.

A significant diurnal variation in tPA was observed, as in the Swedish MONICA Study (Eliasson et al 1993; Eliasson 1995). These results from cross-sectional population samples confirm the results of small longitudinal studies (Andreotti et al 1988; Angleton et al 1989); and mirror changes in plasma PAI levels. On multivariate analysis, time of day remained a significant determinant of tPA antigen in the

present study. Hence reference ranges for tPA antigen should be time specific (e.g. morning or afternoon ranges); and the diurnal variation in tPA should be considered in the design of studies of tPA antigen.

Unlike PAI activity, cigarette-smoking (both current and ex-smoking) was significantly associated with higher tPA antigen levels (Table 3.5). This effect was not dose-dependent (Table 3.6), and was stronger in men, possibly because they inhale more than women (Table 3.5). For unknown reasons, tPA antigen was not analysed in relation to smoking habit in the Swedish MONICA Study (Eliasson 1995); and there is little previously published information on tPA antigen levels in chronic smokers (Nilsson et al 1991). An association of tPA antigen with cigarette smoking was also observed on univariate analysis in male patients with angina pectoris in the ECAT Angina Pectoris Study (ECAT Angina Pectoris Study Group, 1993). Acute smoking increases tPA (and VWF) levels, probably due to acute endothelial disturbance (Allen et al 1984); and the chronic effect of smoking on both tPA and VWF in the present study may reflect chronic endothelial disturbance.

Diabetic subjects had non-significant elevations of tPA antigen in the present study (Table 3.7); however the number of diabetic subjects was small. Like PAI activity, tPA antigen correlated with other variables of the "insulin resistance syndrome" (Reaven 1988): body mass index and triglyceride (Figures 3.1-3.3), blood pressure, and glucose and low HDL cholesterol in women (Table 3.11). For unknown reasons, tPA antigen was not analysed in relation to these variables in the Swedish MONICA study (Eliasson 1995). However, the results of the present study are consistent with recent reports from two other

large studies: the ECAT Angina Pectoris Study (ECAT Angina Pectoris Study Group, 1993) and the ARIC Study (Iso et al 1993).

Unlike PAI activity, both history of hypertension (Table 3.8) and ischaemic heart disease (angina or myocardial infarction) (Tables 3.9 and 3.10), were significantly associated with increased tPA antigen levels. The association with myocardial infarction was subsequently studied in a larger, case-control study (Chapter 4).

Other cardiovascular risk factors (Table 3.11) associated with tPA antigen on univariate analysis were (as noted above) components of the insulin resistance syndrome; and serum GGT. On multivariate analysis, serum triglyceride and GGT remained significant independent deteminants of tPA in both men and women. These associations mirror those of PAI activity. In the ARIC Study, tPA antigen was positively associated with alcohol intake in Japanese men, but not in Caucasians (Iso et al 1993). Alcohol stimulates tPA release both in vitro and in vivo (Hendriks et al 1994). Whether or not triglycerides stimulate tPA release is not known; although as noted previously VLDL stimulates PAI-1 release from endothelium (Stiko-Rahm et al 1990).

VWF antigen

Like PAI-1 and tPA, von Willebrand factor antigen is an endothelial product; its associations in the present study were therefore examined to see if they resembled the associations of PAI and tPA. Plasma levels of VWF antigen were similar to those reported in the literature, including two other recent large population samples:

the Caerphilly Study (Elwood et al 1993) and the ARIC Study (Conlan et al 1993). As with PAI and tPA, VWF showed significant associations with several cardiovascular risk factors in the general population. On multivariate analysis, the independent determinants of VWF antigen were age; triglyceride (negative), GGT, thiocyanate and HDL cholesterol (negative) in men; and body mass index and alcohol consumption (both negative) in women. The associations of PAI, tPA and VWF with alcohol consumption (GGT) in men, and body mass index in women, may reflect generalised endothelial disturbance. However the inverse associations of VWF with triglyceride in men, and with alcohol consumption in women, are quite different from the associations of these variables with PAI and tPA. Therefore only some associations of PAI and tPA with cardiovascular risk factors reflect generalised endothelial disturbance. VWF showed stronger correlations with tPA antigen than with PAI activity (Table 3.12), perhaps because PAI-1 is synthesised by hepatocytes as well as endothelial cells.

In the present study, VWF levels rose significantly with age (Table 3.1) in both sexes. These results are very similar to those in the two other recent population samples (Conlan et al 1993; Elwood et al 1993). There was a sex difference with age: younger women tended to have higher levels than men, while older men had higher levels than women (Table 3.1).

A diurnal variation in VWF was seen in men (Table 3.4), but was not significant on multivariate analysis.

As previously discussed, cigarette smoking increased VWF levels (as well as tPA antigen levels) in men (Table 3.5), perhaps due to endothelial disturbance. The effect of smoking on VWF levels was significant on multivariate analysis in both the present study and the ARIC Study (Conlan et al 1993).

Diabetic subjects (although few) had higher levels of VWF antigen compared to non-diabetic subjects (Table 3.7). This is consistent with many previous studies (Osterman and van de Loo 1986) and with the ARIC population study (Conlan et al 1993). These elevations did not appear part of the "insulin resistance syndrome", because VWF showed inverse correlations with body mass index, triglyceride and glucose; and only a weak correlation with blood pressure (Table 3.11).

Von Willebrand factor was not associated with history of hypertension (Table 3.8), but was higher in men with ischaemic heart disease (angina or myocardial infarction) (Table 3.9 and 3.10). The association with myocardial infarction was subsequently studied in a larger, case-control study (Chapter 4).

Other cardiovascular risk factors (Table 3.11) showing associations with VWF antigen on univariate analysis were GGT in men ; and total cholesterol in women. However cholesterol was not a significant association on multivariate analysis.

Fibrin D-dimer

Plasma fibrin D-dimer levels are a marker of increased fibrin turnover (conversion of fibrinogen to fibrin by the coagulation system, followed by lysis of fibrin by fibinolytic enzymes including tPA). D-dimer antigen was measured in the present study as a marker of in vivo fibrinolysis, and was compared to levels of PAI, tPA and VWF.

Although high levels of PAI activity might be expected to inhibit fibrinolysis in vivo, there was only a weak, non-statistically significant, inverse correlation between PAI activity and D-dimer antigen (r=-0.03 in men and r=-0.07 in women; Table 3.12). tPA antigen showed a modest positive correlation with D-dimer (r=0.14 in men and 0.16 in women; $p<0.01$; Table 3.12). VWF antigen showed a stronger correlation with D-dimer (r=0.29 in men and 0.22 in women; $p<0.001$; Table 3.12). Therefore elevated D-dimer levels appeared to correlate better with this marker of endothelial disturbance than PAI or tPA levels. This might reflect fibrin formation on damaged vessel walls, followed by appropriate local endogenous fibrinolysis.

Plasma D-dimer increased with age in each sex (Table 3.1) and on multivariate analysis correlated most strongly with age. D-dimer also correlated with fibrinogen, VWF and tPA; and with white cell count in men and red cell aggregation in women (Table 3.12). It is therefore possible that the increase in D-dimer with age reflects increased fibrin turnover associated with age-related increases in fibrinogen levels, endothelial disturbance, tPA release, and inflammatory reactions. These factors might in turn reflect underlying

atherosclerosis (Al-Zahrani et al 1992; Smith et al 1993; Lee et al 1995; Woodburn et al 1995). When multivariate analysis of D-dimer was repeated including fibrinogen and VWF, VWF was a more important determinant of D-dimer than age in men; while red cell aggregation replaced age as a determinant of D-dimer in women.

A sex difference was observed in D-dimer before the menopause, with higher levels in premenopausal women (Table 3.1). This difference disappeared after the menopause (Table 3.3), hence one possible cause is uterine (menstrual) fibrin turnover. This might also reflect lower PAI activity in premenopausal women (Table 3.1).

A diurnal variation in D-dimer was seen in men (Table 3.4) but was not significant on multivariate analysis.

D-dimer levels were not significantly associated with diabetes or hypertension, but were higher in men who smoked or who had a history of angina or myocardial infarction (Tables 3.5-3.10). The latter association was studied further in a larger case-control study (Chapter 4).

D-dimer levels also correlated inversely with body mass index, triglyceride, and glucose in men; and possitively with blood pressure and thiocyanate. In women, D-dimer correlated with blood pressure, total cholesterol, and glucose (Table 3.11). Only the inverse association with triglyceride in men was significant on multivariate analysis: this may reflect the association of triglyceride with PAI activity, which might inhibit fibrin lysis.

Interleukin-6

It has already been noted that PAI, tPA, VWF and D-dimer correlated with red cell aggregation (a global measure of the acute phase protein reaction) and with fibrinogen, an acute phase protein. Studies of acute-phase protein reactions (e.g. surgery) have shown acute-phase behaviour of PAI-1 and tPA antigen (Juhan-Vague et al 1988). It has recently been shown that the cytokine, interleukin-6 (IL-6) is a major promotor of the acute phase reaction. Serum IL-6 levels were therefore measured in a random subsample of 139 samples in the present study, to see if they correlated with PAI activity, fibrinogen or red cell aggregation in the general population. As expected, a strong correlation was observed between IL-6 levels and red cell aggregation, a global measure of the acute-phase protein reaction ($r=0.37$; $p<0.001$). IL-6 also correlated with fibrinogen ($r=0.23$; $p<0.005$); the synthesis of fibrinogen by hepatocytes is known to be IL-6 dependent (de Boer et al 1991). Interestingly, the correlation of PAI activity with IL-6 was equally strong ($r=0.24$; $p<0.005$), suggesting that IL-6 may also mediate hepatic synthesis of PAI-1. De Boer et al (1991) showed that in vitro, hepatoma cell lines increased PAI-1 synthesis when exposed to interleukin-1 (IL-1) and tumor necrosis factor (TNF), but not to IL-6. However, Kruithof (1993) reported that injection of IL-6 in vivo in baboons led to strong increases in PAI-1 and tPA.

This preliminary study suggests that cytokines such as IL-6 mediate some of the variance of PAI-1 levels in the general population. Further studies are required to investigate this hypothesis, and to determine if IL-6 antagonists may reduce high PAI-1 levels and thus stimulate endogenous fibrinolysis.

CHAPTER 4

A CASE CONTROL STUDY OF PLASMA PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN ACTIVATOR ANTIGEN, FIBRIN D-DIMER ANTIGEN, VON WILLEBRAND FACTOR ANTIGEN AND FIBRINOGEN IN SURVIVORS OF PREMATURE MYOCARDIAL INFARCTION

INTRODUCTION

As discussed in Chapter 1, a number of studies have shown associations of plasma plasminogen activator inhibitor (PAI) or tissue plasminogen activator (tPA) with ischaemic heart disease (IHD) in cross sectional studies; as well as the predictive value of several hæmostatic variables for both primary and secondary myocardial infarction (MI). Fibrinogen is both a primary (Meade et al 1980, 1986) and secondary (Martin et al 1991) predictor of IHD events, while PAI activity (Hamsten et al 1987), tPA antigen and VWF antigen (Jansson et al 1991 a,b; 1993) have also been shown to predict recurrent MI. Several studies have compared individuals with ischaemic heart disease (IHD) and controls for levels of PAI, tPA and VWF (Table 1.5); but none have used controls from a random population study (see Chapter 1). In this study, an attempt was made to define the degree of chronic disturbance in PAI, tPA, von Willebrand factor (VWF), fibrin D-dimer and fibrinogen in survivors of premature MI, comparing them to age-matched controls from a simultaneous random sample of the population (Second Glasgow MONICA survey), which was described in Chapter 3. Red cell aggregation was also measured as a marker of reactant plasma proteins.

SUBJECTS AND METHODS

The cases for the study were consecutive survivors of premature (i.e. before the age of 60 years) myocardial infarction (determined by World Health Organisation (WHO) criteria: WHO 1976), who were admitted to the University Medical Unit, one of four general medical units in one of three hospitals in North Glasgow. These patients were studied in 1990, 1 to 6 years after infarction, to avoid "acute phase" reactions. Patients were selected from unit records by Dr PM Balendra and Professor GDO Lowe. Of 171 patients who were invited to attend for study, 100 (59%) attended, 74% of whom were male and 26% female. These cases underwent the same risk factor assessment used in the concurrent Second Glasgow MONICA Survey (Table 4.1). PAI activity, tPA antigen, VWF antigen, D-dimer antigen, fibrinogen and red cell aggregation were also measured as decribed in Chapter 2.

358 age matched (40-65 years) individuals from the Second Glasgow MONICA survey database were used as the control group. These subjects had no evidence of cardiovascular disease as defined by history of myocardial infarction, angina, hypertension, stroke or diabetes.

Statistical analysis was performed by Dr A J Lee, Cardiovascular Epidemiology Unit, University of Dundee, with input from Professor GDO Lowe and myself. Groups were compared by t-tests, Wilcoxon rank sum tests, or tests for differences in proportions (smoking categories), as appropriate. Multivariate logistic regression

Table 4.1
Conventional risk factors in cases of myocardial infarction and in population controls (mean values with SD in brackets, or % smokers). *p<0.05, **p<0.01, ***p<0.001

	Men		Women	
	Cases	Controls	Cases	Controls
Age (years)	56.1 (5.6)	51.3 (7.4) ***	56.4 (5.9)	52.1 (7.4) ***
Body mass index (kg/m²)	27.2	26.0	28.0	25.3
Diastolic blood pressure (mmHg)	82 (11)	78 (12) **	81 (13)	77 (13)
Cholesterol (mmol/l)	6.94 (1.22)	6.75 (1.22)	7.44 (1.46)	6.93 (1.39)
HDL cholesterol (mmol/l)	1.06 (0.26)	1.21 (0.36) ***	1.16 (0.38)	1.44 (0.46) **
Triglyceride (mmol/l)	2.12 (1.20)	1.97 (1.25)	2.47 (1.71)	1.54 (0.85) *
Glucose (mmol/l)	6.25 (2.65)	5.20 (0.98) **	5.84 (1.96)	5.25 (2.48)
Smokers				
current	50.0%	47.6%	64.0%	48.1%
ex-	44.6%	30.1%	32.0%	20.1%
never	5.4%	22.3%	4.0%	31.8%

analysis was performed for each of PAI, tPA, VWF, fibrinogen and red cell aggregation, to examine whether or not differences in cases versus controls were independent or not of standard risk factors: age, body mass index (BMI), diastolic blood pressure, total and high density lipoprotein (HDL) cholesterol, triglyceride, glucose, and two dummy variables representing current and ex-smoking habit respectively. Results for men and women were analysed separately because of sex differences in both risk factors and haematological variables.

RESULTS

In both sexes, haematological variables are shown as medians for PAI and D-dimer, and as means for the other variables (Table 4.2). PAI, tPA, VWF, D-dimer, fibrinogen and red cell aggregation were all significantly higher in survivors of premature MI compared to controls, with the exception of tPA in men.

Conventional risk factors, including age, BMI, diastolic blood pressure (DBP), cholesterol, HDL cholesterol, triglyceride, glucose and smoking were also analysed. As expected, some of these were higher in cases than in controls (Table 4.1).

A multivariate logistic regression analysis was then performed on cases versus controls, adjusting for age, BMI, DBP, cholesterol, HDL cholesterol, triglyceride, glucose, current smoking and ex smoking. PAI in men; D-dimer, fibrinogen, and red cell aggregation; and VWF in women remained significantly higher in cases compared with controls. tPA no longer showed a statistically significant

Table 4.2
PAI, tPA, vWF, D-dimer, fibrinogen and red cell aggregation in cases of myocardial infarction and in population controls (mean with SD in brackets; medians with IQR in brackets for PAI and D-dimer)
*** p<0.05; ** p<0.01; *** p<0.001**

	Men			Women		
	Cases		Controls	Cases		Controls
PAI (% pool)	113 (114)	***	94 (49)	127 (86)	***	92 (50)
tPA (ng/ml)	8.6 (3.8)		8.0 (3.7)	7.8 (3.6)	*	6.4 (3.6)
VWF (iu/dl)	124 (40)	**	112 (44)	136 (51)	***	102 (39)
D-dimer (ng/ml)	90 (63)	**	57 (40)	105 (53)	***	61 (41)
Fibrinogen (g/l)	2.98 (0.72)	***	2.52 (0.69)	3.48 (0.68)	***	2.55 (0.68)
Red cell aggregation (units)	4.5 (1.2)	***	3.9 (1.2)	5.2 (1.4)	***	3.9 (1.3)

association with MI when these other risk factors were taken into account (Table 4.3).

DISCUSSION

This population-controlled study showed survivors of premature MI to have chronic (1-6 years post-infarct) abnormalities in PAI activity, tPA antigen (in women), VWF antigen , D-dimer antigen, fibrinogen and red cell aggregation, in addition to a higher frequency of several standard risk factors, when compared to controls without evident cardiovascular disease. Multivariate analysis suggested that the increases in cases in PAI (in men); D-dimer, fibrinogen, red cell aggregation; and VWF (in women) remained statistically significant after adjustment for age and standard cardiovascular risk factors.

The increase observed in plasma PAI activity in survivors of premature myocardial infarction in this study is consistent with previous reports prior to 1990 when this study was performed (see Table 1.5 in Chapter 1). It also suggests that standard risk factors, such as body mass index or triglyceride which are associated with PAI-1 (Chapter 3), do not fully account for this increase, at least in men. When these risk factors were included in a mulivariate analysis, the association of PAI with myocardial infarction became non significant in women. These findings may have been influenced by the small number of women who were studied compared to men (26 versus 74). A larger study of women is therefore indicated to exclude an independent association of PAI activity with MI in women.

Table 4.3

Statistical significance of case control differences on logistic regression analysis, including the 9 conventional risk factors in Table 4.1. NS = not significant (p>0.05).

	Men	Women
PAI	***	NS
tPA	NS	NS
vWF	NS	**
D-dimer	**	*
Fibrinogen	***	***
Red cell aggregation	***	***

In these survivors of premature MI, the increase in plasma tPA antigen was less marked than the increase in PAI activity (Table 4.2). It was not statistically significant in men; of only borderline significance in women ($p<0.05$); and not significant on multivariate analysis in either sex. These findings are consistent with previous reports prior to 1990, when this study was performed; some of which found increased tPA antigen while others did not (see Table 1.5 in Chapter 1). The strong relationship of tPA antigen with several standard cardiovascular risk factors (Chapter 3) may explain why its association with myocardial infarction was not significant in multivariate analysis.

There are no previously reported studies of increased levels of plasma D-dimer in survivors of myocardial infarction, although increased levels have been observed in acute MI or unstable angina (Chapter 1). The present study shows for the first time that plasma D-dimer antigen levels are significantly increased in both male and female survivors of premature myocardial infarction (Table 4.2). When multivariate analysis including cardiovascular risk factors was performed, these increases remained statistically significant. This is consistent with the lack of correlation of D-dimer with risk factors which was found in the population survey reported in Chapter 3.

The increase in plasma VWF antigen levels in survivors of premature myocardial infarction is consistent with the two previous published reports (Hamsten et al 1985; Schmitz-Huebner et al 1988). On multivariate analysis, the association of VWF with previous myocardial infarction remained statistically significant in women but not in men (Table 4.2). Similarly, Hamsten et al (1985) observed

higher levels of VWF in female survivors of myocardial infarction than in controls, but no significant difference in men.

Many previous studies have shown increased plasma fibrinogen levels in IHD (reviewed by Ernst and Resch 1993, and Lowe et al 1995), and similar increases found in the present study of survivors of myocardial infarction are consistent with these previous observations. This increase in fibrinogen may also explain in part the increase in red cell aggregation in these individuals. As discussed in Chapter 3, it is possible that the associations of PAI, tPA, VWF, D-dimer, fibrinogen and red cell aggregation with myocardial infarction may be partly due to chronic inflammation (?in arteries) and release of cytokines such as interleukin-6. Further studies are required to address this possibility.

The associations of plasma PAI, tPA, VWF, D-dimer and fibrinogen with myocardial infarction may also be due to underlying coronary artery atherosclerosis, and this is in keeping with the recently published results of the ECAT Angina Pectoris Study (1993). In this study 3,000 patients undergoing coronary angiography were examined, and it was found that those with coronary atherosclerosis had higher levels of PAI activity, tPA antigen and fibrinogen than those with no coronary atherosclerosis. In that study however, neither PAI activity nor tPA antigen were related to the extent or grade of atherosclerotic stenosis; although fibrinogen was.

Atherosclerosis is a generalised disease and although it occurs in the coronary arteries, the majority of atherosclerotic lesions are found in the peripheral arteries supplying the lower limbs. Studies in our laboratory have shown that PAI, tPA, VWF, D-dimer and

fibrinogen are also associated with peripheral arterial disease (Reid 1991; Smith et al 1993; Lee et al 1995), and that D-dimer and fibrinogen are also associated with the extent of disease (Lee et al 1995; Woodburn et al 1995).

The association of PAI, tPA, VWF, D-dimer and fibrinogen with previous myocardial infarction observed in the present study suggests the possibility that increased levels of these variables might be consequences of arterial disease. To investigate the possibility that these increased levels might precede the onset of detectable cardiovascular disease requires prospective studies. Chapter 5 reports the predictive value of these variables in a cohort of men mostly free from detectable cardiovascular disease (the Caerphilly Heart Study); and Chapter 6 reports the predictive value of these variables in a cohort of men and women with peripheral arterial disease (the Edinburgh Claudication Study).

CHAPTER 5

PROSPECTIVE STUDY OF THE PREDICTIVE VALUE OF PLASMA PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN ACTIVATOR ANTIGEN, D-DIMER ANTIGEN AND VON WILLEBRAND FACTOR ANTIGEN FOR ISCHÆMIC HEART DISEASE EVENTS: THE CAERPHILLY HEART STUDY

INTRODUCTION

Interest in the role of measurements of the fibrinolytic system in the prediction of ischæmic heart disease (IHD) has grown in recent years. This is partly due to the results from the Northwick Park Heart Study which have shown an association between low fibrinolytic potential of blood, as measured by the dilute whole blood clot lysis time, and the incidence of IHD (Meade et al 1986). In studies using more specific measurements of the fibrinolytic system, high plasminogen activator inhibitor (PAI) was a risk factor for recurrent infarction in young male survivors of myocardial infarction (Hamsten et al 1987); as were tissue plasminogen activator (tPA) and von Willebrand factor (VWF) (Jansson et al 1991 a, b;1993).

In 1992, there were no published studies of the predictive value of PAI, tPA, fibrin D-dimer or VWF in healthy persons. Discussions with Dr JWG Yarnell in 1992 indicated that stored, unthawed citrated plasma samples were available from the Caerphilly Heart Study examination in 1984-1986, on over 2000 men, of whom over 100 had since experienced ischæmic heart disease events on follow-up. Funding was therefore obtained to perform assays of PAI, tPA, D-

dimer and VWF in these samples, and to examine their predictive value for ischæmic heart disease events. The predictive value of fibrinogen, plasma viscosity and white cell count for ischæmic heart disease events in this cohort has already been reported (Yarnell et al 1991).

SUBJECTS AND METHODS

Study Population

The Caerphilly Heart Study is a study of risk factors (including hæmatological risk factors) for IHD in middle-aged men in Caerphilly, South Wales (Yarnell et al 1991). The original cohort of 2512 men aged 45 to 59 years was recruited between 1979 and 1983, and since then they have been re-examined at five-yearly intervals. The men whose stored samples were studied were those seen at the first re-examination between 1984 and 1988, when they were aged 49 to 65 years. Men of the same age who had moved into the defined geographical area since the original recruitment were also eligible to be examined. A total of 2398 men attended the study clinic and a fasting blood sample was obtained from 2223 (93%) of them.

The men were invited to attend afternoon or evening clinics, where a detailed medical and lifestyle history was obtained, the London School of Hygiene and Tropical Medicine (LSHTM) chest pain questionnaire was administered, a full 12 lead electrocardiogram (ECG) was recorded, and weight and blood pressure measured. The men were then invited to return, fasting, to an early morning clinic where a blood sample was taken.

Blood Collection, Storage and Analysis

Blood was taken between 07.00h and 10.00h for 91% of the men. It was taken before 07.00h for 7% and between 10.00h and 11.00h for the remaining 2%. The blood was collected into evacuated containers and syringes using a 19-gauge butterfly needle and Sarstedt monovette adaptors. Venous stasis was removed as soon as blood flow commenced. Centrifugation was carried out within the hour, and citrated plasma stored at -70°C. One batch of samples was unavailable for the current analysis so that VWF, tPA, and D-dimer antigens were measured on 1998 fasting samples. Another batch had been thawed on one occasion and was therefore unsuitable for assay of PAI activity, which was measured on 1569 samples. I performed these measurements during 1994, when the plasma had been stored for between 6 and 10 years, using the methods described in Chapter 2.

Incident IHD

Follow-up was performed by Mr P Sweetnam, Dr P Elwood, Dr JWG Yarnell and colleagues in the MRC Epidemiology Unit (South Wales), Cardiff at an average interval of 61 months. All men were flagged with the National Health Service Central Registry (NHSCR), and death certificates coded to International Classification of Disease (ICD) 410-414 inclusive were used as the definition of fatal IHD. Some questions about admission to hospital with chest pain were added to the LSHTM questionnaire. These, together with lists from Hospital Activity Analysis (HAA) of all men admitted to local hospitals with a

diagnosis of ICD 410-414, were used as the basis for a search of hospital notes for events meeting standard WHO criteria (WHO 1976) for acute myocardial infarction (MI). Finally, the appearance on the follow-up ECG of major or moderate Q-waves (Minnesota codes 1-1 or 1-2) when there were no Q-waves on the recruitment ECG was taken as evidence that a non-fatal MI had occurred during the follow-up period.

Using these definitions there were 129 major incident IHD events among the 1998 men who had measurements of tPA, D-dimer and VWF antigens, and 103 events among the 1569 men who had PAI activity measured. The average annual incidence of IHD events was 1.3%.

Statistical Methods

Statistical analysis was performed by Mr P Sweetnam in Cardiff after discussions with myself and Professor G D O Lowe. Adjusted mean differences (Table 5.1) were obtained by analysis of covariance, in which fibrin D-dimer was transformed to logarithms because of its positively skewed distribution. The remainder of the analysis was performed using multiple logistic regression analysis with the occurrence, or not, of a major incident IHD event as the dependent variable. The hæmostatic factors were treated in two ways. First, their distributions were divided into equal `fifths' using the four quintiles, and the results presented as the odds of major incident IHD in each `fifth' relative to a baseline `fifth' which was always taken as the 20% of men with the lowest levels. Second, the hæmostatic factors were entered into the same models as continuous variables to provide a test

Table 5.1

Mean levels and age-adjusted mean differences (with 95% confidence intervals) between men who developed major IHD and those who did not.

	No IHD		IHD		Age-adjusted mean*
	n	Mean* (SD)	n	Mean* (SD)	difference (95% CI)
PAI (%)	1466	134 (56)	103	143 (60)	+9.8 (-1.5 to +21.1) p=0.09
t-PA (ng/ml)	1869	11.6 (4.1)	129	12.6 (4.4)	+0.9 (+0.2 to +1.7) p=0.02
D-dimer (ng/ ml)	1869	70.7	129	89.6	+0.21 (+0.11 to +0.30) p<0.0001
VWF (iu/dl)	1868	119.2 (41.5)	129	128.0 (43.7)	+7.4 (+0.1 to +14.8) p=0.05

* D-dimer was transformed to logarithms. The transformed back mean values are therefore geometric means. The age-adjusted mean difference is given on the logarithmic scale.

for the trend in the relative odds of IHD. When used as a continuous variable, D-dimer was again transformed to logarithms. Other risk factors included in multivariate analysis were age, smoking habit, diastolic blood pressure, body mass index, total and HDL cholesterol, personal history of diabetes, family history of myocardial infarction before age 55 years, and time of blood sampling.

Men with evidence of ischæmia at recruitment were not excluded from the analysis. Reasons for this are given in detail elsewhere (Yarnell et al 1991). Briefly, 31% of these men had some evidence of ischæmia at baseline, as judged from the LSHTM chest pain questionnaire or the ECG. Exclusion of such a large group, among whom just over half of the major incident events occurred, did not seem satisfactory. Instead, we included standardised measures of angina, history of severe chest pain and ECG ischæmia at baseline as three covariates in the logistic regression analysis. This is likely to be a conservative procedure in that it may underestimate the association between the hæmostatic factors and incidence of IHD.

RESULTS

Mean plasma levels of PAI activity and tPA antigen were higher in the present study than in the Glasgow MONICA study (Table 5.1), probably because most men were sampled in the early morning (Chapter 3). Plasma levels of PAI, tPA, VWF antigen and D-dimer antigen were all higher among the men who developed major IHD, as shown in Table 5.1. The age adjusted mean difference for PAI activity of 9.8% pool units (95% CI from -1.5 to +21.1% pool units), which was not statistically significant ($p>0.05$). The age adjusted mean difference

in tPA was 0.9 ng/ml with a 95% CI of 0.2 to 1.7 ng/ml (p=0.02). Much the strongest association of IHD was with D-dimer. Geometric mean D-dimer was 89.6 ng/ml among the men who developed major IHD compared with 70.7 ng/ml amongst those who did not (p<0.0001). The age adjusted mean difference in log (D-dimer) of +0.21 implies that the age adjusted geometric mean level was 23% higher, with a 95% CI from 12% to 35%. The age adjusted mean difference for VWF was 7.4 iu/dl (95% +0.1 to +14.8) (p=0.05).

Table 5.2 and figure 5.1 show incidence and relative odds of IHD by `fifths' of PAI activity. There was some suggestion of a trend of increasing incidence of IHD with increasing PAI activity in both the unadjusted results and on adjustment for age and smoking habit. However the trend is not statistically significant, and was much reduced on adjusting for risk factors and evidence of ischæmia at baseline. Further controlling for tPA, with which PAI was strongly correlated (r=+0.45) removed any suggestion of a trend.

For tPA antigen (Table 5.3, figure 5.1) the incidence of IHD increased from 4.5% in the 20% of men with the lowest levels to 7.8% in the 20% with the highest levels. Unadjusted, the trend for incidence of IHD to increase with increasing tPA was statistically significant (p=0.009) and remained so on adjusting for age and smoking habit (p=0.03). However, the trend became non-significant after adjusting for other risk factors and evidence of ischæmia at baseline.

Table 5.4 and figure 5.1 show a very strong trend (p<0.0001) for incidence of IHD to increase from 3.0% in the 20% of men with the lowest levels of D-dimer antigen to 11.7 % among the 20% with the

Table 5.2

Incidence and relative odds of IHD according to 'fifths' of the level of PAI activity

	'Fifths' of PAI activity (range in %pool units)					
	1 (<87)	2 (87-110)	3 (111-141)	4 (142-183)	5 (>183)	p for trend
Incidence of IHD						
Number(%)	14(4.5%)	25(8.0%)	16(5.2%)	22(7.1%)	24(7.8%)	
Relative odds of IHD						
Model:						
PAI alone	1.0	1.83	1.15	1.61	1.78	0.16
PAI, age, smoking	1.0	1.86	1.22	1.71	1.86	0.14
+other risk factors	1.0	1.88	1.13	1.38	1.53	0.48
+tPA	1.0	1.77	1.01	1.18	1.25	0.87

Table 5.3
Incidence and relative odds of IHD according to 'fifths' of the level of tPA antigen

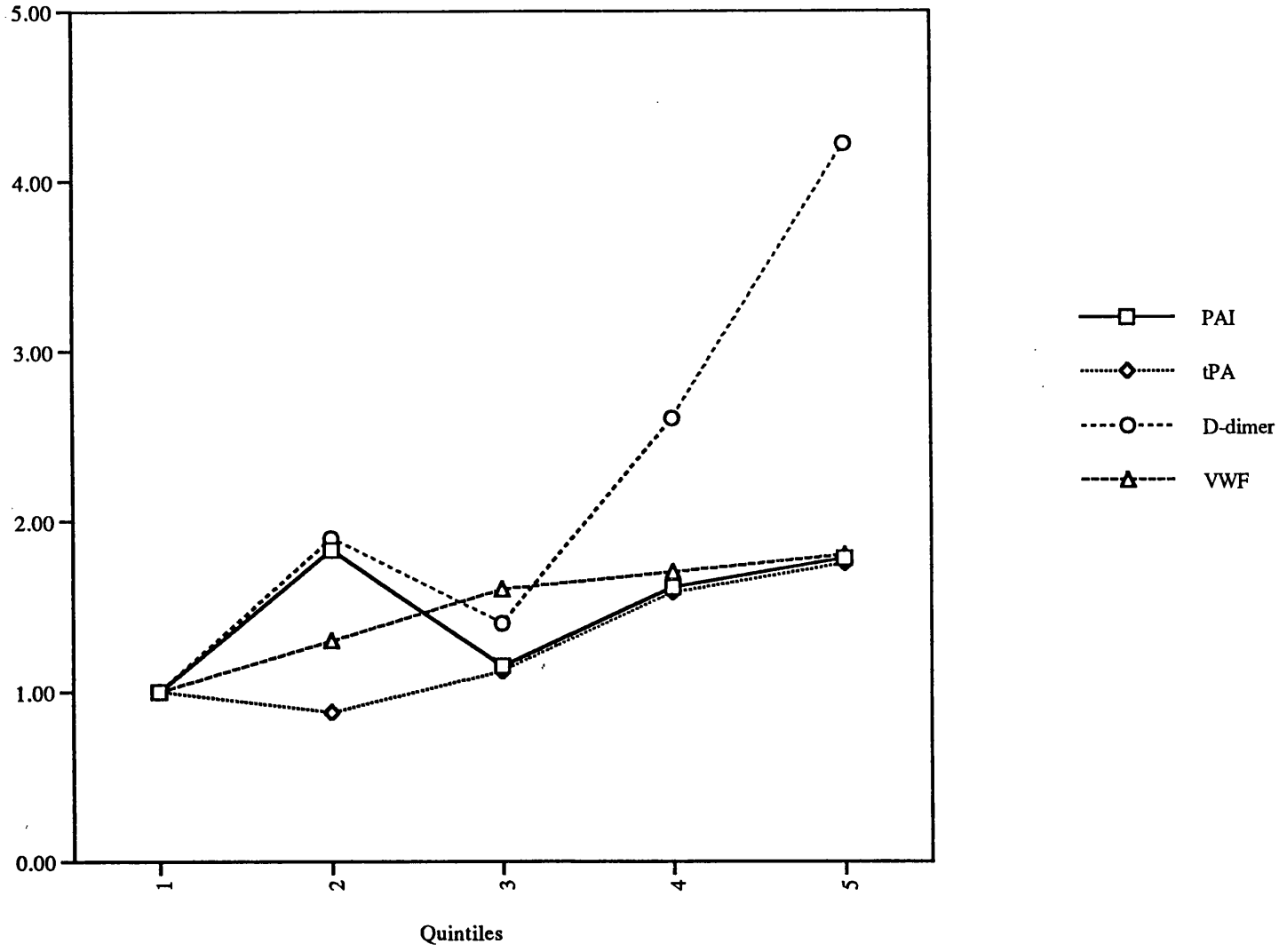
	'Fifths' of tPA antigen (range in ng/ml)					
	1 (<8.2)	2 (8.2-10.3)	3 (10.4-12.3)	4 (12.4-14.8)	5 (>14.8)	p for trend
Incidence of IHD						
Number(%)	14(4.5%)	25(8.0%)	16(5.2%)	22(7.1%)	24(7.8%)	
Relative odds of IHD						
Model:						
tPA Ag alone	1.0	0.88	1.12	1.58	1.75	0.009
tPA Ag, age, smoking	1.0	0.87	1.14	1.51	1.58	0.03
+other risk factors	1.0	0.77	0.93	1.16	1.14	0.34

Table 5.4

Incidence and relative odds of IHD according to 'fifths' of the level of fibrin D-dimer antigen

	'Fifths' of D-dimer antigen (range in ng/ml)					
	1 (<47)	2 (47-61)	3 (62-78)	4 (79-106)	5 (>106)	p for trend
Incidence of IHD						
Number(%)	12(3.0%)	22(5.6%)	16(4.2%)	29(7.6%)	46(11.7%)	
Relative odds of IHD						
Model:						
D-dimer alone	1.0	1.90	1.40	2.61	4.22	<0.0001
D-dimer, age, smoking	1.0	1.89	1.34	2.41	3.81	<0.0001
+other risk factors	1.0	1.88	1.43	2.19	3.53	0.0003
+fibrinogen	1.0	1.88	1.42	2.16	3.48	0.0008

Figure 5.1 Relative Odds of IHD by Haematological Variables



highest levels. Unadjusted, the relative odds increased fairly steadily to 4.22 (95% CI 2.23 to 7.98). Controlling for all risk factors and evidence of ischæmia at baseline reduced the trend, but it remained highly statistically significant (p=0.0003) and the relative odds in the top 20% were still 3.53 (95% CI, 1.82 to 6.85). The further addition of plasma fibrinogen (Yarnell et al 1991) to the model made no material difference to the association between D-dimer and incident IHD.

Table 5.5 and figure 5.1 show incidence and relative odds of IHD by `fifths' of VWF antigen. There is a clear trend of increasing incidence of IHD with increasing VWF in both the unadjusted results, and on adjustment for risk factors.

DISCUSSION

In this study, we have found strong evidence for an independent association between plasma fibrin D-dimer and incident IHD. D-dimer is a breakdown product formed when plasmin acts on cross-linked fibrin and can therefore be considered to be an indirect measure of fibrin turnover. The present study therefore suggests that increased fibrin turnover is a risk predictor for ischæmic heart disease.

Since we performed the present study, Ridker et al (1994a) have also associated elevated levels of D-dimer with increased risk of IHD, although in their study, D-dimer did not appear to be an independent predictor when other risk factors were included in multivariate analysis. However, the study of Ridker et al (1994a) was a nested case-control study in a selected group of United States physicians, whereas the present study was a cohort study in an unselected

population. We found that D-dimer was still a very strong predictor of IHD after controlling for a large set of risk factors and for baseline evidence of ischæmia. Fully adjusted relative odds of IHD increased fairly steadily to 3.53 and the test for trend was highly statistically significant (p=0.0003). This association between D-dimer and incident IHD was found both in the 69% of men with no evidence of ischæmia at recruitment and among the 31% who did have such evidence. The group of risk factors adjusted for included fibrinogen, so that the association between D-dimer and IHD does not arise simply because both are associated with fibrinogen (Yarnell et al 1991).

Our finding of an independent predictive value of plasma VWF antigen for IHD is consistent with the recent reports of Meade et al (1994a) in healthy men, of Thompson et al (1995) in patients with angina pectoris, and of Janssen et al (1991a) in myocardial infarction. These findings all support a role for this marker of endothelial disturbance in prediction of ischæmic heart disease.

The present study found only a weak, non-significant relationship between PAI activity and incident ischæmic heart disease. Hamsten and colleagues (1987) found that PAI activity was a risk factor for recurrent MI in men with a first myocardial infarction before age forty-five years. Meade and colleagues (1986, 1993a) found that low fibrinolytic activity, as measured by the dilute whole blood clot lysis time, was a predictor of IHD in men initially aged 40 to 54 years. They found no association in men aged 55 to 64 years at recruitment. None of the men in the present study were younger than 45 at baseline, but one third were aged 45 to 56 years. There was no suggestion in our data of a stronger association between PAI activity

and incident IHD among these younger men. Our results are consistent with those of Ridker et al (1992) who found no association between PAI antigen and incident IHD among the apparently healthy United States doctors in the Physician's Health Study. Furthermore, both Jansson et al (1991b;1993) and Thompson et al (1995) found no association between PAI activity and subsequent cardiovascular events among subjects with angina pectoris. It therefore appears that PAI activity may only predict ischæmic heart disease in young men.

We found a significant (p=0.009) trend for incident IHD to increase with increasing levels of plasma tPA antigen, such that relative odds of IHD in the 20% of men with the highest levels of tPA antigen were 1.75 (95% CI 0.99 to 3.10). However, that trend was largely abolished on adjusting for other cardiovascular risk factors, in particular serum lipids, with which tPA antigen is correlated (Chapter 3). This finding is remarkably similar to that of Ridker et al (1993a,b). Jansson et al (1991a; 1993) and Thompson et al (1995) also found a positive association of tPA with ischæmic heart disease events, but in their large groups of patients with severe angina pectoris, the association remained after adjusting for other risk factors. These contradictory findings may be related to the fact that the mean difference in tPA between the group who developed IHD events and the group who did not was 1.6 ng/ml in the study of Jansson et al (1993) and 1.9 ng/ml in the study of angina patients by Thompson et al (1995), but only 1.2 ng/ml in the study of Ridker et al (1993a) and 0.9 ng/ml in the current study. Furthermore, an independent association of tPA with prevalent ischæmic heart disease was not found in our case-control study (chapter 4).

It is necessary to consider the limitations and possible biases in the current study. The most serious potential limitation is that the plasma samples were stored for between six and ten years before analysis. There are at least three reasons for assuming that long-term storage has not affected our results. Firstly, the levels obtained are similar to those reported from other studies, and are similar (allowing for diurnal variation) to those found when measuring samples after short-term storage in healthy persons in our laboratory (chapter 3). Secondly, the associations that we found between PAI, tPA, D-dimer, VWF and other cardiovascular risk factors in the present study were very similar to those reported by our own laboratory in the Glasgow MONICA study in Chapter 3 (unpublished data). Thirdly, errors introduced by storage would be likely to obscure associations rather than to generate them, and we have found associations between these variables and incident IHD which are similar to those reported from other studies. Another potential problem was that one batch of samples had been thawed on one occasion. Because thawing can affect PAI activities, this was not measured on this sample set. Differences in mean tPA, D-dimer and VWF levels between thawed and unthawed samples were trivial (data not shown).

Because of the weak associations between D-dimer or VWF antigen levels and standard cardiovascular risk factors (chapter 3), they may add significantly to predicting the risk of ischæmic heart disease in the general population. Their independence as risk predictors is consistent with their independent associations with prevalent myocardial infarction in the case control study reported in chapter 4. Taken together, the results of the studies reported in

chapter 4 and the present chapter support roles for increased endothelial disturbance and fibrin turnover in ischæmic heart disease.

The predictive value of D-dimer and VWF antigens for ischæmic heart disease in patients with established arterial disease (claudication) are considered in Chapter 6.

CHAPTER 6

PROSPECTIVE STUDY OF THE PREDICTIVE VALUE OF PLASMA FIBRIN D-DIMER ANTIGEN, VON WILLEBRAND FACTOR ANTIGEN, AND FIBRINOGEN FOR THE PROGRESSION OF PERIPHERAL ARTERIAL DISEASE AND THE RISK OF ISCHÆMIC HEART DISEASE IN CLAUDICANTS; THE EDINBURGH CLAUDICATION STUDY.

INTRODUCTION

In patients with clinically evident ischaemic heart disease (IHD), longitudinal studies have shown that an increased risk of major cardiovascular events is related to several abnormalities in fibrinolysis and coagulation when measured at baseline. These include fibrinogen (Cooper and Douglas 1991; Martin et al 1991), tissue plasminogen activator antigen (tPA) (Jansson et al 1991b), von Willebrand factor (VWF) (Jansson et al 1991a) and plasminogen activator inhibitor (PAI) (Hamsten et al 1987).

In a previous case control study from this department and from the Wolfson Unit for Prevention of Peripheral Vascular Diseases, University of Edinburgh, we have shown that peripheral arterial disease in a population was associated with increased fibrinogen, increased fibrin turnover as measured by fibrin D-dimer and increased endothelial disturbance as shown by higher levels of VWF. Raised levels of PAI-1 were also observed (Smith et al 1993). Funding was therefore obtained for a prospective study of patients with intermittent claudication which aimed to relate D-dimer, VWF and

fibrinogen to the progression of peripheral arterial disease (as measured by the ankle-brachial pressure index (ABPI), and the risk of ischæmic heart disease (IHD).

SUBJECTS & METHODS

The Edinburgh Claudication Study was performed in collaboration with Drs FGR Fowkes and E Housley of the Wolfson Unit for Prevention of Peripheral Vascular Diseases, University of Edinburgh. 742 consecutive patients with intermittent claudication diagnosed in the Peripheral Vascular Clinic in the Royal Infirmary of Edinburgh were followed up for 1 year. This is the only such clinic in Edinburgh. Patients were excluded from the study if they had rest pain, gangrene, ulceration or impending angioplasty or arterial surgery. 617 patients without such exclusions were then entered into the study, which was in keeping with the power calculation that 600 patients would be needed to detect a 9% difference in mean fibrinogen level between those who would and those who would not have a coronary event during the 1 year follow-up.

The London School of Hygiene and Tropical Medicine (LSHTM) questionnaire on intermittent claudication and angina was completed by each patient at the outset of the study. This questionnaire also included details of smoking and diabetic status. Pressure measurements were made in one leg and arm, from which the Ankle Brachial Pressure Index (ABPI) was derived. This index has been shown to be as good an indicator of peripheral arterial disease as other, more invasive measurements (Fowkes et al 1988). The laboratory research assistants were trained in the Department of

Medicine in the standard methods of blood sampling prior to the study. Venous blood was withdrawn for measurement of VWF, D-dimer, haematocrit and fibrinogen, which were assayed as described in chapter 2. The same physical investigations were carried out on the patients 1 year later, but no blood was taken. Deaths were identified from the General Practitioners, relatives and from the Scottish NHS Central Registry. Cardiovascular events identified at the clinic and cardiovascular deaths were confirmed by reviewing medical records and were classified by WHO criteria (WHO 1976).

Statistical analysis was performed by Dr R Elton, Dept. of Public Health Sciences, University of Edinburgh, with input from Dr FGR Fowkes, Professor GDO Lowe and myself. The Wilcoxon rank sum test was used to determine associations between each baseline factor and IHD events. To calculate the odds ratios, the range of values of laboratory tests was divided at the median, or into quintiles. Confidence intervals were calculated by the exact method. The odds ratios approximated to relative risk because of the low number of events. Multiple logistic regression analysis was used to test the significance of sets of factors adjusted for one another, and confidence intervals for odds ratios were calculated with the normal approximation. The association between change in the ABPI and baseline factors was tested by Wilcoxon rank sum test or Spearman rank correlation. Multiple linear regression was used to test for association between final ABPI and sets of factors adjusted for one another and for baseline ABPI.

RESULTS

During the year of follow-up, 32 patients died and 558 returned for the 1 year examination. Ischæmic heart disease events occurred in 36 patients, 15 of which were fatal. Of the non-fatal events, 4 were incident angina and 17 were myocardial infarction. The relationships between the baseline factors and ischæmic heart disease events over the following year are shown in Table 6.1. This shows that fatal ischæmic heart disease events were significantly related to plasma fibrinogen and to plasma D-dimer. D-dimer was the only variable to show a relationship to total ischæmic heart disease events (p=0.0006).

When stepwise multiple logistic regression analysis was carried out (Table 6.2), age and fibrinogen were seen to be independent predictors of ischæmic heart disease death, while these adjustments resulted in D-dimer and other factors losing statistical significance. However, all ischæmic heart disease events were independently predicted by male sex and D-dimer level. Figure 6.1 shows the relative risks for combined ischæmic heart disease events for fifths of D-dimer expressed relative to the lowest quintile. The fourth and fifth quintile groups showed an increased relative risk of 3.3 (95% CI 0.9-14.4) and 4.4 (95% CI 1.3-19.0), respectively.

Plasma VWF showed a non-significant trend to prediction of ischæmic heart disease events (Table 6.1).

The mean reduction in ABPI was 0.010 (SE 0.007). Progression of arterial disease, as measured by a fall in the ABPI, was greater in

Table 6.1

Relative risks (95% confidence intervals) for fatal and non-fatal IHD events within 1 year in relation to hæmatological factors at baseline

	Relative risks (95% CI)		
	Fatal (n=15)	Non-fatal (n=21)	Total (n=36)
Hæmatocrit	0.2 (0.02-1.0)	1.1 (0.4-2.9)	0.6 (0.3-1.3)
Fibrinogen	2.1 (0.7-7.8)*	0.8 (0.3-2.0)	1.2 (0.6-2.4)
von Willebrand factor	2.1 (0.7-8.1)	1.0 (0.4-2.5)	1.3 (0.6-2.8)
D-dimer	3.0 (0.9-13.1)**	2.2 (0.8-6.5)	2.6 (1.2-5.9)†

Relative risks are based on higher relative to lower values divided at the median. Wilcoxon rank sum *p<0.05; **p<0.01; †p<0.01

Table 6.2

Results of stepwise multiple logistic regressions for IHD events

	Predictor	Estimate	se	p
IHD death	Age	0.090	0.036	0.010
	Fibrinogen	0.782	0.252	0.007
Fatal and non-fatal IHD events	Sex	0.953	0.401	0.028
	D-dimer	0.0023	0.0012	0.048

Variables: age, sex, hæmatocrit, plasma viscosity, blood viscosity, von Willebrand factor, D-dimer. The non-significant variables ($p>0.05$) are not included in the table.

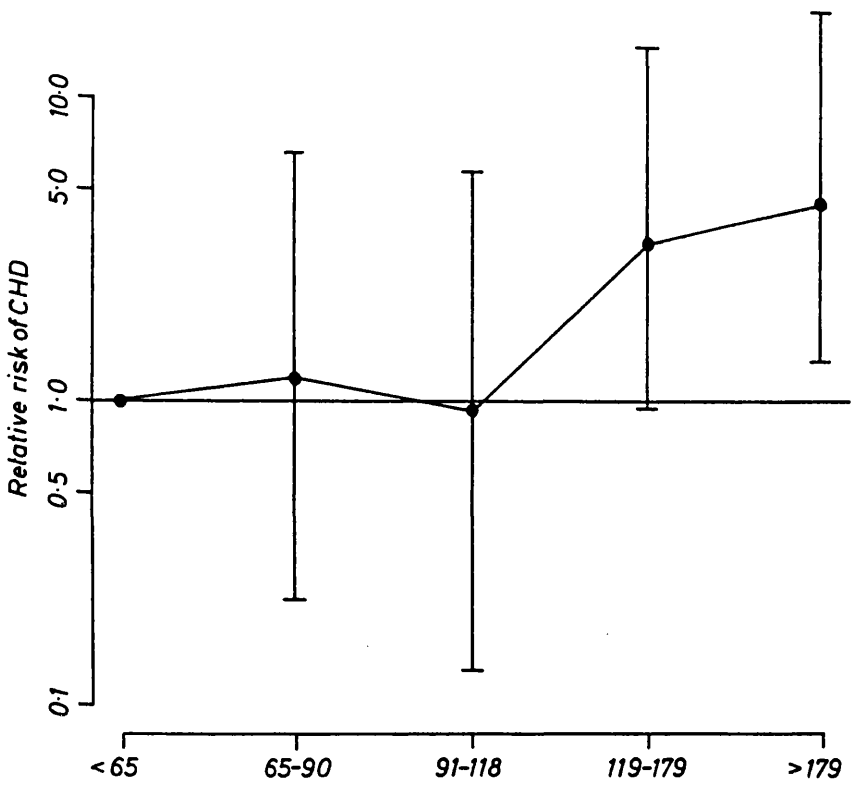


Figure 6.1 Relative risks (95%CI) for fatal and non-fatal IHD within one year for upper four quintiles of D-dimer relative to lowest quintile

smokers than non-smokers after 1 year, but showed no significant correlation with any of the haematological factors measured on univariate analysis. However a stepwise multiple regression showed that only current cigarette smoking (p=0.0004), age (p=0.0051) and D-dimer levels (p=0.0077) were each independently associated with change in final ABPI (Table 6.3).

DISCUSSION

Patients with intermittent claudication have an increased risk of critical limb ischaemia, amputation and cardiovascular events, especially ischaemic heart disease (IHD) events. Previous studies have described higher plasma levels of fibrinogen, D-dimer and VWF in patients with peripheral arterial disease when compared to controls (Smith et al 1993). These variables may play a part in atherogenesis and thrombogenesis.

In this study, plasma fibrinogen levels and age were shown to be the most important independent predictors of ischaemic heart disease deaths in claudicants. This was also observed in the Northwick Park prospective study of patients with stable intermittent claudication (Banerjee et al 1992) and provides further evidence that plasma fibrinogen concentration is both a primary (Ernst and Resch 1993) and a secondary (Cooper and Douglas 1991; Martin et al 1991; Banerjee et al 1992) risk factor for arterial disease.

The findings of this study that plasma fibrin D-dimer levels were predictive of fatal and non-fatal IHD events in claudicants, and that D-dimer had predictive value for the progression of peripheral arterial

Table 6.3

Final results of stepwise multiple regression for prediction of final ankle brachial pressure index (ABPI)

Predictor	Estimate	se	p
Initial ABPI	0.673	0.037	<0.0001
Current smoking	-0.054	0.015	0.0004
Age	-0.0023	0.0008	0.0051
D-dimer	-0.00017	0.00006	0.0077

Variables: age, sex, initial ABPI, current cigarette smoking, hæmatocrit, fibrinogen, plasma viscosity, blood viscosity, von Willebrand factor, D-dimer. The non-significant variables ($p>0.05$) are not included in the table.

disease (as measured by the fall in ABPI) have not previously been reported. The predictive value of D-dimer for IHD in claudicants is consistent with its predictive value for IHD in the Caerphilly Heart Study cohort, described in Chapter 5; and with the recent results of the PLAT study (Cortellaro et al 1993). Increased D-dimer levels have been found in patients with peripheral arterial disease, which correlated with clinical severity (Al-Zahrani et al 1992) and with the extent of disease as measured by the ABPI (Reid 1991; Smith et al 1993; Woodburn et al 1994). This suggests that the increased fibrin turnover in these patients is related to the extent of their arterial disease. These findings are consistent with ongoing fibrin formation and lysis contributing to the progression of both coronary and peripheral atherosclerosis (the Rokitansky-Duguid hypothesis: Duguid 1946).

In this study, a non-significant trend was observed for a predictive value of plasma VWF antigen for IHD events. This is consistent with the results of the Caerphilly Heart Study (Chapter 5) and with the results of studies of survivors of myocardial infarction or angina patients (Jansson et al 1991b; Thomson et al 1995).

CHAPTER 7

PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN AACTIVATOR ANTIGEN AND VON WILLEBRAND FACTOR ANTIGEN IN NON-INSULIN DEPENDENT DIABETIC PATIENTS WITH AND WITHOUT MICROALBUMINURIA.

INTRODUCTION

People with non-insulin dependent diabetes mellitus (NIDDM) have been shown to have increased plasma PAI activity, tPA antigen and VWF antigen levels compared to non-diabetic subjects (Chapters 1 and 3). It has been established that microalbuminuria is a marker for early renal damage and mortality in insulin-dependent diabetes (Viberti et al 1978, 1982). In non-insulin dependent diabetics, albuminuria is associated with increased mortality, and subclinical increases in albumin excretion (microalbuminuria) have been shown to be predictive of death from cardiovascular disease (Mogenson et al 1984; Jarrett et al 1984; Schmitz and Vaeth 1988). Standard cardiovascular risk factors do not completely explain the high prevalence of cardiovascular disease in NIDDM with microalbuminuria (Allawi and Jarrett 1990). Several studies have suggested that haemostatic and fibrinolytic changes may contribute to the pathogenesis of diabetic complications (Osterman and van de Loo 1986; Small et al 1987, 1989; Gough and Grant 1991).

AIMS OF STUDY

In this study the primary aim was to determine if any differences could be found in plasma levels of PAI, tPA, VWF or fibrinogen between NIDDM patients with, and those without, microalbuminuria; and to compare these two groups of patients with matched non-diabetic controls. A second aim was to see if these variables were correlated with two measures of free radical activity, which has been shown to be abnormal in NIDDM subjects (Kaji et al 1985), and which may be implicated in vascular endothelial damage (Blake et al 1985, Stringer et al 1989).

SUBJECTS AND METHODS

24 NIDDM patients were studied; 12 who had microalbuminuria and 12 who had not. 12 matched non-diabetic control subjects were also studied. Patients and controls were selected by Dr. A Collier. Microalbuminuria was determined with an immunoturbimetric assay (Spooner et al 1987), and was defined as an albumin-creatinine ratio of > 3.5 with urinary protein concentration <200mg/L. The mean urinary albumin excretion rates from three timed overnight urine collections ranged from 42 to 140 g/min. None of the patients had any clinical or electrocardiographic evidence of ischaemic heart disease, and all had easily palpable peripheral pulses. Two of the diabetics were taking a cardioselective ß-blocker; otherwise none of the patients were taking medication other than sulphonylureas. In all patients the blood pressure was less than 140/90mmHg. No patient had proliferative retinopathy, but ten patients without

microlbuminuria and all patients with microalbuminuria had background retinopathy. Total cholesterol concentrations were <6.5mM, and triglyceride concentrations were <2.0mM. All patients and controls were venesected in the fasting state between 09.00h and 10.00h. Blood glucose was measured using a glucose oxidase method on a Perspective analyser (American Monitor UK, Burgess Hill, UK), and HbA_1 by gel electrophoresis (Corning, Halstead, UK).

PAI activity, tPA antigen, Von Willebrand factor antigen and fibrinogen were measured as described in Chapter 2.

Linoleic acid (PL-9, 12-LA) and its diene conjugate (PL-9, 11-LA) were measured by high-performance liquid chromatography (HPLC) (Cawood et al 1983; Iverson et al 1985). The ratio was used as a measure of free radical activity. Lipid peroxides were also measured using HPLC and were expressed as malondialdehyde (MDA) (Collier et al 1990). These assays were performed by Mr A G Rumley in the Department of Biochemistry. Gartnavel General Hospital, Glasgow.

Statistical analysis was performed by Dr A Collier. Results are presented as medians and ranges. The three groups were compared using the Kruskall-Wallis analysis of variance, and the possible influences of glycaemic control (as measured by HbA_1 and plasma glucose) and albumin excretion on the variables measured were assessed by multiple regression analysis.

RESULTS

All three groups of subjects were closely age matched, and the diabetic groups were matched for disease duration and glycaemic control (Table 7.1). Increases in plasma PAI activity, tPA antigen, VWF antigen and fibrinogen were found in diabetic subjects when compared with controls ($p<0.05$). In patients with microalbuminuria, further increases were seen in tPA ($p=<0.03$) and in VWF ($p<=0.03$) when compared to normoalbuminuric diabetic patients (Table 7.2). There was no difference in the concentration of linoleic acid (PL-9, 12-LA) between the control subjects and the two diabetic groups. The concentration of the non-peroxide isomer of linoleic acid (PL-9, 11-LA) was higher in the microalbuminuric diabetic group than in control subjects ($p<0.05$). The molar ratio (9, 11-LA: 9, 12-LA) was elevated in both the normoalbuminuric ($p<0.05$) and the microalbuminuric ($p<0.05$) diabetic groups compared with controls; there was no difference between the two diabetic groups (Table 7.2). The concentration of MDA was greater in the microalbuminuric diabetic group compared with the normoalbuminuric diabetic group ($p<0.05$) and the control subjects ($p<0.001$). The concentration of MDA was also elevated in the normoalbuminuric patients compared with the control subjects ($p<0.01$).

No significant correlation was observed between any of the haemostatic or fibrinolytic parameters and either measures of free radical activity, rate of albumin excretion, or glycaemic control.

Table 7.1

Clinical details of control subjects and diabetic patients

	Age (yr)	*n* (M/F)	Duration of Diabetes (yr)	Plasma glucose (mM)	HbA_1 (%)
Controls	50 (39-64)	6/6			
Diabetics					
Normo albuminuric	54 (45-65)	5/7	11 (8-16)	8.2 (6.9-10.7)	9.1 (7.8-10.9)
Micro albuminuric	57 (47-65)	7/5	14 (9-19)	8.0 (6.8-11.1)	9.5 (7.6-11.6)

Results are medians with ranges in parentheses.

The concentrations of hæmatological factors and free radical markers in control subjects and diabetic patients

	PAI (% normal pool)	tPA (ng/ml)	VWF (iu/dl)	Fibrinogen (g/L)	PL-9, 11-LA'/ PL-9, 12-LA (%)	MDA (μM)
Controls	109 (81-220)	6.0 (3.0-8.5)	80 (42-124)	2.6 (1.7-3.5)	1.5 (1.1-2.1)	1.9 (1.1-2.5)
Diabetics						
Normo albuminuric	119 (92-243)	12.5 (4.0-21.0)	81 (51-104)	2.9 (2.1-5.1)	2.1 (0.7-4.2)	2.4 (2.0-2.7)
Micro albuminuric	160 (87-350)	14.0 (10.0-27.5)	110 (64-192)	3.2 (2.3-5.4)	2.3 (1.7-5.5)	3.0 (2.0-4.0)

Values are medians with ranges in parentheses. PL-9, 11-LA, linoleic acid; PL-9, 11-LA', nonperoxide isomer - conjugated diene of linoleic acid; MDA, malondialdehyde.

DISCUSSION

This study confirms the increases in PAI activity, tPA antigen, VWF antigen and fibrinogen in NIDDM patients shown in previous studies (Osterman and van de Loo 1986; Small et al 1989; Kruithof et al 1988; Gough and Grant 1991) and it also shows that these disturbances are more marked in patients with microalbuminuria. There is little published information on the relationship between these variables and albuminuria. Schmitz and Ingerslev (1990) and Stehouwer et al (1992) observed that elevation in VWF was more marked in NIDDM subjects with microalbuminuria than in those without. Gough and Grant (1991) reported a correlation between urinary albumin level and plasma PAI-1 activity and antigen. The present study confirms and extends these findings. The elevations in PAI, tPA and VWF may be due to endothelial disturbance in diabetic subjects: there is evidence for both morphological and functional abnormalities of endothelium in diabetics. Micraoalbuminuria reflects renal glomerular damage, perhaps as part of generalised endothelial disturbance. It is therefore possible that the greater elevation in the endothelial products, tPA and VWF, in NIDDM subjects with microalbuminuria reflects more widespread endothelial disturbance.

The elevation of PAI activity and fibrinogen may be partly due to release of IL6 and other cytokines when monocytes adhere to damaged endothelium (as discussed in Chapter 3).

Elevations in PAI, tPA, VWF and fibrinogen are potential cardiovascular risk factors in non-diabetic subjects (Chapters 5 and 6). In diabetics, fibrinogen and VWF have been shown to predict

cardiovascular events (Breddin et al 1986; Kannel et al 1990; Stehouwer et al 1992). Elevations in these variables may therefore be one possible causal link between microalbuminuria and cardiovascular risk in NIDDM, by increasing the tendency to thrombosis.

Free radical formation, including that produced by activated neutrophils and endothelial cells, is one potential mechanism by which endothelial disturbance may occur in diabetes and its vascular complications (Dormandy 1983; Stringer et al 1989). The results of the present study confirm previous work suggesting increased free radical formation in NIDDM (Jennings et al 1987; Collier et al 1990). Although measures of free radical formation were more elevated in patients with microalbuminuria, there were no significant correlations between these measures and haemostatic factors. A similar lack of correlation was observed between lipid peroxides or diene conjugates and increased levels of PAI, tPA, VWF and fibrinogen in our study of cases of peripheral vascular disease and controls (Smith et al 1993). These data therefore do not support the hypothesis that increased free radical formation is related to haemostatic changes. However further work is required to confirm or reject this hypothesis.

Recently, Shearman et al (1992) reported that exercise in claudicants induced free radical formation and also induced microalbuminuria. They therefore suggested that leg ischaemia might cause free radical- induced renal glomerular damage. Exercise can also induce microalbuminuria in diabetics (Viberti et al 1978).

Endothelial release of PAI-1, tPA and VWF can also be stimulated by insulin-induced hypoglycaemia in insulin-dependent

diabetic patients, and in non-diabetics; and by acute exercise in diabetics (more likely in the younger, more active, less obese IDDM patients than in NIDDM patients) and in normal subjects (Prowse and MacGregor 1988). The last two chapters of the present thesis therefore compared the effects of insulin-induced hypoglycaemia and acute exercise in IDDM patients and in matched non-diabetic controls.

CHAPTER 8

EFFECTS OF INSULIN-INDUCED HYPOGLYCÆMIA ON PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN ACTIVATOR ANTIGEN, FIBRIN D-DIMER ANTIGEN, VON WILLEBRAND FACTOR ANTIGEN AND FIBRINOGEN IN INSULIN-DEPENDENT DIABETIC PATIENTS AND NON-DIABETIC CONTROLS.

INTRODUCTION

In humans, catecholamines (adrenaline and noradrenaline) are released in response to acute hypoglycæmia (Gader et al 1974). This catecholamine release has a number of effects on the blood, including decreased plasma volume and increases in haematocrit (Hilsted et al 1985), white cell and erythrocyte counts (Frier et al 1983, 1988) and platelet aggregation (Hutton et al 1979). Few studies have looked at the effects of acute hypoglycæmia, which stimulates catecholamine release, on fibrinolysis or hæmostasis. Adrenaline has been shown to increase fibrinolytic activity (Macfarlane and Biggs 1948), and factor VIII activity (Ingram 1961) and to mediate VWF and tPA release from endothelium via ß adrenergic mechanisms (Cryer 1980; Grant et al 1987; Prowse and MacGregor 1988). Arginine vasopressin release may also play a role (Fisher et al 1987); while growth hormone probably does not (Lowe et al 1982). Hyperinsulinaemia without hypoglycaemia does not result in changes in VWF or global fibrinolytic activity, excluding a direct effect of insulin on these variables (Grant et al 1987). DDAVP, a synthetic analogue of arginine vasopressin, has been shown to increase factor VIII, VWF and fibrinolytic activity (Mannucci

et al 1977), and can be used in the treatment of acquired and congenital bleeding disorders.

As discussed in Chapters 1 and 3, non-insulin dependent diabetic (NIDDM) patients have increased levels of PAI, tPA, VWF and fibrinogen. In insulin dependent diabetes mellitus (IDDM) increasing levels of VWF are seen, but there are conflicting reports on changes in fibrinolytic activity, PAI and tPA (Fuller et al 1979; Gough and Grant 1991). In IDDM, it has been suggested that insulin-induced hypoglycaemia may be one mechanism causing increased release of VWF from endothelium and therefore increased plasma VWF levels (Lowe et al 1982). The effects of insulin on PAI and tPA in diabetic and non-diabetic subjects are conflicting (Prowse and MacGregor 1988; Grant et al 1987; Gough and Grant 1991). Insulin has been shown to cause the release of PAI-1 from cultured hepatoma cells (Alessi et al 1988). PAI-1 levels in obese non-diabetics and in NIDDM have also been shown to correlate with endogenous insulin levels (Juhan-Vague et al 1988). However a study looking at short-term hyperinsulinaemia without hypoglycaemia in diabetics showed no effect on the euglobulin clot lysis time, a global test of fibrinolytic activity (Grant et al 1987).

AIM OF STUDY

The aim of this study was to examine the effects of acute hypoglycaemia on levels of PAI activity, tPA antigen, and VWF antigen (as markers of endothelial disturbance), as well as D-dimer and fibrinogen. Normal subjects were compared with insulin- dependent diabetics in order to determine if there were any differences in the basal levels and/or pattern of change between the two groups.

SUBJECTS AND METHODS

Six normal healthy male volunters were used as controls. They were aged between 21-30 years, had a normal body mass index and none were taking medication. The diabetic patients aged 20-39 years (mean age 27 years) were recruited by Dr. M Fisher from the diabetic clinic at Glasgow Royal Infimary. To confirm the absence of endogenous insulin, plasma concentrations of C-peptide were measured and found to be <0.3 nmol/l, both fasting and in response to 1.0 mg intravenous glucagon. The diabetic patients had poor glycaemic control, with glycated haemoglobin values of >10% (normal <8.5%). Four of the patients had diabetes of less than 5 years duration, one had duration of diabetes of 17 years, and one of 25 years (mean duration 8 years.) Direct optical ophthalmoscopy detected mild diabetic retinopathy in the two patients with the longer duration of disease. No clinical evidence of peripheral neuropathy was found and none of the patients showed laboratory evidence of microalbuminuria based on a latex agglutination test (Spooner et al 1987).

Subjects were maintained supine after an overnight fast and an indwelling cannula was inserted into an anticubital vein, and kept patent with 150mM saline. At 06.00h an intravenous bolus of short acting insulin (Human Actrapid; Novo, Basingstoke, Hants.) 0.15 u/kg body weight was given and the time to the onset of the acute autonomic response (R) was recorded. This reaction is manifest as an abrupt rise in heart rate and the onset of autonomic symptoms (such as sweating) and neuroglycopenic symptoms including confusion and

drowsiness. In all subjects, R coincided with the nadir of blood glucose. Blood glucose levels were monitored using a Reflolux II glucometer (BCL, Lewes, East Sussex). It was necessary to know the nadir for each individual, as there is a biological variation which must be taken into account when deciding the times of subsequent sampling during the recovery phase from hypoglycaemia. The expected variation was found in the time to reach the blood glucose nadir with a range of 25-45 minutes. To allow for this individual response and make comparisons meaningful, blood sampling during the recovery phase was timed from R. All subjects gave written informed consent to the study, which was approved by the hospital's Ethical Committee.

Blood glucose was measured with a Beckman auto-analyser (Beckman Instruments Inc., Fullerton, California) using the glucose oxidase method. Plasma levels of PAI activity, tPA antigen, VWF antigen, D-dimer antigen, fibrinogen and fibrin plate lysis area were measured as described in Chapter 2. Plasma levels of adrenaline and noradrenaline were measured in the MRC Blood Pressure Unit at the Western Infirmary, Glasgow by radioenzymic assays (Ball et al 1986).

Statistical analysis was performed by Dr. M Fisher using the Starview 512 package (Brainpower Inc., Calabasas, CA, USA) on an Apple Macintosh SE Computor (Apple Computer UK Ltd., Hemel Hempstead, Herts., UK). Changes within a group were compared using Student's t-test for paired data. Comparisons of basal values between the two groups were made using Student's t-test for unpaired data, and comparisons between the groups in response to hypoglycaemia were made by using analysis of variance for repeated measures. The

results are presented as means and SEM. P values of less than 0.05 were considered significant.

RESULTS

Evidence of hypoglycaemia was seen in all subjects with increased heart rate, sweating and moderate neuroglycopenia. The mean time to the acute autonomic response (R) showed no differences in the two groups: normal subjects 26±2 minutes and diabetic patients 30±4 minutes.There were no diferences in heart rate or blood presure responses between the two groups.

Blood Glucose

Similar baseline concentrations of blood glucose were found in patients and controls with a mean of 4.8±0.2 mmol/l in controls and 4.6±0.4mmol/l in patients. The nadir of blood glucose achieved by the two groups was also similar (Figure 8.1).

Catecholamines

In the control group the plasma adrenaline concentrations rose from 0.1±0.1 to 2.4±0.2 nmol/l at R'+15 min (Student's t-test, $p<0.001$). The rise in the diabetic group was less, reaching a maximum of 1.8±0.4 nmol/l (analysis of variance, $p<0.05$). The plasma concentration of noradrenaline increased in response to hypoglycaemia in both groups and no significant difference was observed between the two groups. (Figure 8.2).

Fibrin Plate Lysis Area

Global fibrinolytic activity as measured by the fibrin plate lysis area (FPLA) was higher in the control group than the diabetics (143± 15mm^2 vs 124± 15mm^2 p=0.357). The FPLA rose significantly in the control group to 193± 26mm2 at R +15min (p<0.01); and similarly in the diabetic group to 155± 11mm^2 (analysis of variance p=0.67).

PAI activity.

Baseline PAI was not significantly different in the control and diabetic groups. PAI activity showed a significant fall in controls, dropping from 117±9% to 95±16% at R' (p<0.05) The response of the diabetic patients was not significantly different with a fall from 117±20 % to 82±11 % (analysis of variance p=0.26) (Figure 8.3).

TPA antigen

Basal concentrations of tPA antigen were significantly reduced in the diabetic patients compared to controls, at 3.4±0.7 vs 8.5±1.3 ng/ml (p<0.01). The increase in response to hypoglycaemia was lower than in the control group, but this was not statistically significantly different. tPA antigen levels rose to 11.1 ± 2.5 ng/ml at R+15min in controls, and to 5.3±2.2 ng/ml in diabetics (analysis of variance, p= 0.26) (Figure 8.3).

VWF antigen

The basal levels of VWF antigen in patients and controls were not significantly different, although higher in the diabetics (75±9iu/dl for controls and 104±25iu/dl for patients). There was an increase in VWF levels in both groups in response to hypoglycaemia, and the increase in the diabetic group was significantly greater than the controls (analysis of variance, $p<0.02$). A maximum of 130±30iu/dl was seen in patients at R + 60mins as compared to 109±8iu/dl in the controls (Figure 8.4).

D-dimer antigen

No significant changes were observed in D-dimer levels in response to hypoglycaemia in either group, with concentrations in the control subjects of 36±6 ng/ml basally, 35 ± 5 ng/ml at R, 35 ± 7 ng/ml at R+15min, and 33 ± 8 ng/ml at R+60min; and concentrations in the diabetic patients of 38 ± 4 ng/ml basally, 36 ± 5 ng/ml at R, and 35 ± 5 ng/ml at R+15min, and 35 ± 5 ng/ml at R+60min.

Fibrinogen

Baseline fibrinogen levels were not significantly different in the control and diabetic groups; although they tended to be higher in diabetics. Fibrinogen levels did not change in response to hypoglycaemia (Figure 8.4).

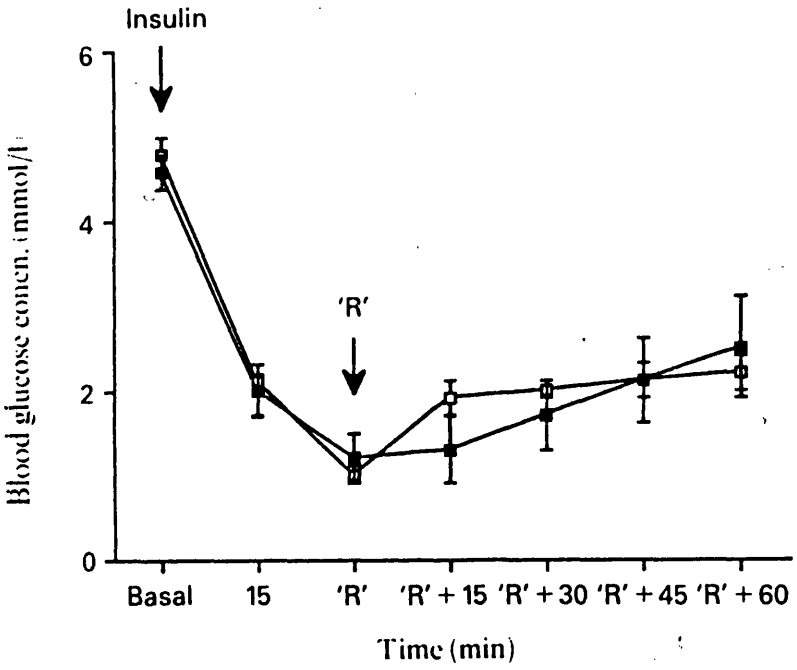


Figure 8.1 Blood glucose in diabetic patients (solid squares) and non-diabetic controls (open squares). Data are presented as means and SEM. R = onset of autonomic response.

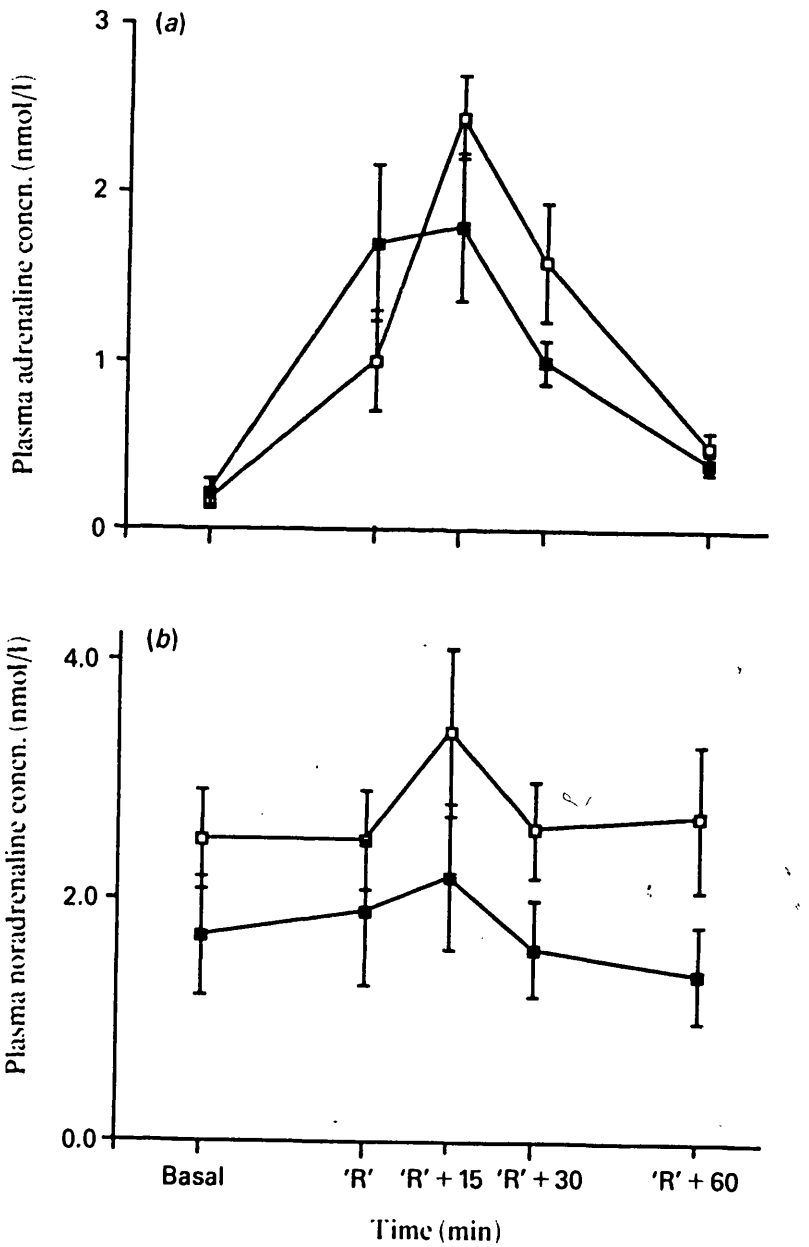


Figure 8.2 Plasma adrenaline and nonadrenaline in diabetic patients (solid squares) and non-diabetic controls (open squares). Data are presented as means and SEM. R = onset of autonomic response.

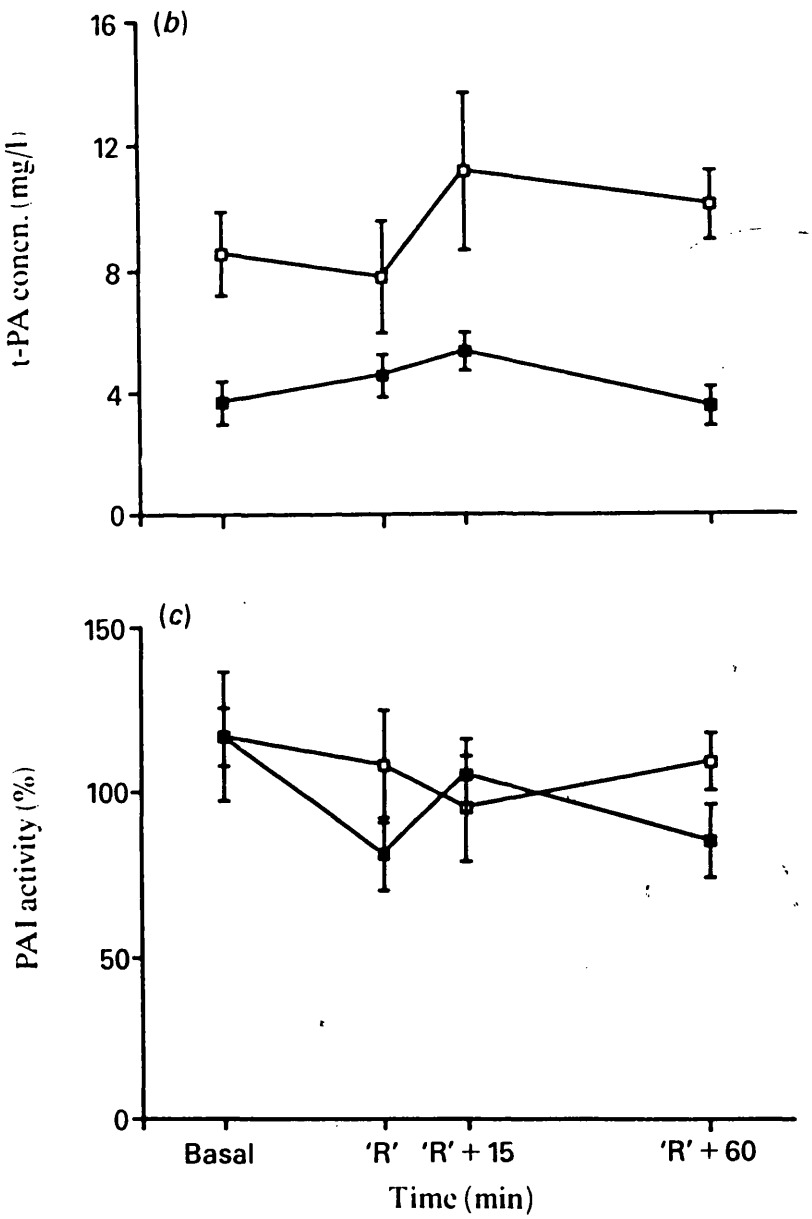


Figure 8.3 tPA antigen and PAI activty in diabetic patients (solid squares) and non-diabetic controls (open squares). Data are presented as means and SEM. R = onset of autonomic response.

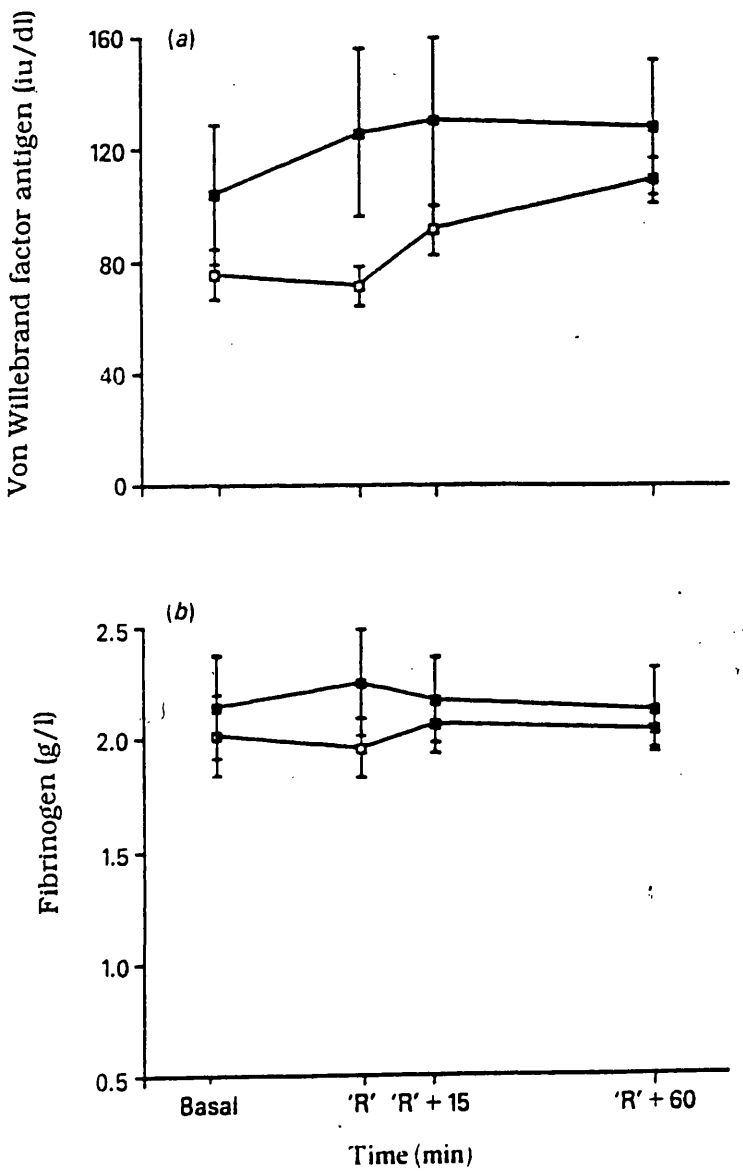


Figure 8.4 Von Willebrand factor and fibrinogen in diabetic patients (solid squares) and non-diabetic controls(open squares). Data are presented as means and SEM. R = onset of autonomic response.

DISCUSSION

In this study, patients with insulin-dependent diabetes mellitus (IDDM) showed a tendency to higher levels of VWF antigen and fibrinogen than in non-diabetic controls, in accordance with previous, larger studies (Osterman and van de Loo 1986). Studies of PAI activity and tPA antigen have shown conflicting results in IDDM (Grant and Medcalf 1990). In the present study, IDDM patients had significantly lower tPA antigen levels, similar levels of PAI activity, and a tendency to lower global fibrinolytic activity, as measured by the fibrin plate lysis area.

In this study, the expected changes in fibrinolysis were observed during acute hypoglycaemia. Total plasminogen activator as measured by the fibrin plate lysis area was increased in both controls and diabetic subjects. tPA antigen was seen to increase and PAI activity to fall in both groups. These findings are consistent with the endothelial release of tPA and the formation of PAI-tPA complexes and clearance from the circulation. Only one previous study (Dalsgaard-Nielsen et al 1982) compared changes in fibrinolysis in insulin-dependent diabetics and non-diabetic controls, using the euglobulin clot lysis time as a global test of fibrinolytic activity. They found a greater increase in fibrinolytic activity ("potential") in controls than in diabetics. The global fibrinolytic response to hypoglycaemia in the present study (as measured by the fibrinolytic activity of the euglobulin fraction on fibrin plates) likewise appeared lower in diabetics although the difference was not statistically significant (Figure 8.3). Likewise, there was a trend for lower tPA antigen increments in diabetics, although this difference also was not statistically significant (Figure 8.3). This

may be a manifestation of endothelial disturbance. On the other hand, VWF release was greater in diabetics (Figure 8.4).

These changes appeared maximal at the time of peak plasma catecholamine activity (Figure 8.1 and 8.2), consistent with the postulated ß adrenergic mechanism of tPA release from endothelium (Prowse and MacGregor 1988).

Despite the hypoglycaemia induced increases in VWF and tPA antigen observed in this study, there was no detectable increase in fibrin formation or lysis, as measured by D-dimer levels. This observation is consistent with previous reports (Marsh and Gaffney 1982).

In conclusion, this study (and that of Dalsgaard-Nielsen et al 1982) suggests that IDDM patients may have impaired endothelial function, as measured by a lesser release of tPA in response to insulin. The power of these studies was limited by their small sample size, which is understandable because few persons are prepared to volunteer for insulin-induced hypoglycaemic stress. The ability of these studies to demonstrate differences betwen subject groups is also limited by the imprecision of timing of maximal autonomic response (R). Nevertheless, the significant acute increase in VWF antigen in IDDM patients suggests that insulin independent hypoglycaemia may be one mechanism for the chronic elevation in plasma VWF in IDDM (Lowe et al 1982). The lower baseline level of tPA antigen in IDDM in the present study is unexplained; although as noted above decreased endothelial release of tPA may be another manifestation of endothelial disturbance in this condition.

CHAPTER 9

THE EFFECTS OF SUBMAXIMAL EXERCISE ON PLASMINOGEN ACTIVATOR INHIBITOR ACTIVITY, TISSUE PLASMINOGEN ACTIVATOR ANTIGEN, VON WILLEBRAND FACTOR ANTIGEN AND FIBRINOGEN IN INSULIN-DEPENDENT DIABETIC PATIENTS AND NON-DIABETIC SUBJECTS.

INTRODUCTION

Patients with insulin-dependent diabetes mellitus (IDDM) may have impaired endothelial response (release of tPA) to insulin-induced hypoglycæmia (Chapter 8). Acute submaximal exercise also releases tPA (and VWF) from endothelium, again via ß adrenergic mechanisms (Prowse and MacGregor 1988). Cash and McGill (1969) showed that IDDM patients had an impaired increase in global fibrinolytic activity (as measured by the euglobulin clot lysis time) after acute exercise compared to non-diabetic matched controls. There are no reported studies of the effects of exercise on specific fibrinolytic variables (PAI or tPA) or VWF in IDDM compared to non-diabetic controls.

AIM OF STUDY

The aim of this study was to investigate changes in PAI activity, tPA antigen, VWF antigen and fibrinogen in response to submaximal exercise in IDDM patients and in matched non-diabetic control subjects.

SUBJECTS AND METHODS

Eight control subjects and eight age matched insulin-dependent diabetics (20-35yrs) were enrolled in the study by Dr. A Collier. All were male. The diabetic patients were poorly controlled (mean HbA_1 10.4 SEM 0.4 %; normal range 5-8%), and duration of diabetes ranged from 4-13 years.

After an overnight fast, an indwelling cannula was inserted into an antecubital vein at 08.00h. In order to prevent hypoglycæmia, patients were given a reduced dose of short-acting insulin 20 min prior to a standard (40G carbohydrate) breakfast.

Resting and treadmill electrocardiography were performed using the Bruce protocol. Control subjects and diabetic patients were exercised until tiredness or until the development of limiting symptoms. Standard 12 lead electrocardiograms were performed before and immediately after exercise, while during the test the heart rate and electrocardiogram were monitored continuously in three electrocardiographic leads. All those involved in the study had normal resting electrocardiograms with no abnormal changes during or after exercise. All subjects gave written informed consent to the study, which was approved by the hospital's Ethical Committee.

Blood samples were taken before and at 0, 15, 30, 60 and 120 minutes after exercise. PAI activity, tPA antigen, VWF antigen, fibrinogen and hæmatocrit were measured as described in Chapter 2. D-dimer levels were not measured because of the lack of change

following hypoglycæmia (Chapter 8) or after exercise as shown in a previous study (Marsh and Gaffney 1982). Glucose, glycated haemoglobin, catacholamines and cortisol were also measured as described in Chapter 8.

Statistical analysis was performed jointly by Dr. A Collier and myself. Changes within a group were compared using Student's t-test for paired data, and basal values between groups using Students's t-test for unpaired data. Comparisons between the groups in response to hypoglycæmia were made using analysis of variance for repeated measures. The results are presented as mean ± SEM.

RESULTS

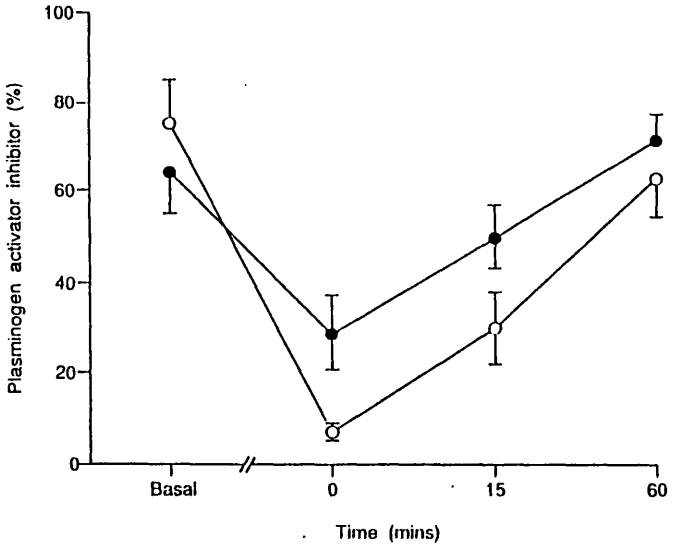
There was no significant difference in the duration of exercise between the control and study groups (15-18mins). There were also no differences in the heart rate and blood pressure measurement between the control subjects and the diabetic patients (data not shown).

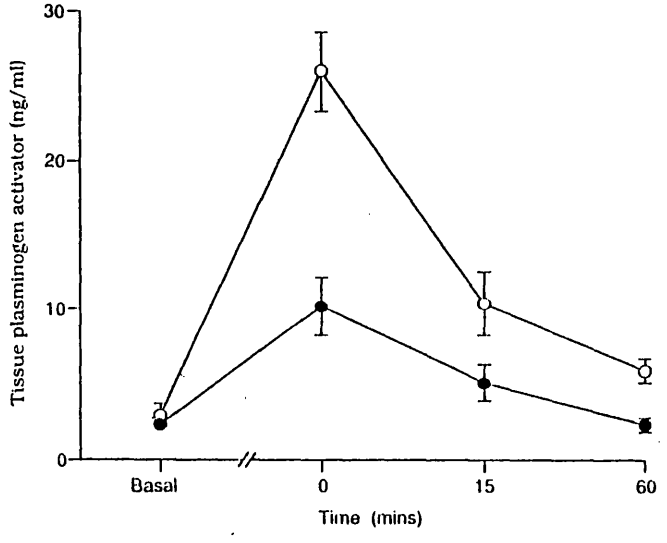
Blood Glucose

As expected, there were significant differences between diabetics and control subjects at all time points in the study (Figure 9.1).

Catecholamines and cortisol

There were no significant differences in noradrenaline, adrenaline (Figure 9.1) or cortisol (Figure 9.3) in reponse to exercise between the two groups.





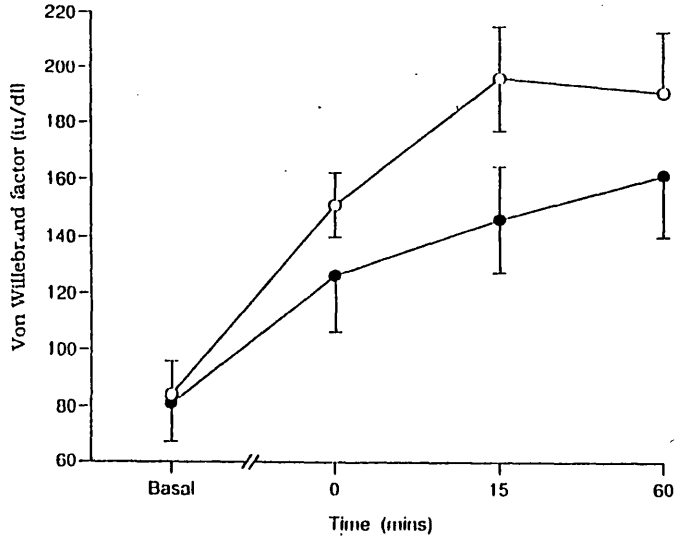
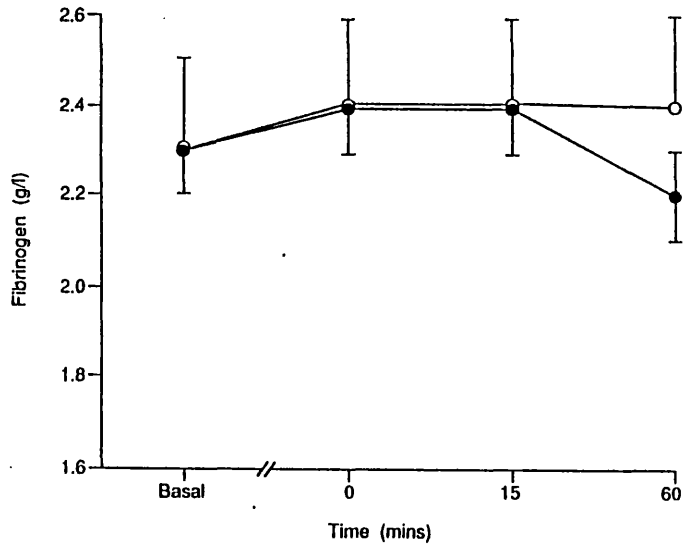
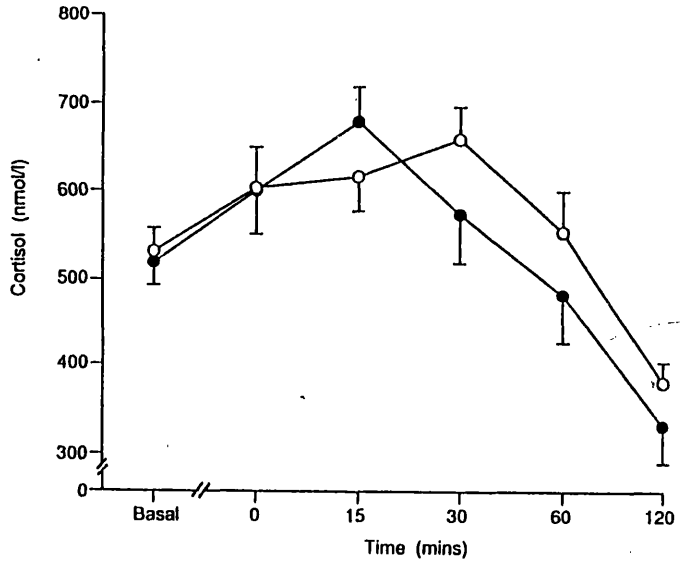


Figure 9.2 Changes in PAI activity, tPA antigen and VWF in response to acute exercise in diabetic patients (solid circles) and non-diabetics (open circles). Data given as mean and SEM.





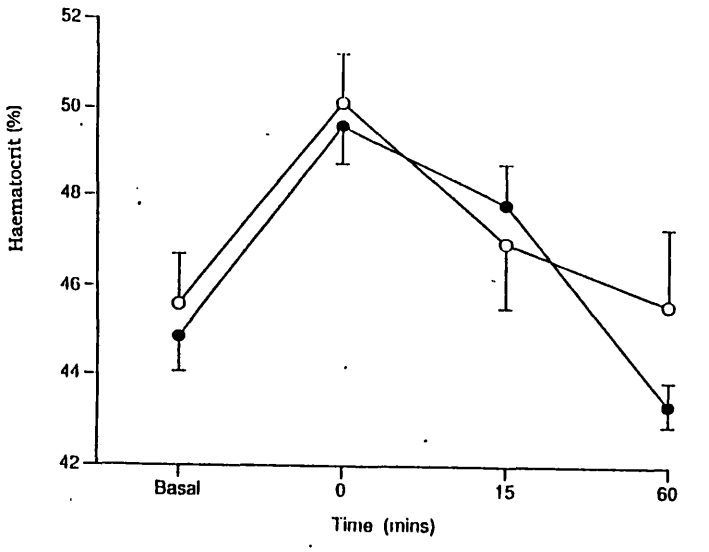


Figure 9.3 Changes in fibrinogen, haematocrit and cortisol in response to acute exercise in diabetic patients (solid circles) and non-diabetics (open circles). Data given as mean and SEM.

PAI activity

Baseline PAI activity was similar in the controls and diabetic group (75 ± 9.9% in controls; 70 ± 7.4% in diabetics), but the fall in PAI activity at the end of the exercise period was more marked in the controls; 75 ± 10% to 7 ± 2% ($p< 0.002$) in controls compared to 75 ± 10% to 29 ± 8%($p<0.02$) in diabetics (Figure 9.2).

tPA antigen

Basal concentrations of tPA were similar in the control and IDDM groups (3.0 ± 0.8 vs 2.4 ± 0.2 ng/ml). However at the end of the period of exercise, tPA antigen levels rose in the control subjects to 26.0 ± 2.6 ng/ml compared to only 10.2 ± 1.9 ng/ml in the diabetic group (analysis of variance $p=0.0019$) (Figure 9.2).

VWF antigen

There was no significant difference between baseline VWF levels in the two groups. There was no statistically significant difference between the two groups in the response of VWF to exercise (Figure 9.2), although a slightly greater increase was observed in the control group, from a basal level of 84 ± 12 iu/dl to a maximum of 186 ± 18 iu/dl 15 mins after exercise ($p<0.0001$). In the diabetic group, the VWF levels rose from a basal level of 81 ± 13 iu/dl to a maximum of 161 ± 21 iu/dl, 60 mins after exercise ($p<0.001$).

Fibrinogen

There was no significant change in the plasma fibrinogen levels in response to exercise in either of the two groups, nor any significant difference in the baseline levels (Figure 9.3).

Hæmatocrit

The hæmatocrit rose significantly from baseline after exercise ($p<0.05$), with a similar increase in both IDDM and control groups. No difference was observed in baseline levels (Figure 9.3).

DISCUSSION

In this study, acute submaximal exercise produced the expected increase in tPA antigen, in both controls and IDDM groups (Prowse and MacGregor 1988). PAI activity levels fell in both groups, consistent with complex formation between tPA and PAI and their clearance from the circulation (Prowse and MacGregor 1988). In the diabetic group, exercise caused a lesser increase in tPA levels, and henceforth a lesser decrease in PAI activity compared with the control subjects. These results are consistent with the study of Cash and McGill (1969) which observed a lower global fibrinolytic response to exercise in IDDM patients; and suggest decreased endothelial response (tPA release) to exercise in IDDM, which may also explain the non-significant trend to lower VWF release in response to exercise. Alternatively, the difference may reflect differences in hepatic blood

flow, which clears tPA from the circulation (de Boer et al 1993). The differences in tPA release cannot be explained by different degrees of hæmoconcentration (increased hæmatocrit, due to decreased plasma volume) in the two study groups (Figure 9.3); nor by different catecholamine or cortisol responses (Figures 9.1 and 9.2).

The smaller increase in tPA release in IDDM patients compared to controls demonstrated in this study are similar to the lower tPA release provoked by insulin-induced hypoglycæmia (Chapter 8), which again may reflect an impaired endothelial response to catecholamine increase.

In conclusion, we have observed that exercise causes smaller increases in tPA antigen, and possibly in VWF antigen, in IDDM patients compared to controls. These differences are consistent with endothelial dysfunction in IDDM.

CHAPTER 10

GENERAL DISCUSSION AND SUGGESTIONS FOR FURTHER WORK

Plasma plasminogen activator inhibitor (PAI) activity

Astrup and Permin (1947) originally proposed that impaired fibrinolysis may lead to decreased removal of arterial fibrin deposits, and thus atherosclerosis and arterial thrombosis. As discussed in Chapter 1, recent studies have confirmed that fibrin plays an important part in atherosclerosis and thrombosis. Plasma levels of plasminogen activator inhibitor (PAI) activity (which in non-pregnant persons is mainly PAI-1 activity) is the main determinant of plasma fibrinolytic potential which it inhibits. High levels of PAI activity may therefore be a causal factor in atherosclerosis and thrombosis. This possibility was supported by the findings of Hamsten et al (1987) of high PAI activity in survivors of premature myocardial infarction, which predicted further infarction.

The study of laboratory and biological (longitudinal) variability in PAI activity (Chapter 2) found that both were acceptable when measured over a 4-week period, over a range of normal and raised levels in patients with arterial disease (intermittant claudication). The study of variability in a population sample (Chapter 3) showed that diurnal variation , serum triglycerides, alcohol intake (in men) and

diabetes mellitus had significant effects on plasma PAI activity. These findings are consistent with other studies.

In the case control study of previous myocardial infarction, PAI activity was significantly higher in infarct survivors (Chapter 4). In men, this was not explained by standard risk factors such as triglyceride levels. These findings confirm and extend previous findings of high PAI activity in men with ischaemic heart disease (IHD). However PAI activity was not a predictor of IHD events when measured in stored plasma samples from the prospective Caerphilly Heart study (Chapter 5). This negative finding might be explained by prolonged sample storage although this is unlikely (see discussion in Chapter 5). While it is possible that PAI-1 activity does play a role in IHD, it appeares from this study, and that of Ridker et al (1992), that it is not a risk predictor for IHD events.

In the population study (Chapter 3), PAI activity correlated with interleukin-6, a cytokine involved in the acute-phase protein reaction. Further studies are suggested to determine whether or not this is a direct effect in man, as suggested by the experimental studies of Kruithof (1993) in baboons. Antagonists of IL-6 may be a potential approach to lowering elevated PAI activity levels, and thereby stimulating endogenous fibrinolysis in man.

Plasma PAI activity may be determined not only by environmental factors and cytokines, but also by genetic variation at the PAI-1 locus. Dawson and Henney (1991) identified a guanosine insertion / deletion polymorphism, -675 base pairs upstream from the start of transcription of the PAI-1 gene, where one allele had a

sequence of four guanosines (4G) and the other had five guanosines (5G). 4G homozygotes had higher plasma PAI activity than 4G/5G heterozygotes or 5G/5G homozygotes, whether or not they had ischaemic heart disease (Dawson and Henney 1992; Ye et al 1995). In non-insulin-dependent diabetes mellitus, recent studies suggest that the 4G genotype is more frequent in those with ischaemic heart disease and is also associated with increasing PAI levels with plasma glucose or triglyceride (Mansfield et al 1995). Further studies might therefore investigate the combination of PAI levels, genotypes, glucose intolerance, lipid levels and markers of the acute phase reaction in cardiovascular disease and diabetes. High PAI-1 levels were observed in NIDDM patients with microalbuminuria (Chapter 7), consistent with endothelial damage in such patients. At present, there is evidence that high plasma PAI-1 levels can be reduced by weight reduction, exercise, or metformin treatment of NIDDM (Gris et al 1990; Juhan-Vague and Alessi 1993). Reduction in PAI-1 levels is one possible mechanism for the cardiovascular benefits of these treatments.

Plasma tissue plasminogen activator (tPA) antigen

As discussed in Chapter 1, elevated tPA antigen levels are associated with elevated PAI-1 levels, probably due to circulating tPA-PAI-1 complexes. They may also reflect endothelial disturbance. and may be predictive of IHD.

The study of laboratory and biological (longitudinal) variability in tPA antigen (Chapter 2) found that both were acceptable when measured over a 4-week period, and that both were of a similar magnitude to PAI activity. The study of variability in a population

sample (Chapter 3) confirmed that tPA antigen correlated with PAI activity (r = 0.3-0.4) and with several cardiovascular risk factors including age, male sex, time of day, cigarette-smoking, triglyceride, alcohol consumption and (in men) haematocrit and red cell aggregation. Comparison with PAI activity, VWF levels and red cell aggregation suggested that some of these associations may reflect circulating PAI-tPA compexes, and others may reflect endothelial disturbance or acute-phase reactions.

In the case-control study of previous myocardial infarction, tPA antigen was significantly higher in infarct survivors (Chapter 4). However this was not statistically significant on multivariate analysis, suggesting that other risk factors accounted for this difference. Likewise, the significant predictive value of tPA antigen for IHD events in the Caerphilly Heart Study (Chapter 5) was accounted for by other risk factors on multivariate analysis, as in the study of Ridker et al (1993a,b). On the other hand, three recent studies have found that tPA antigen was an independent predictor of IHD events in patients with angina, who have higher tPA levels (Jansson et al 1993; Ridker et al 1994b; Thompson et al 1995). These findings suggest that there may be a threshold at which high plasma tPA levels predict thrombotic events, and that endothelial disturbance (which releases tPA) may play a role. Further studies are suggested to establish the clinical predictive value of tPA antigen in patients with arterial disease.

The studies of diabetic patients showed higher tPA antigen levels in NIDDM patients with microalbuminuria, which again suggests endothelial disturbance in such patients (Chapter 7).

Younger patients with IDDM and minimal complications had impaired endothelial release of tPA antigen in response to insulin-induced hypoglycaemia (Chapter 8) or exercise (Chapter 9) compared to matched non-diabetic controls. This was accompanied by a lesser fall in plasma PAI activity levels (the fall probably reflecting formation of tPA-PAI-1 complexes). Impaired tPA release may be a more sensitive indicator of endothelial disturbance than elevated tPA levels. Further studies of release of endothelial tPA in response to exercise are suggested in other groups of patients at increased risk of IHD, for example patients with hyperlipidaemia or hypertension.

Plasma von Willebrand factor (VWF) antigen

As discussed in Chapter 1, plasma levels of VWF antigen may be another marker of endothelial disturbance in atherosclerosis and diabetes mellitus. The study of laboratory and biological (longitudinal) variability of VWF (Chapter 2) found that while laboratory variability was similar to PAI activity and tPA antigen, longitudinal variability was much lower. This may reflect its longer circulation half-life, and is advantageous in defining an individual person's mean level accurately from a single sample.

The study of variability in a population sample (Chapter 3) showed relationships of VWF to age, cigarette-smoking and diabetes mellitus. Unlike PAI activity and tPA antigen, VWF antigen was not related to variables of the "insulin resistance syndrome". This suggests that elevated levels of PAI-1 and tPA antigen in this syndrome may reflect increased hepatic synthesis of PAI-1 (?in response to

triglyceride-rich VLDL), rather than increased release of PAI-1 /tPA from endothelium.

The case-control study of previous myocardial infarction showed that VWF antigen was higher in cases. On multivariate analysis, VWF antigen was significantly higher in female cases but not male cases. On the other hand, VWF was a significant independent predictor of IHD events in the Caerphilly Heart Study (Chapter 5), and showed a trend to prediction of fatal IHD events in the Edinburgh Claudication Study (Chapter 6). Recently, Meade et al (1994a) found that VWF was also a significant independent predictor of IHD events in the Northwick Park Heart Study. Woodburn (1995) also found that VWF was an independent predictor of peripheral arterial graft occlusion. Taken together, these observations suggest that further studies of VWF antigen in prediction of IHD would be worthwhile. Raised VWF levels may reflect underlying endothelial disturbance, for example due to atherosclerosis. They may also promote thrombosis by increasing platelet adhesion and aggregation.

NIDDM patients with microalbuminuria also had higher VWF levels than those without microalbuminuria (Chapter 7), again suggesting endothelial disturbance. As with tPA antigen, IDDM patients showed a lesser release of VWF antigen than non-diabetic controls to insulin-induced hypoglycaemia (Chapter 8) and to exercise (Chapter 9). This again suggests endothelial disturbance in such patients. Future studies of both tPA and VWF release in response to exercise are suggested in other groups at increased risk of IHD, for example patients with hyperlipidaemia or hypertension.

Plasma fibrin D-dimer antigen

Plasma levels of PAI and tPA indicate the fibrinolytic potential of plasma, but do not measure ongoing lysis of fibrin in vivo. In the studies reported in this thesis, plasma D-dimer antigen was measured as a marker of fibrin formation and cross-linking (through activation of the coagulation system) followed by fibrin lysis by plasmin to yield cross-linked degradation products possessing D-dimer antigen.

The study of laboratory and biological (longitudinal) variability in plasma D-dimer antigen (Chapter 2) found that laboratory variability was higher than for PAI, tPA or VWF (16%); however biological variability was low over 4 weeks (1%). These stable levels of D-dimer may reflect its longer circulating half-life, and as with VWF may be advantageous in that an individual person's level may be determined accurately from a single plasma sample.

The study of variability in a population sample (Chapter 3) showed increasing D-dimer antigen with age and with previous history of angina in men. Addition of other haematological variables to models showed that VWF, red cell aggregation and fibrinogen were also associated with D-dimer. Fibrin turnover may therefore increase with age because of underlying arterial disease, endothelial disturbance, acute-phase reactions, or increasing plasma levels of fibrinogen from which fibrin is formed.

The case-control study of previous myocardial infarction showed that D-dimer was higher in cases, and that this was not explained by

other risk factors (Chapter 4). D-dimer was also the strongest independent predictor of IHD events in the Caerphilly Heart Study (Chapter 5). Cortellaro et al (1993) and Ridker et al (1994a) recently obtained similar results. These results may reflect the association of D-dimer with underlying atherosclerosis (Reid 1991; Al-Zahrani et al 1993; Smith et al 1993; Lee et al1995; Woodburn et al 1995). D-dimer levels predicted progression of atherosclerosis in the Edinburgh Claudication Study (Chapter 6). These findings are consistent with the Rokitansky-Duguid hypothesis: that fibrin formation contributes to coronary and peripheral atherosclerosis (Duguid 1946).

We have recently found that D-dimer levels are raised in atrial fibrillation, in which there is an increased risk of thromboembolism from the heart (Lip et al 1995). Warfarin, which decreases the risk of thrombosis, normalised D-dimer levels in these patients. Persons with raised D-dimer levels may therefore benefit from warfarin in prevention of thrombosis. Further studies are suggested to explore this possibillity.

It is possible that increased D-dimer levels reflect the body's response to increased formation of intravascular fibrin, rather than the activation of the fibrinolytic system. The effect of warfarin in lowering D-dimer levels (Lip et al 1995) is consistent with this hypothesis. On the other hand, acute increase in tPA levels in response to insulin-induced hypoglycaemia did not result in increased D-dimer levels (Chapter 8). Likewise, Marsh and Gaffney (1982) found no increase in D-dimer levels following release of tPA by acute exercise. In these studies, lack of intravascular fibrin may be the

explanation of why increase in tPA levels is not followed by increased D-dimer levels.

Conclusion

Recent advances in laboratory technology (chromogenic activity and ELISA assays) have allowed measurement of specific components of fibrinolysis and of endothelial products. The studies reported in this thesis suggest that the assays of PAI activity, tPA antigen, VWF antigen and D-dimer antigen are potentially useful in studying their roles in cardiovascular disease and diabetes mellitus. The results of these studies suggest several lines of future research, and also suggest several considerations for interpretation of their plasma levels.

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