PLASMINOGEN ACTIVATOR INHIBITOR-1 AND CARDIOVASCULAR RISK

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ABSTRACT

Subjects with diabetes mellitus suffer from widespread premature atherosclerosis especially coronary heart disease. Diminished fibrinolysis, attributed to increased concentrations of plasminogen activator inhibitor-1 (PAI-1), may explain in part the accelerated atherosclerosis and thrombosis observed in diabetic subjects. In this thesis the determinants of PAI-1 in non-insulin-dependent diabetic subjects (NIDDM) and subjects post myocardial infarction were studied, with special focus on proinsulin-like molecules, cytokines and PAI-1 gene-environment interactions.

PAI-1 activity was examined in a cross-sectional study of 146 NIDDM subjects using specific assays of insulin and intact and des 31,32 proinsulin, and measures of insulin resistance, relating these measurements to serum lipids and hypoglycaemic therapy. Proinsulin levels were higher on sulphonylurea therapy, but no difference in PAI-1 activity was observed. The importance of proinsulin in relation to serum PAI-1 was however highlighted by a cross-over study, where the role of 4 months therapy with sulphonylurea or insulin on PAI-1 was studied. There were no differences observed in glycaemic control, insulin sensitivity or lipids, but sulphonylurea therapy was associated with higher levels of PAI-1 antigen and proinsulin compared to insulin.

In a cross-sectional study, a difference in PAI-1 activity according to a common insertion (5G)/ deletion (4G) polymorphism in the promoter region of the PAI-1 gene was demonstrated. In this study serum triglyceride, and its interaction with the promoter 4G/5G polymorphism were important determinants of PAI-1 activity.

PAI-1 gene-environment interactions and the role of cytokines, were explored by studying subjects during the course of acute myocardial infarction and after six month follow-up. Acutely, proinsulin emerges as an important determinant of PAI-1 activity. Non-survivors had higher PAI-1 activity day one post infarction compared to survivors. At six months, subjects with the 4G/4G polymorphism had higher PAI-1 antigen compared to 5G/5G subjects, there were no differences observed in levels of proinsulin-like molecules or cytokines.
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<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<td>AER</td>
<td>Albumin excretion rate</td>
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<td>AMI</td>
<td>Acute myocardial infarction</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Apo</td>
<td>Apoprotein</td>
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<td>ASO</td>
<td>Allele specific oligo melting</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>bp</td>
<td>Base pair</td>
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<td>CHD</td>
<td>Coronary heart disease</td>
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<td>CK</td>
<td>Creatine kinase</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>FPG</td>
<td>Fasting plasma glucose</td>
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<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HepG2</td>
<td>Hepatoma cell line</td>
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<td>HOMA</td>
<td>Homeostasis Model Assesment</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<td>IDL</td>
<td>Intermediate density lipoprotein</td>
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<td>IEMA</td>
<td>Immunoenzymometric assay</td>
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<td>IGF-1</td>
<td>Insulin like growth factor-1</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<td>I</td>
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<td>IRI</td>
<td>Immunoreactive insulin</td>
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<td>ITT</td>
<td>Insulin tolerance test</td>
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kb Kilobase
LDL Low density lipoprotein
MCR-G Metabolic clearance rate of glucose
mRNA Messenger ribonucleic acid
NADPH Nicotinamide adenine dinucleotide phosphate
NIDDM Non-insulin dependent diabetes mellitus
OGTT Oral glucose tolerance test
PAI-1 Plasminogen activator inhibitor-1
RFLP Restriction fragment length polymorphisms
SU Sulphonylurea
STR Subscapular to triceps ratio
TGF-β Transforming growth factor-β
TNF-α Tumour necrosis factor-α
t-PA Tissue plasminogen activator
VLDL Very low density lipoprotein
WHO World Health Organisation
WHR Waist to hip ratio
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CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 Introduction
Subjects with diabetes mellitus suffer from widespread premature atherosclerosis especially coronary artery disease (CHD). Conventional risk factors such as smoking, obesity, blood pressure and serum lipids fail to fully explain this excess risk (Kannel et al, 1979; Pyörälä et al, 1987). Diminished fibrinolysis, attributed to increased concentrations of plasminogen activator inhibitor-1 (PAI-1), may help bridge this gap and explain in part the accelerated atherosclerosis and thrombosis observed in diabetic subjects.

1.2 The fibrinolytic pathway
The fibrinolytic system is our natural defence against thrombosis, and a balance exists between plasminogen activators and inhibitors (Figure 1.1). Impaired fibrinolysis can be caused by either diminished endothelial release of tissue plasminogen activator (t-PA) or increased levels of PAI-1. PAI-1 is a 50 kDa glycoprotein, and a serine protease inhibitor. Since it was first identified in 1983, evidence suggests that it is the major regulator of the fibrinolytic system (Chmielewska et al, 1983). It binds and rapidly inhibits both single and two chain t-PA and urokinase (uPA). PAI-1 and t-PA rapidly form an inactive reversible complex followed by a slower transition to a stable irreversible complex.

1.3 Molecular forms of PAI-1
PAI-1 in blood exists in 3 different molecular forms: latent (or inactive), active, and PAI-1 complexed to t-PA (Declerck et al, 1990) (Figure 1.2). Active PAI-1 accounts for 60% of PAI-1 in plasma and 4% of platelet PAI-1, whereas latent PAI-1 accounts for 3-13% of plasma PAI-1 and 90% of platelet PAI-1 (Declerck et al, 1988). PAI-1 is secreted in an active form, but under normal physiological conditions is rapidly inactivated to the latent form. In vitro, latent PAI-1 can be reactivated by denaturants.
Figure 1.1 The fibrinolytic pathway

Figure showing the fibrinolytic pathway with the following components:
- t-PA
- u-PA
- PAI-1
- Plasminogen
- Plasmin
- Fibrinogen
- X-L Fibrin
- FDP's
- Thrombin
- Xa
- Prothrombin

Key enzymes and molecules:
- TGF-B
- Latent TGF-B
- Active TGF-B
- Extrinsic pathway
- Intrinsic pathway

Diagram includes arrows indicating the flow and interactions between these components.
Figure 1.2  The activity and concentration of the main components of the fibrinolytic system as measures in assays.
or negatively charged phospholipids (Heckman et al, 1985), but it is not known whether latent PAI-1 can be reactivated in vivo. Plasma clearance of active t-PA is faster than the clearance of t-PA/PAI-1 complex. High levels of active PAI-1 convert more t-PA into t-PA/PAI-1 complex, effectively slowing the clearance of total t-PA antigen. This explains why high levels of PAI-1 activity are associated with an increase in total t-PA (Chandler et al, 1997). Vitronectin is the PAI-1 binding protein, and in blood vitronectin increases the stability of PAI-1 (Wiman et al, 1988). The PAI-1-vitronectin complex is readily dissociated by t-PA (Wiman et al, 1988). PAI-1 activity evaluates free active PAI-1, whereas PAI-1 antigen assays evaluate not only free active PAI-1, but also inactive PAI-1 complexed with t-PA, and latent PAI-1 released by platelets in vivo (Booth et al, 1988).

1.4 Sites of PAI-1 expression

The origin of plasma PAI-1 under normal and pathological conditions is unknown. The major source of PAI-1 synthesis is thought to be from hepatocytes and endothelial cells, but there are also contributions from platelets and smooth muscle cells. The highest concentration of PAI-1 is found in the liver and spleen, with the brain and myocardium containing very small amounts (Simpson et al, 1991). The concentration of PAI-1 increases dramatically during endotoxaemia. In endotoxin-treated mice, there is widespread expression of PAI-1 mRNA in all tissues, with the highest level of induction in the liver (Sawdey et al, 1991). The large pool of PAI-1 in platelets acts as an important physiological source of PAI-1 (Booth et al, 1988). Activated platelets at the site of a clot release their pool of PAI-1 from their α granules (Torr-Brown et al, 1995). Recent interest has focused on the ability of adipose tissue to contribute to the elevated PAI-1 levels observed in obesity. In vitro, adipocytes express PAI-1, and this expression is augmented by TGF-β (Lundgren et al, 1996). In mice, PAI-1 mRNA is detected in mature adipocytes, and both TNF-α and endotoxin significantly increase PAI-1 mRNA in adipose tissue, levels peaking at 3-8 hours (Samad et al, 1996). These studies suggest a novel mechanism to explain the increased PAI-1 levels in obesity.
1.5 Evidence for PAI-1 as a risk factor for atherosclerosis and thrombosis: Abnormal expression of PAI-1 in vivo

Vascular haemostasis results from a careful balance between the coagulation and fibrinolytic system. Net fibrinolytic activity is the result of balance between the plasminogen activators and PAI-1. Patients with little or no detectable PAI-1 have lifelong bleeding diatheses (Schleef et al, 1989; Fay et al, 1992), whereas inherited elevation of PAI-1 is associated with familial thrombosis (Schved et al, 1991; Berdeaux et al, 1991). Elevated levels of PAI-1 are a risk for thrombotic problems, such as recurrent myocardial infarction (Hamsten et al, 1987) and deep vein thrombosis following hip replacement (Eriksson et al, 1989). Transgenic mice carrying human PAI-1 cDNA under the control of the mouse metallothionine promoter are born with high levels of PAI-1 and develop thrombotic problems in their extremities (Erickson et al, 1990). Finally, mice deficient in the PAI-1 gene exhibit a mild hyperfibrinolytic state and greater resistance to venous thrombosis (Carmeliet et al, 1993).

Increased PAI-1 gene expression in atherosclerotic human arteries

Defective fibrinolysis, whether due to elevated PAI-1 activity or reduced t-PA activity, not only predisposes individuals to thrombotic events, but may play a role in the formation and progression of atherosclerotic plaques. Increased synthesis of PAI-1 has been demonstrated in components of the atherosclerotic plaque, and this may lead to fibrin deposition during plaque rupture, contributing to the progression of the lesion (Chomiki et al, 1994; Padró et al, 1995). PAI-1 within the plaque inhibits plasmin formation, which plays an important role in cleaving extracellular matrix proteins, directly or via activation of metalloproteinas. This may lead to stabilisation and further growth of the plaque.

1.6 Diminished fibrinolysis in diabetes

Abnormalities in the fibrinolytic system have been described in both insulin dependent (IDDM) and non-insulin dependent diabetes (NIDDM). In NIDDM, most studies have demonstrated that there is diminished fibrinolysis due to elevated levels of PAI-1 (Auwerx et al, 1988). In IDDM, however the results are mixed, and diminished,
normal or enhanced fibrinolysis have all been reported. In this thesis, only subjects with NIDDM were studied.

In subjects with NIDDM, a variety of risk factors independently associate with diminished fibrinolysis, (obesity, hypertension, dyslipidaemia, hyperinsulinaemia and insulin resistance), tend to converge. Is the diminished fibrinolysis in diabetes just a reflection of this cluster? Numerous studies have attempted to dissect out the independent contribution of the above risk factors in determining fibrinolytic activity in diabetes, but this task has been hampered by the complex relationships that exists between them. In non-diabetic subjects, insulin resistance is paralleled by increased insulin concentrations and both correlate with triglyceride levels, so any one or more of these three variables may explain inter-relationships with PAI-1 activity. By contrast, in NIDDM, insulin resistance and concentrations of insulin and triglyceride are less tightly interdependent, and in this situation concentrations of proinsulin-like molecules may also play a significant role in determining PAI-1 activity.

1.7 Regulation of PAI-1 expression in diabetes

PAI-1 expression is affected by a large number of substances in vivo and in vitro, and evidence suggests that the regulatory mechanism is at the post-transcriptional level. A large amount of data on PAI-1 regulation comes from in vitro studies, predominantly in endothelial cells and the hepatoma cell line, HepG2.

NIDDM subjects have elevated PAI-1 levels, what are the main substances regulating PAI-1 levels in diabetes? Is it possible to dissect out the relative importance of the components of the insulin resistance syndrome? Attempts for example, to dissect out the independent effects of the insulin-like molecules on PAI-1 are hampered by the problem that most interventions studies (such as weight loss, exercise or the oral hypoglycaemic agent metformin), which result in a change in one molecule, result in a parallel change in them all. This highlights the need for in vitro experiments to establish their relative contributions. Recent in vivo and in vitro studies will be discussed below. These are summarised in Tables 1.1 and 1.2 respectively.
<table>
<thead>
<tr>
<th>Reference:</th>
<th>Intervention:</th>
<th>Subjects</th>
<th>Effect on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PAI-1 activity</td>
</tr>
<tr>
<td>Gris et al, 1990</td>
<td>Exercise, weight reduction, smoking cessation</td>
<td>Normal</td>
<td>↓</td>
</tr>
<tr>
<td>Folsom et al, 1993</td>
<td>6 month weight loss</td>
<td>Obese</td>
<td>↓</td>
</tr>
<tr>
<td>Gough et al, 1994</td>
<td>12 month exercise</td>
<td>NIDDM</td>
<td>↑</td>
</tr>
<tr>
<td>Grant et al, 1991</td>
<td>2 hr infusion of: TG insulin glucose</td>
<td>Normal</td>
<td>↓</td>
</tr>
<tr>
<td>Vuorinen-Markkola et al, 1992</td>
<td>3 hr insulin infusion</td>
<td>Normal</td>
<td>↓</td>
</tr>
<tr>
<td>McCormack et al, 1993</td>
<td>ITT OGTT</td>
<td>Obese</td>
<td>↓</td>
</tr>
<tr>
<td>Barriocanal et al, 1995</td>
<td>3 hr TG + low dose insulin infusion</td>
<td>Normal</td>
<td>↑</td>
</tr>
<tr>
<td>Grant et al, 1991</td>
<td>Metformin</td>
<td>NIDDM</td>
<td>↓</td>
</tr>
<tr>
<td>Nagi et al, 1993</td>
<td>Metformin</td>
<td>NIDDM</td>
<td>↓</td>
</tr>
<tr>
<td>Jain et al, 1993</td>
<td>8 wks Insulin vs SU therapy- poor control</td>
<td>NIDDM</td>
<td>↓</td>
</tr>
<tr>
<td>Nordt et al, 1995</td>
<td>1 hr infusion of: insulin proinsulin C-peptide</td>
<td>Rabbits</td>
<td>↑*</td>
</tr>
</tbody>
</table>

PAI-1: plasminogen activator inhibitor-1; ITT: insulin tolerance test; OGTT: oral glucose tolerance test; PI: proinsulin; TG: triglyceride; SU: sulphonylurea. * also increase in PAI-1 mRNA in aorta and liver.
Table 1.2
PAI-1 synthesis in vitro:

<table>
<thead>
<tr>
<th>Reference:</th>
<th>Cell type:</th>
<th>insulin</th>
<th>Intact PI</th>
<th>31,32 PI</th>
<th>64,65 PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alessi et al, 1988</td>
<td>HepG2, HUVEC</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kooistra et al, 1989</td>
<td>Human hepatocytes</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grant et al., 1991</td>
<td>HepG2</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schneider et al, 1991</td>
<td>HepG2</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schneider et al, 1992</td>
<td>Porcine aortic endothelial cells</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>▲*</td>
</tr>
<tr>
<td>Anfosso et al, 1993</td>
<td>‘Resistant’ HepG2 †</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klassen et al, 1993</td>
<td>Human aortic endothelial cells</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nordt et al, 1994</td>
<td>HepG2, HUVEC</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>Alessi et al, 1995</td>
<td>HepG2, Porcine aortic endothelial cells</td>
<td>▲</td>
<td>▲</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HUVEC</td>
<td>▲</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HepG2: Human hepatoma cell line; HUVEC: Human umbilical vein endothelial cell; PI: proinsulin
* Split proinsulin obtained by exposing intact proinsulin to trypsin. † HepG2 cells preincubated with high dose insulin
1.7.1 The role of insulin:
Hyperinsulinaemia has been associated with CHD in non-diabetic subjects (Pyörälä 1985). In NIDDM subjects plasma PAI-1 activity parallels the extent of hyperinsulinaemia, and insulin has been implicated as a major physiological regulator of PAI-1 (Juhan-Vague et al, 1989). A strong relationship \((r=0.44, p<0.001)\) was observed between plasma insulin and PAI-1 in the ECAT study in 1484 patients with angina (Juhan-Vague et al, 1993). The relationship of hyperinsulinaemia and insulin resistance with elevated PAI-1 activity is yet to be elucidated. Despite population correlations of insulin concentration with PAI-1 activity, and \textit{in vitro} effects of insulin on PAI-1 production, a direct effect of insulin on stimulating PAI-1 activity \textit{in vivo} in humans has not been shown, either by intravenous infusions of insulin, or by an oral glucose load with the aim of producing portal hyperinsulinaemia (Table 1.1).

Numerous problems arise, however, in attempting to explore the insulin-fibrinolytic axis \textit{in vivo}. Firstly, plasma insulin levels achieved in clamp studies, although physiological, are much lower than those employed \textit{in vitro}. Secondly, these studies are normally commenced in the morning, and the diurnal variation of PAI-1 levels (peak 4-6 a.m and nadir at about 6 p.m) (Angleton et al, 1989), might mask any possible increase observed due to insulin. Thirdly the studies have been of relatively short duration, and more prolonged insulin infusions may be required to stimulate PAI-1 production, especially in the liver. Furthermore, insulin may be stimulating local endothelial production of PAI-1 which may not be reflected in plasma levels. It is, however difficult to isolate the direct action of insulin alone \textit{in vivo} on PAI-1, as insulin infusions result in changes in other metabolic variables such as free fatty acids, which in turn may alter PAI-1 levels. It is possible that the lack of PAI-1 stimulation shown in clamp studies is because portal vein insulin is the important factor in directly stimulating hepatocyte PAI-1 production. Mildly hyperglycaemic NIDDM subjects have portal and peripheral hyperinsulinaemia and high PAI-1 levels, and IDDM subjects have low portal vein insulin concentrations and low or normal PAI-1 levels. Insulin may also indirectly stimulate PAI-1 via its effect on plasma triglyceride levels.
Despite the lack of PAI-1 stimulation by intravenous insulin in humans, a one hour infusion of insulin in rabbits increased plasma PAI-1 activity (3.6 and 3.8 fold respectively) and PAI-1 mRNA levels in aorta and liver peaking at 3 hours and returning to baseline in 24 hours (Nordt et al, 1995). Rather than stimulating PAI-1 production, insulin may play a role in changing the molecular forms of PAI-1 in plasma or platelets hence influencing fibrinolytic activity. There has been no study to date examining this possible effect.

Thus in humans there is little evidence that interventions resulting in increased concentrations of insulin \textit{in vivo} increase PAI-1. What effect does a reduction in insulin concentrations have on PAI-1? These studies are summarized in Table 1.1. Reducing insulin levels and insulin resistance by exercise, weight loss and the drug metformin has been shown to reduce PAI-1 activity (Table 1.1).

A substantial amount of data on the role of insulin in PAI-1 production comes from \textit{in vitro} studies, these are summarised in Table 2, but studies on both liver cells and endothelial cells should be interpreted with caution. The hepatoma cell line HepG2, which is used extensively to study PAI-1 expression, retains the capacity to synthesise proteins involved in fibrinolysis but the regulatory mechanisms may not be similar to normal hepatocytes. Human hepatocytes, however, have been shown to produce PAI-1 in response to insulin (Kooistra et al, 1989). After several passages \textit{in vitro} endothelial cells may be subject to different regulatory mechanisms and may not represent endothelial cell function \textit{in vivo}.

1.7.2 The role of proinsulin

a. Chemistry of proinsulin and its conversion products

Insulin synthesis in the \(\beta\) cell involves the cleavage of proinsulin at two sites, to form insulin and C-peptide (Figure 1.3). Two breaks occur in the amino acid chain, producing 32,33 split proinsulin and 65,66 split proinsulin. After chain cleavage, the two basic amino acids are removed by the action of a carboxypeptidase, producing des 31,32 and des 64,65 proinsulin. The development of assays using highly specific
Figure 1.3
Proinsulin biosynthesis

b. Proinsulin-like molecules in clinical studies

In NIDDM subjects, approximately 30% of fasting immunoreactive insulin concentrations (detected by non-specific assays using polyclonal antibodies) consists of proinsulin-like molecules (mainly intact proinsulin and des 31,32 proinsulin) (Yudkin 1993). The elevated PAI-1 levels in these subjects may, therefore, be a consequence of precursors of insulin rather than insulin itself. Evidence is again derived from in vivo and in vitro data (Tables 1.1 and 1.2 respectively). In a study of 51 NIDDM subjects des 31,32 proinsulin concentrations correlated more closely with cardiovascular risk factors, including PAI-1, than specific levels of insulin (Nagi et al, 1990). An intervention study in 11 NIDDM subjects, investigating the effect of altering levels of proinsulin-like molecules (suppression with exogenous insulin and stimulation with sulphonylureas) on cardiovascular risk factors including PAI-1, showed a fall of 43% in levels of intact proinsulin, 20% in those of des 31,32 proinsulin and 14% in PAI-1 activity on insulin therapy (Jain et al, 1993). The fall in proinsulin concentrations correlated with that of PAI-1. No change occurred in levels of apoproteins or lipoproteins.

There may be grounds for suspecting a role for proinsulin in thrombogenesis in NIDDM from clinical studies. In a study using human proinsulin, 4 fatal and 2 non-fatal myocardial infarctions occurred during 2 years of follow-up among 68 proinsulin treated NIDDM subjects versus none in the insulin treated group (Galloway et al, 1992). As well as infusing insulin, Nordt et al (1995), assessed the effect of proinsulin infusions in rabbits. Intravenous proinsulin infusions, led to an increase in plasma PAI-1 activity, and in PAI-1 mRNA in aorta and liver

1.7.3 The role of insulin resistance.

In non-diabetic subjects insulin concentrations correlate closely with insulin resistance,
in NIDDM subjects however, this relationship is dissociated. PAI-1 levels have shown an inverse correlation with insulin sensitivity in NIDDM subjects. In a group of nine obese non-diabetic and NIDDM subjects, insulin sensitivity, measured by a hyperinsulinaemic-euglycaemic clamp, showed a significant negative correlation with PAI-1 antigen concentrations (Potter van Loon et al, 1993). Increased PAI-1 production \textit{in vitro} has been demonstrated in HepG2 cells rendered insulin resistant by pre-incubation with insulin to reduce insulin receptor numbers (Anfossa et al, 1993). It is not apparent whether insulin sensitivity is an independent determinant of PAI-1 levels or whether other factors, as components of the 'insulin resistance' or Reaven's syndrome, could explain the association.

1.7.4 The role of glucose
Hyperglycaemia is an additional risk factor for impaired fibrinolysis. Increased glucose concentrations can directly increase PAI-1 production from human umbilical vein endothelial cells (HUVEC) (Maiello et al, 1992) and porcine aortic endothelial cells (Nordt et al, 1993). In NIDDM a significant relationship also exists between circulating PAI-1 activity and glucose concentrations (Nagi et al, 1996), but not with long-term glycaemic control as judged by HbA1c measurement (Auwerx et al, 1988; Mansfield et al, 1994).

1.7.5 The role of lipids
It has been proposed that insulin resistance or hyperinsulinaemia could influence the synthesis of PAI-1 via their effects on lipid metabolism. Dyslipidaemia is common in NIDDM subjects, in particular high triglyceride (TG) and low serum high density lipoprotein levels. Hypertriglyceridaemia has been associated with an increased risk of CHD in prospective and cross-sectional studies. In the WHO Multinational Study (West et al, 1983) and in the Paris Prospective Study (Fontbonne et al, 1989), total triglyceride concentrations were the risk factor that showed the strongest correlation with occurrence of CHD. In a seven year follow-up study in 313 NIDDM subjects, low HDL and HDL\textsubscript{2} cholesterol, high total VLDL, VLDL cholesterol, and high VLDL TG were powerful risk indicators for CHD (Laakso et al, 1993). The risk of CHD
events was two-fold in NIDDM subjects with high total TG levels. Could the link between hypertriglyceridaemia and CHD be partly explained by attenuated fibrinolysis? PAI-1 activity correlates significantly with serum TG concentrations in subjects with obesity (Vague et al, 1986), CHD (Mehta et al, 1987), hypertriglyceridaemia (Asplund-Carlson et al, 1993) and NIDDM (Juhan-Vague et al, 1989). Dietary reductions in serum TG are associated with reduced PAI-1 levels (Mussoni et al, 1992). However acute intravenous administration of TG, as Intralipid, does not increase PAI-1 activity (Table 1.1).

In vitro studies have demonstrated the effect of various lipoproteins on PAI-1 synthesis (Stiko-Rahm et al, 1990; Tremoli et al, 1993). VLDL from hypertriglyceridaemic patients increases endothelial cell production of PAI-1 to a greater degree than that from normo-triglyceridaemic subjects, this effect being abolished by antibody directed against the apoB receptor (Stiko-Rahm et al, 1990). Oxidised LDL also stimulates endothelial cell PAI-1 synthesis (Latron et al, 1991; Kugiyama et al, 1993) as does lipoprotein(a) (Lpa) (Etingin et al, 1991). Lp(a), LDL and HDL suppressed constitutive and stimulated t-PA secretion from HUVEC in a dose dependent manner (Levin et al, 1994). But it is not only PAI-1 which may be controlled by alterations in lipid concentrations: the interaction of lipoproteins with cell-surface binding sites may interfere with endothelial t-PA production. Lp(a) may modulate fibrinolysis by a unique mechanism. It consists of an LDL particle covalently bonded to apolipoprotein(a), which has structural homology to the plasminogen kringle 4. Lp(a) may impair fibrinolysis by competing with tPA or plasminogen for binding to fibrin (Zysow et al, 1993). Transgenic mice expressing the human Apo(a) gene are refractory to t-PA mediated thrombolysis (Palabricia et al, 1995).

Insulin resistance in NIDDM normally refers to resistance to insulin-mediated glucose uptake. Resistance may also exist, however, to the antilipolytic effect of insulin resulting in increased non-esterified fatty acids (NEFA) levels, which in turn may increase PAI-1 levels (Bastard et al, 1995).
1.8 The genetics of PAI-1

1.8.1 Structure of the PAI-1 gene

The human PAI-1 gene is approximately 12.2 kb in length, contains 9 exons and 8 introns and is located on the long arm of chromosome 7 (Bosma et al, 1988; Klinger et al, 1987) (Figure 1.4). Transcription of the PAI-1 gene results in two species of mRNA, an unstable 3.2 kb form, and a relatively more stable 2.2 kb form, which differ only in the length of their 3'untranslated regions, and are formed by alternative cleavage and polyadenylation (Bosma et al, 1988 and 1991; Loskutoff, 1991). Evidence suggests that the mRNAs are independently regulated: those regulators involved in the acute phase response preferentially increasing the 3.2 kb message (Schleef et al, 1988; van den Berg et al, 1988).

1.8.2 The promoter region of the PAI-1 gene

Most of the regulation of the PAI-1 gene is through the activation or repression of transcription. The promoter region of the gene is responsible for driving transcription and contains sites to which transcriptional regulators can bind. The first 805bp of the 5' flanking region of the PAI-1 gene constitutes the promoter region (Fig 1.4). Functional studies in which the 5' flanking region is attached to a reporter gene, provide information on the ability of different sections of the gene to promote transcription in response to different stimuli in cell culture (Zonneveld et al, 1988; Riccio et al, 1988). A 260-bp fragment, from -189 to +71 is sufficient to confer some of the PAI-1 tissue specific expression, and regions of the promoter responsive to dexamethasone and TGF-β have been localised (Zonneveld et al, 1988; Riccio et al, 1988; Westerhausen et al, 1991).

1.8.3 Genetic variation in the PAI-1 gene

The large degree of inter-individual and intra-individual variation in plasma PAI-1 activity may be due to common genetic variation within the PAI-1 gene. The following polymorphisms in the PAI-1 gene have been identified and studied (Fig 1.4):
Figure 1.4 The PAI-1 gene.

(CA) repeat polymorphism

4G/5G polymorphism

HindIII RFL polymorphism
(I) Restriction fragment length polymorphisms.

Two restriction fragment length polymorphisms (RFLP) in the PAI-1 gene, a common *HindIII* and a rare *XmnI* RFLP have been identified, both showing Mendelian inheritance (Klinger et al, 1987). Data suggests that the *HindIII* RFLP may lie in the 3' flanking sequence of the PAI-1 gene. This RFLP has been associated with altered plasma PAI-1 levels in a sample of young myocardial infarct survivors and in an aged matched control population (Dawson et al, 1991).

(ii) (CA)n repeat polymorphisms.

A (CA)n repeat polymorphism has been identified in intron 3 of the PAI-1 gene and is associated with altered levels of PAI-1: lower n values predicting higher PAI-1 levels (Dawson et al, 1991). This polymorphism is in linkage disequilibrium with the *HindIII* RFLP. A further (CA)n dinucleotide repeat sequence lies in the promoter of the PAI-1 gene from 172 to 153 bp 5' to the site of initiation of transcription (Mansfield et al, 1994). It is atypical of dinucleotide repeat polymorphisms described previously in that it has only two common alleles, the less common having an allele frequency of 0.01.

(iii) Insertion (5G)/deletion (4G) polymorphism in the promoter.

Dawson et al (1993) have described a common single base pair insertion (5G)/deletion (4G) polymorphism, 675 base pairs upstream from the start of transcription of the PAI-1 gene (Fig. 1.4). In a sample of 107 young patients with previous myocardial infarction, subjects with the 4G allele had higher plasma PAI-1 activity levels compared to subjects with the 5G allele, but this association was weaker in healthy controls. In both patients and controls, individuals homozygous for the 4G allele showed a positive correlation between plasma fibrinogen levels (an acute phase reactant), and plasma PAI-1 activity, while those with one or more 5G alleles showed a weak or negative relationship. This is consistent with findings from *in vitro* expression studies which show that the 4G allele responds to interleukin 1 (IL-1) (a mediator of the acute-phase response) by increasing transcription from the PAI-1 promoter whereas the 5G allele does not (Dawson et al, 1993). It has been shown that hepatic nuclear proteins bind differentially to the 4G or 5G sequence in the promoter.
region of the PAI-1 gene (Dawson et al, 1993; Eriksson et al, 1995). The insertion of an extra guanine at -675 in the PAI-1 promoter creates a binding site for proteins which may have a direct effect in repressing PAI-1 transcription during an acute phase response.

1.8.4 Population studies on genetic variation in the PAI-1 gene.
Table 1.3 summarises data available at the time work for this thesis was conducted, on population studies examining the frequency of the above polymorphisms of the PAI-1 gene. Prior to the work presented in this thesis, there had been no study examining the role of the promoter 4G/5G polymorphism in a diabetic population.

1.8.5 The concept of gene environment interaction
The plasma level of a particular protein in an individual is the result of the interactions between genetic variations at a number of different loci and the interactions between these variations and environmental factors. An individual with a high-risk genotype may develop severe and early disease (e.g. CHD) if exposed to a high-risk environment (e.g. smoking, obesity or hyperlipidaemia) compared to an individual with a low risk genotype. Situations where the high-risk environment is unavoidable such as in diabetes, puts individuals with high-risk genotypes under greater threat.

Genetic polymorphisms of haemostatic and fibrinolytic factors are associated not only with inter-individual differences in plasma levels but also with response to environmental factors. Gene environmental interactions have been described for factor VII, fibrinogen and PAI-1.

The major environmental agent modulating factor VII level is dietary fat intake (Lane et al, 1992). There are two common polymorphisms of the factor VII gene, the presence or absence of a decanucleotide sequence in the promoter region (P0/P10)(Marchetti et al, 1993) and a G to A nucleotide substitution that gives rise to either an arginine or a glutamine residue at amino acid 353 (R353Q) (Green et al, 1991). In most subject groups tested, the P0 allele is associated with R353 and it is not
Table 1.3
Summary of clinical studies on polymorphisms in the PAI-1 gene.

<table>
<thead>
<tr>
<th>Polymorphism:</th>
<th>Allele</th>
<th>Subjects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>Post MI</td>
</tr>
<tr>
<td>HindIII RFLP</td>
<td>1</td>
<td>0.46</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>Promoter 4G/5G</td>
<td>4G</td>
<td>0.47</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>5G</td>
<td>0.53</td>
<td>0.49</td>
</tr>
</tbody>
</table>
clear which, if either polymorphism is functional. The R353/P0 allele is associated with elevated factor VII antigen and activity (Green et al, 1991; Lane et al, 1992), with a higher correlation between factor VII coagulant activity and plasma triglyceride levels, and with a dramatic rise in factor VII activity following a lipid rich meal (Lane et al, 1992; Silveira et al, 1994).

The major environmental agent modulating fibrinogen levels is cigarette smoking. There are numerous polymorphisms at the fibrinogen locus which comprises three genes, each coding one of the fibrinogen polypeptides a, b, g. The A allele of the b-fibrinogen G/A-455 polymorphism is associated with elevated levels of fibrinogen, and is associated with a larger effect of smoking on fibrinogen levels (Green et al, 1993). This allele has recently been found to be associated with the extent of atherosclerosis (Behague et al, 1996). PAI-1 gene-environment interactions will be discussed and further studied in chapters 5 and 6 of this thesis.

1.9 Clinical implications of diminished fibrinolysis in diabetes

1.9.1 Diabetic macrovascular disease

Whether diminished fibrinolysis is a cause or effect of CHD is still disputed, but evidence is emerging more strongly in favour of the former. Evidence from the Northwick Park Heart Study suggests a strong independent relationship between reduced fibrinolytic activity, as measured by dilute clot lysis time, and increased risk of CHD in men (Meade et al, 1993). Elevated PAI-1 has been demonstrated in young survivors of myocardial infarction, and predisposes this group to reinfarction (Hamsten et al, 1985 and 1987). Diabetic subjects have higher morbidity and mortality from acute myocardial infarction (AMI) (Yudkin et al, 1987; Jacoby et al, 1992). This may in part be explained by NIDDM subjects having higher PAI-1 levels on admission with acute myocardial infarction (Gray et al, 1993), and showing less enzymatic evidence of reperfusion following thrombolytic therapy compared to non-diabetic subjects (Gray et al, 1993). NIDDM subjects have higher platelet PAI-1 compared to non-diabetics (Jokl et al, 1994), making platelet-rich thrombi more resistant to thrombolysis.
In apparent contrast to the proposed role for PAI-1, higher t-PA antigen concentration was associated with increased risk of future myocardial infarction in the Physicians Health Study (Ridker et al, 1993), t-PA antigen also predicted long term mortality in a longitudinal study of patients with angina and angiographically documented CHD (Jansson et al, 1993), and was an independent predictor of AMI in 3000 subjects in the ECAT study (Thompson et al, 1995). The increased t-PA antigen, may reflect increased complex formation by t-PA and PAI-1, and hence reflect high PAI-1 activity. Alternatively, elevated t-PA antigen may be a result of prevalent endothelial dysfunction. Juhan-Vague et al (1996), analysed the results of the ECAT study, a prospective multicenter study of 3034 patients with angina pectoris followed for two years (ECAT angina pectoris study group, 1993). Baseline measurements included 10 fibrinolytic variables, and the results were analysed in relation to the subsequent incidence of myocardial infarction or sudden coronary death. Factors involved in the insulin resistance syndrome strongly affected PAI-1 and to a lesser extent t-PA antigen. t-PA antigen was influenced primarily by inflammation and endothelial cell damage.

1.9.2 Diabetic microvascular disease

Changes in components of the fibrinolytic system may also play an important role in microangiopathy. Plasmin and uPA are activators of latent metalloproteinasises, such as collagenases, responsible for proteolysis of extracellular matrix proteins, so that increased PAI-1 levels may lead to the basement membrane thickening observed in diabetic microangiopathy (Miskin et al, 1990).

In the presence of diabetic retinopathy, PAI-1 levels are further elevated in NIDDM (García Frade et al, 1990). In IDDM there is a tendency for elevation of PAI-1 levels with worsening retinopathy (Haitas et al, 1984), although this finding is not found consistently, and in a study of 50 IDDM subjects there was no relationship between diabetic microvascular complications and fibrinolytic parameters (Walmsley et al, 1991). A cross-sectional study of 454 IDDM subjects showed no association of PAI-1 levels with diabetic complications (Maser et al, 1995).
1.9.3 Hypoglycaemic therapy

As mentioned above, glucose can directly increase PAI-1 production from vascular endothelium (Maiello et al, 1992; Nordt et al, 1993). It is important that hypoglycaemic therapy does not worsen the increased cardiovascular risk observed in NIDDM subjects by altering PAI-1. The results of the UGDP study, suggesting an increased cardiovascular mortality on sulphonylureas, are not widely accepted (University Group Diabetes Program, 1970). Could hypoglycaemic therapy affect PAI-1 levels directly or via elevation of serum proinsulin? In a cross-over design, the effect of therapy with sulphonylureas or with insulin for eight weeks on PAI-1 activity was studied in 11 poorly controlled NIDDM subjects (Jain et al, 1993). Without altering glycaemic control, insulin treatment was associated with reduced PAI-1 activity, this reduction correlating with the suppression of concentrations of proinsulin-like molecules. Metformin (1,1-dimethylbiguanide) therapy has been shown to reduce PAI-1 in NIDDM subjects (Grant et al, 1991; Nagi et al, 1993). Long-term prospective studies, however, on the effects of hypoglycaemic therapy on fibrinolysis are lacking.

1.9.4 Lipid lowering therapy

Lowering triglyceride levels in NIDDM patients with drugs that concomitantly reduce PAI-1 by a pathway independent of their lipid lowering effect might go further towards reducing the incidence of atherosclerosis and thrombosis. Pharmacological concentrations of gemfibrozil inhibit basal, and growth factor inducible, augmentation of PAI-1 synthesis in HepG2 cells (Fujii and Sobel, 1992) Recently niacin has been shown not only to decrease constitutive synthesis of PAI-1 by HepG2 cells, but also to attenuate the induction of PAI-1 by transforming growth factor-β (a potent stimulus for PAI-1), hence reducing atherothrombosis by clot-associated mitogens (Brown et al, 1995). These studies also suggest that a pathological link exists between regulation of lipoprotein and PAI-1 gene expression.
1.10 Summary

Accumulating evidence suggests that impaired fibrinolysis plays an important role in the increased incidence of atherosclerosis and thrombosis in diabetic patients, and is particularly important in relation to CHD. For this reason it is important to understand the determinants of the increased level of PAI-1 observed in diabetes. In NIDDM, dissecting out the independent effects of lipids, insulin resistance and insulin-like molecules on PAI-1 activity is hampered by the close inter-relationships that exist between them.

1.11 Aims of this thesis:

1. To study the determinants of PAI-1 activity in a cross-sectional study of NIDDM patients, using specific assays of insulin, intact and des 31,32 proinsulin, and insulin sensitivity.

2. In a cross-sectional fashion to determine the effect of various treatments of NIDDM and ethnicity on PAI-1 activity.

3. In a cross-over study to investigate whether vigorous treatment with sulphonylurea and insulin produce greater stimulation and suppression of proinsulin-like molecules, and whether in consequence these produce larger changes in PAI-1 levels.

4. To determine the frequency of the 4G/5G polymorphism of the PAI-1 gene in a sample of NIDDM subjects, and to examine its association with plasma PAI-1 activity and its interaction with other determinants of PAI-1.

5. To study PAI-1 gene -environment interaction in detail by studying subjects after acute myocardial infarction.
CHAPTER 2

METHODOLOGY

This chapter will outline the background to the methodology used in the different studies in this thesis. The following topics will be discussed:

2.1 Measurement of fibrinolytic activity
2.2 Measurement of plasma insulin, proinsulin-like molecules and C-peptide
2.3 Measurement of insulin sensitivity and beta cell function
2.4 Measurement of plasma glucose, glycated haemoglobin and fructosamine
2.5 Measurement of serum lipids
2.6 Measurement of albumin excretion rate
2.7 Measurement of cytokines

2.1 Measurement of fibrinolytic activity

2.1.1 Background

Early epidemiological and clinical studies of fibrinolytic function were performed using methods that simultaneously measured the function of fibrinolytic activators and inhibitors such as euglobulin clot lysis time (Pechet, 1965) or fibrin plate lysis (Åstrup and Mullertz, 1952). Recently sensitive and specific assays for specific components of the fibrinolytic system, including t-PA and its fast acting inhibitor PAI-1, have led to an improved understanding of the role of the fibrinolytic system in various disease states. These assays are based on antigen antibody interactions or on the interactions of plasminogen activators with specific chromogenic substrates, and are outlined below.

2.1.2 Sample collection
It is essential that blood samples for measurement of fibrinolytic activity are collected with care into tubes containing inhibitors of the platelet release reaction, as antigen measures have shown that only 8% of PAI-1 is found in plasma and 92% is released from lysed platelets (Macy et al, 1993). Blood samples for measurement of components of the fibrinolytic system (PAI-1, t-PA) and fibrinogen were therefore collected into ice cold tubes containing sodium citrate (9 parts of blood were mixed with 1 part of 3.8% sodium citrate), centrifuged for 15 mins at 3000g and 4°C and the separated plasma was then stored at -10°C until analysed. Due to the diurnal variation of PAI-1 levels (peak 4-6 a.m and nadir at about 6 p.m) (Angleton et al, 1989), samples for all studies in this thesis were taken in the morning between 8-10 a.m.

2.1.3 Assays associated with fibrinolysis

2.1.3.1. Plasminogen activator inhibitor-1 activity

PAI-1 is a plasma protein that rapidly and specifically inhibits t-PA and urokinase. Functional assays depend on the addition of excess levels of t-PA to the plasma sample and measurement of residual t-PA activity. These assays have the advantage of measuring functionally active PAI-1 but also detect other inhibitors of t-PA such as α2-antiplasmin, PAI-2, and plasminogen activator binding proteins, and have proved difficult to standardize. For instance, what constitutes an excess of added t-PA activity? How long and under what conditions should the t-PA and PAI-1 be incubated? How should the t-PA activity be measured? Should single or two-chain t-PA be used? Chandler et al (1989) standardized the measurement of PAI-1 activity in plasma using single chain t-PA at a concentration of 5 IU.ml⁻¹, incubated for 15 mins at 37°C followed by acidification and measurement of residual t-PA activity by an amidolytic method. They showed that t-PA must be added in excess, but the addition of too much reduces the assay precision as only a small fraction of the original t-PA is inhibited, whereas addition of too little t-PA will result in underestimation of PAI-1 activity. Numerous commercial kits are available for determination of PAI-1 activity. A multicentre evaluation of commercial kit methods was performed by Gram et al (1993). A non-commercial reference method described by Verheijen et al (1982) was
used based on titration of plasma PAI with eight different concentrations of single-chain t-PA. Individual kit method results deviated significantly from the reference method and from each other. There was an inverse correlation observed between PAI-1 activity and the CV, with a CV of 100% for low PAI-1 activity and a CV of 16% for high PAI-1 activity. The study concluded that at present it is not possible to transfer to clinical practice the results from scientific studies.

In the cross-sectional study in this thesis (Chapter 3) the Kabi Coatest PAI-1 kit was used. However we subsequently found that PAI-1 activity measured using this kit was poorly reproducible. In the multicentre study mentioned above (Gram et al, 1993), results with this kit showed the greatest deviation from the reference method. All samples from the crossover study were measured by this method, which was acceptable for relative comparisons. However greater accuracy and precision was observed with the Biopool Spectrolyse pL kit (Umeå, Sweden). We evaluated this kit and it was used in all subsequent studies in this thesis. The characteristics of both of these kits will be described.

2.1.3.1.a Kabi Coatest PAI-1 activity kit

This kit determines the capacity of plasma to inhibit single chain human t-PA. PAI-1 is measured in arbitrary units (AU) where one AU of PAI-1 is defined as the amount of the inhibitor which inhibits one IU of t-PA.ml⁻¹ plasma. The assay described below uses a 10 minute incubation period as Speiser et al (1986) showed that t-PA inhibition was maximal after 10 mins incubation at 25°C and Korninger et al (1985) found that on prolonged incubation there was a gradual loss of t-PA activity mainly due to α₂ antiplasmin whereas the initial rapid loss was due to PAI-1.

**Assay principle**

A fixed amount of tissue plasminogen activator (t-PA) was added in excess to undiluted plasma where part of it rapidly forms a complex with the fast acting inhibitor PAI-1. Plasminogen is then activated to plasmin by the residual t-PA in the presence of a stimulator (fibrinogen fragments). The amount of plasmin formed is directly proportional to the residual t-PA activity and inversely proportional to the PAI-1
activity in the sample.

**Assay method**

Equal volumes of citrated plasma and single chain t-PA (concentration 40 IU.ml\(^{-1}\)) were incubated for 10 mins at 25\(^{\circ}\)C and diluted with 4 ml sterile water. Standards containing 40, 30, 20, 10, 0 AU.ml\(^{-1}\) of PAI-1 were prepared by mixing two standards containing 0 and 40 AU.ml\(^{-1}\) PAI-1 respectively in varying proportions. 100 \(\mu\)l of the incubator solution or standard was then incubated with 50 \(\mu\)l of a stimulator solution (fibrin fragments) and 100 \(\mu\)l of plasminogen-chromogenic substrate reagent (Plasminogen R + chromogenic substrate (\(<\text{Glu-Phe-Lys-pNA.HCL}\) + Tris buffer) for 70 mins at 37\(^{\circ}\)C. The amount of plasmin was determined by measuring the amidolytic activity of plasmin on the chromogenic substrate (\(<\text{Glu-Phe-Lys-pNA.xHCL}\) and the release of p-nitroaniline (pNA) was determined at 405 nm. The absorbance of the standards against their concentration of PAI-1 was plotted using a linear regression programme and the PAI-1 concentration of the unknown sample was then calculated from the standard curve.

**Assay characteristics**

The assay allows detection of 5 AU.ml\(^{-1}\) of PAI-1. There is no influence from variations in \(\alpha_2\) antiplasmin concentrations between 0-150\% and heparin concentrations below 4 IU.ml\(^{-1}\) do not affect the assay. The intra-assay coefficient of variation (CV) was 6.5\% and the inter-assay CV 8.5\%.

**2.1.3.1.b Biopool Spectrolyse pL kit**

This kit determines the capacity of plasma to inhibit single-chain human melanoma t-PA. The results are expressed as U.ml\(^{-1}\) and the effect of secondary inhibitors is diminished by acidification of plasma.

**Assay principle**

The assay is a two-stage indirect enzymatic assay. In stage one, a fixed amount of t-PA is added to the plasma sample and allowed to react with the PAI-1 present. The sample is then acidified to destroy \(\alpha_2\) antiplasmin and other plasmin inhibitors. In stage two the residual t-PA activity is measured.
**Assay method**

Stage one: Equal volumes of citrated plasma and single chain t-PA (concentration 40 IU.ml\(^{-1}\)) were incubated for 15 min at 25°C. Standards containing 40, 30, 20, 10, 0 AU.ml\(^{-1}\) of PAI-1 were prepared. The samples were then incubated in 100\(\mu l\) of 1.0 mol.l\(^{-1}\) sodium acetate buffer, pH 3.9, for 20 min at 37°C to destroy \(\alpha_2\) antiplasmin activity. Stage two: The outermost wells in a micro test plate are filled with water to obtain uniform temperature in all sample wells. To measure residual t-PA activity 200\(\mu l\) of ice cold Glu-plasminogen, poly-D-lysine (stimulator), D-But-CHT-Lys-pNA (chromogenic substrate) and imidazole were added to 20\(\mu l\) of standard or sample. The plate was then sealed with adhesive tape and floated in a water bath at 37°C for 90 min. The residual t-PA activity in the sample catalyzes the conversion of plasminogen to plasmin which in turn hydrolyses the chromogenic substrate, the amount of colour developed being proportional to the t-PA activity in the sample. The release of pNA was determined at 405nm. The absorbance of the standards against their concentration of PAI-1 was plotted using a linear regression programme and the PAI-1 concentration of the unknown sample was then calculated from the standard curve.

**Assay characteristics**

PAI-1 activity in the range 8 to 28 U.ml\(^{-1}\) can accurately be measured in undiluted plasma samples and 16 to 56 U.ml\(^{-1}\) in plasma dilute 1:2 in PAI depleted plasma. The intra-assay CV was 6.0% and inter-assay CV 6.5%.

The Biopool kit was compared to the Kabi kit, by measuring 20 samples using both kits. There was a strong correlation between the values obtained (\(r=0.97; p<0.0001\)) (Figure 2.1).

**2.1.3.2 Plasminogen activator inhibitor-1 antigen**

As described in chapter 1, PAI-1 is synthesised in an active form which spontaneously converts to a latent, inactive form that can be reactivated in vivo (Vaughan *et al*, 1990). Recently an inactive, but cleavable form of PAI-1 has been identified (Declerck *et al*, 1993).
Figure 2.1  A comparison of PAI-1 activity using Biopool and Kabi kits

\[ r = 0.97 \]
\[ p < 0.0001 \]
Various polyclonal and monoclonal antibodies have been produced against PAI-1 and have been applied for the construction of immunological assays. The use of different antibodies in different enzyme linked immunoassays (ELISA) may alter the specificity towards the various forms of PAI-1 (e.g. latent, active, complexed to t-PA or complexed to the PAI-1 binding proteins). Commercial kits for determination of PAI-1 antigen based on different reagents may therefore not be measuring similar forms of PAI-1. The particular form of increased PAI-1 in plasma may depend on the disease state and hence the origin of the increased PAI-1, e.g. either from endothelial cells (active) or from platelets (latent) (Alessi et al, 1990). It is important for interpretation of data to know which forms are being measured. PAI-1 antigen was measured using the Biopool TintElize kit. This is an ELISA for the quantitative determination of endothelial-type PAI-1 in plasma. It also detects active and latent forms of PAI-1. The t-PA/PAI-1 complexes are recovered with about the same efficiency as free PAI-1.

**Assay principle**

The assay is based on the ELISA described by Declerck et al (1988) using the same mouse monoclonal 'capture' antibody (MA-7D4B7), and detected with a polyclonal anti-PAI-1 antibody-conjugate which has been selected to make the assay insensitive to variations in PAI-1 conformation. Active, latent and tPA-complexed forms are equally recognised by this assay. Rheumatoid factor(s) in plasma can bridge coat with conjugate antibodies causing a non-specific, high response. To overcome this problem each sample was analyzed in two adjacent wells: one contains normal mouse IgG (N-well) and the other contains mouse anti PAI-1 IgG (A-well). The A-well measures non-specific effects and the N-well is specific for PAI-1 plus the same non-specific response as the adjacent A-well. The difference in absorbance between the N-well and the A-well is highly PAI-1 specific.

**Assay method**

PAI-1 antigen standards (40, 20, 10, 0 ng.ml⁻¹ PAI-1) were prepared from PAI-1 standard plasma, the PAI-1 antigen content of which was based on amino acid quantitation of PAI-1 using the known amino acid composition and a molecular weight of 52,000 D. PAI-1 antigen standards and samples of citrated plasma were added to
micro-test wells coated with monoclonal anti-human PAI-1 antibodies (MA-7D4B7), which captures the PAI-1 in the sample. A second anti-PAI-1 antibody, conjugated to peroxidase, was then added and the solution was incubated for 2 h at room temperature. The conjugated antibody binds to the free antigenic determinants on the immobilized PAI-1 forming a coat antibody-PAI-1 antigen-conjugated antibody "sandwich". Unbound conjugated antibody and other unbound materials were washed away and a peroxidase substrate (Orthophenylenediamine) was added. The substrate becomes coloured through catalytic oxidation by peroxidase-conjugated antibody and hydrogen peroxidase. The intensity of the colour is directly proportional to the amount of PAI-1 in the sample. The enzymatic reaction was stopped by adding sulphuric acid. The absorbance was measured at 492 nm. The difference in absorbance between the N-well and the A-well was calculated for all standards and samples and this difference, denoted \( \Delta A \), represents the PAI-1 specific response. A standard curve was obtained by plotting \( \Delta A \) against the amount of PAI-1 in the standard samples using a linear regression programme. The amount of PAI-1 in the test samples was obtained by comparing the sample \( \Delta A \) with the standard curve.

**Assay characteristics**

The assay quantitates both free (active and latent) and complexed forms of PAI-1. The t-PA/PAI-1 and u-PA/PAI-1 complexes are recovered with about the same efficiency as free PAI-1. The detection limit is about 2 ng/ml PAI-1. The within assay CV was 4.5% (\( n=10 \)) and the between assay CV was 7.5% (\( n=12 \)).

### 2.1.3.3 Tissue plasminogen activator antigen

T-PA was measured using the Biopool TintElize kit which quantifies human single-chain and two-chain t-PA antigen.

**Assay principle**

The assay is a two-site ELISA, using quenching and normal antibodies as a control for t-PA specificity. This ensures that t-PA antigen measurements are unbiased by the presence of e.g. rheumatoid factor(s) and anti-goat antibodies.

**Assay method**

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t-PA antigen standards (30, 24, 18, 12, 6, 3, 1.5 and 0 ng.ml⁻¹) and the unknown samples were added to two adjacent micro-test wells: one contains normal goat IgG (N well) and the other contains goat anti human t-PA IgG (A well). During the incubation period (3 h at room temperature) the t-PA in the sample binds to the anti t-PA immunoglobulin G. A second anti-t-PA antibody conjugated to horseradish peroxidase is then added to the wells and incubated for a further hour. This binds to free antigenic determinants on the t-PA molecules present forming a coat antibody-t-PA antigen-conjugated antibody "sandwich". Unbound conjugated antibody and other unbound materials are then washed away. A substrate (Orthophenylenediamine Dihydrochloride) is added. The amount of colour developed from substrate catalytically oxidised by the enzyme, peroxidase, is proportional to the amount of t-PA in the sample. The enzymatic reaction is stopped by adding sulphuric acid and the absorbance is read at 492 nm. The difference in assay response between the two wells is highly t-PA specific. To obtain a standard curve the difference in absorbance between the N well and the A well, denoted ΔA, was plotted against the known concentration of t-PA in the standard using a linear regression program. The amount of t-PA in the sample was determined by comparing the sample ΔA with the standard curve.

Assay characteristics
The assay measures t-PA antigen in the range 0.03 to 0.6 ng t-PA per well. Maximum sensitivity of the assay is 1.5 ng.ml⁻¹ sample. The assay detects both active and complexed forms of single-chain and two-chain t-PA. No cross reaction with urokinase is observed. Heparin (<8 U.ml⁻¹) does not influence the determination. The immunoreactivity of single-chain and two-chain t-PA in complex with α₂ antiplasmin, PAI-1, and PAI-2 is around 90%. The within assay CV was 6% (n=10) and the between assay CV 10% (n=9).

2.1.3.4 Fibrinogen
Fibrinogen was measured according to the Clauss method (1957) using the Boehringer Mannheim kit.
Assay principle

The clotting time of plasma after the addition of thrombin in excess is proportional to the concentration of fibrinogen.

Assay method

Human fibrinogen solution (0.4 gl⁻¹) was diluted to give standard concentrations of 0.1, 0.2, 0.3 and 0.4 gl⁻¹. 0.2 ml of each concentration was added to a separate glass tube with 0.2 ml of thrombin (250 NIHI units.ml⁻¹) and placed in a water bath at 37°C and the time to the onset of clotting was recorded in duplicate. The clotting time was plotted against the known fibrinogen concentration on logarithmic graph paper to obtain a standard curve. Citrated plasma was diluted 1/10 with barbitone buffer solution and 0.2 ml of the diluted plasma was added to 0.2 ml of thrombin in a glass test tube, placed in a water bath at 37°C and the time to the onset of clotting was recorded in duplicate. The concentration of fibrinogen in the unknown plasma samples was calculated by reading the concentration of fibrinogen corresponding to the clotting time from the standard curve and correcting for dilution.

Assay characteristics

The intra-assay CV was 1.4%(n=20) and inter-assay CV 3.0%(n=10).

2.2 Measurement of plasma insulin and proinsulin-like molecules

2.2.1 Background

As mentioned in Chapter 1, in NIDDM subjects proinsulin-like molecules contribute around 30% of fasting immunoreactive insulin (IRI) (Yudkin, 1993). Recent developments in assay technology have enabled proinsulin and its intermediate conversion products to be distinguished from insulin.

A variety of assay methods have been previously employed to measure proinsulin concentrations, including radioimmunoassay of molecules separated by gel filtration (Horwitz et al, 1975 and Mako et al, 1977) or by immunoprecipitation using antibodies to C-peptide (Saad et al, 1990). Radioimmunoassays using guinea pig antiserum specific for human proinsulin (Yoshioka et al, 1988) have been used. Polyclonal
proinsulin antibodies have been used in two-site immunoradiometric (IRMA) assays (Heding et al, 1977) and in enzyme-linked immunosorbent assays (Hartling et al, 1986). Highly specific and sensitive monoclonal antibodies to proinsulin-like molecules have been described (Sobey 1989).

2.2.2 Measurement of plasma insulin

2.2.2.a. Radioimmunoassay for insulin (RIA)

Plasma insulin was measured by radioimmunoassay using a commercial kit, based on a polyclonal antibody (Novo Nordisk, Copenhagen, Denmark).

Assay principle

Insulin in samples and standards compete with the ^125I-insulin tracer for binding sites on the antibody. After incubation the amount of tracer bound to the antibody was inversely proportional to the amount of insulin in the sample or standard. The bound and free fraction was separated using a polyethylene glycol solution, and centrifuged. The radioactivity in the precipitate was counted and standard curve drawn, from which the concentration of insulin in the samples was interpolated.

Assay method

Insulin standards (0 to 100 µU.ml⁻¹) were prepared by serial dilution of a known insulin standard. Unlabelled insulin, i.e. the standard or unknown sample, was preincubated with a surplus of insulin antibody. After 20 hr incubation ^125I-insulin, was added and after a further 20 to 24 hr incubation the free and antibody bound insulin are separated by the addition of 95% ethanol. After mixing the antibody bound insulin precipitates immediately while the free ^125I-insulin stays in solution. After centrifugation the free ^125I-insulin was discarded and the antibody bound ^125I-insulin was determined by measuring the radio-activity in the precipitate (NE 1600 gamma counter). A standard curve of counts per minute against insulin concentration (µU.ml⁻¹) was plotted and the insulin concentration (µU.ml⁻¹) of the unknown samples was determined.

Assay characteristics

The within assay CV was 5%(n=21) and the between assay CV was 9.6%(n=8).
2.2.2.2. Enzymoimmunoradiometric (IEMA) assay for insulin

The application of enzyme amplification systems to immunoassays has enabled the development of enzyme immunoassays with high sensitivity and speed (Stanley et al, 1985, Self et al, 1985). These methods have been employed in the development of a highly specific and sensitive assay for insulin using monoclonal antibodies.

Assay principle (Figure 2.2.1)

Plasma insulin was measured using specific monoclonal antibodies (Sobey et al, 1989) in a two site amplified IEMA (Mohamed-Ali et al, 1992). The monoclonal antibodies used, 14B and 3B1, belong to the immunoglobulin G class (Serono Diagnostics, Woking, UK). 14B does not cross react with human proinsulin and binds to insulin around the C-peptide A-chain junction at the 65,66 residues, while 3B1 binds to the B chain around residues 6 to 8. 14B is specific for human insulin and was used as the 'capture' antibody, 3B1 was conjugated to alkaline phosphatase and used as the 'signal' antibody. As 14B and 3B1 bind to separate non-overlapping epitopes on the insulin antigen, a 'sandwich' of immobilised antibody, insulin and conjugate is formed on the surface of the well. The amount of bound conjugate was dependent on the concentration of insulin in the standard or sample. The enzyme activity of the bound fraction was measured in an end point enzyme amplified assay.

Assay method

Insulin standards were prepared from known concentrations of insulin antigen (International reference standard, Potters Bar, South Mims, UK). 14B was coated onto a microtitre plate by overnight incubation at 4°C in Tris/HCL, pH 9.0. The plate was washed with Tris, pH 8.2, to remove excess antibody, and non specific sites blocked with 1% bovine serum albumin (BSA)/ Tris, pH 8.2 for 1 h at 37°C. 25μl of insulin standards and samples were added to each well and incubated overnight at 4°C following which 100μl of 3B1 conjugated to alkaline phosphatase was added. The unbound conjugate was washed off four times with Tris 0.05% Tween 20, and the plates dried at room temperature.
Figure 2.2 Enzymoimmunometric assay for plasma insulin

2.2.1 Insulin plate immunoassay

2.2.2 Enzyme Amplification for Colour Development
For colour development, 100μl of substrate (NADP⁺) was added to each well and incubated at room temperature for 20 min. NADP⁺ was dephosphorylated to NAD⁺ by the bound alkaline phosphatase. 100μl of amplifier (alcohol dehydrogenase and diaphorase in amplification buffer containing phosphate, ethanol, BSA and p-iodonitrotetrazolium violet) was added to each well and incubated for 10 min at room temperature. NAD⁺ was reduced by ethanol, and the NADP formed reduced the tetrazolium salt to regenerate NAD⁺ and produce a formazan dye (Figure 2.2.2). The rate of reduction of the tetrazolium salt was directly proportional to the concentration of the NAD⁺ originally formed by the enzyme in the bound conjugate. The reaction was terminated with 0.3M H₂SO₄ and the absorbance measured at 490nm. The concentration of insulin was read from a standard curve, plotted using the insulin concentrations in the standards.

**Assay characteristics**

The assay was sensitive to 2.0 pmol.1⁻¹, the intra-assay CV was 8.7%(n=20) and inter-assay CV 12.0%(n=12). Cross-reactivity with intact proinsulin was less than 5%, and recovery from spiked plasma on 8 separate occasions, was 96% (Mohamed-Ali et al 1992).

### 2.2.3 Microplate immunoradiometric assay (IRMA) for Intact and des 31,32 proinsulin

**Assay principle**

Intact and des 31, 32 proinsulin were measured using a two site monoclonal immunoradiometric assay (IRMA) modified to a microplate method (Mohamed-Ali et al, 1996).

**Assay method**

Purified monoclonal antibodies 3B1 and A6 were purchased from Serono Diagnostics (Woking, Sussex), and anti C-peptide antibody, PEP 001, from Novo Nordisk, (Denmark). Plates were coated with 200μl per well 3B1 antibody diluted to 2μg.ml⁻¹ in 0.01M Tris buffer, pH 9.0, with 0.15 M NaCl, and incubated overnight at +4°C. Coating antibody was recovered and non-specific binding blocked by washing 4 times
with 0.01M Tris with 5% sucrose, 0.45% NaCl and 0.1% BSA, pH 8.5, and dried at 37°C for 1 hr. Standards covering the range 0.25 to 125 pmol.l⁻¹ were prepared for both antigens. 125μl of standards, controls or samples were added following addition of 25μl of 0.01M Tris containing 1% BSA, 0.6% normal mouse serum and aprotinin (200KIU.l⁻¹). The normal mouse serum was used to reduce the interference due to human anti-mouse antibodies and heterophilic antibodies in the samples. Aprotinin, a protease inhibitor was used to minimise sample degradation during incubation. Plates were mixed for 1 min and incubated overnight at +4°C. Plated were washed 3 times with Tris/Tween was buffer and last traces aspirated. 100μl per well of iodinated antibody (A6-I¹²⁵ for the intact proinsulin assay and PEP001- I¹²⁵ for the des 31,32 proinsulin assay) diluted in 0.01 M Tris with 1.0% BSA to contain approximately 20,000cpm was added to all wells, mixed and incubated overnight at +4°C. Plated were washed 4 times with wash buffer. The radioactivity of individual wells was determined on a NE 1600 gamma counter (Nuclear Enterprises Ltd., Edinburgh, UK). The curve fitting algorithm used the 4PL-model with concentration (linear) on the x-axis and response on the y-axis.

**Assay characteristics**

The sensitivities of the assays were determined by measuring the zero dose standard 20 times. The standard corresponding to the mean response of the zero standard plus 3 standard deviations was defined as the limit of detection of the assay. The specificity was determined by spiking samples with the related molecules, human insulin up to 2500 pmol.l⁻¹, IGF-1 up to 250 pmol.l⁻¹, C-peptide up to 250 nmol.l⁻¹ and the proinsulin-like molecules up to 250 pmol.l⁻¹. The cross-reactivity of each of these metabolites was ascertained. The precision of the assays was obtained from the coefficient of variation of the quality control samples and these were defined for three levels in within batch and between batch assays.

1- **Intact Proinsulin:**

The detection limit of the assay was 0.25 pmol.l⁻¹. The intra-assay CV was 6.3%(n=20) and inter-assay CV 9.8%(n=12). There was no cross-reaction with insulin but 78% cross-reaction with des 64,65 proinsulin. Recovery of intact
proinsulin added to human plasma was 93% (range 83-100%).

2- Des 31,32 proinsulin

The detection limit of the assay was 0.125 pmol.l⁻¹. The intra-assay CV was 8.6%(n=20) and inter-assay CV 12.3%(n=12). There was no cross-reaction with insulin but 59% and 54% with intact and des 64,65 proinsulin. Recovery of des 31,32 proinsulin added to human plasma was 89% (range 84-99%).

2.2.4 C-peptide

Plasma C-peptide was measured using a radioimmunoassay kit for human C-peptide (Biodata S.p.A, Italy). C-peptide in samples and standards compete with the ¹²⁵I C-peptide tracer for binding sites on the antibody. After incubation the amount of tracer bound to the antibody was inversely proportional to the amount of C-peptide in the sample or standard. The bound and free fraction was separated using a polyethylene glycol solution, and centrifuged. The radioactivity in the precipitate was counted and standard curve drawn, from which the concentration of C-peptide in the samples was interpolated.

Assay characteristics

The detection limit of the assay was 0.1 ng.ml⁻¹, with intra-assay CV of 3.5% and inter-assay CV 7.8%.

2.3 Measurement of insulin sensitivity and beta cell function

2.3.1 Background

Insulin sensitivity (IS), defined as the ability of a given amount of insulin to dispose of a glucose load, can be assessed by many methods. The simplest are the oral glucose tolerance test with simultaneous determination of glucose and insulin levels and the intravenous insulin tolerance test, in which the slope of decline of glucose levels may be taken as measure of IS. However these methods have their limitations due to variations in insulin levels and hormonal counter-regulation. The "gold standard" for measurement of insulin sensitivity is the euglycaemic hyperinsulinaemic clamp, in which, during a 2 hour insulin infusion, basal glucose concentrations are maintained
by infusing appropriate amounts of glucose. The mean glucose infusion rate in the second hour is a measure of IS (De Fronzo et al, 1979). Harano et al, (1977) described a method for the IS test using a simultaneous continuous infusion of glucose, insulin and somatostatin to inhibit endogenous insulin secretion. The glucose level at 2 hours reflects IS. Heine et al (1985) found significant correlations of insulin sensitivity measured using the euglycaemic hyperinsulinaemic clamp, with insulin sensitivity measured by this technique, with (r=0.90) and without somatostatin (r=0.83). For our crossover study we employed the modified Harano technique i.e. without somatostatin for the measurement of IS.

For population studies however, the above methods of glucose and insulin infusions are time consuming. In our cross-sectional study we used Homeostasis Model Assessment (HOMA) to derive an estimate of IS (Matthews et al, 1985). The model can also be used to estimate b-cell function (BCF). The steady-state basal plasma glucose and insulin concentrations are determined by their interaction in a feed-back loop. HOMA is a computer solved model to predict the homeostatic concentrations which arise from varying degrees of BCF and insulin resistance. The estimate of IS obtained with HOMA correlate with the euglycaemic clamp (r=0.88, p<0.0001), and the estimate of BCF with that derived from using the hyperglycaemic clamp (r=0.61, p<0.01) (Matthews et al, 1985). Values are expressed as a percentage of a normal population with 100% being taken as normal for IS and BCF. As the model was originally developed using immunoreactive insulin, we multiplied our IEMA insulin values by a correction factor (1.17) prior to using the computer program. The computer program used was developed from the original HOMA formula (Levy J, personal communication).

2.3.2 Modified Harano technique (Figure 2.3)

After an overnight fast the right antecubital vein was cannulated for infusions of insulin and glucose, and the left distal forearm vein cannulated retrogradely and warmed in an insulating blanket at 50°C, for venous sampling of arterialized blood. Each subject was
Figure 2.3 The modified Harano technique for measuring insulin sensitivity.

Metabolic Clearance Rate Glucose (MCR-G) = \frac{\text{glucose infusion rate}}{\text{mean glucose concentration}} \frac{(120-150 \text{ min})}{(120-150 \text{ min})}
given a 150 min continuous infusion of glucose (6mg.kg⁻¹.min⁻¹) and 50mU.kg⁻¹.h⁻¹ human soluble insulin (Actrapid, Novo Nordisk, Basingstoke, Hants, UK) in 50 mls of Haemaccel (Hoechst), as separate infusions using Travenol Flowgard pumps (Baxter Health Care, Compton, Newbury, UK). Blood glucose was measured at baseline and every 15 min during the procedure (Reflolux, Boehringer, Lewes, Sussex, UK). Arterialised blood samples were withdrawn from the insulated arm every 5 min for the last 30 min for assay of plasma glucose by the glucose oxidase method (Beckman Glucose II analyzer, Beckman, Brea, CA, USA). Steady state plasma glucose was calculated from the mean of the seven samples taken from 120-150 mins. It was possible to achieve steady state plasma glucose concentrations in nearly all subjects. Glucose measurements during the insulin sensitivity test are shown in 20 NIDDM subjects post glibenclamide therapy (Figure 2.4a) and post insulin therapy (Figure 2.4b). The mean CV for the steady state glucose concentrations were 2.9% in subjects taking glibenclamide and 2.1% in subjects taking insulin. Insulin sensitivity was calculated as the metabolic clearance rate (MCR) of glucose by dividing the glucose infusion rate by the steady state plasma glucose concentration and expressed as ml.kg⁻¹.min⁻¹.

2.3.3 Homeostasis Model Assessment (HOMA)

We employed fasting plasma glucose and specific insulin levels in the HOMA model to derive an estimate of IS and BCF. Because the physiological relationship between glucose concentrations and insulin secretion no longer applies in insulin treated patients it is not possible to measure IS and BCF in such patients. The computer program used was developed from the original HOMA formula (Levy J, personal communication).

2.3.4 Comparison of insulin sensitivity measured using the modified Harano method and HOMA

In 19 poorly controlled NIDDM subjects, on diet alone, insulin sensitivity was measured using the modified Harano method, and the HOMA model. The correlation observed between the 2 measurements was (r=0.78, p<0.0001)(Figure 2.5).
Figure 2.4a Glucose measurements during insulin sensitivity test (Modified Harano). 20 subjects studied post glibenclamide therapy.

Figure 2.4b Glucose measurements during insulin sensitivity test (Modified Harano). 20 subjects post insulin therapy.
Figure 2.5  Correlation between measurements of insulin sensitivity using the modified Harano method and the HOMA model.

\[ r = 0.78 \]

\[ p < 0.0001 \]
2.4 Measurement of plasma glucose, glycated haemoglobin and fructosamine

2.4.1 Glucose
Plasma glucose was measured by a glucose oxidase method using a Beckman glucose analyser (Beckman, Brea, CA, USA), with inter-assay CV of 1.8% (n=14).

2.4.2 Glycated Haemoglobin (HbA1)
HbA1 was measured by agar gel electrophoresis (Corning, Halstead, UK). The normal range was 6.5-8.5% with an intra-assay CV of 3% and inter-assay CV of 5%.

2.4.3 Fructosamine
Serum Fructosamine was measured using a commercial kit (Roche), using a Cobas-Fara centrifugal analyser. The normal range is 2.05-2.85 mM. The intra-assay CV was <3%.

2.5 Measurement of serum lipids

2.5.1 Total Triglyceride
Plasma triglyceride was measured using GPO-PAP kit (Boehringer Mannheim, Lewes, E.Sussex, UK) (inter-assay CV 1.9%).

2.5.2 Cholesterol
Plasma cholesterol were measured by an enzymatic, colorimetric method using Kit C system high performance CHOD-PAP method (Boehringer Mannheim, Lewes, E.Sussex, UK) (inter-assay CV 1.6%). HDL cholesterol was measured in solution after precipitation of LDL and VLDL using sodium heparin and manganese chloride (inter-assay CV 5.5%). LDL-cholesterol was calculated using the Friedwald formula (Friedwald et al, 1972).

2.5.3 Apolipoproteins
Apoproteins A1, A2 and B were analysed by immunoturbometric assays (Mount JN
1988), using a Cobas Bio Centrifugal analyser (Hoffmann-La Roche, Switzerland), with inter-assay CV's of 2.8%, 3.1% and 2.7% respectively.

2.5.4 Lipoprotein (a)

Lp (a) was measured by a 1-step sandwich ELISA (Immunozym Lp (a), Immuno).

*Assay principle*

The wells of the ELISA test strips are coated with monospecific, polyclonal anti-apo(a)-antibodies. Diluted samples are incubated with the conjugate, which consists of a monospecific and monovalent anti-apo(a)-Fab-fragment coupled with peroxidase (anti-apo(a)-peroxidase-conjugate). During the incubation time Lp(a) particles and free apo(a) are bound at the solid phase and marked by the conjugate. In a second incubation step the substrate reaction takes place. The colour intensity is directly proportional to the Lp(a) concentration in the sample.

*Assay method*

100µl of conjugate solution and 100µl of diluted sample (calibrator or control) was added to each well. The test strips were covered with foil, and incubated at room temperature for 2 hours. The strips were washed with buffer solution (Tris/HCl, pH 8.4) and 200µl of substrate solution (tetra-methyl-benzidine in ethanol/DMSO) added to each well. The strips were incubated for 30 min and the reaction stopped with 2mol.l⁻¹ sulphuric acid. Optical density was measured at 450nm in a plate reader, and the Lp(a) concentrations determined from a reference curve.

*Assay characteristics*

The intra-assay CV was 5% and inter-assay CV 9.1%.

2.6 Measurement of albumin excretion rate

Each subject collected 3 separate timed overnight urine samples. The albumin excretion rate (AER) (µg.min⁻¹) was calculated by multiplying the albumin concentration (µg.ml⁻¹) by the urine volume (ml) and dividing this by the time taken to collect the sample (min). A mean of the three AERs was taken.
Assay method
Urinary albumin was measured by an in-house modified, competitive radioimmunoassay (Chesham et al, 1986), and validated against a commercial radioimmunoassay \( r=0.96; \) Pharmacia-LKB, Milton Keynes, Bucks, UK.

Assay characteristics
The inter-assay CV was 7.8 \( (n=24) \) % and intra-assay CV < 5\%\( (n=11) \).

2.7 Measurements of cytokines

2.7.1 Interleukin-1 (IL-1β)

Assay principle
IL-1β was measured using an enzyme-linked immunosorbent assay (R&D Systems, Oxford, UK).

Assay method
A monoclonal antibody specific for IL-1β was coated onto a microtiter plates. Standards and samples were pipetted into the wells and any IL-1β present bound to the immobilized antibody. After washing away any unbound proteins, an enzyme-linked polyclonal antibody specific for IL-1β was added to the wells to sandwich the IL-1β immobilization during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution (lyophilized NADPH) was added to the wells. After an incubation period of 2 hours on a microtitre plate shaker, an amplifier solution (alcohol dehydrogenase and diaphorase) was added to the wells and colour developed in proportion to the amount of IL-1β bound in the initial step. The enzyme amplification system for colour development is similar to that described for the specific insulin assay (IEMA) in section 2.2.2.b (Figure 2.2). The colour development was stopped with sulphuric acid.

Assay characteristics
The IL-1β assay had a limit of detection of 0.1 pg.ml\(^{-1}\), an intra-assay CV of 8.0\%(n=20) and an inter-assay CV of 9.7\%(n=5).
2.7.2 Interleukin-6 (IL-6)

Assay principle
IL-6 was measured using an enzyme-linked immunosorbent assay, that was specific for the total amount (i.e. bound and unbound) of IL-6 (R&D Systems, Oxford, UK).

Assay method
A monoclonal antibody specific for IL-6 was coated onto a microtiter plates. The assay methodology is as described for IL-1β.

Assay characteristics
The IL-6 assay had a limit of detection of 0.09 pg.ml⁻¹, an intra-assay CV of 5.3%(n=20) and an inter-assay CV of 9.2%(n=5).

2.7.3 Tumour necrosis factor-α (TNF-α)

Assay principle
TNF-α was measured using an enzyme-linked immunosorbent assay, that was specific for the total amount (i.e. bound and unbound) of TNF-α (R&D Systems, Oxford, UK).

Assay method
A monoclonal antibody specific for TNF-α was coated onto a microtitre plates. The assay methodology is as described for IL-1β.

Assay characteristics
The TNF-α assay had a limit of detection of 0.1pg.ml⁻¹, an intra-assay CV of 6.9%(n=20) and an inter-assay CV of 8.4%(n=4).

Assays for fibrinolytic parameters were performed by myself, as were insulin sensitivity measurements. Assay of insulin, proinsulin-like molecules, cytokines, and AER were performed by Dr Vidya Mohammed-Ali at the department of Medicine, Whittington Hospital. Lipid assays and measurement of HbA1 and fructosamine were performed by Chemical Pathology at the Whittington Hospital. Lipoprotein assays were performed by Dr Lumb at the department of Chemical Pathology, St. Thomas’s Hospital.
CHAPTER 3

DETERMINANTS OF PLASMINOGEN ACTIVATOR INHIBITOR-1
ACTIVITY IN NIDDM SUBJECTS AND ITS RELATION TO
HYPOGLYCAEMIC THERAPY AND ETHNIC GROUP

3.1 Introduction

PAI-1 activity is raised in NIDDM subjects and may contribute to their excess risk of cardiovascular disease. In this study we examined the determinants of PAI-1 activity in 146 NIDDM subjects using specific assays of insulin, intact and des 31,32 proinsulin and measures of insulin resistance, relating these measurements to serum lipids and hypoglycaemic therapy.

3.1.1 A cluster of variables determine PAI-1 activity in NIDDM


In a group of nine non-diabetic and diabetic subjects, PAI-1 antigen correlated significantly with insulin resistance as measured by a hyperinsulinaemic euglycaemic clamp (Potter van Loon et al, 1993). In non-diabetic subjects insulin resistance is paralleled by increased insulin concentrations and both correlate with triglyceride levels, so any one or more of these three variables may explain inter-relationships with
PAI-1 activity. By contrast in NIDDM, insulin resistance and concentrations of insulin and triglyceride are less tightly interdependent, and in this situation concentrations of proinsulin-like molecules may also play a significant role in determining PAI-1 activity (Nagi et al, 1990).

3.1.2 PAI-1 and hypoglycaemic therapy in NIDDM

Diabetic treatment may also affect PAI-1 activity, either directly or via one of the cluster of variables known to affect PAI-1 as mentioned above.

3.1.2.1 Sulphonylureas

Chlorpropramide and tolbutamide, both first generation sulphonylureas, were the first oral hypoglycaemic drugs reported to enhance blood fibrinolysis (Fearnley et al, 1960). The fibrinolysis-enhancing effect was reversed within 3-4 weeks. By combining a biguanide with a sulphonylurea, it was demonstrated that the fibrinolysis-enhancing effect could be sustained for as long as 3 years in subjects with CHD, while temporary effects were seen when each drug was given alone (Fearnley et al, 1967). It is of interest that in a pilot study, patients treated with the combination therapy had a low incidence of re-infarction when followed for 36 months post infarction (Chakrabarti and Fearnley, 1972).

The interest in sulphonylureas lay dormant for many years following the results of the University Group Diabetes Program study (UGDP study group, 1970), which demonstrated increased mortality on tolbutamide. Two studies then reported that gliclazide, a second generation sulphonylurea, produced an increase in both blood fibrinolytic activity and the activity of vessel wall plasminogen activator (Chan et al, 1982, Almér, 1984). Further studies showed that gliclazide could increase endothelial cell t-PA dependent fibrinolysis in subjects with IDDM with no residual β-cell function (Gram et al, 1988). A rise in plasma t-PA activity has also been demonstrated in subjects changed from tolbutamide (a first generation sulphonylurea) to gliclazide (Gram et al, 1989). The reported effects of gliclazide in these studies were all
independent of the metabolic state of the patient. The basic pharmacological mechanism for this action remains unclear. Glipizide, another second generation sulphonylurea, causes a five fold increase in t-PA release from cultured bovine aortic endothelial cells on an insulin-free medium (Kuo et al, 1988). This effect was more potent than tolbutamide, and involved increased RNA and protein synthesis.

Jain et al (1993) compared the effect of therapy with sulphonylureas or with insulin on PAI-1 activity in 11 poorly controlled NIDDM subjects. Without altering glycaemic control, insulin treatment was associated with reduced PAI-1 activity, this reduction correlating with the suppression of concentrations of proinsulin-like molecules. This study suggested that the higher PAI-1 activity observed on sulphonylurea treatment, may have a direct correlation with higher levels of proinsulin-like molecules observed in these subjects.

3.1.2.1 Metformin

Metformin, a biguanide antihyperglycaemic agent, has been shown to reduce PAI-1 activity levels in normal subjects with obesity (Vague et al, 1987). Nagi and Yudkin, (1993), investigated the effect of metformin in NIDDM subjects in a 12 week, double blind, placebo controlled study. Metformin treatment was associated with improved glycaemic control, lower insulin resistance and lower risk factors for cardiovascular disease including PAI-1. Grant et al (1996) reported an improvement in fibrinolysis in NIDDM subjects treated with metformin for 6 months. This effect was independent of the dose of metformin used.

3.1.3 Ethnic differences in PAI-1

The risk of cardiovascular disease and NIDDM differs between racial groups. Pima Indians living in Arizona and South Asian immigrants living in the UK have a higher prevalence of NIDDM (Knowler et al, 1978, Mather et al, 1985), and both populations are hyperinsulinaemic and insulin resistant compared to Caucasians. South Asian subjects living in the UK, despite lower cholesterol concentrations and lower smoking
rates have considerably higher cardiovascular mortality than native Europeans (Marmot et al, 1985). Nagi et al, (1996), found higher levels of PAI-1 activity in non-diabetic Pima Indians than in non-diabetic Europeans, but significantly lower PAI-1 levels in diabetic Pimas than in diabetic Europeans or Asians. No association was found between the higher prevalence of ischaemic heart disease in Asians subjects and PAI-1 levels.

An interesting study by Frist et al, (1995), showed that cultured human umbilical vein endothelial cells (HUVEC) from black American subjects express significantly higher levels of t-PA and lower levels of PAI-1 than cultured HUVECs from white American subjects reflecting an apparent increased fibrinolytic potential in cultured HUVECs derived from black Americans. This finding may provide an explanation for the lower incidence of CHD observed in black American subjects.

3.2 Aims of the study

We investigated PAI-1 activity in 146 well characterised NIDDM subjects. The aims of this study were:

1. To examine the determinants of PAI-1 activity in a sample of NIDDM subjects using specific assays of insulin, intact and des 31,32 proinsulin and measures of insulin sensitivity.

2. To determine the effect of treatment of NIDDM on PAI-1 activity and proinsulin-like molecules.

3. To determine the effect of ethnic group on PAI-1 activity and proinsulin-like molecules.
3.3 Subjects and methods:

3.3.1 Subjects

146 NIDDM subjects (66 male, 80 female) attending the outpatient clinic at the Whittington Hospital were studied. Subjects had been diagnosed between the ages of 25-65 yrs, and were part of the UK Prospective Diabetes Study Group (UKPDS, 1994). At the start of the UKPDS 10 years ago, after a 3 month run-in period, subjects were randomised to treatment with diet alone, insulin, or oral hypoglycaemic agents. At the time of our study, subjects were on the following treatments: insulin (n=36), sulphonylurea (n=46), sulphonylurea plus metformin (n=27), metformin (n=11), and diet alone (n=26). This treatment differed from initial randomisation, and our analyses were based on current treatment rather than initial randomisation. The sample comprised: 86 Caucasian, 26 Afro-Caribbean and 34 Asian subjects. All patients gave informed verbal consent and the study was approved by the ethical committee of Islington Health Authority.

3.3.2 Methods

Patients attended the diabetic clinic after an overnight fast. Weight and height were recorded with the patients wearing light clothing and without shoes. Waist measurements were taken as the minimum circumference in cm at or just above the umbilicus, and hip girth was recorded at the site of the greater trochanter. BMI (kg.m\(^{-2}\)) and waist hip ratio (WHR) were calculated. Blood pressure was measured using a Takeda automatic sphygmomanometer (A and D, Tokyo, Japan). A mean of three measurements were taken at 5 minute intervals.

Fasting venous blood samples were taken without stasis between 8 and 10 am to overcome the diurnal variation in PAI-1 activity. Samples for PAI-1 activity and antigen were collected in tubes containing 3.8% sodium citrate, centrifuged at 2500g for 15min at 4°C and stored at -70°C. Assay methodology for the measurement of fibrinolytic parameters, insulin-like molecules, insulin sensitivity and lipids are outlined in Chapter 2.
3.4 Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS). Unless specified, data are presented as mean (SD). Specific insulin, intact proinsulin, des 31,32 proinsulin, insulin sensitivity, beta cell function and triglycerides were logarithmically transformed as their distributions were skewed, and their results are shown as geometric mean (SD). The differences in PAI-1 activity between the treatment groups and ethnic groups were analysed by ANOVA. Multiple regression analysis, with logarithmic transformation of skewed data, was used to study the relationship of PAI-1 with other variables while controlling for confounding variables. Statistical significance was taken as p<0.05.

3.5 Results

3.5.1 Clinical characteristics overall and in each treatment group

The sample consisted of 146 NIDDM subjects (66 male, 80 female) with a mean age of 59.0 (7.8) years and mean BMI 28.5 (4.9) kg.m$^{-2}$. The number of subjects and clinical characteristics of individual treatment groups are shown in Table 3.1.

3.5.2 Fibrinolytic parameters, insulin-like molecules, insulin sensitivity and lipids in each treatment group

The biochemical characteristics of individual treatment groups are shown in Table 3.2. There was no significant difference in PAI-1 activity between the 5 treatment groups (ANOVA p=0.37; Table 3.2). However there were significant differences in levels of insulin, intact and des 31,32 proinsulin (Table 3.2, Figures 3.1-3.3). The levels of intact proinsulin were lowest in the insulin treated subjects and highest in the sulphonylurea plus metformin group. As expected, the immunoreactive and specific insulin levels in the insulin treated subjects were significantly higher than in all other groups.
Table 3.1
Clinical characteristics of each treatment group

<table>
<thead>
<tr>
<th></th>
<th>Treatment Groups</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insulin</td>
<td>SU</td>
<td>SU + Metformin</td>
<td>Metformin</td>
<td>Diet</td>
<td>ANOVA</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>36</td>
<td>46</td>
<td>27</td>
<td>11</td>
<td>26</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.7 (7.2)</td>
<td>59.4 (8.6)</td>
<td>58.9 (6.6)</td>
<td>55.5 (8.8)</td>
<td>59.9 (7.8)</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>29.1 (7.2)</td>
<td>27.7 (3.9)</td>
<td>29.6 (4.7)</td>
<td>30.0 (5.6)</td>
<td>27.0 (5.5)</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>WHR</td>
<td>0.96 (0.09)</td>
<td>0.95 (0.08)</td>
<td>0.98 (0.10)</td>
<td>0.99 (0.10)</td>
<td>0.96 (0.09)</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128.5 (20.3)</td>
<td>133.0 (21.0)</td>
<td>129.2 (24.4)</td>
<td>125.1 (24.3)</td>
<td>123.7 (16.5)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.2 (10.1)</td>
<td>77.1 (12.5)</td>
<td>73.2 (10.2)</td>
<td>75.9 (14.0)</td>
<td>73.6 (8.9)</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>

Data are arithmetic means ± SD. SU: sulphonylurea.
Table 3.2
PAI-1 activity, metabolic characteristics and levels of insulin, proinsulin-like molecules and insulin sensitivity in each treatment group

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Insulin</th>
<th>SU</th>
<th>SU + Metformin</th>
<th>Metformin</th>
<th>Diet</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>36</td>
<td>46</td>
<td>27</td>
<td>11</td>
<td>26</td>
<td>p</td>
</tr>
<tr>
<td>FPG (mmol.l⁻¹)</td>
<td>9.8 (5.3)</td>
<td>9.9 (4.6)</td>
<td>10.1 (3.4)</td>
<td>8.6 (2.7)</td>
<td>8.5 (3.9)</td>
<td>0.30</td>
</tr>
<tr>
<td>PAI-1 activity (AU.ml⁻¹)</td>
<td>14.3 (9.4)</td>
<td>13.6 (9.1)</td>
<td>17.4 (9.4)</td>
<td>12.0 (10.3)</td>
<td>13.1 (9.4)</td>
<td>0.37</td>
</tr>
<tr>
<td>*IRI (pmol.l⁻¹)</td>
<td>123.9 (2.0)</td>
<td>75.9 (2.3)</td>
<td>66.2 (2.6)</td>
<td>56.5 (3.5)</td>
<td>64.5 (2.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l⁻¹)</td>
<td>141.2 (2.2)</td>
<td>50.4 (2.0)</td>
<td>41.7 (1.9)</td>
<td>27.2 (2.5)</td>
<td>44.3 (2.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l⁻¹)</td>
<td>4.2 (2.8)</td>
<td>12.2 (2.1)</td>
<td>13.0 (2.1)</td>
<td>5.4 (2.6)</td>
<td>6.5 (2.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>*des₃₁,₃₂ proinsulin (pmol.l⁻¹)</td>
<td>2.2 (2.7)</td>
<td>4.6 (2.1)</td>
<td>5.1 (2.1)</td>
<td>2.1 (2.5)</td>
<td>3.1 (2.1)</td>
<td>0.004</td>
</tr>
<tr>
<td>*Proinsulin Ratio</td>
<td>4.3 (3.0)</td>
<td>23.9 (1.7)</td>
<td>28.6 (1.7)</td>
<td>20.7 (2.0)</td>
<td>17.0 (2.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>*Insulin sensitivity (HOMA)%</td>
<td>-</td>
<td>38.5 (2.0)</td>
<td>46.0 (2.0)</td>
<td>73.1 (2.4)</td>
<td>45.9 (2.4)</td>
<td>0.10</td>
</tr>
<tr>
<td>*Beta-cell function (HOMA)%</td>
<td>-</td>
<td>38.9 (2.3)</td>
<td>32.0 (2.3)</td>
<td>31.3 (2.5)</td>
<td>47.8 (3.0)</td>
<td>0.37</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l⁻¹)</td>
<td>1.4 (1.7)</td>
<td>1.4 (1.8)</td>
<td>1.6 (1.8)</td>
<td>2.0 (1.7)</td>
<td>1.3 (1.8)</td>
<td>0.14</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>5.2 (1.0)</td>
<td>5.4 (1.1)</td>
<td>5.2 (1.1)</td>
<td>5.2 (1.1)</td>
<td>5.5 (1.0)</td>
<td>0.85</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.3)</td>
<td>1.0 (0.3)</td>
<td>1.1 (0.3)</td>
<td>1.2 (0.3)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± SD and * Geometric means x/l± SD for skewed data
Proinsulin ratio: ratio of intact plus des 3₁,₃₂ proinsulin to total insulin-like molecules; SU: sulphonylurea.
Figure 3.1 Insulin levels in the five treatment groups

The box and whisker indicates the 10th, 25th, 50th, 75th and 90th percentiles.
Figure 3.2 Intact proinsulin in the five treatment groups

The box and whisker indicates the 10th, 25th, 50th, 75th and 90th percentiles.
Figure 3.3 Des 31,32 proinsulin in the five treatment groups

The box and whisker indicates the 10th, 25th, 50th, 75th and 90th percentiles.
### 3.5.3 Correlation of PAI-1 with clinical and biochemical parameters in each treatment group

The correlations between PAI-1 activity, BMI, WHR, insulin, intact and des 31,32 proinsulin, insulin sensitivity, beta-cell function and serum lipids for the whole population and for individual treatment groups are shown in Table 3.3. In the whole sample, PAI-1 activity showed strong correlations with insulin sensitivity (excluding insulin treated patients) and serum triglyceride concentrations. There were also correlations with specific insulin, intact and des 31,32 proinsulin levels. These relationships were also evident in the individual treatment groups, but because of smaller numbers few of the correlations were statistically significant.

### 3.5.4 Fibrinolytic parameters, insulin-like molecules, insulin sensitivity and lipids in the three ethnic groups

The clinical and biochemical characteristics of the three ethnic groups are shown in Table 3.4. There were significant differences in BMI, with Asian subjects having the lowest and Caucasian subjects highest BMI. Asian subjects were also significantly younger, hence ANOVA p values for all fibrinolytic parameters, insulin-like molecules and lipids comparing the 3 ethnic groups were corrected for age and BMI (Table 3.4). Asian subjects had significantly higher levels of intact and des 31,32 proinsulin, and a higher proinsulin ratio compared to the other ethnic groups. Afro-Caribbean subjects had the lowest level of triglyceride and PAI-1 activity, but only the lower levels of triglyceride reached statistical significance due to the lower SD of the variable.

### 3.5.5 Correlation of PAI-1 with clinical and biochemical parameters in each ethnic group

The correlations between PAI-1 activity and other variables for the individual ethnic groups are shown in Table 3.5. PAI-1 activity correlated with insulin sensitivity in all ethnic groups, but with proinsulin-like molecules in Caucasian subjects only. PAI-1 activity did not correlate with serum triglyceride levels in Afro-Caribbean subjects who had the lowest triglyceride levels.
Table 3.3
Correlation of variables with PAI-1 in each treatment group

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>All</th>
<th>Insulin</th>
<th>Sulphonylurea</th>
<th>Sulphonylurea + Metformin</th>
<th>Metformin</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>146</td>
<td>36</td>
<td>46</td>
<td>27</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>BMI (kg.m²)</td>
<td>0.26**</td>
<td>-0.03</td>
<td>0.26</td>
<td>0.10</td>
<td>0.72*</td>
<td>0.40*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.17</td>
<td>0.27</td>
<td>0.05</td>
<td>0.27</td>
<td>-0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>FPG</td>
<td>0.32***</td>
<td>0.47**</td>
<td>0.36*</td>
<td>0.19</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>†HRI (pmol.l⁻¹)</td>
<td>0.28**</td>
<td>0.15</td>
<td>0.27</td>
<td>0.32</td>
<td>0.43</td>
<td>0.43*</td>
</tr>
<tr>
<td>†Specific insulin (pmol.l⁻¹)</td>
<td>0.29***</td>
<td>0.22</td>
<td>0.29</td>
<td>0.34</td>
<td>0.61</td>
<td>0.50*</td>
</tr>
<tr>
<td>†Intact proinsulin (pmol.l⁻¹)</td>
<td>0.24**</td>
<td>0.10</td>
<td>0.28</td>
<td>0.33</td>
<td>0.33</td>
<td>0.40*</td>
</tr>
<tr>
<td>†des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>0.30***</td>
<td>0.17</td>
<td>0.30*</td>
<td>0.31</td>
<td>0.51</td>
<td>0.47*</td>
</tr>
<tr>
<td>†Insulin sensitivity (HOMA)%</td>
<td>-0.42*** ‡</td>
<td>-</td>
<td>-0.35*</td>
<td>-0.35</td>
<td>-0.62*</td>
<td>-0.54**</td>
</tr>
<tr>
<td>†Beta-cell function (HOMA)%</td>
<td>-0.01‡</td>
<td>-</td>
<td>-0.20</td>
<td>-0.04</td>
<td>0.35</td>
<td>0.14</td>
</tr>
<tr>
<td>†Triglyceride (mmol.l⁻¹)</td>
<td>0.39***</td>
<td>0.57***</td>
<td>0.37**</td>
<td>0.34</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>0.11</td>
<td>-0.15</td>
<td>0.50***</td>
<td>-0.14</td>
<td>-0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>-0.26**</td>
<td>-0.31</td>
<td>-0.04</td>
<td>-0.54**</td>
<td>-0.001</td>
<td>-0.35</td>
</tr>
</tbody>
</table>

† Values log transformed prior to analysis
‡ 36 subjects on insulin excluded (n=110 for these correlations)
* p < 0.05  ** p < 0.01  *** p < 0.001

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Table 3.4
Variables in the 3 ethnic groups.

<table>
<thead>
<tr>
<th></th>
<th>Caucasian n=86</th>
<th>Asian n=34</th>
<th>Afrocaribbean n=26</th>
<th>anova p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>60.2 (8.0)</td>
<td>55.9 (7.5)</td>
<td>57.4 (6.2)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>BMI (kg.m⁻²)</strong></td>
<td>29.6 (5.2)</td>
<td>25.5 (7.5)</td>
<td>28.3 (3.9)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>0.96 (0.09)</td>
<td>0.95 (0.07)</td>
<td>0.96 (0.09)</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>130.0 (22.0)</td>
<td>121.6 (17.1)</td>
<td>135.2 (20.4)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>74.1 (11.1)</td>
<td>75.7 (12.6)</td>
<td>78.2 (7.3)</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Fasting glucose (mmol.l⁻¹)</strong></td>
<td>10.2 (4.8)</td>
<td>8.5 (2.8)</td>
<td>9.0 (4.3)</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>PAI-1 activity (AU.ml⁻¹)</strong></td>
<td>15.3 (9.8)</td>
<td>13.0 (9.9)</td>
<td>11.9 (7.4)</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>Specific insulin (pmol.l⁻¹)</strong></td>
<td>58.0 (2.5)</td>
<td>56.3 (1.9)</td>
<td>60.3 (3.0)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Intact proinsulin (pmol.l⁻¹)</strong></td>
<td>7.9 (2.4)</td>
<td>10.6 (2.5)</td>
<td>5.6 (3.1)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>des 31,32 proinsulin (pmol.l⁻¹)</strong></td>
<td>3.4 (2.3)</td>
<td>4.5 (2.6)</td>
<td>2.4 (2.3)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Proinsulin ratio %</strong></td>
<td>15.1 (2.7)</td>
<td>20.0 (2.5)</td>
<td>10.5 (3.9)</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Insulin Sensitivity (HOMA)%†</strong></td>
<td>33.6 (2.6)</td>
<td>35.6 (1.8)</td>
<td>34.0 (2.8)</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Triglyceride (mmol.l⁻¹)</strong></td>
<td>1.5 (1.6)</td>
<td>1.6 (1.8)</td>
<td>1.1 (1.7)</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol.l⁻¹)</strong></td>
<td>5.5 (1.2)</td>
<td>5.4 (0.9)</td>
<td>5.2 (1.3)</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mmol.l⁻¹)</strong></td>
<td>1.1 (0.3)</td>
<td>1.0 (0.3)</td>
<td>1.2 (0.3)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± SD and * Geometric means ×/÷ SD for skewed data.
† 36 subjects on insulin excluded (Caucasian n=65, Asian n=25, Afro-Caribbean n=19 for these values).
The lower p values are corrected for age and BMI. Boldface p values are significant.
Table 3.5

Correlations of PAI-1 activity with variables in the three ethnic groups.

<table>
<thead>
<tr>
<th></th>
<th>Caucasian</th>
<th>Asian</th>
<th>Afro-Caribbeans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=86</td>
<td>n=34</td>
<td>n=26</td>
</tr>
<tr>
<td></td>
<td>(60%)</td>
<td>(23%)</td>
<td>(17%)</td>
</tr>
<tr>
<td>Specific insulin (pmol.l⁻¹)</td>
<td>0.31**</td>
<td>0.15</td>
<td>0.40*</td>
</tr>
<tr>
<td>Intact proinsulin (pmol.l⁻¹)</td>
<td>0.23*</td>
<td>0.31</td>
<td>0.24</td>
</tr>
<tr>
<td>des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>0.32**</td>
<td>0.32</td>
<td>0.24</td>
</tr>
<tr>
<td>Triglyceride (mmol.l⁻¹)</td>
<td>0.38***</td>
<td>0.46**</td>
<td>0.25</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>0.14</td>
<td>0.25</td>
<td>-0.27</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>-0.27*</td>
<td>-0.20</td>
<td>-0.39*</td>
</tr>
<tr>
<td>Insulin sensitivity (HOMA)%†</td>
<td>-0.42**</td>
<td>-0.37*</td>
<td>-0.61**</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
*** p < 0.001
† 36 subjects on insulin excluded (Caucasian n=65, Asian n=25, Afro-Caribbean n=19 for these correlations)
3.5.6 Multiple regression analyses

Multiple regression analysis of variables associated with PAI-1 activity in the three ethnic groups are shown in Table 3.6, and in subjects not on insulin therapy in Table 3.7. In Caucasian and Asian NIDDM subjects triglyceride levels are an important determinant of PAI-1 activity. This relationship is not observed in Afro-Caribbean subjects, where serum insulin levels appear more important (Table 3.6). Including the HOMA model as an estimate of insulin sensitivity in the model, and hence excluding insulin treated subjects, insulin sensitivity displaces triglyceride levels as a major determinant of PAI-1 activity in Asian subjects. This appears to be the case in Afro-Caribbean subjects, but does not reach statistical significance (Table 3.7).

3.6 Discussion

3.6.1 Determinants of PAI-1 activity

PAI-1 levels are raised in subjects with NIDDM. The aim of our study was to examine the determinants of PAI-1 activity in a sample of diabetic patients, and to dissect out the contribution of hypoglycaemic therapy, concentrations of insulin and triglycerides, and insulin sensitivity. This study has shown that triglyceride concentrations and insulin sensitivity are the strongest correlates of fasting PAI-1 activity. This correlation is present even after allowing for the effects of variables that are known to influence PAI-1 activity, such as fasting plasma proinsulin-like molecules, age, WHR and BMI.

3.6.2 The effect of hypoglycaemic therapy

Cardiovascular disease accounts for as much as 75% of all deaths in NIDDM subjects, and PAI-1 activity may play an important role in this excess cardiovascular risk. It is important that hypoglycaemic treatment does not worsen this risk by elevating PAI-1 levels directly or indirectly via other known variables. In a previous cross-over study Jain et al (1993), demonstrated that suppression of proinsulin-like molecules in NIDDM subjects treated with insulin resulted in a parallel decrease in PAI-1 activity. In this study, proinsulin-like molecules were lower in insulin treated patients than in patients treated with sulphonylurea, metformin and diet treatment alone.
Table 3.6
Multiple regression analyses of variables associated with PAI-1 activity in the three ethnic groups

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Variables in the model</th>
<th>$\beta$</th>
<th>SE of $\beta$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian n=86</td>
<td>Age</td>
<td>-0.025</td>
<td>0.12</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>3.99</td>
<td>1.94</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.56</td>
<td>0.23</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>3.86</td>
<td>1.91</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>0.49</td>
<td>1.17</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>-0.52</td>
<td>2.07</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>2.99</td>
<td>2.25</td>
<td>0.19</td>
</tr>
<tr>
<td>Asian n=34</td>
<td>Age</td>
<td>0.13</td>
<td>0.26</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>-6.59</td>
<td>5.33</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>-0.23</td>
<td>0.62</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>7.12</td>
<td>3.00</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>3.21</td>
<td>2.97</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>2.60</td>
<td>3.67</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>-1.10</td>
<td>3.76</td>
<td>0.77</td>
</tr>
<tr>
<td>Afro-Caribbean n=26</td>
<td>Age</td>
<td>0.06</td>
<td>0.25</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>-1.60</td>
<td>3.00</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>-1.15</td>
<td>0.58</td>
<td>-1.97</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>6.69</td>
<td>3.73</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>3.32</td>
<td>1.49</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>1.65</td>
<td>3.15</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>0.83</td>
<td>4.48</td>
<td>0.86</td>
</tr>
</tbody>
</table>

The figures shown are regression coefficient $\beta$ and SE of $\beta$ and p values of the variables in the model. PAI-1 is the dependent variable.
### Table 3.7
Multiple regression analyses of variables associated with PAI-1 activity in the three ethnic groups, in subjects not on insulin therapy.

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>Variables in the model</th>
<th>$\beta$</th>
<th>SE of $\beta$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caucasian</strong></td>
<td>Age</td>
<td>0.15</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>n = 65</strong></td>
<td>Sex</td>
<td>2.84</td>
<td>2.34</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.82</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>0.17</td>
<td>2.43</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>-8.38</td>
<td>10.92</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>1.89</td>
<td>2.69</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>-0.01</td>
<td>2.79</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity</td>
<td>-10.49</td>
<td>11.58</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Asian</strong></td>
<td>Age</td>
<td>0.17</td>
<td>0.31</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>n = 25</strong></td>
<td>Sex</td>
<td>-14.60</td>
<td>6.93</td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.623</td>
<td>0.68</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>1.55</td>
<td>3.58</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>-67.21</td>
<td>25.06</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>2.43</td>
<td>4.18</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>-5.45</td>
<td>6.98</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity</td>
<td>-77.61</td>
<td>26.88</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td><strong>Afro-Caribbean</strong></td>
<td>Age</td>
<td>-0.07</td>
<td>0.25</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>n = 19</strong></td>
<td>Sex</td>
<td>-6.32</td>
<td>2.53</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>-0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>0.75</td>
<td>3.75</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>-33.97</td>
<td>19.93</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Intact proinsulin</td>
<td>-2.92</td>
<td>2.59</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Split proinsulin</td>
<td>1.04</td>
<td>3.24</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity</td>
<td>-39.23</td>
<td>21.32</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The figures shown are regression coefficient $\beta$ and SE of $\beta$ and p values of the variables in the model. PAI-1 is the dependent variable.
Despite this difference there was no significant difference in PAI-1 activity between the groups.

A cross-sectional study however has limited power in detecting such differences, as large inter-subject variation in PAI-1 activity exists. This may be overcome when subjects are studied in a longitudinal or cross-over fashion. In a previous cross-over study Nagi et al, (1993) demonstrated a reduction in PAI-1 activity on metformin treatment, but again this was not evident in the current study, perhaps because of the small number of subjects in the metformin only treatment group and the cross-sectional nature of the study. Another shortfall of this study is that despite the original randomisation to hypoglycaemia therapy in the UKPDS, treatments in our patients were non-randomised, as over the years subjects had failed on diet therapy alone and were commenced on either insulin or sulphonylurea treatment, or failed on oral therapy and were switched to insulin.

### 3.6.3 The influence of ethnic group

Asian subjects had higher levels of proinsulin-like molecules and triglyceride, and from our previous studies we would have predicted a higher level of PAI-1 activity. Although a trend was observed, this was not statistically significant due to limited power of a cross-sectional study when large inter-individual variations in PAI-1 activity exist. Afro-Caribbean subjects had the lowest level of serum triglyceride and PAI-1 activity. Due to the limited power of our study the difference in PAI-1 activity was not statistically significant. This observation is in keeping with previous studies that have suggested enhanced fibrinolytic potential in black subjects possibly accounting for their lower rate of CHD. A polymorphism in the PAI-1 gene in the 3 ethnic groups will be further discussed in Chapter 5.

### 3.7 Conclusion

In the population studied, after entering all factors known to be associated with PAI-1 activity into a multiple regression equation, triglyceride level, along with insulin
sensitivity, appeared the strongest correlates of PAI-1 activity. The relationship of hyperinsulinaemia and insulin resistance with elevated PAI-1 activity is yet to be unravelled. Despite population correlations of insulin concentration with PAI-1 activity, and in vitro effects of insulin on liver cell PAI-1 production, a direct effect of insulin on stimulating PAI-1 activity in vivo has not been shown (Landin et al, 1991, Grant et al, 1990). Reducing insulin levels and insulin resistance by exercise, weight loss (Gris et al, 1990) and metformin (Nagi and Yudkin, 1993) reduces PAI-1 activity. Increased PAI-1 production in vitro has been demonstrated in insulin resistant HepG2 cells, an effect which was inhibited by metformin (Anfossa et al, 1993).

Several studies to date have shown a correlation between serum triglyceride concentrations and PAI-1 activity, and it has been proposed that insulin resistance or hyperinsulinaemia could influence the synthesis of PAI-1 via their effects on lipid metabolism. In vitro studies have demonstrated the effect of various lipoproteins on PAI-1 synthesis as discussed above, and VLDL from hypertriglyceridaemic patients increases endothelial cell production of PAI-1 to a greater degree than that from normotriglyceridaemic subjects, this effect being abolished by antibody directed against the apoB receptor (Stiko-Rahm et al, 1990). The strong independent association of triglyceride levels with PAI-1 activity in this study are in keeping with the above observations on serum triglycerides.
CHAPTER 4

THE EFFECT OF INSULIN VERSUS SULPHONYLUREA THERAPY ON CARDIOVASCULAR RISK FACTORS AND FIBRINOLYSIS IN TYPE 2 DIABETES

4.1 Introduction

4.1.1 Glycaemic control and the risk of coronary heart disease in NIDDM

The underlying mechanisms for accelerated atherogenesis in NIDDM are poorly understood. Although NIDDM is associated with a clustering of risk factors favouring atherogenesis (e.g. high total triglyceride and low HDL cholesterol levels, and a high prevalence of obesity and hypertension), population-based prospective studies have repeatedly shown that only a small proportion of the excess risk for coronary heart disease (CHD) in NIDDM can be explained by the effect of NIDDM on the levels of known cardiovascular risk factors (Pyörälä et al, 1987).

Recently there has been debate on the role of hyperglycaemia as a cause of the excess CHD in NIDDM, the evidence however is limited. In the WHO Multinational study of Vascular Disease in Diabetes (West et al, 1983), the relationship between fasting plasma glucose levels and the prevalence of different manifestations of atherosclerotic vascular disease was assessed in a pooled population of 3583 diabetic patients from nine different populations. The plasma glucose level failed to show any relation to the prevalence of major Q-wave changes on the electrocardiogram. On the other hand, a review of six prospective population-based studies, including a large number of NIDDM patients by Laakso and Kuusisto (1996), have indicated that poor glycaemic control (assessed by fasting hyperglycaemia or glycosylated HbA1 levels) increases the risk for CHD, stroke and amputation independently of other risk factors.

4.1.2 Hypoglycaemic therapy

There is no convincing evidence that strict glycaemic control delays or prevents
atherosclerotic complications of diabetes. It is hence important that hypoglycaemic therapy does not worsen cardiovascular risk, but there is little information available on the effects of different modes of therapy for NIDDM on cardiovascular risk factors. The results of the UGDP study, suggesting an increased cardiovascular mortality on sulphonylureas, are not widely accepted (UGDP Study Group, 1970). Insulin treatment in NIDDM has been shown to reduce VLDL-triglyceride and elevate HDL-cholesterol concentrations (Taskinen et al, 1988), but it is difficult to separate out direct effects of insulin from indirect effects through insulin-induced improvements in glycaemic control and thereby on insulin resistance.

4.1.3 Previous studies on hypoglycaemic therapy in NIDDM
In Chapter 3, in a cross-sectional study of 146 NIDDM subjects, we demonstrated that insulin therapy is associated with lower concentrations of proinsulin-like molecules compared to those seen in patients treated with sulphonylurea or metformin. A previous cross-over study (Nagi et al, 1993) demonstrated a reduction in PAI-1 activity on metformin. Jain et al (1993), compared, in cross-over design, the effect of therapy with sulphonylureas or with insulin, without changes in glycaemia or insulin sensitivity on PAI-1 activity in 11 poorly controlled NIDDM subjects. Without attempting to alter glycaemic control, insulin treatment was associated with reduced PAI-1 activity. While this study suggested differences between treatment regimens on levels of PAI-1 and proinsulin (but not lipids), it was unable to dissect out relative effects of changes in glycaemia, insulin sensitivity and therapeutic regimen on these variables.

4.2 Aims of the study
The aims of this cross-over study were:

i- To separate the contributions of improved control versus treatment regimen on cardiovascular risk factors, including PAI-1 and proinsulin-like molecules in NIDDM subjects.
ii- To investigate whether more vigorous treatment with sulphonylurea and insulin produce greater stimulation and suppression of proinsulin-like molecules, and consequently larger changes in PAI-1, than found in the previous study where poor glycaemic control was unaltered.

iii- To study a larger number of patients for a longer duration of time with a washout period between therapies.

4.3 Subjects and methods

4.3.1 Subjects

Twenty NIDDM subjects attending the diabetic clinic at the Whittington Hospital were studied. NIDDM was diagnosed according to WHO criteria (WHO, 1985). All subjects were poorly controlled on diet alone and were studied prior to the commencement of additional hypoglycaemic therapy. Subjects with a previous history of ischaemic heart disease, thromboembolic disease or clinical or biochemical evidence of renal or hepatic disease were excluded. Subjects were on no other medication, including aspirin, at time of study, and all gave informed written consent. The study was approved by the ethical committee of the Islington Health Authority.

4.3.2 Study Design

Subjects were given insulin (once or twice daily pre-mixed human soluble and isophane 30:70) (Humulin M3, Lilly, IN, USA) or sulphonylurea (glibenclamide), once or twice daily, each for a period of 16 weeks, in a randomised cross-over study, with a four week washout period between treatments. Subjects were asked to perform fasting and pre-supper home blood glucose monitoring, and dosage adjustments were made over the telephone every three to four days. In addition, each subject was seen weekly for the first four weeks, and then monthly to adjust therapy and to measure fasting plasma glucose concentrations in order to achieve normoglycaemia (fasting plasma glucose <6mmol.l$^{-1}$). Insulin was commenced at 10 U/day for subjects of ideal body weight, with dosage increase of 25% for each 10% over ideal body weight, and the dose was increased by 2 U/day according to home blood glucose monitoring (mean dose
27.3 ± 11.7 U/day). Glibenclamide was started at 2.5 mg/day, and increased by 2.5 mg increments according to home blood glucose monitoring to a maximum of 20 mg/day (mean dose 8.8 ± 5.6 mg/day).

Subjects were studied on four occasions (Figure 4.1):

Visit 1: first baseline (B1)
Visit 2: after 16 weeks treatment with sulphonylurea or insulin
Visit 3: after a 4 week washout period (second baseline, B2)
Visit 4: after 16 weeks treatment with insulin or sulphonylurea

At each visit subjects attended the clinical investigation unit of the Department of Medicine, UCLMS, Whittington Hospital between 0800 and 0900 after an overnight fast. When on treatment, subjects were advised to omit their evening and morning dose of sulphonylurea or insulin prior to the visit to avoid hypoglycaemia during the insulin sensitivity test. Weight and height were recorded with the patients wearing light clothing and without shoes. Waist measurements were taken as the minimum circumference in centimeters at or just above the umbilicus, and hip girth was recorded at the site of the greater trochanter. Skin-fold measurements were performed with Holtain callipers (Holtain, Crymych, Dyfedd, UK), including three measurements of subscapular and triceps skin fold. Body mass index (BMI) (kg.m⁻²), waist-to-hip ratio (WHR) and subscapular-to-triceps ratio (STR) were calculated. After 10 mins rest, blood pressure (BP) was recorded twice in the right arm using a random zero sphygmomanometer (Hawksley, Lancing, W.Sussex, UK), with diastolic BP recorded at phase V of the Korotkoff sounds. The mean of two BP readings were taken.

Fasting venous blood samples were taken without venous stasis for measurement of plasma glucose, glycated haemoglobin (HbA₁), fructosamine, insulin, proinsulin-like molecules, serum lipids and apoproteins. Samples for fibrinogen, PAI-1 activity and PAI-1 and t-PA antigens were collected in tubes containing 3.8% sodium citrate,
Figure 4.1  Crossover design to investigate the effect of sulphonylureas versus insulin on cardiovascular risk (B1: 1st baseline, B2: second baseline)
centrifuged at 3000g for 10 min at 4°C and stored immediately at -70°C. After fasting samples were taken, subjects underwent a modified Harano measurement of insulin sensitivity. Methodology for the above assays, and for insulin sensitivity measurements are described in Chapter 2.

4.4 Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS), and are expressed as mean ± (SD) for normally distributed data and as geometric mean ×/± (SD) for skewed data. The variables after sulphonylurea (SU) treatment were compared with those after insulin (I) treatment and with first baseline (B1), and the differences were analysed by paired Student's t-test for normally distributed data and by Wilcoxon's rank sum test for skewed data. The following were tested for:

1- **Treatment effect** by calculating the difference and average of the observations after B1 to I (d₁ and a₁) and B1 to SU (dSU and aSU) therapy for each subject, and averaging these for each group (d₁, a₁, dSU and aSU respectively.)

2- **Period effect** using a two sample t-test comparing d₁ with dSU.

3- **Treatment-period interaction** by a two sample t-test comparing a₁ and aSU.

ANOVA was used to study the independent relationship between two variables while allowing for confounding variables using logarithmic transformation of skewed variables. Statistical significance was taken as p < 0.05.

4.5 Results

4.5.1 Clinical characteristics (Table 4.1)

Twenty NIDDM subjects (12 male, 8 female) with a mean age of 55.9 ± 8.3 years and mean duration of diabetes 4 (0.25-10) years completed both phases of the study. Two subjects dropped out of the study, one due to renal calculi and another after deciding against insulin therapy.
TABLE 4.1

Clinical characteristics of subjects at both baselines and 16 weeks post sulphonylurea and insulin therapy.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (B1)</th>
<th>Baseline (B2)</th>
<th>Post SU</th>
<th>Post I</th>
<th>p(SUvsI)</th>
<th>p(SUvsB1)</th>
<th>p(IvsB1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>29.2 (3.8)</td>
<td>30.4 (4.3)</td>
<td>30.8 (4.3)</td>
<td>30.4 (4.3)</td>
<td>0.30</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WHR</td>
<td>0.99 (0.11)</td>
<td>0.96 (0.12)</td>
<td>0.93 (0.08)</td>
<td>0.93 (0.09)</td>
<td>0.92</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>STR</td>
<td>1.66 (0.67)</td>
<td>1.82 (0.80)</td>
<td>1.90 (0.79)</td>
<td>2.22 (1.00)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.005</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>135.5 (19.4)</td>
<td>128.6 (19.3)</td>
<td>135.9 (19.5)</td>
<td>136.8 (23.4)</td>
<td>0.79</td>
<td>0.91</td>
<td>0.94</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.1 (15.3)</td>
<td>79.3 (12.7)</td>
<td>80.7 (14.1)</td>
<td>84.6 (14.0)</td>
<td>0.14</td>
<td>0.84</td>
<td>0.16</td>
</tr>
<tr>
<td>AER* (µg.min⁻¹)</td>
<td>8.8 (2.0)</td>
<td>9.3 (2.2)</td>
<td>8.6 (2.0)</td>
<td>7.0 (1.9)</td>
<td>0.32</td>
<td>0.79</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Data are arithmetic means (± SD) or *geometric means (×/⁻ SD) for skewed data. Significance of difference between sulphonylurea and insulin therapy, and with baselines has been assessed using Student's t-test for normally distributed data and Wilcoxon rank for skewed data.
Because it was felt unethical to withdraw dietary and monitoring recommendations in the four weeks washout period, values for fasting glucose, fructosamine and HbA1c, following the washout period (second baseline B2) did not return to the initial pretreatment range. Hence all comparisons on the effects of treatment with insulin or sulphonylurea were made with the first baseline (B1) (Fleiss, 1986). Analyses showed no period effect, and no treatment-period interaction, implying that the difference between B1 and B2 did not preclude analysis as a cross-over design (Hills and Armitage, 1979). BMI was higher on both treatments than at B1, but did not differ between treatments. There were no changes compared to B1 or differences between the two therapies in blood pressure or AER.

4.5.2 Glycaemic control and insulin sensitivity (Table 4.2)
Treatment with insulin or sulphonylurea produced similar improvement in glycaemia, as judged by fasting glucose, HbA1c, fructosamine, and in metabolic clearance rate of glucose as compared to B1. Levels of glucose, post-glucose load, were similar on both treatments (Fig 4.2).

4.5.3 Insulin-like molecules: fasting (Table 4.2) and post-glucose load (Figures 4.2 and 4.3)
Insulin concentrations, both fasting and stimulated, were higher on treatment than at B1, but did not differ significantly between treatments. Fasting and post-glucose load intact proinsulin concentrations were higher on sulphonylurea than on insulin or B1. Fasting des 31,32 proinsulin concentrations did not differ significantly between treatments or compared to B1, however 30, 60 and 120 min values on both insulin and sulphonylurea were higher than B1. Fasting C-peptide levels were higher on sulphonylurea therapy compared to B1, but did not differ significantly between the two treatments. Samples were not collected for C-peptide post glucose load. All differences in concentrations of insulin-like molecules persisted on correcting for BMI and for sequence of treatment.
<table>
<thead>
<tr>
<th></th>
<th>Baseline (B1)</th>
<th>Post SU</th>
<th>Post I</th>
<th>p (SU v I)</th>
<th>p (SU v B1)</th>
<th>p (I v B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mmol.l(^{-1}))</td>
<td>12.8 (3.5)</td>
<td>9.1 (2.5)</td>
<td>8.9 (1.9)</td>
<td>0.77</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>HbA1 (%)</td>
<td>11.7 (2.1)</td>
<td>8.5 (0.9)</td>
<td>8.6 (1.2)</td>
<td>0.63</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fructosamine (mmol.l(^{-1}))</td>
<td>2.5 (0.7)</td>
<td>1.9 (0.5)</td>
<td>2.0 (0.3)</td>
<td>0.77</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCR-glucose* (ml.kg(^{-1}.)min(^{-1}))</td>
<td>1.86 (1.4)</td>
<td>2.36 (1.4)</td>
<td>2.27 (1.4)</td>
<td>0.37</td>
<td>0.0005</td>
<td>0.07</td>
</tr>
<tr>
<td>Insulin* (pmol.l(^{-1}))</td>
<td>26.4 (2.2)</td>
<td>38.6 (1.9)</td>
<td>42.7 (2.0)</td>
<td>0.20</td>
<td>0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>Proinsulin* (pmol.l(^{-1}))</td>
<td>9.8 (2.3)</td>
<td>12.8 (2.7)</td>
<td>8.0 (2.1)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.19</td>
</tr>
<tr>
<td>Des 31,32 proinsulin* (pmol.l(^{-1}))</td>
<td>3.6 (2.6)</td>
<td>3.4 (3.5)</td>
<td>2.9 (3.8)</td>
<td>0.88</td>
<td>0.88</td>
<td>0.99</td>
</tr>
<tr>
<td>Proinsulin ratio* (%)</td>
<td>33.0 (1.6)</td>
<td>28.2 (1.7)</td>
<td>20.8 (1.5)</td>
<td>0.05</td>
<td>0.22</td>
<td>0.002</td>
</tr>
<tr>
<td>C-peptide* (ng.ml(^{-1}))</td>
<td>0.7 (0.3)</td>
<td>0.9 (0.4)</td>
<td>0.8 (0.4)</td>
<td>0.10</td>
<td>0.0007</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Data are arithmetic means (± SD) or geometric means (×/÷ SD) for skewed data. Significance of difference between sulphonylurea and insulin therapy and with baseline has been assessed using Student's t-test for normally distributed data and Wilcoxon rank for skewed data. Proinsulin ratio: ratio of intact plus des 31,32 proinsulin to total insulin-like molecules.
Figure 4.2 Glucose and insulin concentrations post glucose load, at baseline and post insulin and sulphonylurea therapy.
Figure 4.3 Intact and des 31,32 proinsulin concentrations post glucose load, at baseline and post insulin and sulphonylurea therapy.
4.5.4 Fibrinolytic parameters (Table 4.3, Figure 4.4)

PAI-1 activity and antigen were higher on sulphonylurea therapy than B1. PAI-1 antigen was significantly higher on sulphonylurea therapy than on insulin. There were no significant differences in t-PA antigen or fibrinogen at the end of the two treatment phases.

4.5.5 Lipid and lipoproteins (Table 4.4)

There were no significant differences in lipoprotein or apoprotein concentrations between treatments or baseline.

4.6 Discussion

The increased risk of cardiovascular disease in NIDDM appears to be independent of duration of diabetes and glycaemic control and it is important that hypoglycaemic therapy in the long term improves and not worsens this risk. This study differs from previous studies on the effect of hypoglycaemic therapy on cardiovascular risk factors, in that subjects were studied at baseline, having never been subjected to any form of therapy apart from diet alone, and then were reinvestigated in a cross-over fashion with a four week washout period. By achieving similar improvements in glycaemia and insulin sensitivity on both treatments it was possible to dissect out the contributions of improved control versus treatment regimen. This study demonstrated that sulphonylurea therapy results in higher circulating levels of proinsulin and of PAI-1 activity and antigen compared to baseline, and additionally, in higher levels of PAI-1 antigen and intact proinsulin compared to insulin therapy. There were no differences in other insulin resistance syndrome variables between sulphonylurea and insulin treatment. This data supports the previous cross-sectional study (Chapter 3) of 146 NIDDM subjects, where subjects on sulphonylurea therapy had higher levels of proinsulin as compared to subjects treated with insulin, and the previous cross-over study were insulin therapy in NIDDM subjects lowered both proinsulin and PAI-1 activity (Jain et al, 1993). What are the possible consequences, if any, of increased levels of proinsulin and PAI-1 in NIDDM patients treated with sulphonylureas?
TABLE 4.3

Fibrinolytic parameters at first baseline and 16 weeks post treatment with sulphonylurea and insulin.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (B1)</th>
<th>Post SU</th>
<th>Post I</th>
<th>p (SU v I)</th>
<th>p (SU v B1)</th>
<th>p (I v B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 activity (AU.l⁻¹)</td>
<td>19.9 (1.8)</td>
<td>23.7 (2.4)</td>
<td>22.2 (2.1)</td>
<td>0.54</td>
<td>0.02</td>
<td>0.22</td>
</tr>
<tr>
<td>PAI-1 antigen (ng.ml⁻¹)</td>
<td>32.2 (1.8)</td>
<td>47.6 (2.0)</td>
<td>36.5 (2.2)</td>
<td>0.05</td>
<td>0.006</td>
<td>0.35</td>
</tr>
<tr>
<td>t-PA antigen (ng.ml⁻¹)</td>
<td>11.3 (2.9)</td>
<td>12.5 (2.4)</td>
<td>12.4 (3.3)</td>
<td>0.21</td>
<td>0.09</td>
<td>0.15</td>
</tr>
<tr>
<td>Fibrinogen (mg.dl⁻¹)</td>
<td>256.4 (43.9)</td>
<td>239.2 (31.0)</td>
<td>243.3 (46.8)</td>
<td>0.79</td>
<td>0.13</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Data are geometric means (x/± SD) for skewed data. Significance of difference between sulphonylurea and insulin therapy and with baseline has been assessed using the Wilcoxon rank test. SU: Sulphonylurea; I: Insulin
Figure 4.5 PAI-1 activity at baseline and post sulphonylurea and insulin therapy.

![Box plot of PAI-1 activity](image1)

Figure 4.6 PAI-1 antigen at baseline and post sulphonylurea and insulin therapy.

![Box plot of PAI-1 antigen](image2)
### TABLE 4.4
Lipid and lipoprotein concentrations at first baseline and 16 weeks post sulphonylurea and insulin.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (B1)</th>
<th>Post SU</th>
<th>Post I</th>
<th>p (SU v I)</th>
<th>p (B1 v S)</th>
<th>p (B1 v I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mmol.l⁻¹)</td>
<td>5.9 (1.4)</td>
<td>5.9 (1.3)</td>
<td>5.8 (1.1)</td>
<td>0.76</td>
<td>0.82</td>
<td>0.59</td>
</tr>
<tr>
<td>Total Triglyceride (mmol.l⁻¹)</td>
<td>1.61 (1.94)</td>
<td>1.66 (1.98)</td>
<td>1.67 (1.88)</td>
<td>0.65</td>
<td>0.74</td>
<td>0.68</td>
</tr>
<tr>
<td>VLDL-Cholesterol (mmol.l⁻¹)</td>
<td>0.35 (2.52)</td>
<td>0.39 (2.12)</td>
<td>0.36 (2.68)</td>
<td>0.56</td>
<td>0.95</td>
<td>0.56</td>
</tr>
<tr>
<td>VLDL-Triglyceride (mmol.l⁻¹)</td>
<td>0.83 (2.55)</td>
<td>0.93 (2.26)</td>
<td>0.90 (2.59)</td>
<td>0.91</td>
<td>0.51</td>
<td>0.33</td>
</tr>
<tr>
<td>IDL-Cholesterol (mmol.l⁻¹)</td>
<td>0.13 (2.00)</td>
<td>0.12 (2.00)</td>
<td>0.12 (1.70)</td>
<td>0.53</td>
<td>0.95</td>
<td>0.28</td>
</tr>
<tr>
<td>IDL-Triglyceride (mmol.l⁻¹)</td>
<td>0.09 (2.08)</td>
<td>0.08 (1.81)</td>
<td>0.08 (1.74)</td>
<td>0.54</td>
<td>0.49</td>
<td>0.32</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol.l⁻¹)</td>
<td>3.03 (1.34)</td>
<td>2.98 (1.37)</td>
<td>2.75 (1.25)</td>
<td>0.08</td>
<td>0.76</td>
<td>0.10</td>
</tr>
<tr>
<td>HDL2 (mmol.l⁻¹)</td>
<td>0.47 (0.27)</td>
<td>0.46 (0.23)</td>
<td>0.50 (0.18)</td>
<td>0.29</td>
<td>0.60</td>
<td>0.58</td>
</tr>
<tr>
<td>HDL3 (mmol.l⁻¹)</td>
<td>0.77 (0.19)</td>
<td>0.83 (0.21)</td>
<td>0.85 (0.20)</td>
<td>0.66</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>ApoA1 (g.l⁻¹)</td>
<td>1.34 (0.25)</td>
<td>1.30 (0.24)</td>
<td>1.35 (0.26)</td>
<td>0.06</td>
<td>0.75</td>
<td>0.76</td>
</tr>
<tr>
<td>ApoA2 (g.l⁻¹)</td>
<td>0.53 (0.14)</td>
<td>0.53 (0.11)</td>
<td>0.52 (0.12)</td>
<td>0.68</td>
<td>0.81</td>
<td>0.46</td>
</tr>
<tr>
<td>ApoB (g.l⁻¹)</td>
<td>1.21 (0.36)</td>
<td>1.20 (0.31)</td>
<td>1.20 (0.32)</td>
<td>0.93</td>
<td>0.64</td>
<td>0.93</td>
</tr>
<tr>
<td>Lp(a) (mg.dL⁻¹)</td>
<td>20.00 (4.61)</td>
<td>16.77 (6.42)</td>
<td>18.58 (5.55)</td>
<td>0.93</td>
<td>0.64</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Data are geometric means (×/±SD) for skewed data. Significance of differences between sulphonylurea, insulin therapy and baseline has been assessed using the Wilcoxon rank test. SU: Sulphonylurea; I: Insulin
In NIDDM, proinsulin-like molecules may contribute up to 60% of immunoreactive insulin, and in previous studies these concentrations correlated more closely with cardiovascular risk factors, including PAI-1, than specific levels of insulin (Nagi et al, 1990). Proinsulin induces PAI-1 synthesis \textit{in vitro}, acting via the insulin receptor (Nordt et al, 1994), and intravenous infusions of proinsulin increase plasma PAI-1 activity, and PAI-1 mRNA in aorta and liver (Nordt et al, 1995). A study examining the effects of therapeutic human proinsulin was terminated due to an increased incidence of cardiovascular events (Galloway, 1990), and it could be postulated that this was due to diminished fibrinolytic activity. Sulphonylurea therapy in NIDDM subjects may predispose to increased cardiovascular events, as suggested by the UGDP study, by increasing proinsulin production and hence PAI-1.

Gliclazide, a second generation sulphonylurea, has been shown to increase t-PA activity in subjects with NIDDM (with no change observed in PAI-1 activity), the effect being independent of metabolic control (Gram et al, 1989). This study did not measure t-PA activity, and differs from the above in that the aim was to improve glycaemic control. The mechanism for the independent effect of gliclazide on t-PA activity is unknown. Although none of the studies have measured proinsulin-like molecules, it may be a direct effect on endothelial t-PA production.

Reduced fibrinolysis will predispose the individual to an increased risk of atherogenesis and thrombosis. PAI-1 levels are elevated in NIDDM and in subjects with CHD, in both groups it is a predictor of reinfarction (Hamsten et al, 1987). Recently the DIGAMI (Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction) study demonstrated insulin-glucose infusions, followed by intensive multidose insulin treatment reduced mortality in NIDDM patients with acute myocardial infarction by a factor of one third, and the effect persisted for at least 3.5 years (Malmberg et al, 1996; Malmberg et al, 1997). The DIGAMI study postulated that the improvement in mortality seen on insulin therapy may be due to lower PAI-1 activity, although this was not tested.
The study described in this chapter showed that levels of PAI-1 antigen were higher on sulphonylurea compared to insulin therapy, but PAI-1 activity and t-PA antigen did not differ significantly between treatments. This may in part be explained by an increase in BMI in subjects on both therapies. This was a consequence of strict diabetic control, and occurred despite dietetic advice. Although this increase was similar on both treatments, and corrected for in our statistical analyses, this may have accounted for the increase in PAI-1 activity at the end of the study on both insulin and sulphonylurea therapy. PAI-1 activity is closely associated with BMI, the possible decrease in PAI-1 activity on insulin therapy might have been counteracted by an increase in BMI, PAI-1 antigen conversely, still remained significantly raised in subjects on sulphonylurea therapy.

The concept of portal versus peripheral insulin levels in determining PAI-1 activity must also be considered. The increase in PAI-1 activity on sulphonylurea could be due to an increased level of portal insulin. Previous studies have attempted to dissect out this difference, by administering peripheral insulin infusions (Grant et al, 1990; McCormack et al, 1993), and attempting to stimulate portal insulin with an oral glucose tolerance test (McCormack et al, 1993; Nagi et al, 1996; Seljeflot et al, 1994). None of the studies have been able to show a convincing elevation of PAI-1. In contrast, some studies demonstrate that oral glucose reduces PAI-1 activity. These studies have been criticised for their short duration of action, but no study has directly implicated portal insulin. Although portal insulin levels and hepatic PAI-1 production may have contributed to our findings, we have no evidence to support this theory. In subjects with insulin dependent diabetes (IDDM), the data on PAI-1 activity is conflicting, although most studies show a decrease in PAI-1 activity. This effect may be due to low portal and hepatic insulin concentrations in IDDM subjects.

PAI-1 in blood exists in three different molecular forms: latent (or inactive), active, and PAI-1 complexed to t-PA (Declerck et al, 1990). The PAI-1 antigen assay measures total antigen and is unable to differentiate between the latent, active and
complexed PAI-1 forms, while the t-PA assay measures t-PA complexed to PAI-1 and PAI-2. Most t-PA antigen is bound to PAI-1 and relates inversely to fibrinolysis (Chandler et al, 1997). Relative proportions of latent, active and complexed PAI-1 are influenced by age, obesity, time of sample and disease process (Andreotti et al, 1990; Alessi et al, 1990), and it is possible that hypoglycaemic therapy alters the amount of PAI-1 present in different molecular forms. Thus far, there has been no study examining the effect of diabetic therapy on the relative concentrations of the various molecular forms. Furthermore, the clinical relevance of latent antigen on overall fibrinolytic potential needs to be evaluated.

4.7 Conclusion

In conclusion this study showed that 16 weeks treatment with sulphonylurea or insulin:

1. Resulted in equal improvement in glycaemia and insulin sensitivity.
2. On sulphonylurea therapy subjects had higher fasting and post-glucose load levels of intact proinsulin compared to B1 and insulin.
3. PAI-1 antigen was higher on sulphonylurea than insulin therapy.
4. There were no differences in concentrations of total, VLDL and IDL cholesterol and triglyceride, LDL, HDL2 and HDL3 cholesterol, ApoA1, A2 and B1 or Lp(a) with improved glycaemia or between therapies.

These results may help explain the improved mortality observed post myocardial infarction in insulin treated NIDDM subjects in the DIGAMI study. The results of the UK Prospective Diabetes Study (UKPDS study group, 1991) are anxiously awaited. This study will ultimately answer the question of the effects of long term therapy with sulphonylurea and insulin on the incidence of cardiovascular events.
CHAPTER 5

THE STUDY OF A POLYMORPHISM IN THE PAI-1 GENE, AND THE ROLE OF PAI-1 GENE-ENVIRONMENT INTERACTIONS

5.1 Introduction

Gene variability is the source of inter-individual differences and could explain the variation in susceptibility to atherosclerosis and myocardial infarction. Characterization of the molecular variability of candidate genes could allow identification of populations at particular risk. The plasma level of a particular protein observed in an individual at any one time will be the result of interaction between genetic variation and several different environmental factors. Understanding the interactions of the PAI-1 gene in the context of thrombotic disease is thus of major importance.

5.1.2 PAI-1 Genetics

The background to the genetics of PAI-1 have been discussed in section 1.8. Studies of various polymorphisms of the PAI-1 gene, have demonstrated an association between variation in the PAI-1 locus and PAI-1 levels. This chapter focuses on the insertion (40)/deletion (50) polymorphism in the promoter of the PAI-1 gene.

5.1.3 PAI-1 4G/5G promoter polymorphism

Dawson et al (1993) have described a common single base pair insertion (5G)/deletion (4G) polymorphism 675 base pairs upstream from the start of transcription of the PAI-1 gene. The 4G/5G polymorphism is common in the general population with the two alleles having approximately equal frequency. Analysis of the binding of human hepatoma cell line derived nuclear proteins to the two allele sequences suggests that the 5G allele contains an additional protein binding site which is not present in the 4G allele (Dawson et al, 1993). The effect of this 4G/5G polymorphism on transcription was investigated by transient transfection of the HepG2 human hepatoma cell line with
constructs comprising the bacterial chloramphenicol acetyltransferase (CAT) coding sequence driven by the two different allelic sequences of the PAI-1 promoter (-805 to +83). Quantification of the CAT mRNA produced from these constructs showed that HepG2 cells transfected with the 4G allele produced 6 times more mRNA than the 5G allele in response to stimulation with IL-1, while there was little or no difference between the alleles under basal conditions (Dawson et al, 1993). This suggests that the 4G/5G polymorphism is functional, and that the difference in sequence at position -675 influences transcription of the PAI-1 gene under stimulation with IL-1.

5.1.4 Population studies on the PAI-1 4G/5G promoter polymorphism

As would be predicted from the higher promoter activity, in a sample of 107 young patients with previous myocardial infarction, subjects with the 4G allele had higher plasma PAI-1 activity levels compared to subjects with the 5G allele, but this association was weaker in healthy controls (Dawson et al, 1993). In patients and controls individuals homozygous for the 4G allele showed a positive correlation between plasma fibrinogen levels (an acute phase reactant), and plasma PAI-1 activity, while those with one or more 5G allele showed a weak or negative relationship. These observations have been confirmed in the ECTIM (Etude Cas-Temoins de l'Infarctus du Myocarde) study, a case controlled study of myocardial infarction based in Belfas, Lille, Stasbourg and Toulouse to investigate environmental and genetic factors which contribute to differences in mortality and incidence of CHD. Homozygosity for the 4G allele was associated with higher PAI-1 activity (Ye et al, 1995). There has been no study prior to the work in this thesis on the PAI-1 promoter polymorphism in diabetic subjects.

5.2 Aims of the study

1. To determine the frequency of the 4G/5G promoter polymorphism of the PAI-1 gene in a sample of NIDDM subjects.
2. To determine whether NIDDM subjects homozygous for the 4G allele have higher levels of PAI-1 activity.

3. To examine any interaction of the 4G/5G polymorphism with other determinants of PAI-1.

5.3 Subjects

146 NIDDM subjects (66 male, 80 female) attending the out patient department at the Whittington Hospital were studied. The sample comprised 86 Caucasian, 26 Afro-Caribbean and 34 Asian subjects. Characteristics of the subjects are outlined in Chapter 3.

5.4 Genetic Methodology

All solutions used in the preparation and manipulation of DNA samples were prepared with the use of double distilled H₂O and autoclaved. Similarly all plasticware used was autoclaved or was sterile.

5.4.1 DNA preparation from blood samples

Genomic DNA was prepared from whole blood collected in EDTA tubes using a standard small-scale technique. 400μl of freshly prepared 0.17M NH₄Cl was added to 100μl of whole blood in a screw capped Eppendorf tube. The sample was mixed well by inverting and left at room temperature for 20 min. It was then spun in a microfuge for 1 min. The pellet was resuspended and washed three times in cold 0.9% NaCl. After the final wash the pellet was resuspended in 200μl of 0.05M NaOH, boiled for 10 min and neutralised with 25μl 1M Tris pH 8.0.

5.4.2 Polymerase chain reaction (PCR)

The 890bp region of the PAI-1 gene was amplified using PCR. Conditions for the PCR were standard (Saiki et al 1988) using 200ng of genomic DNA as a template and 200ng of each primer in a 50μl volume. The primer sequences were as follows:
The PCR contained 200\mu M each of deoxyguanosine-5'-triphosphate, deoxythymidine-5'-triphosphate, deoxycytosine-5'-triphosphate and deoxyadenosine-5'-triphosphate, 10mM Tris-HCl (pH 8.3), 1.0mM MgCl$_2$, 50mM KCl, 5\mu l dimethyl sulphoxide and 200ng Taq polymerase. Samples were overlaid with paraffin oil and underwent the following thermal cycles:

93\degree C - 3 min
93\degree C - 1 min
55\degree C - 1 min 29 cycles
72\degree C - 2 min
72\degree C - 5 min

5.4.3 Agarose gel electrophoresis:

Agarose gel electrophoresis was used to check the PCR prior to further genotyping. A solution of 1\% agarose, 10\% TAE buffer and 0.025\mu g/ml of ethidium bromide was prepared and boiled in a microwave oven until the agarose had fully dissolved. The solution was allowed to cool in cold water and poured into a perspex gel former containing the appropriate size of comb to form wells. The gel was left to set at room temperature. Once set, the comb was removed and the gel placed in a horizontal tank. 5\mu l of each PCR product was placed in the wells and the gel was run in 1\times TAE buffer and ethidium bromide at an appropriate voltage until the desired separation of DNA bands was achieved. A 1Kb DNA size marker was used. The DNA fragments were observed under UV transillumination and photographed. The size of the band was compared to the size marker to ensure that the correct segment had been amplified in the PCR reaction.

5.4.4 Allele specific oligomelting (ASO)

5.4.4.1 Southern blotting and hybridisation.
PCR products from the subjects were run on a large agarose gel (20 × 20cm), prepared as above. The gel was denatured (in 0.5M NaOH, 1.5M NaCl) for 60 min on a rotary shaker. Two pieces of Hybond N+ filters (Amersham, UK) were cut to the same size as the gel, and placed on the gel smoothing it down to remove air bubbles. The gel and filters were then placed between two stacks of paper towels (approximately 30 cm) and covered with cling film. A 500g weight was placed on top to ensure good contact between the gels and the filters and to keep the towels in place. This was left overnight.

After blotting, the towels were removed. Before the filter was removed the position of the wells were marked on the filter with an indelible marker. To alkali fix the Southern blots, the filters were placed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 1 hour and then rinsed in 5 × SSC (20 × SSC: 0.3M NaCl, 0.3M sodium citrate).

**Hybridisation:**

**Hybridisation solution:**

- 5 × SSPE (3M NaCl, 0.2M NaH₂PO₄, 20 mm EDTA)
- 5 × Denharts solution (0.1% Ficoll, 0.1% bovine serum albumin 0.1% polyvinylpyrrolidone)
- 0.1% Sodium Dodecyl Sulphate (SDS)

**Hybridisation:**

Each 20 × 20 filter was cut into two pieces, rolled and placed in a separate 50ml Falcon tube. One tube was labelled 4G, the other 5G. 4 mls of hybridisation solution were added to the tubes and the filters were pre-hybridized in a rotary oven at 42°C for 10 min. Two end labelled oligonucleotides (see below) were then hybridized separately to one of the filters in hybridization solution at a temperature 2-3°C below their calculated melting temperature, ie 42°C for at least one hour.
Oligonucleotides used for the 4G/5G polymorphism:

4G allele \(\text{CACGTGGGGAGTCA}\) \(T_m=46^\circ\text{C}\)

5G allele \(\text{ACGTGGGGAGTCA}\) \(T_m=46^\circ\text{C}\)

### 5.4.4.2 5' end labelling of oligonucleotide

Labelling mixture:

- \(^{32}\text{P}\) labelled ATP \(3\mu\text{l}\)
- T4 kinase \(1\mu\text{l}\)
- PNK buffer \(2\mu\text{l}\)
- oligonucleotide \(1\mu\text{l}\)
- distilled water \(13\mu\text{l}\)

**TOTAL VOLUME:** \(20\mu\text{l}\) per tube

A labelling mixture was used in a total volume of \(20\mu\text{l}\), placed in two separate Eppendorf tubes and incubated at 37°C for 30 min. The labelled oligonucleotides were separated from nucleotides using a Sephadex G25 column, spun at 1000rpm for three min, producing a probe with minimal nucleotide contamination.

### 5.4.4.3 Washing of filter

The radioactive hybridization solution was discarded and the filter removed and placed in a bath of 0.1\% SDS, 5×SSPE prewarmed to 65°C for 15 min. The filter was then removed and placed next to a slow X-ray film at -70°C for 24 hours. The film was developed and if necessary washed further or exposed for a longer time. All genotyping was done independently by at least two individuals. Figure 5.1 shows a section of the ASO autoradiograph. Lane A shows the 4G allele, and lane B the 5G allele.
Figure 5.1
Allele specific oligomelting autoradiograph demonstrating the 4G/5G polymorphism.
Lane (A) 4G
Lane (B) 5G
5.4.5 Direct DNA sequencing

5.4.5.1 Introduction

In order to check the genotyping performed by the ASO method, 12 random samples were selected for direct DNA sequencing. The chain terminating method of Sanger et al (1977), with modified T7 DNA polymerase (Sequenase, USB) using Dynabeads M-280 Streptavidin (Dynal AS, Oslo, Norway) was used. This chain-termination method of sequencing involves the synthesis of a DNA strand by a DNA polymerase \textit{in vitro} using a single stranded DNA template. Synthesis is initiated at only the one site where an oligonucleotide primer anneals to the template (Figure 5.2). The synthesis reaction is terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation. The chain-terminating nucleotide analogs are the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs), which lack the -OH group necessary for DNA chain elongation. When mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalyzed polymerisation will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information. A radioactively labelled nucleotide is also included in the synthesis, so the labelled chains of various length can be visualised by autoradiography after separation by acrylamide gel electrophoresis.

5.4.5.2 Method

a) Dynabeads M-280 Streptavidin

The Dynabeads were used as a magnetic solid phase for the capture and purification of the amplified DNA.

1- The Dynabeads were washed in binding and washing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2.0M NaCl)

2- Immobilisation of the PCR product:

40\mu l of prewashed beads was added to 40\mu l of the PCR product (PCR reaction as mentioned above) and incubated for 15 min.
Figure 5.2 The chain termination method of direct DNA sequencing.

Chain-terminating (dideoxy) nucleotides can not form bond with dNTP

DNA polymerase + ddCTP + 4 dNTPs +

A series of labelled strands results, the length of which in this case is dependent on the location of a G base relative to the end of the DNA.
3- Melting the DNA duplex:

The Eppendorf tube containing the immobilized product, was placed in a magnetic separator (Dynal MPC), the supernatant removed, and the beads washed with 40µl of binding and washing buffer. The supernatant was again removed and the beads resuspended in 8µl of freshly prepared 0.1M NaOH solution and incubated at room temperature for 10 min.

4- Separating the DNA strands:

Using the magnetic separator the NaOH supernatant (containing the biotinated strand) was removed and the beads (with the immobilised biotinated strand) washed with 50µl 0.1M NaOH, once with 40µl wash buffer, and once with 50µl TE buffer.

b) Sequencing reaction

Annealing template and primer:

Primer DS3 (346 ng/µl) 1µl
Reaction buffer 2µl
DNA 7µl
TOTAL VOLUME: 10µl

The capped centrifuge tube was heated to 65°C for 2 min and allowed to cool at room temperature. Once below 30°C, annealing was complete and the tube was placed on ice.

Labelling reaction:

To the annealed template-primer the following was added on ice:

Template-primer 10.0µl
DTT 0.1M 1.0µl
Diluted labeling mixture 2.0µl
[α-35S]dATP 0.5µl
Diluted Sequenase 2.0µl

The above mixture was incubated at room temperature for 5 min.
Termination reaction:

2.5 μl of the ddGTP termination mixture was warmed in a capped tube for 1 min and 3.5μl of the labelling mixture added and incubated for a further 5 min. 4μl of Stop Solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) supplied in the kit, was added and the mixture was stored on ice until it was loaded on the sequencing gel. Prior to loading the samples were heated to 75°C for 2 min and loaded immediately.

5.4.5.3 Polyacrylamide gel electrophoresis

Two glass electrophoresis plates were cleaned with detergent, distilled water and then ethanol. One plate was siliconized with Repelcote (BHD) to prevent the gel sticking to both plates. The two plates were taped together to prevent leaking, with 0.4mm spacers. 100mls of 6% acrylamide solution was prepared: 42g urea, 10mls 10 ×TBE (89mM Tris-borate, 2mM EDTA at pH8.3) and 20mls of a 30% stock of 19:1 bis acrylamide solution (obtained from Severn Biotech Ltd). The top of the plates were raised from the horizontal and the acrylamide solution was poured between the plates with a 50ml syringe avoiding bubble formation. Two 0.4mm sharks tooth combs were inserted upside down and clipped tightly to form a horizontal surface at the top of the gel. The gel was left to set for one hour, after which the tape was removed and the gel placed in a vertical apparatus. The combs were removed and 1×TBE running buffer added. The top surface of the gel was cleaned with buffer using a pipette and the combs replaced. Samples were loaded in 0.5 volume formamide dye. The gel was run at a constant power of 60W for approximately three hours until the required size separation was achieved. Once run the gel was transferred to 3M filter paper and dried under vacuum at 80°C for at least one hour. It was then autoradiographed overnight.

Figure 5.3 shows a section of the direct sequence autoradiograph with the 12 samples, and figure 5.4 shows an enlarged section demonstrating a run of 5 Gs.
Figure 5.3
Direct sequence autoradiograph.
Arrow points to a run of 5G’s. This section is enlarged in figure 5.4.
Figure 5.4

Enlarged section of direct sequence autoradiograph showing run of 5G's
5.5 Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS). Skewed data were logarithmically transformed, and their results shown as geometric mean ×/ΔSD. Differences in frequencies of categorical variables (genotype) between groups were assessed by χ² test. The differences in PAI-1 activity between the genotypic groups were analysed using ANOVA. To investigate possible interactions between PAI-1 genotype and other determinants of PAI-1, regression slopes between genotype groups were compared, and factorial ANOVA was used to assess the significance of any interaction.

5.6 Results

Results of PAI-1 genotyping on the total population and in the individual ethnic groups are shown in Tables 5.1 and 5.2. The sample consisted of 86 Caucasians, 34 Asians and 26 Afro-Caribbeans. The PAI-1 4G/5G polymorphism was in Hardy-Weinberg equilibrium in the Caucasian and Afro-Caribbean subgroups, but differed significantly (χ² = 7.9, p < 0.02) in the Asian subgroup, due to an excess of 4G/5G heterozygotes (Table 5.2). Genotype of a random sample was confirmed by sequence analysis, and all samples were assessed independently by two people, ensuring errors in genotyping were an unlikely cause of the apparent excess of heterozygotes. The frequency of the 4G allele was similar in the Caucasians and Asians (0.53 and 0.46 respectively), however in the Afrocaribbeans the frequency was 0.23, with a complete absence of 4G/4G homozygotes.

Since the association of PAI-1 levels with 4G/5G genotype was homogeneous in all three ethnic groups, and since evidence suggests that this relationship is causal, the three ethnic groups were pooled to increase the statistical power of the analysis. Table 5.3 shows levels of variables in the three 4G/5G genotype groups. In the group as a whole there was a significant difference in PAI-1 activity between the three genotypes, the mean PAI-1 activity being 6.1AUml⁻¹ higher in those with the 4G/4G than those homozygous for the 5G allele.
Table 5.1

PAI-1 4G/5G genotype in males and females, with corresponding allelic frequencies.

<table>
<thead>
<tr>
<th></th>
<th>Males (n=80)</th>
<th>Females (n=66)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequencies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4G</td>
<td>0.42</td>
<td>0.52</td>
</tr>
<tr>
<td>5G</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td>$\Sigma \chi^2(p) 2 df$</td>
<td>0.03 (NS)</td>
<td>0.19 (NS)</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>4G/4G</td>
<td>14 (17.5%)</td>
<td>18 (27.3%)</td>
</tr>
<tr>
<td>4G/5G</td>
<td>39 (48.8%)</td>
<td>32 (48.5%)</td>
</tr>
<tr>
<td>5G/5G</td>
<td>27 (33.7%)</td>
<td>16 (24.2%)</td>
</tr>
</tbody>
</table>

Table 5.2

PAI-1 4G/5G genotype in the three ethnic groups, with corresponding allelic frequencies.

<table>
<thead>
<tr>
<th></th>
<th>Caucasian (n=86)</th>
<th>Asian (n=34)</th>
<th>Afro-Caribbean (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allele Frequencies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4G</td>
<td>0.53</td>
<td>0.46</td>
<td>0.23</td>
</tr>
<tr>
<td>5G</td>
<td>0.47</td>
<td>0.54</td>
<td>0.77</td>
</tr>
<tr>
<td>$\Sigma \chi^2(p) 2 df$</td>
<td>3.04 (NS)</td>
<td>7.88 (&lt;0.02)</td>
<td>2.44 (NS)</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>4G/4G</td>
<td>29 (33.7%)</td>
<td>3 (8.8%)</td>
<td>0</td>
</tr>
<tr>
<td>4G/5G</td>
<td>34 (39.6%)</td>
<td>25 (73.5%)</td>
<td>12 (46.2%)</td>
</tr>
<tr>
<td>5G/5G</td>
<td>23 (26.7%)</td>
<td>6 (17.7%)</td>
<td>14 (53.8%)</td>
</tr>
</tbody>
</table>
Table 5.3
Variables in the three 4G/5G genotype groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>4G/4G n=32 (22%)</th>
<th>4G/5G n=71 (48.6%)</th>
<th>5G/5G n=43 (29.4%)</th>
<th>anova p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 activity (AU.ml(^{-1}))</td>
<td>18.3 (10.0)</td>
<td>13.7 (9.1)</td>
<td>12.2 (8.7)</td>
<td><strong>0.016</strong></td>
</tr>
<tr>
<td>*Specific insulin (pmol.l(^{-1}))</td>
<td>61.6 (3.0)</td>
<td>51.7 (2.4)</td>
<td>68.1 (2.4)</td>
<td>0.28</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l(^{-1}))</td>
<td>7.2 (2.4)</td>
<td>9.4 (2.5)</td>
<td>6.4 (2.8)</td>
<td>0.11</td>
</tr>
<tr>
<td>*des 31,32 proinsulin (pmol.l(^{-1}))</td>
<td>3.4 (2.5)</td>
<td>3.6 (2.4)</td>
<td>3.1 (2.5)</td>
<td>0.66</td>
</tr>
<tr>
<td>*Insulin sensitivity (HOMA)%(\dagger)</td>
<td>49.5 (2.4)</td>
<td>44.2 (2.2)</td>
<td>42.0 (1.9)</td>
<td>0.76</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l(^{-1}))</td>
<td>1.6 (1.9)</td>
<td>1.4 (1.8)</td>
<td>1.4 (1.9)</td>
<td>0.48</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l(^{-1}))</td>
<td>5.6 (0.8)</td>
<td>5.4 (1.1)</td>
<td>5.0 (1.1)</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l(^{-1}))</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.3)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± SD and * Geometric means \(\times/\div\) SD for skewed data
\(\dagger\) 36 subjects on insulin excluded (4G/4G n=22; 4G/5G n=59; 5G/5G n=29)
There was no significant difference in triglyceride concentrations between the three groups. PAI-1 activity in the 4G/4G genotype subjects showed a strong significant correlation with serum triglycerides ($r=0.65$, $p<0.0001$) (Table 5.4). This relationship was weaker in the 4G/5G group ($r=0.30$, $p=0.025$), and did not reach statistical significance in the 5G/5G group ($r=0.28$, $p=0.18$).

Multiple regression analysis showed that the effect of serum triglyceride concentrations on PAI-1 activity differed according to genotype ($p=0.04$ for genotype triglyceride interaction). This is illustrated by the difference in slopes of the regression lines of triglycerides on PAI-1 in the three different genotypes (Figure 5.5). Correlation between all other variables and PAI-1 activity showed no major or consistent differences between genotypes (Table 5.4). A multiple regression model, including factors known to correlate with PAI-1 activity (fasting plasma specific insulin, intact and des 31,32 proinsulin, triglyceride, WHR, BMI, age, treatment, ethnicity and genotype) explained $36\%$ ($R^2=0.36$, $F$ ratio=$3.5$, $p<0.0001$) of the variation in PAI-1 activity. In this model insulin sensitivity and triglyceride concentrations explained the greatest variation in PAI-1 activity. The genotype effect appeared to be mediated mainly by the 4G genotype-triglyceride interaction as, employing a stepwise model, this interaction remained significant when both triglyceride and genotype were included, but the 4G genotype was displaced from the model by the interaction term (Table 5.5). There were no significant interactions between genotype and either insulin, insulin resistance or proinsulin-like molecules.

5.7 Discussion

Genetic variation in the PAI-1 gene is associated with altered levels of PAI-1 activity in healthy individuals and in CHD patients (Dawson et al, 1991). This is the first report to show a similar association in subjects with NIDDM. The allele frequency in these patients was similar to that described in subjects with coronary heart disease (CHD) and controls (Dawson et al, 1993), and, as in subjects with CHD, the 4G/4G genotype was associated with higher PAI-1 activity levels (Table 5.3).
Table 5.4  
Correlations of PAI-1 activity with variables in the three 4G/5G genotype groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>4G/4G (n=32)</th>
<th>4G/5G (n=71)</th>
<th>5G/5G (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific insulin (pmol.I^-1)</td>
<td>0.33</td>
<td>0.30*</td>
<td>0.27</td>
</tr>
<tr>
<td>Intact proinsulin (pmol.I^-1)</td>
<td>0.17</td>
<td>0.20</td>
<td>0.39*</td>
</tr>
<tr>
<td>des 31,32 proinsulin (pmol.I^-1)</td>
<td>0.31</td>
<td>0.23</td>
<td>0.37*</td>
</tr>
<tr>
<td>Triglyceride (mmol.I^-1)</td>
<td>0.65***</td>
<td>0.30*</td>
<td>0.28</td>
</tr>
<tr>
<td>Total cholesterol (mmol.I^-1)</td>
<td>0.06</td>
<td>0.19</td>
<td>-0.15</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.I^-1)</td>
<td>-0.38*</td>
<td>-0.29*</td>
<td>-0.25</td>
</tr>
<tr>
<td>Insulin sensitivity (HOMA)%^†</td>
<td>-0.43</td>
<td>-0.45**</td>
<td>-0.45*</td>
</tr>
</tbody>
</table>

*  p < 0.05  
**  p < 0.01  
*** p < 0.001  
†  36 subjects on insulin excluded (4G/4G n=22; 4G/5G n=59; 4G/5G n=29; for these correlations)
Figure 5.5 Plot of PAI-1 activity vs triglyceride in the three PAI-1 genotypes

The slope (B) and SE of the regression line between PAI-1 activity and triglyceride concentrations are as follows:

- 4G/4G, B = 10.3, SE = 2.0
- 4G/5G, B = 5.3, SE = 2.0
- 5G/5G, B = 3.8, SE = 2.0

Difference in slope 4G vs not 4G/4G: F = 4.21, p = 0.04
Table 5.5
Multiple regression analysis with PAI-1 activity as the dependent variable, correcting for age and BMI

<table>
<thead>
<tr>
<th>Variable added:</th>
<th>4G/4G</th>
<th>Triglyceride</th>
<th>Interaction</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b(SE)p</td>
<td>b(SE)p</td>
<td>b(SE)p</td>
<td></td>
</tr>
<tr>
<td>4G/4G</td>
<td>4.63(1.8)</td>
<td>0.10</td>
<td>0.013</td>
<td>0.10</td>
</tr>
<tr>
<td>4G/4G + Triglyceride</td>
<td>4.15(1.7)</td>
<td>5.7(1.3)</td>
<td>0.018</td>
<td>0.0001</td>
</tr>
<tr>
<td>4G/4G + Triglyceride +interaction</td>
<td>1.69(2.1)</td>
<td>3.9(1.5)</td>
<td>5.8(2.8)</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The figures shown are the regression coefficient b (SE b) and p values of the variables already in the model shown on the vertical axis, with the dependent variable PAI-1 activity, when adding sequentially the variables shown on the horizontal axis. BMI: body mass index; interaction: interaction of the 4G/4G genotype with serum triglyceride.
The striking difference in allele frequency in the Afro-Caribbeans, with absence of 4G/4G homozygotes, requires confirmation in a larger study.

An interesting and novel finding was the strong association of PAI-1 activity in this group with triglyceride levels. Genetic variation considered alone did not contribute significantly to variation in PAI-1 levels in multiple regression analysis but considered as an interaction with serum triglyceride concentrations had a significant effect. It has been shown that hepatic nuclear proteins bind differentially to the 4G or 5G sequence in the promoter region of the PAI-1 gene. The insertion of an extra guanine at -675 in the PAI-1 promoter creates a binding site for proteins which may have a direct effect in repressing PAI-1 transcription during an acute phase response (Dawson et al, 1993). One possible mechanism to explain the genotype/triglyceride interaction may be that triglyceride levels or composition may alter the levels of these binding proteins, and hence PAI-1 production (Figure 5.6).

In conclusion, this study demonstrates that PAI-1 genotype/triglyceride interaction and insulin sensitivity are the major determinants of PAI-1 activity in NIDDM. The influence of fasting plasma specific insulin, immunoreactive insulin and proinsulin-like molecules on PAI-1 activity is less clear, and any influence may be secondary through their effect on serum triglyceride levels. The influence of triglyceride on PAI-1 appears genotype dependent. The 4G/5G polymorphism in the promoter of the PAI-1 gene may determine the effect of triglyceride on PAI-1 activity. It may therefore be possible to target individuals with unfavourable genotypes for early interventional lipid lowering therapy to reduce their overall risk of atherogenesis and thrombosis.
Figure 5.6
The PAI-1 promoter showing the site of the 4G/5G variable site.
CHAPTER 6

PAI-1 ACTIVITY FOLLOWING MYOCARDIAL INFARCTION: THE ROLE OF ACUTE PHASE REACTANTS, INSULIN-LIKE MOLECULES AND PROMOTER (4G/5G) POLYMORPHISM IN THE PAI-1 GENE

6.1 Introduction

6.1.1 PAI-1 and myocardial infarction

Elevated PAI-1 levels have been demonstrated in various subgroups with an increased risk of myocardial infarction, including obese subjects (Vague et al, 1986; Landin et al, 1990), non-diabetic premenopausal obese women (Vague et al, 1989), subjects with angina pectoris (Juhan-Vague et al, 1989), subjects with hypertension (Landin et al, 1990) and with NIDDM (Juhan-Vague et al, 1989). PAI-1 activity is elevated in subjects admitted with acute myocardial infarction (AMI) (Almer et al, 1987; Gray et al, 1993), and elevated levels on admission with AMI is an important determinant of reperfusion following thrombolytic therapy (Sane et al; Gray et al, 1993). Hamsten et al (1987), have shown that elevated PAI-1 activity is an important predictor of recurrent infarction in young survivors of a first myocardial infarction.

The ECAT study, a multicenter European study which included 3034 patients, showed that during the two year follow up, 106 patients suffered from a coronary event, and the increased risk was associated with higher baseline t-PA and PAI-1 antigen levels (ECAT study, 1993). Clinical manifestations of ischemic heart disease result from the progressive development of an atherosclerotic plaque and thrombus formation. Fibrin deposition in an obstructed coronary artery leads to AMI, but fibrin deposition on the vessel wall may also have a more subtle role by initiating endothelial cell injury and stimulating cell proliferation (Thompson and Smith, 1989). Hypofibrinolysis due to elevated levels of PAI-1, may hence be an important risk factor for ischemic heart disease.
6.1.2 PAI-1 as an acute phase reactant

PAI-1 is an acute phase protein and increased plasma PAI-1 levels are found during acute phase responses such as AMI (Gray et al, 1993), after surgery (Mellbring et al, 1985) and in endotoxemia and septicaemia (Kruithof et al, 1993; Colucci et al, 1985). PAI-1 levels increase up to five-fold in response to endotoxin and lipopolysaccharide \textit{in vivo} and \textit{in vitro} (Emeis et al, 1986; Suffredini et al, 1989) and to interleukin-1 (IL-1) \textit{in vitro} (Emeis et al, 1986). IL-1, tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and transforming growth factor-\(\beta\) (TGF-\(\beta\)) stimulate PAI-1 production in endothelial cells and HepG2 cells (van Hinsbergh et al, 1988; De Boer et al 1991). Regulation of PAI-1 by TGF-\(\beta\) is thought to be important in angiogenesis, wound healing and in modifying the extra-cellular matrix (Sporne et al, 1987). TGF-\(\beta\) is also an acute phase mediator in the liver, and mediates acute phase hepatic PAI-1 production (Mackiewicz et al, 1990).

6.1.3 Myocardial infarction and the acute phase response

Myocardial infarction is associated with inflammation. Inflammatory lesions seen in specimens hours to days after the onset of infarction may reflect, in part, the healing process. Recently interest has focused on the pathogenic role of inflammation in early myocardial ischaemia.

Myocardial necrosis produces an inflammatory process resulting in migration of neutrophils into the region of injury within 24 hours with a maximal response in four to five days. The inflammation results in the hepatic production of acute phase proteins such as C-reactive protein (CRP). CRP doubles four to eight hours after the onset of pain and continues to increase to levels several hundred fold above baseline two to four days post MI (Smith et al, 1977; Kushner et al, 1981). Pietilä et al (1996), reported a significant association between mortality six months after AMI and peak levels of serum CRP during the acute phase, in the absence of a significant association with infarct size estimated from peak levels of creatine kinase (CK). Thus, the prognostic significance of peak CRP levels may in part be related to the extent of myocardial necrosis and partly to the unknown individual determinants of the intensity of the acute phase response.
Pannitteri et al (1997), studied the increase in levels of cytokines post AMI. There was a marked rise in plasma levels of IL-6, with peak levels occurring at 20-30 hrs after the onset of AMI. Post infarction levels of IL-6 correlated with levels of CK-MB, and hence reflected the extent of myocardial damage. There was also a moderate increase in IL-8 post AMI, which preceded the increase in IL-6, but no increase in IL-1-β or TNF-α were observed. Miyao et al (1993), observed a rise in IL-6 post AMI, with peak levels occurring 3-7 days post AMI. On admission, there was a positive correlation between peak levels of IL-6 and CRP, but not with peak levels of CK-MB. Maury and Teppo (1989), demonstrated a rise in TNF-α concentrations post AMI, with levels peaking at 70 hrs. There was no correlation however with levels of CK-MB.

PAI-1 levels have been associated with neutrophil count and C-reactive protein (CRP) (Woodhouse et al, 1997). Higher CRP levels predicted coronary events in the ECAT study (Thompson et al, 1995). In the a nested case control study based on 16 year follow up of men from the Multiple Risk Factor Intervention Trial who were initially free of clinical coronary disease, also found that CRP was a predictor of subsequent CHD death (Kuller et al, 1995). CRP concentration is a prognostic marker in unstable angina, with higher levels associated with poor outcome (Liuzzo et al, 1994).

6.1.4 PAI-1 gene-environment interaction

As mentioned in Chapter 5, different polymorphisms of the PAI-1 gene have been described. The polymorphism most likely to be functional is the 4G/5G polymorphism in the promoter of the PAI-1 gene. In normal subjects, subjects with coronary heart disease and subjects with NIDDM, the 4G allele is associated with higher PAI-1 levels, and in vitro, produces six times more PAI-1 mRNA than the 5G allele in response to IL-1 (Dawson et al, 1993). In NIDDM subjects the 4G allele is also associated with a stronger correlation of PAI-1 with triglyceride levels (as shown in Chapter 5).

This chapter will explore the variation in PAI-1 response to an acute phase stimulus (AMI), and relate these changes to the 4G/5G polymorphism in the PAI-1 gene and to cytokine levels. We hypothesise that this variation may be the consequence of gene-
environment interactions and that the PAI-1 genotype may be responsible for differences in response to acute phase stimuli not only *in vitro* but also *in vivo*.

### 6.2 Aims of the study

i- To test the predictive value of the levels of PAI-1, insulin-like molecules and cytokines taken day 1 post AMI to outcome at six months.

ii- To follow the time course of plasma concentrations of PAI-1 and cytokines during admission with AMI and to compare this to levels at six months.

iii- To test the hypothesis, *in vivo*, that subjects homozygous for the 4G allele of the PAI-1 gene have an exaggerated response to acute phase stimuli compared to the 4G/5G and 5G/5G group, resulting in greater and more persistent elevation of PAI-1 levels during admission with AMI.

### 6.3 Subjects and methods

**Subjects**

*a- Baseline study Subjects and methods:*

123 consecutive patients admitted to the Whittington Hospital with confirmed AMI (World Health Organisation Criteria; WHO, 1976) were studied. All patients included in the study were given thrombolysis with streptokinase (1.5mU) on admission. Patients gave informed written consent, and the study was approved by the ethical committee of the Islington Health Authority. A fasting venous blood sample (20mls) was taken without stasis between 8 and 9 am to overcome the diurnal variation in PAI-1 activity on the morning following admission, day 1 (12-24 hrs post AMI), on day 2 (24-48 hrs post AMI) and on day 5. Samples were also collected for measurement of insulin-like molecules, IL-1β, IL-6, TNF-α, lipids and glucose.

*b- Follow-up study:*

Subjects were re-called six months following their initial admission with AMI. Weight, height, waist measurements and hip girth were recorded. BMI (kg.m⁻²), and waist-to-hip ratio (WHR) were calculated. Fasting blood was taken, between 8 and 10 am for
measurement of fibrinolytic variables, insulin-like molecules, lipids and acute phase markers. Subjects then had a 75g oral glucose tolerance test. General Practitioners of the subjects who did not attend for follow-up were contacted to establish mortality data.

Methods:
The assay methodology used in this study are described in Chapter 2. DNA was prepared from blood using a standard small-scale technique. An 890bp region of the PAI-1 promoter from each subject was amplified using the polymerase chain reaction, and allele specific oligo melting was used in genotype determination as described in Chapter 5.

6.4 Statistical analysis
Data were analysed using the Statistical Package for Social Sciences (SPSS). Data are presented as mean (SD) for normally distributed data and geometric mean (SD) for skewed data. PAI-1 activity on day one after AMI was compared to that on day 2, day 5, and at 6 months after AMI and related to the concentrations of cytokines and insulin-like molecules on each occasion. The comparison of levels of variables between the three genotypic groups were analysed using analysis of variance (ANOVA) followed by paired t-tests. Multiple regression analysis was used to study the relationship of PAI-1 with other variables while controlling for confounders. P< 0.05 was considered as statistically significant.

6.5 Results
6.5.1 Clinical Characteristics of patients at presentation and follow-up
The sample consisted of 123 subjects (89 men and 34 women) admitted with AMI. At six months, 60 subjects (49 men and 11 women) attended for a follow-up visit, 12 had died (4 men and 8 women), and the remaining 51 non-attenders were confirmed alive by their General Practitioner. Clinical and biochemical characteristics of all subjects during their hospital stay on days 1, 2, and 5 post AMI are shown in Table 6.1. Table 6.2 shows clinical and biochemical characteristics on days 1, 2, and 5, and at 6 months of the 60 subjects only, who attended for a follow-up visit at six months.
Table 6.1
Variables post myocardial infarction (all patients, n=123).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mmol.l(^{-1}))</td>
<td>8.9 (4.7)</td>
<td>7.3 (3.3(^{c}))</td>
<td>6.6 (2.0(^{a}))</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l(^{-1}))</td>
<td>54.9 (2.2)</td>
<td>50.8 (2.1(^{c}))</td>
<td>51.0 (2.2(^{c}))</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l(^{-1}))</td>
<td>8.2 (2.1)</td>
<td>6.3 (2.0(^{c}))</td>
<td>6.3 (1.9(^{c}))</td>
</tr>
<tr>
<td>*Des 31,32 proinsulin (pmol.l(^{-1}))</td>
<td>3.4 (2.8)</td>
<td>2.3 (2.9(^{c}))</td>
<td>2.3 (2.9(^{c}))</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l(^{-1}))</td>
<td>1.8 (1.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l(^{-1}))</td>
<td>5.6 (1.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l(^{-1}))</td>
<td>1.3 (0.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*PAI-1 activity (AU.ml(^{-1}))</td>
<td>21.4 (3.1)</td>
<td>12.0 (3.1(^{c}))</td>
<td>13.7 (2.3(^{b}))</td>
</tr>
<tr>
<td>*PAI-1 antigen (ng.ml(^{-1}))</td>
<td>41.7 (1.4)</td>
<td>35.1 (1.4(^{c}))</td>
<td>37.5 (1.3(^{c}))</td>
</tr>
<tr>
<td>*Fibrinogen (pmol.l(^{-1}))</td>
<td>187.9 (1.8)</td>
<td>397.8 (1.4(^{c}))</td>
<td>518.0 (1.3(^{c}))</td>
</tr>
<tr>
<td>*IL-1 (pg.ml(^{-1}))</td>
<td>0.64 (2.1)</td>
<td>0.62 (2.0)</td>
<td>0.52 (2.5(^{b}))</td>
</tr>
<tr>
<td>*IL-6 (pg.ml(^{-1}))</td>
<td>11.9 (1.9)</td>
<td>8.6 (1.7(^{c}))</td>
<td>7.1 (1.8(^{c}))</td>
</tr>
<tr>
<td>*TNF-(\alpha) (pg.ml(^{-1}))</td>
<td>2.8 (2.0)</td>
<td>2.5 (2.0)</td>
<td>2.6 (1.7)</td>
</tr>
<tr>
<td>*CRP</td>
<td>2.5 (3.7)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± (SD) and * Geometric means ×/÷ (SD) for skewed data.

a: p < 0.05 vs day 1                b: p < 0.01 vs day 1                c: p < 0.001 vs day 1

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| Table 6.2 |
|-----------------|--------|--------|--------|
|                | Day 1  | Day 2  | Day 5  |
| Fasting plasma glucose (mmol.l⁻¹) | 8.3 (3.6) | 6.5 (2.3)<sup>c</sup> | 6.9 (2.0) |
| *Specific insulin (pmol.l⁻¹)      | 60.6 (2.0) | 51.7 (2.1) | 57.9 (1.8) |
| *Intact proinsulin (pmol.l⁻¹)     | 8.5 (1.9) | 6.0 (2.0)<sup>c</sup> | 6.6 (1.8)<sup>b</sup> |
| *Des 31,32 proinsulin (pmol.l⁻¹)  | 3.4 (2.9) | 2.4 (2.8)<sup>b</sup> | 2.8 (2.7)<sup>a</sup> |
| *Triglyceride (mmol.l⁻¹)          | 1.8 (1.8) | -      | -      |
| Total cholesterol (mmol.l⁻¹)      | 5.5 (1.4) | -      | -      |
| HDL cholesterol (mmol.l⁻¹)        | 1.3 (0.3) | -      | -      |
| *PAI-1 activity (AU.ml⁻¹)         | 24.8 (2.0) | 9.7 (3.8)<sup>c</sup> | 12.7 (2.3)<sup>c</sup> |
| *PAI-1 antigen (ng.ml⁻¹)          | 43.2 (1.4) | 35.3 (1.4)<sup>c</sup> | 37.1 (1.3)<sup>b</sup> |
| *Fibrinogen (pmol.l⁻¹)            | 198.9 (1.8) | 385.7 (1.3)<sup>c</sup> | 517.5 (1.3)<sup>c</sup> |
| *IL-1 (pg.ml⁻¹)                   | 0.58 (2.0) | 0.59 (2.0) | 0.52 (2.6)<sup>a</sup> |
| *IL-6 (pg.ml⁻¹)                   | 12.3 (1.7) | 9.7 (1.7)<sup>b</sup> | 7.8 (1.9)<sup>c</sup> |
| *TNF-α (pg.ml⁻¹)                  | 2.8 (1.9) | 2.2 (2.2)<sup>b</sup> | 2.5 (1.7)<sup>a</sup> |
| *CRP                            | 2.1 (3.6) | -      | -      |

Data are arithmetic means ± (SD) and *Geometric means × (SD) for skewed data.

<table>
<thead>
<tr>
<th>a:</th>
<th>p &lt; 0.05 vs day 1</th>
<th>d:</th>
<th>p &lt; 0.05 vs day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>b:</td>
<td>p &lt; 0.01 vs day 1</td>
<td>e:</td>
<td>p &lt; 0.01 vs day 5</td>
</tr>
<tr>
<td>c:</td>
<td>p &lt; 0.001 vs day 1</td>
<td>f:</td>
<td>p &lt; 0.001 vs day 5</td>
</tr>
</tbody>
</table>

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6.5.2 Fibrinolytic parameters (Tables 6.1 and 6.2)(Figure 6.1)

PAI-1 activity was high on day 1 post AMI, at 5 days levels decreased and were significantly lower than on day 1. In the 60 subjects followed up, levels of PAI-1 activity at six months, although lower, were not significantly different from day 5 post AMI. PAI-1 antigen levels however, continued to decrease, and levels at six months were significantly lower than those on day 5 (Table 6.2). Fibrinogen levels were low on day 1 post AMI due the effect of thrombolysis with streptokinase, levels continued to rise to day 5, levels at six months were however significantly lower than day 5.

6.5.3 Insulin-like molecules and cytokines (Tables 6.1 and 6.2)(Figure 6.2)

Levels of insulin-like molecules decreased from day 1 to day 5 post AMI. Although levels of IL-1 were similar on day 5 to those at six months, levels of IL-6 continued to decrease, and were significantly lower at six months compared to day 5 (Tables 6.1 and 6.2).

6.5.4 Correlation of variables with PAI-1 activity (Table 6.3)

Table 6.3 shows the correlation coefficients of variables with PAI-1 day 1 and day 5 post AMI in all subjects and in the 60 attenders at six months. In all subjects, on day 1 post AMI, PAI-1 activity correlated with levels of intact proinsulin ($r=0.38$, $p<0.0001$), and des 31,32 proinsulin ($r=0.24$, $p=0.008$), but not with levels of cytokines, lipids or glucose. Similar correlations were observed in the 60 follow-up subjects. At six months however, PAI-1 activity correlated with levels of IL-1 only ($r=0.31$, $p=0.04$).

6.5.5 Survivors versus non-survivors (Table 6.4)

At six months, 60 patients (50%) attended for follow-up, 51 non-attenders (40%) were confirmed alive, and there were 12 non-survivors (10%). All non-survivors died of cardiac related causes, confirmation was obtained from the patients' medical records. Non-survivors had higher PAI-1 activity day 1 post-MI compared to the survivors (t-test, $p=0.01$). There were no differences in levels of cytokines or insulin-like molecules between the two groups (Table 6.4).
Data shown as geometric Mean (SE).
Dotted line: All subjects (n=123)
Solid line: Follow-up subjects only (n=60).
Figure 6.2
IL-1 and IL-6 levels post myocardial infarction

Data shown as geometric mean (SE)
Dotted line: All subjects (n=123)
Solid line: Follow-up subjects only (n=60)
Table 6.3
Correlation of variables with PAI-1 activity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 1 (n=123)</th>
<th>Day 1 (n=60)</th>
<th>6 months (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mmol.l⁻¹)</td>
<td>0.19*</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>Specific insulin (pmol.l⁻¹)</td>
<td>0.13</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>Intact proinsulin (pmol.l⁻¹)</td>
<td>0.38***</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>Des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>0.24**</td>
<td>0.27*</td>
<td>0.07</td>
</tr>
<tr>
<td>Triglyceride (mmol.l⁻¹)</td>
<td>0.15</td>
<td>0.20</td>
<td>0.14</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>0.12</td>
<td>0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-1 (pg.ml⁻¹)</td>
<td>0.02</td>
<td>0.07</td>
<td>0.31*</td>
</tr>
<tr>
<td>IL-6 (pg.ml⁻¹)</td>
<td>0.01</td>
<td>0.19</td>
<td>0.02</td>
</tr>
<tr>
<td>TNF-α (pg.ml⁻¹)</td>
<td>0.08</td>
<td>0.24</td>
<td>0.002</td>
</tr>
<tr>
<td>CRP</td>
<td>0.05</td>
<td>0.05</td>
<td>-</td>
</tr>
</tbody>
</table>

*  p<0.05  
** p<0.01  
*** p<0.001
Table 6.4  
Variables in the survivors and non-survivors (at six months), on day 1 post myocardial infarction.

<table>
<thead>
<tr>
<th>Non-survivors</th>
<th>Survivors</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=12</td>
<td>n=111</td>
<td>p</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol.l⁻¹)</td>
<td>10.8 (4.7)</td>
<td>8.7 (4.7)</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l⁻¹)</td>
<td>39.1 (5.1)</td>
<td>56.9 (1.9)</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l⁻¹)</td>
<td>11.1 (2.7)</td>
<td>7.9 (2.0)</td>
</tr>
<tr>
<td>*Des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>5.3 (2.5)</td>
<td>3.3 (2.9)</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l⁻¹)</td>
<td>2.2 (1.5)</td>
<td>1.8 (1.8)</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>6.4 (2.1)</td>
<td>5.5 (1.3)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>1.3 (0.4)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>*PAI-1 activity (AU.ml⁻¹)</td>
<td>35.3 (1.8)</td>
<td>20.2 (3.2)</td>
</tr>
<tr>
<td>*PAI-1 antigen (ng.ml⁻¹)</td>
<td>46.3 (1.4)</td>
<td>41.2 (1.5)</td>
</tr>
<tr>
<td>*Fibrinogen (pmol.l⁻¹)</td>
<td>163.0 (2.1)</td>
<td>190.9 (1.8)</td>
</tr>
<tr>
<td>*IL-1 (pg.ml⁻¹)</td>
<td>0.59 (1.8)</td>
<td>0.65 (2.1)</td>
</tr>
<tr>
<td>*IL-6 (pg.ml⁻¹)</td>
<td>12.8 (1.5)</td>
<td>11.8 (1.9)</td>
</tr>
<tr>
<td>*TNF-α (pg.ml⁻¹)</td>
<td>3.0 (1.6)</td>
<td>2.7 (2.0)</td>
</tr>
<tr>
<td>*CRP</td>
<td>3.7 (2.9)</td>
<td>2.4 (3.0)</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± (SD) and * Geometric means x/÷ (SD) for skewed data.
6.5.6 Diabetic versus non-diabetic subjects (Table 6.5).

At follow-up all subjects had an oral glucose tolerance test. 45 subjects (75%) had normal glucose tolerance, 7 subjects (12%) had impaired glucose tolerance (IGT), and 8 subjects (13%) had diabetes. Subjects with normal glucose tolerance had significantly lower levels of intact and split proinsulin compared to subjects with IGT or NIDDM. They also had lower levels of PAI-1 antigen and activity, but this did not reach statistical significance. There were no differences in levels of cytokines between the 2 groups.

6.5.7 PAI-1 promoter 4G/5G polymorphism (Tables 6.6 and 6.7)

In total 118 patients were genotyped. Subjects with the PAI-1 4G/4G polymorphism had higher levels of insulin and PAI-1 antigen six months post myocardial infarction compared to the other genotypes. Although similar trends were observed in levels of proinsulin-like molecules, PAI-1 activity and IL-1, with 4G/4G subjects having higher levels, this did not reach statistical significance. There were no significant differences in PAI-1 activity, antigen or cytokines between the three genotypes on day 1 post AMI.

6.6 Discussion

PAI-1 activity is elevated in AMI. In this study 123 patients were studied post myocardial infarction. PAI-1 levels were elevated post myocardial infarction, and by the time of discharge from hospital (day 5), PAI-1 activity had reached levels similar to that observed at six months, although PAI-1 antigen levels continued to decrease following discharge. Non-survivors at six months had higher levels of PAI-1 day 1 post AMI compared to survivors.

The mechanism behind the association between PAI-1 activity and myocardial infarction is not fully understood. One possibility is that elevated PAI-1 is a primary risk factor for myocardial infarction, but as yet there is no consensus on the prognostic value of PAI-1 (Juhan-Vague et al, 1993). The ECAT study (ECAT angina pectoris study, 1993) however showed that t-PA antigen, and PAI-1 antigen and activity predicted an increased incidence of myocardial infarction and sudden death in patients with angina.
Table 6.5
Variables in subjects with normal glucose tolerance and in subjects with impaired glucose tolerance and diabetes six months post myocardial infarction.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=43)</th>
<th>IGT + NIDDM (n=15)</th>
<th>t-test</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mmol.l⁻¹)</td>
<td>5.2 (0.6)</td>
<td>7.9 (3.0)</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l⁻¹)</td>
<td>48.7 (2.0)</td>
<td>66.4 (2.3)</td>
<td></td>
<td>0.24</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l⁻¹)</td>
<td>5.0 (1.8)</td>
<td>9.5 (2.6)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>*Des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>1.9 (2.5)</td>
<td>4.7 (3.6)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l⁻¹)</td>
<td>1.4 (1.5)</td>
<td>1.9 (1.7)</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>5.9 (1.2)</td>
<td>6.1 (1.2)</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>1.3 (0.3)</td>
<td>1.3 (0.4)</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td>*PAI-1 activity (AU.ml⁻¹)</td>
<td>8.3 (4.0)</td>
<td>12.9 (3.9)</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>*PAI-1 antigen (ng.ml⁻¹)</td>
<td>28.4 (1.5)</td>
<td>31.7 (1.4)</td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>*Fibrinogen (pmol.l⁻¹)</td>
<td>393.8 (1.3)</td>
<td>447.2 (1.2)</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>*IL-1 (pg.ml⁻¹)</td>
<td>0.35 (2.8)</td>
<td>0.43 (2.1)</td>
<td></td>
<td>0.44</td>
</tr>
<tr>
<td>*IL-6 (pg.ml⁻¹)</td>
<td>3.2 (2.3)</td>
<td>3.0 (2.3)</td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>*TNF-α (pg.ml⁻¹)</td>
<td>1.8 (1.8)</td>
<td>1.6 (2.5)</td>
<td></td>
<td>0.70</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± (SD) and * Geometric means ×/÷ (SD) for skewed data.
Table 6.6
Variables in the three PAI-1 genotypes day 1 post myocardial infarction (all patients, n=118).

<table>
<thead>
<tr>
<th></th>
<th>4G/4G n=22</th>
<th>4G/5G n=64</th>
<th>5G/5G n=32</th>
<th>ANOVA (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mmol.l⁻¹)</td>
<td>8.7 (3.0)</td>
<td>8.6 (3.8)</td>
<td>9.9 (6.0)</td>
<td>0.50</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l⁻¹)</td>
<td>62.8 (1.8)</td>
<td>52.3 (2.4)</td>
<td>52.6 (2.4)</td>
<td>0.64</td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l⁻¹)</td>
<td>9.1 (2.2)</td>
<td>8.3 (1.9)</td>
<td>7.4 (2.3)</td>
<td>0.58</td>
</tr>
<tr>
<td>*Des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>4.0 (3.1)</td>
<td>3.2 (2.7)</td>
<td>3.3 (3.0)</td>
<td>0.70</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l⁻¹)</td>
<td>2.2 (2.1)</td>
<td>1.7 (1.7)</td>
<td>1.8 (1.6)</td>
<td>0.25</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>6.2 (1.4)</td>
<td>5.3 (1.3)</td>
<td>5.9 (1.4)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>1.3 (0.3)</td>
<td>1.2 (0.4)</td>
<td>1.3 (0.4)</td>
<td>0.62</td>
</tr>
<tr>
<td>*PAI-1 activity (AU.ml⁻¹)</td>
<td>26.5 (2.0)</td>
<td>19.0 (3.5)</td>
<td>21.8 (3.1)</td>
<td>0.49</td>
</tr>
<tr>
<td>*PAI-1 antigen (ng.ml⁻¹)</td>
<td>44.3 (1.5)</td>
<td>39.9 (1.5)</td>
<td>43.3 (1.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>*Fibrinogen (pmol.l⁻¹)</td>
<td>150.7 (2.0)</td>
<td>209.8 (1.8)</td>
<td>173.1 (1.8)</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>*IL-1 (pg.ml⁻¹)</td>
<td>0.71 (1.8)</td>
<td>0.65 (2.3)</td>
<td>0.61 (1.9)</td>
<td>0.50</td>
</tr>
<tr>
<td>*IL-6 (pg.ml⁻¹)</td>
<td>14.1 (1.6)</td>
<td>11.8 (1.7)</td>
<td>12.4 (1.4)</td>
<td>0.39</td>
</tr>
<tr>
<td>*TNF-α (pg.ml⁻¹)</td>
<td>2.6 (2.0)</td>
<td>2.7 (2.2)</td>
<td>2.9 (1.7)</td>
<td>0.82</td>
</tr>
<tr>
<td>*CRP</td>
<td>2.0 (3.3)</td>
<td>2.9 (4.0)</td>
<td>2.3 (3.3)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± (SD) and * Geometric means ×/÷ (SD) for skewed data.
### Table 6.7
**Variables in the three PAI-1 genotypes 6 months post myocardial infarction (n=57).**

<table>
<thead>
<tr>
<th></th>
<th>4G/4G n=10</th>
<th>4G/5G n=34</th>
<th>5G/5G n=13</th>
<th>ANOVA (p*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mmol.l⁻¹)</td>
<td>6.6 (2.7)</td>
<td>6.2 (2.2)</td>
<td>5.2 (0.8)</td>
<td>0.45</td>
</tr>
<tr>
<td>*Specific insulin (pmol.l⁻¹)</td>
<td>71.3 (2.3)</td>
<td>56.3 (1.9)</td>
<td>44.0 (2.3)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>*Intact proinsulin (pmol.l⁻¹)</td>
<td>8.3 (3.0)</td>
<td>6.0 (2.2)</td>
<td>5.2 (1.9)</td>
<td>0.29</td>
</tr>
<tr>
<td>*Des 31,32 proinsulin (pmol.l⁻¹)</td>
<td>6.3 (3.7)</td>
<td>2.7 (2.7)</td>
<td>1.7 (2.8)</td>
<td>0.19</td>
</tr>
<tr>
<td>*Triglyceride (mmol.l⁻¹)</td>
<td>1.7 (1.7)</td>
<td>1.6 (1.5)</td>
<td>1.7 (1.5)</td>
<td>0.10</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>6.2 (1.4)</td>
<td>5.8 (1.1)</td>
<td>5.7 (1.3)</td>
<td>0.49</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l⁻¹)</td>
<td>1.2 (0.3)</td>
<td>1.3 (0.4)</td>
<td>1.2 (0.4)</td>
<td>0.40</td>
</tr>
<tr>
<td>*PAI-1 activity (AU.ml⁻¹)</td>
<td>18.3 (3.5)</td>
<td>7.6 (5.6)</td>
<td>9.5 (2.9)</td>
<td>0.25</td>
</tr>
<tr>
<td>*PAI-1 antigen (ng.ml⁻¹)</td>
<td>30.2 (1.5)</td>
<td>29.6 (1.5)</td>
<td>27.9 (1.3)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>*Fibrinogen (pmol.l⁻¹)</td>
<td>393.5 (1.4)</td>
<td>411.9 (1.3)</td>
<td>395.4 (1.3)</td>
<td>0.88</td>
</tr>
<tr>
<td>*IL-1 (pg.ml⁻¹)</td>
<td>0.60 (2.2)</td>
<td>0.34 (2.6)</td>
<td>0.33 (2.6)</td>
<td>0.33</td>
</tr>
<tr>
<td>*IL-6 (pg.ml⁻¹)</td>
<td>2.8 (1.6)</td>
<td>3.6 (2.3)</td>
<td>3.3 (2.6)</td>
<td>0.67</td>
</tr>
<tr>
<td>*TNF-α (pg.ml⁻¹)</td>
<td>2.1 (1.7)</td>
<td>1.9 (1.9)</td>
<td>1.7 (1.5)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Data are arithmetic means ± (SD) and * Geometric means ×/÷ (SD) for skewed data.
* ANOVA p values are corrected for age and BMI.
An alternative explanation is that metabolic or other factors associated with myocardial infarction or coronary heart disease cause elevated PAI-1 activity. The increase in PAI-1 activity in AMI is at least in part an inflammatory acute phase reaction. PAI-1 is an acute phase protein and increased plasma levels are found during acute phase responses such as AMI, after surgery, and in endotoxemia and septicaemia. There have been numerous in vitro studies examining the effect of cytokines on PAI-1 production in both endothelial and HepG2 cells (Emeis et al, 1986; van Hinsbergh et al, 1988; de Boer et al, 1991), but their role in vivo is unclear. In this study there were no correlations observed between the cytokines measured (IL-1, IL-6 and TNF-α) and PAI-1 activity or antigen immediately post AMI. At six months however PAI-1 activity correlated with IL-1 levels. The significance of this finding is not clear. There were no differences in cytokine levels between the survivors and non-survivors day 1 post AMI despite a difference in PAI-1 activity.

In the acute setting day 1 post myocardial infarction, proinsulin-like molecules and not cytokines, showed the strongest relationship with PAI-1 activity, highlighting the importance of these molecules in determining PAI-1 activity. The majority of patients in this study were non-diabetic, and hence had low levels of proinsulin-like molecules, despite this a significant relationship between PAI-1 activity and proinsulin-like molecules was shown. At six months, subjects with IGT or NIDDM had significantly higher levels of proinsulin-like molecules compared to controls, but the differences in PAI-1 activity and antigen did not reach statistical significance. In NIDDM subjects, elevated concentrations of proinsulin-like molecules are associated with deleterious changes in recognised cardiovascular risk factors (Nagi et al, 1990). Similar relationships have been shown in non-diabetic subjects (Haffner et al, 1993). It is possible that the link between proinsulin and cardiovascular disease may be reduced fibrinolytic activity due to elevated PAI-1 activity. Recently a significant relationship between insulin and proinsulin-like molecules and haemostatic factors including PAI-1, was shown in young non-diabetic men with and without coronary disease (Båvenholm et al, 1995). The relationships with PAI-1 were stronger in patients than in controls. This may be due to the interaction of proinsulin with other risk factors, such as abnormal lipoproteins, cytokines or endothelial damage,
that influences PAI-1 levels.

It has been suggested that proinsulin and like molecules act to increase PAI-1 expression via the insulin receptor. However the binding of proinsulin-like molecules to these receptors is ten fold lower than that of insulin (Peaky et al, 1985). *In vitro* work has shown that both intact and split proinsulin increase expression of PAI-1 mRNA in bovine aortic endothelial cells and that this effect was independent of insulin and not mediated via the insulin or insulin-like growth factor (IF) receptor (Schneider et al, 1992). Nordt et al (1995) showed that intravenous infusion of proinsulin in rabbits leads to an increase in plasma PAI-1 activity, and increased PAI-1 mRNA in aorta and liver. These *in vitro* and *in vivo* studies suggest that proinsulin augments PAI-1 expression independently of insulin. Recently a study has suggested the presence of a high affinity independent proinsulin receptor in human IM-9 lymphoblasts (Jehle et al, 1996).

In addition to environmental factors mentioned above, there is evidence suggesting that PAI-1 levels are related to sequence variations in the PAI-1 gene. Levels of PAI-1 have been found to relate to genotype at three polymorphisms of the PAI-1 gene (Dawson et al, 1991; Dawson et al, 1993). Interest has focused on the 4G/5G polymorphism due to its position in the important promoter region of the PAI-1 gene. In Chapter 5, higher levels of PAI-1 were found in subjects with the 4G/4G genotype, intermediate levels in 4G/5G subjects and lowest levels in subjects with the 5G/5G genotype. Support for the 4G/5G polymorphism having a functional role comes from *in vitro* studies. Dawson et al (1993), studied the PAI-1 4G/5G polymorphism in HepG2 cells. Cells with the 4G allele produced six times more PAI-1 mRNA than the 5G allele in response to IL-1. More recently analysis of nuclear protein binding at the promoter of the PAI-1 gene, has demonstrated binding of a transcription enhancing nuclear protein to the 4G and 5G allele and of a transcription inhibiting protein that competes for the same binding area but only on the 5G allele (Eriksson et al, 1995). In this way the 4G/5G polymorphism may have a functional role in determining basal PAI-1 production.
The aim of this study was to test the above observations *in vivo*, using AMI as the acute phase stimulant. The hypothesis tested *in vivo*, was that subjects homozygous for the 4G allele have an exaggerated response to acute phase stimuli compared to 4G/5G and 5G/5G subjects. This was not the case. Their were no differences observed in the levels of PAI-1 activity, antigen or cytokines between the three PAI-1 genotypes, during the course of AMI. At six months follow-up however subjects with the PAI-1 4G/4G polymorphism, had significantly higher levels of PAI-1 antigen. 4G/4G subjects, also had higher levels of PAI-1 activity, IL-1, and insulin-like molecules, but due to the small number of subjects in each group, this did not reach statistical significance. There were no differences in the frequency of PAI-1 genotype in survivors versus non-survivors at six months, but the small numbers prevent any conclusions being drawn. The relatively small size of this study indicates the requirement for larger studies to confirm these findings and investigate the role of PAI-1 genotype in relation to cytokines further. More accurate methods for the determination of cytokines levels may be needed, such as determining monocyte cytokine levels rather than plasma levels.

In conclusion, high PAI-1 activity day one post AMI was a significant predictor of mortality at six months. This is an important finding with therapeutic implications. Patients with elevated PAI-1 activity on admission, could be targeted for aggressive therapy with early intervention to improve prognosis. In theory such patients could be treated with higher dose thrombolytic therapy, to counteract their high initial PAI-1 levels. In practise this is hampered by the lack of fast assays available for PAI-1 which can be employed in clinical practice. Subjects with the PAI-1 4G/4G polymorphism could be targeted for more aggressive follow-up, to lower their PAI-1 levels, and in theory reduce their risk of re-infarction. This study needs to be repeated in larger number of patients, associating levels of PAI-1, cytokines and PAI-1 genotype with extent of coronary artery disease ideally as measured by angiography.
CHAPTER 7
CONCLUSION

This work has explored the role of PAI-1 as a risk factor for cardiovascular disease. Clinical manifestations of coronary heart disease result principally from the progressive development of atherosclerotic plaques and subsequent thrombus formation. Fibrin deposition results not only in acute occlusion of coronary arteries, but also incorporates into the vessel wall, playing a major role in the progression of atherosclerotic lesions. Impairment of the fibrinolytic system mainly due to elevated levels of PAI-1 increases fibrin deposition, and is a prime candidate for the development of athero-thrombosis. Subjects with NIDDM suffer from premature accelerated atherosclerosis. Understanding the underlying mechanisms responsible for the accelerated disease in these subjects is of vital importance in devising prevention strategies.

In Chapter 3, the determinants of PAI-1 activity were explored in a cross-sectional fashion in NIDDM subjects attending the diabetic clinic. An attempt was made to dissect out the roles of insulin resistance variables, diabetic therapy and ethnic group, in determining PAI-1 levels. PAI-1 activity is associated with components of the insulin resistance syndrome, and dissecting out the contribution of individual components of the syndrome in determining PAI-1 is hampered by the close relationship that exists between them. In this study however, serum triglyceride levels were one of the strongest correlates of fasting PAI-1 activity. This study also revealed that NIDDM subjects on sulphonylurea therapy had higher levels of intact proinsulin than subjects treated with insulin. This did not however result in higher PAI-1 activity in sulphonylurea patients due to the limited power of a cross-sectional study. Previous in vitro and in vivo studies however, point to the importance of proinsulin as an important determinant of PAI-1 activity, and this theme was further explored in Chapter 4. Asian subjects in this study had highest, and Afro-Caribbean subjects the lowest levels of proinsulin-like molecules and triglyceride compared to Caucasian subjects. A higher level of PAI-1 activity would have been predicted, but again the power of the study was limited by its cross-sectional nature. This study need confirmation with larger number of patients in each ethnic group, the finding
may help to explain the very high incidence of coronary heart disease in Asian subjects with NIDDM.

In order to pursue the finding of higher levels of proinsulin observed in NIDDM subjects treated with sulphonylureas observed in Chapter 3, a crossover study was performed. The results confirmed results from previous studies, and showed that NIDDM subjects treated with sulphonylureas had higher levels of proinsulin and PAI-1 antigen compared to insulin treated NIDDM subjects. As the increased risk of cardiovascular disease in NIDDM appears to be independent of duration of diabetes and glycaemic control, it is important that hypoglycaemic therapy in the long term improves and not worsens this risk. Are NIDDM subjects treated with sulphonylureas at greater risk of atherosclerosis and thrombosis compared to subjects treated with insulin or metformin? Recently the DIGAMI (Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction) study has renewed interest in this question. The DIGAMI study demonstrated that insulin-glucose infusions, followed by multidose insulin therapy, reduced mortality in NIDDM patients with acute myocardial infarction by a factor of a third, this effect persisting for 3.5 years. Was this reduction in mortality due to a lower PAI-1 activity in insulin treated patients? Unfortunately PAI-1 levels were not measured. The results of the UKPDS are anxiously awaited. This study will ultimately answer the question of the effects of long term therapy with sulphonylurea and insulin on the incidence of cardiovascular events.

Chapter 5 explored the importance of PAI-1 gene environment interaction in determining PAI-1 levels. The importance of a common polymorphism in the promoter of the PAI-1 gene was shown. NIDDM subjects with the PAI-1 4G/4G genotype, had higher levels of fasting PAI-1 activity. This finding confirmed similar findings in non-diabetic subjects with coronary heart disease. An interesting and novel finding was the interaction of the PAI-1 4G/5G polymorphism with serum triglyceride levels. In subjects with the 4G/4G polymorphism, serum triglyceride levels showed the strongest correlation with PAI-1 activity. This finding may have important therapeutic implications: it may be possible to target individuals with unfavorable genotypes for early interventional lipid-lowering
therapy to reduce their overall risk of atherogenesis and thrombosis- an exciting prospect.

In Chapter 6, gene-environment interactions were further explored in the setting of acute myocardial infarction. High PAI-1 activity on day 1 post myocardial infarction was an important predictor of mortality 6 months post myocardial infarction. Subjects with the PAI-1 4G/5G polymorphism, had higher PAI-1 levels 6 months post myocardial infarction compared to the other PAI-1 genotypes. With development of more efficient, faster PAI-1 assays for use in clinical practise, and more rapid methods of DNA screening for polymorphisms, one could postulate that subjects admitted with high PAI-1 levels and adverse genotype could be selected for more aggressive intervention. This intervention may be in the form of early angioplasty or coronary artery grafting, or aggressive treatment with lipid lowering agents, to prevent the rapid acceleration of atherothrombosis in these individuals- an exciting prospect for the future.
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PUBLICATIONS ARISING FROM WORK IN THIS THESIS

PAPERS:


ABSTRACTS

1. Panahloo A, Mohamed-Ali V, Yudkin JS.

2. Panahloo A, Mohamed-Ali V, Yudkin JS.
   The relationship between serum triglyceride, proinsulin-like molecules and plasminogen activator inhibitor-1 in non-insulin-dependent diabetes. *Diabetic
Polymorphism in the plasminogen activator inhibitor-1 (PAI-1) gene as a strong

Proinsulin-like molecules relate to insulin resistance in non-insulin-dependent

Effect of sulphonylurea vs. insulin on fibrinolysis in type 2 diabetes: a crossover

Sulphonylurea therapy stimulates basal and post glucose load levels of proinsulin.

The effect of hypoglycaemic therapy in subjects with non-insulin dependent

Proinsulin-like molecules -possible determinants of fibrinolytic activity. *J


**ORAL PRESENTATIONS**

1. Panahloo A, Mohamed-Ali V and Yudkin JS.

Plasminogen activator inhibitor-1 is strongly associated with insulin resistance in NIDDM subjects.

Plenary oral presentation BDA Southampton, Sept 1993


Proinsulin-like molecules relate to insulin resistance in non-insulin-dependent diabetes.

Oral presentation BDA, Lancaster, Sept 1994