

Cardiovascular risk factors in Asian and Caucasian subjects with and without Type 2 diabetes: studies using new highly specific assays for insulin, intact proinsulin and des 31,32^{split} proinsulin.

by

Dinesh K. Nagi, MBBS, MRCP (UK)

of

Department of Medicine,
University College London Medical School,
Whittington Hospital, G Block, Archway Wing,
Archway Road, London, UK.

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ANITA, SUNIL, YASMIN, SANJAY,
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CHAPTER 1: ABSTRACT

Hyperinsulinaemia is implicated in the pathogenesis of coronary artery disease and is related to a number of risk factors for coronary artery disease. However, the biological significance of the role of hyperinsulinaemia and insulin resistance in the pathogenesis of coronary artery disease remains controversial and far from established. It has been speculated for a long time that proinsulin-like molecules may cross-react in radioimmunoassays for insulin though this has not been demonstrated.

In this dissertation an effort has been made to analyse the relationships of cardiovascular risk factors, blood pressure, dyslipidaemia and of plasminogen activator inhibitor (PAI-1) activity, with concentrations of insulin, intact proinsulin and des 31,32 proinsulin. Insulin, proinsulin and des 31,32 proinsulin were assayed employing highly specific monoclonal antibody-based assays. In a preliminary study sera from 51 subjects with type 2 diabetes (non-insulin-dependent) mellitus was analysed to measure insulin concentrations by a conventional radioimmunoassay using a polyclonal antibody and also by a highly specific monoclonal-antibody based assay. This study revealed that proinsulin-like molecules cross-react in conventional radioimmunoassay for insulin with a potency very similar to that of insulin. In this study, cardiovascular risk factors also correlated with concentrations of immunoreactive insulin, but some of these relationships became weaker or insignificant when insulin concentrations measured by a highly specific assay was employed to study these relationships. Both intact proinsulin and des 31,32 proinsulin concentrations were related to a number of cardiovascular risk factors including PAI-1 activity. These observations raised the possibility that proinsulin-like molecules may have role in the pathogenesis of macrovascular disease in type 2 diabetic subjects. Asian subjects with type 2 diabetes had higher concentrations of des 31,32 proinsulin. Based on these preliminary results,

further studies were performed to reanalyse these observed relationship, with inclusion of non-diabetic and diabetic subjects from two different ethnic groups (Asians and caucasian). Subsequently intervention studies using metformin (Diethyl biguanide) and insulin treatment in subjects with type 2 diabetes were performed to study the effect of these treatments on insulin resistance, insulin and proinsulin-like molecules, plasminogen activator inhibitor and on risk factors for cardiovascular disease.

These studies revealed that non-diabetic Asian subjects had a preferential central distribution of fat, lower levels of physical activity and higher prevalence of ischaemic heart disease than age- and sex-matched caucasian subjects. Asian subjects showed significant 2-hr post glucose load hyperinsulinaemia compared to caucasian non-diabetic subjects. Concentrations of intact proinsulin but not of des 31,32 split proinsulin were also significantly higher in Asian non-diabetic subjects than caucasians, however the ratio of proinsulin-like molecules to insulin was similar in both ethnic groups. There was no ethnic differences in PAI-1 activity or other cardiovascular risk factors among diabetic and non-diabetic subjects from two ethnic groups. Significant and consistent relationships were observed between the concentrations of proinsulin like-molecules and levels of certain risk factors for cardiovascular disease in all the studies. The relationships of intact proinsulin and des 31,32 proinsulin with risk factors for cardiovascular disease were either similar to or stronger in magnitude than insulin concentrations measured by a specific assay. These relationships were highly significant in subjects with type 2 diabetes. No ethnic differences were noted in the relationship of insulin and proinsulin-like molecules to cardiovascular risk factors. There was no significant association of insulin or proinsulin-like molecules to electrocardiographic evidence of coronary artery disease, however the number of subjects with coronary artery disease was small.

Using a specific assay for insulin, subjects with type 2 diabetes who are treated with diet alone, had significantly higher fasting insulin concentrations than non-diabetic subjects. The relationship of fasting insulin concentrations with fasting and 2-hr plasma glucose however was inverted U shaped, suggesting a declining β -cell function as hyperglycaemia worsens. Subjects with diabetes had significantly lower 30-min post load insulin concentrations compared to non-diabetic subjects suggesting a defective beta cell response in subjects who have type 2 diabetes. We have confirmed that concentrations of proinsulin and des 31,32 proinsulin are elevated in diet treated subjects with type 2 diabetes, though the proportion of these molecules varied in different studies. Likely explanations for these results are the heterogeneity of diabetes and different population of subjects included in these studies.

Intervention studies demonstrated that conventional treatment modalities such as insulin or metformin treatment in type 2 diabetic subjects is associated with a reduction in the concentrations of proinsulin-like molecules. In both intervention studies the reduction in proinsulin-like molecules was significantly related to each other suggesting a close relationship in the mechanisms which control their processing and secretion. Metformin treatment was associated with improvements in insulin sensitivity, and reduction in the concentrations of proinsulin-like molecules, cardiovascular risk factors and of PAI-1 activity, but there were no significant associations between reductions in concentrations of proinsulin-like molecules and those of cardiovascular risk factors. However during insulin treatment, reductions in PAI-1 activity was significantly associated with changes in concentrations proinsulin-like molecules. The associations observed between proinsulin-like molecules and cardiovascular risk factors especially PAI-1 activity in these studies raise the possibility of a role of proinsulin-like molecules in the pathogenesis of cardiovascular disease particularly in subjects with type 2 diabetes and warrants further studies.

CHAPTER 2: INTRODUCTION AND REVIEW OF LITERATURE

2.1 Coronary artery disease

Coronary artery disease is one of the leading causes of morbidity and mortality in industrialised nations. A number of studies have indicated both type 1 and type 2 diabetes to be a risk factors for coronary artery disease. Although accelerated atherosclerosis is one of the main causes of excessive morbidity and mortality from macrovascular disease in subjects with diabetes, other pathogenetic mechanisms may also play an important role.

2.2 Diabetes and coronary artery disease

2.2.1 Post-mortem and angiographic studies:

Patients with diabetes suffer from a generalised and premature atherosclerosis. This was first evident from the findings of large autopsy studies which confirmed a three to four fold excess of coronary atherosclerosis among subjects with diabetes than non-diabetic subjects. Data from the International Atherosclerosis Project of 23000 coronary artery autopsies showed that diabetes was associated with increased atherosclerotic lesions in both men and women (Robertson et al, 1968). Studies also confirmed that subjects with diabetes had more severe atherosclerosis in the coronary arteries (Robertson et al, 1968; Vigorita et al, 1980). Coronary angiographic studies found that subjects with diabetes and impaired glucose tolerance (IGT) had more severe atherosclerosis of coronary arteries (Hamby et al, 1976; Dortimer et al, 1978), while others found no difference in the severity and extent of atherosclerotic lesions, although subjects with diabetes tended to have more severe symptoms (Verska et al, 1975; Waller et al, 1980).

2.2.2 Population studies of mortality and morbidity

Studies from Boston, Massachusetts showed that after the advent of insulin, cardiovascular and renal disease were the leading causes of mortality (Entmacher et al, 1964; Marks et al, 1971; Kessler, 1971). When data were analysed by the age of onset of diabetes, in subjects with age at onset of more than 20 years, macrovascular disease was the predominant cause of mortality. The proportion of deaths from macrovascular disease was unrelated to duration of diabetes in subjects in whom onset of disease occurred after 40 years of age (Kessler, 1971).

In Framingham, where subjects were followed for a period of sixteen years, it was apparent that subjects with non-insulin dependent diabetes in general show excess mortality from all causes especially coronary artery disease and the risk was 1.7 times in males and 3.3 times in females compared to non-diabetic population (Garcia et al, 1974; Kannel et al, 1979). Macrovascular disease in general account for upto 50-75 % of all deaths in subjects with type 2 diabetes (Panzram, 1987).

Studies demonstrated an excess of electrocardiographic abnormalities (Reunanen et al, 1979; Ducimetiere et al, 1979) and symptoms of arterial disease in subjects with newly discovered type 2 diabetes (Reunanen et al, 1979; Sakuma et al 1976, Stamler et al, 1979a). In a follow-up study of 3822 hospitalised subjects with diabetes in Oslo, Norway, coronary artery disease was the commonest cause of death, and the mortality ratio of subjects with diabetes, below 70 years of age, was 4.3 for men and 8.6 for women compared to a non-diabetic population (Westlund et al, 1969). However these differences were less marked in subjects aged 70 years or more. A population based survey showed that coronary heart disease mortality (CHD) was 2.8 times higher in subjects with diabetes (Pell and D'Alonzo, 1970). Similarly studies from Bedford showed that subjects with non-insulin dependent diabetes and impaired glucose tolerance showed increased mortality compared with non-diabetic subjects (Jarrett et

al, 1982). This study also showed that mortality was similar between men and women with diabetes, whereas in non-diabetic cohorts men had excess mortality from CHD. The Whitehall survey showed that increased risk of coronary artery disease was similar between subjects with known diabetes and those with newly diagnosed diabetes (Fuller et al, 1983), indicating that the risk of heart disease perhaps antedates the onset of clinical diabetes. Among those with type 2 diabetes there was no relationship with known duration of clinically diagnosed diabetes and CHD mortality. There was also a linear relationship between cardiovascular mortality and 2-hr plasma glucose concentrations among male subjects without diabetes (Jarrett et al, 1982).

Even the earliest evidence of impaired glucose tolerance is associated with increased risk and mortality from coronary artery disease, a phenomenon observed in the Whitehall study in the United Kingdom (Fuller et al, 1983) and also in a study from Chicago (Stamler et al, 1979 b). It is well established that individuals with impaired glucose tolerance from various populations have elevated levels of risk factors for cardiovascular disease (CVD) (Keen et al, 1965; Ostrander et al, 1965) and this risk translates into CVD end points in longitudinal studies (Fuller et al, 1983; Jarrett et al, 1982; Stern et al; 1985). In the Paris Prospective Study, in subjects with previously known diabetes, CHD mortality was three times higher than in non-diabetic subjects while subjects with newly diagnosed diabetes and those with impaired glucose tolerance had 2.1 and 1.9 times excess mortality than non-diabetic subjects respectively (Eshcwege et al, 1985). It is noticeable that in this study in subjects with impaired glucose tolerance mortality rates were similar to subjects with newly diagnosed diabetes. Subjects with impaired glucose tolerance showed increased prevalence of CVD risk factors and hyperinsulinaemia.

A number of studies have also compared the prevalence of coronary artery disease,

using standardised methods such as Rose questionnaire for angina pectoris and Minnesota coding for electrocardiographic abnormalities, in subjects with diabetes and those without. In Paris Prospective Survey the prevalence of ECG abnormalities was 2.6 times higher in diabetics than non-diabetics. In a Finnish study the prevalence of CHD symptoms was nearly three times as high as in subjects with diabetes (Pyörälä et al, 1987).

The real evidence of increased coronary artery disease can be assessed only if an increase in the number of new cases of coronary artery disease can be shown in subjects with diabetes during prospective studies. In Framingham, during a period of 18 years, the incidence of all new cases of CHD was 1.7 times higher in males and 3.3 times higher in females (Garcia et al, 1974; Kannel et al, 1979). The results were similar for angina pectoris and for sudden death, the two other common manifestations of CHD.

Asian immigrant populations have been shown to have excess mortality from coronary artery disease compared to the caucasian population in the UK (Tunstal Pedoe et al, 1975; Marmot et al, 1985; McKeigue et al, 1989; Balarajan et al, 1984). Population surveys amongst Asian immigrants from other areas of the world have also shown similar trends (Danaraj et al, 1959; Wyndham et al, 1982; Sorokin et al, 1975). The underlying cause for this excess morbidity and mortality is not explained on the basis of conventional risk factors such as smoking, blood pressure, and serum cholesterol (McKeigue et al, 1985; Beckles et al, 1986; Miller et al, 1988). Asian subjects in the U.K also suffer from an excess of diabetes but even taking this higher prevalence of diabetes into account a large proportion of the cardiovascular mortality remains unexplained (McKeigue et al, 1989, Beckles et al. 1986).

Population surveys have examined whether high rates of diabetes and the excess mortality from coronary artery disease in Asian subjects in the UK are related to syndrome of insulin resistance and its associated metabolic sequelae, a concept popularised by Reaven (Reaven, 1988). These studies showed a strong tendency towards a central distribution of obesity in Asian subjects which was associated with higher fasting and post glucose load insulin concentrations, higher total triglyceride and low HDL-cholesterol and higher blood pressure than caucasian subjects (McKeigue et al, 1988; McKeigue et al, 1991; McKeigue et al, 1992). However little is known about the cause of central obesity and the mechanisms by which central obesity can be related to coronary artery disease in this population.

2.3 Coronary artery disease and cardiovascular risk factors in subjects with diabetes

The pathology of atherosclerotic vascular disease in diabetic subjects is generally believed to be similar to that seen in subjects without diabetes, though a minority seem to continue to subscribe to the view that there may be a specific diabetic macrovascular disease affecting heart muscle (Regan et al, 1978; Ledet et al, 1979; Fein et al 1985). Studies have demonstrated that the relationship of known risk factors to subsequent development of coronary artery disease is similar in diabetic and non-diabetic population (Kannel et al, 1979). There have been number of studies examining the relationship of known risk factors for CHD in non-diabetic subjects, and the effects of diabetes on such risk factors. In subjects with type 2 diabetes, conventional risk factors such as smoking, obesity, physical activity, blood pressure, serum lipids, fail to explain the excess of coronary artery disease (Garcia et al, 1974; Fuller et al, 1983; Reunanen et al, 1979). Therefore, there may be other as yet unknown risk factors which directly or indirectly may explain the excess of cardiovascular disease in a diabetic population or the interaction between various risk factors for coronary artery disease may be different for subjects with diabetes (Pöyrälä

et al, 1987).

There is also a graded increase in levels of certain cardiovascular risk factors with increasing glucose intolerance even at levels below that defined as IGT. In Mauritian Asian Indians, fasting plasma triglyceride, fasting and 2-hr plasma glucose, fasting serum insulin, and systolic and diastolic blood pressure rise linearly and significantly for both sexes with increasing concentrations of 2-hr plasma glucose (Dowse et al, 1991). A similar pattern has previously been shown in whites in Rancho Bernado (Barrett-Connor et al, 1984).

However some cast doubt on the causal relationship of type 2 diabetes with excess coronary artery disease and suggest that the occurrence of the two together is coincidental with diabetes developing in subjects already at high risk of coronary artery disease (Jarrett, 1984). This view is supported by studies showing a congregation of risk factors for coronary artery disease in subjects destined to develop type 2 diabetes and coronary artery disease later in life (Haffner et al, 1990; DeFronzo et al, 1991).

2.3.1 Plasma triglyceride

The role of triglyceride as an independent risk factors for coronary artery disease has been a matter of considerable controversy over the last decade (Hulley et al, 1980; Carlson et al, 1981; Lippel et al, 1981; Castelli, 1986). A strong association between plasma triglyceride concentrations and ECG abnormalities was observed in the WHO Multinational Study of Vascular Disease in diabetics, (West et al, 1983) an association which persisted after controlling for the effect of age, sex, cholesterol, blood pressure and relative body weight. However, in most prospective studies which showed a relationship between plasma triglyceride and coronary artery disease in a univariate analysis, such associations were no longer significant in a multivariate analysis

including HDL-cholesterol and LDL-cholesterol. However, it is difficult to interpret such a multivariate analysis because of a close relationship between plasma triglyceride and HDL-cholesterol. Therefore one can not rule out the possibility of a role of triglyceride in the pathogenesis of coronary artery disease.

The concentrations of plasma triglyceride and VLDL are elevated in subjects with IGT (Capaldo et al, 1983), type 2 diabetes at the time of diagnosis and in those with long duration of diabetes (Uusitupa et al, 1988; Barrett-connor et al, 1982; Laakso et al, 1985). These raised levels of triglyceride seen in subjects with type 2 diabetes and IGT are not due to concomitant obesity often seen in these subjects (Uusitupa et al, 1988; Barrett-Connor et al, 1982; Laakso et al, 1985). The elevated plasma levels are usually not normalised even after adequate control of hyperglycaemia, and qualitative abnormalities still persist (Bagdade et al, 1990). Elevated triglyceride levels are a results of increased hepatic production of VLDL and perhaps also due to impaired clearance of VLDL due to decreased activity of lipoprotein lipase activity (Nikkila et al, 1981). Women with type 2 diabetes show a greater increase in plasma triglyceride concentrations than male diabetic subjects (Uusitupa et al, 1988; Walden et al, 1984; Gordon et al, 1977). Therefore, in subjects with type 2 diabetes and IGT, the disturbed triglyceride metabolism may make it an even more important risk factor for IHD. Indeed the finding of Paris Prospective Study showed an independent association between plasma triglyceride and coronary artery disease in subjects with diabetes and impaired glucose tolerance (Fontbonne et al, 1989).

2.3.2 Serum cholesterol and LDL-cholesterol:

Cross sectional, prospective and intervention studies have clearly established total and LDL-cholesterol as a risk factors for coronary artery disease in non-diabetic subjects (Lipid Research Clinic Program, 1975, 1984; Castelli et al, 1986; Anderson et al, 1987;

Carlson et al, 1988; Menninen et al, 1988; Brown et al, 1990). The finding of the Framingham Study showed that the effects of total cholesterol on development of coronary artery disease were similar in subjects with and without diabetes (Kannel et al, 1979). The predictive value of serum cholesterol on CHD death was found to be similar in subjects with and without diabetes, both in the Whitehall and the Finnish Social Insurance Study population (Jarrett et al, 1985; Reunanen et al, 1979).

The results of studies of plasma cholesterol levels in subjects with type 2 diabetes have been variable, some reporting elevated (Herman et al, 1977; Mattock et al, 1979; Taylor et al, 1981; Taskinen et al, 1982) and others normal concentrations (Barrett-Connor et al, 1982; Gordon et al, 1977; Durrington et al, 1980; Simpson et al, 1979; Laakso et al, 1985; Uusitupa et al, 1986) compared to non-diabetic subjects of similar age and sex. Similar results are also published from subjects with IGT, where some studies have found elevated (Yano et al, 1982; Barrett-Connor et al, 1984) while others normal (Capaldo et al, 1983) levels of LDL-cholesterol.

Recently, multiple subclasses of LDL have been recognised (Shen et al, 1981). These qualitative abnormalities of LDL particle have been shown to be associated with CHD (Austin et al, 1988; Musliner et al, 1988; Krauss et al; 1987). It has been shown that high triglyceride levels are associated with increased LDL particle density in subjects with coronary artery disease undergoing coronary angiography, compared with controls (Crouse et al, 1985). Perhaps most significantly, in non-obese normal male subjects, increased WHR is associated with increased LDL particle density, independently of body mass index (Harris Peebles et al, 1989; Terry et al, 1989). In obese women, LDL particle size was found to correlate negatively with fasting insulin levels (Barakat et al, 1990). Similarly LDL size and density but not total cholesterol are related to hyperinsulinaemia and insulin resistance (Reaven et al, 1993, Sheu et al, 1993). In

subjects with IGT and type 2 diabetes, LDL particle size was smaller than in the normal glucose tolerant subjects (Barakat et al, 1990). Also in subjects with type 2 diabetes, both poor glycaemic control and hypertriglyceridaemia are associated with increased levels of atherogenic dense LDL particles, compared to normal controls, even when there is no difference between the groups in terms of total or LDL cholesterol (James et al, 1991). It is therefore, possible that these qualitative abnormalities can have a deleterious effect on coronary artery disease in subjects with type 2 diabetes and IGT who are insulin resistant.

2.3.3 HDL-cholesterol

Studies in normal subjects have shown HDL-cholesterol to be a strong independent risk factor for CHD (Miller et al, 1975, Gordon et al, 1977). The effect of HDL-cholesterol on CHD seem to be similar in subjects with and without diabetes as was observed in Framingham (Gordon et al, 1977). Most studies of the relationship of HDL-cholesterol with CHD in subjects with diabetes have been of a cross-sectional nature. Welborn and Laakso have shown an inverse association between HDL-cholesterol and CHD in subjects with type 2 diabetes (Welborn et al, 1984; Laakso et al, 1985). It is now well established that subjects with type 2 diabetes particularly those with poor glycaemic control have low HDL-cholesterol concentrations than subjects of similar age and sex (Nikkila, 1984; Laakso et al, 1985; Barrett-Connor et al, 1982). Similar results of low HDL-cholesterol were reported in subjects with IGT (Ganda et al, 1985), while one study found no difference in HDL-cholesterol concentrations between subjects with and without IGT (Capaldo et al, 1983).

2.3.4 Blood pressure

Both systolic and diastolic blood pressure are risk factors for coronary artery disease in non-diabetic populations, and findings of the DuPont County Study after 10 year and

the Framingham study after 20 years follow-up showed that the impact of hypertension on CHD morbidity and mortality was similar in subjects with and without diabetes (Pell et al, 1970; Kannel et al, 1979, MRFIT study, 1982). The results from the Finnish Social Servants Study also showed that after at least six years of follow-up of subjects aged 40-60 years, hypertension was a risk factor for coronary artery disease in both diabetic and non-diabetic subjects (Aromaa et al, 1984).

The prevalence of hypertension is higher in subjects with than non-diabetic subjects (Ostrander et al, 1965; Pell et al, 1967; Garcia et al, 1974; Ingelfelter et al, 1976; Dupree et al, 1980; Modan et al, 1985) and this high prevalence is unexplained by concomitant obesity (Uusitupa et al, 1985; Pell et al, 1967; Jarrett et al, 1982; Modan et al, 1985) or renal disease (Drury et al, 1993). A number of possible mechanisms have been suggested for the link between hypertension and type 2 diabetes hyperinsulinaemia and insulin resistance being one of these (Modan et al. 1985, Zavaroni et al, 1989). Similarly studies have also shown that subjects with IGT have an increased prevalence of hypertension (Jarrett et al, 1982; Yano et al, 1982; Barrett-Connor et al, 1984; Cederholm et al, 1985; Modan et al, 1985).

2.3.5 Haemostatic and fibrinolytic factors:

There is increasing evidence to implicate that abnormalities of coagulation, haemostatic and fibrinolysis are associated with occurrence of coronary artery disease. Subjects with coronary artery disease show attenuation of fibrinolysis (Chakrabarti et al, 1968). Preliminary results of a prospective study from Northwick park showed that factor VII and factor VIII were predictors of CHD mortality (Meade et al, 1980). Subjects in this study, who died of coronary artery disease also showed depressed fibrinolysis, an association which was lost on longer follow-up. More recently, increased levels of t-PA inhibition has been shown to predict recurrence of acute myocardial infarction

(Hamsten et al, 1985, 1987). Subjects with type 2 diabetes have been shown to have reduced fibrinolysis compared to non-diabetic subjects (Fearnley et al, 1963) and abnormalities of coagulation (Bern et al, 1980; Christie et al, 1984). A recent large cross-sectional study found that depressed fibrinolysis is associated with CHD, diabetes, higher triglyceride, smoking and impaired cardiac pump function (ECAT Angina Pectoris Study Group, 1993). Fibrinolysis is markedly depressed in subjects with type 2 diabetes and is due to an increase in PAI-1 activity (Auwerx et al, 1988; Wamsley et al, 1991).

Among non-diabetic subjects, higher fibrinogen is associated with coronary artery disease (Nicolaidis et al, 1977; Wilhelmsen et al, 1984; Stone & Thorp, 1985; Yarnell et al, 1991). Prospective studies from Goteborg, Framingham and Leigh showed an association of raised fibrinogen with coronary artery disease (Wilhelmson et al, 1984; Kannel, 1987; Stone & Thorp, 1985). Studies in middle aged subjects with diabetes showed that high plasma fibrinogen at baseline was associated with increased subsequent coronary artery disease and CHD mortality (Schmechel et al, 1984).

2.4 Hyperinsulinaemia and its relationship with coronary artery disease

2.4.1 Non-diabetic subjects

The first suggestions that abnormal insulin/glucose homeostasis may be encountered, not only in subjects with glucose intolerance, but also in subjects with normal glucose tolerance, and that this is somehow associated with AVD, were made nearly 30 years ago. By the early 1960's, the association between IGT and coronary artery disease (CAD), hypertension, and peripheral vascular disease, was widely documented (Ostrander et al, 1965). Nikkila et al. found that 55% of non-obese survivors of myocardial infarction had a hyperinsulinaemic response to an oral glucose tolerance

test, and 10% a deficient response (Nikkila et al, 1965). This contrasted with fasting insulin levels, which did not differ from a control group. IGT was also found in 29% of these subjects- a finding supported by many other studies from this period. These authors also noted the correlation between obesity and plasma insulin concentrations- a subject to which we shall return in detail. At the same time, Peters and Hales demonstrated both fasting and stimulated hyperinsulinaemia in 7 subjects 6-17 months after myocardial infarction, compared to 7 normal controls (Peters and Hales, 1965). A relationship between IGT and the prevalence of CAD was also found in the general population in a sample of subjects from the Bedford study (Keen et al, 1965). In subjects with essential hypertension and peripheral vascular disease, hyperinsulinaemia also has been found in the presence of normal glucose tolerance (Welborn et al, 1963). Therefore, by the mid 1960's it was clear that insulin resistance, hyperinsulinaemia, and IGT were all associated with atherosclerosis, and this soon led to the explicit hypothesis that insulin might play a primary role in atherogenesis (Stout et al, 1969). In his 1988 Banting lecture, Reaven suggested a primary role for insulin resistance per se, in the pathogenesis of dyslipidaemia and hypertension in subjects with type 2 diabetes, and in non-diabetic subjects, and therefore in the pathogenesis of AVD in the general population (Reaven et al, 1988). This hypothesis is attractive because it explains the commonly observed clustering of risk factors, and has been influential in setting the agenda for much research. According to Reaven, insulin resistance, hyperinsulinaemia, IGT, elevated VLDL triglyceride, decreased HDL cholesterol, and hypertension, were all to be viewed as a syndrome with insulin resistance as the antecedent. Since the original hypothesis by Stout in 1969, that insulin may be linked to atherogenesis (Stout and Vallance-Owen, 1969; Stout, 1987) several populations based and experimental studies have tried to explore this possibility.

Three population studies in non-diabetic subjects have demonstrated a relationship

between hyperinsulinaemia and coronary artery disease. In the Helsinki Policemen Study, 5 year follow up data showed that both fatal and non-fatal myocardial infarction were commoner in those who had highest fasting, 1-hr, 2-hr and total insulin response to oral glucose load. Multivariate analysis showed that 1-hr and 2-hr post glucose insulin levels were independent predictors of coronary artery disease (Pyörälä et al, 1979). In the Paris Prospective Study there was an association between 2-hr insulin concentrations following oral glucose load and coronary artery disease but in a multivariate analysis only fasting insulin was associated with coronary artery disease (Ducimetière et al, 1980, Eschwège et al, 1985). In a study in Busseleton total cardiac deaths in men of 60-69 years, but not in women, were related significantly to fasting plasma insulin concentrations (Welborn et al, 1979), and on multivariate analysis the association of post load insulin and CHD mortality was lost.

In summary these three population surveys provide evidence of an association between hyperinsulinaemia and ischaemic heart disease though results of these studies differ from each other in details. Secondly, HDL-cholesterol concentrations, a strong predictor of coronary artery disease were not measured in these studies. In a recent study from Pima Indians, a population predisposed to high rates of type 2 diabetes, endogenous insulin was not associated with ECG abnormalities of resting electrocardiographs in a cross-sectional and prospective analyses (Liu et al, 1993). However despite high prevalence rates of diabetes, the prevalence of CHD in non-diabetic Pima Indians is extremely low compared to caucasian population and may have confounded the results. However, the relationship of hyperinsulinaemia with CHD is far from established and there are strong proponents of the argument against a role for insulin causing atherogenesis (Jarrett, 1988; Durrington, 1992).

2.4.2 Subjects with type 2 diabetes:

2.4.2.1 Cross-sectional studies

Studies relating insulin to atherosclerosis are indeed few in diabetic populations. In studies of type 2 diabetes, subjects with atherosclerotic vascular disease were found to have higher plasma insulin levels (Kashyap et al, 1970; Santen et al, 1972). In the Shwabing study, both diabetic and non-diabetic subjects with macrovascular disease had higher fasting C-peptide levels than those without (Standl et al, 1985). In the same study macrovascular disease was commoner in those subjects who had highest C-peptide levels, whether expressed independently or in relation to body mass index. In Japanese and Japanese Americans with type 2 diabetes, hyperinsulinaemia was reported to be associated with increased occurrence coronary artery disease (Fujimoto et al, 1989). In subjects with type 2 diabetes treated with insulin, insulin dose was a significant predictor of myocardial infarction after 5 year follow-up (Janka et al, 1987).

In 247 subjects with newly diagnosed type 2 diabetes, fasting and post load insulin concentrations were associated with ECG abnormalities (Hillson et al, 1984). Elevated concentrations of both insulin and C-peptide are related cross-sectionally to ECG abnormalities in Japanese American men (Bergstrom et al, 1988). Recently a study from Finland has shown insulin to be associated with coronary artery disease and acute myocardial infarction in obese (BMI >27) diabetic men but not in normal subjects particularly when corrected for body mass index (Ronnemaa et al, 1990). In this study significant univariate associations were seen between various insulinogenic measures and triglyceride levels. However, in Pima Indians no relationship of either endogenous or exogenous insulin was seen with ECG abnormalities of CHD among subjects with type 2 diabetes (Liu et al, 1993)

2.4.2.2 Prospective studies

In the Oxford Study, referred to above, of 247 newly diagnosed subjects with type 2 diabetes, after five year follow-up fasting plasma insulin were higher in those subjects who developed ECG abnormalities than those whose ECGs remained normal (Hillson et al, 1984). However, in Bedford in subjects with borderline diabetes, a low 2-hr plasma insulin was associated with increased risk of CHD after a 10 year follow-up (Jarrett et al, 1982). In Pima Indians of Arizona, neither endogenous nor exogenous insulin predicted ECG abnormalities in subjects with type 2 diabetes (Liu et al, 1993).

2.5 Hyperinsulinaemia and risk factors for cardiovascular disease

There is a close relationship between insulin, triglyceride metabolism and HDL-cholesterol metabolism (Olefsky et al, 1974; Orchard et al, 1983; Laakso et al, 1987; Zavaroni et al, 1989). Insulin plays an important part in regulating levels of plasma triglycerides through its influence on VLDL triglyceride production. This is particularly the case in the subjects with diabetes as there is excess of substrate availability (Olefsky et al, 1974; Greenfield et al, 1980). Recently an inverse relationship between HDL-cholesterol and insulin has been shown (Cambien et al, 1987; Laakso et al, 1985, 1990). It is suggested that insulin, through its effects on triglyceride and HDL-cholesterol can accelerate atherogenesis (Reaven, 1988). Similarly decreased HDL-cholesterol and increased VLDL in subjects with IGT is also related to endogenous hyperinsulinaemia, which reflect insulin resistance (Sheng et al, 1986). The effects of insulin on cholesterol and LDL-cholesterol is not so clear. However, insulin increases the number of LDL receptors on cultured skin fibroblasts (Chait et al, 1979). However insulin treatment in subjects with type 2 diabetes is associated with lowering of plasma triglyceride and an increase in HDL-cholesterol, changes which are likely to be associated with reduced risk of coronary artery disease (Taskinen et al, 1988).

Hypertriglyceridaemia also appears to inhibit LDL receptor binding in cultured skin fibroblasts (Hiramatsu et al, 1985), providing another possible link between cholesterol metabolism on the one hand and insulin resistance on the other due to the relationship of insulin resistance with high triglyceride levels. As discussed previously, LDL particle which is more atherogenic and is linked to hyperinsulinaemia or insulin resistance, may also play an important role in the pathogenesis of coronary artery disease.

A recent study, however, has reported that in the absence of diabetes, hypertension and obesity, measures of insulinaemia are not consistently related to blood pressure or to measures of lipid metabolism and coagulation, and are thus poor predictors of other cardiovascular risk factors. The same study found that the dominant association of fasting and post glucose load insulinaemic measures were with triglyceride, especially in women, with less frequent graded differences between quintiles observed for cholesterol, systolic and diastolic blood pressures (Winocour et al, 1989). In the San-Antonio Heart study the association of hyperinsulinaemia with risk factors for coronary artery disease was assessed in a population based study of 614 Mexican Americans without diabetes (Haffner et al, 1986). Forty three subjects subsequently developed diabetes during follow up. These subjects had higher baseline values of several risk factors, including fasting and 2-hr glucose concentrations, fasting insulin, total and LDL-cholesterol, and body mass index, and lower levels of HDL-cholesterol. These differences persisted after adjustments for obesity or level of glycaemia, but were not significant when corrected for plasma insulin concentrations. When subjects with IGT at baseline were excluded the more atherogenic risk profile persisted. These findings provide credence to the theory that subjects destined to develop diabetes may be at increased risk of coronary artery disease many years before onset of diabetes and that this may be related to fasting hyperinsulinaemia. The relationship of hyperinsulinaemia with adverse changes in lipid and lipoproteins is likely to be a

reflection of underlying insulin resistance as studies have shown a direct relationship between insulin resistance and serum lipoprotein (Abbott et al, 1987, Garg et al, 1988) as well as plasma HDL-cholesterol and triglyceride concentrations (Laws et al, 1992).

2.6 Hyperinsulinaemia and hypertension

Hypertension is an important risk factor for cardiovascular disease and hyperinsulinaemia and or insulin resistance is implicated in the regulation of blood pressure (Ferrannini et al, 1990). Subjects with type 2 diabetes have increased prevalence of hypertension which is not related entirely to the presence of diabetic renal disease (Drury et al, 1983). Hypertensive subjects have higher insulin levels in response to oral glucose when compared to normotensive controls and this hyperinsulinaemia is not related to age or therapy for hypertension (Welborn et al, 1966; Berglund et al, 1976; Asch et al, 1991). In an Israeli population, there was a highly significant relationship between hypertension and glucose intolerance independent of the effects of age, sex, obesity and antihypertensive treatment. In a subgroup fasting insulin and post glucose insulin were significantly elevated in hypertensive subjects. The highest insulin levels were found in subjects who had the combination of hypertension, obesity and glucose intolerance (Modan et al, 1985). These findings point to the fact that the presence of hypertension is associated with a state of peripheral insulin resistance. Insulin levels have been shown to correlate with both systolic and diastolic blood pressures (Manicardi et al, 1986; Bonora et al, 1987) and with insulin resistance as measured by euglycaemic clamp (Ferrannini et al, 1987). In a study of newly diagnosed type 2 diabetic subjects hyperinsulinaemia correlated with blood pressure and the association was independent of the effects of obesity (Uusitupa et al, 1987).

Intervention studies in non-diabetic obese subjects with dietary treatment aimed at

weight reduction have shown a fall in blood pressure as well as insulin concentrations, there being a significant relationship between changes in insulin levels and changes in blood pressures (Rocchini et al, 1987). In a study of young men with untreated essential hypertension who were neither obese nor had diabetes, insulin stimulated glucose uptake measured using euglycaemic clamps was markedly impaired. Insulin resistance observed in these subjects affected only glucose but not lipid metabolism and was localised to skeletal muscle and not being seen at the hepatic level (Ferrannini et al, 1987). In a study of 16 subjects with type 2 diabetes - 8 with and 8 without hypertension, insulin resistance, measured with the euglycaemic clamp technique, correlated with mean arterial blood pressure, whereas the fasting insulin level showed no association (Gans et al, 1991).

However recent studies from the Pima Indians a population characterised by obesity, insulin resistance, hyperinsulinaemia and a high prevalence of diabetes, have failed to show any association between insulin and blood pressure (Saad et al, 1991). Similarly results of studies from Mauritius indicate that there may be ethnic variability in the relationship of insulin to blood pressure (Dowse et al, 1993). There are several mechanisms by which hyperinsulinaemia might be related to hypertension. These include the effects of insulin on renal sodium reabsorption (DeFronzo et al, 1981) and enhanced sympathetic activity (Landsberg 1986). In summary, essential hypertension has been shown to be associated with both hyperinsulinaemia and hypertension. The insulin resistance of essential hypertension manifests itself as a selective inability of insulin to stimulate glycogen synthesis in skeletal muscle. The relationship if any of proinsulin-like molecules with hypertension is not known.

2.7 Hyperinsulinaemia, insulin resistance, PAI-1 and coronary artery disease

The fibrinolytic system is a complex enzyme cascade which leads, in its final step, to

the conversion of plasminogen to plasmin- a step essential for degradation of fibrin. A simplified scheme is shown in figure 2.1. While tissue plasminogen activator (t-PA) and urokinase are the two main activators of this system, PAI-1 is the major circulating inhibitor of t-PA (Auwerx et al, 1988). Raised levels of PAI-1, therefore, by inhibiting fibrinolysis can either initiate or propagate thrombotic events. Fibrin deposition is universal in human atherosclerotic plaque (Thompson and Smith, 1989; Smith et al, 1990; Bini et al, 1989) and it may contribute to the growth of plaque tissue by a variety of mechanisms such as stimulation of cell proliferation (Naito et al, 1992) and by its capacity to bind low density lipoprotein hence leading to its accumulation in the atherosclerotic plaque (Smith et al, 1976; Smith and Cochran, 1990). The role of arterial fibrin deposition in the occlusion of coronary arteries by way of thrombus formation is well recognised. Hypofibrinolysis, therefore, by decreased removal of fibrin could initiate and also play part in the progression of atherothrombosis.

Hypofibrinolysis has previously been shown to be associated with obesity (Shaw et al, 1965), hypertriglyceridaemia (Andersen et al, 1981) and diabetes (Fearnley et al, 1963). Recently this defect in fibrinolysis has been attributed to increased activity of PAI-1, a fast acting inhibitor of tPA (Auwerx et al, 1988; Wiman et al, 1990). Several studies have shown that PAI-1 activity is elevated in subjects with coronary artery disease (Paramo et al, 1985; Hamsten et al, 1985; Mehta et al, 1987) and in men under 45 years of age with myocardial infarction PAI-1 appears to be independent risk factor for recurrent myocardial infarction (Hamsten et al, 1987). PAI-1 activity levels have been shown in a prospective study as best predictor of progression of atherosclerosis but only in subjects with impaired glucose tolerance (Bavenholm et al, 1990).

Studies have also shown that a strong correlation exists between PAI-1 activity and

► **Fibrinolytic pathway**

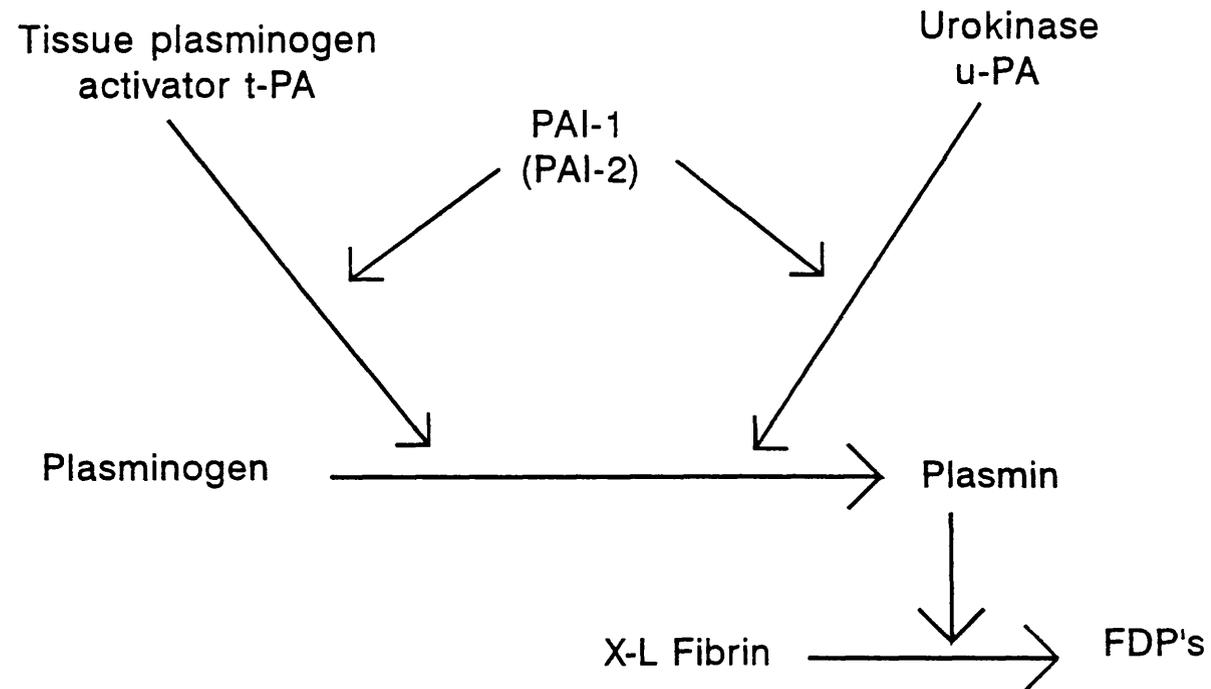


Figure 2.1: Schematic representation of the fibrinolytic pathway and its major inhibitors

body mass index (Vague et al, 1989; Landin et al, 1990) centrality of obesity as measured by waist-to-hip ratio (Landin et al, 1990; Sundell et al, 1989) and triglyceride levels (Mehta et al, 1987; Hamsten et al, 1987; Vague et al, 1987; Sundell et al, 1989). PAI-1 levels are elevated in subjects with type 2 diabetes (Auwerx et al, 1988; Juhan-Vague et al, 1989; Almer et al, 1988; Gram et al, 1988; Wamsley et al, 1990) while insulin dependent diabetic subjects seem to have normal or low levels (Wamsley et al, 1990). Similarly hypertensive men also have higher PAI-1 levels (Landin et al, 1990) and PAI-1 activity correlate with systolic blood pressure (Sundell et al, 1989). PAI-1 levels show correlation with fasting insulin concentration in normal subjects with a wide range of body mass index (Vague et al, 1986), non-diabetic obese women (Juhan-Vague et al, 1987), subjects with type 2 diabetes (Juhan-Vague et al, 1989) and with angina pectoris (Juhan-Vague et al, 1989). Similar findings have been reported in non-obese hypertensive subjects (Landin et al, 1990), and obese children (Legnani et al, 1988). This relationship of PAI-1 with insulin seems to be independent of other risk factors.

Insulin has been shown to increase PAI-1 production from HepG2 cell lines and this effect was not due to cell proliferating effect of insulin but to increase in levels of PAI-1 mRNA (Alessi et al, 1988; Kooistra et al, 1989, Grant et al, 1990). On the other hand in-vivo studies have failed to show stimulatory effects of insulin on PAI-1 levels during short term infusion (Grant et al, 1990; Potter Van Loon et al, 1990). The mechanism by which insulin may influence PAI-1 release from hepatic cells may be direct or through its effects on lipoprotein metabolism. There is a relationship between PAI-1 activity and triglyceride levels. Purified VLDL triglyceride have been shown to stimulate the PAI-1 secretion from endothelial cells from umbilical vein, this stimulating effect being dependent on the binding of lipoproteins to ApoB/E receptor on the cells and was abolished in the presence of antibody against ApoB/E receptors (Stiko-Rahm

et al. 1990). Similar finding of increased PAI-1 synthesis from cultured endothelial cells under the effects of native and modified LDL have been described (Latron et al, 1990; Tremoli et al, 1993) have been have been described recently. There is a strong possibility that insulin may affect PAI-1 synthesis from hepatocytes and that insulin resistance and consequent hyperinsulinaemia may increase the risk of coronary artery disease by enhancing thrombus formation due to decreased fibrinolysis by increasing levels of PAI-1.

The beneficial effect of exercise on insulin resistance is well known (Koivisto et al, 1986). Physical training and weight loss due to dietary modifications has been shown to improve fibrinolytic activity (Sundell et al, 1989; Huisveld et al, 1990; Folsom et al, 1993; Gris et al, 1990) and it is likely this improvement may be mediated by increase in insulin sensitivity due to weight loss. The relationship between PAI-1 and insulin was further strengthened by intervention studies aiming to reduce insulin concentrations by reducing insulin resistance. Fasting is associated with reduction in fasting insulin concentrations and a reduction in PAI-1 levels (Vague et al, 1986). The significant relationship between PAI-1 and insulin and a simultaneous reduction in both these parameters by various interventions may suggest that insulin might directly or indirectly influence circulating PAI-1 activity.

2.8 Hyperinsulinaemia, obesity, body fat distribution, and coronary artery disease.

Obesity is associated with increased mortality from coronary artery disease (Hubert et al, 1983) but it is not clear as to whether this is an independent association (Tumilehto et al, 1987). Obesity is an insulin resistance state (Caro, 1991) and is associated with impaired glucose tolerance, hypertension and hyperlipidaemia and the relationship of obesity with ischaemic heart disease may therefore be an indirect one.

Both non-diabetic and diabetic obese subjects show fasting hyperinsulinaemia and exaggerated insulin responses to oral glucose compared to their non-obese counterparts (Bagdade et al, 1968, 1974). Conversely caloric restriction and weight reduction in obese subjects is associated with lowering of fasting insulin concentrations and a reduced insulin response to glucose challenge (Olefsky et al, 1974).

Attention has been focussed on body fat distribution with obesity being categorised as central or abdominal and peripheral or gluteal (Vague, 1956). There is an positive independent association between upper body obesity with cardiovascular disease and glucose intolerance, independent of body mass index, in both male and female sex (Larsson et al, 1984; Lapidus et al, 1984, Ohlson et al, 1985; Lundgren et al, 1989). This relationship of upper body obesity with cardiovascular disease and glucose tolerance is due the excess accumulation of intraabdominal fat which plays a central role in glucose and lipid metabolism (Fujioka et al, 1987; Bjorntörp, 1990; Pouliot et al, 1982). Although obesity is associated with hyperinsulinaemia (Pederson, 1989) the closest association is with upper body obesity (Stern et al, 1986, Peiris et al, 1986). In women, upper body obesity is associated with higher glucose and insulin response to oral glucose test (Kissebah et al, 1982) and presence of impaired glucose tolerance did not change this relationship. Increasing waist-to-hip ratios were accompanied by increasing fasting insulin levels (Evans et al, 1984). How upper body obesity is associated with hyper insulinaemia and insulin resistance remains to be understood completely although there are suggestions that this may be due do decreased hepatic insulin extraction or metabolic clearance rate of insulin (Yki-Järvinen et al, 1985, Peiris et al, 1986).

Similarly in subjects with diabetes a central distribution of fat is associated with increased frequency of macrovascular disease, hypertension, higher triglyceride and

low HDL-cholesterol (Van-Gall et al, 1988) and in the Paris Prospective Study, in subjects with IGT and type 2 diabetes, a central distribution of body fat was associated with coronary artery disease (Fontbonne et al, 1992). In summary upper body obesity is associated with coronary artery disease, diabetes, hyperinsulinaemia, hypertension and hypertriglyceridemia independent of body mass index (BMI).

2.9 Physical activity and Cardiovascular disease

Physical activity has been demonstrated to be associated with reduced risk of heart attacks and increased longevity in non-diabetic populations (Leon et al, 1987; Paffenberger et al, 1993; Sandvik et al, 1993). Physical activity has been shown to be associated with a significantly improved CVD risk factor profile (Cooper et al, 1976; Lehtonen et al, 1978; Gibbons et al, 1983). Studies in Mauritian women showed that diabetes, impaired glucose tolerance, 2-hr plasma glucose, fasting and 2-hr insulin and serum triglyceride were higher in inactive women, and HDL-cholesterol lower, than a group with higher physical activity (Zimmet et al, 1991, Dowse et al, 1991). In this study the results of physical activity on cardiovascular risk factors were shown to be mediated via its effects on insulin-glucose metabolism (Zimmet et al, 1991).

2.10 Physical activity and diabetes

The effect of exercise on carbohydrate metabolism suggests that exercise may be an important protective factor for the development of Type 2 diabetes (Koivisto et al, 1986, Rosenthal et al, 1993). Cross-sectional studies strongly support the association of physical activity with a low prevalence of type 2 diabetes in physically active men and this association was independent of age, BMI, and urban living (Helmrich et al, 1991; Manson et al, 1991). Recently physical activity has been shown to be an independent risk factor for type 2 diabetes in Hindu Asian Indians, Creole men, and Hindu women living in Mauritius (Dowse et al, 1991). Studies from Pima Indians have

also shown a significant and negative relationship between physical activity and upper body fat distribution, suggestive of a mechanisms through which physical activity may be related to development of diabetes (Kriska et al, 1993).

2.11 Congregation of multiple risk factors

Hyperinsulinaemia is associated with individual risk factors for cardiovascular disease and a number of studies have shown that among populations there is a clustering of risk factors such as hyperinsulinaemia, obesity, impaired glucose tolerance, hypertension, high plasma triglyceride and low HDL-cholesterol (Florey et al, 1976; Burke et al, 1986; Cambien et al, 1987; Zavaroni et al, 1989, 1992; Haffner et al, 1992; Fontbonne et al, 1992; DeFronzo et al, 1992, Zimmet et al, 1984). Reaven has suggested that the underlying mechanism for the congregation of these risk factors is the presence of insulin resistance (Reaven 1988). It seems that a low level of physical activity may play an important part in determining the levels of several components of hyperinsulinaemia insulin resistance syndrome (Zimmet, 1991).

2.12 Proinsulin-like molecules in Type 2 diabetic subjects

Insulin is produced in the pancreatic beta cells from its precursor molecule proinsulin (Fig 2.2) (Steiner and Oyer, 1967), which in itself arises from its precursor preproinsulin (Steiner et al, 1989). Conversion of proinsulin to insulin and C-peptide occurs within the secretory granules and the steps involved in its conversion are shown in figure 2.3 (Rhodes et al, 1992; Rhodes and Alacron, 1994). The first step in proinsulin conversion is its cleavage by an endoprotease which occurs either at the junction of B-chain/C-peptide or at the C-peptide/A-chain junction. This is followed by the removal of C-terminal basic amino acids by enzyme carboxypeptidase H. This process leads to two split proinsulin conversion intermediates, which are subjects to further action of these enzymes leading to the generation of mature insulin molecule

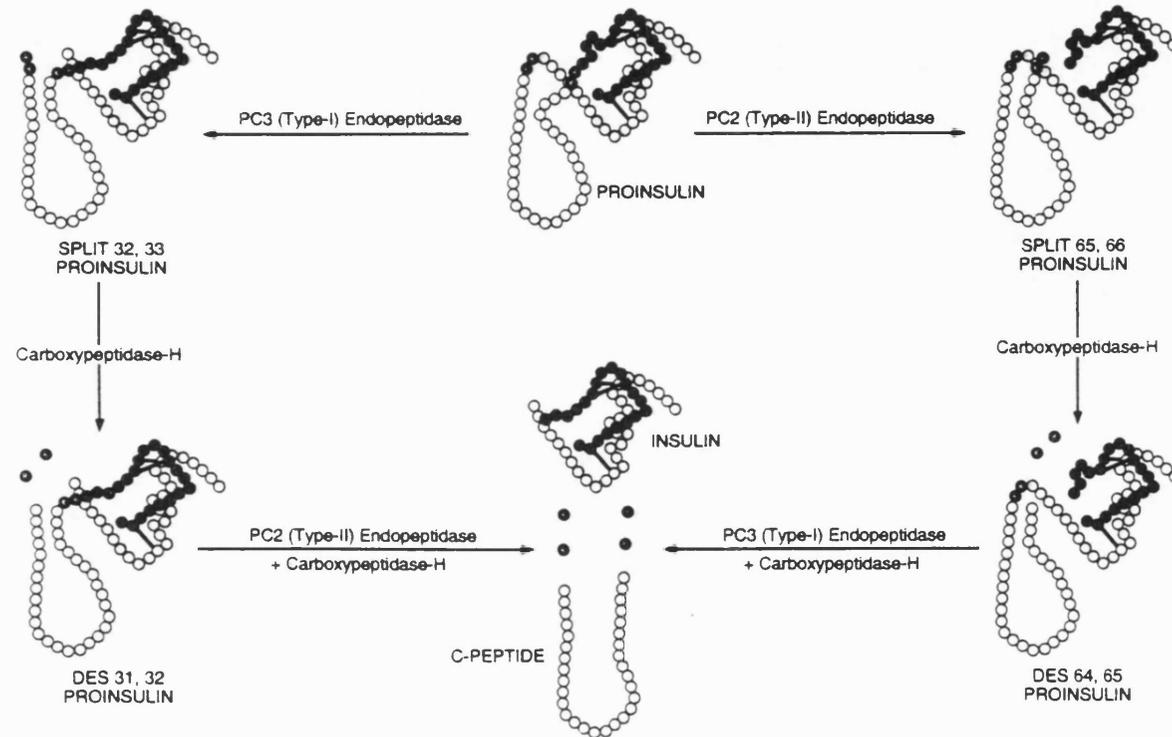


Figure 2.3: Scheme for enzymatic processing of proinsulin to insulin through its conversion intermediates. K=Arginine, R=Lysine

and C-peptide (Hutton et al, 1991). The contents of the granules are secreted normally in response to an appropriate stimulus and this process represents a regulated pathway of insulin release, which appears to be the major pathway of insulin release (Rhodes and Halban, 1987; Halban, 1991).

During the process of insulin secretion in normal subjects, a small amount of proinsulin escapes in to the circulation (Robbins et al, 1984), but because of the longer half life of proinsulin due to slow clearance of proinsulin from the plasma compared to insulin (Rubenstein et al, 1972; Sonksen et al, 1973; Revers et al, 1984), peripheral concentrations of proinsulin are higher than is reflected by their relative secretion. In normal subjects 10% to 15 % of the circulating insulin like immunoreactivity is made up by proinsulin (Horwitz et al, 1975). Several studies have shown a disproportionate elevation of circulating proinsulin concentration in type 2 diabetic subjects (Duckworth et al, 1972; Gordon et al, 1974; Mako et al, 1977; Ward et al, 1987; Yoshioka et al, 1988; Temple et al, 1989). The proportion of proinsulin-like molecules in these studies showed a large variation (table 2.1) and may be due to differences in assay specificities or subject heterogeneity. High concentrations are thought to result from the secretion of the contents of immature secretory granules which contain higher ratio of proinsulin to insulin under the influence of hyperglycaemic stimulus.

Recently the use of highly specific assays have permitted the measurement of metabolites of intact proinsulin in the circulation (Sobey et al, 1989) and elevated concentrations of des 31,32 proinsulin have been reported in obese subjects with type 2 diabetes this seeming be the major circulating metabolite of proinsulin (Temple et al, 1989).

2.12.1 Effects of proinsulin-like molecules

2.12.2 Receptor binding studies

Biological effects of proinsulin result from its interaction with the insulin receptor, since several studies have failed to show peripheral conversion of proinsulin to insulin (Challoner et al, 1970; Kitabchi et al, 1972; Freychet, 1974). Modifications of intact proinsulin molecules, even in the form of single peptide bond cleavage, leads to dramatic increases in the relative potencies of animal proinsulin (Chance, 1970). Modifications at the junction of C-peptide with the A chain of insulin leading to the formation of 65,66 split proinsulin or des 64,65 proinsulin, results in an increase in affinity for receptor binding ranging from 11 to 27 times over that of intact proinsulin. Modification near the B chain of insulin resulting in the formation of 32,33 split proinsulin or des 31,32 proinsulin resulted approximately in a five fold increase in receptor affinity relative to intact proinsulin. It would seem that modification of the COOH- terminal is the preferential pathway for proinsulin conversion to its intermediate forms (Peavy et al, 1985).

Intact proinsulin shows receptor binding, in rat lymphocyte, adipocytes and hepatocyte which is of the order of 0.5-2.0 % of that of insulin (Peavy et al, 1985). Studies of Tillil et al in mongrel dogs showed that after equimolar injection of proinsulin and its intermediates, the biological activity of was dependent on the metabolic clearance rate of these molecules and des 64,65 proinsulin was more potent than intact proinsulin and des 31,32 proinsulin with intact proinsulin being least potent of the three (Tillil et al, 1990).

2.12.3 Biological effects

2.12.3.1 Carbohydrate metabolism

Insulin precursor molecules seem to have a longer half life than insulin. The half life of

intact proinsulin is 42 min while that of des 31,32 proinsulin is about 18 min (Tillil et al, 1990). Metabolic clearance studies in mongrel dogs have shown that after equimolar injections of proinsulin metabolites, des 64,65 proinsulin seem to be cleared more rapidly, followed by des 31,32 proinsulin with intact proinsulin being cleared considerably more slowly than insulin (Tillil et al, 1990). At high concentrations each derivative produced the same maximal glucose lowering response. At lower concentrations, however the relative potencies seem to parallel the receptor binding and metabolic clearance of these molecules. In normal humans the blood glucose lowering ability of intact proinsulin is 8% of that of insulin, while in contrast proinsulin mediated suppression of hepatic glucose production was 12%, suggesting that proinsulin may have a preferential effect on hepatic glucose metabolism in humans (Revers et al, 1984) and dogs (Lavelle-Jones et al, 1987). Other in-vivo studies in humans also confirmed this hepatospecificity of proinsulin (Glauber et al, 1986, 1987; Davis et al, 1991, 1992).

2.12.3.2 Effects on Lipids

Proinsulin treatment has been shown to reduce plasma triglyceride levels and increase HDL₂-cholesterol in type 2 diabetic subjects (Drexel et al, 1988; Winocour et al, 1991), while one study demonstrated no effect on lipids despite significant and equal reductions in plasma glucose among subjects treated with intact proinsulin and neutral isophane insulin (Huisman et al, 1989). Studies in which proinsulin treatment led to a reduction in lipids, the lipid lowering effect seems to be a specific effect of proinsulin rather than secondary to improved glycaemic control as improvement in glycaemic control using Isophane insulin led to opposite changes in plasma triglyceride levels (Drexel et al, 1988).

Table 2.1: Proinsulin-like molecules (fasting) as a proportion of immunoreactive insulin.

Proinsulin-like molecules (%)		Reference (year)
Non-diabetics	Diabetic	
56	45	Duckworth et al, (1972)
-	38 ± 8	Gordon et al, (1974)
12 ± 3	14 ± 9	Mako et al, (1977)
15 ± 5	32 ± 14	Ward et al, (1987)
16 ± 8	24 ± 8	Yoshioka et al, (1988)
11 ± 6	20 ± 11	Saad et al, (1990)
24 ± 8 (non-obese)	50 ± 13 (non-obese)	Temple et al, (1989)
28 ± 6 (obese)	49 ± 27 (obese)	

Modified from Yudkin, 1993.

2.12.3.3 Effects on PAI-1 activity

Intact proinsulin has about 20% of the activity of insulin in stimulating PAI-1 synthesis from isolated liver tumor cells (Alessi et al, 1988). Further in-vitro studies on pig aortic endothelium suggests that intact proinsulin and split proinsulin both increased PAI-1 activity, by increased expression of PAI-1 mRNA (Schneider et al, 1992). The potency of proinsulin in stimulating PAI-1 activity was of same magnitude as that of insulin. Furthermore the effects of proinsulin were not mediated by its interaction with the insulin receptor and could not be attenuated by insulin and IGF-1 (Schneider et al, 1992). It seems that split forms of proinsulin may have a stronger biological potency than intact proinsulin, because of their relative receptor binding is of higher magnitude than that of intact proinsulin (Peavy et al, 1984).

2.12.3.4 Effect of interventions on proinsulin-like molecules

Dietary treatment in subjects with type 2 diabetes has been shown to lower the concentrations of insulin precursor molecules and normalise the raised proinsulin to insulin ratio (Yoshioka et al, 1989) and concentrations of intact and 32,33 split proinsulin (Davies et al, 1993). Sulphonylurea treatment has been shown to stimulate the production of proinsulin leading to increased concentrations of these molecules in the circulation (Elkeles et al, 1982), while long term treatment had no effect (Duckworth et al, 1974). Studies from Pima Indian subjects show that concentrations of proinsulin like molecules were significantly higher in subjects on sulphonylurea treatment and lower in those currently being treated with insulin than subjects on no drug treatment for diabetes (Nagi et al, 1994). One study has shown suppression of these molecules during hyperinsulinaemic clamp (Koivisto et al, 1986). Recently, intensified insulin treatment has been shown to normalise raised concentrations of proinsulin in subjects with type 2 diabetes (Lindstrom et al, 1992). Although there is large body of evidence to show that raised proinsulin concentrations reflect β -cell

dysfunction and hyperglycaemia is responsible for disproportionate release of proinsulin-like molecules, the role of insulin resistance in determining concentrations of these molecules is not clear.

2.13 Controversies and unanswered questions

The major question remains as to why is insulin related to risk factors for cardiovascular disease and what does this association reflect? Is it insulin per se which has deleterious effects on cardiovascular risk factors or that the primary culprit is insulin resistance with insulin as an innocent bystander (Zimmet, 1993).

For obvious reasons, the majority of epidemiological studies on the insulin resistance syndrome have not measured insulin resistance directly, but have employed insulin concentrations as surrogates for insulin resistance. This assumption may have led to the confusion which at present surrounds the role of insulin resistance and hyperinsulinaemia in the pathogenesis of AVD.

Insulin resistance in the presence of normal beta cell function will lead to increasing concentrations of plasma insulin, in order to maintain normoglycaemia (Matthews et al, 1985). Thus, in epidemiological work, fasting insulin concentrations in subjects with normal glucose tolerance have been used as surrogate indices of insulin resistance. These assumptions may be reasonable, although there are several difficulties - e.g. insulin concentrations to be measured are often close to the limit of assay sensitivity, and insulin-precursor molecules may cross-react in the assays, further reducing their sensitivity. The major difficulty arises when applying such assumptions to subjects with glucose intolerance, and to insulin concentrations following glucose load.

In subjects with impaired glucose tolerance, fasting hyperinsulinaemia may be correctly

regarded as a compensatory phenomenon to insulin resistance, as shown by prospective studies on Pima indians, in which the transition from normal to IGT is accompanied by a rise, and the transition from IGT to diabetes, by a fall in fasting insulin concentrations (Saad et al, 1991), implying that the relationship between insulin resistance and insulin concentrations is lost in this context. Increasing hyperglycaemia may be the cause of the subsequent fall in insulin levels, as the beta cells become exhausted (Saad et al, 1989; Rosetti, 1990). Embodying this concept of a rise and fall in islet beta cell function, DeFronzo has coined the term "Starling's law of the Pancreas" (DeFronzo, 1988).

The concept of using glucose-stimulated 2-hr insulin concentrations, or insulin area-under-curve, as surrogates for insulin resistance is supported in subjects with normal glucose tolerance, where insulin resistance correlated with a hyperinsulinaemic response to the oral glucose tolerance test ($r = -0.60$) (Hollenbeck et al, 1987). However, insulin levels in the non-fasting state, are not exclusively dependent upon insulin resistance, in either diabetic or non-diabetic subjects. Bruce et al. demonstrated a defective first phase insulin response in subjects with type 2 diabetes, which was associated with subsequent hyperglycaemia, hyperinsulinaemia, and reduced suppression of glucagon (Bruce et al, 1988). Recently, Mitrakou et al. have extended this work to subjects with IGT, showing that the hyperinsulinaemia 2 hours after a glucose challenge is also explained by a defective first phase insulin release. This causes more sustained hyperglycaemia, which in turn leads to higher insulin levels at 2 hours, independently from insulin resistance (Mitrakou et al, 1992). Thus, despite an apparent association between insulin resistance and hyperinsulinaemia after oral glucose challenge, insulin resistance may not be the exclusive cause of hyperinsulinaemia in this situation, nor of the increased insulin area-under-curve measurements - an interpretation supported by Hollenbeck and Reaven's observation

that only 36% of the variation in insulin area-under-curve response to a glucose load can be explained by insulin resistance (Hollenbeck et al, 1987).

However, in insufficiently large groups of subjects, and in the absence of any reference to the blood glucose concentration, fasting insulin concentrations may bear little relationship with insulin resistance, as measured by the euglycaemic clamp technique, perhaps because of the assay problems and type 2 error. This is borne out by the observations that fasting insulin concentrations alone are not significantly correlated with insulin resistance in either lean (Ferrannini et al, 1987), or obese subjects, with or without essential hypertension (Manicardi et al, 1986).

In normoglycaemic subjects, fasting insulin concentrations would be expected to parallel insulin resistance, but it remains uncertain whether insulin resistance or hyperinsulinaemia is the primary abnormality. Beta cell hypersecretion may be viewed as the result of an insulin resistance defect predominantly in skeletal muscle. However, insulin resistance also has been shown to increase in response to experimental hyperinsulinaemia in normal human subjects under euglycaemic clamp conditions (Rizza et al, 1985), and to be a function of the insulin inter-pulse interval (Peiris et al, 1992), suggesting that hyperinsulinaemia may contribute to, as well as result from insulin resistance, such that the primary defect might lie in the islet beta cell rather than in skeletal muscle.

Therefore, there is a complex interplay between insulin resistance and insulin concentrations in the fasting state, and although we can make reasonable deductions about the former from the latter, it does not follow that insulin resistance in skeletal muscle is the sole determinant of fasting hyperinsulinaemia.

A final problem is the observation that insulin concentrations may have been overestimated because of the poor specificity of many insulin assays, in which insulin precursor molecules may have cross-reacted with insulin (Temple et al, 1990). This observation raises further questions about the relationship of insulin with cardiovascular risk factors observed in previous studies. Furthermore elevated concentrations of proinsulin-like molecules in subjects with type 2 diabetes also raise the possibility of a role for proinsulin-like molecules in subjects with type 2 diabetes in determining levels of cardiovascular risk factors.

We believe that the extent of the problem of cross-reaction of proinsulin-like molecules in insulin assays is likely to be less in non-obese non-diabetic subjects, in whom only about 10-15% of the total immunoreactive insulin is proinsulin, and conventional insulin assays would tend systematically to overestimate insulin concentrations in both study subjects and their normal controls.

Therefore, although both insulin resistance and hyperinsulinaemia have been implicated in the natural history of AVD, the relationship between them is not simply one of immediate cause-and-effect, and that the dependency of insulin concentrations on insulin resistance does not preclude the possibility either that an abnormality of beta cell secretion initiates insulin resistance, or that some common antecedent is responsible for both of them. In attempting to use insulin concentrations as surrogate indices of insulin resistance, we need to be aware of these pitfalls.

In non-diabetic subjects fasting insulin concentrations increase in parallel with insulin resistance hence it is difficult to answer this question in non-diabetic subjects due to problems of co-linearity. However in subjects with type 2 diabetes, insulin resistance and insulin concentrations do not rise in parallel due to beta cell dysfunction, while

proinsulin-like molecules show progressive rise in response to β -cell dysfunction and worsening hyperglycaemia (Leahy et al, 1991; Leahy, 1993).

Therefore, in this dissertation, We have performed a series of clinical studies to analyse the relationship of insulin resistance, proinsulin-like molecules with PAI-1 and also other cardiovascular risk factors in subjects with type 2 diabetes. We have performed cross-sectional and intervention studies to examine:

- i Cross-reaction of proinsulin-like molecules with insulin immunoassays.
- ii The relationship of proinsulin-like molecules with PAI-1 and cardiovascular risk factors in subjects with type 2 diabetes.
- iii Proinsulin and insulin responses to oral glucose tolerance test in Asian and caucasian non-diabetic and diabetic subjects with reanalyses of the previously observed relationships of proinsulin-like molecules with PAI-1.
- iv Intervention studies, using metformin and insulin treatment to modify insulin resistance and the effects of such a modification on proinsulin-like molecules and cardiovascular risk factors including PAI-1.

CHAPTER 3: MATERIAL AND METHODS

3.1 Methods

3.1.1 Body Mass Index (BMI)

was calculated as weight in kg/height in meters squared ($BMI = \text{kg}/\text{m}^2$). Height and weight were recorded without shoes and wearing light clothing.

3.1.2 Anthropometric measurements

Waist circumference measurement was recorded in triplicate using a steel tape at the site of minimum girth between the subcostal margin and the anterior superior iliac spine while hip circumference was recorded at the site of the greater trochanter. Waist-hip-ratio was calculated from the mean of three measurements for each. Skinfold thickness measurements were performed using Holtain calipers (Holtain Ltd, Crosswell, Crymych, Dyfed, Wales). Subscapular skinfold was measured at the inferior angle of the scapula along the natural lines of skinfold. Triceps skinfold thickness was recorded in the left arm posteriorly midway between the tip of acromion and the olecranon process, over the belly of the triceps muscle. Subscapular to triceps skinfold ratio was calculated using the mean of three measurements for each.

3.1.3 Blood pressure

was recorded after 15 mins rest with subjects in the sitting position and on the right side using a random zero sphygmomanometer (Hawksley Ltd, Lancing, Sussex, England). Diastolic blood pressure was recorded at phase 5 Korotkoff sounds. The mean of the two recorded blood pressure readings was taken for statistical analyses.

3.1.4 Ankle and Brachial systolic pressures

were recorded using an ultrasonic blood flow detector (Vasculascope) and ankle to brachial pressure ratios were calculated.

3.1.5 Plasma glucose

Blood for plasma glucose analyses was collected in sodium fluoride containing vacutainer bottles and plasma was separated within one hour of sample collection. Plasma glucose was analysed using glucose oxidase (Beckman analyser, Beckman Instruments, Brea, California, USA). The interassay CV for measurement of glucose was 2.2%.

3.1.6 Glycated haemoglobin

was measured using electroendosmosis (Corning Ltd, Halstead, Essex, UK). The intraassay CV of this assay is 3% and interassay CV is 5.5%. The normal range for our laboratory is 6.5-8.5%.

3.1.7 Lipids

Serum triglyceride

was measured by an enzymatic method (Bucolo et al, 1973) using a triglyceride PAP kit (Roche Diagnostica, Welwyn Garden City, Herts, UK). The interassay CV of this assay is 1.2%.

Total serum cholesterol

was measured by an enzymatic method (Roeschlau et al, 1974) (CHOD-PAP method, Boehringer Mannheim Diagnostics and Biochemicals Ltd, Bell Lane, Lewes, Sussex, UK). The interassay CV of this assay is 4%. High density lipoprotein cholesterol (HDL-cholesterol) was measured using the same methods as used to measure total cholesterol but after precipitation of VLDL and LDL with magnesium chloride

phosphotungstate reagent. The inter assay CV is 5.4%. LDL-cholesterol was calculated using the Friedewald equation (Friedewald et al, 1972).

3.1.8 Plasma Lipoproteins

were separated by ultracentrifugation and standard enzymatic methods were used to measure the cholesterol and triglyceride content of each fraction. Apoproteins A1, A2, and B were analysed by immunoturbidimetric assays (Mount et al, 1988) using a Cobas Bio Centrifugal analyser. These assays were performed at the Department of Endocrinology and Chemical pathology, St Thomas's Hospital London.

3.1.9 Plasminogen activator inhibitor (PAI-1)

was measured by a spectrophotometric method (Chmielewska et al, 1983) using a chromogenic substrate (S-2251) employing a kit (Kabi Vitrum, Uxbridge, Middlesex, UK), which was modified to a microplate method. The intraassay CV is 4.5% and interassay CV is 8.5%. Results are expressed in arbitrary units (AU/ml), one unit of inhibitor being defined as the amount which inhibits one international unit of tissue plasminogen activator.

Principle of the assay

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

Sample collection PAI-1

Samples were collected without venous stasis and using a wide bore needle to avoid platelet aggregation. Previous studies have suggested that platelet are an important source of PAI-1 (Booth et al, 1985) and their activation during venous stasis and venepuncture has been shown to affect PAI-1 levels (Urden et al, 1987; Chandler et al, 1989). 4.5 ml of blood was collected into pre-chilled tubes containing 0.5 ml of 3.8% sodium citrate solution and samples were immediately cold centrifuged and separated into aliquots and frozen at -80°C.

Assay performance and characteristics

The assay for PAI-1 was modified to a microplate assay and experiments were performed to check performance under different assay conditions. The effects of temperature and incubation times on the modified assay performance were studied extensively. Some of these experiments are described below. After an incubation period the reaction was stopped using 20% acetic acid and plates were read using Dynatech MR/7000 microplate reader (Dynatech, Billingshurst, West Sussex, England).

Effect of incubation temperature on standard curve for PAI

After initial experiments to modify the assay onto a microplate, it was clear that the assay was extremely temperature sensitive with wide variations in assay performance due to small changes in temperature and incubation times. An incubator with chamber variation of less than 1°C (Memmert, BDH, Dagenham, England) was used. Experiments therefore were done to study the effect of temperature variation for a constant incubation time. Standard curves were constructed at four different temperatures (Fig 3.1). With each degree change of incubation temperature, there was an significant shift in the slope and the intercept of the standard curve leading to a wide variation in the assay performance.

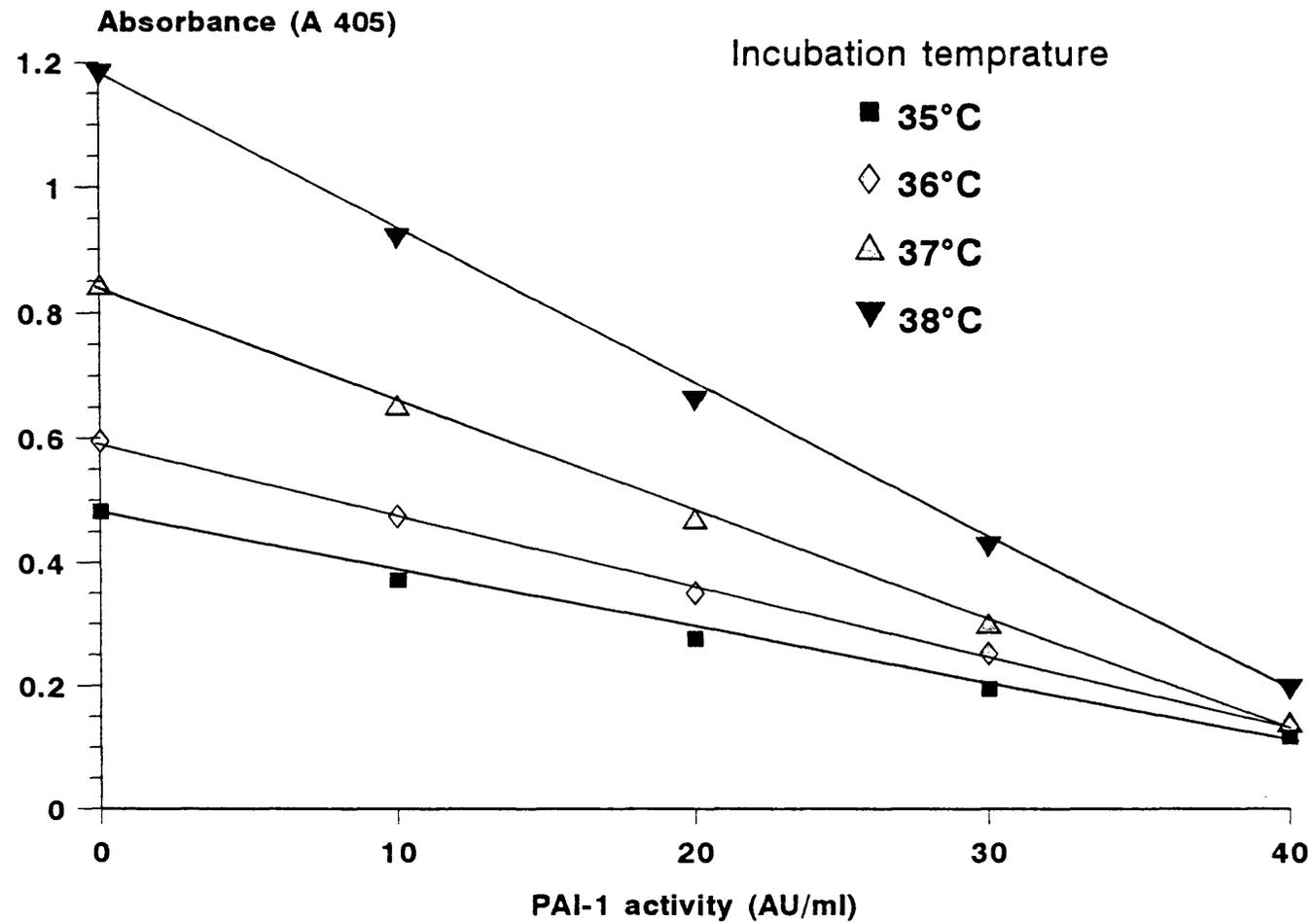


Figure 3.1: Effect of different incubation temperatures on the standard curve for PAI-1
Incubation period 90 min

Effect of incubation time on standard curve for PAI

Effects of varying incubation time period on the slope of the standard curve is shown in figure 3.2 and was found to be similar to the one seen for the effect of temperature. Assay performance when modified to a microplate was optimum and most reproducible at 37°C with less than 1°C variation in temperature for an incubation period of 90 min.

3.1.10 Plasma beta thromboglobulin (β TG) and platelet factor 4 (PF4):

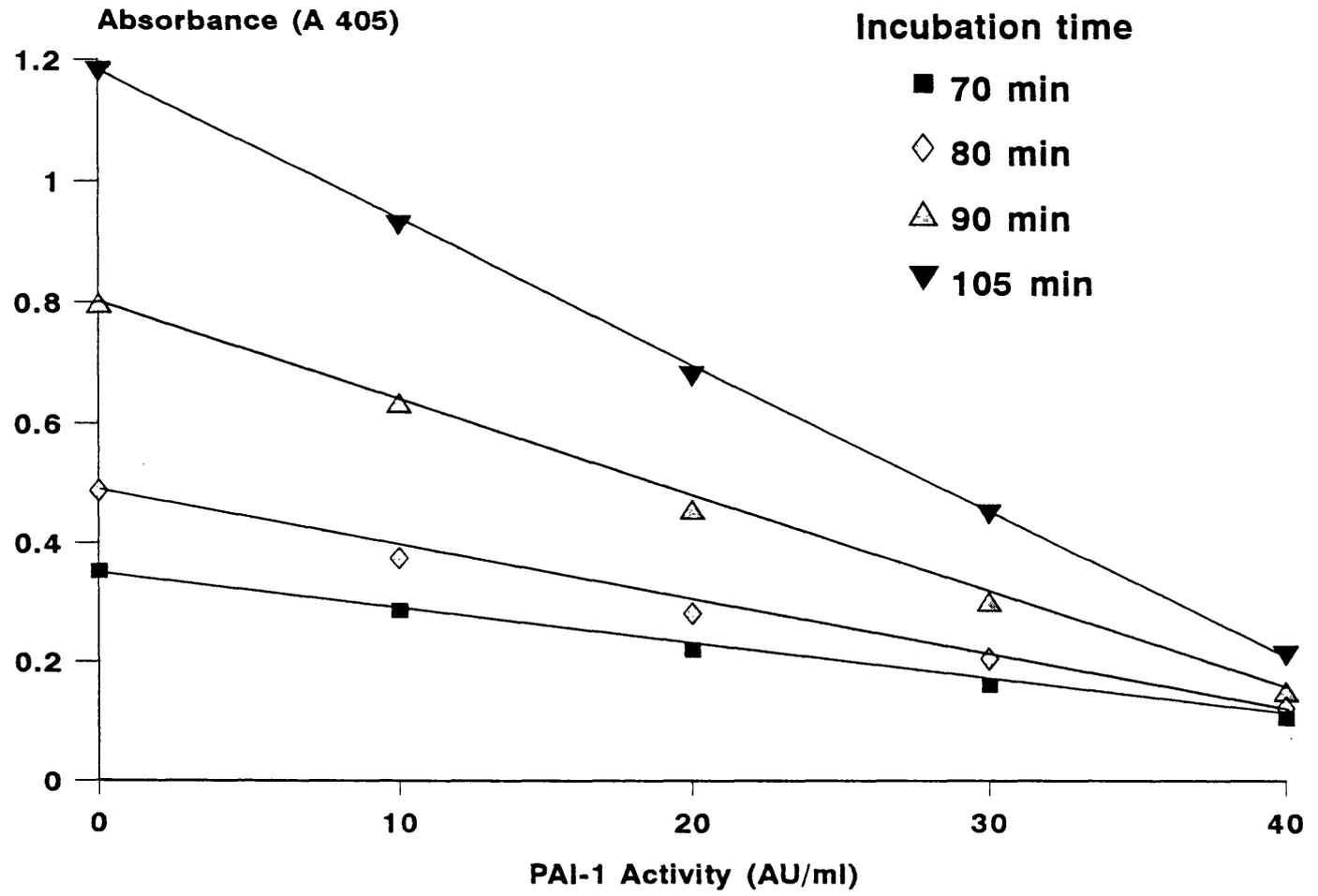
were determined using radioimmunoassay kits (Amersham, Aylesbury, Bucks, and Abbotts Diagnostics, Walsingham, Berks, UK respectively). Samples were collected in special tubes (containing EDTA and Theophylline) and without venous stasis to avoid platelet aggregation. A previous study (Kaplan et al, 1981) has shown that to overcome in-vitro platelet aggregation data only from those samples where ratio of β TG to PF4 ratio is ≥ 3.0 should be used. All samples from each subject were analysed in the same batch. The interassay CV for β TG was 9% and for PF4 was 12.3%.

3.1.11 Fibrinogen

was analysed by Von Clauss method (BCL, Lewes, Sussex, UK). Samples for these assay were stored at -40°C and were analysed in the same batch. Interassay CV of this assay is 1.4%.

3.1.12 Platelet aggregation studies

Spontaneous platelet aggregation in whole blood was performed over a 60 min period with the use of an Ultra-Flo blood platelet counting method (Lumley et al, 1981). Aliquotes (900 μ l) of whole blood were shaken under 5% CO₂ in air in a waterbath at 37°C for 60 min and platelet counts were measured at 10, 20, 30 and 60 mins. Platelet aggregation responses to agonists in whole blood were determined by



**Figure 3.2: Effect of different incubation times on the standard curve for PAI-1
Incubation temperature (37°C)**

measuring the percentage of platelets remaining 3 mins after adding four concentrations ADP to determine dose response curve.

Agonist induced platelet aggregation studies on platelet rich plasma (PRP) were performed using four concentrations of adenosine diphosphate (ADP) as previously described (Born, 1962), expressing sensitivity as EC50 (agonist concentration producing 50% of maximum aggregation).

3.1.13 Immunoreactive Insulin

Insulin concentrations were measured using a polyclonal antibody- based assay (Novo Pharmaceuticals, Basingstoke, Hampshire, UK) (Heding, 1972). ^{125}I was used as tracer. ^{125}I -insulin (Porcine Tyr A19) and anti-porcine insulin guinea pig sera were (M 8170) diluted in phosphate buffer (FAM solution, 0.04M, pH 7.4) containing human albumin fraction 5 (1g/l) (Sigma Chemicals, Poole, Dorset, England) and thiomersal (0.24g/l). All standards and samples were dissolved and diluted in phosphate buffer containing NaCl (0.6g/l), human albumin (60g/l) and this solution is called NaFAM solution. Freeze dried insulin antibody (1.0 ml) was reconstituted using sterile water and subsequently diluted with 59 ml of NaFAM solution to a concentration of 1:450,000.

Radioimmunoassay procedure

Duplicates of 100 μl of insulin standards (0 to 50 μl of insulin/ml) or samples were added to 100 μl of anti-insulin guinea pig serum diluted to 1:450,000. After incubation for 20 to 24 hrs at 4°C, 100 μl of ^{125}I -insulin was added. After another incubation period of 20 to 24 hr at 4°C, 1.6 ml of ethanol was added in order to separate the free and the antibody-bound insulin. After thorough mixing and centrifugation for 10 mins at 2500 rpm, the supernatant was discarded and pellets were washed with a solution containing ethanol (960 ml), NaFAM (18ml) and distilled water (162ml) and centrifuged

again and the supernatant discarded. The radioactivity was counted in the pallet using a gamma counter. Concentrations of the unknown were read from the standard curve, and samples with concentrations which fell outside the straight part of the standard curve were repeated. Studies were performed to measure intra and interassay coefficients of variation of the assay and were found to be 5.0% and 9.6% respectively. Samples from Steady State Plasma Insulin (SSPI) during Insulin Sensitivity Test were diluted 1:5 in NaFAM solution and then assayed as described above.

3.1.14 Specific insulin, intact proinsulin and des 31,32 proinsulin

were measured by new monoclonal antibody-based highly specific two-site immunoradiometric assays (IRMAs) (Sobey et al, 1989) and also by modification of these assay to a microplate ELISA called enzymoimmunometric assay (EIMA) (Mohamed-Ali et al, 1992, 1993; Gould et al, 1993). Methods for these assays have been published but are described briefly here.

Preparation and selection of antibodies

Purified antibodies were purchased from Serono Diagnostics (Woking, Sussex, England), following their original development in Prof CN Hales laboratories (Department of Biochemistry, University of Cambridge, England). Mouse monoclonal antibodies reacting with human insulin and intact proinsulin were obtained by fusing mouse spleen cells with NSO mouse myeloma cells. The mouse spleen cells used to produce monoclonal antibody A6 were obtained from a mouse immunised by injecting 20 μg of human proinsulin emulsified with Freund's complete adjuvant. To obtain monoclonal antibody 14B a mouse was immunised with subcutaneous injecting 100 μg of human insulin emulsified with Freund's complete adjuvant. The monoclonal antibody 3B1 obtained from injecting BALB/c mice subcutaneously with 10 μg of biosynthetic human proinsulin in complete Freund's adjuvant.

The monoclonal antibodies A6 and 14B were selected to bind either intact human proinsulin or human insulin respectively to the exclusion of the other. Antibodies for cellulose immunoabsorption were partially purified from ascitic fluid by precipitation with 40% (v/v) saturated $(\text{NH}_4)_2\text{SO}_4$. Antibodies for iodination were further purified on a DEAE-Affigel Blue (BioRad) column and eluted with a NaCl gradient from 0 to 500mM. The antibodies were shown to be pure on electrophoresis on a 10% SDS/polyacrylamide gel.

Preparation of cellulose immunoabsorbant

Partially purified antibodies were coupled to finely divided aminocellulose by diazotisation, reacting 100 mg of protein with 100 mg of cellulose. The resulting immunoabsorbant contained approximately 200 μg of protein per mg of cellulose and had the following binding capacities: cellulose linked 3B1 bound approximately 4 μg of insulin per mg of cellulose, and cellulose linked A6 and 14B bound approximately 2.5 μg of proinsulin and insulin respectively per mg of cellulose.

Iodination of antibodies

Purified monoclonal antibodies were iodinated by iodogen method (Salacinski et al, 1981) using 20 μg of antibody and 0.5 mCi of Na^{125}I (Amersham International, Aylesbury, Buckinghamshire, UK) with 10 μg of iodogen in 50 μl of 0.5M-sodium phosphate, pH 7.5 for 15 min at room temperature. Unreacted iodide was separated on a 1cm x 25cm column of Sephadex G-25 (Pharmacia, Milton-Keynes, England) eluted with 0.05 M-sodium phosphate barbitone buffer, pH 8.0. The specific radioactivity of antibodies was 2.5-3.0 $\mu\text{Ci}/\text{pmol}$ (18-22 $\mu\text{Ci}/\mu\text{g}$).

Standards, recovery and dilutions

Standards were made up in 0.05 M-sodium phosphate barbitone buffer, pH 8.0,

containing 5%(w/v) bovine serum albumin and stored at -20°C until used. Recovery was assessed by dilution of known quantities of the appropriate hormones in plasma sample. Recovery of human insulin (50-100pM) was 78% (range 69-91%) at 1:4 dilution of normal plasma sample. Under the same conditions recoveries of intact, des 31,32 proinsulin (5-100 pM) were 77% (60-91%) and 75% (70-80%) respectively. Similarly plasma samples were diluted to ensure that these dilutions were parallel to the standard curve.

Intra and interassay variation

The intra-assay precision of the assay as determined by replicate analysis of samples gave coefficients of variation of < 10% over the concentration ranges 20-476 pM, 22-930 pM, 8-230 pM for insulin, intact proinsulin and des 31,32 proinsulin respectively. The interassay precision (coefficient of variation) determined from the mean of duplicate assay in different assay was < 15% over a concentration range 23-127 pM and 2-209.4 pM for insulin and intact proinsulin. Precision was also estimated by the method of Ekins (Ekins, 1983) from the precision of duplicates in at least 10 assays. Coefficients of variation were < 15% over the concentration ranges 3-240 pM, 0.5-50 pM and 12-30 pM for insulin, intact proinsulin and des 31,32 split proinsulin.

Two-site assay for human proinsulin

Iodinated monoclonal antibody A6 (50 μ l, approximately 20,000 cpm, containing 12% (v/v) of normal mouse serum) was added to either unknown plasma sample, intact proinsulin standard or quality control plasma (200 μ l), mixed and incubated for 24 h at 4°C. Cellulose bound monoclonal antibody 3B1 (50 μ l, binding approximately 30 ng of insulin/50 μ l) was then added, mixed and incubated for a further 24-hr at 4°C. At the end of this period, 0.5 ml of ice-cold 0.025M-sodium barbitone buffer, pH 8.0 was added to each tube and mixed. The tubes were centrifuged at 8300 g (Beckman

microfuge) for 3 mins at room temperature and supernatant removed by aspiration. The pellets were washed again by the addition of ice-cold sodium barbitone buffer containing Tween 20 (0.5%, v/v) and centrifuged again at 8300 g for 3 mins and the supernatant was removed by aspiration. The pellets were counted for 10 mins in a Nuclear Enterprise 1600 Gamma Counter. Standard curves and unknowns were calculated by using the Nuclear Enterprise RIA Software and an Apple IIe computer.

Two-site assay for human insulin

The assay was carried out as above using iodinated monoclonal antibody 3B1 and cellulose bound 14B. The cross reactivities with intact proinsulin and des 31,32 split proinsulin were $5.3 \pm 1.4\%$ and $5.0 \pm 1.0\%$ respectively. The assay detected 2.3 pM of insulin.

Two-site assay for des 31,32 proinsulin

This assay was carried out using cellulose bound antibody 3B1 and iodinated anti C-peptide antibody PEP 001 (Novo-Nordisk, Cambridge, England). Human insulin at concentration 500 pM did not react in this assay. The cross reactivities of intact proinsulin was $84.0 \pm 2.0\%$. The assay detected 2.5 pM of des 31,32 proinsulin.

All the assays described above were developed in Prof CN Hales laboratories (Department of Biochemistry, University of Cambridge, England) and were used to measure insulin, intact proinsulin and des 31,32 proinsulin in studies described in chapters 4 and 7. These were modified as described below by Vidya Mohammed-Ali, validated against the original assays and were used to measure insulin, intact proinsulin and des 31,32 proinsulin in studies described in chapters 5 and 6.

Modifications of insulin assay

The above assays were modified to an end-point amplified enzyme immunoassay for insulin (IEMA) using same antibodies (Mohamed Ali et al, 1992). Monoclonal antibodies 14B and 3B1 were used in a two site assay with 14B as a trap antibody, and detection with a 3B1-alkaline phosphatase conjugate. The signal was amplified by co-factor cycling via the alcohol dehydrogenase /diaphorase system for increased sensitivity. The assay for insulin was sensitive to 2.0 pM. Intrassay coefficient of variation (CV) for the same plasma sample was 8.7% for concentrations above 15pM with an interassay CV of 12%. Cross-reactivity with intact proinsulin was less than 5% and the recovery of insulin from spiked plasma carried out on 8 separate occasions was 96% (88-105%).

Modification of intact and des 31,32 proinsulin assay

Original assays as described by Sobey et al for intact and des 31, 32 proinsulin required large amount of antibodies. These assays were modified to a microplate method (Mohamed Ali et al, 1993) using previously characterised antibodies 3B1, A6 and anti C-peptide antibody PEP001. Plates were coated with 3B1 (1µg/ml), a sandwich formed with intact proinsulin or des 31,32 proinsulin, and detection by ¹²⁵I labelled A6 or PEP001 respectively and radioactivity of individual wells counted. Detection limit defined as mean + 3SD of zero signal for intact and des 31,32 proinsulin were 0.25 pM and 0.125 pM compared to 2.5 pM for previously described IRMAs. Intra- and interassay CVs were 6.3 and 9.8% respectively for intact proinsulin and 8.6 and 12.3% for des 31,32 proinsulin. There was no cross-reaction with insulin in either assay but 78% cross-reaction with des 64-65 proinsulin in the intact proinsulin assay and 59% and 54% with intact and des 64-65 proinsulin in des 31,32 proinsulin assay. Therefore des 31,32 proinsulin concentrations were calculated by subtracting the cross-reactivity of measured proinsulin. Recovery of intact or des 31,32 proinsulin added to human plasma (3-33 pM) was 93% (83-101%) and 89% (84-99%)

respectively. These microplate IRMAs for proinsulin and des 31,32 proinsulin were thus more sensitive, and relatively easy for preparation of immobile phase and for sample throughput than the original assays described by Sobey et al.

3.1.15 Plasma C-peptide

Plasma C-peptide was measured using a radioimmunoassay kit for human C-peptide (Antiserum K6, Novo Pharmaceuticals, Basingstoke, Hants, UK) (Heding, 1975). The intra-assay CV was 4.7% and inter-assay CV 8.8%. This assay has 75% cross reactivity with human proinsulin, but because the usual concentrations of proinsulin in diabetic subjects are approximately 4% of those of C-peptide, this cross reaction is of little relevance.

Radioimmunoassay procedure

Duplicates of 100 μ l of standard solution (0 to 0.5 pmol/ml of C-peptide) or samples were added 100 μ l of anti-C-peptide guinea pig serum diluted to 1:18,000. After incubation for 20 to 24 hrs at 4°C, 100 μ l of ¹²⁵I-C-peptide was added and after another incubation period of 20 to 24 hr at 4°C, 1.6 ml of 96% ethanol (v/v) was added in order to separate the free and the antibody bound insulin. After thorough mixing and centrifugation for 10 min at 2500 rpm, the supernatant was discarded and pallets were washed with a solution containing 96% ethanol (960 ml), NaFAM (18ml) and distilled water (162ml) and centrifuged again for 10 min and supernatant discarded. The radioactivity was counted in the pallet using a gamma counter. Concentrations of the unknown were read from the standard curve, and samples with concentrations which fell outside the straight part of the standard curve were repeated. Studies were performed to measure intra- and inter-assay coefficients of variation of the assay and were found to be 4.7% and 8.8% respectively.

3.1.16 Insulin resistance

3.1.16.1 Description of the method

Insulin resistance was measured using a modification of Harano method (Harano et al, 1977). Subjects were given a fixed amount of 20% glucose (6mg/kg/min) and soluble insulin 50 mU/kg/h as separate intravenous infusions. Soluble human insulin (Actrapid, Novo Nordisk, Basingstoke, Hants, UK), was used in 100ml of sodium chloride solution (154 mM) and infused through a Travenol Floguard pump (Baxter Health Care, Compton, Newbury, Berks, UK). Infusions were continued for a total period of 150 mins and arterialised samples were taken from the opposite arm every 5 mins for the last 30 mins from 120 to 150 mins. Steady state plasma glucose (SSPG) was calculated from the mean of seven samples taken between 120 to 150 mins and this reflected insulin resistance. Metabolic clearance rate was calculated by dividing the glucose infusion rate by steady state plasma glucose.

The insulin sensitivity test (IST) without pancreatic suppression is a simple way to quantify insulin resistance and previous studies have shown that insulin resistance thus measured correlates with that measured using euglycaemic insulin clamp ($r=0.83$) (Heine et al, 1985). There have been no studies to assess reproducibility of this test to see if it is suitable to be used in intervention studies to measure insulin resistance. Therefore, studies were performed to assess the reproducibility of this test.

3.1.16.2 Validation and reproducibility studies of IST

Initial experiments were done to see if it is possible to achieve a steady state glucose and a steady state insulin concentration in subjects with type 2 diabetes who are heterogenous in respect to body mass index and fasting plasma glucose, and who may have different metabolic clearance rates of insulin. 21 diet controlled subjects were studied twice at three month intervals.

The clinical and biochemical details of the subjects are shown in table 3.1. There were no significant differences between the various measurement during these two visits other than body mass index which was minimally but significantly lower at the second visit. Fasting plasma glucose and fasting insulin concentrations were similar at two visits. Steady state plasma insulin concentrations did not differ significantly from each other during the two visits.

i Steady state plasma insulin (SSPI) during IST

Insulin concentrations were measured in the fasting state and then at 120, 135, 150 min following infusion of glucose and insulin. A satisfactory steady state insulin concentrations (CV of <10% during period of steady state) was achieved in most experiments (median CV 4.3%, range 1.6-13%), only on one occasion (1/42 = 2.4%) did the CV of steady state insulin concentrations exceeding 10%. There was however a huge inter-individual variation in the actual steady state insulin concentrations achieved (Fig 3.3). The intra-individual variation between steady state plasma insulin when each subject underwent the test twice was 4.8% (range 1.6-13%).

There was a highly significant relationship ($r_s=0.79$) between fasting plasma insulin and steady state plasma insulin concentrations, suggesting that given a constant infusion of insulin, steady state plasma insulin is a function of the prevailing fasting insulin concentrations (Fig 3.4).

It is generally believed that to compare insulin resistance between subjects using this method one has to achieve similar steady state plasma insulin concentrations and this prerequisite is achieved during euglycaemic clamp and during Insulin Sensitivity Test.

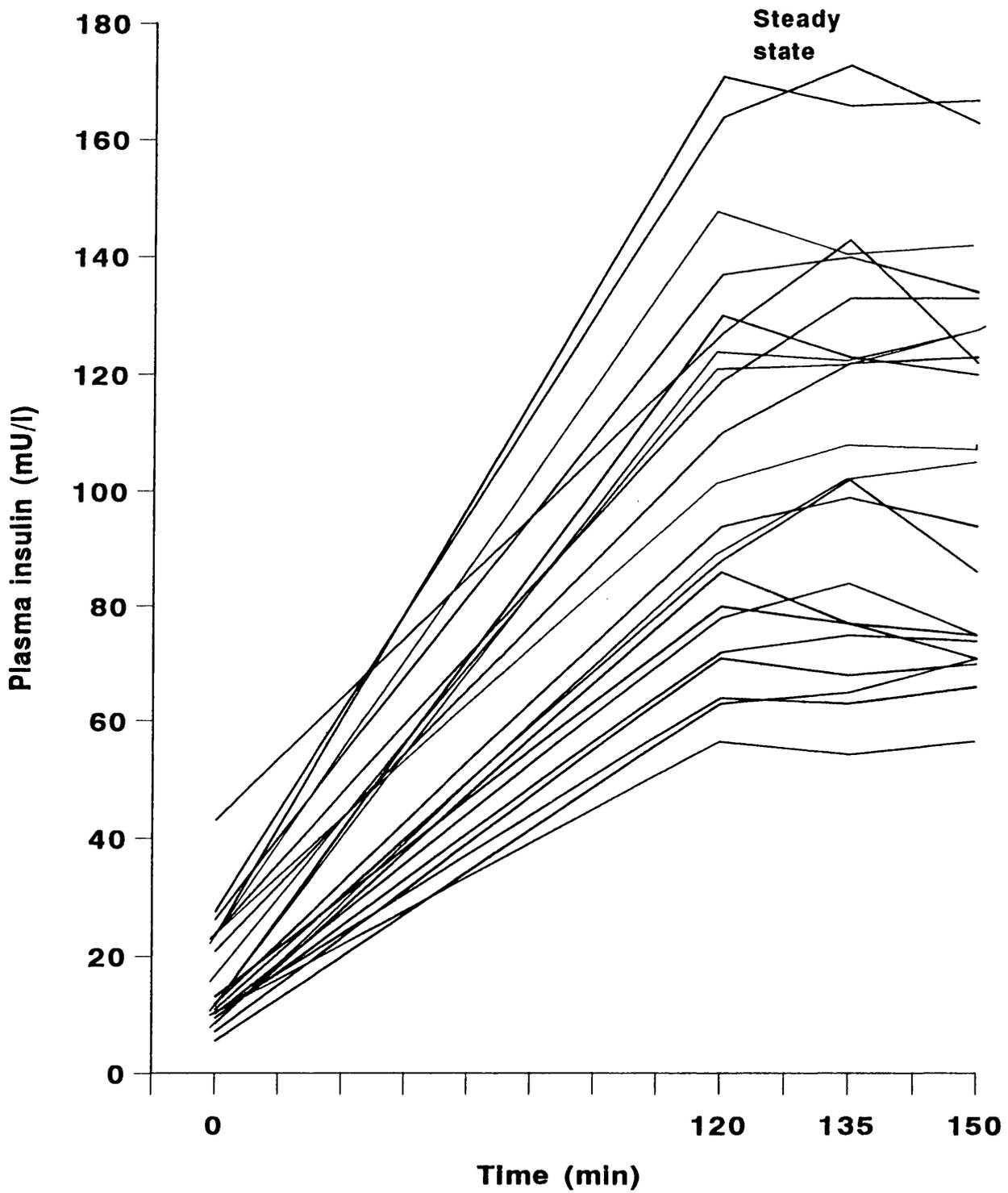


Figure 3.3: Fasting and steady state plasma insulin (SSPI) concentrations during Insulin sensitivity test

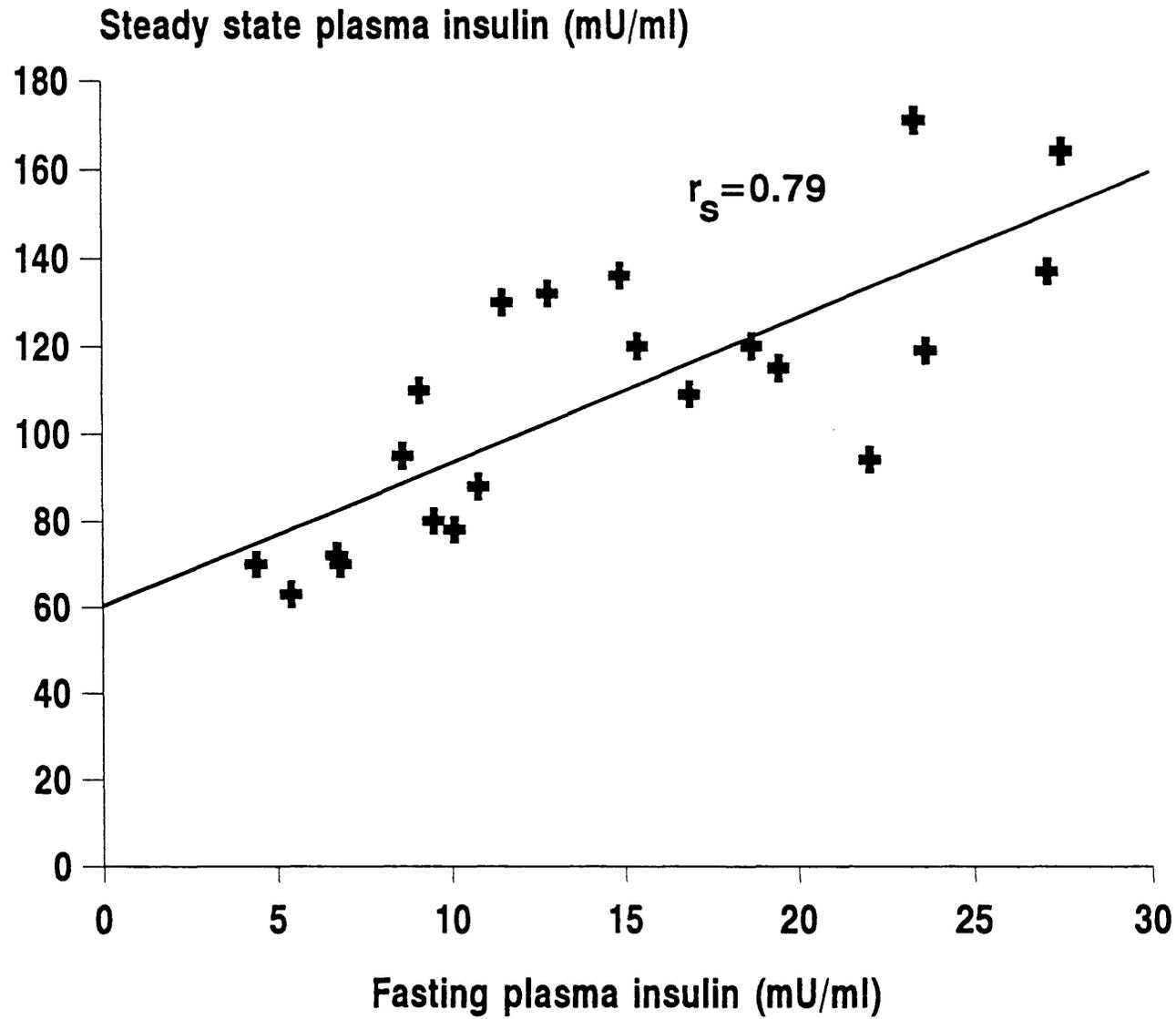


Figure 3.4: Relationship between fasting insulin concentrations and steady state plasma insulin during insulin sensitivity test.

Table 3.1: Clinical and biochemical details of subjects at visit 1 and visit 2

Variable	Visit 1 n=21	Visit 2 n=21	P value
Body mass index (kg/m ²)	28.3 ± 5.2	27.9 ± 5.3	<0.02
Fasting plasma glucose (mmol/l)	11.0 ± 3.5	11.6 ± 4.6	ns
Fasting plasma insulin (µu/l)	16.6 ± 9.2	17.2 ± 9.8	ns
Steady state plasma insulin (µu/l)	107.8 ± 34.6	112.8 ± 33.7	ns
Steady state plasma glucose (mmol/l)	11.8 ± 5.1	12.3 ± 4.9	ns
Metabolic clearance rate of glucose (ml/kg/min)	3.4 ± 1.6	3.2 ± 1.3	ns

Data shown as Mean ± SD

with pancreatic suppression (Greenfield et al, 1981). However it is obvious from the above experiments that it is not possible to achieve a similarly constant SSPI in between individuals during IST without pancreatic suppression. However this inter-individual variation in SSPI is not of great significance when studying effects of intervention where subjects act as their own controls, but is important when comparing different subjects in terms of insulin resistance using IST without pancreatic suppression.

ii Steady state plasma glucose (SSPG) during IST

Steady state plasma glucose concentrations reflects insulin resistance, i.e. the higher the SSPG the higher the insulin resistance (Greenfield et al, 1981). It was possible to achieve a steady state in plasma glucose concentrations in nearly all experiments (Fig 3.5) and coefficient of variation for SSPG was $3.6 \pm 2.1\%$. There was a significant relationship between fasting plasma glucose concentrations and SSPG ($r=0.40$, $p=0.039$), at the first visit but not at the second visit ($r=0.32$, $p=0.11$).

iii Reproducibility of IST

The metabolic clearance rate (MCR) of glucose at both visits is shown in figure 3.6. It is apparent that the variation in the MCR of glucose from first to second visit was a random one in either direction and there was no significant change in the mean MCR of glucose in whole group from first to second visit. In figure 3.7 MCR of glucose at the second visit is shown as a percent change from that at the first visit.

The mean coefficients of variation (CV) were calculated for various measures of insulin resistance and are shown in table 3.2. The coefficient of variation of SSPG during IST without pancreatic suppression is high but compares well to the reported coefficient of variation for SSPG during IST with pancreatic suppression and MCR of glucose

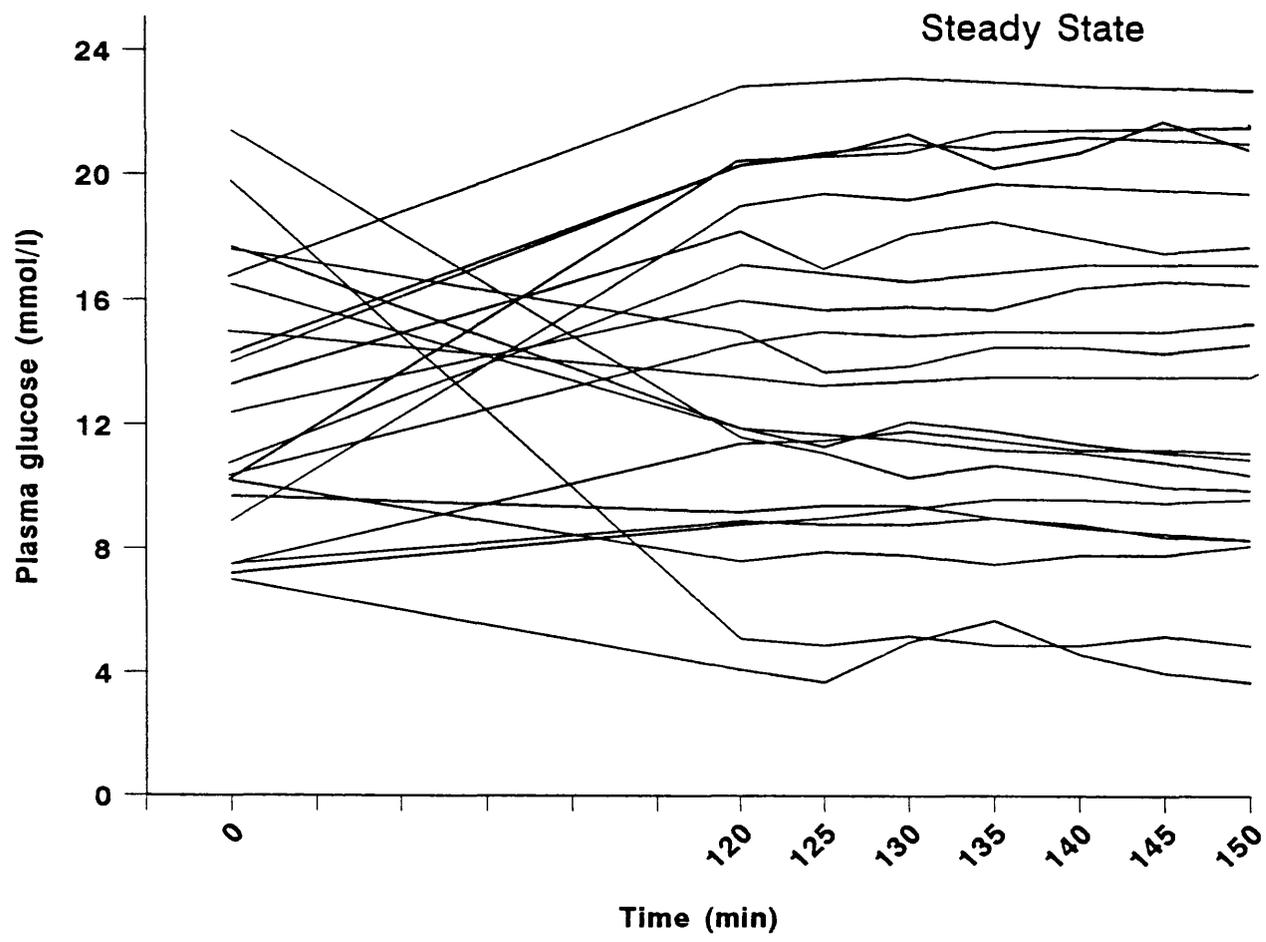


Figure 3.5: Steady state plasma glucose (SSPG) during insulin sensitivity test

MCR glucose (ml/kg/min)

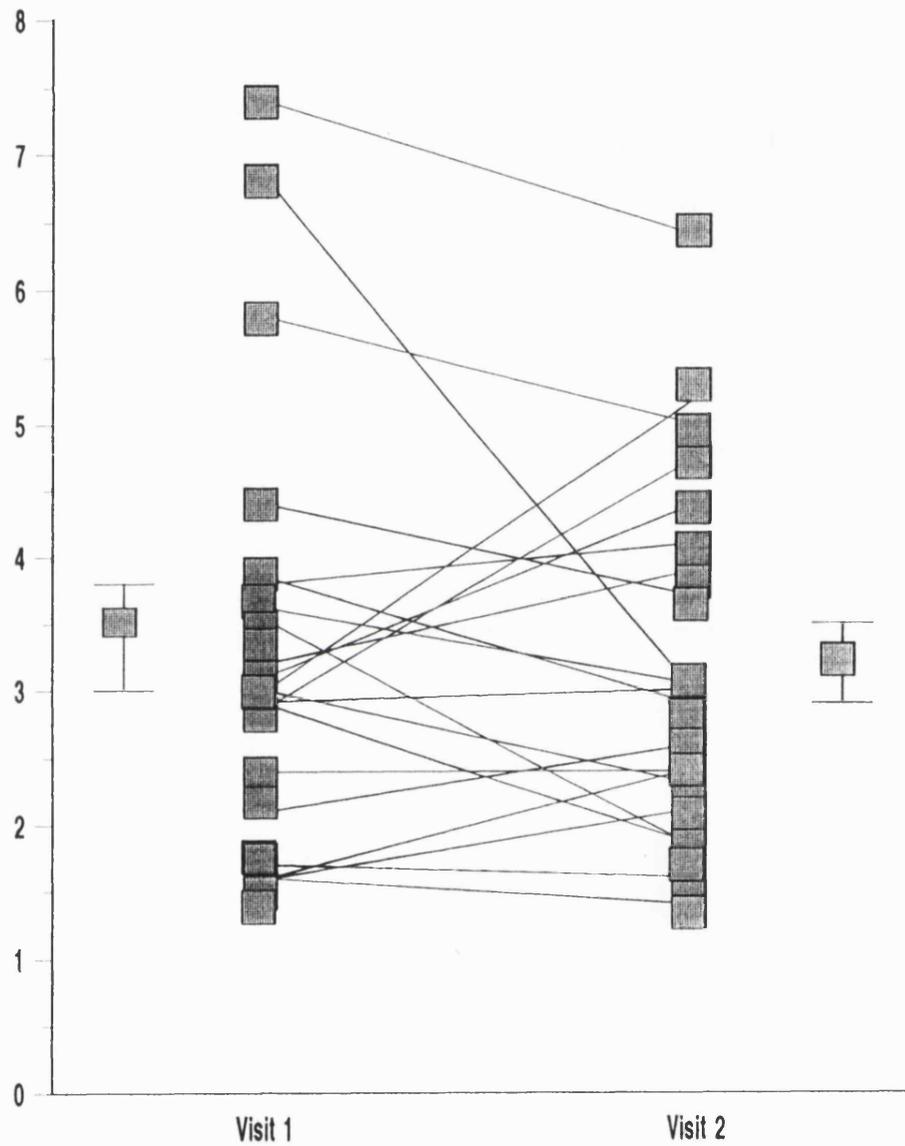


Figure 3.6: Metabolic clearance of glucose (MCR) at visit 1 and visit 2

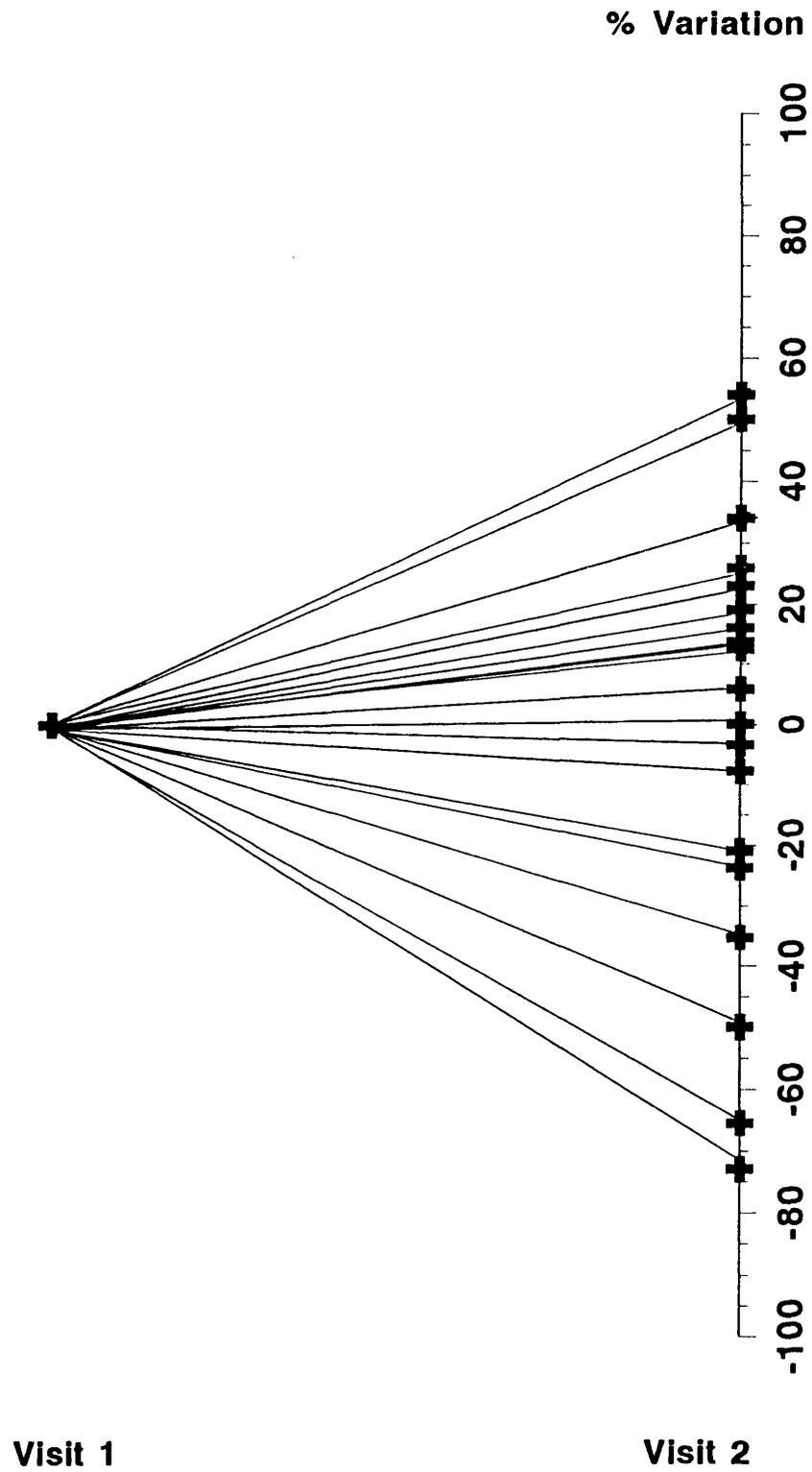


Figure 3.7: Percent variation in MCR of glucose from visit 1 to visit 2

measured by the hyperinsulinaemic euglycaemic clamps (CV = 19%) (Greenfield et al, 1981).

3.1.17 Beta-cell function and insulin resistance

were also estimated by Homeostasis Model Assessment (HOMA), which employs fasting concentrations of glucose along and those of insulin or C-peptide to derive estimates of β -cell function and insulin sensitivity, based on a mathematical model (Matthews et al, 1985). While it is recognised that the absolute values of these estimates depend upon the insulin assay employed, the model is still valid for comparing within subject changes in estimates between therapy periods and also to compare populations provided the insulin measurements were done using the same assay. Moreover, the use of C-peptide concentrations in the model along with fasting glucose circumvents the problems of variability from using different insulin assays with different specificities.

3.1.18 Physical Activity

Physical activity was assessed from a brief questionnaire asking about occupational and leisure time physical activity and was graded as follows;

GRADE

- 0 No regular leisure time physical activity and a sit down job.
- 1 No organised physical activity during leisure time, but 3 to 4 hours per day of walking or standing.
- 2 Sporadically involved in physical activities (such as weekend jogging, cycling, tennis, swimming etc)
- 3 Consistent job activities such as lifting or stair climbing/ and or participating regularly in recreational activities such as jogging, cycling, basketball at least three times a week for 30 to 60 min per session.

Table 3.2: CV's of various measures of insulin resistance.

Variable	% Variation
Fasting plasma glucose	16
Steady state plasma glucose	24
Fasting plasma insulin	27
Steady state plasma insulin	9
MCR glucose (ml/kg/min)	24

It is acknowledged that this method provides a rather crude index of physical activity. However we observed this method to be a satisfactory one for two reasons; firstly when used in a large study sample (n=310) of diabetic and non-diabetic subjects, expected associations of physical activity using this method with diabetes were observed (unpublished observations, DKN). Secondly we found a good correlation of physical activity measured using this method with a well validated method to measure physical activity (Kriska et al 1993).

3.1.19 Ischaemic heart disease:

Resting 12 lead electrocardiographs were coded using Minnesota coding. Ischaemic heart disease (IHD) was diagnosed by the use of The Whitehall criteria, defined as presence of one or more of the following Minnesota codes: abnormalities of Q/QS waves (1.1-1.3); S-T depression (4.1-4.4); T-wave inversion or flattening (5.1-5.3); or left-bundle-branch block (7.1) (Fuller et al, 1983).

3.3.20 Urine albumin and creatinine:

Urine albumin was measured by an in-house modified competitive enzymeimmunoassay (Gould et al, 1993), validated against a commercial radioimmunoassay (Pharmacia-LKB, Milton Keynes, Bucks, UK) (Chesham et al, 1986) ($r=0.96$). Urine creatinine by measured by Jaffe's method. Urine albumin to creatinine ratio was calculated.

3.2 Personal role in various measurements:

I was responsible for setting up and validating the Insulin Sensitivity Test in the department where these studies were conducted and also studied the reproducibility of this test. I had a major role in setting up the PAI-1 activity assays in the department which was subsequently used in the studies described in this dissertation.

Assays for immunoreactive insulin, C-peptide, PAI-1 activity, β TG and PF4 in all the studies were performed by myself. Studies of platelet function in whole blood and for platelet rich plasma, used in Chapter 6 were mostly performed by myself with some help from Dr Stavros Pappas.

Assays for total cholesterol, HDL-cholesterol, total triglyceride and fibrinogen were performed in the Department of Chemical Pathology, Whittington Hospital, London, while those for lipoprotein analysis in Chapter 7 were performed at Department of Chemical Pathology, St Thomas's Hospital, London.

Assays for insulin and proinsulin like molecules for studies in Chapter 4 and 7 were performed in Prof Nick Hales Laboratory in Department of Biochemistry, Addenbrook's Hospital, Cambridge, and those for studies in Chapter 5 and 6 by modification of these assays by Vidya Mohamed-Ali in the Department of Medicine, Whittington Hospital, London.

CHAPTER 4: RELATIONSHIP OF CONCENTRATIONS OF PROINSULIN-LIKE MOLECULES WITH CARDIOVASCULAR RISK FACTORS IN ASIAN AND CAUCASIAN SUBJECTS WITH TYPE 2 DIABETES

4.1 Introduction

The measurement of insulin in plasma by radioimmunoassay (Yalow & Berson, 1959) made it possible to investigate the role of insulin in the aetiology of type 2 diabetes. The subsequent discovery of proinsulin (Steiner & Oyer, 1967) and the finding that proinsulin is present in human plasma (Roth et al, 1968) raised the important question of insulin assay specificities. It was generally believed, though never experimentally confirmed, that proinsulin like molecules may cross react in radioimmunoassays for insulin which employed polyclonal antibodies.

In normal subjects, during the process of insulin secretion from the pancreas, for each molecule of insulin released, an equivalent amount of C-peptide is also released into the portal circulation (Polonsky et al, 1984). Small amounts of intact proinsulin (about 5%) escapes into the circulation in normal subjects (Duckworth et al, 1972; Mako et al, 1977). However, due to its half life being longer than that of insulin (Starr et al, 1974), at any given time point the steady state levels of proinsulin are approximately 10-15% of insulin in the circulation. Therefore, this question of assay specificity is unlikely to represent a major problem in non-diabetic subjects. However fasting proinsulin concentrations are known to be raised in subjects with type 2 diabetes, both at fasting and during an oral glucose tolerance test (Duckworth et al, 1972; Gordon et al, 1974; Mako et al, 1977; Ward et al, 1987; Yoshioka et al, 1988; Temple et al, 1989).

To date no information is available on proinsulin-like molecules in non-diabetic and diabetic Asian subjects in whom previous studies have demonstrated hyperinsulinaemia (Mohan et al, 1986; McKeigue et al, 1992, Knight et al, 1993) and increased insulin

resistance compared with caucasian subjects (Sharp et al, 1987; McKeigue et al, 1990).

4.2 Study design, methods and aims

In this preliminary investigation fasting blood samples from 51 (25 Asian, 26 Caucasian) subjects with type 2 diabetes were examined for concentrations of insulin and the insulin precursor molecules, intact and des 31,32 proinsulin, and for risk factors for cardiovascular disease . Subjects were recruited at random from a busy district general hospital diabetic clinic and attended the Clinical Investigation Unit at the Whittington Hospital between 8-10 am after an overnight fast. Insulin, intact proinsulin and des 31,32 proinsulin were measured using sensitive and highly specific monoclonal antibody-based assays and concentrations were compared with those of total immunoreactive insulin (IRI) measured using a conventional insulin radioimmunoassay.

The aims of this study were, firstly, to look at the contribution made by intact proinsulin and des 31,32 proinsulin to the hyperinsulinaemia of type 2 diabetes; secondly, to analyse the relationship of concentrations of insulin and its precursor molecules to those of standard cardiovascular risk factors; and thirdly, to study the contribution of insulin precursor molecules to previously reported differences in insulin levels between the Asian and caucasian subjects.

4.3 Results

4.3.1 Clinical and biochemical characteristics

These are shown in table 4.1. Asian subjects were significantly younger, lighter and had worse glycaemic control than caucasians. Asian subjects had significantly longer duration of diabetes but were similar in terms of treatment category for diabetes.

4.3.2 Contribution of intact proinsulin and des 31,32 proinsulin to the hyperinsulinaemia of subjects with type 2 diabetes.

The major circulating insulin precursor molecule was des 31,32 proinsulin, comprising a mean concentration of $47.8\% \pm 12.4\%$, while concentrations of insulin and intact proinsulin were $37.7\% \pm 15.0\%$ and $12.4\% \pm 5.6\%$ respectively of the sum of three insulin-like molecules. In these subjects the concentration of "IRI" were $99.1\% \pm 25.9\%$ of the sum of all insulin-like molecules. Insulin concentrations measured by the specific assay correlated significantly with IRI concentrations ($r = 0.84$; $p < 0.001$) (Fig 4.1). Similarly there was a significant relationship between IRI and the sum of insulin and proinsulin-like molecules ($r = 0.88$; $p < 0.001$) (Fig 4.2).

4.3.3 Concentrations of insulin precursor molecules and cardiovascular risk factors in the two ethnic groups

These results are shown in table 4.2. Asian subjects had significantly higher concentrations of the sum of all insulin like molecules and those of des 31,32 proinsulin than caucasians but when these concentrations were compared using analysis of variance to include age, gender, and treatment into the analysis these differences were no longer significant. There were no effects of treatment categories or gender on the concentrations of these molecules. The percentage of insulin, intact proinsulin and des 31,32 proinsulin according to body mass index are shown in table 4.3. There were no significant differences in the concentrations of these molecules between obese and non-obese diabetic subjects. There were no significant differences between Asian and caucasian subjects in the concentrations of total triglyceride, total and LDL-cholesterol, HDL-cholesterol or plasminogen activator inhibitor (PAI-1) activity.

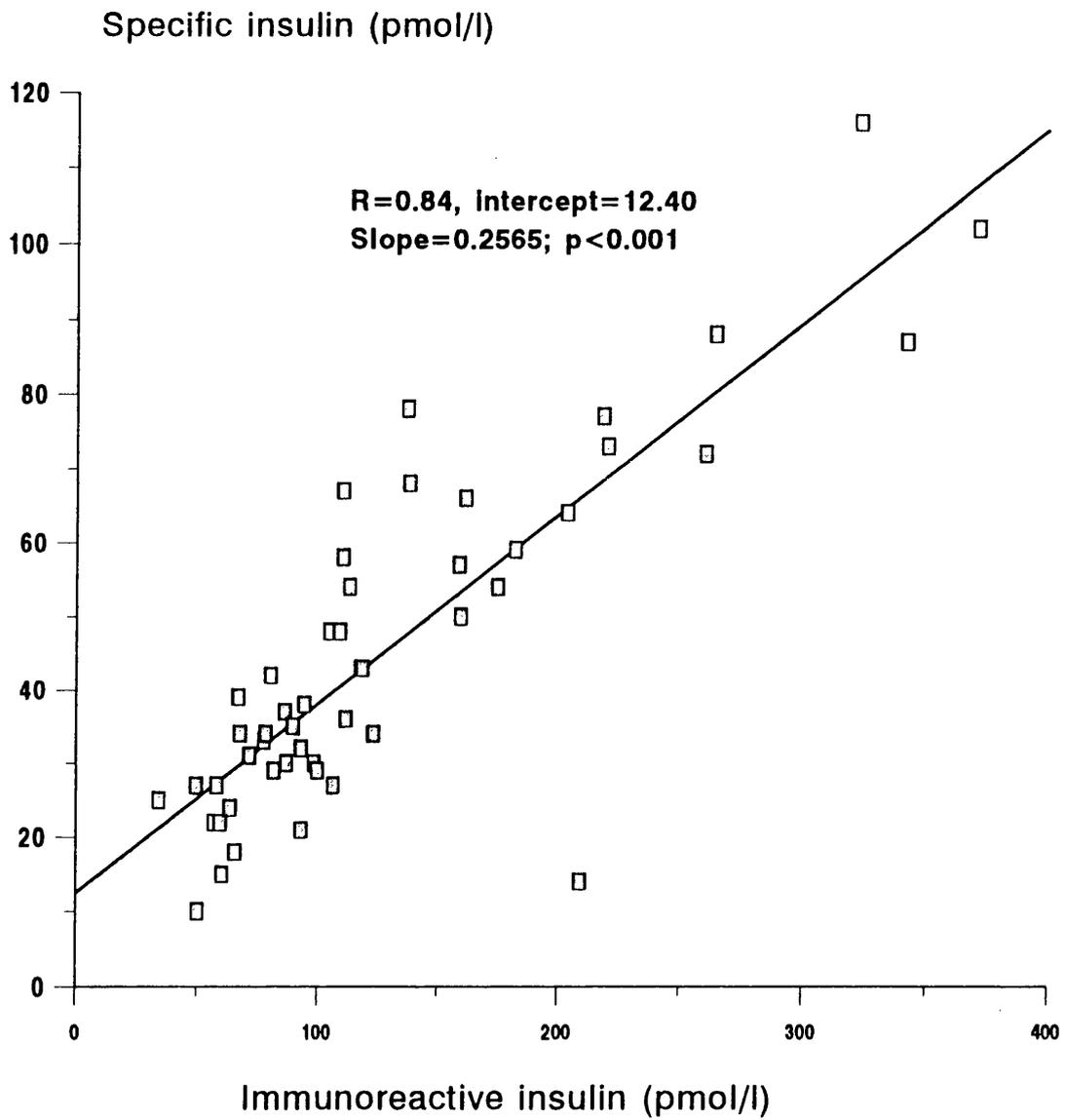


Figure 4.1: Relationship of fasting concentrations of specific insulin with IRI in subjects with type 2 diabetes

Sum of insulin, intact proinsulin
and des 31,32 proinsulin (pmol/l)

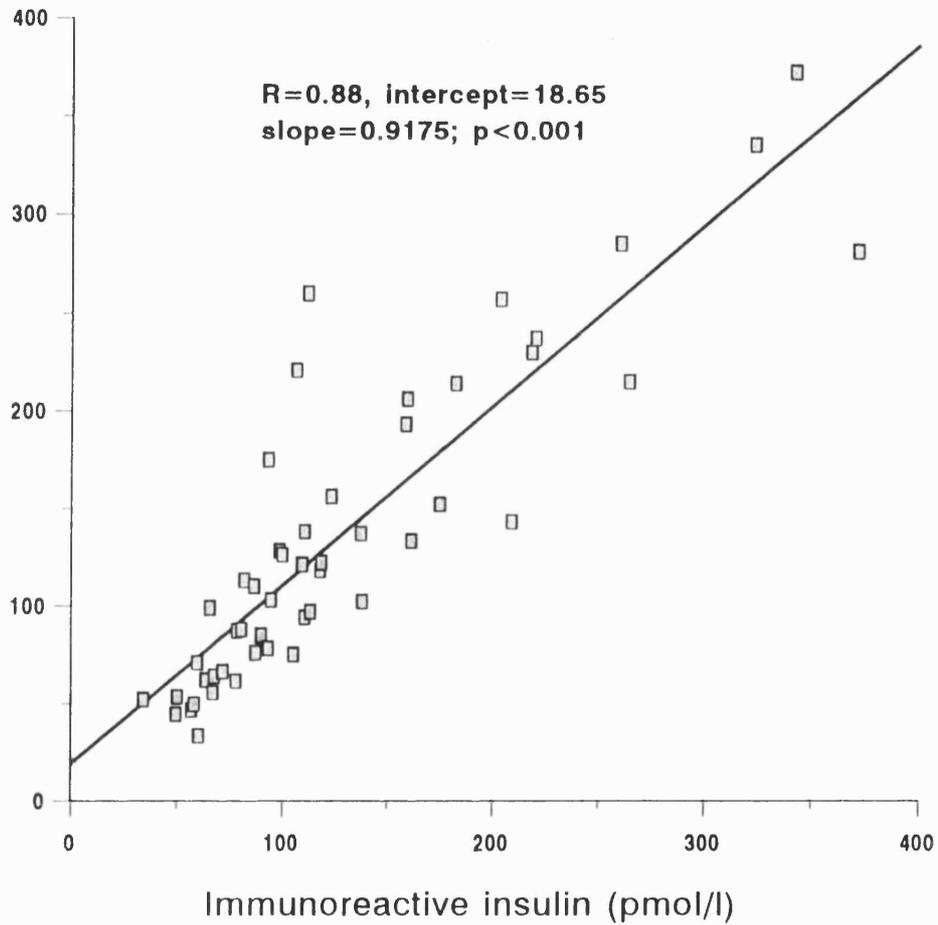


Figure 4.2: Relationship of fasting concentrations of IRI with insulin-like molecules in subjects with type 2 diabetes

Table 4.1: Clinical characteristics of subjects type 2 diabetes

Variables	Asians n= 25	Caucasians n= 26	Significance of difference
Age (yrs)	50 ± 9	55 ± 8	0.02
Gender (M, F)	17,8	12,14	ns
Treatment			
Diet alone	11	9	
Diet + Metformin	1	4	ns
Diet + Sulphonylurea	10	9	
Diet + both	3	4	
Duration of diabetes (yrs)	4.0 (0.5-6)	2.7 (0.75-14)	ns
Fasting plasma glucose (mmol/l)	10.2 ± 3.6	8.6 ± 2.6	0.08
Glycated haemoglobin (%)	9.8 ± 2.2	8.6 ± 1.6	0.01
Body mass index (kg/m ²)	24.0 (18.6-40.0)	27.2 (21.4-38.4)	0.04
Waist-hip-ratio	0.92 ± 0.06	0.88 ± 0.07	ns
Subscapular-triceps-ratio	1.23 ± 0.20	1.32 ± 0.31	ns
Systolic blood pressure	120 (100-160)	122 (91-180)	ns
Diastolic blood pressure	78 (64-98)	74 (51-110)	ns

Values are expressed as mean ± SD for normally distributed data or as median (range) for skewed data

Table 4.2: Concentrations of insulin, insulin-like molecules, lipids and plasminogen activator inhibitor in Asian and caucasian subjects with type 2 diabetes

Variables	Asian n = 25	Caucasian n = 26	Significance of difference
Specific insulin (pmol/l)	45.5 (10-116)	34.0 (14-78)	ns
Intact proinsulin (pmol/l)	23.0 (2.4-52)	13.4 (1.6-46)	0.07
Des 31,33 proinsulin (pmol/l)	66.0 (15-237)	37.5 (11-167)	0.01
*Sum (pmol/l)	130.0 (44-372)	91.0 (33-285)	0.04
Immunoreactive insulin (pmol/l)	112 (34-372)	93 (57-261)	ns
Total cholesterol (mmol/l)	5.67 ± 1.12	5.96 ± 1.13	ns
Total triglyceride (mmol/l)	2.0 (0.5-22.4)	1.6(0.5-10.0)	ns
HDL-cholesterol (mmol/l)	0.97 ± 0.17	0.98 ± 0.20	ns
LDL-cholesterol (mmol/l)	3.71 ± 0.90	4.17 ± 1.15	ns
PAI-1 (AU/ml)	10.2 (5-51)	9.2 (4-42)	ns

Values are expressed as mean (SD) for normally distributed data or as median (range) for skewed data

*Sum = (Specific insulin + intact proinsulin + des 31,32 proinsulin)

4.3.4 Relationship of insulin and insulin precursor molecules with cardiovascular risk factors

There were significant positive relationships between the concentrations of IRI and those of total cholesterol and total triglyceride, and a negative relationship with HDL-cholesterol (table 4.4). The relationships of IRI with PAI-1 and LDL-cholesterol were not significant. However when these relationships were reanalysed employing concentrations of insulin measured by the specific assay only the relationship with triglyceride remained significant. Concentrations of des 31,32 proinsulin in these subjects correlated positively with those of cholesterol, triglyceride and plasminogen activator inhibitor, and negatively with those of HDL-cholesterol. The relationships between concentrations of these molecules and these risk factors were little affected by including gender, age, race and body mass index in the analysis, other than that with HDL-cholesterol which were weakened in such an analysis (table 4.5). Concentrations of IRI and those of specific insulin showed significant positive correlations with both systolic and diastolic blood pressure while intact proinsulin and des 31,32 proinsulin correlated with diastolic blood pressure only. In a multiple regression analysis to control for other confounding factors, when concentration of IRMA insulin were entered into the model after age, gender, race and body mass index, the relationship with diastolic blood pressure was not significant (F change = 2.51 (5,43 df), $p=0.12$) but both intact proinsulin and des 31,32 proinsulin were significantly related to diastolic blood pressure in separate models ($F = 4.40$ (5,43 df), $p=0.042$; $f = 4.67$ (5,43 df), $p=0.036$) respectively. When all these insulin like molecules were entered into the model the significance of F change was the largest for des 31,32 proinsulin and neither of the other molecules improved the model significantly (table 4.6). In a similar analysis using systolic blood pressure as the dependent variable, none of the insulin like molecules made a significant contribution to the model when entered after age, gender, race and body mass index.

Table 4.3: Percentage of fasting specific insulin, intact proinsulin and des 31,32 proinsulin concentrations in non-obese and obese subjects with type 2 diabetes

Variables	Non-obese (BMI¹ < 27)	Obese (BMI¹ ≥ 27)	Significance of difference
Number	29	22	ns
Specific insulin	41 (12-70)	34 (10-62)	ns
Intact proinsulin	14 (3-29)	15 (8-21)	ns
Des 31,32 proinsulin	44 (25-77)	52 (31-69)	ns

Values shown as median (range)

¹ BMI = Body mass index

Table 4.4: Correlation coefficients of insulin and proinsulin-like molecules in all subjects

Variables	IRI	Specific insulin	Des 31,32 proinsulin	Intact proinsulin
Age	-0.22	-0.06	-0.42 ¶	-0.32 *
Body mass index	0.30 *	-0.26 *	0.22	0.14
Waist-hip-ratio	0.16	-0.03	0.19	0.18
Subscapular-triceps-ratio	0.08	-0.02	-0.03	0.05
Fasting glucose	0.09	0.03	0.17	0.26 *
Total cholesterol	0.25 *	0.17	0.23 *	0.21
Total triglyceride	0.42 §	0.36 ¶	0.33 ¶	0.29 *
HDL-cholesterol	-0.30 *	-0.06	-0.29 *	-0.29 *
LDL-cholesterol	0.11	0.01	0.09	0.11
PAI-1	0.14	0.08	0.26 *	0.13
Systolic blood pressure	0.24 *	0.29 *	0.14	0.17
Diastolic blood pressure	0.48 §	0.42 §	0.31 *	0.33 ¶

All analyses were performed using Spearman rank correlation.

IRI = immunoreactive insulin

*p<0.05 ¶ p<0.01 § p<0.001

Table 4.5: Partial correlation coefficients \ddagger of insulin and proinsulin-like molecules in all subjects

Variables	IRI	Specific insulin	Des 31,32 proinsulin	Intact proinsulin
Total cholesterol	0.28*	0.14	0.31*	0.25
Total triglyceride	0.37§	0.30*	0.29*	0.25
HDL-cholesterol	-0.13	-0.04	-0.08	-0.11
LDL-cholesterol	0.19	0.02	0.21	0.18
PAI-1 activity	0.12	0.04	0.21	0.14
Systolic blood pressure	0.33*	0.28*	0.25	0.23
Diastolic blood pressure	0.47§	0.33¶	0.28	0.30*

\ddagger All analyses were performed using Spearman rank correlation and adjusted for age, sex, ethnicity and body mass index.

IRI = immunoreactive insulin

* $p < 0.05$ ¶ $p < 0.01$ § $p < 0.001$

Table 4.6: Multiple regression analysis for determinants of diastolic blood pressure

Variables in Model	Variable added	F Change (df)	Multiple r²	Significance
	Sex	0.01 (1,47)	0.0002	ns
Sex	Race	1.33 (2,46)	0.028	ns
Sex, Race	Age	2.36 (3,45)	0.077	ns
Sex, Race, Age	BMI	4.71 (4,44)	0.166	0.036
Sex, Race, Age, BMI	des 31,32 proinsulin	6.91 (5,43)	0.277	0.012
Sex, Race, Age, BMI, des 31,32 proinsulin	specific insulin	0.56 (6,42)	0.288	ns
Sex, Race, Age, BMI, des 31,32 proinsulin, specific insulin	intact proinsulin	0.31 (7,41)	0.296	ns

Variables with skewed distribution (insulin, intact proinsulin, des 31,32 proinsulin) were log transformed to normalise their distribution.

4.4 Discussion

Insulin is produced in the pancreatic beta cells from its precursor molecule proinsulin. During the process of insulin secretion in normal subjects a small amount of proinsulin also escapes into the circulation. Several studies have shown a disproportionate elevation in the circulating proinsulin concentrations in various disease states including subjects with type 2 diabetes (Ward et al, 1987; Yoshioka et al, 1988; Temple et al, 1989). High concentrations of proinsulin in subjects with type 2 diabetes are thought to result from the secretion from the pancreas of the contents of immature secretory granules which contain a higher ratio of proinsulin to insulin. Recently the use of highly specific assays have permitted the measurements of split forms of proinsulin in the circulation (Sobey et al, 1989; Temple et al, 1990) and elevated concentrations of des 31,32 proinsulin have been reported in both obese and non-obese subjects with type 2 diabetes (Temple et al, 1989). Numerous previous studies have also demonstrated that subjects with type 2 diabetes show insulin resistance and hyperinsulinaemia (Saad et al, 1989; DeFronzo et al, 1991; Reaven, 1988).

The results of this study indicate that high concentrations of intact and des 31,32 proinsulin in the plasma of these subjects with type 2 diabetes produce a large discrepancy between specific insulin concentrations and those measured by a conventional radioimmunoassay employing polyclonal antibodies. That the cross-reaction of proinsulin-like molecules in the insulin radioimmunoassay used in this study was a more generalised problem associated with other commercially available kits to measure insulin, was shown by a study of temple et al, in which proinsulin-like molecules reacted with a potency equal to insulin in three commercially available kits commonly used to measure insulin (Temple et al, 1990). Hence it is likely that most if not all insulin radioimmunoassays using polyclonal antibodies have the potential

problems of cross-reactivity with proinsulin-like molecules. The measurement of intact proinsulin and des 31,32 proinsulin as "insulin" in conventional radioimmunoassays would suggest that hyperinsulinaemia reported in some of the previous studies of type 2 diabetic subjects (Turner et al, 1982; Reaven, 1988; Laakso et al, 1987; Gerich, 1988; Saad et al, 1989; DeFronzo et al, 1991) may be a result of overestimation of insulin because of this cross reaction. The considerable confusion in literature concerning plasma insulin concentrations in type 2 diabetes may in part be due to the result of this variable cross-reactivity of insulin RIAs with proinsulin-like molecules.

Proinsulin and its metabolites have a low insulin-like biological activity on a molar basis (Revers et al, 1984; Bergenstal et al, 1984; Glauber et al, 1987; Davis et al, 1991) and their confusion with insulin would lead to erroneous conclusions regarding the insulin status of an individual.

The concentrations of total proinsulin-like molecules in this study was higher ($62.3 \pm 15.1\%$) than that reported in previous studies of subjects with type 2 diabetes (Table 2.1) (Duckworth et al, 1972; Gordon et al, 1974; Mako et al, 1977; Ward et al, 1987; Yoshioka et al, 1988; Temple et al, 1989; Saad et al, 1990) and also than our own subsequent studies reported later in the dissertation (chapters 5,6,7). None of the subjects in this study were included in the subsequent studies and therefore these results may represent true population heterogeneity, previously well described in subjects with type 2 diabetes. However, an outside possibility may be that this high proportion of proinsulin-like molecules was due to some systematic error in the assays to measure these molecules, as these assays use the same three antibodies, albeit in different combinations. As the sum of these three insulin-like molecule correlated highly with IRI ($r = 0.88$), overestimation of proinsulin-like molecules would only be possible if insulin was being underestimated at the same time and to a similar proportion in each

subject. We feel that as assays for insulin, intact proinsulin, and des 31,32 proinsulin were performed in separate batches, the likelihood of such a systematic error is indeed extremely remote.

Hyperinsulinaemia and insulin resistance are associated with elevated concentrations of triglyceride and low concentrations of HDL-cholesterol in both non-diabetic and diabetic subjects (Laakso et al, 1987). More recently, hyperinsulinaemia has been shown to be associated with increased concentrations of plasminogen activator inhibitor in obese non-diabetic subjects (Juhan-Vague et al, 1987; Vague et al, 1986) and also in subjects with angina due to ischaemic heart disease (Juhan-Vague et al, 1987). Elevated levels of insulin have been reported in essential hypertension and also hypertensive diabetic subjects (Bonora et al, 1987; Ferrannini et al, 1987; Mbanya et al, 1989).

In this study we have found that in these subjects about two thirds of the concentration of IRI is made up by intact proinsulin and des 31,32 proinsulin. It is also apparent that the relationships of IRI concentration with those of triglyceride, total cholesterol and HDL-cholesterol were no longer significant when insulin concentration measured by a highly specific assay were used in the analyses, suggesting that the relationships with IRI are at least in part a reflection of their relationship with insulin precursor molecules which were being measured as insulin in the conventional RIA for insulin. No significant relationships were apparent between concentration of IRI or specific insulin and those of PAI-1, while des 31,32 proinsulin did however show a significant relationship with plasminogen activator inhibitor.

Diastolic blood pressure was related to the concentrations of all insulin-like molecules and that of des 31,32 proinsulin appeared to be the most significant in a multiple

regression analysis. Previous studies have shown that hyperinsulinaemia may correlate with systolic and diastolic blood pressure (Christleib et al, 1985; Fournier et al, 1986; Bonora et al, 1987; Haffner et al, 1988). In this population, however, we found no independent relationship of insulin concentrations with systolic blood pressure. One of the possible explanations for this lack of association is an extremely low prevalence of hypertension in this study population resulting in a narrow range of blood pressure readings. Secondly, it is possible that such a relationship was not significant due to the smaller sample size of this study.

Even though intact and des 31,32 proinsulin are the major circulating insulin-like molecules, and their concentrations showed significant associations with a number of cardiovascular risk factors, these associations do not necessarily represent a cause and effect relationship. One can only, at present, speculate the possible underlying mechanisms for such an association.

Receptor binding studies in rat hepatocytes, adipocytes and lymphocytes have shown that intact proinsulin has receptor binding which is of the order of 0.5 to 2.0% of that insulin (Peavy et al, 1985). However metabolic studies in human subjects have demonstrated that intact proinsulin appears to exert a more profound metabolic effect than in-vitro studies would suggest, with about 8% of the biological activity of insulin for peripheral glucose uptake, about 12% on suppression of hepatic glucose production (Revers et al, 1984) and 20% of the action of insulin in stimulating PAI-1 synthesis from Hep-G2 cell lines (Alessi et al, 1988). Receptor binding studies have also shown that des 31,32 proinsulin has a ten-fold receptor affinity than intact proinsulin in isolated rat adipocytes and about five fold greater affinity in IM-9 lymphocytes (Peavy et al, 1985). A recent study in dogs demonstrated that the hypoglycaemic activity of proinsulin metabolites varied with their metabolic clearance rate. The ascending order

of activity was intact proinsulin, des 31,32 proinsulin and des 64,65 proinsulin (Tillil et al, 1990). Thus in subjects with type 2 diabetes, such as in this study, if the molar ratio of des 31,32 proinsulin and intact proinsulin to insulin is 2:1, insulin precursor molecules might in theory have a substantial metabolic effect in concert with insulin, particularly on glucose and lipid metabolism at the hepatic level.

Subsequent to the findings of this study, we have reconfirmed these associations of proinsulin-like molecules with cardiovascular risk factors in non-diabetic (Mohamed-Ali et al, 1992) and diabetic subjects (Chapters 5,6,7 in this dissertations). In the San Antonio Heart Study, findings similar to our observations in this study were reported in 113 non-diabetic subjects, in whom both fasting and 2-hr post load proinsulin concentrations as measured by a radioimmunoassay showed stronger relationships with cardiovascular risk factors such as total triglyceride, total cholesterol and HDL-cholesterol than did insulin (Haffner et al, 1993). A subsequent study, comparing fasting plasma proinsulin concentrations in subjects with and without angiographic evidence of coronary artery disease with those without and found no significant differences between these groups. However the sample size in that study was very small (Ratner et al, 1993).

Hyperinsulinaemia has been implicated in the cation exchange abnormalities that characterise hypertension, obesity and glucose intolerance (Halkin et al, 1988) perhaps through its action on Na/H exchange (Rosic et al, 1985). Insulin leads to renal sodium retention (DeFronzo et al, 1981) and also may have an effect on sympathetic nervous system (Landsberg et al, 1986). Finding that des 31,32 proinsulin concentrations correlate with levels of diastolic blood pressure in these subjects suggests that these molecules may have similar actions to that of insulin on the renal tubule or on sympathetic nervous system. Alternatively both insulin and proinsulin may be a marker

for the predisposition to coronary artery disease and reflect a common underlying antecedent. However such speculations would require experimental confirmation in future studies.

4.5 Summary and conclusions

This study, the first ever to employ highly specific assays to explore the relationships of insulin, intact proinsulin and its metabolite des 31,32 proinsulin with cardiovascular risk factors, suggested that

1. A conventional RIA for insulin using a polyclonal antibody, cross-react with intact proinsulin and des 31,32 proinsulin with a potency equal to that of insulin. Thus the insulin status of an individual, particularly with non-insulin dependent diabetes, determined using these conventional immunoassays is likely to be misinterpreted due to the measurement of insulin precursor molecules as "insulin." This finding is of great relevance in studying the insulin status of the individual as the biological potency of proinsulin and its metabolites, on a molar basis, is much less than that of insulin.
2. Concentrations of intact proinsulin and des 31,32 proinsulin are elevated in subjects with type 2 diabetes and could represent upto two thirds of the total circulating insulin like immunoreactivity.
3. Elevated concentrations of these molecules are associated with risk factors for cardiovascular disease, suggesting that the previously described associations between hyperinsulinaemia and elevated concentrations of these risk factors in subjects with type 2 diabetes may partly be a consequence of the elevated levels of these precursor molecules.

4. A significant relationship between PAI-1 activity and des 31,32 proinsulin concentrations and not those of insulin was observed suggesting that this molecule may have some role to play in regulation of plasma PAI-1 in subjects with type 2 diabetes.

5. Asian subjects had higher concentrations of des 31,32 proinsulin than caucasian subjects, although these differences were no longer significant when age, gender and treatment were taken into account. Because of this observation, and previous studies that Asian subjects demonstrate hyperinsulinaemia and increased insulin resistance, these observations merit further studies of age- and sex-matched non-diabetic and diabetic subjects to explore the possibilities of ethnic differences in the concentrations of insulin precursor molecules and their relationship to cardiovascular risk factors.

Hyperinsulinaemia has been linked to cardiovascular disease, both in population studies and in groups of non-diabetic subjects at high risk of cardiovascular disease (Pyörälä et al, 1979; Welborn et al, 1979; Ducimetière et al, 1980; McKeigue et al, 1988, 1990; Haffner et al, 1992). The concept of the Insulin Resistance Syndrome or Syndrome X, a combination of hyperinsulinaemia, central obesity, glucose intolerance, increased VLDL triglyceride, low HDL-cholesterol and hypertension, with insulin resistance as the underlying cause for these related variables, has been introduced recently (Reaven, 1988; Zimmet, 1992). It remains to be seen whether elevated concentrations of insulin precursor molecules are of any relevance in determining the concentrations of these risk factors in non-diabetic subjects

CHAPTER 5: INSULIN, PROINSULIN-LIKE MOLECULES AND THEIR RELATIONSHIP WITH CARDIOVASCULAR RISK FACTORS IN ASIAN AND CAUCASIAN NON-DIABETIC AND DIABETIC SUBJECTS

5.1 Introduction

In our observations in the previous chapter, we found that Asian subjects with type 2 diabetes had higher concentrations of des 31,32 proinsulin but these differences were not significant after taking into account confounding variables such as age, sex, bmi and treatment. Previous studies of Asian subjects have reported that Asians have hyperinsulinaemia and also increased insulin resistance, and these observations merit further exploration using specific assays for insulin and proinsulin-like molecules.

Studies have shown that insulin concentrations are significantly related to PAI-1 activity in both non-diabetic and diabetic subjects (Vague et al, 1986; Juhan-Vague et al, 1987; Vague et al, 1989; Juhan Vague et al, 1989). The relationship of insulin with PAI-1 was put to question by our observations in the previous chapter in a study of 51 subjects with type 2 diabetes where concentrations of insulin as measured by a conventional assay (immunoreactive insulin) correlated with PAI-1 but this correlation was no longer significant when insulin concentrations measured by a highly specific assay were used to study this relationship. In the that study we also observed that both intact and des 31, 32 proinsulin were related to number of cardiovascular risk factors such as blood pressure, total triglyceride and HDL-cholesterol. However in this study the number of subjects included was rather small and also did not include non-diabetic subjects.

We now extend our observations to a study of subjects with normal glucose tolerance (NGT), those with impaired glucose tolerance (IGT) and subjects with type 2 diabetes

the non-diabetic population were used. Subjects attended the clinical investigation wing of the Academic Unit of Diabetes and Endocrinology between 8 and 10 am after an overnight fast for an oral glucose tolerance test (OGTT) and a limited examination which included anthropometric measurements, recording of blood pressure and a 12 lead resting electrocardiograph. During the OGTT each subject underwent a questionnaire to assess smoking, alcohol intake and physical activity. Alcohol intake was converted into number of units of alcohol intake daily. Physical activity was assessed from a brief questionnaire asking about both occupational and leisure time physical activity

A total of 196 Asian subjects (103 males, 93 females) were contacted and 113 returned the questionnaire expressing a desire for participation. Six subjects were excluded from the study (2 already known to have diabetes, 3 subjects taking OCP and 1 taking thiazide diuretic) and 3 failed to attend. These were age- and sex-matched with caucasian subjects. Caucasian subjects who responded by returning the questionnaire were sent appointments. 91 caucasian subjects responded and agreed, of whom five were excluded and 4 failed to attend. Thus 82 took part in the study.

A standard 75g OGTT was performed in 104 Asian and 82 caucasian non-diabetic subjects and 34 Asian and 47 caucasian diabetic subjects who were recruited from diabetic clinic. Diabetes and IGT was diagnosed by WHO criteria (WHO, 1985). Subjects with previously diagnosed diabetes were classified as diabetic even if their 2-hr plasma glucose on recent OGTT was < 11.1 mmol/l. Plasma glucose was analysed at five time points after glucose load (0, 30, 60, 90, 120 mins) and plasma insulin, at 0, 30 and 120 mins, and plasma intact proinsulin and des 31,32 proinsulin at 0 min alone.

treated with diet alone. As in the previous study in Chapter 4, highly specific assays to measure insulin and proinsulin-like molecules were used to examine the relationship of cardiovascular risk factors and PAI-1 activity to insulin, intact proinsulin and des 31,32 proinsulin concentrations.

5.2 Aims of the study

The aims of this study were, firstly, to look at the contribution made by intact proinsulin and 31,32 proinsulin to the hyperinsulinaemia in subjects with NGT and type 2 diabetes; secondly, to analyse the relationship of concentrations of insulin and its precursor molecules to those of standard cardiovascular risk factors in subjects with varying degree of glucose intolerance; and thirdly, to study the contribution of insulin precursor molecules to previously reported differences in insulin levels in the Asian and caucasian subjects.

These aims, therefore, were broadly very similar to the study in Chapter 4 but with following differences:

1. Inclusion of subjects with NGT and IGT from both ethnic groups.
2. Subjects in this study also underwent a standard 75 gm oral glucose tolerance test (OGTT) with measurement of insulin concentrations during OGTT.
3. Diabetic subjects included were treated with diet alone, to avoid the confounding effects of treatment for diabetes on PAI-1 activity and other variables.
4. Variables, such as exercise, smoking habits and alcohol intake, with a potential to confound cardiovascular risk factors and insulin concentrations, were also quantified.

5.3 Study population

Exclusion Criteria

1. Age less than 35 or more than 70
2. History of ischaemic heart disease
3. Thiazide treatment
4. Taking oral contraceptive pill (OCP)
5. Pregnancy
6. Unable to give informed consent.
7. Albustix positive albuminuria.

Study of non-diabetic subjects

Asian subjects: living in London were selected at random and were mailed letters with an invitation to participate in the study and to return a simple questionnaire to assess their eligibility for entry into the study. Those who agreed and returned the questionnaire were sent an appointment to attend for an oral glucose tolerance test and a limited physical examination.

Caucasian subjects: were selected at random from the computer register of a group practice in Islington, London. These were age and sex-matched with the Asian subjects and were sent letters by their own general practitioner encouraging them to participate in the study. Those eligible and willing to take part were contacted and sent appointments.

Study of diabetic subjects

Cross-sectional studies: Subjects were selected from a busy district general hospital diabetic clinic. The selection process was random and similar exclusion criteria as for

5.4 Statistical analysis

Values are expressed as mean \pm SD for normally distributed data or median (range) for skewed data. To compare the two ethnic groups unpaired t-tests for normally distributed data and Mann Whitney-U tests for skewed data were used. Relationships between two variables was assessed using Pearson correlation analysis after log transforming the non-normally distributed data. Multiple regression analyses were used to study the relationship of variables while controlling for others. Multiple logistic regression analysis was used to study the association of various risk factors with ischaemic heart disease.

5.5 Results

5.5.1 Prevalence of diabetes and impaired glucose tolerance

Of the 104 Asian screened, 16 (15.4%) had IGT and 6 (5.8%) diabetes, while of 82 caucasians subjects 13 (15.9%) and 2 (2.4%) had IGT and diabetes respectively. The crude prevalence of diabetes or impaired glucose was not significantly different between caucasian and Asian subjects ($\chi^2 = 1.24$, df 1, p = ns; $\chi^2 = 0.08$, df 1, p = ns) respectively. Data from subjects with newly diagnosed diabetes (6 Asians, 2 caucasians) were grouped with those from subjects with previously known diabetes (34 Asians, 47 caucasians) for analyses as there were no significant differences between these subjects.

5.5.2 Comparison of Asian vs caucasian subjects

5.5.2.1 Anthropometric measurements

Asian subjects with normal glucose tolerance were significantly thinner, yet had significantly higher subscapular-triceps skinfold ratio (STR) than caucasians (Table 5.1). Asian subjects with type 2 diabetes were significantly younger, thinner than caucasian subjects (Table 5.2). There were no significant differences in the body fat distribution

between Asian and caucasian diabetic subjects. Asian subjects with IGT were similar to caucasians with IGT in most respects and because of the small numbers any differences between Asian and caucasian subjects with IGT did not achieve statistical significance.

5.5.2.2 Exercise, smoking and alcohol consumption

Among non-diabetic subjects, the number of subjects who performed higher grades of exercise was significantly higher in caucasian group ($\chi^2 = 7.6$, df 3, $p = 0.01$; Table 5.1). However, no significant differences were observed in the exercise patterns between Asian and caucasian subjects with diabetes (Table 5.2).

Among non-diabetic subjects the number of current smokers was significantly higher in the caucasian group, and caucasian smokers smoked a significantly higher number of cigarettes than Asian subjects. The number of subjects who consumed alcohol was significantly higher in caucasians as was the daily amount of alcohol consumed (Table 5.1). The differences for smoking and for alcohol in subjects with diabetes was similar to that seen among non-diabetic subjects from both ethnic groups (Table 5.2).

5.5.2.3 Plasma glucose, insulin responses to OGTT and fasting intact and des 31,32 proinsulin

Biochemical characteristics of these subjects are shown in table 5.3 for non-diabetic and table 5.4 for diabetic subjects. Asian non-diabetic subjects had similar fasting plasma glucose and 2-hr plasma glucose but significantly lower 30-min post load glucose and lower glycated haemoglobin concentrations than caucasian non-diabetic subjects (Table 5.3). There were no significant differences between Asian and caucasian subjects with diabetes in terms of their fasting plasma glucose and post load glucose and glycated haemoglobin concentrations (Table 5.4).

Although fasting insulin concentrations were not significantly different between the two groups, Asian non-diabetic subjects had significantly higher 2-hr plasma insulin concentrations than caucasian subjects (Fig 5.1 a). Fasting intact proinsulin concentrations were also significantly higher in Asians non-diabetic subjects (Fig 5.2 a). These differences in insulin and intact proinsulin concentrations were even more significant when analysed using ANOVA controlling for age, sex, BMI, WHR and physical activity. No significant differences were apparent in concentrations of des 31,32 proinsulin between Asian and caucasian non-diabetic subjects (Fig 5.2 b). Fasting plasma C-peptide concentrations in Asian non-diabetic subjects were lower, although the difference did not achieve statistical significance (Table 5.3).

Asians subjects with diabetes also had significantly higher 2-hr post load insulin concentrations than caucasians, however the differences among subjects with impaired glucose tolerance were not significant due to small number of subjects (Fig 5.1 b, c). There were no significant differences in fasting intact or des 31,32 proinsulin concentrations among diabetic subjects or subjects with IGT between Asian and caucasians.

The percent of insulin and of proinsulin like molecules of the total sum of insulin-like molecules (insulin + intact proinsulin + des 31,32 proinsulin) by diabetes and ethnicity is shown in table 5.5. The proportion of sum comprising proinsulin-like molecules was similar among caucasian and Asian non-diabetic [median 12.5% range (1.6-47.2)% vs 12.5% (3.0-53.2)%, $p = ns$) and diabetic subjects [median 18.4% range (3.5-61.0) vs 22.8 (2.2-55.0)%, $p = ns$) respectively.

Table 5.1: Clinical characteristics of caucasian and Asian non-diabetic subjects

Variable	Caucasian (n = 67)	Asian (n = 82)	Significance of difference
Age	49.3 ± 8.1	48.2 ± 8.0	ns
Sex (M, F)	31, 36	40, 42	ns
Body mass index (kg/m ²)	25.7 ± 3.7	24.4 ± 3.5	0.04
Waist-hip-ratio	0.874 ± 0.081	0.854 ± 0.109	ns
Subscapular-triceps-ratio	1.17 ± 0.41	1.36 ± 0.69	0.047
Systolic blood pressure	118 ± 16	114 ± 15	ns
Diastolic blood pressure	73 ± 10	72 ± 10	ns
Current smokers (n %)	31 (46%)	13 (16%)	0.001
Number of cigarettes	20 (5-50)	10 (3-20)	0.001
Alcohol (n %)	42 (63%)	7 (9%)	0.001
Alcohol intake (units)	10 (1-60)	4 (2-18)	0.001
Exercise	25 (37%)	26 (32%)	ns
Exercise grade 0	0/67 (0%)	0/82 (0%)	
1	34/67 (51%)	54/82 (66%)	0.01
2	26/67 (39%)	27/82 (33%)	
3	7/67 (10%)	1/82 (1%)	
Ischaemic heart disease	3/67 (4.5%)	15/82 (18.3%)	0.009

Data shown as mean ± sd or median (range).

Table 5.2: Clinical characteristics of caucasian and Asian subjects with type 2 diabetes

Variable	Caucasian (n = 49)	Asian(n = 40)	P value
Age	57.1 ± 7.2	52.5 ± 8.1	0.006
Sex (M, F)	38,11	30,10	ns
Body mass index (Kg/m ²)	27.8 ± 2.8	26.4 ± 4.3	0.07
Waist-hip-ratio	0.929 ± 0.085	0.933 ± 0.085	ns
Subscapular-triceps-ratio	1.78 ± 0.89	1.86 ± 0.76	ns
Systolic blood pressure	131 ± 18	125 ± 18	ns
Diastolic blood pressure	80 ± 10	79 ± 17	ns
Current smokers n(%)	20 (41%)	6 (15%)	0.008
Number of cigarettes	20 (7-45)	15 (4-60)	0.01
Alcohol	28 (57%)	10 (25%)	0.002
amount of alcohol (units)	9 (1-140)	8 (3-120)	0.01
Exercise	10 (20%)	10 (25%)	ns
Exercise Grade 0	25 (51%)	22 (55%)	
1	11 (22%)	10 (25%)	ns
2	13 (27%)	6 (15%)	
3	0 (0%)	2 (5%)	
Ischaemic heart disease	5/49 (10%)	7/40 (18%)	ns

Data shown as mean ± sd or median (range).

Table 5.3: Biochemical characteristics of caucasian and Asian non-diabetic subjects

Variable	Caucasian (n = 67)	Asian (n = 82)	Significance of difference
Fasting glucose (mmol/l)	5.1 ± 0.5	5.1 ± 0.5	ns
30-min glucose (mmol/l)	8.4 ± 1.6	7.9 ± 1.5	0.031
120-min glucose (mmol/l)	5.1 ± 1.4	5.4 ± 1.1	ns
Glycated haemoglobin (%)	7.3 ± 1.3	6.9 ± 1.1	0.043
Fasting C-peptide (nmol/l)	0.51 ± 0.44	0.45 ± 0.14	<0.08
Fasting insulin (pmol/l)	30 (12-65)	37 (17-89)	ns
30-min insulin (pmol/l)	274 (121-506)	319 (111-572)	ns
120-min insulin (pmol/l)	186 (73-471)	274 (99-552)	0.005
Fasting intact proinsulin (pmol/l)	2.1 (1.4-4)	2.7 (1.4-5.8)	<0.02
Fasting des 31,32 proinsulin (pmol/l)	2 (0.5-6.4)	2.1 (0.4-5.8)	0.87
Total triglyceride (mmol/l)	1.2 (0.3, 4.9)	1.1 (0.3, 5.3)	ns
Total cholesterol (mmo/l)	5.9 ± 1.5	5.8 ± 1.1	ns
LDL-cholesterol (mmol/l)	3.9 ± 1.5	3.8 ± 0.9	ns
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.4 ± 0.4	ns
PAI-1 (AU/ml)	16.5 ± 6.4	17.5 ± 7.2	ns
Urine albumin/creatinine ratio	0.3 (0.02, 7.3)	0.3 (0.03, 3.1)	ns

Data shown as mean ± sd or median (range).

Table 5.4: Biochemical characteristics of caucasian and Asian subjects with type 2 diabetes

Variable	Caucasian (n = 49)	Asian (n = 40)	Significance of difference
Fasting glucose (mmol/l)	8.6 ± 2.8	9.3 ± 3.5	ns
30-min glucose (mmol/l)	13.4 ± 3.4	13.9 ± 4.8	ns
120-min glucose (mmol/l)	15.4 ± 5.6	15.5 ± 5.6	ns
Glycated haemoglobin (%)	8.8 ± 1.9	9.6 ± 2.5	ns
Fasting C-peptide (nmol/l)	0.82 ± 0.35	0.70 ± 0.27	ns
Fasting insulin (pmol/l)	57 (13-288)	87 (19-267)	ns
30-min insulin (pmol/l)	115 (39-228)	131 (32-368)	ns
120-min insulin (pmol/l)	148 (51-477)	210 (55-535)	0.008
Fasting intact proinsulin (pmol/l)	8 (3.3-18.2)	8.9 (3.6-26.9)	ns
Fasting des 31,32 proinsulin (pmol/l)	5.4 (1.6-18.6)	6.9 (1.6-11.7)	ns
Total triglyceride (mmol/l)	2.0 (0.5, 9.0)	1.9 (0.9, 5.9)	ns
Total Cholesterol (mmol/l)	7.0 ± 1.5	6.4 ± 1.2	0.05
LDL-Cholesterol (mmol/l)	4.7 ± 1.3	4.3 ± 1.1	ns
HDL-Cholesterol (mmol/l)	1.2 ± 0.4	1.1 ± 0.3	0.06
PAI-1 (AU/ml)	23.1 ± 6.6	22.8 ± 7.3	ns
Urine albumin/creatinine ratio	0.4(0.03, 16.1)	0.4(0.05, 15.6)	ns

Data shown as mean ± standard deviation or median (range).

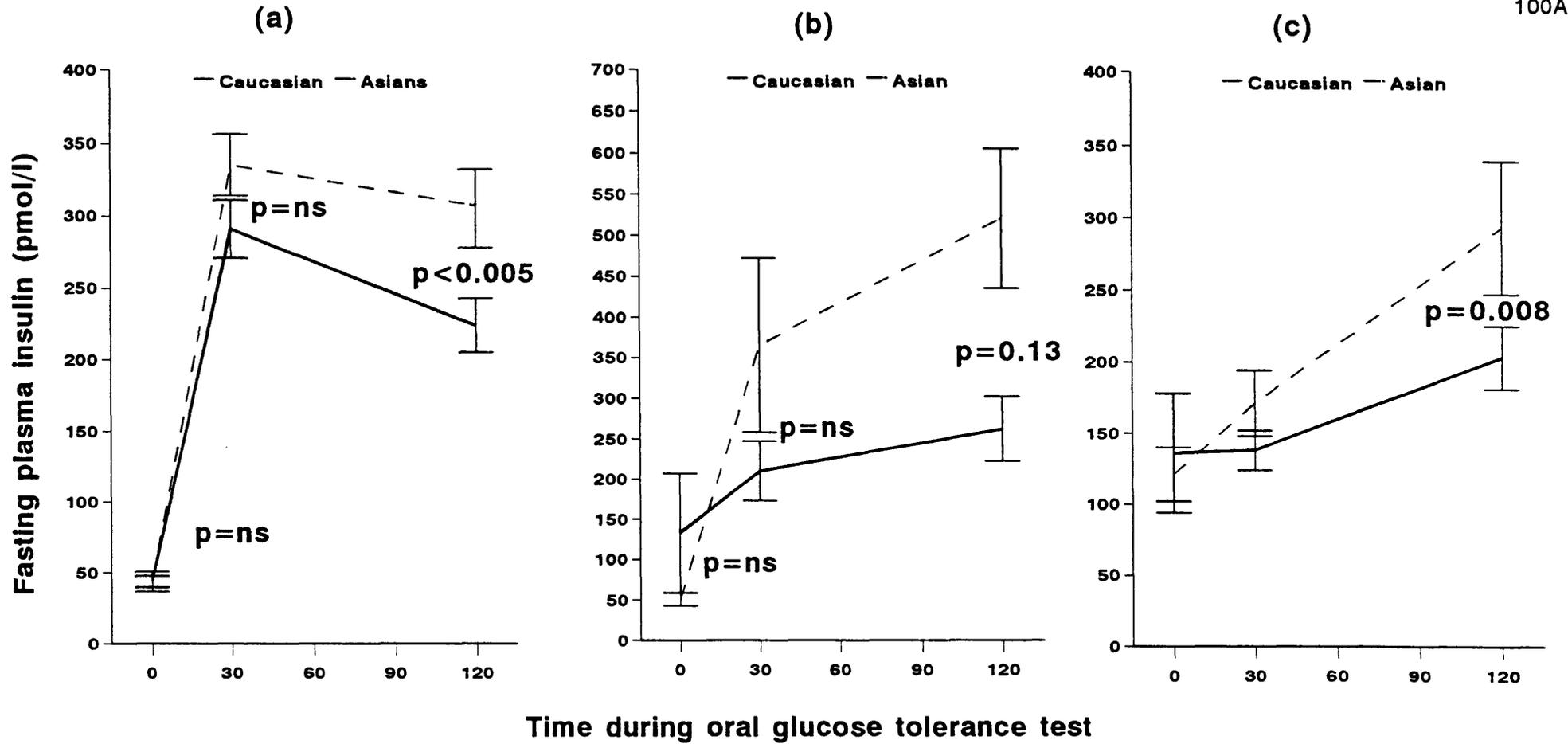


Figure 5.1: Insulin responses to oral glucose in Asian and caucasian (a) normal subjects and those with (b) IGT and (c) type 2 diabetes

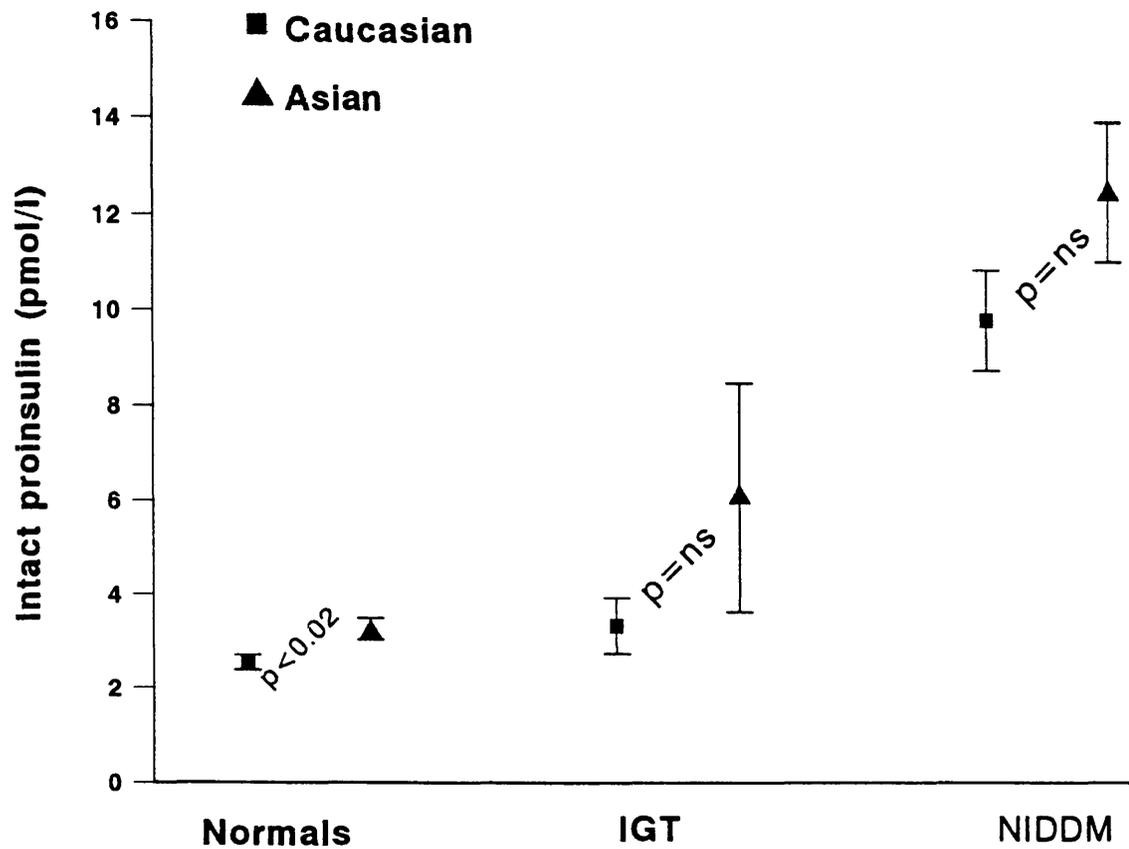


Figure 5.2 (a): Fasting intact proinsulin concentrations in Asian and caucasian normals, IGT and type 2 diabetes

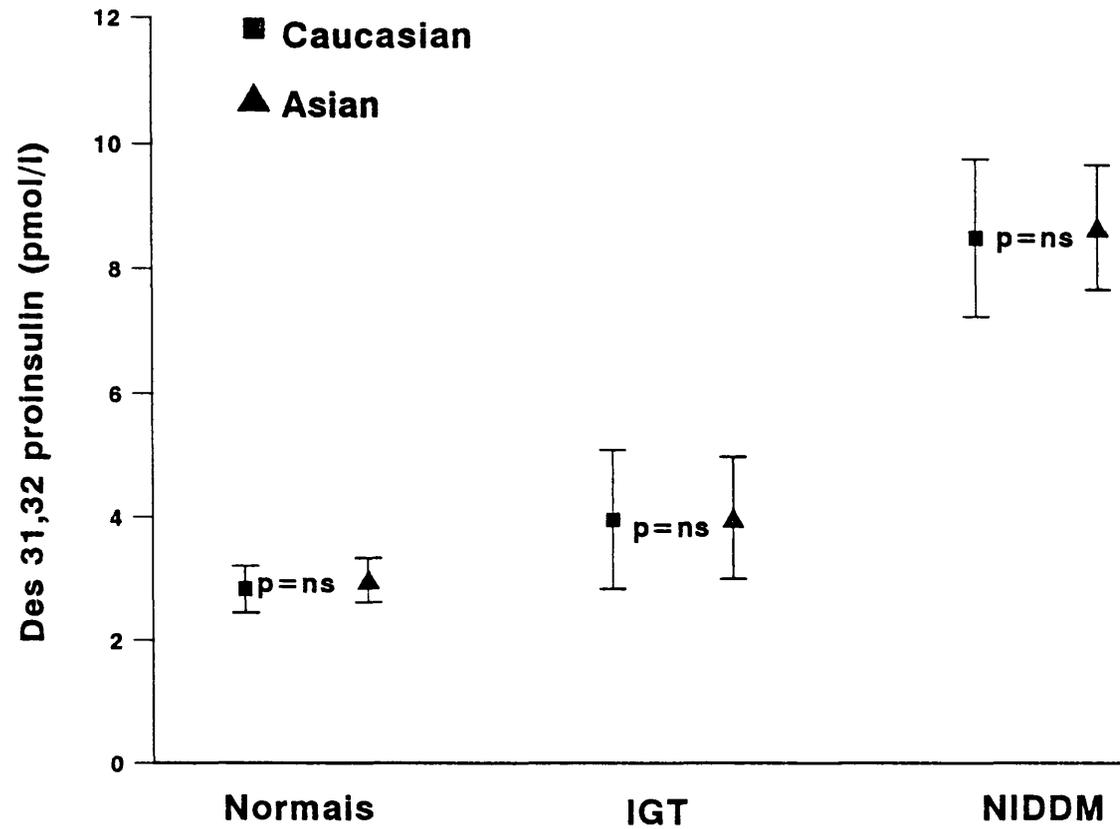


Figure 5.2 (b): Fasting des 31,32 proinsulin concentrations in Asian and caucasian normals, IGT and type 2 diabetes

5.5.2.4 Insulin/C-peptide ratio

Insulin/C-peptide ratios, as an index of hepatic insulin extraction were significantly higher in Asian non-diabetic subjects than caucasians [median 0.01 range (0.02-0.47) vs 0.07 (0.02-0.68), $p=0.003$]. However these differences were no longer significant after taking into account age, sex, body mass index and waist-hip-ratio. No significant differences were seen among diabetic subjects between the two ethnic groups.

5.5.2.5 Ischaemic heart disease

Electrocardiographic evidence of ischaemic heart disease was significantly more frequent in the Asians than caucasians but only among non-diabetic subjects (Table 5.1).

5.5.2.6 Cardiovascular risk factors, and PAI-1 activity

Asian subjects, diabetic and non-diabetic were similar to caucasians regarding most cardiovascular risk factors (Tables 5.3, 5.4). Caucasian subjects with diabetes, however, had borderline higher total cholesterol ($p=0.05$) and HDL-cholesterol concentrations ($p=0.06$) than Asian subjects but were similar with regard to other cardiovascular risk factors such as systolic and diastolic blood pressure, total triglyceride, and urine albumin/creatinine ratios. PAI-1 activity was also similar between Asian and caucasian subjects among subjects with different categories of glucose intolerance (Fig 5.3). There were no significant differences in any of the measured plasma lipids, or PAI-1 activity among subjects with and without IHD among normal subjects.

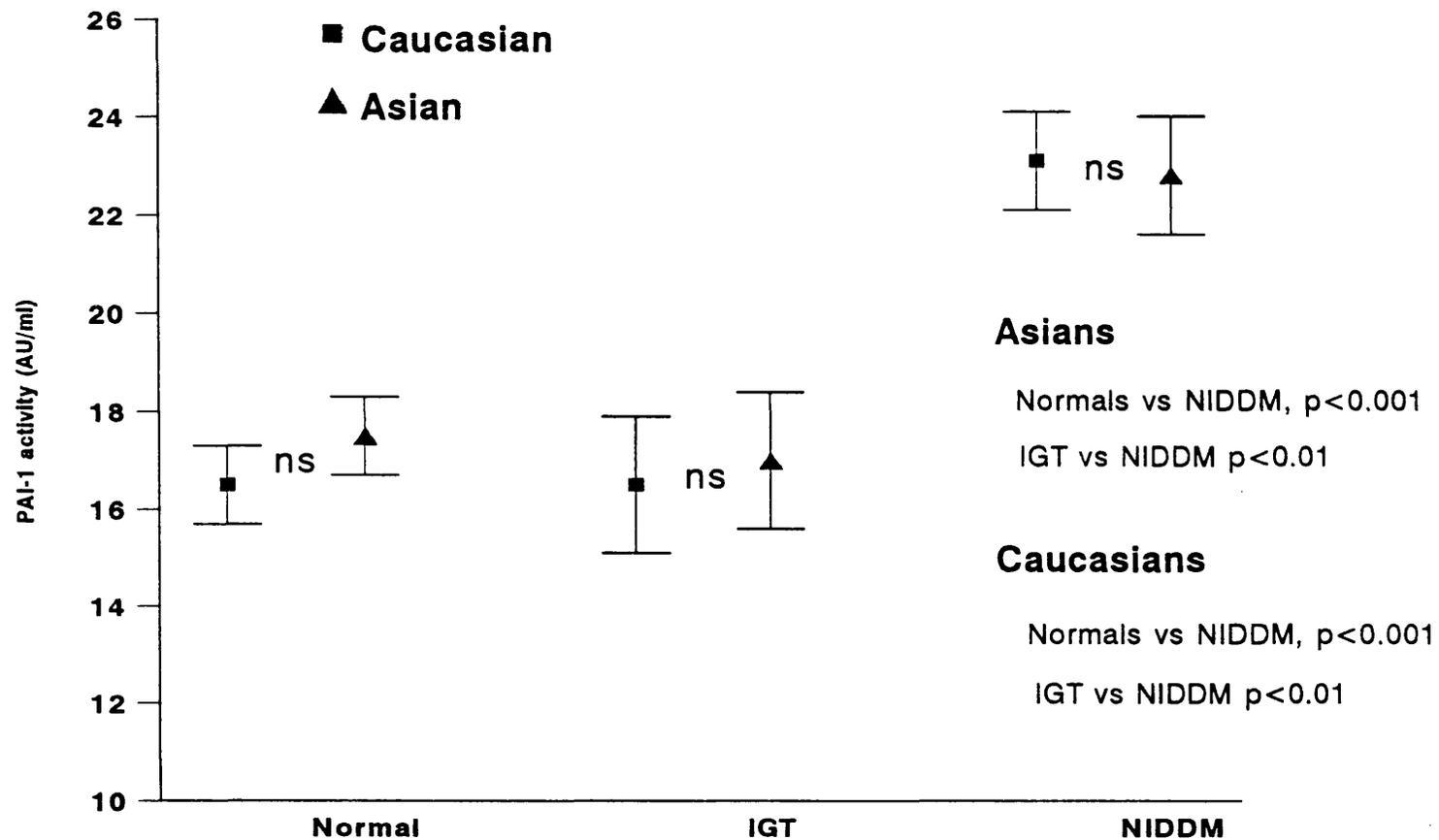


Figure 5.3: PAI-1 activity in Asian and caucasian subjects

Table 5.5: Insulin, proinsulin-like molecules as percent of sum* in Asian and caucasian subjects.

Variables	Caucasian		Asian	
	Non-diabetic	Diabetic	Non-diabetic	Diabetic
Insulin (%)	85 (53-98)	80 (39-96)	86 (47-97)	77 (45-98)
Intact proinsulin (%)	5.6 (0.9-16.8)	10.0 (1.6-44.6)	6.4 (1.2-21)	10.3 (1.2-39.5)
Des 31,32 proinsulin (%)	5.6 (0.7-42.8)	6.8 (1.3-24.9)	4.5 (0.3-47.8)	8.8 (0.4-25)
Proinsulin-like molecules /Sum (%)	12.5 (1.6-47.2)	18.4 (3.5-61.0)	12.5 (3.0-53.2)	22.8 (2.2-55.0)

* Sum = (Insulin + intact proinsulin + des 31,32 proinsulin)

Data shown as median and range

5.5.3 Diabetic vs non-diabetic subjects

5.5.3.1 Anthropometric measurements

Subjects with diabetes were significantly more obese, had higher waist-hip-ratio, and higher subscapular-to-triceps skinfold ratios (STR) in both ethnic groups than subjects with normal glucose tolerance (Tables 5.6, 5.7).

5.5.3.2 Exercise, smoking and alcohol consumption

Diabetic subjects in both ethnic groups reported lower levels of physical activity than non-diabetic subjects ($\chi^2 = 46.4$, $df = 3$, $p < 0.001$ for caucasians, and $\chi^2 = 58.4$, $df = 3$, $p < 0.001$ for Asians). In both ethnic groups there was a significantly negative relationships between physical activity and BMI but not between WHR or STR and physical activity. There were no significant differences in the number of subjects who were current smokers between diabetic and non-diabetic subjects among both ethnic groups. However Asian diabetic subjects on average smoked significantly more cigarettes ($p < 0.02$) than non-diabetic subjects. The number of subjects who consumed alcohol was also significantly higher among Asian diabetic ($\chi^2 = 8.4$, $df = 1$, $p = 0.004$) than asian non-diabetic subjects, while no such differences were apparent in caucasians.

5.5.3.3 Plasma glucose, insulin responses to OGTT and fasting intact and des 31,32 proinsulin

Data comparing subjects with different categories of glucose tolerance is shown in table 5.6 for caucasians and table 5.7 for Asians. Fasting plasma glucose concentrations were higher in subjects with IGT and diabetes compared to subjects with NGT, but glycated haemoglobin was higher only in subjects with diabetes than those with IGT and NGT, there being no significant differences among subjects with NGT and IGT.

Subjects with diabetes in each ethnic group had significantly higher fasting plasma insulin, but lower 30 min insulin concentrations (Fig 5.4) than non-diabetic subjects. However, 2-hr post load insulin concentrations were not significantly different between non-diabetic and diabetic subjects. Subjects with type 2 diabetes also had higher fasting plasma C-peptide, intact proinsulin and des 31,32 proinsulin concentrations than non-diabetic subjects in both ethnic groups.

The ratio of proinsulin-like molecules to total insulin-like molecules was significantly higher in subjects with type 2 diabetes than normal subjects in each ethnic groups (Asians median 22.8% vs 12.5%, $p=0.015$; caucasians median 18.4% vs 12.5%, $p=0.023$). In all subjects both intact and des 31,32 proinsulin concentrations were strongly and positively correlated with concentrations of fasting plasma glucose and glycated haemoglobin while the correlation of fasting insulin concentrations with those of plasma glucose concentrations was much weaker (Table 5.8).

The relationship of fasting insulin and proinsulin-like molecules to fasting and 2-hr glucose was analysed in subjects with diabetes after categorising diabetic subjects into groups of approximately equal numbers, by severity of fasting and 2-hr post load glucose concentrations. In such an analysis, an inverted U shaped relationship of insulin concentrations with fasting plasma glucose (Fig 5.5) and 2-hr glucose concentrations was observed (Fig 5.6). The concentrations of proinsulin-like molecules did not increase in a linear fashion by severity of fasting plasma glucose concentrations (Fig 5.7) but did so in relation to severity of 2-hr plasma glucose concentrations (Fig 5.8).

The ratio of plasma insulin to C-peptide concentrations, as a measure of hepatic insulin extraction, was similar among non-diabetic and diabetic subjects.

Table 5.6: Clinical and biochemical characteristics of caucasian subjects

Variable	Non-diabetic (n= 67)	IGT (n= 13)	Diabetic (n= 49)	p value		
				NGT vs IGT	NGT vs diabetics	IGT vs diabetics
Age	49.3 ± 8.1	51.9 ± 6.7	57.1 ± 7.2	ns	<0.001	0.02
Body mass index (kg/m ²)	25.7 ± 3.7	26.2 ± 6.4	27.8 ± 2.8	ns	<0.001	ns
Waist-hip-ratio	0.874 ± 0.081	0.906 ± 0.084	0.929 ± 0.085	ns	<0.001	ns
Subscapular-triceps-ratio	1.17 ± 0.41	1.35 ± 0.40	1.78 ± 0.89	ns	<0.001	0.01
Systolic blood pressure	118 ± 16	121 ± 21	131 ± 18	ns	<0.001	ns
Diastolic blood pressure	73 ± 10	76 ± 13	80 ± 10	ns	<0.001	ns
Fasting glucose (mmol/l)	5.1 ± 0.5	5.9 ± 0.8	8.6 ± 2.8	<0.01	<0.001	<0.001
30-min glucose (mmol/l)	8.4 ± 1.6	9.6 ± 1.8	13.4 ± 3.4	<0.03	<0.001	<0.001
120-min glucose (mmol/l)	5.1 ± 1.4	8.5 ± 0.9	15.4 ± 5.6	<0.0	<0.001	<0.001
Glycated haemoglobin (%)	7.3 ± 1.3	7.2 ± 1.1	8.8 ± 1.9	ns	<0.001	0.007
Fasting C-peptide (nmol/l)	0.51 ± 0.44	0.43 ± 0.15	0.82 ± 0.35	ns	<0.001	0.008
Fasting insulin (pmol/l)	30 (12-65)	36 (8-276)	57 (13-288)	ns	<0.001	ns
Fasting proinsulin (pmol/l)	2.1 (1.4-4.0)	2.6 (1.7-6.9)	8 (3.3-18.2)	ns	<0.001	<0.001
Des 31,32 proinsulin (pmol/l)	2.0 (0.5-6.4)	2.6 (1.0-9.3)	5.4 (1.6-18.6)	ns	<0.001	<0.001
Total triglyceride (mmol/l)	1.2 (0.3-4.9)	1.4 (0.9-4.0)	2.0 (0.5-9.0)	ns	<0.001	ns
Total cholesterol (mmol/l)	5.9 ± 1.5	6.0 ± 1.2	7.0 ± 1.5	ns	<0.001	0.02
LDL-cholesterol (mmol/l)	3.9 ± 1.5	3.9 ± 0.9	4.7 ± 1.3	ns	0.002	0.04
HDL-cholesterol (mmol/l)	1.3 ± 0.4	1.3 ± 0.3	1.2 ± 0.4	ns	0.04	ns
PAI-1 (AU/ml)	16.5 ± 6.4	16.5 ± 4.8	23.1 ± 6.6	ns	<0.001	<0.01
Urine Alb/Creat Ratio	0.3 (0.02-7.3)	0.2 (0.08-5.5)	0.4 (0.03-16.1)	ns	ns	ns

Data shown as mean ± standard deviation or median (range)

*Significance of difference between three groups analysed by Students t-test

Table 5.7: Clinical and biochemical characteristics of Asian subjects

Variable	Non-Diabetic (n = 82)	IGT (n = 16)	Diabetic (n = 40)	P value		
				NGT vs IGT	NGT vs diabetics	IGT vs diabetics
Age	48.2 ± 8.0	51.4 ± 8.4	52.5 ± 8.1	ns	0.006	ns
Body mass index (kg/m ²)	24.4 ± 3.5	25.3 ± 3.5	26.4 ± 4.3	ns	0.006	ns
Waist-hip-ratio	0.854 ± 0.109	0.873 ± 0.093	0.933 ± 0.085	ns	<0.001	0.02
Subscapular-triceps-ratio	1.36 ± 0.69	1.68 ± 1.1	1.86 ± 0.76	ns	<0.001	ns
Systolic blood pressure	114 ± 15	116 ± 14	125 ± 18	ns	<0.001	0.04
Diastolic blood pressure	72 ± 10	73 ± 10	79 ± 17	ns	<0.001	0.05
Fasting glucose (mmol/l)	5.1 ± 0.5	5.5 ± 0.8	9.3 ± 3.5	0.05	<0.001	<0.001
30-min glucose (mmol/l)	7.9 ± 1.5	9.2 ± 1.7	13.9 ± 4.8	0.001	<0.001	<0.001
120-min glucose (mmol/l)	5.4 ± 1.1	8.9 ± 0.9	15.5 ± 5.6	<0.001	<0.001	<0.001
Glycated haemoglobin (%)	6.9 ± 1.1	7.3 ± 0.9	9.6 ± 2.5	ns	<0.001	<0.001
Fasting C-peptide (nmol/l)	0.45 ± 0.14	0.52 ± 0.10	0.70 ± 0.27	ns	<0.001	0.005
Fasting insulin (pmol/l)	37 (17-89)	46.5 (13-103)	131 (32-368)	ns	<0.001	<0.02
Fasting proinsulin (pmol/l)	2.7 (1.4-5.8)	4.3 (2.0-5.0)	8.9 (3.6-26.9)	0.052	<0.001	<0.001
Des 31,32 proinsulin (pmol/l)	2.1 (0.4-5.8)	2.5 (1.3-8.0)	6.9 (1.6-11.7)	ns	<0.001	<0.001
Total triglyceride (mmol/l)	1.1 (0.3-5.3)	1.5 (0.2-2.3)	1.9 (0.9-5.9)	ns	<0.001	0.01
Total cholesterol (mmol/l)	5.8 ± 1.1	6.1 ± 1.3	6.4 ± 1.2	ns	<0.01	ns
LDL-cholesterol (mmol/l)	3.8 ± 0.9	4.1 ± 1.1	4.3 ± 1.1	ns	<0.005	ns
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.3	1.1 ± 0.3	ns	<0.001	0.01
PAI-1 (AU/ml)	17.5 ± 7.2	17.0 ± 5.2	22.8 ± 7.3	ns	<0.001	<0.003
Urine Alb/Creat ratio	0.3 (0.03-3.1)	0.3 (0.12-2.8)	0.4 (0.05-15.6)	ns	ns	ns

Data shown as mean ± standard deviation or median (range)

*Significance of difference between three groups analysed by Students t-test

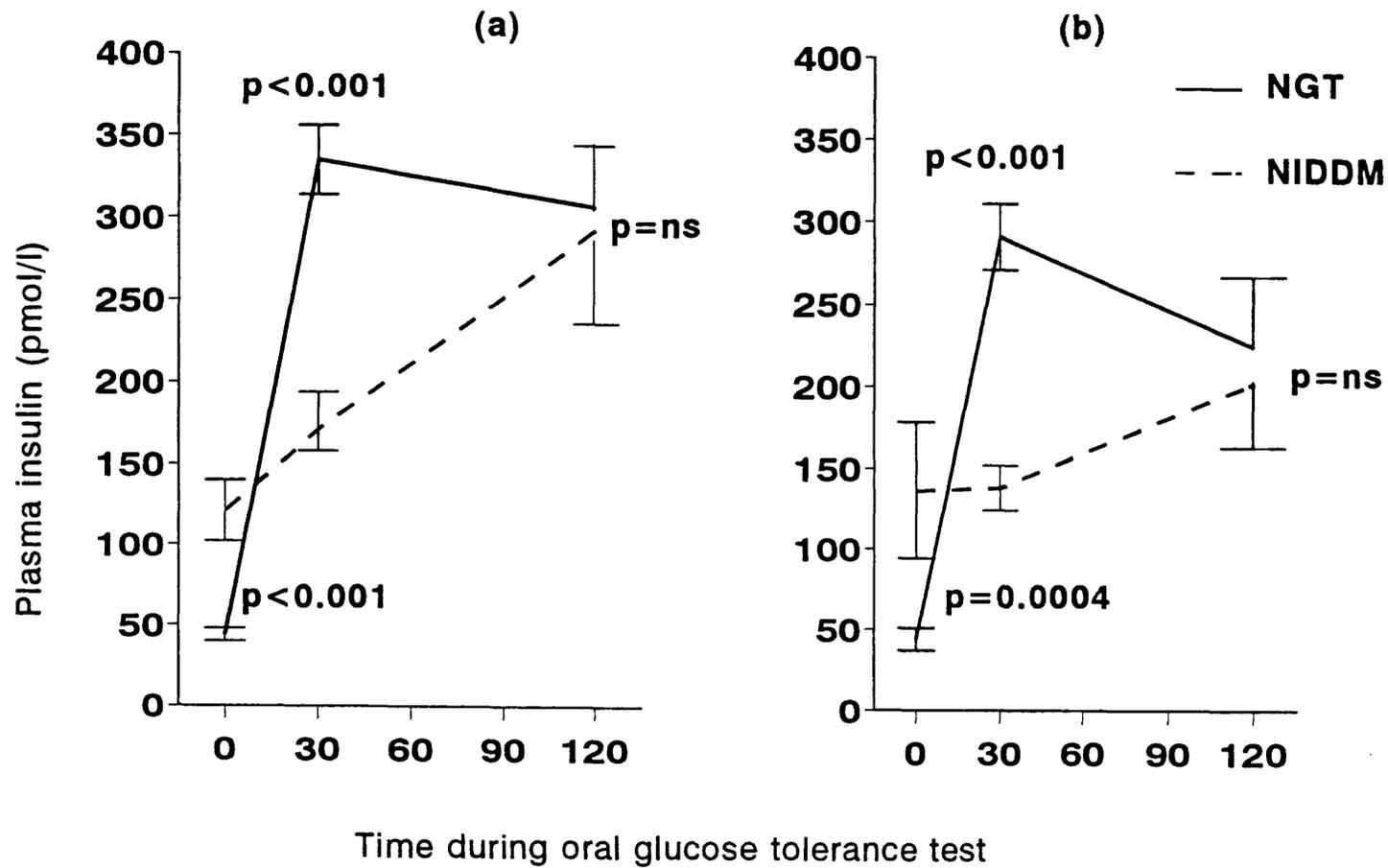


Figure 5.4: Insulin response to oral glucose in (a) Asian and (b) caucasian subjects with normal glucose tolerance (NGT) and type 2 diabetes. Data shown as mean \pm sem

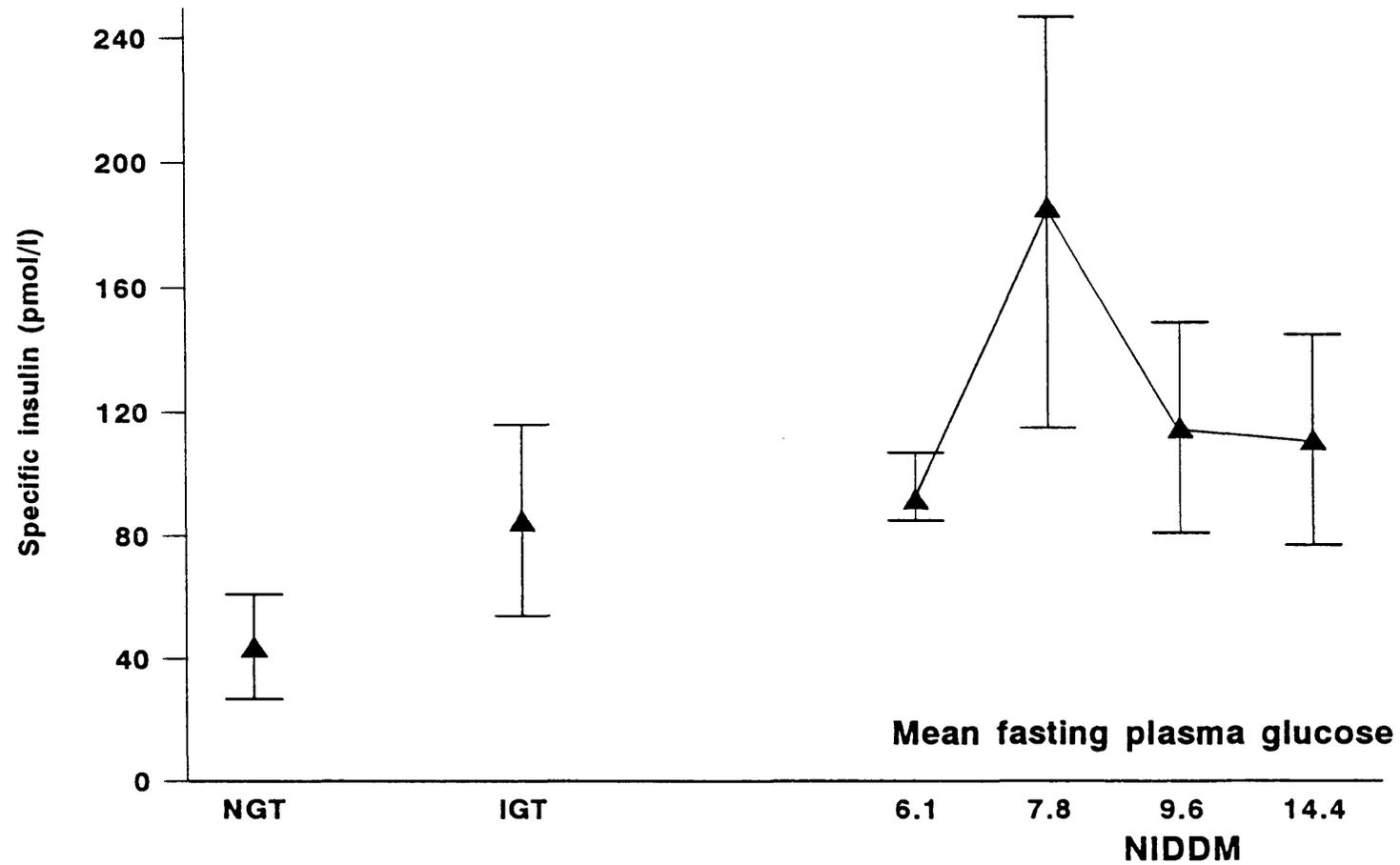


Figure 5.5: Fasting concentrations of specific insulin in subjects with NGT, IGT and type 2 diabetes. Subjects with diabetes divided into four groups by severity of fasting plasma glucose

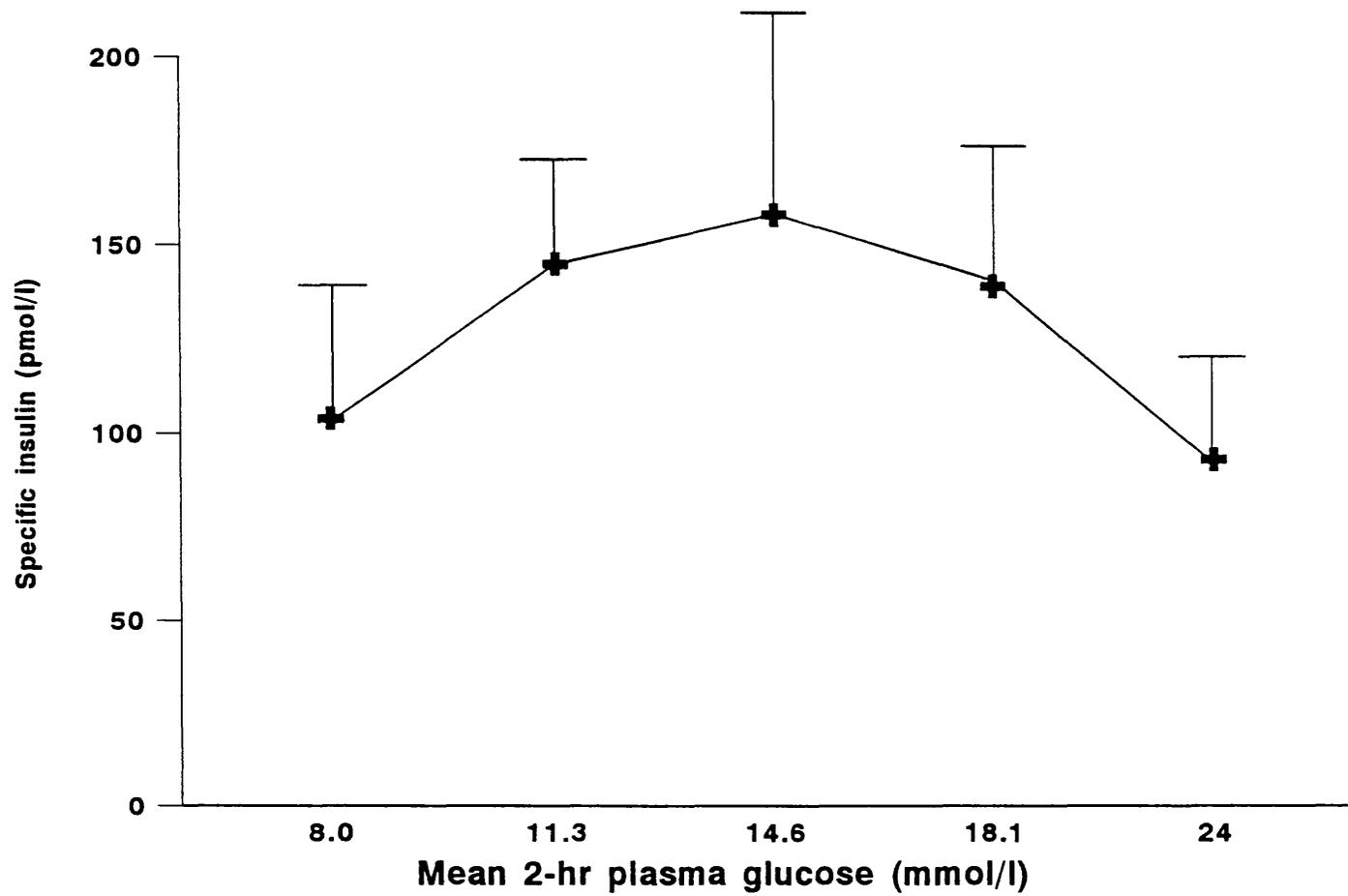


Figure 5.6: Fasting concentrations of specific insulin in subjects with type 2 diabetes by severity of 2-hr plasma glucose concentrations

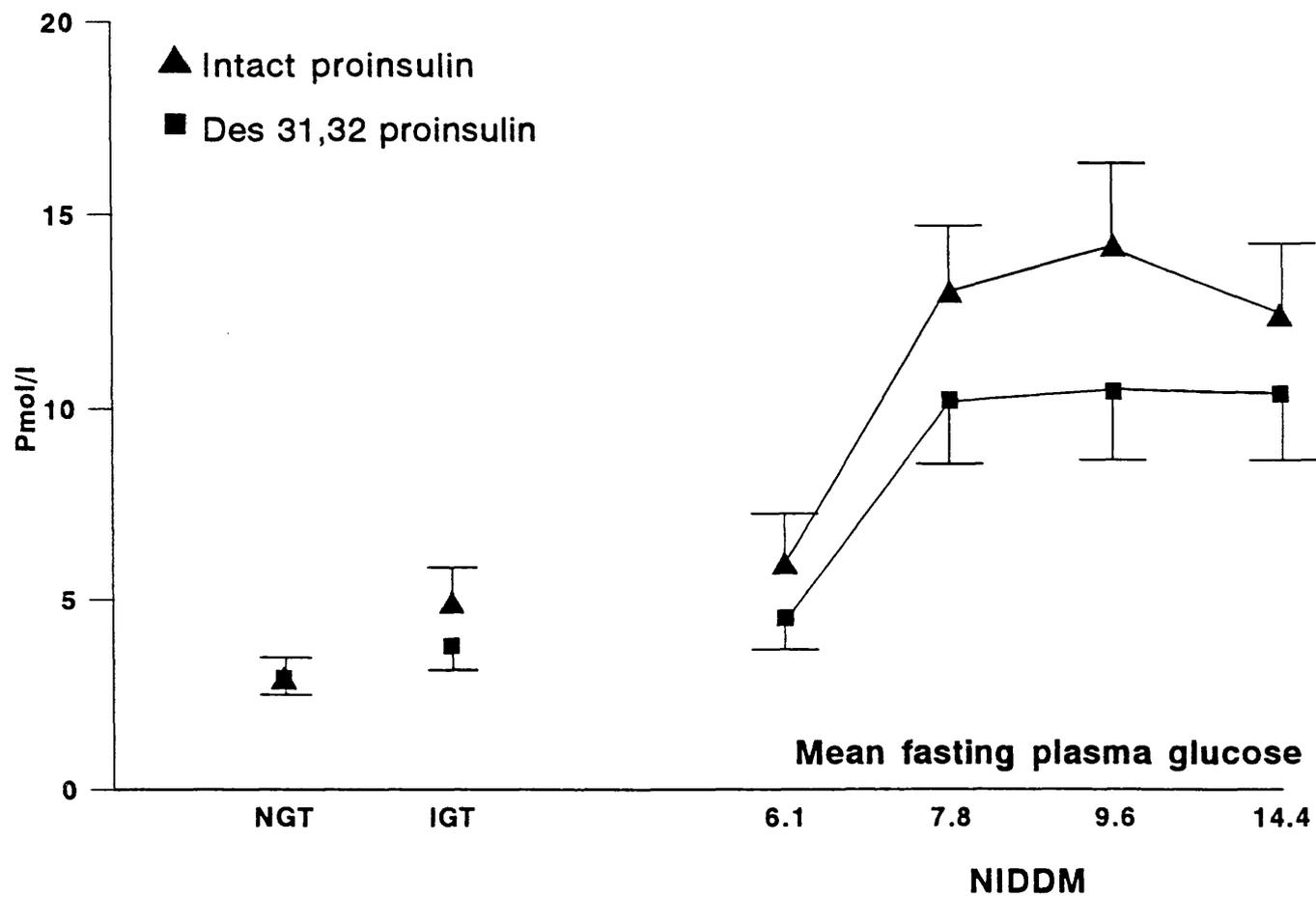


Figure 5.7: Fasting concentrations of proinsulin-like molecules in subjects with NGT, IGT and type 2 diabetes. Subjects with diabetes divided into four groups by severity of fasting glucose

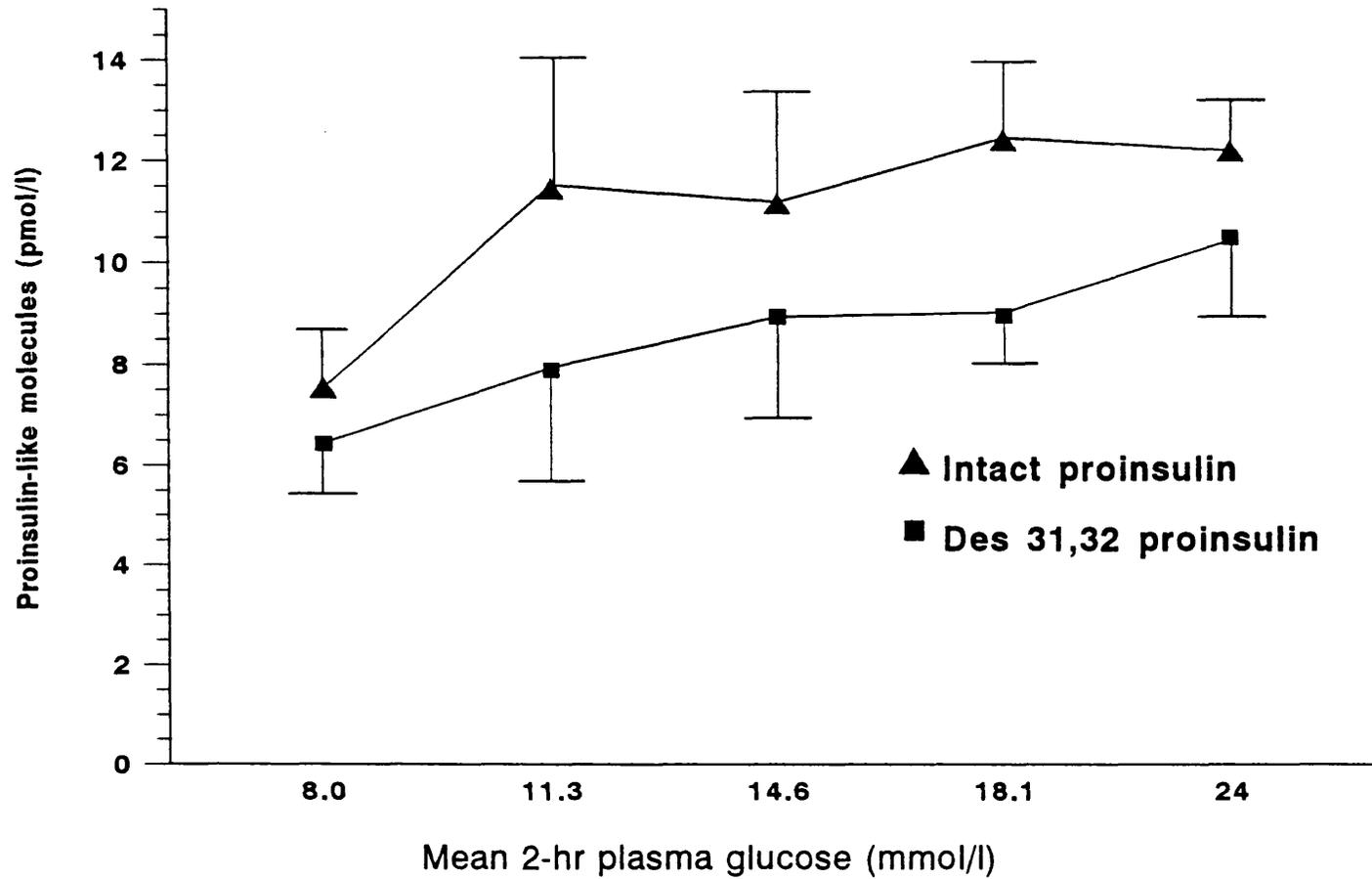


Figure 5.8: Fasting concentrations of proinsulin-like molecules in subjects with type 2 diabetes by severity of 2-hr plasma glucose

5.5.3.4 Cardiovascular risk factors

Subjects with type 2 diabetes had significantly higher total triglyceride, higher total and LDL-cholesterol, but lower HDL-cholesterol than non-diabetic subjects. Systolic and diastolic blood pressures were also higher in subjects with diabetes. Activity of PAI-1 was significantly higher in subjects with diabetes than subjects with NGT and IGT among both ethnic groups (fig 5.3). However PAI-1 activity was similar in subjects with normal and impaired glucose tolerance.

5.5.4 Relationship of insulin, intact proinsulin and des 31,32 proinsulin with cardiovascular risk factors

The correlation coefficients of various cardiovascular risk factors with insulin, proinsulin and des 31,32 proinsulin are shown in all subjects and for different ethnic groups in table 5.8. In all subjects, in a bivariate analysis, concentrations of proinsulin-like molecules were significantly associated with age, body mass index and waist-hip-ratios. Further analyses of the relationships of cardiovascular risk factors with specific insulin and proinsulin-like molecules were performed after controlling for the effects of age sex, BMI and WHR in a multiple regression analyses.

5.5.4.1 Relationship with body mass index and waist-hip ratio

In caucasian non-diabetic subjects neither insulin nor proinsulin like molecules showed any significant association with BMI or WHR. In caucasian diabetic subjects, both fasting insulin ($p=0.04$) and fasting proinsulin like molecules ($p=0.008$) however were related significantly to BMI but not to WHR. These associations with BMI persisted after controlling for WHR.

In Asians fasting insulin concentrations were related significantly to BMI ($p=0.01$) and WHR ($p=0.02$) in non-diabetic subjects but not in diabetics. The relationship of BMI with

insulin in non-diabetic subjects was weakened after controlling for the effect of WHR.

Proinsulin like molecules were not related to either BMI or WHR in Asian non-diabetic subjects but significantly in diabetic subjects (BMI $p=0.002$, WHR $p=0.004$). Once again the association of BMI to proinsulin like molecules was weakened when controlled for the effects of WHR but that of WHR became insignificant when controlled for BMI.

5.5.4.2 Relationship with systolic and diastolic blood pressure

In all subjects, insulin concentrations, intact and des 31,32 proinsulin concentrations were significantly associated with both systolic and diastolic blood pressures (Table 5.8). However after controlling for age, sex, ethnicity, BMI and WHR, the relationship of insulin with diastolic blood pressure became insignificant while that of intact proinsulin and des 31,32 proinsulin persisted (Table 5.8a)

5.5.4.3 Relationship with plasma triglyceride

Insulin, intact and des 31,32 proinsulin showed significant relationship and of similar magnitude to plasma triglyceride in all subjects. The relationship of these molecules with triglyceride persisted after controlling for age, sex, ethnicity, BMI and WHR.

5.5.4.4 Relationship with total cholesterol, HDL-cholesterol and LDL-cholesterol.

In all subjects insulin concentrations were significantly and positively related to total and LDL-cholesterol and negatively with HDL-cholesterol. Intact proinsulin and des 31,32 proinsulin concentrations were related positively to total and LDL-cholesterol and negatively to HDL-cholesterol (Table 5.8). The relationships of intact and des 31,32 proinsulin with total, HDL-cholesterol and LDL- cholesterol were similar to those for insulin.

Table 5.8: Relationship[■] of cardiovascular risk factors with insulin, proinsulin and des 31,32 proinsulin in caucasian and Asian diabetic and non-diabetic subjects.

Variables	Insulin			Intact proinsulin			Des 31,32 proinsulin		
	All	Caucasian	Asian	All	Caucasian	Asian	All	Caucasian	Asian
Age	0.10	0.15	0.08	0.25§	0.32§	0.22*	0.16*	0.21*	0.11
Body mass index	0.28§	0.26¶	0.33§	0.32§	0.30§	0.38§	0.35§	0.36§	0.36§
Waist-hip-ratio	0.29§	0.30¶	0.30§	0.39§	0.44§	0.37§	0.37§	0.46§	0.32§
SBP	0.23§	0.26¶	0.21¶	0.32§	0.36¶	0.32§	0.30§	0.29¶	0.31§
DBP	0.23§	0.20*	0.27¶	0.33§	0.33¶	0.36§	0.34§	0.34¶	0.34§
Triglyceride	0.37§	0.30¶	0.46§	0.42§	0.41§	0.44§	0.48§	0.51§	0.44§
Cholesterol	0.21§	0.24¶	0.20*	0.23§	0.26¶	0.23*	0.28§	0.33§	0.23*
HDL-cholesterol	-0.24§	-0.16	-0.32§	-0.25§	-0.23*	-0.15	-0.23§	-0.19*	-0.26¶
LDL-cholesterol	0.18¶	0.20*	0.14	0.18¶	0.19*	0.21*	0.22¶	0.24*	0.21*
PAI-1 activity	0.28§	0.35¶	0.21*	0.40§	0.57§	0.25*	0.39§	0.57§	0.25*
Fasting glucose	0.22§	0.21*	0.24¶	0.60§	0.65§	0.58¶	0.44§	0.44§	0.45¶
2-hr glucose	0.26§	0.30¶	0.24*	0.64§	0.71§	0.60¶	0.46§	0.48§	0.45¶
HbA1	0.18¶	0.19*	0.18*	0.47§	0.43§	0.50¶	0.36§	0.31¶	0.40¶
Alb/Creat ratio	0.04	-0.023	0.08	0.15*	0.08	0.19*	0.16*	0.09	0.20*

* $p < 0.05$, ¶ $p < 0.01$, § $p < 0.001$, SBP = systolic blood pressure, DBP = diastolic blood pressure

■ relationship analysed by Pearsons correlation after log transforming the skewed data

Table 5.8 a: Relationship[■] of systolic and diastolic blood pressures with insulin, and proinsulin-like molecules in all subjects

Variables	Insulin	Intact proinsulin	Des 31,32 proinsulin
Systolic blood pressure	0.15 *	0.22 §	0.20 §
Diastolic blood pressure	0.12	0.20 §	0.22 §

■ Partial correlation coefficients, adjusted for age, sex, ethnicity, BMI and WHR. Relationship analysed by Pearsons correlation after log transforming the skewed data

* $p < 0.05$, ¶ $p < 0.01$, § $p < 0.001$,

5.5.4.5 Relationship with urine albumin creatinine ratio

Proinsulin-like molecules were related to urine albumin/creatinine ratio in all subjects, while this relationship was not true for insulin concentrations.

5.5.5 Physical activity and cardiovascular risk factors

Non-diabetic subjects with higher grades of physical activity had lower serum cholesterol (ANOVA $p=0.03$) but there were no significant associations between physical activity and triglyceride, HDL-cholesterol, PAI-1, systolic or diastolic blood pressure.

5.5.6 Relationship of PAI-1 with cardiovascular risk factors, insulin and proinsulin-like molecules.

The correlation coefficients between PAI-1 and other variables is shown in table 5.9 for all subjects. In all subjects in a univariate analysis, PAI-1 activity was significantly related to age, BMI, WHR, STR, fasting C-peptide and fasting insulin (Fig 5.9), intact proinsulin, des 31,32 proinsulin concentrations, serum triglyceride (Fig 5.10), systolic and diastolic blood pressure, fasting plasma glucose (Fig 5.11) and 2-hr post load glucose concentrations (Fig 5.12) and weakly to urine albumin/creatinine ratio.

Controlled for age, sex and ethnicity the association of PAI-1 activity with WHR became insignificant (Table 5.10, Model 1) while that of BMI persisted (Table 5.10, Model 2). The relationship of BMI with PAI-1 activity also remained significant when controlled for WHR (Table 5.10, Model 3) but became insignificant when controlled for insulin and triglyceride concentrations (Table 5.10, Model 4)

After controlling for the effects of age, sex, ethnicity, BMI and WHR, PAI-1 activity was significantly related to fasting insulin (partial $r=0.22$, $p<0.001$), intact proinsulin

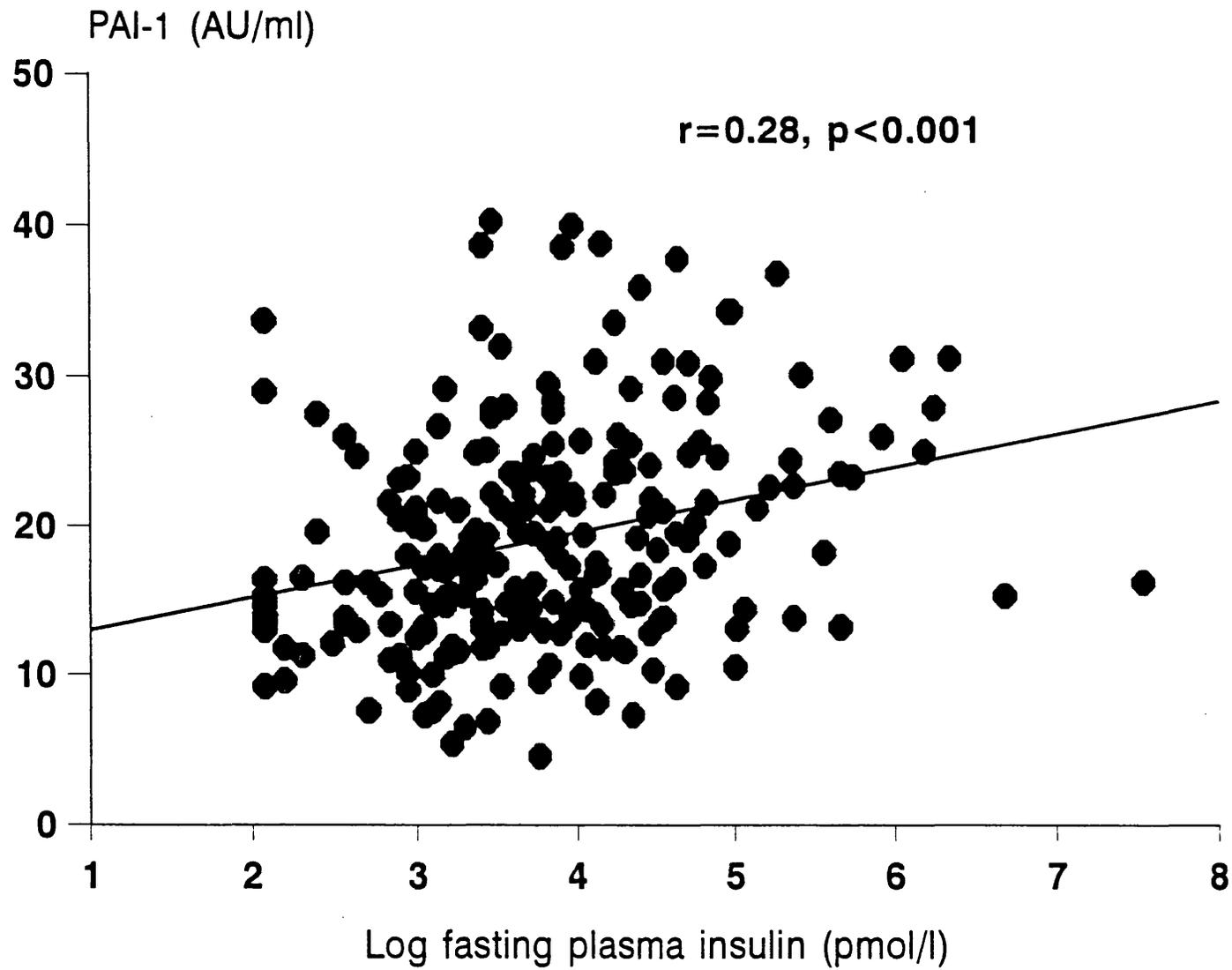


Figure 5.9: Relationship of PAI-1 activity with fasting plasma insulin concentrations

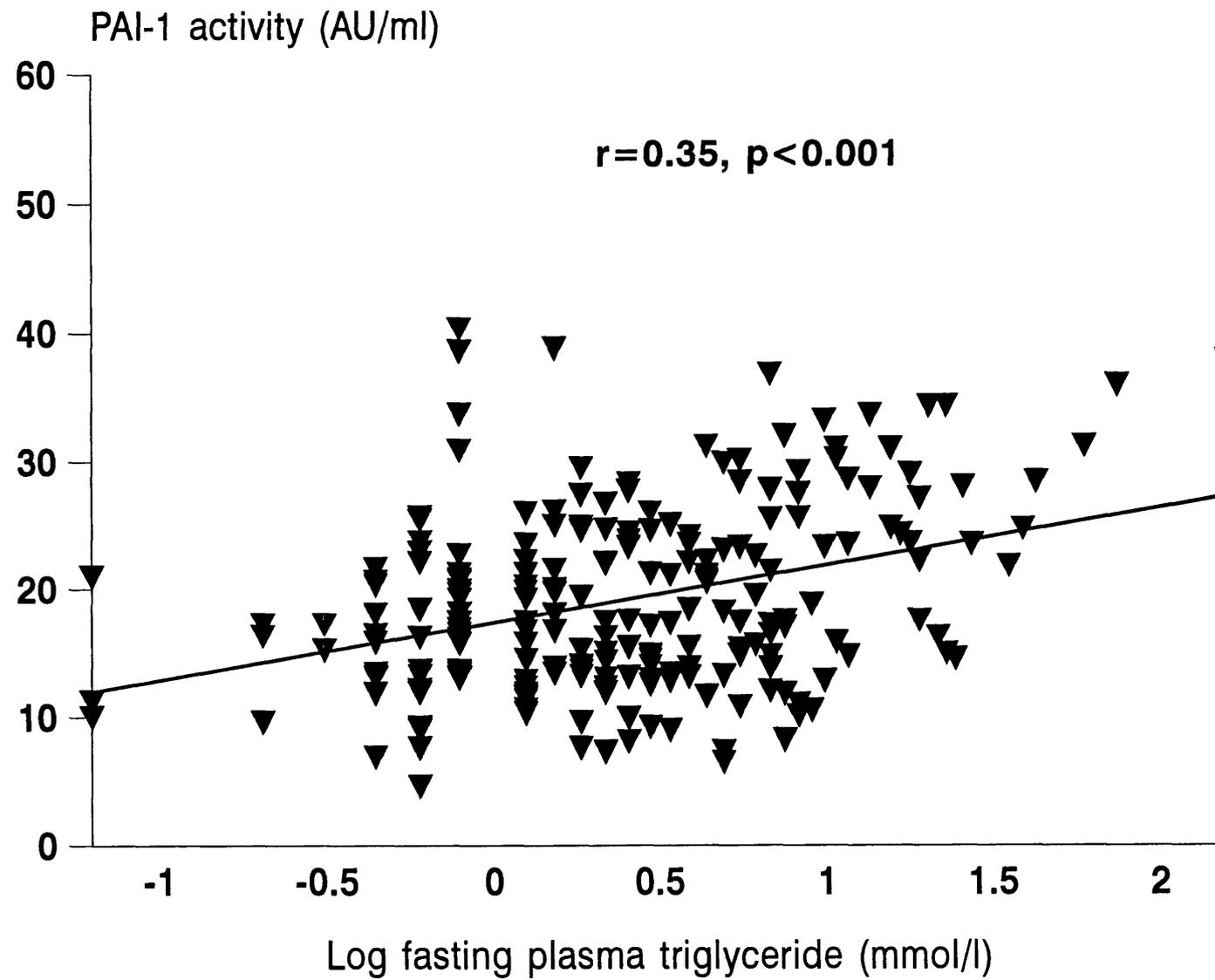


Figure 5.10: Relationship of PAI-1 activity with fasting plasma triglyceride concentrations

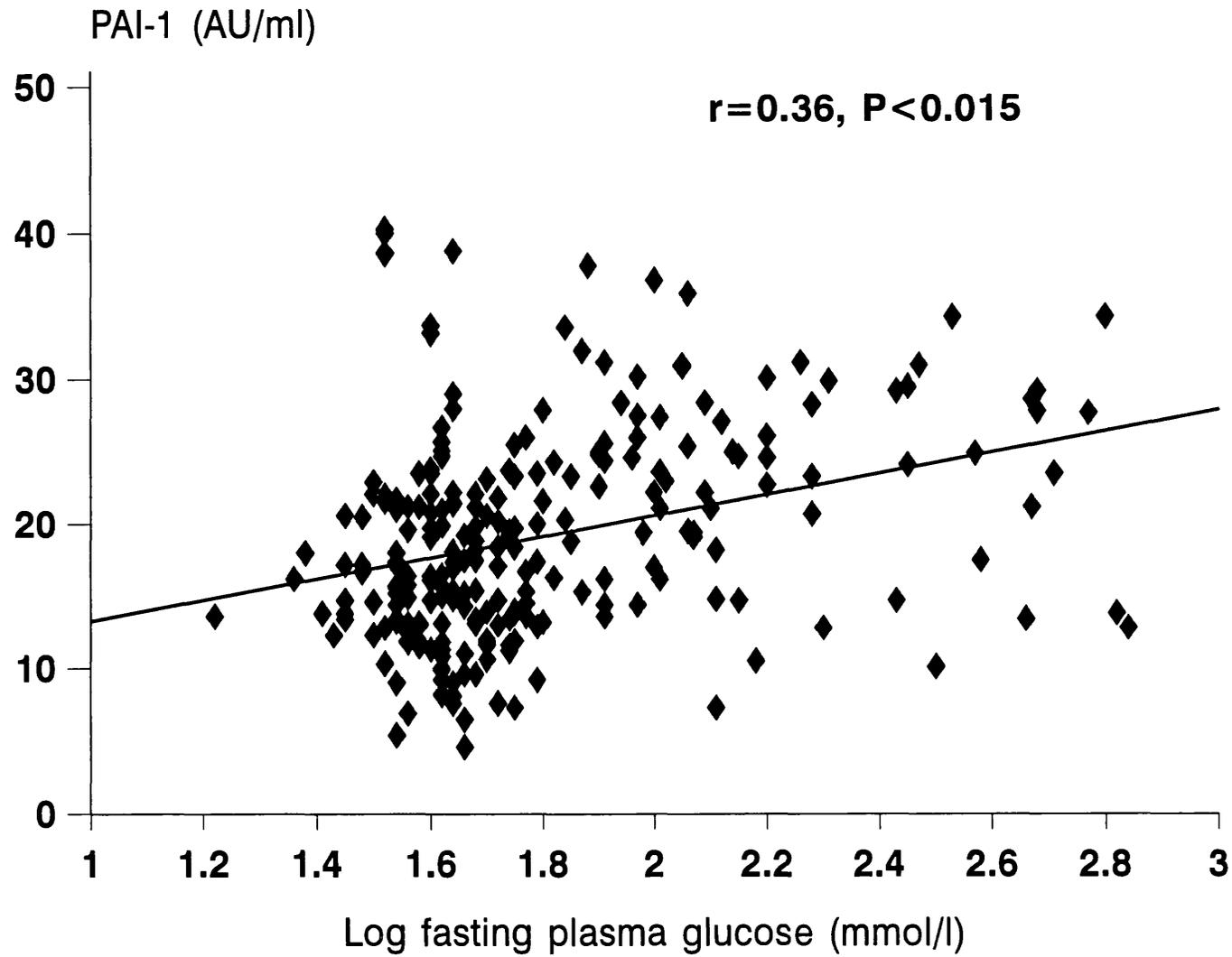


Figure 5.11: Relationship of PAI-1 activity with fasting plasma glucose concentrations

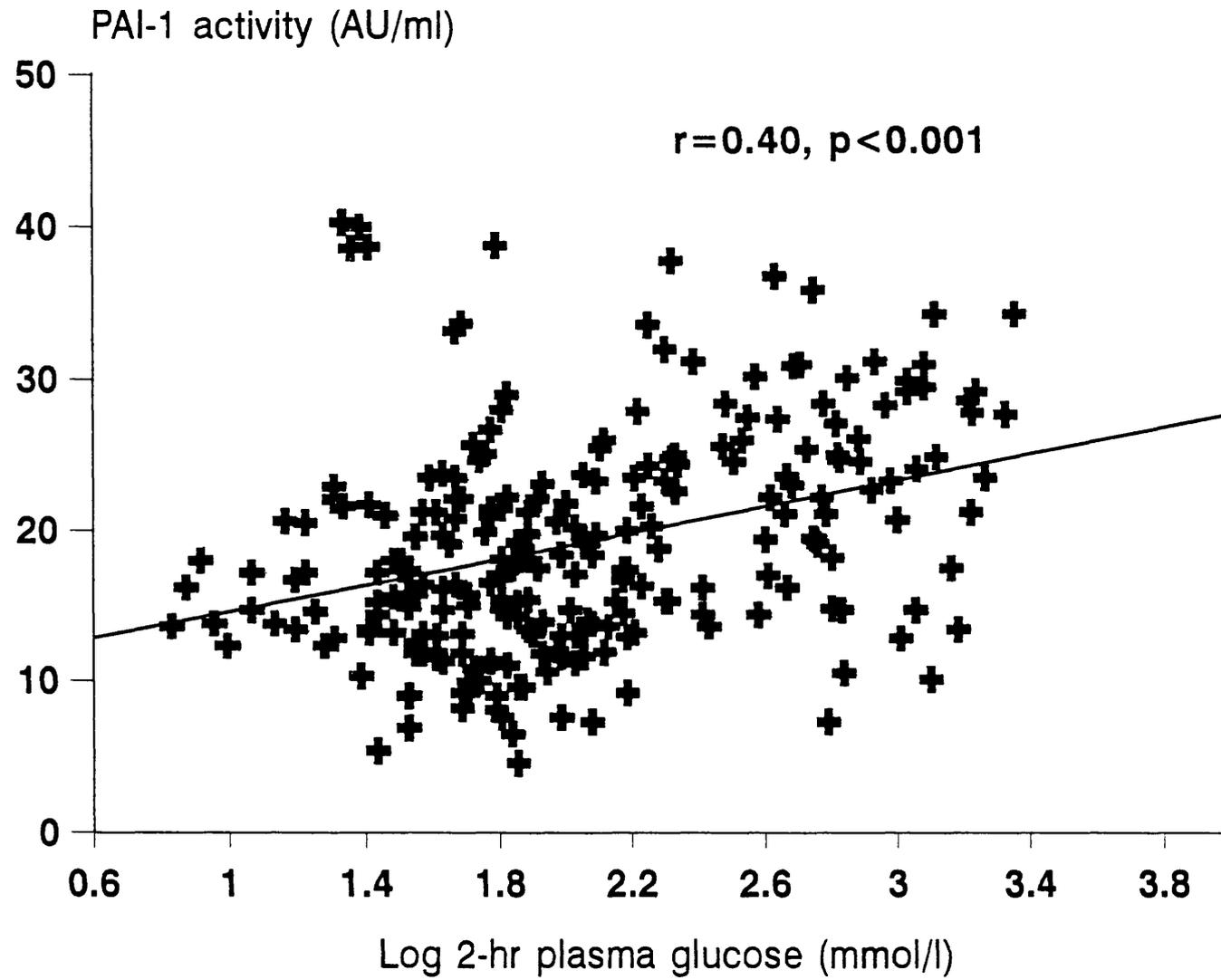


Figure 5.12: Relationship of PAI-1 activity with 2-hr plasma glucose concentrations

Table 5.9: Relationships[■] of PAI-1 activity with study variables in all subjects

Variables	All Subjects (n = 249)¶	Non-diabetic (n = 163)	Diabetic (n = 86)
Age	0.14¶	0.14	-0.20
Body mass index	0.25§	0.14*	0.27§
Subscapular-triceps ratio	0.13*	0.02	0.03
Waist-hip-ratio	0.12¶	0.04	0.04
Insulin	0.28§	0.11	0.24*
Intact proinsulin	0.41§	0.10	0.32¶
Des 31,32 proinsulin	0.39§	0.17	0.41§
C-peptide	0.33§	0.10	0.27¶
Triglyceride	0.35§	0.10	0.43¶
Fasting glucose	0.33§	-0.00	0.15
2-hr glucose	0.40§	0.06	0.22*
HbA1	0.24§	0.04	0.06
Systolic blood pressure	0.18*	0.05	0.06
Diastolic blood pressure	0.15*	0.03	0.15

■ Relationship analysed by Pearsons correlation after log transforming the skewed data.

* $p < 0.05$ ¶ $p < 0.01$ § $p < 0.001$

Table 5.10: Multiple regression analyses to determine the associations of PAI-1 to WHR and BMI

MODELS				
Variables in the models	Estimate	SE	T value	p value
Model 1				
Waist-hip-ratio *	9.1830	6.5083	1.41	0.15
Model 2				
Body mass index *	0.4048	0.1057	3.83	0.0002
Model 3				
Body mass index §	0.4046	0.1150	3.54	0.0005
Model 4				
Body mass index –	0.1622	0.1081	1.50	0.13

* Adjusted for age, sex and ethnicity

§ Adjusted for age, sex, ethnicity and WHR

– Adjusted for age, sex, ethnicity, insulin and triglyceride

(partial $r = 0.36$, $p < 0.001$), des 31,32 proinsulin (partial $r = 0.33$, $P < 0.001$), fasting triglyceride concentrations (partial $r = 0.30$, $p < 0.001$), fasting glucose (partial $r = 0.31$, $p < 0.001$) and 2-hr glucose concentrations (partial $r = 0.36$, $p < 0.001$) and also to diabetes as a categoric variable.

To examine if a combination of variables explained the observed association of PAI-1 with diabetes two models were constructed. In the first model diabetes as a categoric variable was added after age, sex and ethnicity (Table 5.12, model 1). In such a model diabetes was significantly associated with PAI-1 activity. In the second model, variables BMI, WHR, insulin, intact proinsulin, des 31,32 proinsulin and triglyceride were then forced into the model (table 5.12, model 2). Inclusion of these variables in such a model reduced the significance of association of PAI-1 with diabetes, but nevertheless it still remained statistically significant ($T = 2.05$, $p = 0.042$). However the addition of 2-hr plasma glucose into the model after triglyceride reduced the significance of association of diabetes with PAI-1 activity ($T = 1.3$, $p = 0.21$).

We also assessed, using similar multiple regression analysis techniques, whether the association of diabetes with various cardiovascular risk factors can be explained by combination of insulin, intact proinsulin and des 31,32 proinsulin. In such analyses, insulin-like molecules when forced into the model before diabetes as a categoric variable, either abolished the significance of the associations of diabetes (triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, and diastolic blood pressure) or reduced the significance of the association (systolic blood pressure, $p = 0.042$ and PAI-1 activity, $p = 0.022$).

When results were analysed separately in non-diabetic (normal+IGT) and diabetic subjects, different results were apparent and the estimates of PAI-1 activity controlled

for several variables is shown in table 5.11. In the non-diabetic subjects PAI-1 activity was related significantly only to body mass index and to no other variables measured in this study. In non-diabetic subjects the relationship of body mass index with PAI-1 became insignificant after controlling for age, sex, whr, fasting insulin and fasting serum triglyceride concentrations. However, PAI-1 activity in diabetic subjects was significantly associated with, body mass index and not to WHR and also to fasting insulin, intact proinsulin, des 31,32 proinsulin, C-peptide and 2-hr plasma glucose following oral glucose load. However, after adjusting for age, sex, ethnicity, BMI and WHR, PAI-1 activity was only related to fasting intact proinsulin, des 31,32 proinsulin, plasma triglyceride and 2-hr post load glucose concentrations, and not to fasting insulin (Table 5.11).

5.5.7 Ethnic differences in the association of PAI-1 activity with other measures

The relationship of PAI-1 with BMI ($r = 0.41$, $p < 0.001$) and WHR ($r = 0.30$, $p < 0.001$) was stronger in caucasian subjects, while in Asian subjects PAI-1 was only weakly related to BMI ($r = 0.24$, $p < 0.05$) and showed no relationship to WHR ($r = 0.04$, $p = \text{ns}$). Similarly PAI-1 was weakly but significantly related to systolic blood pressure ($r = 0.19$, $p < 0.05$) in Asian subjects while the relationship in caucasian subjects was insignificant ($r = 0.05$, $p = \text{ns}$). However there was a considerable overlap of confidence intervals for these correlations between the two ethnic groups.

Table 5.11: Covariate adjusted estimates of correlations of PAI-1 activity in all subjects, non-diabetics and with type 2 diabetes

	Non-diabetic subjects			Diabetic subjects			All subjects		
	Estimate	standard error	p value	Estimate	standard error	p value	Estimate	standard error	p value
Body mass index¶	0.1833	0.1172	0.12	0.5075	0.2026	0.014	0.404	0.105	0.002
Waist-hip-ratio¶	-1.6817	7.5222	0.82	10.1161	10.701	0.34	9.183	6.508	0.15
SBP§	0.0114	0.0348	0.74	0.0086	0.0418	0.83	0.046	0.027	0.08
DBP§	-0.0353	0.0513	0.49	0.0540	0.0713	0.45	0.387	0.117	0.001
Fasting insulin§	0.7192	0.7803	0.35	1.0370	0.7373	0.16	1.756	0.526	0.001
Fasting intact proinsulin§	0.5584	1.0478	0.59	2.5189	1.1665	0.03	3.282	0.597	0.0001
Fasting des 31,32 proinsulin§	1.0114	0.6055	0.09	2.5408	0.8522	0.003	2.354	0.458	0.0001
Triglyceride§	1.3059	1.1155	0.24	5.7334	1.3371	0.001	4.267	0.876	0.001
Fasting glucose§	0.0025	0.8690	0.99	0.3423	0.2356	0.15	0.840	0.170	0.0001
2-hr glucose§	0.1507	0.2903	0.60	0.2765	0.1365	0.046	0.486	0.076	0.0001
Urine albumin/Creatinine ratio§	0.6148	0.3875	0.11	-0.3202	0.4434	0.47	0.351	0.302	0.24

¶ Adjusted for age, sex and ethnicity, § Adjusted for age, sex, ethnicity, BMI and WHR

SBP= systolic blood pressure, DBP= diastolic blood pressure

Table 5.12: Multiple regression analyses to determine the associations of PAI-1 to diabetes

MODELS				
Model 1	R² = 0.16, df = 4, F = 9.34, p = 0.0001			
Variables in the model	Estimate	SE	T value	p value
intercept	16.034	3.8035	4.22	0.0001
age	0.0173	0.0541	0.32	0.74
sex	0.5577	0.8934	0.62	0.53
ethnicity	0.5083	0.8685	0.59	0.55
diabetes (yes, no)	6.0419	0.9775	6.18	0.0001
Model 2	R² = 0.28, df = 10, F = 6.11, p = 0.0001			
Variables in the model	Estimate	SE	T value	P value
intercept	22.349	7.4545	3.00	0.0031
age	-0.0378	0.0585	-0.65	0.51
sex	0.2030	1.2677	0.16	0.87
ethnicity	0.3291	0.9455	0.35	0.72
waist-hip-ratio	-12.421	6.9148	-1.80	0.07
body mass index	0.1467	0.1169	1.25	0.21
triglyceride	2.8351	0.9775	2.90	0.004
intact proinsulin	1.0626	1.1656	0.91	0.36
des 31,32 proinsulin	1.0019	0.6538	1.53	0.12
insulin	0.1057	0.5729	0.18	0.85
diabetes (yes, no)	2.8997	1.4164	2.05	0.042

5.5.8 Discussion

The main results of this study show that using a highly specific assay to measure insulin, Asian non-diabetic subjects show hyperinsulinaemia following an oral glucose tolerance test compared with age and sex matched caucasian subjects, results which are not explained by differences in body mass index and centrality of obesity between Asian and caucasian subjects. A previous study has shown that in normoglycaemic subjects, post glucose load insulin concentrations correlate positively and significantly with insulin resistance as measured by euglycaemic clamps (Hollenbeck et al, 1987). Hyperinsulinaemia after an oral glucose load in Asian non-diabetic subjects may, therefore, be the consequence of underlying insulin resistance. Similar results of hyperinsulinaemia in response to oral glucose have previously been reported in non-diabetic Asian subjects resident in London U.K (McKeigue et al, 1991), Asian men surviving acute myocardial infarction (Hughes et al, 1989) and non-diabetic Asians from Bradford UK (Knight et al, 1992) and Coventry (Simmons et al, 1993) in the U.K. However all these studies used insulin assays which were not specific for insulin. A recent study of non-diabetic first degree relatives of Asian subjects with type 2 diabetes showed higher insulin concentrations during OGTT than in normal controls (Gelding et al, 1994). In the same study Asian subjects were also hyperinsulinaemic compared to a caucasian group, although the two ethnic groups were not well matched and had small numbers of subjects. Previous studies in Asian subjects with MODY also showed that these subjects had very high IRI responses to glucose load compared to those reported in European populations (Mohan et al, 1984), though no direct comparison with caucasian subjects were made in this study. A previous study found no differences in insulin response among Asians and European non-diabetic subjects (Rubenstein et al, 1969).

Higher insulin concentrations in Asians following an oral glucose load, may however, be due to an enhanced sensitivity of pancreatic β -cells to glucose in this population. Higher fasting molar ratio of insulin to C-peptide in Asian non-diabetic subjects is suggestive of lower hepatic extraction of insulin in this ethnic group and the observed differences in insulin response to oral glucose load in Asian subjects may in part be due to this phenomenon. However one has to be careful of the pitfalls using C-peptide/insulin molar ratio as an index of hepatic insulin extraction (Polonsky et al, 1984).

Asian non-diabetic subjects also showed a tendency toward low levels of physical activity and more central distribution of fat, both of which are known to decrease insulin sensitivity (Koivisto et al, 1986; Despres et al, 1988). However differences in the physical activity patterns or centrality of obesity between Asian and caucasian non-diabetic subjects did not explain the higher insulin concentrations in Asian subjects. The results of this study are novel in that this study employed assays which are highly specific and are able discriminate between insulin and insulin precursor molecules.

Asian subjects with type 2 diabetes also had significantly higher 2-hr post glucose load insulin concentrations, results similar to those in non-diabetic subjects. However no ethnic differences in insulin precursor molecules were found among subjects with type 2 diabetes. In the only previous study comparing Asian and caucasian subjects with diabetes, Asians showed both fasting and post glucose load hyperinsulinaemia, using conventional assays for insulin (Mohan et al, 1986).

Exaggerated insulin response to an oral glucose load, similar to seen in the current study, has previously been reported, albeit using assays which were not specific, in Pima Indians (Aronoff et al, 1977) and Nauruans (Zimmet et al, 1979), ethnic groups

with high susceptibility to type 2 diabetes. A recent prospective study in Pima Indians has shown that exaggerated insulin response to intravenous glucose and oral glucose has been shown to predict future diabetes in non-diabetic Pima Indians, though not to same degree as does insulin resistance (Lillioja et al, 1993). In this study there was a 25 fold increase in incidence of diabetes between the 10th and 90th percentile of insulin resistance, whereas the increase between the 10th and 90th percentile of acute insulin response was 3 fold even when insulin resistance was taken into account. Whether exaggerated insulin response to oral glucose load plays a significant role in the development of future diabetes in Asian subjects is unknown and can be answered only in prospective studies.

The prevalence of electrocardiographic evidence of ischaemic heart disease was significantly higher in Asian non-diabetic subjects than their caucasian counterparts despite the fact that this population had significantly lower rates of smoking than caucasians. Similar finding of high prevalence of IHD has recently been reported from Asians in Mauritius (Tuomilehto et al, 1993). There were no significant differences in cardiovascular risk factors including PAI-1 activity among Asian and caucasian non-diabetic subjects other than a tendency towards a more central distribution of fat in Asian subjects. These results are different from those of previous studies which have shown that Asian non-diabetic subjects had higher plasma triglyceride and low total and HDL-cholesterol concentrations (McKeigue et al, 1991; Knight et al, 1992). It is likely that the number of subjects in our study is not big enough to show the differences observed in previous studies. However the results of this study are similar to those of McKeigue et al showing a central distribution of fat in Asian subjects (McKeigue et al, 1991).

Electrocardiographic abnormalities in Asians were significantly associated with waist-

hip ratio and body mass index but not to any of the measured cardiovascular risk factors including PAI-1 activity. Previous studies have shown that central obesity is an independent predictor of ischaemic heart disease (Larsson et al, 1984; Lapidus et al, 1984). Neither fasting insulin nor 2-hr insulin concentrations following oral glucose load showed any association with ECG evidence of ischaemic heart disease, results which are very similar to those recently reported in subjects from Mauritius and Nauru, two other populations with high prevalence of type 2 diabetes (Collins et al, 1993). However the number of subjects with ischaemic heart disease in this study is too small to argue for or against the role of hyperinsulinaemia or insulin resistance acting through conventional cardiovascular risk factors and PAI-1 activity in this population, and therefore these results can not be generalised.

This study also shows that subjects with type 2 diabetes, treated with diet alone, have significant fasting hyperinsulinaemia compared to normal subjects in both ethnic groups. However, insulin concentrations at 30-min after oral glucose load were significantly lower in subjects with diabetes compared to non-diabetic subjects. These results are in agreement with the published results using specific assays for insulin similar to the one employed in the present study (Temple et al, 1989; Davies et al, 1993). However our results differ from those of Temple et al and Davies et al in two respects. Firstly subjects with type 2 diabetes in our study showed significantly higher fasting insulin concentrations than non-diabetic subjects. Secondly, there was a clear overlap of insulin concentrations at 30-min between subjects with normal glucose tolerance and those with type 2 diabetes, thereby failing to confirm the previous findings of Temple et al, which showed a clear demarcation in 30-min insulin response between non-diabetic and subjects with type 2 diabetes. These differences between our findings and those of Temple et al and Davies et al may be due to heterogeneity of type 2 diabetes, and different populations included in these studies.

Fasting insulin concentrations were related to severity of fasting and 2-hr post load glucose and demonstrated an inverted U shaped curve, which has been previously shown, using conventional assays for insulin, in cross-sectional and longitudinal studies (DeFronzo et al, 1988; Zimmet et al, 1987; Saad et al, 1989).

We have also confirmed that concentrations of intact proinsulin and des 31,32 proinsulin are elevated in subjects with diet treated subjects with type 2 diabetes both in Asian and caucasian subjects. However, the proportion of proinsulin-like molecules was much less than found in our previous observations (Chapter 4), where subjects with diabetes on average had a proportion of proinsulin like molecules comprising $62 \pm 15\%$ of the sum of insulin and proinsulin-like molecules. However subjects in the current study were diet treated and had only mild to moderate degree of fasting hyperglycaemia which may explain some of the differences. Other possible explanations for these results is the heterogeneity of diabetes and different populations of subjects included in these studies.

We observed a significant association of low levels of physical activity with type 2 diabetes in both ethnic groups, results which are in accordance with the previous studies showing low physical activity to be a predictor of future diabetes in caucasian (Helmrich et al, 1991; Manson et al, 1991), and Asian subjects (Dowse et al, 1991). It is highly unlikely that the lower levels of physical activity observed in subjects with type 2 diabetes in the current study were due a consequence of the development of type 2 diabetes, as these subjects were healthy and free of any major complications known to restrict physical activity. A significant association of elevated concentrations of serum cholesterol with low levels of physical activity was also observed in non-diabetic subjects which was only partly explained by body mass index and waist-hip ratio and may, therefore, reflect a direct effect of physical activity on serum cholesterol

concentrations. Similar findings have previously been reported from Asian Indians in Mauritius (Zimmet et al, 1991; Dowse et al, 1991).

The relationships of insulin concentrations, as measured by a highly specific and sensitive assay, with cardiovascular risk factors were generally similar in both ethnic groups. We have once again, in a much larger study sample which included diabetic and non-diabetic subjects, observed significant associations of intact proinsulin and des 31,32 proinsulin with cardiovascular risk factors, similar to or stronger than those seen for insulin. These findings are consistent with our observation in subjects with diabetes (Chapter 4, 6, 7), and more recently by others in non-diabetic subjects (Haffner et al, 1993), and subjects with type 2 diabetes (Davies et al, 1993; Panahaloo et al, 1994). However the biological importance of these associations remains unclear. In-vitro and animal studies have shown that intact proinsulin and its metabolites have biological actions similar to that of insulin on carbohydrate, and lipid metabolism (Galloway et al, 1992) and also on plasminogen activator inhibitor-1 expression (Schneider et al, 1992). However the biological potency of these molecules is much less than that of insulin on a molar basis. Given that in non-diabetic subjects the molar concentrations of these molecules is approximately 1/10 that of insulin, it is highly unlikely that they have a major causal role to play in determining cardiovascular risk factors in non-diabetic subjects. A more likely explanation might be that these associations of concentrations of insulin and proinsulin-like molecules with cardiovascular risk factors are not causal and instead represent associations through some common antecedent, possibly insulin resistance or a low birth weight (Hales et al, 1992).

Studies have shown low birth weight to be associated with cardiovascular mortality (Barker et al, 1993), glucose intolerance (Phipps et al, 1993) and raised proinsulin-like molecules in adult life (Hales et al, 1991). More recently low birth weight was also

associated with higher insulin resistance, as measured by a short insulin tolerance test, in adult life (Phillips et al, 1994) in caucasian subjects. However these authors in their subsequent publication found that subjects with low birth weight had normal insulin secretion (Phillips et al, 1994). Recently, similar findings have been reported from the San-Antonio Heart Study, where subjects with low birth weight showed many of the features of Insulin Resistance Syndrome in adult life and poor health outcomes (Valdez et al, 1994). It is, therefore, possible that the relationships of proinsulin-like molecules with cardiovascular risk factors first reported by us and subsequently by others (Davies et al, 1993, Haffner et al, 1993), may be partly due to a common association of low birth weight with increased insulin resistance and hence higher concentrations of proinsulin-like molecules, and increased risk of type 2 diabetes and ischaemic heart disease in adult life (Barker et al, 1993).

In subjects with diabetes, however, where concentrations of these molecules are usually much higher, these molecules may still partly contribute to the biochemical disturbances, particularly of lipids and PAI-1 activity, commonly reported to be associated with insulin resistance and/or "hyperinsulinaemia" seen in these subjects. This hypothesis is supported by in-vitro studies on pig aortic endothelium suggesting that intact proinsulin and split proinsulin both increased PAI-1 activity, and a parallel increased expression of PAI-1 mRNA. The potency of proinsulin in stimulating PAI-1 activity was of the same magnitude as that of insulin. Furthermore the effects of proinsulin were not mediated by its interaction with the insulin receptor and could not be attenuated by insulin or IGF-1 (Schneider et al, 1992). It seems that split forms of proinsulin may have a higher potency than that of intact proinsulin, because their receptor binding is of higher magnitude than that of intact proinsulin (Galloway et al, 1992).

The results of this study also demonstrate that PAI-1 activity is elevated in subjects with type 2 diabetes who are treated with diet alone and without severe hyperglycaemia. These findings are similar to number of previous studies of subjects with type 2 diabetes (Auwerx et al, 1988; Wamsley et al, 1990; Garcia Frade et al, 1990; Gough et al, 1993), while different from other who found no such difference (Grant et al, 1989; Rydzewski et al, 1990; Ho et al, 1991). However this is the first study investigating PAI-1 activity in large number of diet treated subjects with relatively good glycaemic control and without the confounding effect of treatment for diabetes and presence of microvascular and significant macrovascular complications, which may have effects on circulating PAI-1 activity (Hamsten et al, 1985; Garcia-Frade et al, 1990; Rydzewski et al, 1990).

PAI-1 activity in subjects with IGT, however, was similar to that in normal subjects, although the number of subjects with IGT was small. Subjects with IGT were also similar to normal subjects in most aspect other than having slightly but significantly elevated fasting plasma glucose and des 31,32 proinsulin concentrations. Impaired glucose tolerance was diagnosed following a single OGTT and not confirmed by a repeat OGTT and it is likely that a large proportion of these subjects would have normal glucose tolerance on repeat OGTT (Olefsky et al, 1971; Harding et al, 1972) and subjects diagnosed to have IGT on the basis of two OGTTs are less likely to revert to NGT (Nagi et al, 1992).

The results of previous studies of the relationship between PAI-1 and glycaemic control are inconsistent, some reporting a positive relationship (Gunnarsson et al, 1980; Van wersch et al, 1990) while others showed an insignificant relationship (Auwerx et al, 1988; Garcia Frade et al, 1990; Juhan Vague et al, 1989). In a recent study, in a group of subjects admitted to hospital with acute myocardial infarction, PAI-1 was

related to plasma glucose concentrations at the time of admission (Gray et al, 1993). The finding of the current study show that fasting plasma glucose concentrations have an association with PAI-1 activity when data were analysed in all subjects, and that PAI-1 activity was significantly related to 2-hr plasma glucose concentrations among diabetic subjects. The association of diabetes with PAI-1 was not fully explained by insulin, proinsulin-like molecules, triglyceride, and may suggest a direct effect of hyperglycaemia which is hallmark of diabetes, on PAI-1 activity. The underlying mechanism of this association is not entirely clear but it clearly is independent of the effects of BMI, serum triglyceride and insulin concentrations. A recent study has shown that human endothelial cells cultured in elevated glucose concentrations show increased expression of both tPA and PAI-1 (Maiello et al, 1992), and yet another study of IDDM subjects reported normalisation of impaired enzyme kinetics of fibrinolysis on lowering blood glucose levels (Geiger et al, 1985), suggesting that glucose may have a direct effect on PAI-1 expression from endothelial cells in-vitro and probably also in-vivo. These studies and our findings may provide evidence for an underlying mechanism for elevated levels of PAI-1 in hyperglycaemic states.

In a univariate regression, PAI-1 was related to BMI and WHR, results which are similar to previous studies (P Vague et al, 1989; Landin et al, 1990; Auwerx et al, 1988; J Vague et al, 1989). This relationship remained significant only with BMI and not with WHR, after controlling for age, sex and ethnicity. These findings suggest that overall adiposity, rather than its distribution, has an independent association with PAI-1 in the group of subjects studied. Furthermore the association of BMI with PAI-1 became insignificant after controlling for fasting insulin and triglyceride, suggesting that the effects of obesity on PAI-1 are mediated through its relationship with triglyceride and insulin concentrations. These results are similar to previous published data where the effect of obesity disappeared after controlling for insulin concentrations (Vague et al,

1986; Juhan-Vague et al, 1989). A previous study found that PAI-1 was related to WHR only in obese (BMI >35) women and not in lean, irrespective of their WHR (Landin et al, 1990) and as such our results are compatible with these previous observations because subjects in the current study were not grossly obese.

The relationship between PAI-1 and plasma insulin concentrations have been reported in non-diabetic (Juhan-Vague et al, 1989) and diabetic subjects (Nagi et al, 1990, Juhan-Vague et al, 1989) though not consistently (Hamsten et al, 1985, 1987). Most if not all conventional radioimmunoassays for insulin also measure proinsulin and its metabolite des 31,32 proinsulin (Temple et al, 1990). We have found that PAI-1 activity was unrelated to plasma insulin concentrations in non-diabetic subjects but only weakly related in diabetic subjects. However PAI-1 activity was significantly and strongly related to proinsulin-like molecules in diabetic subjects. Therefore the association of PAI-1 activity with insulin concentrations, measured a highly specific assay which has no cross reactivity with insulin precursor molecules, is much weaker than previous reports. Our observations (Chapter 4 and Chapter 5) would suggest that this weaker relationship may be consequent upon the assay measuring only insulin and not proinsulin and its precursors which are strongly associated with PAI-1 activity in subjects with type 2 diabetes.

In-vitro, insulin stimulates PAI-1 release from HepG2 cell lines (Alessi et al, 1988; Kooistra et al, 1989; Grant et al, 1991), but not from vascular endothelial cells (Alessi et al, 1988). Insulin infusion over a short period does not increase PAI-1 activity (Grant et al, 1990; Potter van Loon et al, 1990). However one study has shown that during euglycaemic hyperinsulinaemic clamp, concentration of t-PA increased by 75% and those of PAI-1 decreased by 49% after two hours (Landin et al, 1991). The results of these studies are in support of our findings where we have not seen an significant

association of insulin with PAI-1 among non-diabetic subjects.

However the lack of exogenous acute insulin infusions during short term to increase PAI-1 concentrations may be explained by number of reasons. Firstly, it may be due to the inability of exogenous insulin infusion to achieve sufficient level of portal insulinaemia needed to induce secretion of PAI-1 from liver, as PAI-1 concentrations rise presumably due to the effect of endogenous insulin secretion induced after a mixed meal (Medvescek et al, 1990), although one study showed no changes in PAI-1 after OGTT (McCormack et al, 1993). Secondly, a state of prolonged chronic hyperinsulinaemia may be essential for PAI-1 secretion from the liver, or alternatively the presence of both hyperglycaemia and portal insulinaemia may be required to secrete PAI-1 from liver. Finally, it is well established that insulin is secreted normally in a pulsatile fashion and exogenous insulin infusions do not mimic the exact physiological conditions. These explanations are speculative and need confirmation. In addition one has to remember that studies examining the effects of exogenous insulin on PAI-1 both in-vivo and in-vitro can not be equated with the possible effects of endogenous insulin which is a marker of insulin resistance especially in non-diabetic subjects. The lack of relationship of insulin concentrations with PAI-1 activity and an association with concentrations proinsulin-like molecules which are raised in subjects with type 2 diabetes may argue that proinsulin-like molecules, and not insulin regulate PAI-1 activity in subjects with type 2 diabetes.

A significant but weak relationship between triglyceride levels and PAI-1 has previously been noted in non-diabetic and diabetic subjects (Landin et al, 1990; Mehta et al, 1987; Hamsten et al, 1985; Juhan-Vague et al, 1987). In this study the strength of the relationship between PAI-1 and triglyceride was similar to the previous reported studies in subjects with type 2 diabetes. These findings would suggest that triglyceride

levels play an important role in the regulation of PAI-1 in diabetic subjects. This may provide an explanation by which the observed relationship between triglyceride concentrations and macrovascular disease in diabetic subjects may occur (Fontbonne et al, 1989). Recently it has been observed that very low density lipoprotein (VLDL) stimulates PAI-1 secretion by endothelial cells from umbilical vein, and VLDL from subjects with hypertriglyceridaemia was more potent than VLDL from normotriglyceridaemic subjects in stimulating PAI-1 release (Stiko-Rahm et al, 1990).

In subjects with type 2 diabetes, however, serum triglyceride concentrations were the variable most strongly related to PAI-1 activity and could explain 13% of the variation. The association of plasma triglyceride concentrations with PAI-1 activity in subjects with diabetes is also suggestive of a role of insulin resistance, as triglyceride concentrations have been shown to be raised in insulin resistant states, particularly in subjects with type 2 diabetes. Insulin resistance therefore, either directly or through raised plasma triglyceride concentrations, may play an important role in the pathogenesis of macrovascular disease by depressing fibrinolysis. Thrombosis has been shown to be a crucial factor in the evolution of acute coronary events in subjects predisposed to atherosclerosis (Davies et al, 1984; Dewood et al, 1980) and elevated PAI-1 activity may be part of the mechanism where by insulin resistance is associated with coronary heart disease (Vague et al, 1991). Recently, PAI-1 activity has been shown to be elevated in subjects with type 2 diabetes both at the time of admission with acute myocardial infarction and at follow-up 6 to 12 months later and therefore may be a determinant of outcome of acute myocardial infarction (Gray et al, 1993).

PAI-1 activity has been shown to be related to blood pressure (Landin et al, 1990). Our observation of a weak association of PAI-1 with systolic and a significant association with diastolic blood pressure in all subjects, disappeared in a multiple regression

analysis after controlling for insulin and triglyceride concentrations. These results suggests that the relationship of PAI-1 with blood pressure is likely to be an indirect one through its association with obesity, insulin concentrations and raised triglyceride concentrations, features likely to represent underlying insulin resistance.

Asian Indian subjects in the United Kingdom, an immigrant ethnic minority, have a high risk of coronary artery disease and increased prevalence of non-insulin-dependent diabetes (Marmot and Adelstein, 1985; Mather et al, 1985). Asian subjects also exhibit hyperinsulinaemia and increased insulin resistance (Mohan et al, 1986; Sharp et al, 1987) which may be related to their excess risk of glucose intolerance and perhaps also to excess risk of coronary heart disease (McKeigue et al, 1988). However, we found no differences in the concentrations of various risk factors and PAI-1 activity between Asian and caucasian subjects in different categories of glucose tolerance. However Asian subjects showed features of central obesity and were physically less active than caucasians.

5.5.9 Summary and conclusions

In conclusion the findings of this study indicate;

1. Hyperinsulinaemia in response to an oral glucose load in Asian non-diabetic subjects than in age- sex-matched caucasians, using specific assays for insulin.
2. Asian non-diabetic subjects show a central distribution of fat despite significantly lower body mass index than caucasians.
3. low levels of physical activity are seen in Asians than caucasians non-diabetic subjects but not among diabetic subjects.

4. absolute intact proinsulin concentrations are high in non-diabetic Asians but the proportion of proinsulin was similar to that in caucasian subjects.
5. Asian non-diabetic subjects had a higher prevalence of coronary artery disease than caucasians non-diabetic subjects.
6. no significant differences are seen in conventional cardiovascular risk factors such as lipids, systolic and diastolic blood pressure between Asians and caucasian non-diabetic subjects.
7. no ethnic differences in PAI-1 activity between Asian and caucasian subjects exists among subjects with different categories of glucose intolerance.
8. no significant differences between Asian and caucasian diabetic subjects other than a high total and low HDL-cholesterol of borderline significance in caucasian subjects.
9. subjects with type 2 diabetes showed significantly higher concentrations of fasting insulin than non-diabetic subjects in both ethnic groups. However the relationship of fasting insulin concentrations to fasting and 2-hr glucose was of an inverted U shaped, signifying that in subjects type 2 diabetes worsening hyperglycaemia is accompanied by a lower beta cell response and these subjects show insulin deficiency.
10. a lower insulin response at 30 min after oral glucose load was observed in subjects with type 2 diabetes implying an impaired insulin secretion.

11. subjects with type 2 diabetes showed significantly higher concentrations of intact and des 31,32 proinsulin in both ethnic groups, although the proportion of these molecules in subjects with diabetes was much less than in our previous observations.
12. concentrations of both intact and des 31,32 proinsulin showed a progressive rise with increase in 2-hr plasma glucose concentrations.
13. proinsulin like molecules were correlated with concentrations of number of cardiovascular risk factors and these associations were similar or stronger to those seen for specific insulin.
14. PAI-1 activity was elevated in diet treated subjects with diabetes while no differences were found between subjects with normal and impaired glucose tolerance.
15. PAI-1 activity was associated with BMI and not with WHR. The association of BMI with PAI-1 activity was explained by raised insulin and triglyceride concentrations in these subjects, signifying that the effects of obesity on PAI-1 activity are mediated by raised triglyceride and insulin concentrations.
16. a significant association of diabetes with PAI-1 activity was not fully explained by variables such as BMI, WHR, insulin, intact proinsulin, des 31,32 proinsulin and triglyceride concentrations.
17. PAI-1 activity in all subjects was significantly associated with fasting plasma glucose concentrations as well as with 2-hr post load glucose in subjects with

type 2 diabetes.

18. serum triglyceride concentrations were the single most important determinant of PAI-1 in diabetic subjects and could explain 13% variation of plasma PAI-1 activity.

These findings suggest that out of number of factors associated with PAI-1, serum triglyceride concentrations play an important part in the regulation of PAI-1 activity in diabetic subjects and raised PAI-1 activity thus may explain the excess of cardiovascular disease in subjects with IGT and type 2 diabetes reported in recent epidemiological study (Fontbonne et al, 1989). The independent association with BMI confirms the importance of generalised, rather than central obesity in relation to fibrinolysis. An association with plasma glucose concentrations suggests that hyperglycaemia per se may regulate PAI-1 activity. Raised levels of PAI-1 in subjects with type 2 diabetes, therefore, may reflect the combined effects of high serum triglyceride, raised plasma glucose and intact and des 31,32 proinsulin concentrations, and may play an important part in the pathogenesis of CAD by impairing fibrinolysis. However one has to be careful in interpreting these associations as all these may be a reflection of a common antecedent such as underlying insulin resistance, or possibly a low birth weight due to poor intrauterine growth, which has been shown to be associated with higher insulin resistance, raised levels of proinsulin-like molecules and excess of coronary artery disease in adult life.

Asian subjects show high rates of diabetes and features of central obesity which are accompanied by exaggerated insulin response to oral glucose load and may reflect underlying insulin resistance. Low levels of physical activity in Asians and its negative relationship with body mass index suggests that lack of physical activity in this ethnic

group may predispose them to obesity and hence insulin resistance and its metabolic sequelae. However we found no ethnic differences in the PAI-1 activity between Asian and caucasian subjects and these findings suggest that differences in the CHD risk between these two ethnic groups is not due to PAI-1 activity.

Studies have suggested that dietary intake may play a role in determining several components of the insulin resistance and differences in diet between Asians and caucasians may also be relevant but were not assessed in this study. Until a more precise and detailed knowledge of dietary habits in Asians in the UK is known, studies to assess the influence of increase in physical activity on insulin resistance, insulin secretion, and risk factors for cardiovascular disease are urgently needed in this population which has extremely high rates of diabetes and excess mortality from ischeamic heart disease.

CHAPTER 6: THE EFFECTS OF METFORMIN ON RISK FACTORS FOR CARDIOVASCULAR DISEASE AND PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) ACTIVITY IN ASIAN AND CAUCASIAN SUBJECTS WITH TYPE 2 DIABETES.

6.1 Introduction

Metformin, a biguanide antihyperglycaemic agent, has been used for treatment of Type 2 diabetic subjects for over 30 years. It is an effective drug in lowering both fasting and post-prandial plasma glucose (Hermann, 1979; Hermann et al, 1992). The precise mechanism of action of the drug is unclear but it is generally accepted that the antihyperglycaemic effect of metformin is not mediated through increased insulin concentrations (Bailey et al, 1988). Some studies, however, have reported that metformin lowers plasma insulin concentrations in obese non-diabetic subjects (Vague et al, 1987) as well as in subjects with type 2 diabetes (Vague et al, 1970). The effect of metformin on the concentrations of insulin and insulin precursor molecules using highly specific assays has not been studied.

Metformin has been shown to improve insulin sensitivity (Gin et al, 1985; Nosadini et al, 1987) though one study failed to show any effect of metformin on insulin stimulated glucose uptake (Wu et al, 1990). A number of studies have evaluated the effects of this drug on several risk factors for cardiovascular disease in normal and non-insulin-dependent diabetic subjects but nearly all these studies were of limited duration and the majority were not placebo-controlled so that the results remain inconsistent (Bailey et al, 1988). It remains possible that the divergence of the reported effects of metformin on cardiovascular risk factors could have been because most studies did not investigate the effect of metformin on all the conventional risk factors in a single study population.

In order to investigate the effect of metformin on glycaemic control, insulin resistance, hyperinsulinaemia, concentrations of insulin-like molecules, and risk factors for cardiovascular disease, we have performed a double-blind placebo-controlled crossover study in 27 subjects with type 2 diabetes. We have investigated subjects with type 2 diabetes from two different ethnic groups (Asian and caucasian) in order to compare the effects of metformin in subjects with different severity of insulin resistance.

6.2 Aims of the study

The aims were to investigate the effects of metformin monotherapy:

1. on glycaemic control and on insulin resistance and to relate changes in glycaemic control to those in insulin resistance.
2. on plasma concentrations of insulin, intact proinsulin and des 31,32 proinsulin using sensitive and specific assays for these molecules.
3. on risk factors for cardiovascular disease in Type 2 diabetic subjects, and the relationship between changes in these risk factors and those in glycaemic control, insulin resistance, or changes in concentrations of proinsulin-like molecules.
4. and finally to compare the effect of metformin in two ethnic groups previously reported to have different degrees of insulin resistance and are at different risk for cardiovascular disease.

6.3 Study design and methods

All subjects had diabetes diagnosed by World Health Organisation criteria (WHO,

1985). The subjects studied represented a random sample of subjects with type 2 diabetes attending a diabetic clinic, and the study was approved by the Ethical Committee of Islington Health Authority. Before inclusion in the study each subject was screened for complications of diabetes. Subjects with clinical or electrocardiographic evidence of ischaemic heart disease were excluded, as were subjects with a previous history of thromboembolic disease, clinical or biochemical evidence of renal or hepatic disease, untreated proliferative diabetic retinopathy, or albugin-positive proteinuria. Female patients of child-bearing age and patients unable to give fully informed consent were also excluded. After initial screening, all antidiabetic treatment was discontinued and fasting plasma glucose concentrations measured after 2 weeks. Subjects whose fasting plasma glucose concentration was > 15 mmol/l were excluded from entry into the study.

The Study design is shown in figure 6.1. Metformin was given for a total period of twelve weeks duration in a double-blind placebo-controlled design, and the dose was increased stepwise from 850mg once daily for one week to 850mg twice daily (bd) for five weeks and 850mg three times daily (tds) for a further 6 weeks. Baseline assessment took place on the day of inclusion into the study and a similar assessment took place after 12 weeks therapy (phase I). There followed a washout period of two weeks, after which subjects were re-assessed as at entry into the trial, and crossed over to the alternate treatment (phase II), being re-assessed finally at the end of phase II. Subjects were also seen at one week, two weeks and six weeks into each treatment phase for a more limited assessment. Each subject was given a fresh pack of metformin or matched placebo (prepared and supplied by Lipha Pharmaceuticals, West Drayton, Middx, UK) on each visit and the number of tablets returned were counted on each occasion to check for compliance. At each visit patients were fully questioned about symptoms and side effects potentially related to metformin, those

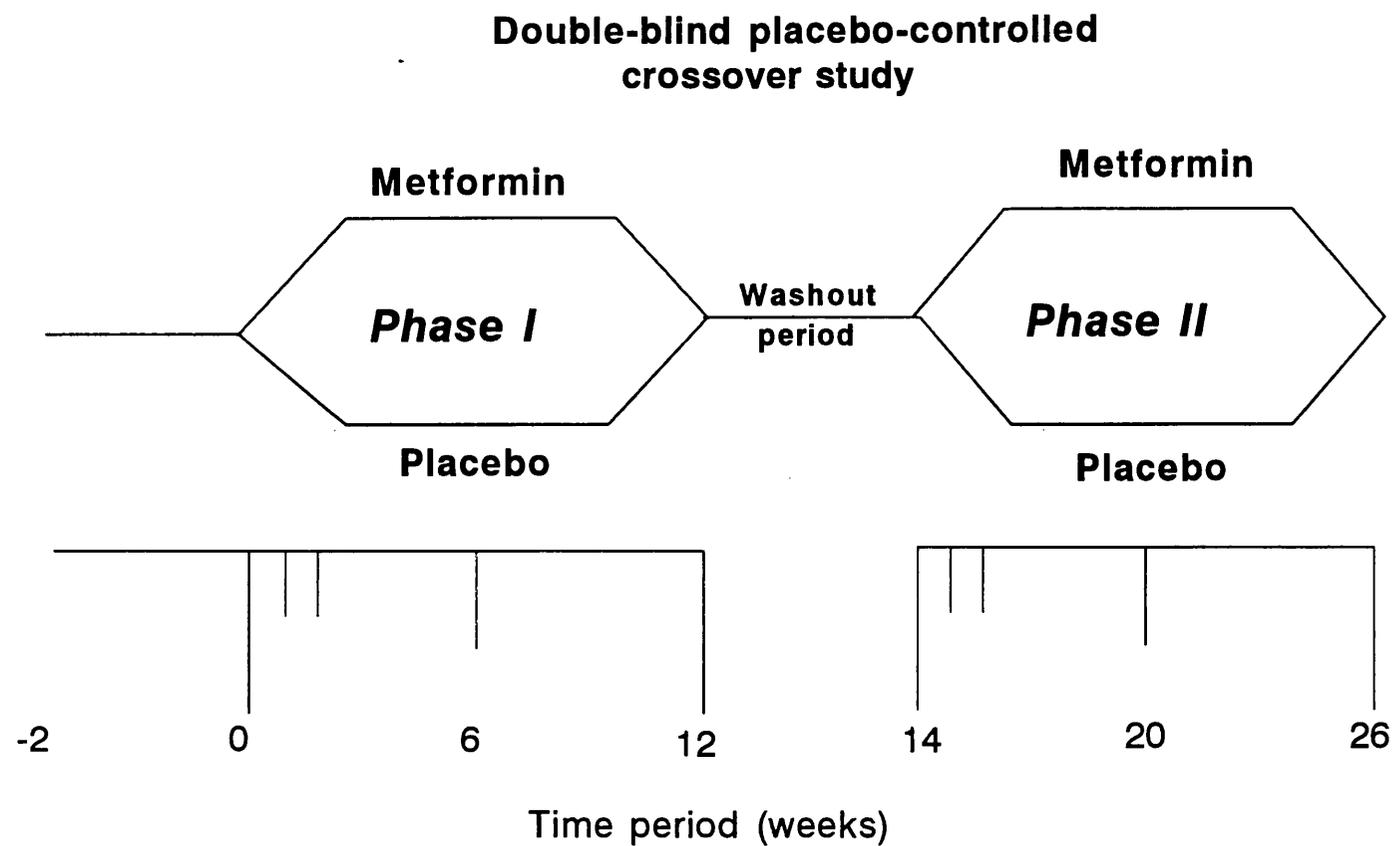


Figure 6.1: Study design to investigate the effects of metformin in subjects with type 2 diabetes

attributable to hyperglycaemia, and also to report any other symptoms. 33 subjects (20 caucasian, 13 Asian) were included into the study and 27 (17 caucasian, 10 Asian) finished the trial successfully.

6.4 Clinical and biochemical investigations

Each subject attended the Clinical Investigation Ward of the Diabetic Unit at the Whittington Hospital between 8 a.m. and 10 a.m. after a 12 hour fast. Weight and height were recorded wearing light clothing and without shoes, and skinfold measurement were performed. Waist-to-hip ratio (WHR) and subscapular-to-triceps skinfold ratio (STR) were estimated and body mass index (BMI) was calculated. Blood pressure was recorded twice after a 30 min period of rest.

Ankle and brachial systolic pressures were recorded using ultrasonic surface blood flow detectors (Vasculoscope) and ankle-to-brachial systolic pressure ratios were calculated. Each subject then had an indwelling venous cannula placed in the antecubital fossa and a venous blood sample was withdrawn for various measurements (blood glucose, glycated haemoglobin, serum fructosamine, lipids, C-peptide, insulin, intact proinsulin, des 31,32 proinsulin, PAI-1, platelet studies in whole blood and platelet rich plasma, β -thromboglobulin (β TG), platelet factor 4 (PF4) and fibrinogen. Samples for C-peptide, insulin, proinsulin, des 31,32 split proinsulin, PAI-1, β TG and PF4 were immediately cold centrifuged at 4°C and stored at -80°C. An insulin sensitivity test was then performed by the modified Harano technique (Heine et al, 1985). An estimate of β -cell function and insulin sensitivity on metformin treatment was also obtained by homeostasis model assessment (HOMA) (Matthews et al, 1985) employing fasting concentrations of C-peptide and those of fasting plasma glucose. Insulin concentrations measured by a conventional radioimmunoassay were termed as "immunoreactive insulin" (IRI) and those by a specific assay as "specific insulin".

6.5 Statistical analysis

Values are expressed as mean (SD) for normally distributed data or as median (range) for skewed data. Changes in different variables between the end of the metformin and the end of the placebo treatment are shown as mean and 95% confidence intervals for normally distributed changes, or as median and range for skewed changes. Differences between variables after metformin treatment were analysed by Student's paired t-test for normally distributed data and by Wilcoxon rank sum test for skewed data. Correlations between changes in different variables were analysed using linear regression analysis for normally distributed data and Spearman rank correlation for skewed data. Analysis of variance was used in order to study the independent relationship between two variables while allowing for confounding variables.

6.6 Results

6.6.1 Subject characteristics, and compliance with treatment

33 subjects (20 Caucasian, 13 Asian) were recruited and 27 satisfactorily completed both phases of the study. One subject withdrew due to gastrointestinal side effects when the dose of metformin was increased from 850mg bd to 850mg tds. Five other subjects could not continue the study due to personal reasons, two during the metformin and three during the placebo phase. Apart from one subject who withdrew from the study due to gastrointestinal upset, two other subjects reported mild gastrointestinal side effects in the form of transient dyspepsia and diarrhoea which abated after 5-7 days while continuing on treatment and both subjects finished the trial successfully.

Compliance was assessed by counting the number of tablets returned and was found to be satisfactory, with 96 to 100% tablet intake during both phases of the study.

Patient characteristics by ethnicity are shown in table 6.1 and table 6.2. Asian subjects were significantly younger and had shorter duration of diabetes than caucasian subjects. At baseline Asian subjects had similar degree of glycaemic control, insulin resistance, fasting insulin and C-peptide concentrations and other cardiovascular risk factors to caucasians. Plasminogen activator inhibitor activity was also similar in the two ethnic groups. Values at baseline and after the washout periods were not significantly different and the order in which treatment was given had no effect on any of the measured variables.

6.6.2 Effect of metformin on body weight and glycaemic control

Metformin treatment led to a highly significant reduction in fasting plasma glucose concentrations at six weeks (mean reduction -2.0 mmol/l, 95% CI -2.7 to -1.3 mmol/l, $p < 0.001$) and 12 weeks (mean reduction -3.1 mmol/l, 95% CI -4.1 to -2.0 mmol/l, $p < 0.001$) compared to placebo (Table 6.3, Fig 6.2 and Fig 6.3). The improvement in the mean fasting plasma glucose concentration was accompanied by a significant reduction in glycated haemoglobin and serum fructosamine concentrations (Table 6.3). However, in two subjects there was a deterioration in the fasting plasma glucose concentrations after 12 week treatment with metformin (Fig 6.2), which was also corroborated by a higher glycated haemoglobin after 12 week treatment with metformin in these two subjects.

TABLE 6.1: Clinical characteristics of subjects at baseline

Variable	All Subjects (n = 27)	Caucasian Subjects (n = 17)	Asian Subjects (n = 10)	Significance of Difference
Gender - M, F	19,8	11,6	8,2	ns
Age (years)	53.5 ± 9.8	56.8 ± 8.9	48.0 ± 8.9	0.023
Duration of diabetes	5.0 (1-20)	6.0 (1-20)	2.5 (1-14)	ns
Body mass index [kg/m ²]	27.6 ± 4.5	28.4 ± 5.1	26.1 ± 3.0	ns
Waist-hip ratio	0.95 ± 0.06	0.94 ± 0.07	0.96 ± 0.04	ns
Subscapular-triceps ratio	1.62 ± 0.37	1.62 ± 0.43	1.62 ± 0.26	ns
Systolic blood pressure	130.4 ± 22.5	130.0 ± 19.6	131 ± 27.9	ns
Diastolic blood pressure	75.7 ± 9.4	75.0 ± 6.6	76.8 ± 13.2	ns
Right ankle/brachial systolic pressure ratio	1.16 ± 0.10	1.18 ± 0.11	1.14 ± 0.09	ns
Left ankle/brachial systolic pressure ratio	1.17 ± 0.15	1.17 ± 0.16	1.18 ± 0.14	ns

Data shown as mean and SD for normally distributed data or median (range) for skewed data. Significance of differences between ethnic groups has been assessed using Student's t-tests for normally distributed data, Mann Whitney-U tests for skewed data and chi squared for categoric data

TABLE 6.2: Biochemical characteristics of subjects at baseline

Variable	All Subjects (n = 27)	Caucasians (n = 17)	Asians (n = 10)	P value
Fasting plasma glucose	12.1 ± 4.3	12.2 ± 4.5	11.8 ± 4.0	ns
HbA1 [%]	10.7 ± 2.2	10.9 ± 2.7	10.3 ± 1.3	ns
Fructosamine [mmol/l]	3.38 ± 0.79	3.46 ± 0.89	3.24 ± 0.57	ns
Fasting "IRI" [pmol/l]	99 (29-213)	77 (29-184)	101 (61-213)	ns
Fasting True insulin [pmol/l]	56 (21-145)	47 (24-145)	71 (21-131)	ns
Fasting C-peptide [nmol/l]	0.59 ± 0.24	0.56 ± 0.22	0.63 ± 0.28	ns
Intact proinsulin [pmol/l]	8.0 (3.6-42.2)	6.4 (3.6-37.2)	10 (4.6-42.2)	ns
Des 31,32 proinsulin [pmol/l]	6.2 (2.7-30.9)	6.0 (2.7-20.1)	6.3 (3.8-30.9)	ns
PAI-1 [AU/ml]	26.5 ± 6.4	25.4 ± 5.4	28.4 ± 7.8	ns
Total cholesterol [mmol/l]	5.9 ± 1.4	6.0 ± 1.6	5.7 ± 0.9	ns
HDL-cholesterol [mmol/l]	1.14 ± 0.32	1.20 ± 0.37	1.04 ± 0.16	ns
LDL-cholesterol [mmol/l]	3.9 ± 1.3	3.9 ± 1.5	3.8 ± 1.0	ns
Total triglyceride [mmol/l]	1.9 (0.6-5.0)	1.8 (0.6-5.0)	1.6 (1.1-3.3)	ns
MCR glucose [ml/kg/min]	2.9 (1.4-7.4)	2.9 (1.4-4.1)	2.9 (1.4-7.4)	ns
Albumin excretion [µg/min]	5.1 (1.0-83.8)	4.3 (1.4-83.8)	5.2 (1.0-23.0)	ns

Data shown as mean and SD for normally distributed data or median (range) for skewed data. Significance of differences between ethnic groups have been assessed using Student's t-tests for normally distributed data, Mann Whitney U tests for skewed data and Chi squared for categorical data.

IRI = immunoreactive insulin, MCR = metabolic clearance rate,

TABLE 6.3: Clinical and biochemical characteristics of subjects 12 weeks after metformin and placebo treatment

Variable	12 weeks after metformin treatment	12 weeks after placebo treatment	Mean and 95% CI for the difference after 12 week of metformin and placebo treatment	P value
Body mass index [kg/mt ²]	27.4 ± 4.6	27.6 ± 4.9	-0.2 (-0.5 to +0.1)	ns
Waist-hip ratio	0.938 ± 0.06	0.941 ± 0.06	-0.003 (-0.011 to +0.005)	ns
Subscapular-triceps ratio	1.57 ± 0.36	1.56 ± 0.32	+0.01 (-0.05 to +0.07)	ns
Fasting plasma glucose [mmol/l]	8.9 ± 2.5	12.0 ± 2.3	-3.1 (-4.1 to -2.0)	<0.0001
HbA1 [%]	9.2 ± 1.4	10.5 ± 2.3	-1.3 (-2.1 to -0.5)	0.003
Fructosamine [mmol/l]	2.75 ± 0.54	3.49 ± 0.82	-0.75 (-0.96 to -0.53)	<0.001
Fasting "IRI" [pmol/l]	97 (25-223)	94 (29-293)	+5 (-89 to +143) *	ns
Fasting specific insulin [pmol/l]	57 (21-218)	79 (19-198)	+10 (-39 to +105) *	ns
Fasting C-peptide [nmol/l]	0.59 ± 0.22	0.59 ± 0.24	0.00 (-0.05 to +0.05)	ns
Intact proinsulin [pmol/l]	4.6 (1.4-43.7)	8.0 (3.2-51.3)	-2.9 (-28.4 to +2.5) *	0.003
Des 31,32 proinsulin [mmol/l]	3.3 (1.6-27.4)	5.6 (2.3-29.8)	-1.6 (-14.1 to +5.4) *	0.013

Values given as mean (SD) for normally distributed data and median (range) for non-parametric data. Difference between placebo and metformin treatment are presented as mean (95% confidence intervals) and calculated as (value on metformin at 12 week - value on placebo at 12 week) i.e. negative value implies a lower level on metformin. For skewed data, differences between 12 week placebo and 12 week metformin are presented as median and range; and are indicated with a *. Significance of difference at baseline and after 12 week treatment with metformin and placebo is assessed by Student's paired t-test for normally distributed data and Wilcoxon rank sum test for skewed data.

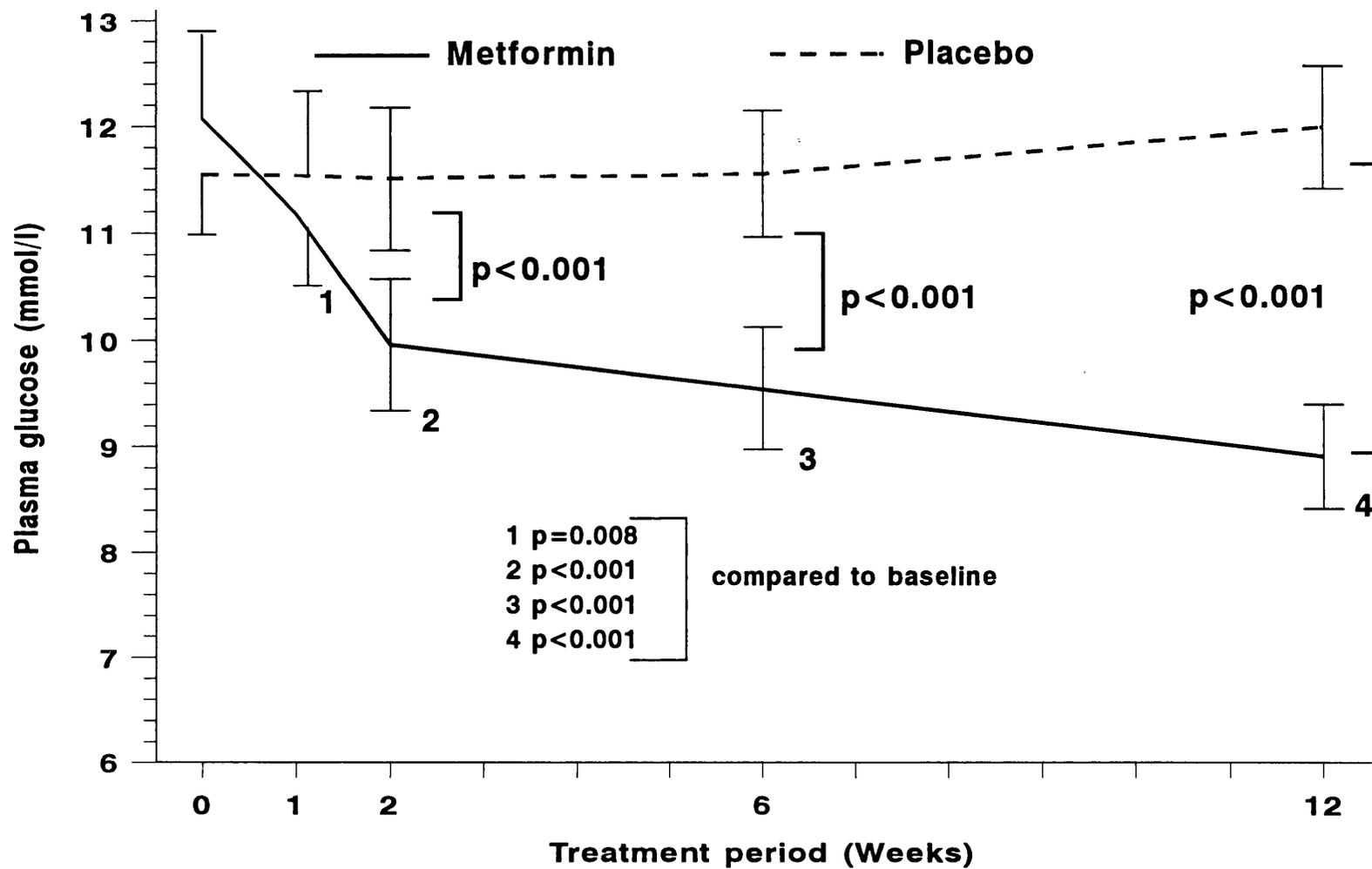


Figure 6.2: Effect of metformin and placebo treatment on fasting plasma glucose concentrations

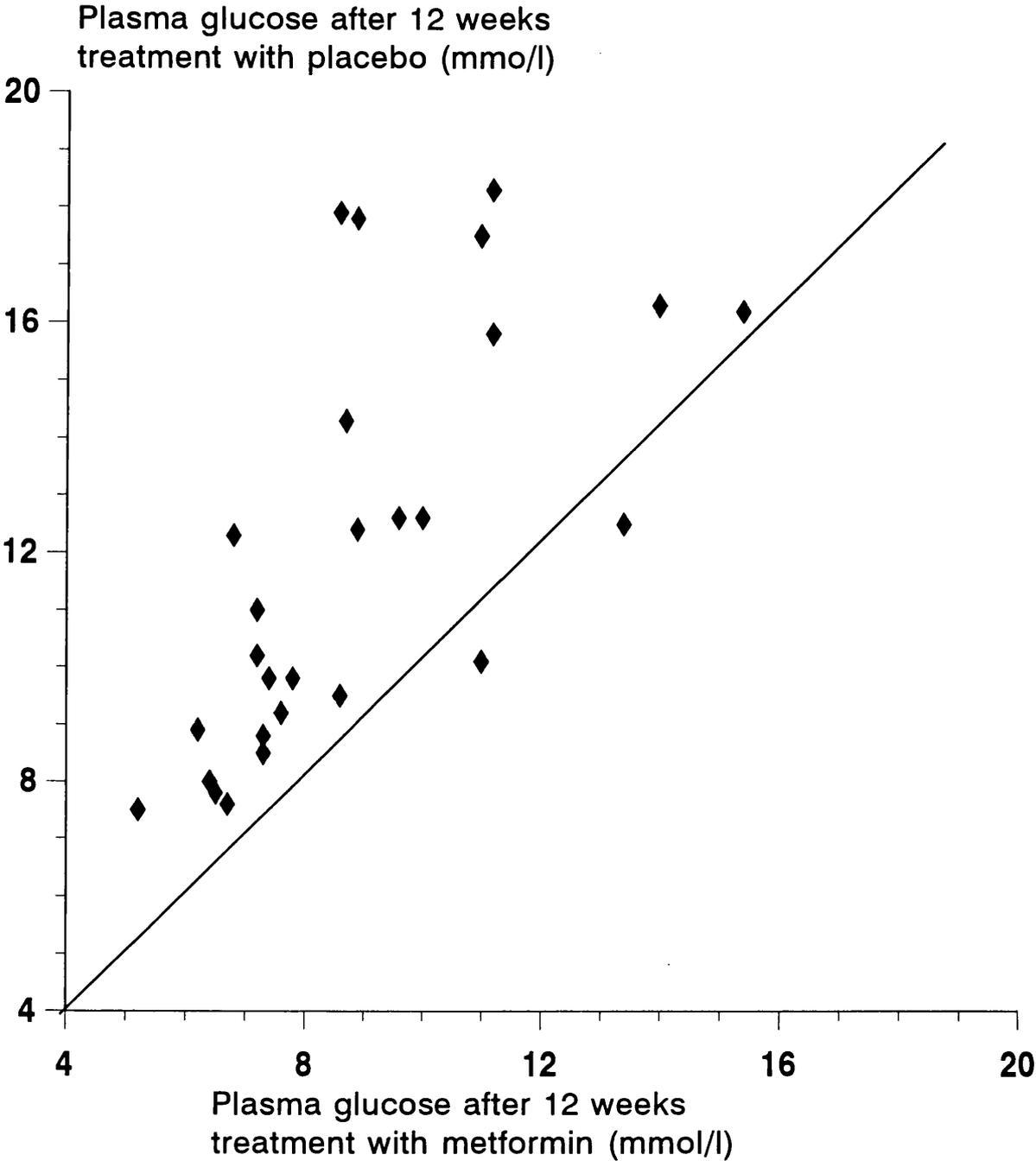


Figure 6.3: Effect of 12 weeks of treatment with metformin and placebo on fasting plasma glucose

There was no significant change in body mass index from baseline to the end of metformin treatment (mean change -0.56, 95% CI +0.2 to -1.32, $p = \text{ns}$) in all subjects. However, the magnitude of weight loss was larger in obese ($\text{BMI} \geq 27 \text{ kg/m}^2$) subjects with mean reduction of 1.1 kg (95% CI +0.3 to -2.5, $p = \text{ns}$) than in lean ($\text{BMI} < 27 \text{ kg/m}^2$) subjects with a mean reduction 0.02 (95% CI +0.38 to -0.42, $p = \text{ns}$).

6.6.3 Effects on insulin resistance and beta cell function

Insulin resistance, expressed as metabolic clearance rate of glucose, showed significant improvement at the end of metformin treatment. Metabolic clearance rate of glucose increased from $2.85 \pm 1.20 \text{ ml.kg}^{-1}.\text{min}^{-1}$ to $3.63 \pm 1.74 \text{ ml.kg}^{-1}.\text{min}^{-1}$, a mean change 0.78 (95% CI +0.05 to +1.34 $\text{ml.kg}^{-1}.\text{min}^{-1}$; $p = 0.036$). The calculations of β -cell function and insulin sensitivity using C-peptide and glucose concentrations in the HOMA model showed that metformin treatment was associated with highly significant improvement in β -cell function from $23.4\% \pm 14.5\%$ to $38.9\% \pm 23.7\%$, (median change +14.0%, range -6.0% to +48.0%, $p < 0.001$), but a much less significant improvement in insulin sensitivity from $58.0\% \pm 21.1\%$ to $63.4\% \pm 25.6\%$, (median change +4.0%, range -25.0% to +43.0%, $p = 0.028$).

6.6.4 Effect on insulin, proinsulin, des 31,32 proinsulin, and C-peptide

There was no change in fasting concentrations of C-peptide, immunoreactive insulin, or specific insulin on metformin treatment. However there were significant reductions in the concentrations of proinsulin-like molecules on metformin treatment (table 6.3): intact proinsulin (median change -30%, range -60% to +55%, $p < 0.001$; Fig 6.4 a) and those of des 31,32 proinsulin (median change -35%, range -57% to +25%, $p < 0.001$; Fig 6.4 b). The reduction in absolute values of both molecules is shown in table 6.3.

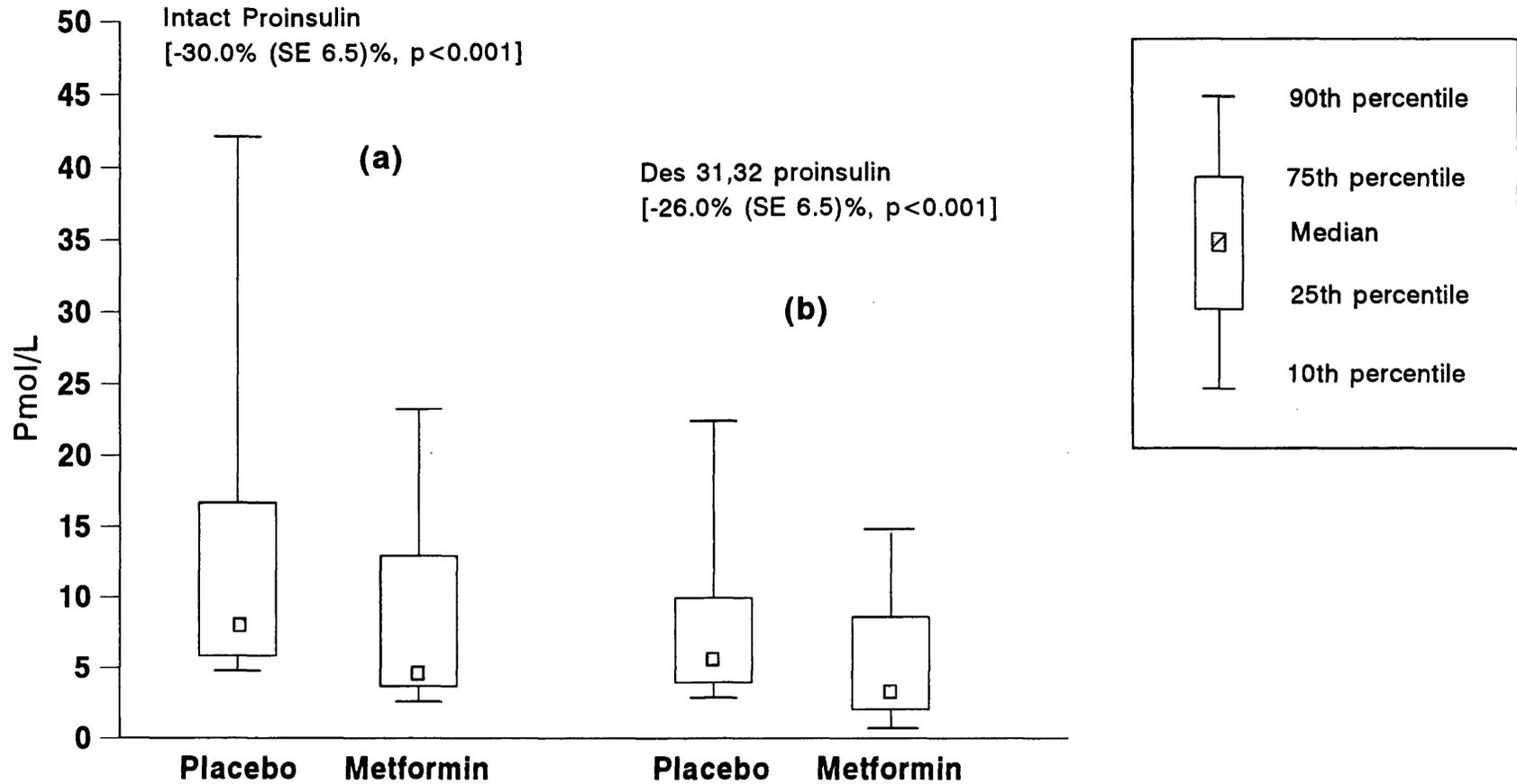


Figure 6.4: The effects of metformin and placebo treatment on (a) intact and (b) des 31,32 proinsulin concentrations in subjects with type 2 diabetes

6.6.5 Effect on cardiovascular risk factors

There was a significant reduction in total cholesterol concentrations after 12 weeks of metformin treatment compared to end of the placebo treatment period, this being accompanied by a significant reduction in LDL-cholesterol, but without any change in HDL-cholesterol concentrations (table 6.4). Similarly, total triglyceride concentrations showed a significant reduction after metformin treatment.

Metformin treatment was also accompanied by a significant reduction in urinary albumin excretion rate (table 6.4) (median change $-2.4 \mu\text{g}/\text{min}$, range -28.0 to $+4.6 \mu\text{g}/\text{min}$, $p=0.004$). There was no significant correlation between changes in albumin excretion and those in fasting plasma glucose concentrations, body mass index, or systolic and diastolic blood pressure. Metformin treatment had no significant effect on mean systolic and diastolic blood pressures, or ankle to systolic brachial pressure ratios (table 6.2).

6.6.6 Effect on PAI-1 and indices of platelet function

Metformin treatment led to a highly significant reduction in PAI-1 activity at 6 weeks and at 12 weeks (table 6.4, fig 6.5,). There were no change in PAI-1 activity in one subject while in six subjects the PAI-1 activity was higher (fig 6.6) after 12 week of metformin treatment. Metformin treatment had no significant effect on fibrinogen levels.

There was no significant difference in in-vivo platelet activation as measured by βTG and PF_4 concentrations after metformin treatment (table 6.5). However to avoid the problems of in-vitro platelet activation data were analysed by excluding subjects with βTG to PF_4 ratio < 3.0 (Kaplan et al, 1981) there were only six subjects with ratio ≥ 3 on both occasions, and eligible for paired t-test thereby making the number too small

TABLE 6.4: Cardiovascular risk factors 12 weeks after metformin and placebo treatment

Variable	12 weeks after metformin treatment	12 weeks after placebo treatment	Mean and 95% CI for the difference after 12 week of metformin and placebo treatment	P Value
Systolic blood pressure [mm Hg]	131.1 ± 19	131.3 ± 17.5	-0.2 (-6.1 to +5.7)	ns
Diastolic blood pressure [mm Hg]	75.6 ± 9.7	76.4 ± 7.5	-0.8 (-4.1 to +2.5)	ns
Rt Ankle/brachial systolic pressure ratio	1.20 ± 0.10	1.17 ± 0.14	+0.03 (-0.03 to +0.09)	ns
Lt Ankle/brachial systolic pressure ratio	1.16 ± 0.13	1.17 ± 0.13	+0.01 (-0.05 to +0.07)	ns
MCR glucose [ml ⁻¹ .kg ⁻¹ .min ⁻¹]	3.2 (1.9-9.5)	2.6 (1.6-6.3)	+0.40 (-0.10 to +1.30)*	0.036
Insulin sensitivity [%]	58 (25-116)	58 (29-138)	+4.0 (-25.0 to +43.0)*	0.03
β-cell function [%]	39 (11-117)	19 (7-73)	+14.0 (-6.0 to +48.0)*	<0.001
Total cholesterol [mmol/l]	5.6 ± 1.1	6.1 ± 1.4	-0.5 (-0.8 to -0.2)	0.002
HDL-cholesterol [mmol/l]	1.14 ± 0.30	1.15 ± 0.38	-0.01 (-0.07 to +0.06)	ns
LDL-cholesterol [mmol/l]	3.6 ± 1.5	4.0 ± 1.3	-0.4 (-0.6 to -0.2)	0.002
Total triglyceride [mmol/l]	1.8 (0.6-3.8)	2.1 (0.8-4.3)	+0.2 (-2.1 to +1.7)*	0.034
Plasminogen activator inhibitor [AU/ml]	19.6 ± 5.8	24.9 ± 7.9	-5.3 (-8.2 to -2.4)	0.001
Albumin excretion [μg/min]	4.2 (0.5-80.9)	6.3 (0.2-76.3)	-2.4 (-28.0 to +4.6)*	0.004

Values given as mean (SD) for normally distributed data and median (range) for non-parametric data. Difference between placebo and metformin treatment are presented as mean (95% confidence intervals) and calculated as (value on metformin at 12 week - value on placebo at 12 week) i.e. negative value implies a lower level on metformin. Significance of difference at baseline and after 12 week treatment with metformin and placebo is assessed by Student's paired t-test for normally distributed data and Wilcoxon rank sum test for skewed data.*For skewed data, differences between 12 week placebo and 12 week metformin are presented as median and range.

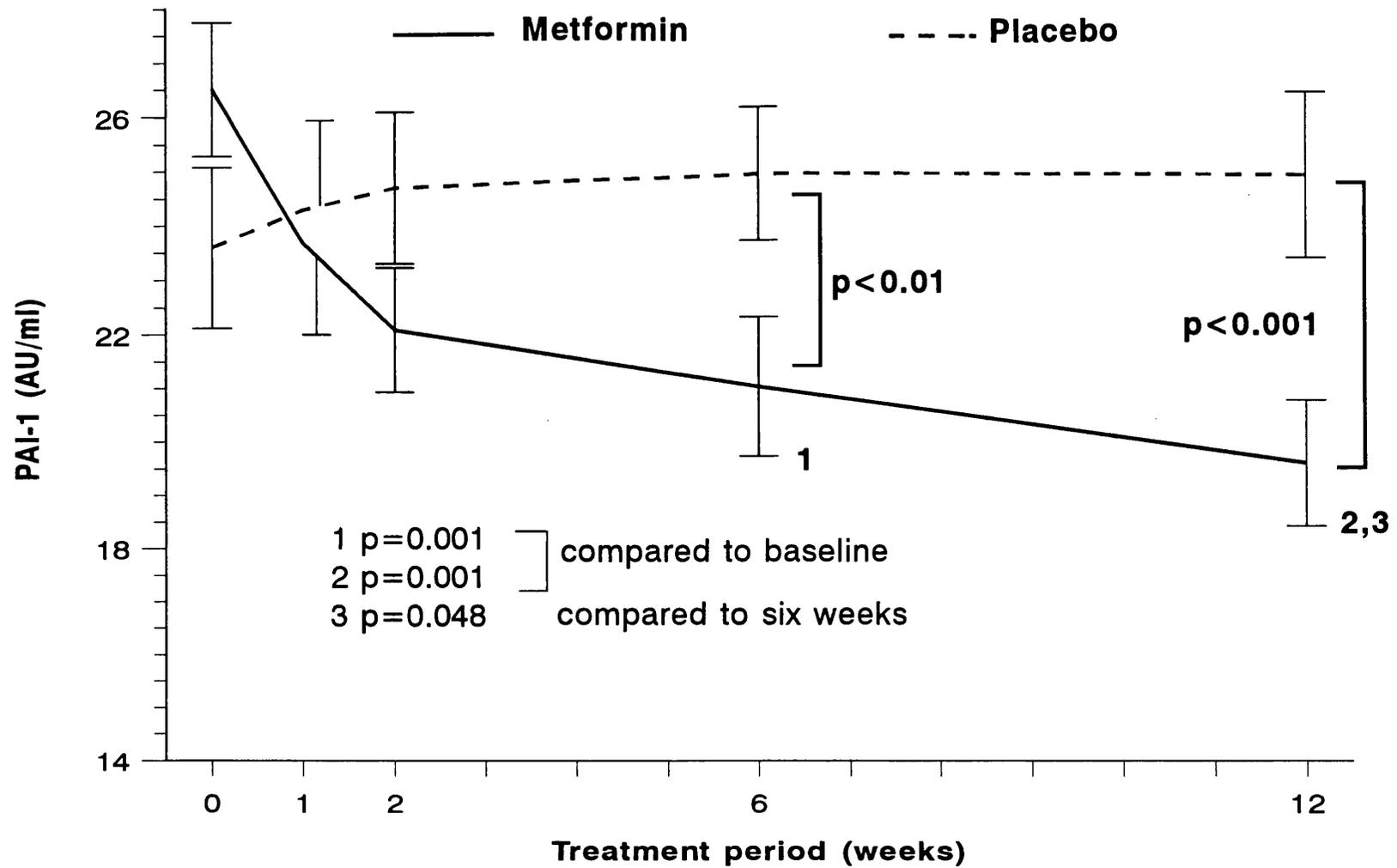


Figure 6.5: Effect of metformin and placebo treatment on PAI-1 activity

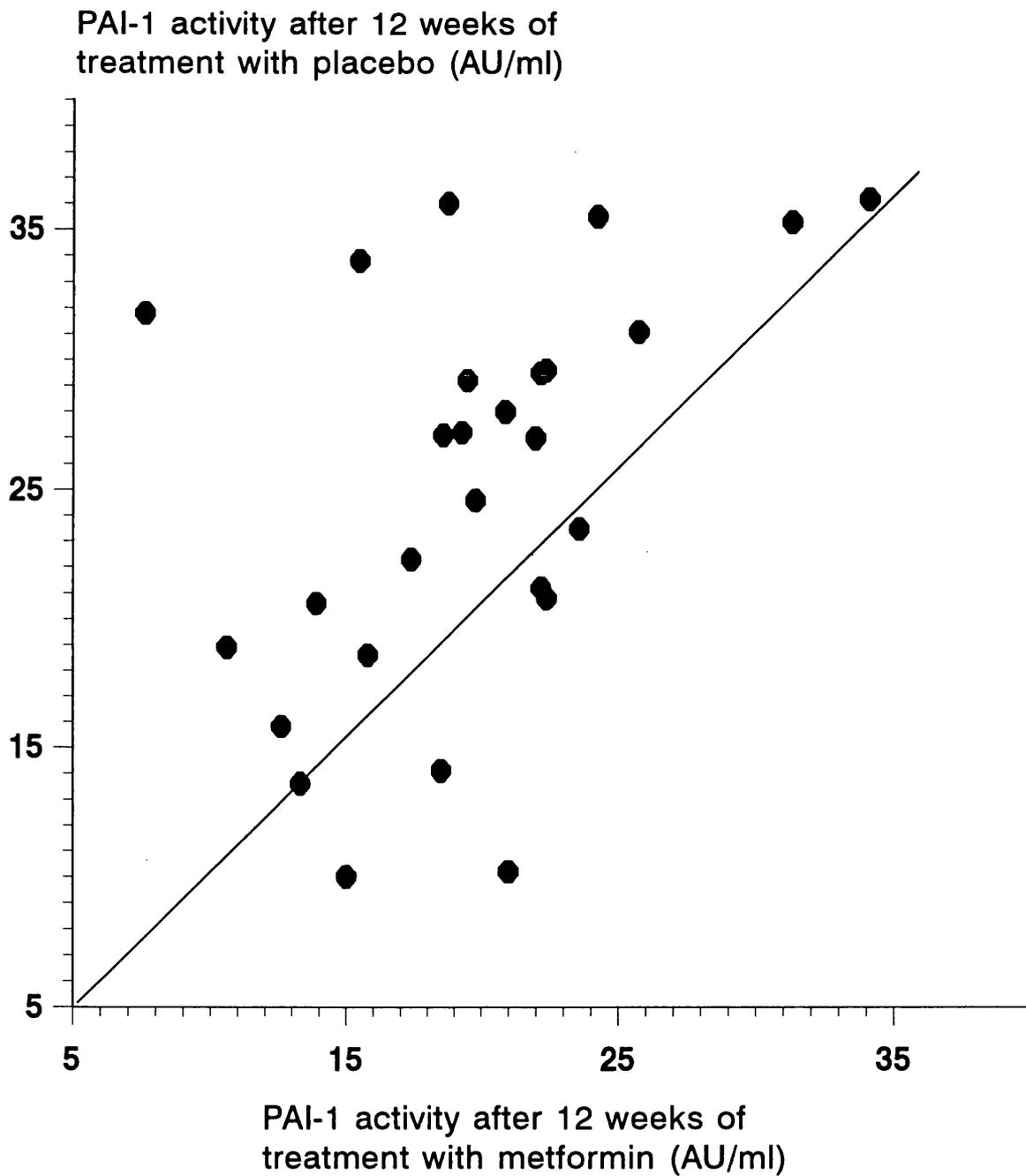


Figure 6.6: Effect of 12 weeks of treatment with metformin and placebo on PAI-1 activity

for any valid comparison, and signifying that despite every effort, in-vitro platelet activation had taken place in a substantial number of samples. No significant changes were observed after metformin treatment in whole blood spontaneous platelet aggregation (fig 6.7) or in adenosine-diphosphate (ADP) induced aggregation in whole blood. Similarly platelet aggregation studies using PRP showed no changes on metformin treatment (table 6.5).

6.6.7 Changes in plasma glucose and risk factors when analysed according to obesity

The changes in fasting plasma glucose concentrations and majority of cardiovascular risk factors on metformin treatment was similar in obese (BMI \geq 27) and non-obese (BMI $<$ 27) subjects. However, in obese subjects there was a significantly greater reduction in plasma triglyceride and PAI-1 activity on metformin treatment than in lean subjects (table 6.6).

6.6.8 Effects of metformin in the two ethnic groups

The effect of metformin on different variables was compared in the two ethnic group. Systolic and diastolic blood pressures tended to fall with metformin in Asian subjects and to rise in caucasian subjects, but the ethnic differences in these treatment changes were not statistically significant. The effects of metformin on glycaemic control or on cardiovascular risk factors, was not significantly different, whether expressed in absolute values or as percentage change, in the two ethnic groups.

6.6.9 Correlations of cardiovascular risk factors with those of IRI, specific insulin, proinsulin-like molecules and insulin resistance

At baseline "immunoreactive insulin" concentrations showed a positive and significant correlation with body mass index, systolic blood pressure, total triglyceride and plasminogen activator inhibitor but not with diastolic blood pressure. Correlations of

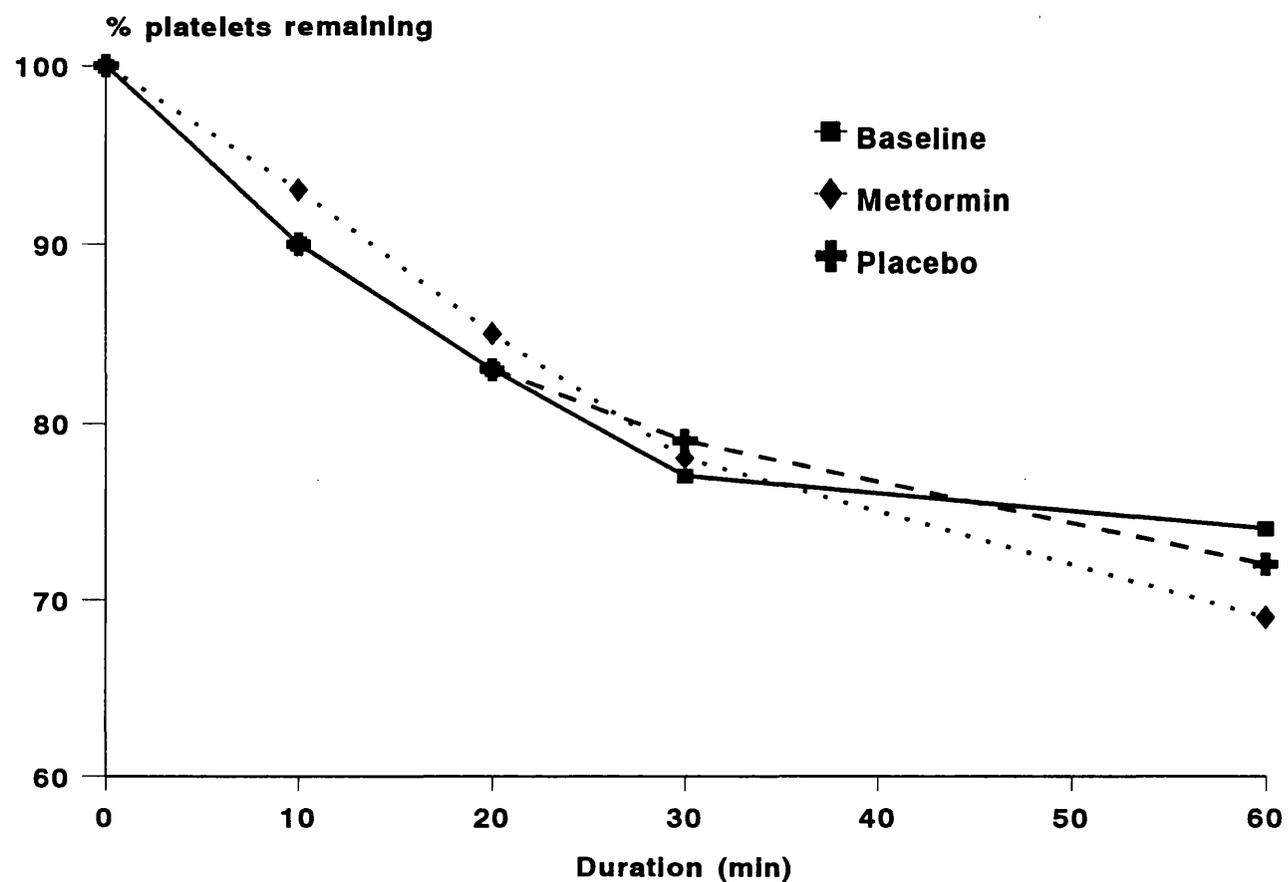


Figure 6.7: Effects of 12 weeks treatment with metformin and placebo on spontaneous platelet aggregation in whole blood

TABLE 6.5: Platelet function and plasma fibrinogen 12 weeks after metformin and placebo treatment

Variable	12 weeks metformin treatment	12 weeks placebo treatment	Mean and 95% CI for the difference after 12 week of metformin and placebo treatment	P value
EC50 Whole Blood	1.2 (0.6-4.0)	2.0 (0.6-7.6)	-0.4 (-6.3 to 1.6)*	ns
EC50 Platelet rich plasma (PRP)	4.4 (4.0-9.0)	4.9 (0.6-8.5)	-0.4 (-7.3 to +4.0)*	ns
Spontaneous whole blood aggregation (%)				
10 min	93 ± 6	90 ± 7	+1.9 (-4.0 to +7.8)	ns
20 min	85 ± 8	83 ± 10	+2.2 (-2.3 to +7.8)	ns
30 min	78 ± 12	79 ± 10	+0.2 (-6.8 to +6.4)	ns
60 min	69 ± 14	72 ± 8	-1.7 (-9.4 to +6.0)	ns
Plasma Fibrinogen [g/l]	3.05 ± 0.33	2.92 ± 0.45	+0.13 (-0.34 to +0.04)	ns
β -Thromboglobulin [ng/ml]	134 (44-635)	212 (38-660)	+11.8 (-601.7 to +423)*	ns
Platelet factor 4 [ng/ml]	95 (5-476)	68 (10-409)	-7.8 (-356.0 to +288.7)*	ns

Values given as mean (SD) for normally distributed data and median (range) for non-parametric data. Difference between placebo and metformin treatment are presented as mean (95% confidence intervals) and calculated as (value on metformin at 12 week - value on placebo at 12 week) i.e. negative value implies a lower level on metformin. For skewed data, differences between 12 week placebo and 12 week metformin are presented as median and interquartile range; these are indicated with a *. Significance of difference at baseline and after 12 week treatment with metformin and placebo is assessed by Student's paired t-test for normally distributed data and Wilcoxon rank sum test for skewed data.

TABLE 6.6: Differences between changes in variables after 12 week of placebo and metformin treatment in obese and non-obese subjects with type 2 diabetes.

Variable	All subjects (n= 27)	Non-obese (BMI < 27) n= 14	Obese (BMI ≥ 27) n= 13	P value
Body weight (Kg)	-0.56 (+0.20 to -1.32)	-0.02 (-0.42 to +0.38)	-1.1 (-2.5 to +0.3)	ns
Fasting plasma glucose (mmol/l)	-3.1 (-4.1 to 2.0)	-3.2 (-5.6 to -1.8)	-2.9 (-4.4 to -1.9)	ns
Glycated haemoglobin (%)	-1.3 (-2.1 to -0.5)	-2.0 (-3.2 to -0.8)	-0.5 (-1.2 to +0.2)	ns
Total triglyceride (mmol/l)	+0.2 (-2.1 to +1.7)*	+0.3 (-0.9 to +0.6)*	+0.1 (-2.1 to +1.7)*	ns
Total cholesterol (mmol/l)	-0.5 (-0.8 to -0.2)	-0.6 (-1.0 to -0.2)	-0.4 (-0.8 to -0.01)	ns
LDL-cholesterol(mmol/l)	-0.4 (-0.6 to -0.2)	-0.5 (-0.9 to -0.1)	-0.3 (-0.5 to -0.03)	ns
PAI-1 (AU/ml)	-5.3 (-8.2 to -2.4)	-3.5 (-6.6 to -0.4)	-7.3 (-12.3 to -2.3)	ns

Difference between placebo and metformin treatment are presented as mean (95% confidence intervals) and calculated as (value on metformin at 12 week - value on placebo at 12 week) i.e. negative value implies a lower level on metformin. * For skewed data, differences between 12 week placebo and 12 week metformin are presented as median and range.

Table 6.7: Correlation coefficients¶ of cardiovascular risk factors with insulin and intact proinsulin, and des 31,32 proinsulin at baseline.

Variables	Immunoreactive insulin	Specific insulin	Intact proinsulin	Des 31,32 proinsulin
Age	-0.16	0.00	-0.12	-0.11
Body mass index	0.62§	0.51¶	0.28	0.37
Waist-hip ratio	0.18	0.11	0.03	0.32
Total triglyceride	0.49¶	0.41*	0.25	0.52¶
Total cholesterol	0.15	-0.03	-0.05	-0.06
HDL-cholesterol	-0.17	-0.31	-0.28	-0.52¶
LDL-cholesterol	0.09	-0.10	-0.07	-0.10
Plasminogen activator inhibitor	0.44*	0.42*	0.00	0.15
Fasting plasma glucose	-0.28	-0.16	0.10	0.23
Glycated haemoglobin	-0.03	-0.17	0.11	0.23
Systolic blood pressure	0.40*	0.29	0.54¶	0.56¶
Diastolic blood pressure	0.31	-0.15	0.43*	0.35

* $p < 0.05$ ¶ $p < 0.01$ § $p < 0.001$

¶ Performed using Spearman correlation coefficients

specific insulin were similar to those of IRI, but there was no significant relationship with systolic blood pressure. Concentrations of specific insulin or IRI were not significantly related to waist-hip-ratio (table 6.7). Correlation of C-peptide were also similar to those observed for IRI, with an additional significant negative correlation with HDL-cholesterol (data not shown).

Intact proinsulin and des 31,32 proinsulin showed no significant relationship with body mass index or waist-hip ratios but both were correlated significantly with systolic blood pressure while intact proinsulin but not des 31,32 proinsulin correlated with diastolic blood pressure. In addition, only des 31,32 proinsulin was related to plasma triglyceride and negatively to HDL-cholesterol. Neither of the proinsulin-like molecules showed any significant relationship to PAI-1 activity at baseline.

6.6.10 Correlations of changes in cardiovascular risk factors with those in insulin, proinsulin-like molecules and insulin resistance

These are shown in table 6.8. Changes in fasting plasma glucose or in glycated haemoglobin concentrations showed no significant correlation with those in body mass index or changes in weight in all subjects. Changes in total triglyceride and total cholesterol correlated with changes in fasting plasma glucose ($r_s = 0.37$; $p = 0.03$ and $r_s = 0.29$; $p = 0.07$ respectively) but not with changes in IRI, specific insulin or insulin resistance. However, there was a significant correlation between the change in insulin resistance (MCR glucose) and that in fasting plasma glucose concentrations ($r_s = 0.48$; $p = 0.007$).

The changes in PAI-1 activity showed no significant correlations with change in fasting IRI ($r_s = 0.11$, $p = 0.28$), specific insulin concentrations or change in insulin resistance ($r_s = -0.19$; $p = 0.172$) in all subjects. However if the same analysis was performed in

subjects in whom fasting plasma glucose had improved by ≥ 2 mmol/l ($n=17$) on metformin treatment, there was a highly significant relationship between changes in insulin resistance and that of changes in PAI-1 ($r_s=-0.53$, $p=0.001$). There was also a significant correlation between changes in PAI-1 and those in BMI ($r_s=0.38$; $p=0.032$) and with also with changes in plasma cholesterol ($r_s=0.43$; $p=0.014$).

There was a significant relationship between changes in concentrations of the proinsulin-like molecules ($r_s=0.80$; $p<0.001$). Changes in des 31,32 proinsulin showed a weak relationship, which was not significant, to changes in fasting plasma glucose concentrations ($r_s=0.37$; $p=0.07$) and changes in body weight ($r_s=0.37$; $p=0.07$) but not to changes in any of the cardiovascular risk factors particularly plasminogen activator inhibitor ($r_s=0.14$; $p=ns$ for intact and $r_s=0.14$; $p=ns$ for des 31,32 proinsulin).

Table 6.8: Correlation coefficients between changes in cardiovascular risk factors after metformin treatment

Variables	ΔTotal cholesterol	ΔLDL-cholesterol	ΔTriglyceride	ΔPAI-1
ΔBody mass index	0.05	0.05	0.24	0.38¶
ΔFasting plasma glucose (FPG)	0.29	0.17	0.37*	0.15
ΔMCR glucose	-0.16	-0.17	0.14	-0.19
ΔSpecific insulin	-0.01	0.01	-0.15	0.07
ΔProinsulin-like molecules	0.18	0.09	-0.07	-0.17

¶ Performed using Spearman correlation coefficients. MCR = metabolic clearance rate

* $p < 0.05$ ¶ $p < 0.01$ § $p < 0.001$

6.6.7 Discussion

Subjects with non-insulin-dependent diabetes have a substantial increase in cardiovascular risk (Garcia et al, 1974; Panzram et al, 1987). This increased risk appears to be independent of the duration of diabetes and the degree of glycaemic control (Jarrett et al, 1982; Jarrett, 1984). Furthermore improvement in glycaemic control in previous studies of subjects with type 2 diabetes has not been associated with any reduction in different risk factors for cardiovascular disease (UGDP, 1977). Thus in these subjects any treatment intended to improve glycaemic control would be beneficial in the long term only if it were also shown to have beneficial effect on risk factors for cardiovascular disease in addition to improvements in glycaemic control, as one of the ultimate aims other than to control symptoms of hyperglycaemia, is to reduce or prevent the incidence of long term complications of diabetes.

Metformin is frequently used either alone or in combination with sulphonylureas for treating subjects with type 2 diabetes. Metformin has been shown in a number of previous studies to be a possible candidate for such a therapeutic potential, perhaps through its beneficial effect on insulin resistance, which has been shown to be associated with deleterious changes in risk factors for cardiovascular disease such as plasma triglyceride, HDL-cholesterol, and blood pressure (Reaven, 1988).

The results of this study indicate that metformin when used up to a dose of 850 mg tds in an incremental dosage schedule and taken with meals, is well tolerated, with only mild and transient side effects even at maximal dosage. Only one subject had to withdraw from the study because of side effects.

In a double-blind placebo-controlled cross-over design we have confirmed the previous observations that metformin significantly improves glycaemic control, with a 20%

reduction in mean fasting glucose concentrations at a dosage of 850mg twice daily. When the dose was increased to 850 mg three times daily, there was a further reduction in fasting plasma glucose concentrations to a total of 26%. However this further reduction (6%) on an increased dose of metformin was not statistically significant when compared with that seen at a dose of 850 mg twice daily, implying no further major advantage as far as glycaemic control was concerned. The improvement in fasting plasma glucose concentrations of 26% is comparable with previous studies, reported between 1970-1992 in subjects with type 2 diabetes using metformin monotherapy (Lord et al, 1983; Prager & Schernthaner, 1983; Trischitta et al, 1983; Fantus & Brosseau, 1986; Parger et al, 1986; Campbell et al, 1987; Nosadini et al, 1987; DeFronzo et al, 1991; Dornan et al, 1991; Wu et al, 1990; Hermann et al, 1991; Reaven et al, 1992).

There was no correlation between the changes in fasting plasma glucose concentrations with those in body mass index or body weight, suggesting that weight reduction is not the major mechanism of action of this drug in lowering plasma glucose concentrations. The reduction in fasting plasma glucose was not significantly different in obese and non-obese subjects, suggesting that the drug is equally effective in both obese and non-obese subjects with type 2 diabetes.

Previous studies have shown that metformin treatment is associated with an average weight loss of 1.2 kg and 1.5 kg respectively in non-obese, and obese subjects with type 2 diabetes (Clarke et al, 1968, 1977). However weight loss is seen particularly in patients who continue to observe a weight reducing diet, and furthermore in those studies where metformin treatment was associated with weight loss, there was no obvious association between the amount of weight loss and the reduction in fasting glucose concentrations in subjects with type 2 diabetes (Hermann et al, 1991). In our

study, we observed no weight change in thin subjects with type 2 diabetes, while obese subjects did on average show 1.1 kg of weight loss although this was not statistically significant. The lack of significant weight loss in our study may have been because subjects were well established on diet before starting treatment and had perhaps achieved a steady body weight before inclusion in this study. Another study employing the same dose and for the same duration as this study, has also shown no significant effect of metformin on body weight (Wu et al, 1990). Our finding, however, does not rule out the possibility that metformin may have a preferential weight reducing effect in obese subjects with type 2 diabetes. Whether this is of any great clinical significance remains uncertain, as the reduction in fasting plasma glucose concentrations were not associated with changes in body weight in this study.

Metformin treatment was associated with significantly improved insulin sensitivity, which correlated significantly with improvements in fasting plasma glucose concentrations. However using the HOMA model, metformin treatment was also accompanied by a significant improvement in β -cell function, and the effect on insulin sensitivity, though significant, was of smaller magnitude. The modified Harano technique used to assess insulin resistance does not quantify insulin resistance in different target tissues, so it is not possible to assess whether the improvement was at hepatic or peripheral level. Previous studies in subjects with type 2 diabetes have confirmed the effect of metformin on both hepatic and peripheral insulin sensitivity (Prager et al, 1986; Nosadini et al, 1987; DeFronzo et al, 1991). Further support for potentiation of insulin action comes from studies in subjects with insulin-dependent diabetes, where metformin treatment was associated with a reduction by 20-50% in insulin requirement (Gin et al, 1982; Pagano et al, 1983).

As the majority of insulin-stimulated glucose uptake occurs in skeletal muscle this is

likely to be the site of action of metformin, though equally a reduction in fasting plasma glucose could have been due to reduced hepatic output of glucose due to improved hepatic insulin sensitivity (Jackson et al, 1987; DeFronzo et al, 1991). However a previous study (Hother-Nielsen et al, 1989) showed no effect of metformin on hepatic insulin action, while confirming its effects on peripheral insulin sensitivity in obese subjects with type 2 diabetes.

The precise mechanism of improved insulin sensitivity due to metformin treatment is unclear, though previous studies have confirmed that it is unlikely to be due to improved receptor binding for insulin (Prager et al, 1983, Lord et al, 1993). Recent in-vivo studies have indicated that metformin increases the recruitment of glucose transporters both independently of the effect of insulin and also on insulin-stimulated recruitment of these transporters (Mathaei et al, 1991), confirming previous suggestions that the major mechanism of action of metformin in improving insulin sensitivity is due to events at a post-receptor level (Jacobs et al, 1986; Fantus et al, 1986; Klip et al, 1990).

The finding of increased β -cell function by the use of HOMA model was unexpected. It is unlikely that metformin has any direct action on the β -cell, and the substantial improvement in β -cell function seen here may have resulted from improved glycaemic control. Previous studies have confirmed an improvement in β -cell function when fasting plasma glucose concentrations are lowered, irrespective of the mode of treatment (Ferner et al, 1988).

Metformin treatment also led to a significant fall in total cholesterol concentrations. Studies conducted over longer periods have shown a small but significant effect of metformin on total cholesterol concentrations in subjects with type 2 diabetes (Taylor

et al, 1982; Haupt et al, 1989; DeFronzo et al, 1991; Hollenbeck et al, 1991; Dornan et al, 1991; Reaven et al, 1992), while other studies have shown no such effect (Campbell et al, 1987; Sirtori et al, 1988). The reduction in total cholesterol concentrations seen in our study group was accompanied by a significant fall in LDL-cholesterol concentrations but without any effect on that of HDL-cholesterol concentrations, although previous studies have shown an increase in HDL-cholesterol with metformin in subjects with type 2 diabetes (Janka, 1985; Groop et al, 1989; Schneider et al, 1990; Wu et al, 1990; Hermann et al, 1991; Reaven et al, 1992).

Total triglyceride concentrations also showed a significant fall after metformin therapy. Previous studies in hypertriglyceridaemic subjects (Montaguti et al, 1979; Fedele et al, 1976) and in normotriglyceridaemic subjects with type diabetes (Taylor et al, 1982; Groop et al, 1989; Haupt et al, 1991; Dornan et al, 1991; Wu et al, 1990; DeFronzo et al, 1991; Reaven et al, 1992) have shown that metformin reduces total triglyceride concentrations. Similarly metformin has also been shown to reduce plasma triglyceride concentrations in obese subjects (Vague et al, 1987), subjects with peripheral vascular disease (Sirtori et al, 1988) and in hyperlipidaemic subjects (Descovich et al, 1978; Schneider et al, 1990; Hollenbeck et al, 1991). The magnitude of triglyceride reduction is much more in hypertriglyceridaemic subjects. Animal studies have shown that the effect of metformin in lowering triglyceride concentrations is due to its effect on VLDL triglyceride (Zavaroni et al, 1985).

We observed no effect of metformin on systolic or diastolic blood pressure or on ankle to brachial systolic pressure ratios. While these results are in accordance with previous findings (Campbell et al, 1988) but do not support some of the earlier studies in which metformin treatment led to a reduction in blood pressure (Landin et al, 1990) and improvements in post ischaemic blood flow, as measured by a strain gauge

plethysmography, in subjects with peripheral vascular disease (Sirtori et al, 1984, 1988).

There were significant reductions in PAI-1 activity at six weeks, and a further reduction at twelve weeks. Previous studies in subjects with type 2 diabetes and non-diabetic subjects has shown that metformin lowers PAI-1 activity (Vague et al, 1987; Grant et al, 1991). As glycaemic control between six and 12 weeks was not significantly different this might suggest an effect of metformin on PAI-1 which is unrelated to its effect on glycaemic control, or possibly a slower effect of metformin on PAI-1 than on plasma glucose. The independent effect of metformin on PAI-1 is further supported by the fact that metformin reduces PAI-1 concentration in non-diabetic subjects without altering glycaemia (Vague et al, 1987). Previous studies in obese non-diabetic subjects have shown that this reduction in PAI-1 is related to reductions in fasting insulin concentrations (Vague et al, 1987) although we failed to confirm this in diabetic subjects. In our population there was no significant change in the mean fasting insulin concentrations, and changes in PAI-1 activity also showed no significant relationship with those in fasting insulin concentration or insulin sensitivity in all subjects.

There was a significant correlation between the fall in PAI-1 and changes in body mass index. In previous studies, weight reduction by other measures has been associated with improved fibrinolysis. It remains possible that mechanism of the reduction in PAI-1 may differ between diabetic and non-diabetic subjects. Previous studies in subjects with type 2 diabetes have shown that metformin improves fibrinolysis (Chakrabarti et al, 1965; Sanananda et al, 1974), and a reduction in PAI-1 activity would now seem to be an important underlying basis for this improvement. We noticed that there was a greater reduction in PAI-1 in obese subjects than in those who were non-obese, probably due to the fact that obese subjects lost more weight than thin subjects.

Thrombosis has been shown to be a crucial factor in the evolution of acute coronary events in subjects predisposed to atherosclerosis (DeWood et al, 1980; Davies et al, 1984) and elevated concentrations of PAI-1 may be part of the mechanism where-by insulin resistance is associated with coronary heart disease (Hamsten et al, 1987; Gray et al, 1993). Thus long term treatment with metformin could reduce the risk of thrombosis through its effect of fibrinolysis by lowering PAI-1 activity.

We have shown no effect of metformin, either independently or secondary to changes in glycaemic control, on platelet aggregation studies in whole blood or platelet rich plasma. A previous study of IDDM subjects (Gin et al, 1989), have shown some beneficial changes in platelet aggregation, while studies in Type 2 diabetic subjects have shown that this effect relates to improvements in glycaemic control (Collier et al, 1989). Similarly no effect of the drug was apparent on markers of in-vivo platelet aggregation such as plasma beta thromboglobulin or platelet factor 4 or on plasma fibrinogen concentrations.

The reductions in urinary albumin excretion rate is an interesting and unanticipated finding and seems to occur along the whole range of albumin excretion values, though most subjects in the study were normoalbuminuric. The lack of significant correlations with changes in body mass index, systolic and diastolic blood pressure, and fasting plasma glucose concentrations would raise the possibility that this might be an independent effect of metformin. A previous study (Campbell et al, 1988) has shown a reduction in urinary albumin excretion rate both on metformin and sulphonylurea treatment which they suggested was secondary to improvement in glycaemic control, although we did not confirm that aspect of their finding. Whatever the mechanisms of reduction in urinary albumin excretion, these results would warrant further controlled trials to look at the effects of various treatment modalities on urinary albumin excretion

in type 2 diabetic subjects with microalbuminuria.

6.8 Summary and conclusions

In conclusion metformin monotherapy in subjects with Type 2 diabetes:

1. Is well tolerated with minimal side effects if given in an incremental dosage schedule and with meals.
2. Significantly improves glycaemic control with 20% reduction in plasma glucose at a dose of 850mg twice daily. Further increase in metformin dosage is associated with small advantages in terms of glycaemic control.
3. Is associated with lowering of intact proinsulin and des 31, 32 proinsulin, but without any changes in specific insulin and C-peptide concentrations.
4. Is associated with improvement in insulin sensitivity assessed by MCR, and by HOMA technique. The significant relationship between changes in insulin sensitivity and changes in fasting plasma glucose would suggest that reduction in insulin resistance may be a major mechanism of action of metformin. Beta cell function also improved on metformin treatment and is likely to be secondary to reduction in fasting plasma glucose, rather than a direct effect of metformin on pancreas.
5. Produced improvement in concentrations of several cardiovascular risk factors including PAI-1, and total and LDL-cholesterol and triglyceride concentrations.
6. Produced a reduction in urine albumin excretion rate, a well recognised cardiovascular risk factor in subjects with type 2 diabetes.

7. had no effect on systolic, diastolic or ankle-to-brachial systolic pressure ratios.
8. had no significant effect on indices of in-vivo and in-vitro platelet function nor on plasma fibrinogen levels.
9. was equally effective and produced similar changes in most variables in Asian and caucasian subjects.
10. Post-hoc subgroup analysis suggested that metformin treatment may have a preferential weight reducing effect in obese subjects and may also have more marked effects on certain cardiovascular risk factors such as plasma triglyceride and plasminogen activator inhibitor in this group.
11. No significant correlations were observed between changes in concentrations of proinsulin-like molecules and those of risk factors for cardiovascular disease including PAI-1 activity.

While these findings suggest that metformin may provide beneficial effect on cardiovascular morbidity and mortality in subjects with type 2 diabetes although the beneficial effects of metformin on cardiovascular risk factors were not related to a reduction in concentrations of proinsulin like molecule. Long term observation studies currently being conducted will provide the long awaited results on the effect of metformin on cardiovascular events (UKPDS, 1983, 1991).

CHAPTER 7: THE EFFECTS OF INSULIN TREATMENT, WITHOUT CHANGING GLYCAEMIC CONTROL, ON THE CONCENTRATIONS OF PROINSULIN-LIKE MOLECULES AND ON RISK FACTORS FOR CARDIOVASCULAR DISEASE INCLUDING PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) IN SUBJECTS WITH TYPE 2 DIABETES.

7.1 Introduction

A number of studies have shown that concentrations of intact proinsulin are raised in subjects with Type 2 diabetes (Duckworth et al, 1972; Gordon et al, 1974; Mako et al, 1977; Ward et al, 1987; Yoshioka et al, 1988; Temple et al, 1989) and dietary treatment leads to a reduction in the concentration of these molecules (Yoshioka et al, 1989). Studies have shown that proinsulin concentrations are increased by sulphonylurea treatment (Elkeles et al, 1982, Davies et al, 1993)), and another has demonstrated that there is 40-50% reduction in the concentration of these molecules during hyperinsulinaemic clamp (Koivisto et al, 1986). Information on the effects of insulin treatment on the concentration of insulin precursor molecules in subjects with Type 2 diabetes is scant.

Previous reports have suggested that non-insulin-dependent diabetic subjects show hyperinsulinaemia in the fasting state and also in response to an oral glucose challenge (DeFronzo, 1983; Gerich et al, 1988). However, using specific 2-site monoclonal antibody based assays, it has been shown that concentrations of intact proinsulin and des 31,32 proinsulin are elevated in subjects with Type 2 diabetes (Temple et al, 1989; Sobey et al, 1989) and that these molecules cross-react in many conventional radioimmunoassays for insulin (Temple et al, 1990).

We have noticed in our previous observations that these insulin precursor molecules

may comprise as much as half of all insulin-like molecules in subjects with Type 2 diabetes. Moreover elevated concentrations of these insulin precursor molecules are associated with deleterious changes in risk factors for cardiovascular disease, including low HDL-cholesterol concentrations, and increased levels of total cholesterol, triglyceride, systolic and diastolic blood pressure and PAI-1. This raises the intriguing possibility that the aetiology of cardiovascular disease in subjects with Type 2 diabetes may partly be related to elevated concentrations of proinsulin-like molecules.

It is clear, however, that imputing any causative role to the insulin precursor molecules in determining cardiovascular risk factors would require a demonstration of reductions in levels of these risk factors as a result of lowering the concentrations of insulin precursor molecules. We speculated that by correcting insulin deficiency with insulin treatment, it may be possible to reduce the concentrations of proinsulin-like molecules, as well as produce beneficial changes in risk factors for cardiovascular disease, and providing an opportunity to investigate any associations between reduction in risk factor for cardiovascular and changes in concentrations of proinsulin-like molecules.

7.2 Aims of the study

The aims of this study therefore were:

1. To relate concentrations of lipoproteins, apoproteins and PAI-1 to measures of insulin, proinsulin-like molecules and insulin resistance.
2. To investigate whether insulin treatment in subjects with Type 2 diabetes, without altering glycaemic control and insulin resistance, is associated with suppression of endogenous insulin and insulin precursor molecules.

3. To study the relationship between changes in concentrations of C-peptide (as an index of endogenous insulin production) of proinsulin-like molecules, and insulin resistance, with observed changes in concentrations of risk factors for cardiovascular disease

7.3 Subjects, materials and methods

7.3.1 Study design

11 type 2 diabetic subjects, diagnosed according to WHO criteria (WHO, 1985), were recruited. All were on treatment with oral hypoglycaemic agents and were in poor glycaemic control (fasting plasma glucose $> 8\text{mmol/l}$), with a mean concentration of HbA_{1c} of $10.5 \pm 3.1\%$. All subjects were free of angina pectoris or intermittent claudication and all had normal electrocardiograms. Patients were studied at baseline and then randomised either to insulin treatment (single or twice daily mixtures of human soluble and isophane insulin 30:70), or continued with their usual sulphonylurea therapy without any changes in dose. After 8 weeks, each subject was reassessed again as at baseline and crossed over to the alternative treatment. There was no washout period between the two treatment phases and the study design is shown in fig 7.1.

Patients were instructed to monitor their capillary blood glucose concentrations twice daily before breakfast and before the evening meal, using visually read reagent strips (BM Glycemie, Boehringer Mannheim, Lewes, Sussex, UK) and treatment was adjusted according to the results of this monitoring, the aim being to maintain fasting and preprandial glucose concentrations unaltered during both limbs of the study. Each subject was seen once weekly at the hospital during the first four weeks of treatment. All subjects were then studied on two further occasions according to the same protocol, after 8 weeks therapy with insulin and with tablets.

All subjects attended the Clinical Investigation Unit, Academic Unit of Diabetes and Endocrinology, Whittington Hospital, after an overnight fast. Height and weight were recorded and body mass index (BMI) was calculated. Subscapular and triceps skinfold measurements were made as previously described and subscapular-to-triceps skinfold ratio (STR) was calculated. Waist and hip girth were measured using a steel tape and waist-to-hip ratio (WHR) was estimated. Each subject then had an indwelling venous cannula placed in the antecubital fossa and a venous blood sample was withdrawn for assay of glucose, insulin, C-peptide, proinsulin-like molecules, PAI-1 and apoproteins and for lipoprotein fractionation. An insulin sensitivity test was then performed by the modified Harano technique. We have observed a within-subject coefficient of variation of less than 25% for the estimate of metabolic clearance rate of glucose using this method (Chapter 3). The study was approved by the Ethical Committee of Islington Health Authority, and written informed consent was obtained.

7.3.2 Statistical Analysis

In order to compare the effects of different treatments, differences between tablet and insulin treatment were analysed using paired Student's t-test for normally distributed data and Wilcoxon's test for skewed data. Correlations were sought using linear regression analysis for normally distributed and Spearman rank for skewed data. Correlations between changes in variables from tablet to insulin treatment were performed using the percentage change in each variable to account for differences at baseline. Values are expressed as mean \pm SD for normally distributed data or as median (range) for skewed data.

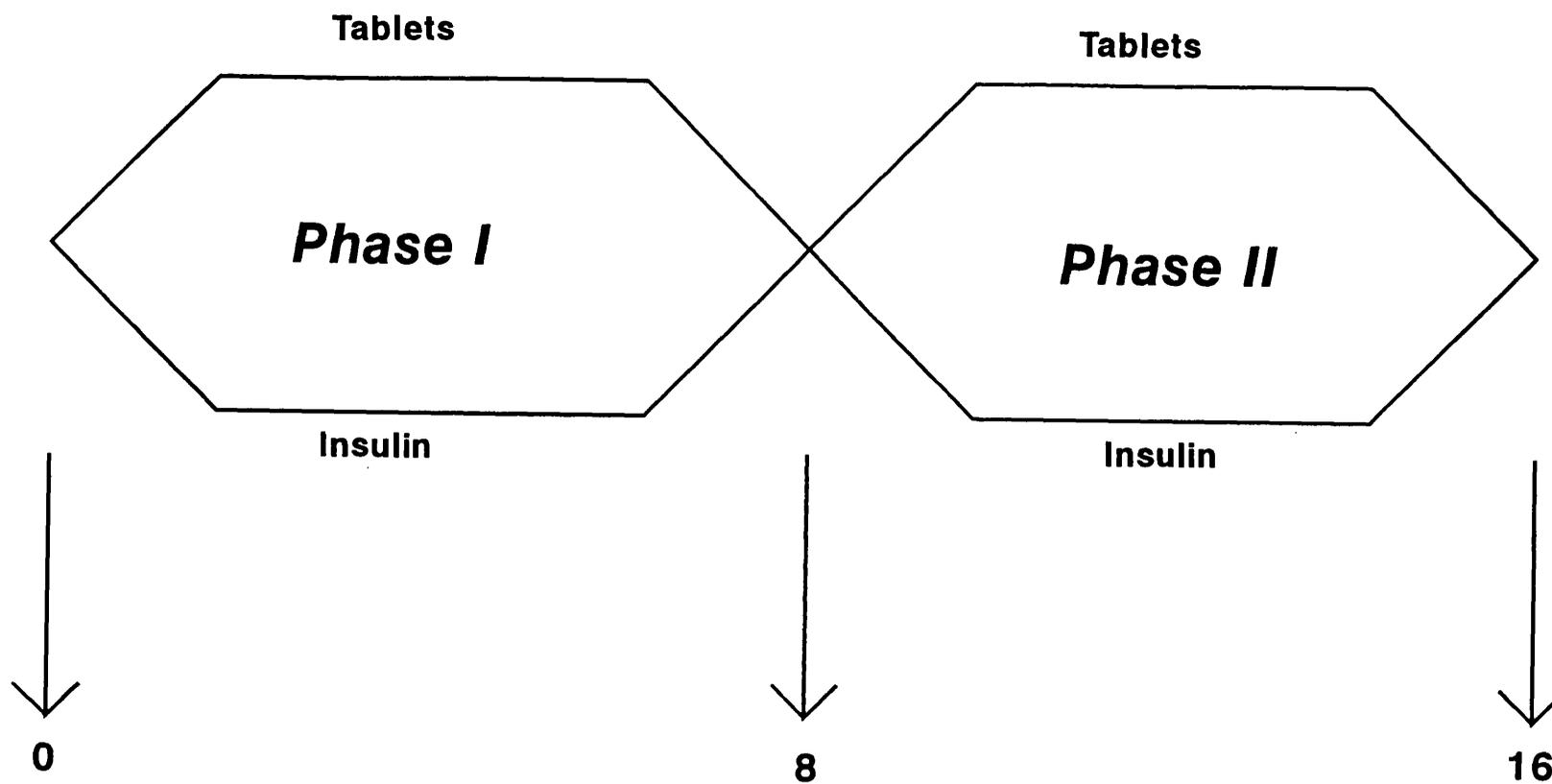


Figure 7.1: Study design to investigate the effects of insulin treatment on insulin precursor molecules and cardiovascular risk factors

7.4 Results

7.4.1 Subjects characteristics

10 men and 1 woman, with a mean age of 56.2 ± 9.7 yrs and a mean duration of diabetes of 7.0 ± 5.8 yrs were studied and their clinical and biochemical characteristics are shown in Table 1. Therapy prior to the study was chlorpropamide (1 pt; dose 200 mg per day) and glibenclamide (6 pts; mean dose 15 mg per day) and tolbutamide (4 patients; mean dose 1.75g per day). The mean insulin dose was 18.6 units/day and only one subject was on twice daily insulin. Subjects were asked to omit their morning insulin injection on the day of assessment. Glycaemic control after tablet and insulin treatment was similar (table 7.1), and there was no difference in body mass index between the two treatment periods. Similarly MCR of glucose remained unchanged between the two therapy periods. At baseline, IRMA insulin represented $74.5\% \pm 8.4\%$ of the sum of all insulin-like molecules, while intact proinsulin and des 31,32 proinsulin comprised $13.4\% \pm 5.9\%$ and $12.1\% \pm 6.3\%$ respectively. The sum of the three insulin-like molecules correlated significantly with the concentration of insulin measured by standard radioimmunoassay ($r=0.98$, $p<0.001$), with the mean sum of these molecules corresponding to $97.8\% \pm 11.7\%$ of the concentration of immunoreactive insulin.

7.4.2 Relationships between insulin, insulin precursor molecules, risk factors for cardiovascular disease and anthropometric measurements at baseline.

Significant correlations were seen at baseline between waist-hip-ratio and intact proinsulin and des 31,32 proinsulin, and C-peptide concentrations, while these relationships were not apparent with body mass index (table 7.2). IRMA insulin, intact proinsulin and C-peptide concentrations correlated positively, and in general significantly, with total and VLDL triglyceride, and negatively with HDL3-cholesterol and Apoprotein A₁ concentrations.

Table 7.1: Clinical and biochemical characteristics of subjects at baseline and after each treatment period.

Variables	At baseline	After 8 weeks of tablet treatment (a)	After 8 weeks of insulin treatment (b)	P value a) vs (b)
Body mass index [kg/m ²]	26.9 ± 5.2	27.2 ± 5.2	27.3 ± 5.0	ns
Waist-to-hip ratio	0.94 ± 0.07	0.95 ± 0.06	0.94 ± 0.06	0.06
Subscapular-to-triceps ratio	2.15 ± 0.50	2.17 ± 0.56	2.16 ± 0.61	ns
Fasting glucose [mmol/l]	14.1 ± 3.8	13.2 ± 2.2	13.8 ± 3.4	ns
Glycated haemoglobin [%]	10.5 ± 3.1	10.3 ± 2.4	10.3 ± 2.0	ns
Specific insulin [pmol/l]	51.0 [13-156]	53.0 [14-177]	62.0 [24-199]	ns
Intact proinsulin [pmol/l]	8.9 [1.4-44.0]	11.0 [3.3-44.0]	5.9 [0.5-27.0]	0.007
Des 31-32 proinsulin [pmol/l]	8.0 [2.8-17.0]	7.7 [4.9-27.0]	6.5 [4.5-19.0]	0.041
Fasting C-peptide [nmol/l]	0.23 ± 0.10	0.30 ± 0.10	0.19 ± 0.10	0.010
Total cholesterol [mmol/l]	6.3 ± 1.1	6.2 ± 1.0	6.0 ± 0.8	ns
LDL-cholesterol [mmol/l]	1.00 ± 0.67	0.86 ± 0.51	0.82 ± 0.58	ns
VLDL-cholesterol [mmol/l]	3.5 ± 1.1	3.7 ± 1.0	3.6 ± 1.0	ns
HDL ₂ -cholesterol [mmol/l]	0.47 ± 0.23	0.54 ± 0.28	0.53 ± 0.35	ns
HDL ₃ -cholesterol [mmol/l]	1.07 ± 0.37	0.91 ± 0.18	0.87 ± 0.28	ns
Total triglyceride [mmol/l]	2.5 ± 1.3	2.3 ± 1.0	2.2 ± 1.2	ns
VLDL-triglyceride [mmol/l]	1.7 ± 1.2	1.5 ± 1.0	1.4 ± 1.0	ns
LDL-triglyceride [mmo/l]	0.38 ± 0.08	0.42 ± 0.13	0.43 ± 0.18	ns
Apoprotein A ₁ [g/l]	1.60 ± 0.28	1.50 ± 0.32	1.60 ± 0.32	ns
Apoprotein A ₂ [g/l]	0.64 ± 0.12	0.60 ± 0.09	0.64 ± 0.16	ns
Apoprotein B [g/l]	1.23 ± 0.22	1.18 ± 0.27	1.20 ± 0.21	ns
PAI-1 [AU/l]	21.7 ± 9.3	24.0 ± 7.6	19.2 ± 4.0	0.022
MCR of glucose [ml/kg/min]	3.6 [1.5-12.3]	3.5 [1.7-10.4]	3.7 [1.3-12.8]	ns

Values are given as mean ± SD for normally distributed data and as a median (range) for skewed data. The comparison of variables between tablet and insulin treatment was performed using paired Student's t-tests for normally distributed data, and Wilcoxon's test for skewed data.

The relationships of IRMA insulin and C-peptide with PAI-1 were significant, while those of proinsulin-like molecules with PAI-1 just failed to achieve significance. Insulin sensitivity, as measured by metabolic clearance rate of glucose using the insulin sensitivity test, showed a significant negative relationships with waist-to-hip ratio and body mass index, and positive relationship with HDL2-cholesterol, and a negative relationship with VLDL-triglyceride, but the relationships with HDL3-cholesterol or PAI-1 were not significant. Concentrations of proinsulin-like molecules at baseline did not significantly correlate with insulin sensitivity. All these correlations were generally similar, but rather weaker, after each of the treatment phases. With insulin treatment, mean values for plasma glucose, HbA_{1c} and insulin resistance remained similar to those for tablet treatment, and no significant changes were observed in BMI or WHR.

7.4.3 Effects on endogenous insulin, insulin precursor molecules, lipids, lipoproteins and PAI-1 activity

There was significant suppression of endogenous C-peptide ($35.0\% \pm 24.2\%$, $p=0.006$; Fig 7.2), intact proinsulin ($43.1\% \pm 36.8\%$, $p=0.03$; Fig 7.3a) and des 31,32 proinsulin ($20.1\% \pm 27.0\%$, $p=0.03$; Fig 7.3b) from tablet to insulin treatment. There were no significant changes in concentrations of any lipoprotein component or of apoproteins, but the activity of PAI-1 fell significantly ($14.3\% \pm 8.3\%$, $p=0.02$) (Fig 7.4). Other variables were unchanged between baseline and tablet treatment (table 7.1).

7.4.4 Relationship between changes in concentrations of C-peptide, insulin and proinsulin-like molecules

There was a close correlation between changes in intact and in des 31,32 proinsulin concentrations with insulin treatment ($r_s=0.83$, $p=0.001$) (Fig 7.5), but that between changes in concentrations of C-peptide and either intact proinsulin ($r_s=-0.41$, $p=0.11$)

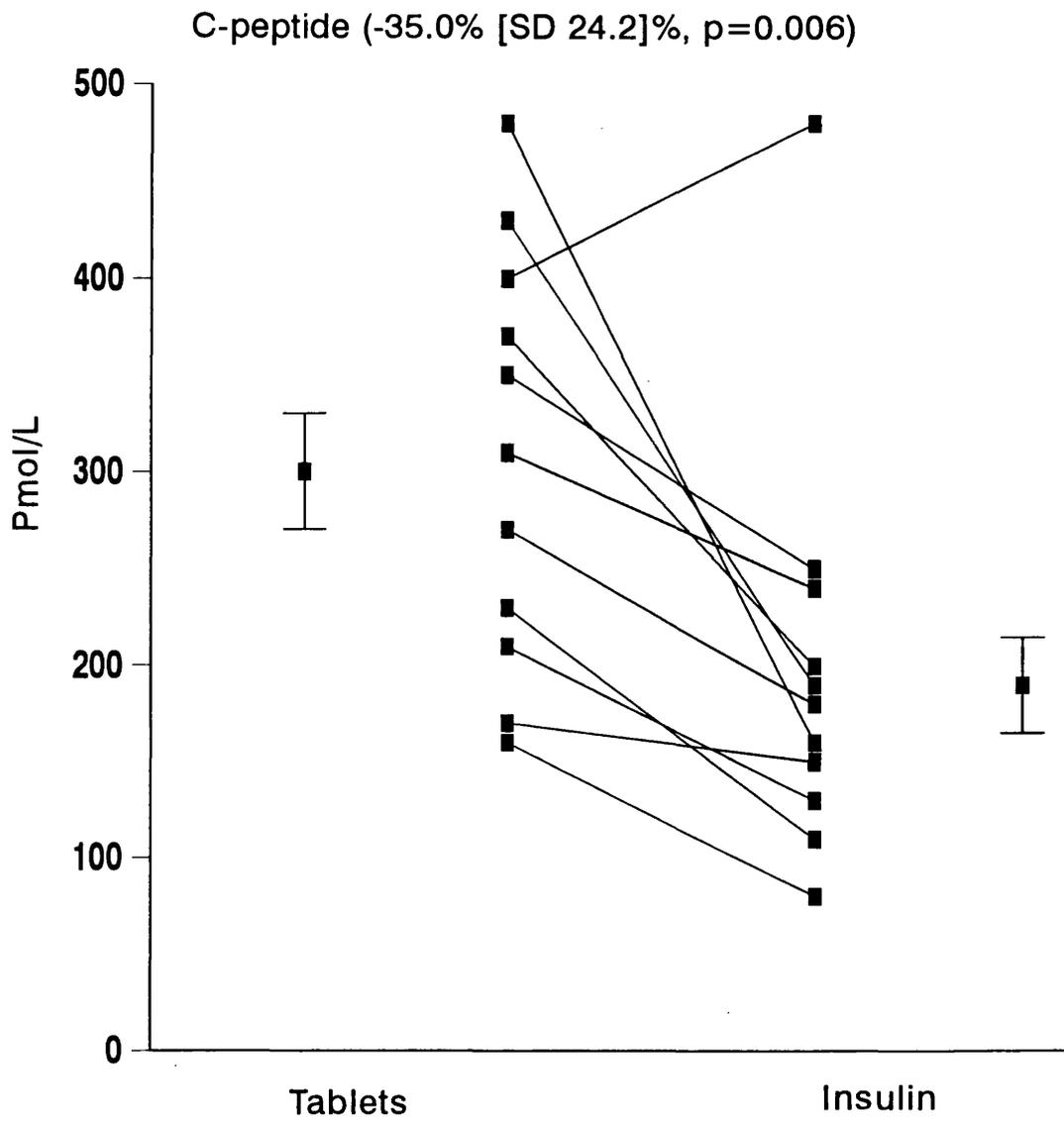


Figure 7.2: The effects of insulin treatment on C-peptide concentrations

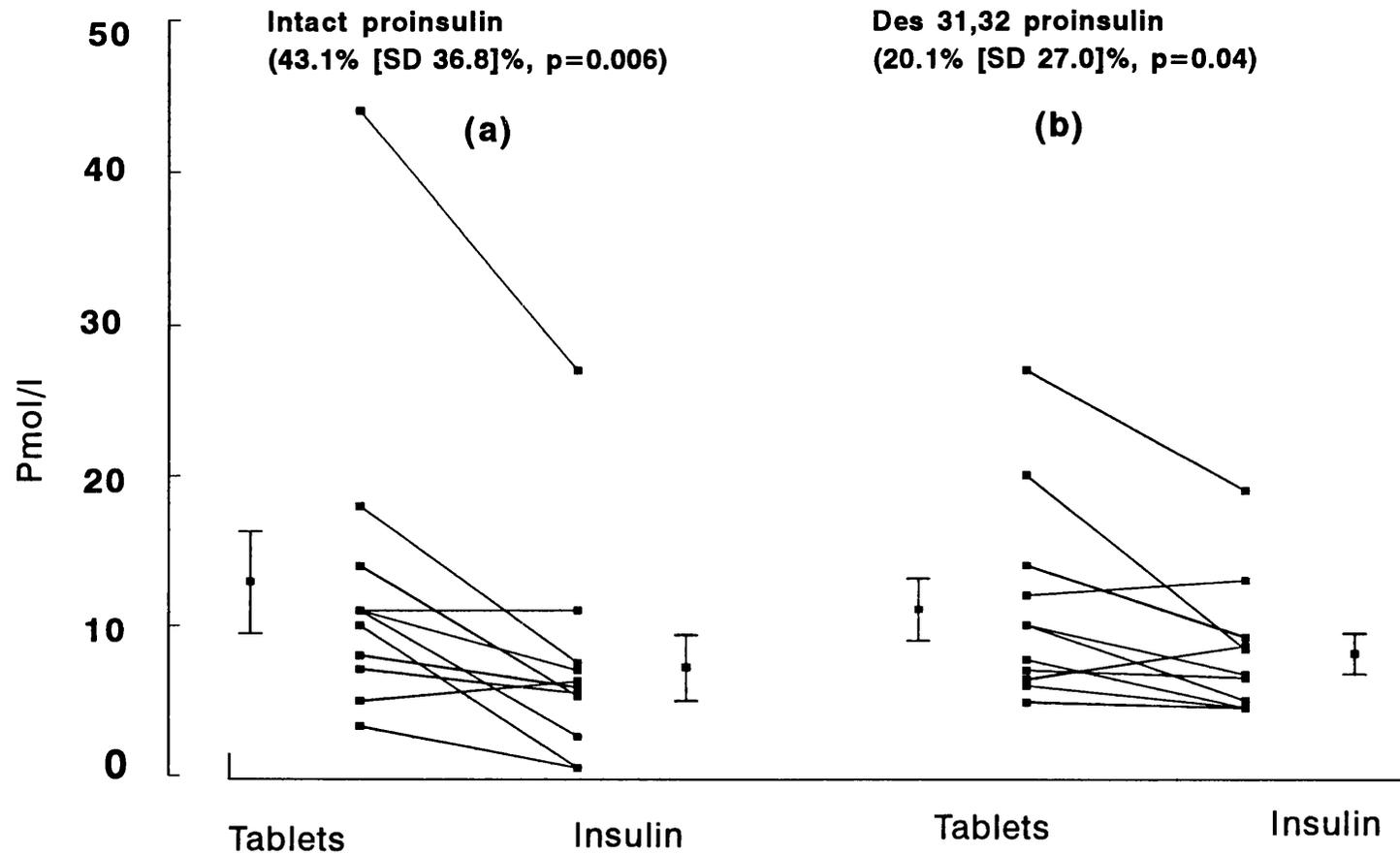


Figure 7.3: The effects of insulin treatment on (a) intact and (b) des 31,32 proinsulin concentrations

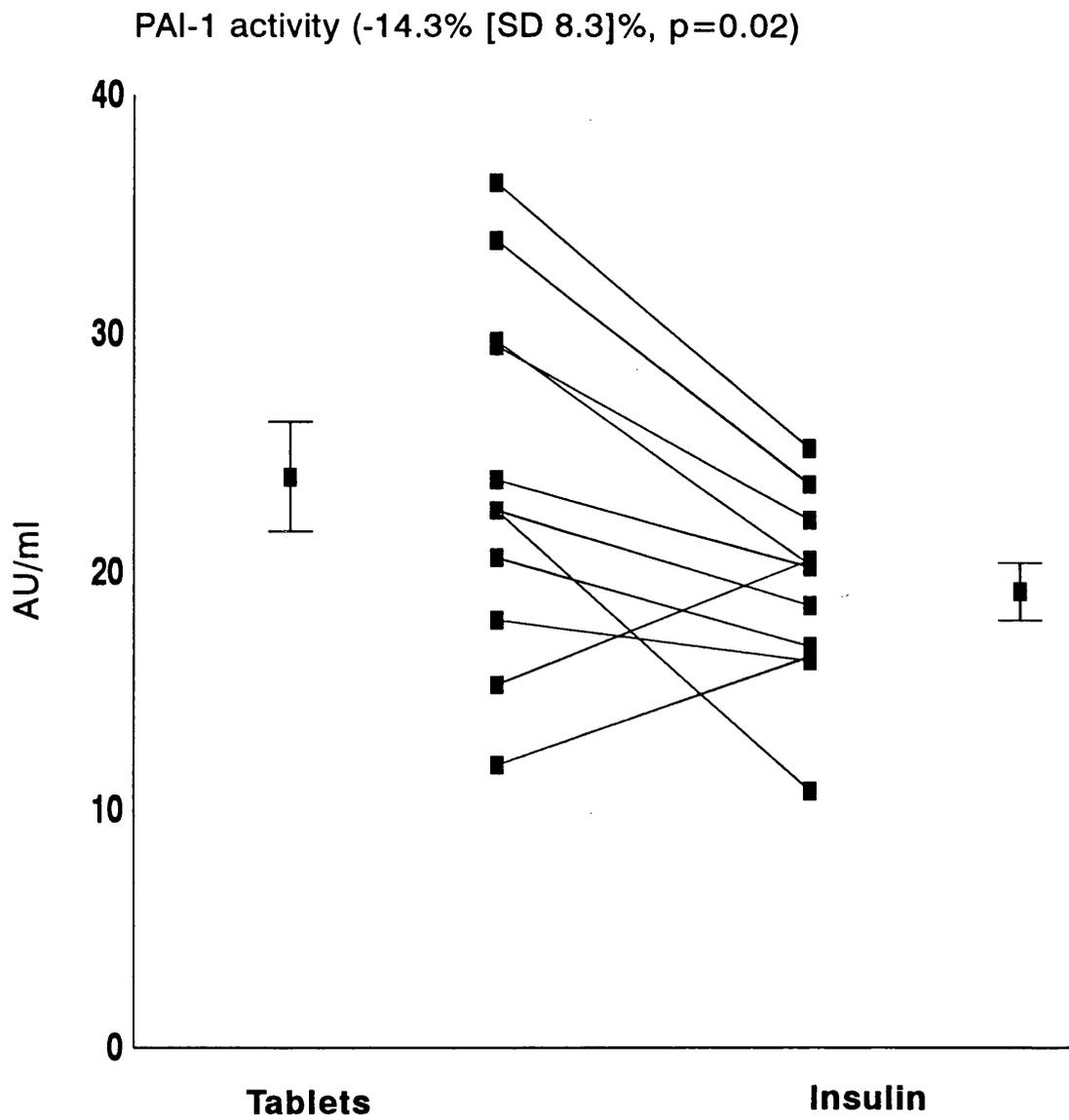


Figure 7.4: The effects of insulin treatment on PAI-1 activity

or des 31-32 split proinsulin ($r_s = -0.27$, $p = 0.21$) were in the opposite direction, although both failed to achieve significance.

7.4.5 Relationships between changes of C-peptide, proinsulin-like molecules, and insulin resistance with changes in risk factors for cardiovascular disease.

The correlations of changes in concentrations of C-peptide, IRMA insulin, intact and des 31,32 proinsulin and in insulin sensitivity (metabolic clearance rate of glucose), as measured by the insulin sensitivity test, with those in concentrations of different cardiovascular risk factors is shown in table 7.3. Changes in intact proinsulin concentration were significantly and positively correlated with those in total and VLDL-triglyceride and PAI-1, and negatively with HDL3-cholesterol and apoprotein A₁. However changes in des 31,32 proinsulin were not significantly correlated with those in any variable. Changes in endogenous insulin production, as evidenced by changes in concentrations of C-peptide, were, paradoxically, positively correlated with those in HDL2-cholesterol concentrations and negatively with those in PAI-1 activity, but there were no significant correlations with any other lipoprotein or apoprotein component. Changes in insulin sensitivity correlated positively with those in HDL2-cholesterol but not with any other risk factor. There were no significant relationships between changes in insulin sensitivity and those in C-peptide or proinsulin-like molecules. Changes in insulin concentrations, as measured by the 2-site monoclonal antibody assay, showed no correlation with any risk factor except that in total cholesterol.

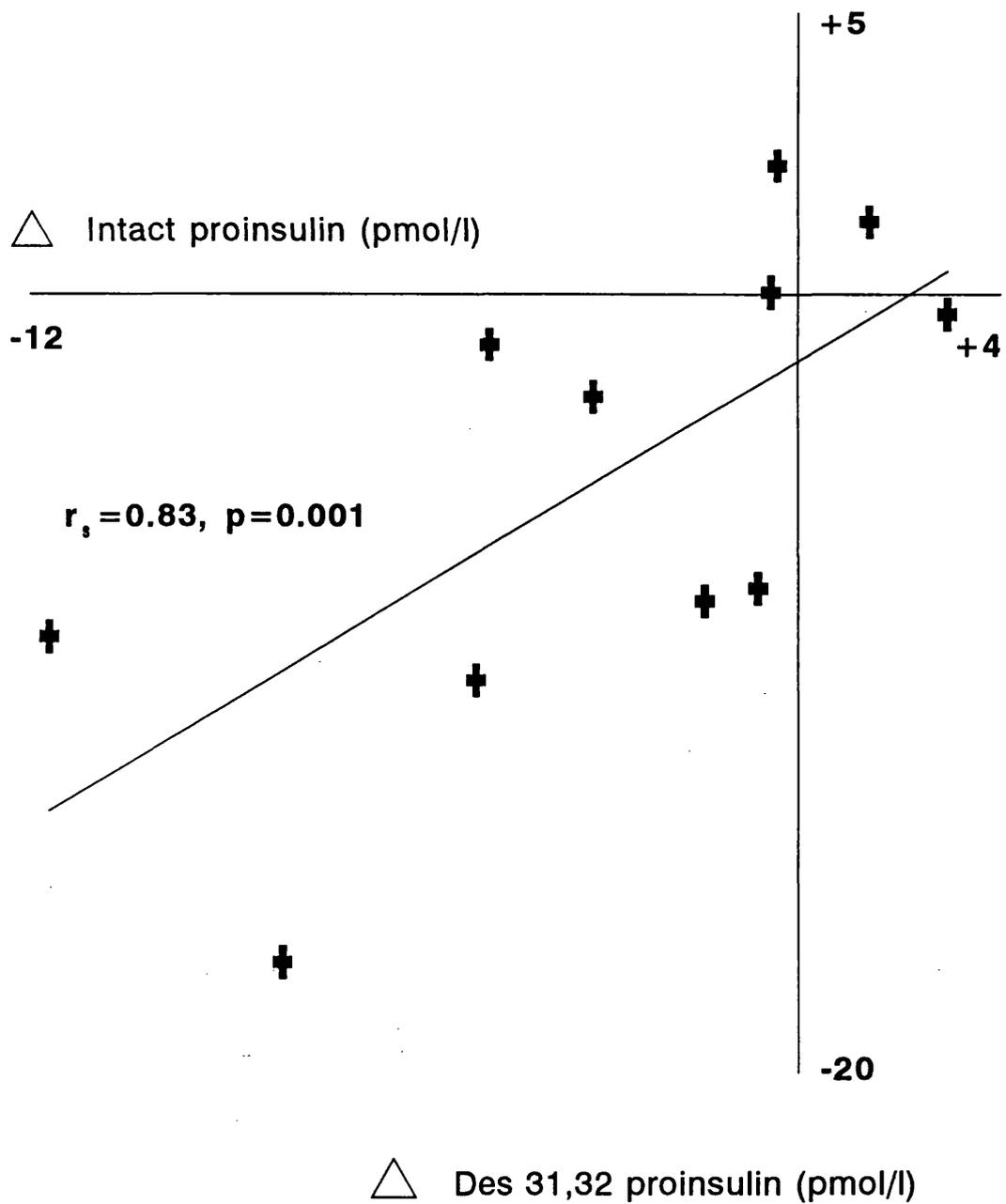


Figure 7.5: Relationship between changes in intact and des 31,32 proinsulin concentrations on insulin treatment

Table 7.2: Correlation coefficients between concentrations of insulin, proinsulin-like molecules and C-peptide, with anthropometric and biochemical variables at baseline.

Variable	Immunoreactive insulin	Specific insulin	Intact proinsulin	Des 31,32 proinsulin	C-peptide	MCR glucose
Body mass index	0.13	0.10	0.13	-0.05	0.47 [p = 0.07]	-0.85 [p < 0.001]
WHR	0.58	0.41	0.73 [p = 0.006]	0.55 [p = 0.04]	0.72 [p = 0.006]	-0.56 [p = 0.03]
STR	0.07	-0.16	0.32	0.11	-0.11	-0.17
FPG	0.14	0.10	0.05	0.29	0.44 [p = 0.09]	-0.47 [p = 0.07]
Glycated haemoglobin	-0.51 [p = 0.05]	-0.46 [p = 0.08]	-0.53 [p = 0.05]	-0.05	-0.17	-0.11
Total cholesterol	-0.10	-0.01	-0.01	-0.18	-0.31	0.18
LDL-cholesterol	-0.11	0.06	-0.01	-0.32	-0.22	0.10
VLDL-cholesterol	0.70 [p = 0.008]	0.60 [p = 0.02]	0.63 [p = 0.02]	0.15	0.44 [p = 0.09]	-0.20
HDL ₂ -cholesterol	-0.10	-0.17	-0.12	0.48 [p = 0.07]	0.05	0.51 [p = 0.05]
HDL ₃ -cholesterol	-0.79 [p = 0.002]	-0.83 [p = 0.001]	-0.56 [p = 0.04]	-0.01	-0.45 [p = 0.08]	0.23
Total triglyceride	0.69 [p = 0.009]	0.56 [p = 0.04]	0.65 [p = 0.02]	0.18	0.66 [p = 0.01]	-0.47 [p = 0.07]
VLDL-triglyceride	0.71 [p = 0.007]	0.56 [p = 0.04]	0.71 [p = 0.007]	0.22	0.69 [p = 0.009]	-0.56 [p = 0.04]
LDL-triglyceride	0.39	0.46 [p = 0.08]	0.22	-0.09	0.12	0.16
Apoprotein A ₁	-0.73 [p = 0.005]	-0.71 [p = 0.007]	-0.53 [p = 0.05]	-0.36	-0.84 [p = 0.001]	0.38
Apoprotein A ₂	-0.38	-0.33 [p = 0.16]	0.35	0.23	-0.41	0.30
Apoprotein B	0.14	0.27	0.23	-0.20	-0.05	-0.02
PAI-1	0.58 [p = 0.04]	0.70 [p = 0.01]	0.41	0.52 [0.06]	0.63 [p = 0.02]	-0.25
MCR glucose	-0.28	-0.11	-0.35	0.00	-0.57 [p = 0.03]	-

All correlations were performed employing Spearman rank correlation analysis. P-values of correlation coefficients are given only where $r > 0.42$ ($p < 0.10$)

Table 7.3: Correlation coefficients between percentage changes in insulin, C-peptide, proinsulin-like molecules and insulin resistance with percentage changes in cardiovascular risk factors.

Variable	Δ Specific insulin	Δ C-peptide	Δ Intact proinsulin	Δ 32,33 split proinsulin	Δ MCR Glucose
Δ Total cholesterol	-0.54 [p = 0.04]	-0.42	-0.23	-0.35	0.05
Δ LDL-cholesterol	-0.39	-0.35	-0.51 [0.05]	-0.32	0.23
Δ VLDL-cholesterol	-0.10	-0.09	0.68 [p=0.01]	0.04	-0.36
Δ HDL ₂ -cholesterol	0.03	0.55 [p = 0.04]	-0.47 [p = 0.07]	-0.07	0.75 [p = 0.005]
Δ HDL ₃ -cholesterol	-0.01	-0.06	-0.57 [p = 0.03]	-0.06	0.32
Δ Total triglyceride	-0.13	-0.08	0.57 [p = 0.03]	-0.05	0.27
Δ VLDL-triglyceride	-0.10	-0.08	0.60 [p = 0.03]	-0.03	-0.34
Δ LDL-triglyceride	-0.43 [p = 0.09]	-0.27	0.13	-0.45 [p = 0.08]	-0.20
Δ Apoprotein A ₁	-0.30	-0.39	-0.54 [p = 0.04]	-0.27	0.19
Δ Apoprotein A ₂	-0.17	-0.19	-0.40	-0.28	0.25
Δ Apoprotein B	-0.31	-0.42	-0.55 [p = 0.04]	-0.29	0.01
Δ Plasminogen activator inhibitor	-0.17	-0.73 [p = 0.006]	0.51 [p = 0.05]	0.34	-0.40
Δ Metabolic clearance rate of glucose	-0.08	0.18	-0.05	0.26	0.26

All correlations were performed employing Spearman rank correlation analysis. P-values of correlation coefficients are given only where $r > 0.43$ ($p < 0.10$)

7.5 Discussion

This study confirms some of the observations of our previous cross sectional study i.e. significant relationships were apparent between concentrations of proinsulin-like molecules and certain cardiovascular risk factors, though a smaller study population meant these relationships were not as significant as in our previous observations. Also intact proinsulin rather than des 31,32 proinsulin appeared to correlate more closely with several risk factors. In the previous study reported in Chapter 4, des 31,32 proinsulin was the major circulating molecule while in this study population the concentrations of intact proinsulin exceeded those of des 31,32 proinsulin. This might explain the differences we observed in these baseline correlations.

The finding of association is clearly not proof of causation, so this intervention study was designed to assess the effects of changes in the concentrations of these molecules on cardiovascular risk factors. The proportion of insulin-like molecules comprising proinsulin and des 31,32 proinsulin in these subjects ($25.9\% \pm 8.6\%$) is smaller than that in our previous study ($62.3\% \pm 15.1\%$). None of the subjects studied here were included in our previous study so it is likely that these differences truly represent population heterogeneity. Employing baseline and tablet therapy observations to calculate intra-individual biological variability in concentrations of insulin-like molecules, these are $18.9\% \pm 17.3\%$ for insulin, $16.2\% \pm 16.1\%$ for intact proinsulin and $23.6\% \pm 20.4\%$ for des 31,32 proinsulin, suggesting that assay imprecision can not contribute substantially to the differences in concentrations of these molecules in this study and the previous study. Moreover the correlation observed between changes in concentrations of intact and des 31,32 proinsulin would be unlikely to be seen if either or both of these assays were imprecise.

In this preliminary investigation, we have shown that insulin treatment, without any

changes in glycaemic control or insulin resistance, is associated with suppression of endogenous insulin production and of proinsulin-like molecules in subjects with type 2 diabetes. More intensive insulin treatment in subjects with Type 2 diabetes has been shown to be associated with improvement in insulin sensitivity (Andrews et al, 1984; Garvey et al, 1985) and in beta cell function (Yki-Järvinen et al, 1988; Kawamori et al, 1989) when glycaemic control improves, but sulphonylurea treatment is able to produce a similar increase in insulin sensitivity to that resulting from exogenous insulin (Firth et al, 1986). In this study we have shown that insulin therapy per se, i.e without changes in glycaemic control, does not produce any change in insulin resistance.

The fall in concentrations of intact proinsulin and of des 31,32 proinsulin showed a close relationship with each other ($r_s=0.83$) but there was an insignificant correlation between the changes in concentrations of these molecules and those in C-peptide. It is possible that patients with severe beta cell failure, and hence with low plasma C-peptide concentrations, would show minimal suppression of C-peptide with insulin, but that in these subjects higher concentrations of proinsulin-like molecules would be suppressed by insulin treatment. However we found a positive relationship between the initial concentrations of C-peptide and of proinsulin-like molecules ($r_s=0.53$, $p=0.05$ for intact proinsulin and $r_s=0.57$; $p=0.03$ for des 31,32 proinsulin) which suggests that proinsulin-like molecules are not released simply as a consequence of, and in proportion to, failing beta cell function. These findings suggest that the mechanisms which regulate insulin release by the pancreas may be controlled separately from those which control proinsulin conversion (Rhodes et al, 1987; Davidson et al, 1988), but at present it can only be speculated how these are altered in subjects with Type 2 diabetes (Porte et al, 1989).

This study was designed to investigate whether evidence derived from cross-sectional

correlations of levels of proinsulin-like molecules and of cardiovascular risk factors could be reinforced by studying the effects of interventions. The lack of significant relationship between changes in concentrations of C-peptide and of proinsulin-like molecules has enabled us to relate changes in concentrations of these molecules independently to those of cardiovascular risk factors, as multiple regression analysis is clearly inappropriate with such small numbers of observations. We have deliberately not analysed relationships between changes from baseline, despite therapy and glycaemic control being unaltered between baseline and tablet treatment, as these two timepoints are not strictly comparable because of possible trial effects.

We have shown that insulin treatment in subjects with Type 2 diabetes is associated with a fall in the concentration of PAI-1, a fast acting inhibitor of tissue plasminogen activator, but not in any lipoprotein or apoprotein fraction. PAI-1 activity is elevated in patients with Type 2 diabetes (Auwerx et al, 1988; Juhan-Vague et al, 1989) and has been implicated in the aetiology of myocardial infarction (Hamsten et al, 1985, 1987). PAI-1 is synthesised in the liver, and in-vitro its synthesis is stimulated by insulin (Alessi et al, 1988; Kooistra et al, 1989; Grant et al, 1991) and proinsulin (Schneider et al, 1992), and thereby may provide a link between hyperinsulinaemia and the thrombotic component of cardiovascular risk. While proinsulin is less potent in stimulating PAI-1 synthesis in-vitro in hepatic cell lines (Alessi et al, 1988), we have observed that proinsulin-like molecules, rather than insulin itself, correlates better with PAI-1 activity in a cross sectional study in subjects with Type 2 diabetes (Chapter 4). In the smaller numbers of subjects in this study, however, at baseline the correlation of PAI-1 with insulin was the stronger. These observations are supported by the fact that intact proinsulin stimulated PAI-1 secretion from bovine endothelial cell (Schneider et al, 1992)

The fall in the activity of PAI-1 showed significant correlations with those in intact proinsulin, but paradoxically, a negative though significant relation with those in C-peptide (table 7.3). These observations would again suggest that in type 2 diabetic subjects proinsulin-like molecules may be as important as endogenous insulin in regulating PAI-1 synthesis. It should be noted, parenthetically, that among the correlations included in table 7.3, it would be expected that 3 should reach conventional levels of significance by chance alone.

We did not show any significant changes in lipoprotein and apoprotein concentrations with insulin treatment suggesting that previous observations of the benefits of insulin therapy on lipoprotein concentrations have resulted mainly from improved glycaemic control (Agardh et al, 1982; Abrams et al, 1982; Taskinen et al, 1990). There were, however, significant correlations between changes in intact proinsulin concentrations and those of VLDL-triglyceride, HDL3-cholesterol and apoprotein A₁. These correlations were not seen with changes in C-peptide concentration, although again there was a paradoxical positive correlation between changes in concentrations of C-peptide and those in HDL2-cholesterol. The lipoprotein abnormalities of Type 2 diabetes appear to relate both to an excessive supply of fatty acids as substrate for triglyceride synthesis, and to alterations in activities of hepatic and adipose tissue lipoprotein lipase (Taskinen et al, 1987; Taskinen, 1990). The relationships we have observed between changes proinsulin-like molecules and those in lipoproteins and apoproteins might suggest that insulin precursor molecules could stimulate hepatic synthesis of VLDL-triglyceride and hepatic lipase activity, thereby producing reciprocal changes in concentrations of VLDL and HDL2-cholesterol (Taskinen, 1990). It should be noted, however, that therapy with human proinsulin in Type 2 diabetes has been shown to produce favourable changes in plasma triglyceride and HDL2-cholesterol concentrations (Drexel et al, 1988). Moreover, despite the observed correlations between changes in concentrations of

proinsulin-like molecules and those in lipoproteins, therapy with insulin produced no significant change in lipoprotein or apoprotein concentrations. It is highly likely that certain of these risk factors could be improved by more intensive insulin treatment, independent of glycaemic control or insulin resistance, if the suppression of proinsulin-like molecules were further suppressed by more intensive treatment.

In this study, insulin sensitivity was measured by the modified Harano technique, which we have shown to provide reproducible measures of insulin resistance, and which correlate with insulin sensitivity measured by euglycaemic clamp (Heine et al, 1985). At baseline, insulin sensitivity showed no correlation with any cardiovascular risk factor except HDL2-cholesterol and VLDL-triglyceride. As the aim of the study was to maintain glycaemic control between the two study periods and insulin sensitivity, as expected, also remained unaltered. Because the individual changes in metabolic clearance rate of glucose were less than the variance of the method, any correlations with changes of this variable may not indicate valid relationships. Nevertheless, changes in insulin sensitivity correlated positively with those in HDL2-cholesterol, but they did not correlate with those in any of the other measured cardiovascular risk factors. These observations may suggest that it is indeed insulin resistance, rather than elevated concentrations of insulin, or of proinsulin-like molecules, that is responsible for the deficiency of adipose tissue lipoprotein lipase activity observed in Type 2 diabetes (Dunn et al, 1984; Taskinen et al, 1990). There was no relationship between changes in proinsulin-like molecules and those of metabolic clearance rate of glucose, arguing against any role for these molecules in determining insulin resistance and vice versa.

7.6 Summary and conclusions

In summary, therefore:

1. Insulin treatment in subjects with type 2 diabetes, without any change in glycaemic control or in insulin resistance, is associated with significant suppression of endogenous insulin secretion and a reduction in the concentrations of insulin, proinsulin and des 31,32 proinsulin.
2. Insulin treatment, even without improving glycaemic control, is also significantly associated with a reduction in PAI-1 activity and may therefore have beneficial effect on cardiovascular events by enhancing fibrinolysis.
3. There were no significant reductions in any other cardiovascular risk factors measured in this study such as lipids and apoproteins, meaning thereby, that in previous studies which showed reductions in lipids and lipoproteins due to different treatment modalities, these changes were most likely to be due to improvement in glycaemic control.
4. There was a highly significant negative relationship between the changes in C-peptide levels and those of PAI-1 activity. The significance of this findings is unclear.
5. The changes in concentration of proinsulin-like molecules correlated with favourable changes in both PAI-1 and other risk factors for cardiovascular disease.

These observations are of interest in the light of observations that a therapeutic trial of proinsulin in type 2 diabetes was stopped prematurely because of an increase in incidence of myocardial infarction in the proinsulin-treated patients after an average

duration of treatment of 19.5 months (Spradlin et al, 1990). Although patients in the proinsulin treated group were in the upper percentile of cardiovascular risk factor group, a comparable number of patients in this group who were on insulin treatment had no events, suggesting a possibility of a causal relationship of human proinsulin with acute myocardial infarction. It must be said, that although this relationship is far from established, the evidence is hard to ignore particularly keeping in mind the known effects of proinsulin on PAI-1 secretion (Alessi et al, 1988; Schneider et al, 1992) and its growth promoting effects (King et al, 1980) in-vitro. Secondly the plasma concentrations of proinsulin during proinsulin treatment ranged from 1400 to 8000 pmol/l (Bergenstal et al, 1984; Glauber et al, 1987) which are on average 10-1000 times as high as those observed in obese and non-obese individual with and without diabetes. As this was a randomised trial and there were no differences at baseline in various cardiovascular risk factors in subjects randomised to proinsulin and isophane insulin treatment, it remains possible that proinsulin treatment may have led to adverse changes in fibrinolysis, leading to excess cardiovascular events in the proinsulin treated group for it is unlikely that proinsulin could accelerate atherosclerotic plaque formation in such a short time. Equally however, it is possible that the smaller number of deaths in the isophane treated group could have been due to a beneficial effect of insulin on fibrinolysis in these subjects, an interpretation which would also be in accordance with the results of this study. These observations also suggest that long term treatment with insulin, with more stringent glycaemic control, could be associated with even more favourable changes in PAI-1 and other risk factors for cardiovascular disease. It would, nevertheless, be hazardous to extrapolate these observations to suggest that beneficial effects on outcome measures might be obtained with insulin therapy, and it is only in the context of a major intervention study where such a hypothesis could be tested (UKPDS, 1993)

CHAPTER 8: CONCLUSIONS

The work described in this dissertation has led to a number of new findings and confirmed others. Firstly, the long held belief that proinsulin and its metabolites may cross-react in conventional insulin immunoassays was confirmed by our observation in Chapter 4 where we showed that proinsulin and its metabolites cross-react with insulin immunoassay with a potency equal to that of insulin. The measurement of proinsulin-like molecules as "insulin" will, therefore, produce erroneous conclusions regarding the insulin status of a subject, as biological activity of these molecules is much less than that of insulin. In our view, this cross-section is probably of less significance in non-obese non-diabetic subjects where the proportion of proinsulin-like molecules is 10-15% of the total insulin like immunoreactivity. However, in subjects with NIDDM and IGT circulating proinsulin-like molecules are elevated and a large proportion of what is measured as "insulin" by conventional insulin immunoassays may be proinsulin-like molecules. The second important implication of this cross-reaction is when measuring fasting concentration of insulin which are usually toward the lower limit of assay sensitivity and measurement of proinsulin-like molecules in this instance may then produce even larger discrepancy.

Secondly, we found that the relationship of immunoreactive insulin concentrations with standard cardiovascular risk factors was weakened or became insignificant when concentrations of insulin measured by specific assays were used to reanalyse these relationship; coupled with the observations of a significant relationship of proinsulin-like molecules with these risk factors, led us to believe that some of the previously observed relationship of insulin with cardiovascular risk factors might be a reflection of the relationship of proinsulin-like molecules with risk factors. We observed a significant relationship of proinsulin-like molecules with PAI-1 activity raising the

possibility that these molecules may play a part in regulating PAI-1 activity, an observation which has been confirmed by in-vitro studies by Schneider et al. and by Nordt.

The relationship of fasting concentrations of proinsulin-like molecules to cardiovascular risk factors at baseline was confirmed subsequently in studies described in Chapters 5, 6 and 7 and also by other workers both in non-diabetic and diabetic subjects.

However, these relationship observed in cross-sectional studies cannot be taken to imply causality and a strong possibility remains that this association is a reflection of some common antecedent. Secondly it also seems biologically implausible that concentration of proinsulin-like molecules, which are of the order of $\approx 10\text{pmol/l}$ or so in non-diabetic subjects, can influence adversely the cardiovascular risks profiles. In these subjects the association of proinsulin-like molecules is most likely to be just an epiphenomenon of a common antecedent. What is the nature of this common antecedent remains unknown. Recent studies suggest that a low birth weight, presumably due to poor intrauterine and neonatal nutrition, is associated with increased cardiovascular mortality, glucose intolerance and increased insulin resistance in adult life. Studies have also shown that low birth weight is associated with hypertension and raised proinsulin-like molecules in adult life and it is possible that low birth weight may represent a common antecedent. These observations would suggest that a low birth weight, irrespective of its cause somehow affect the risk of development coronary artery disease, glucose intolerance and raised proinsulin-like molecules, perhaps by influencing pancreatic β -cell development. However, recently subjects with low birth weight were found to have normal insulin secretion in adult life compared to their normal counterparts, arguing against the role of low birth weight as a major determinant of β -cell function in adult life. However, it remains unknown if low birth

weight is a phenotypic expression of some genetic defect in beta-cell function which would ultimately lead to insulin resistance in adult life or that low birth weight is a phenotype for insulin resistance per se. However of paramount importance are the observations that excess weight gain during adult life was extremely detrimental in subjects who had low birth weight points overwhelmingly towards a strong effect of environmental factors in determining the adverse metabolic sequelae related to low birth weight and or insulin resistance in adult life.

However, in subjects with NIDDM, our observations support the possibility that proinsulin-like molecules could play a role in determining the cardiovascular risk factors, as molar concentrations of these molecules are raised and these molecules may be the major source of circulating insulin-like immunoreactivity. Our observations are further supported by in-vitro studies of the effect of proinsulin and its split products on plasminogen activator-inhibitor-1 expression from endothelial cell lines, which showed that proinsulin and its metabolites stimulate PAI-1 expression and secretion. Studies by Nordt would also suggest that proinsulin may regulate fibrinolysis by its effects on PAI-1 gene (Nordt, 1993). Further support for this relationship we observed between proinsulin-like molecules and PAI-1 activity, and the results of in-vitro studies by others, comes from a randomised trial of proinsulin treatment in subjects with type 2 diabetes. This trial was discontinued due to the possibility of adverse cardiac outcomes in proinsulin treated group. Although the increase in number of cardiac events was only marginal in proinsulin treated group these results cannot be ignored, particularly keeping in mind that proinsulin concentration in proinsulin treated subjects were 800 - 1000 times as high as normally observed in non-diabetic and diabetic subjects and these enormously high concentrations may have adversely affected the fibrinolytic system in these subjects for it is unlikely that proinsulin could propagate atherosclerosis in such a short time. On the contrary, it can be argued that the less

number of cardiac events in NPH insulin treated group were there to beneficial effect of insulin treatment on fibrinolysis, findings which also be in accordance with our finding of reductions in PAI-1 activity on insulin treatment.

We have also shown that both insulin and metformin treatment in NIDDM subjects are associated with suppression of proinsulin-like molecules. As hyperglycaemia has been suggested to be a major mechanism responsible for increase proinsulin/insulin ratio, as well as a preferential release of proinsulin, the reduction seen in these molecules is most likely to be due to the reduction in hyperglycaemia seen on metformin treatment and due to the suppressive effect on pancreatic insulin secretion of exogenous insulin in insulin treated subjects. The relationship in reduction of proinsulin-like molecules and that of PAI-1 activity seen on insulin treatment may lend further support to the theory that proinsulin may be an important regulator of PAI-1 secretion. These observations would suggest that tightening of glycaemic control on insulin treatment may lead to changes in these parameters which may be of larger magnitude than we observed.

We also demonstrated hyperinsulinaemia in response to glucose load on Asian subjects using insulin assay of high specificity. These results point to that Asian subjects are insulin resistance or have an enhanced beta-cell sensitivity to oral glucose compared to caucasian subjects. A preferential central distribution of obesity and lack of physical activity may be responsible for these findings in this ethnic group. The role of exaggerated insulin response in determining future development of diabetes in this population remains to be seen and would require longitudinal studies.

Asian non-diabetic subjects also had high prevalence of electrocardiographic evidence of IHD compared to caucasian subjects, However we did not observe any relationship of either insulin concentrations or PAI-1 activity with IHD in this group. As the number

of subjects who had IHD is small, such a relationship can not be ruled out and studies with large number of subjects are needed in this ethnic group to see if insulin or PAI-1 is associated with IHD in this group. The association of BMI with electrocardiographic changes of IHD suggest that obesity may have a detrimental effect in this ethnic group.

The relationship of diabetes with PAI-1 activity was similar in both ethnic groups and we did not observe any ethnic differences in PAI-1 activity and other conventional cardiovascular risk factors in these populations. There were also no ethnic differences of the effect of metformin on cardiovascular risk factors or glycaemic control and similar benefits were observed in both groups.

Finally we confirmed using specific assays for insulin that the relationship of fasting insulin to hyperglycaemia in type 2 diabetes is inverted U shaped, while concentrations of proinsulin-like molecule are higher as hyperglycaemia worsens. With recent advances in the understanding of enzymology of proinsulin conversion in the β -cell, further studies may reveal the defect which leads to β -cell dysfunction in type 2 diabetes leading to insulin deficiency and hyperproinsulinaemia.

To further elucidate our preliminary observations of the possible role of proinsulin-like molecules in cardiovascular disease in subjects with NIDDM, studies investigating the effects of interventions on these molecules and those on risk factors for coronary artery disease particularly fibrinolysis would be needed. The final proof will come if it is shown that a reduction in proinsulin-like molecules is associated with a reduction in incidence of coronary artery disease which was independent of reduction in other cardiovascular risk factors.

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PUBLICATIONS

The work presented in this dissertation has led to following publications

1. Temple RC, Clark PMS, **Nagi DK**, Schneider AE, Yudkin JS and Hales CN. Radioimmunoassay may overestimate insulin in non-insulin-dependent diabetics. Clin Endocrinol 1990; 32: 689-93.
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3. Jain S, **Nagi DK**, Slavin B, Yudkin JS. Insulin therapy in non-insulin-dependent diabetic subjects suppresses proinsulin-like molecules and plasminogen activator inhibitor (PAI-1) activity. Diabetic medicine 1993; 10: 27-32
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5. **Nagi DK**, Mohamed-Ali V, Walji S, Jain S, Yudkin JS. Hyperinsulinaemia and insulin resistance syndrome-a comparison of caucasian and Asian subjects using specific assays for insulin, intact proinsulin and des 31,32 proinsulin. (Submitted to Diabetologia May, 1994)
6. **Nagi DK**, Mohamed-Ali V, Walji S, Jain S, Yudkin JS. Plasminogen activator inhibitor (PAI-1) in subjects with different categories of glucose intolerance and its relationships to insulin, intact proinsulin and des 31,32 proinsulin. (Submitted to Lancet 15/8/1994)

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1. **Nagi DK**, Hendra TJ, Temple RC, Clark PMS, Schneider A, Hales CN, Yudkin JS. Split proinsulin may be a risk factor for atherogenesis in non-insulin-dependent diabetics subjects. Diabetic Med 1989; 6 (Suppl 1): A34.
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7. **Nagi DK, Hendra TJ, Temple RC, Clark PMS, Schneider A, Hales CN, Yudkin JS.** Comparison of two insulin assays in homeostasis model assessment of insulin resistance and insulin deficiency in type 2 (non-insulin dependent) diabetes. *Diabetologia* 1990; 33 (Suppl): A86.
8. **Nagi DK, Jain S, Garvey S, Walji S and Yudkin JS.** Defective fibrinolysis in non-insulin-dependent diabetes relates to plasma insulin. *Clin Science* 1990; 79 (Suppl 23): 25P.
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17. **Nagi DK, Mohamed-Ali V, Walji S, Jain S, Yudkin JS.** Hyperinsulinaemia and insulin resistance syndrome- a comparison of caucasian and Asian subjects using specific assays for insulin, intact proinsulin, and des 31,32 proinsulin. Accepted BDA meeting, Lancaster, 1994.
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