INSULIN RESISTANCE,
ENDOTHELIAL FUNCTION
AND NON-ESTERIFIED FATTY ACIDS
IN HEALTH AND TYPE 2 DIABETES

A thesis submitted for the degree
of
Doctor of Medicine
to
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by
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ABSTRACT

Type 2 diabetes has been established as a serious cardiovascular risk factor. Insulin resistance plays a major role in its pathogenesis. Associated with insulin resistance is a cluster of metabolic abnormalities— the “insulin resistance syndrome”. An unresolved issue is whether there is a single underlying cause. Recent data suggest that endothelial dysfunction may be intrinsic to the syndrome. Endothelial dysfunction could represent the impact of subclinical disturbance of metabolism. Alternatively, the presence of a common cellular defect could influence both endothelial function and insulin mediated glucose disposal.

This work had two aims: Firstly, to study the relationship between insulin resistance and non-esterified fatty acids (NEFA); Secondly, to assess the relationship between insulin resistance and endothelial function in diabetes. It was hypothesised that a common antecedent links insulin resistance with endothelial dysfunction, cytokines and NEFA being likely candidates.

A large healthy cohort, part of a multicentre project, and 38 patients with uncomplicated type 2 diabetes were studied. Insulin resistance was measured in all subjects using the hyperinsulinaemic clamp technique. In the diabetic cohort endothelial function was assessed with venous-occlusion plethysmography and circulating endothelial cell products.

A strong association between NEFA and insulin resistance was observed with different links in fasting and hyperinsulinaemic states. Insulin resistant subjects had evidence of several components of the insulin resistance syndrome. No evidence for a relationship between insulin resistance and endothelial dysfunction was observed on plethysmography. However, measurement of endothelial cell products showed a significant association between tissue plasminogen activator levels (tPA) and insulin resistance. The association between insulin resistance and tissue plasminogen activator levels was strongly influenced by NEFA metabolism and to a much lesser extent by cytokine concentrations. The results point towards NEFA as a putative signal linking insulin resistance with endothelial dysfunction in diabetes.
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<td>acetylcholine</td>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<td>CVD</td>
<td>cardio-vascular disease</td>
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<td>EGIR</td>
<td>European Group for the Study of Insulin Resistance</td>
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<tr>
<td>FBF</td>
<td>forearm blood flow</td>
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<td>FPG</td>
<td>fasting plasma glucose</td>
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<td>FPI</td>
<td>fasting plasma insulin</td>
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<tr>
<td>HbA1c</td>
<td>glycosylated haemoglobin</td>
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<td>HDL</td>
<td>high-density lipoproteins</td>
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<td>HOMA</td>
<td>Homeostasis Model Assessment</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>IR</td>
<td>insulin resistant</td>
</tr>
<tr>
<td>IS</td>
<td>insulin sensitive</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>L-NMMA</td>
<td>N-monomethyl-L-arginine</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>MIRKO</td>
<td>muscle-specific insulin receptor knockout mice</td>
</tr>
<tr>
<td>M_0</td>
<td>mean glucose infusion rate adjusted for lean body mass</td>
</tr>
<tr>
<td>Mbw</td>
<td>mean glucose infusion rate normalised per kg body weight</td>
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<td>NEFA</td>
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<td>NO</td>
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<td>plasminogen activator inhibitor 1</td>
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<td>sodium nitroprusside</td>
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<td>TM</td>
<td>thrombomodulin</td>
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<td>tPA</td>
<td>tissue plasminogen activator antigen</td>
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<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
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<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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<td>VLDL</td>
<td>very-low-density lipoproteins</td>
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<td>vWF</td>
<td>von Willebrand Factor</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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<td>WHR</td>
<td>waist:hip ratio</td>
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ACKNOWLEDGMENTS

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Chapter 1
Background and introduction

1.1. Diabetes Mellitus

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. Diabetes may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. Often symptoms are not severe, or may even be absent, and hyperglycaemia of sufficient degree to cause pathological and functional changes may be present for a long time before the diagnosis is made.

The effects of diabetes include long-term damage, dysfunction and failure of various organs. Microvascular complications include progressive development of retinopathy, nephropathy, neuropathy and features of autonomic dysfunction. Macrovascular complications include cardiovascular, peripheral vascular and cerebrovascular disease.

Several pathogenic processes are involved in the development of diabetes. These include destruction of the pancreatic beta cells with consequent insulin deficiency, and others resulting in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (1).

1.1.1. Diagnostic criteria for diabetes mellitus

The clinical diagnosis of diabetes is often prompted by symptoms such as mentioned above. A single blood glucose test often establishes the diagnosis in such cases. In uncertain cases an Oral Glucose Tolerance Test is available to establish or exclude the diagnosis. Diagnostic criteria have recently been changed. The major change is the lowering of the diagnostic value of fasting plasma glucose concentration to 7.0 mmol/l and above. This new criterion has been chosen best to correspond to patients who would be diagnosed with the (unchanged from
previous) 2 hour post-load concentration of \( \geq 11.1 \text{ mmol/l} \) previously employed as the WHO cut off for diabetes. This equivalence has been established in several population-based studies (2;3). It also represents an optimal cut-off point to separate the components of bimodal frequency distributions of fasting plasma glucose concentrations seen in several populations. Furthermore, studies have demonstrated an increased risk of microvascular and macrovascular disease in subjects with fasting plasma glucose of 7.0 mmol/l and above (1).

1.1.2. **Classification of diabetes mellitus**

Previous classifications included insulin-dependent diabetes mellitus (IDDM/ type 1), non-insulin-dependent diabetes mellitus (NIDDM/ type 2) impaired glucose tolerance, gestational diabetes mellitus (GDM) and ‘other types’. These represented a compromise between clinical and aetiological classifications, allowing for practicality in everyday clinical use (1). The new proposed classification encompasses both clinical stages and aetiological types of diabetes mellitus and other forms of hyperglycaemia (4). The clinical staging allows for the natural progression of disease, regardless of aetiology. It categorises patients on the actual degree of glycaemia; normoglycaemia vs. hyperglycaemia, with hyperglycaemia being further divided into impaired glucose tolerance or diabetes (Table 1.1). The classification by aetiology incorporates new pathogenic knowledge. It categorises into type 1 (autoimmune or idiopathic), type 2 (predominantly insulin resistant or predominantly insulin deficient), gestational diabetes and ‘other specific types’ (Table 1.2).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Normoglycaemia</th>
<th>Hyperglycaemia</th>
<th>Diabetes Mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal glucose tolerance</td>
<td>Impaired Glucose Tolerance</td>
<td>Not requiring insulin</td>
</tr>
<tr>
<td></td>
<td>Impaired Fasting Glucose</td>
<td>Insulin requiring for control</td>
<td>Insulin requiring for survival</td>
</tr>
</tbody>
</table>

**Type 1**
- Autoimmune
- Idiopathic

**Type 2**
- Predominantly insulin resistance
- Predominantly insulin secretory defects

Other specific types

Gestational diabetes
<table>
<thead>
<tr>
<th>Major types</th>
<th>Aetiology</th>
<th>Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Beta-cell destruction, usually leading to absolute insulin deficiency</td>
<td>▪  Autoimmune</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Idiopathic</td>
</tr>
<tr>
<td>Type 2</td>
<td>May range from predominantly insulin resistance with relative insulin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>deficiency to a predominantly secretory defect with or without insulin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td></td>
</tr>
<tr>
<td>Other specific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Genetic defects of beta-cell function</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Genetic defects in insulin action</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Diseases of the exocrine pancreas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Endocrinopathies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Drug- or chemical-induced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Infections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Uncommon forms of immune-mediated diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Other genetic syndromes sometimes associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  with diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Gestational impaired glucose tolerance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪  Gestational diabetes</td>
</tr>
</tbody>
</table>
1.1.3. Type 2 diabetes mellitus

Type 2 diabetes mellitus has been established as a serious cardiovascular risk factor (mean annual mortality 5.4%) (5). This type of diabetes includes the common major form of diabetes, which results from defects in insulin secretion with almost always a considerable contribution from insulin resistance. By therapeutic requirements type 2 diabetes mellitus is subdivided into requiring insulin for metabolic control and not insulin requiring. By aetiology type 2 diabetes mellitus is either a) predominantly insulin resistant with relative insulin deficiency or b) predominantly insulin deficient with or without insulin resistance.

Insulin resistance is a virtually universal feature of type 2 diabetes mellitus (6), (7). Insulin levels may be normal or elevated. However, the high glucose levels in these patients should result in an even further rise of insulin concentrations if normal beta-cell function was present. Thus insulin secretion is defective, unable to compensate for the insulin resistance (8).

1.2. The Insulin resistance syndrome

1.2.1. Insulin resistance

Resistance to a hormone can be defined as a subnormal biological response to a given concentration of that hormone. Insulin resistance is thought to be the most prevalent form of hormone resistance. Insulin has important effects on carbohydrate, lipid and protein metabolism (Table 1.3). Insulin resistance has therefore not surprisingly been implicated in the pathogenesis of several pathological processes, such as obesity, diabetes mellitus, polycystic ovarian syndrome and essential hypertension (9).

However, strictly, the term insulin resistance refers to a decreased sensitivity of tissues to the action of insulin on glucose homeostasis (resistance to insulin mediated glucose uptake).
Methods for assessment of insulin resistance

Expectations of the ideal method to measure insulin resistance

Insulin resistance may be defined in pharmacological terms as a state in which normal amounts of insulin produce a subnormal biological response (11). This could happen at different levels, e.g. skeletal muscle, liver or adipose tissue.

In epidemiological studies fasting insulin concentrations have been used as surrogate markers of insulin resistance as this only involves a simple fasting blood test. Laakso et al evaluated the correlation between insulin levels (fasting and in response to an oral glucose load) and the degree of insulin resistance (as measured by the euglycaemic hyperinsulinaemic clamp technique) in individuals with varying degrees of glucose tolerance selected from previous population studies and concluded that in population studies, only the fasting insulin level should be used as a marker of insulin resistance, particularly in subjects with abnormal glucose tolerance (12). However, measuring these fasting insulin levels and insulin resistance directly would allow us better to understand the pathophysiology of disease processes and to differentiate between the effects of interventions on insulin resistance vs. insulin secretion.

Methods measuring insulin sensitivity should satisfy at least five requirements: (13):

1) The insulin levels achieved should be high enough to stimulate glucose metabolism and detect possibly small differences in sensitivity of glucose uptake to insulin.
2) The test should distinguish between peripheral and hepatic insulin sensitivity. These pathways may be affected differentially at the early stages of insulin resistance and therefore their separate quantitation may be important. For example, the dose-response curve for stimulation of glucose uptake and suppression of hepatic glucose production (HGP) by insulin in non-diabetic and type 2 diabetic subjects shows that significant differences in glucose uptake between groups are not seen until insulin concentrations have reached 210–280 pmol/l. In contrast, suppression of HGP is seen at much lower insulin levels (14).
3) Measurements should be made under steady-state conditions whenever possible because the available algorithms dealing with the non-steady state are intrinsically ill-conditioned (15). Many of the methods used involve rapid perturbation of the glucose system (even in the absence of clinical hypoglycaemia), which are followed by changes in plasma insulin, plasma glucose and counterregulatory hormones. These in turn can influence insulin sensitivity and thereby measurements.

4) The assumptions made about the body glucose system should be physiologically sound. For example, the minimal model assumes that glucose kinetics are monocompartmental, and that insulin action takes place in a remote compartment (16). The former assumption is untenable (15); and while the latter can be defended for insulin’s peripheral effects it can not for insulin’s hepatic effects.

5) The test should be performed under euglycaemic and not hyperglycaemic conditions. Many of the tests result in a rise in plasma glucose levels. Glucose uptake is then measured during hyperglycaemia. Glucose clearance is influenced by the ambient glucose concentrations, especially at low insulin concentrations (17). Therefore, hyperglycaemia may present a greater problem with methods resulting in only moderate increments of plasma insulin levels. Also, the plasma glucose level will rise to higher levels in glucose-intolerant than in glucose-tolerant subjects.

The available tests, despite their differences in rationale and performance, can provide a satisfactory answer to a definite question: what is the response to a hormone (in individual tissues or at the level of the whole body) under controlled conditions of stimulus (insulin) and substrate (glucose)? However, there is a need for a generally accepted and easily measured and calculated index of insulin resistance.

Insulin is a pleiotropic hormone with a range of actions occurring in multiple tissues (e.g. glucoregulation, antilipolysis, and protein synthesis). The insulin concentration and the shape of the relevant insulin dose-response curve determine the magnitude of each individual action of insulin. Some workers have pointed out that reduced responses to insulin may be due to either reduction in the maximal response (unresponsiveness) or to
right-shifting (insensitivity) of the dose response curve (11;18). Since in general such distinctions cannot be made from the current level of knowledge and can be ascertained only by clamping at multiple levels of insulin the general term insulin resistance is used to include either or both of these anomalies.

**Methods used for measurement of insulin resistance**

Several investigative techniques for estimation of insulin resistance have been developed and are currently in use, but all have limitations and none are suitable for routine clinical use (19). The euglycaemic hyperinsulinaemic clamp technique will be discussed in detail in sections 1.2.1.1.3. and 2.1.5.1.). Other techniques are outlined in table 1.4.

**The euglycaemic hyperinsulinaemic clamp**

Measurement of hormone responsiveness requires a stable relationship between serum hormone concentrations and a measurable hormone-dependent metabolic response. Thus, the relationship could be examined at different hormone concentrations and a dose-response curve constructed.

Generally, the euglycaemic hyperinsulinaemic clamp is considered to be the most direct method and the ‘gold standard’ for the assessment of insulin resistance (37;38). Insulin levels are raised acutely. Simultaneous infusion of glucose is performed in order to maintain euglycaemia. Steady state insulin concentrations are usually achieved within 30 minutes. The maximal hypoglycaemic stimulus usually occurs at 60-120 minutes. The glucose infusion rate requires constant adjustments, in response to frequent bedside serum glucose measurements, until euglycaemia is achieved in steady-state. Thus, the glucose-insulin feedback loop is disrupted and placed under the control of the investigator.

At the dose of insulin used in these clamps, endogenous hepatic glucose output can be assumed to be suppressed by 85-90% in most healthy subjects (39;40). Endogenous secretion of insulin is suppressed by about 40% in most healthy subjects (41). Glucose disposal during such clamps is therefore considered to reflect principally the sensitivity of
skeletal muscle to insulin. The ‘M’ value is then calculated as a measure of insulin sensitivity. If endogenous hepatic glucose production is completely inhibited by an intravenous infusion of insulin the quantity of exogenous glucose required to maintain euglycaemia (M) is a reflection of the net sensitivity of target tissues (mainly skeletal muscle) to insulin.

The clamp technique has been used extensively. When using an exogenous insulin infusion rate of 1 mU/ min/ kg (or 40 mU/ min/ m²), resulting in steady-state levels of 400-500 pmol/ l, the sensitivity and reproducibility of the euglycaemic clamp are sufficient to pick up systematic differences upwards of approximately 10 %.

Measurements are performed at a steady state for both insulin and glucose concentrations. For comparison the CIGMA techniques achieves insulin concentrations between 70 – 140 pmol/ l representing a weak stimulus for peripheral glucose uptake.

The clamp technique can be easily combined with the infusion of labelled glucose to allow measurements of hepatic glucose production and its sensitivity to insulin as well as with indirect calorimetry (to estimate intracellular glucose disposition, i.e. oxidation vs. storage), thereby allowing differentiation between hepatic and peripheral glucose disposal. Despite the acceptance of the euglycaemic clamp as the gold standard measurement technique certain comments are in order. This technique cannot easily be used in clinical practice. The conditions under which measurements are performed are ‘unphysiological’ (sustained hyperinsulinaemia) and do not represent everyday life. It has also been well documented that insulin secretion is pulsatile rather than continuous (42). This is then also reflected in its action. The euglycaemic clamp as a method is unable to duplicate this pulsatility. This criticism also holds true for all other methods of measuring insulin sensitivity.

In our studies the euglycaemic hyperinsulinaemic clamp was used to measure insulin sensitivity and detailed methodology will be described in the relevant chapters (43).
<table>
<thead>
<tr>
<th>Action</th>
<th>Target tissue</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbohydrate metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enhancement of glucose transport (uptake)</td>
<td>All tissues except brain, enterocytes, hepatocytes, renal tubular cells, pancreas</td>
<td>Recruitment of glucose transport proteins from intracellular pool to plasma membrane</td>
</tr>
<tr>
<td>Glucose phosphorylation (initial step for entry of glucose into glycolysis)</td>
<td>Liver, muscle</td>
<td>Induction of glucokinase</td>
</tr>
<tr>
<td>Enhancement of glucose oxidation</td>
<td>Liver, muscle</td>
<td>Activation of pyruvate dehydrogenase</td>
</tr>
<tr>
<td>Enhancement of glycogen synthesis</td>
<td>Liver, muscle</td>
<td>Induction of glycogen synthase</td>
</tr>
<tr>
<td>Stimulation of pentose phosphate shunt</td>
<td>Adipose tissue</td>
<td></td>
</tr>
<tr>
<td>Inhibition of glycogenolysis</td>
<td>Liver</td>
<td>Indirect inhibition via decreased availability of acetyl CoA (decreased allosteric stimulation of pyruvate carboxylase)</td>
</tr>
<tr>
<td>Inhibition of gluconeogenesis</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td><strong>Protein metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enhancement of amino acid transport</td>
<td>Liver, muscle</td>
<td>Direct effect</td>
</tr>
<tr>
<td>Enhancement of protein synthesis</td>
<td>Liver, muscle</td>
<td>Direct effect</td>
</tr>
<tr>
<td>Inhibition of protein degradation</td>
<td>Liver, muscle</td>
<td></td>
</tr>
<tr>
<td><strong>Lipid metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid synthesis</td>
<td>Liver</td>
<td>Acetyl CoA carboxylase, fatty acid synthetase</td>
</tr>
<tr>
<td>Triglyceride synthesis</td>
<td>Liver, adipose tissue</td>
<td>Promotion of esterification by increase of availability of α-glycerophosphate from glycolysis</td>
</tr>
<tr>
<td>Inhibition of lipolysis</td>
<td>Adipose tissue</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>Enhancement of uptake of VLDL and NEFAs</td>
<td>Adipose tissue</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>Promotion of reverse cholesterol transport by HDL</td>
<td>Adipose tissue</td>
<td>Hepatic triglyceride lipase</td>
</tr>
<tr>
<td>Inhibition of ketone body synthesis</td>
<td>Liver</td>
<td>Inhibition of carnitine acyl transferase 1 by increased malonyl CoA</td>
</tr>
<tr>
<td><strong>Electrolytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enhancement of cellular potassium uptake</td>
<td></td>
<td>Na⁺-K⁺ATPase</td>
</tr>
</tbody>
</table>

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Table 1.4: Methods to measure insulin resistance

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Glucose Tolerance Test (OGTT)</td>
<td>Standardised oral glucose load administered, glucose and insulin concentrations are measured</td>
<td>Simple</td>
<td>Substantial test-retest biological variation, not useful in intervention studies if intervention could affect insulin secretion and HGP</td>
<td>(20;21)</td>
</tr>
<tr>
<td>Insulin Suppression Test (IST) with an isolated or quadruple human forearm infusion technique (combined infusion of somatostatin, insulin and glucose)</td>
<td>Endocrine suppression is produced pharmacologically (adrenaline plus propanolol, somatostatin, octreotide) during exogenous hyperglycaemia without changes in the rate of glucose infusion</td>
<td>High degree of reproducibility correlation between SSPG and clamp derived M-values</td>
<td>Insulin-independent glucose disposal and HGP are not taken into account, Inaccurate at extremes of insulin resistance as steady state is achieved at different glucose levels</td>
<td>(22-30)</td>
</tr>
<tr>
<td>Intra venous glucose tolerance test (IVGGT) with minimal model analysis</td>
<td>Intravenous glucose load is administered, glucose and insulin concentrations are measured frequently, complex mathematical modelling with commercially available computer software</td>
<td>Simple clinical procedure, Gives data on insulin-dependent and insulin-independent glucose disposal together with first and second-phase insulin secretion</td>
<td>Only an approximate description of the real system is provided, Considerable discrepancy with clamp derived data, Reproducibility depends on frequency of blood sampling</td>
<td>(31-34)</td>
</tr>
<tr>
<td>Homeostasis model assessment (HOMA)</td>
<td>Very simple, can be applied to large populations</td>
<td>Serum insulin concentrations might cross react with pro-insulin – like molecules in the insulin assays used, validated against the 'clamp' only in small groups</td>
<td></td>
<td>(35;36)</td>
</tr>
</tbody>
</table>
1.2.2. The concept of the insulin resistance syndrome

The association of hyperglycaemia, hypertension and hyperuricaemia was first described as a syndrome by Kylin in 1923 (44). The association of an increased risk for cardiovascular disease with a cluster of metabolic abnormalities was then introduced in the scientific literature as ‘The metabolic syndrome’ -trisindrome métabolique- by Camus (45) and by Crepaldi, who subsequently introduced the term ‘plurimetabolic syndrome’ (46). It was, however, not widely accepted until in 1988 Reaven described this clustering of abnormal glucose tolerance with other cardiovascular risk factors as ‘Syndrome X’ (47). This syndrome has also been characterised as ‘The Insulin Resistance Syndrome’ (48). All these terms represent a multifaceted syndrome characterised by five major abnormalities: impaired glucose tolerance (or diabetes mellitus), hyperinsulinaemia, insulin resistance, dyslipidaemia and hypertension (Table 1.5). Other components have also been included as part of the syndrome: abnormal free fatty acid metabolism (49;50), microalbuminuria (51), hyperuricaemia (52), coagulation disorders (53;54), hyperleptinaemia (55) and most recently increased levels of acute phase proteins and cytokines (56;57). Their relevance and centrality to the syndrome has been widely debated (47;58-63), but no consensus has evolved.

Although the major components of the insulin resistance syndrome are associated, a causal relationship has not been identified (64) and whether it is a true entity or an overlap of multiple common conditions remains unclear (65-67).
Table 1.5: Components of the insulin resistance syndrome

<table>
<thead>
<tr>
<th>Component</th>
<th>Abnormality</th>
<th>Association with the insulin resistance syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Established</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insulin resistance</td>
<td></td>
<td>essential component – defines syndrome (47)</td>
</tr>
<tr>
<td>plasma insulin</td>
<td>Hyperinsulinaemia</td>
<td>ubiquitous marker of insulin resistance, component of Reaven’s syndrome X (47)</td>
</tr>
<tr>
<td>glucose tolerance</td>
<td>impaired glucose tolerance or diabetes mellitus</td>
<td>frequent reflection of insulin resistance, component of Reaven’s syndrome X (47)</td>
</tr>
<tr>
<td>blood pressure</td>
<td>hypertension</td>
<td>essential component (47)</td>
</tr>
<tr>
<td>plasma lipids</td>
<td>increased triglyceride level, decreased HDL-cholesterol</td>
<td>essential components (47)</td>
</tr>
<tr>
<td>body composition</td>
<td>central obesity</td>
<td>strongly associated – included by EGIR, excluded by Zavaroni (1;68-70), not mentioned by Reaven (47)</td>
</tr>
<tr>
<td><strong>Not yet established</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL particle size</td>
<td>small dense</td>
<td>strongly associated with dyslipidaemia and has been suggested as addition to the syndrome</td>
</tr>
<tr>
<td>urinary albumin excretion</td>
<td>microalbuminuria</td>
<td>recently suggested addition to syndrome (51)</td>
</tr>
<tr>
<td>uric acid concentrations</td>
<td>hyperuricaemia</td>
<td>recently suggested addition to syndrome (52)</td>
</tr>
<tr>
<td>endothelial function</td>
<td>dysfunction (e.g. increased vW-Factor, abnormal vascular reactivity)</td>
<td>recently suggested addition to syndrome (71;72)</td>
</tr>
<tr>
<td>coagulation</td>
<td>increased thrombogenicity, increased PAI-1 and fibrinogen</td>
<td>recently suggested addition to syndrome (53;54)</td>
</tr>
<tr>
<td>plasma leptin</td>
<td>hyperleptinaemia</td>
<td>recently suggested addition to syndrome (55)</td>
</tr>
<tr>
<td>NEFA</td>
<td>fasting NEFA increased, decreased NEFA suppressibility</td>
<td>recently suggested addition to syndrome, strongly associated, suggested additions to syndrome, may be mechanistic link between endothelial dysfunction and insulin resistance</td>
</tr>
<tr>
<td>inflammatory markers</td>
<td>increased C-reactive protein</td>
<td>(49;50)</td>
</tr>
<tr>
<td>pro-inflammatory cytokines</td>
<td>increased levels of TNF-alpha and IL-6</td>
<td>(56;57)</td>
</tr>
</tbody>
</table>

EGIR – European Group for the Study of Insulin Resistance; HDL – High density lipoprotein; LDL – Low density lipoprotein; NEFA - non-esterified fatty acids; PAI-1 - Plasminogen activator inhibitor 1; vWF - Von Willebrand Factor
1.2.3. Definition of the insulin resistance syndrome

There is no internationally agreed definition of the insulin resistance syndrome. This, however, is urgently needed to allow for comparison of studies. Recently Alberti and Zimmet for the WHO Consultation (1) described this syndrome as a 'major classification, diagnostic and therapeutic challenge'. They proposed a provisional working definition for the insulin resistance syndrome of glucose intolerance, impaired glucose tolerance or diabetes mellitus and/ or insulin resistance together with two or more of the other components listed below (Table 1.6).

In response to the above proposal, the European Group for the Study of Insulin Resistance (EGIR) has suggested several changes to this definition, mainly including insulin resistance or fasting hyperinsulinaemia as a central and necessary component of the syndrome (70).

To accurately define the insulin resistance syndrome, a clear description of its essential components is needed and internationally agreed criteria for insulin resistance, hyperinsulinaemia and central obesity will be required. The operational definition of insulin resistance usually employed is the amount of glucose disposal under laboratory conditions of standardised glycaemia and insulinaemia (e.g. in an hyperinsulinaemic euglycaemic clamp) (39). In practice such measurements of insulin action can be made only over a very short period. One needs to take into account that an individual’s insulin resistance is not fixed, but influenced by even short term alterations of the conditions under which they are being measured, e.g. exercise, diet, sympathetic tone and stress as well as prescription and non-prescription drugs. Subjects who have recently lost weight or exercised will have lower insulin resistance than subjects who have recently gained weight, been indolent or over-eaten (73). Conversely short-term fasting or low-carbohydrate diets may induce some features of insulin resistance (74). Some authors argue that certain diets may underlie the insulin resistance syndrome (75). Regrettably,
the euglycaemic hyperinsulinaemic clamp as the gold standard for measuring insulin resistance cannot be applied to free-living individuals over a prolonged period of time.

Table 1.6: Provisional working definition by WHO for the insulin resistance syndrome (glucose intolerance, impaired glucose tolerance or diabetes mellitus and/or insulin resistance together with two or more of the other components listed) (1)

1. Impaired glucose regulation or diabetes
2. Insulin resistance (under hyperinsulinaemic euglycaemic conditions, glucose uptake below lowest quartile for background population under investigation)
3. Raised arterial pressure > 160/90 mmHg
4. Raised plasma triglycerides (≥ 1.7 mmol/l; 150 mg/dl) and/or low HDL-cholesterol (≤ 0.9 mmol/l, 35 mg/dl in men; < 1.0 mmol/l, 39 mg/dl in women)
5. Central obesity (waist to hip ratio > 0.9 in men; waist to hip ratio > 0.85 in women) and/or BMI > 30 kg/m²
6. Microalbuminuria (urinary albumin excretion rate > 20 μg/min or albumin:creatinine ratio > 20 mg/g)

1.2.4. The origin of insulin resistance

1.2.4.1. The ‘muscle cell defect’ origin of insulin resistance

It is certain that it is not insulin’s effect on blood flow but a defect at a lower level, e.g. cell or receptor which is mainly responsible for insulin resistance in skeletal muscle. At least part of insulin’s action on blood flow is mediated through an effect on calcium in vascular smooth muscle cells. The increase in calcium induced by angiotensin II is almost obliterated by the acute exposure of vascular smooth muscle cells to insulin (76). Insulin attenuates calcium bursts in vascular smooth muscle cells through several mechanisms, the most important possibly being the voltage-gated calcium channel, with an ancillary role played by Na,K-ATPase (77). Thus insulin acts, at least in part as a calcium channel blocker in vascular smooth muscle cells. Insulin is also known to have pleiotropic, growth-factor like effects, stimulating proliferation and DNA synthesis of vascular smooth muscle cells (78).

In insulin-sensitive cells insulin, after binding to the insulin receptor and activating the
receptor tyrosine kinase, triggers two major pathways for signalling: the phosphoinositol(4,5)-biphosphate (PI3)-dependent pathway and the mitogen-activated protein (MAP)-kinase-dependent pathway. These two pathways are specialised to some degree, in that each mediates some, but not all, of insulin’s effect (79;80). PI3-kinase is mainly responsible for mediating the ‘desirable’ effects of insulin on skeletal muscle, adipocyte and also on the vasculature such as stimulation of NOS and activation of Na,K-ATPase and Glut-4 is responsible for glucose uptake. MAP-kinase is mainly responsible mediating the ‘undesirable’ growth factor-like effects of insulin on the vasculature (81). It has been suggested that these two pathways are in balance in health. Animal experiments suggest that in insulin resistant states expression of MAP-kinase activity to insulin is increased (82). An alteration of insulin action leading to an imbalance of these two pathways could thus possibly lead to prevalence of the more undesirable effects of insulin.

1.2.4.2. The ‘haemodynamic’ origin of insulin resistance

Insulin as a vasodilator

The concept that attenuation of insulin-mediated vasodilatation and decreased capillary recruitment are important in the pathophysiology of insulin resistance has been named the “haemodynamic hypothesis of insulin resistance”.

Most groups have reported a vasodilatory effect of systemically or locally infused insulin (83-88). Baron et al observed that systemic insulin infusion with maintenance of euglycaemia is associated with peripheral vasodilatation (89). These studies generally used pharmacological doses of insulin. However, investigators who have examined the direct effect on vascular tone of more physiological local hyperinsulinaemia have reported different findings. A number of groups reported the absence of any effect of local insulin infusion on vasodilation (88;90-94) or only a weak effect (95).

Furthermore, some authors reported that insulin-mediated vasodilatation in vitro appears to be dependent on local glucose uptake (96). In contrast, Tack et al reported that
vasodilatory response to insulin was preceded by an increase in glucose uptake, but that
the glucose uptake did not determine the vascular response to insulin (87). Cleland et al
performed a 'forearm clamp' by locally infusing glucose and insulin, thus avoiding any
confounding effects of sympathetic stimulation on peripheral blood flow, in 18 healthy
volunteers. Whole body insulin sensitivity was correlated with change in forearm blood
flow, but not with body mass index (BMI), or mean arterial pressure. The authors state
that these data support the concept of a significant functional relationship between
insulin's metabolic and vascular actions, possibly at an endothelial level (97).

Overall, it appears that insulin has not only systemically-mediated but also locally-acting
effects. It is plausible that impairment of either of these functions could lead to
decreasing access of insulin to receptors in target tissues. Assuming that insulin has a
physiological vasodilator action, it is conceivable that impairment of this response might
result in increased vascular resistance. This could link insulin resistance with
hypertension independent of hyperinsulinaemia (98).

Using the leg-perfusion technique during systemic hyperinsulinaemia Baron et al have
extensively studied the relative contributions of decreased glucose delivery and decreased
glucose extraction to insulin-sensitive tissues in insulin resistant states. The finding of
these studies have been consistent in type 1 and type 2 diabetes mellitus and obesity
showing that 'conventional' insulin resistance in these subjects is paralleled by a
decreased ability of insulin to stimulate skeletal muscle blood flow (99-101). In support
of this, sensitivity to insulin-mediated vasodilatation is highly correlated with whole-
body insulin-mediated glucose uptake in healthy subjects (102).

*Effect of increased blood flow on glucose uptake*
It has been widely debated if insulin’s effect on vasculature or its effect on glucose
metabolism is more essential to the development of insulin resistance. Normalisation of
blood flow response to insulin in type 2 diabetes mellitus leads to an improvement of
insulin-stimulated glucose by up to 40% (84). Intra-arterial infusion of methacholine
during hyperinsulinaemia has been reported to increase glucose uptake in normal subjects (103). It has also been reported that an increase in forearm blood flow per se mediated by exogenous bradykinin results in increased glucose uptake (104). However, not all groups have demonstrated an increase in insulin-mediated glucose uptake simply by increasing blood flow (105; 106). Yki-Järvinen et al, using PET scanning, recently reported that whilst bradykinin significantly increased blood flow, glucose uptake in the bradykinin-treated muscles was unchanged, even in insulin resistant subjects (107). Sarabi et al observed that changes in forearm blood flow in response to metacholine, but not SNP, increased glucose uptake (108). Some studies in hypertension have demonstrated reduced glucose uptake in the absence of any differences in insulin-stimulated blood flow (93; 109). However, in several studies Clark et al have demonstrated that important differences exist between perfused and incubated skeletal muscle preparations with regard to their metabolism and control. In the constant-flow perfused rat hindlimb preparation, a group of vasoconstrictors has been identified that enhance muscle metabolism and aerobic contractility. Another group of vasoconstrictors decreases muscle metabolism and aerobic contractility even though perfusate flow remains constant. All effects of both groups of vasoconstrictors are opposed by vasodilators. Because none of the vasoconstrictor effects is evident when isolated muscles are incubated or perfused, involvement of an active vascular system is indicated. Several mechanisms to account for vascular control of perfused skeletal muscle metabolism have been suggested 1) functional vascular shunts, regulated by site-specific vasomodulators, 2) a direct response to a change in the rate of supply of nutrients and removal of products, and 3) a signal substance released by vascular tissue in association with vasoconstriction that interacts with surrounding skeletal muscle cells. Hindlimb perfusion experiments, where perfusion rate is fixed, suggest that changes in distribution of microcirculatory perfusion can modulate substrate uptake (110-112).

Insulin-mediated increase in muscle perfusion may account for approximately 30% of insulin's overall action to stimulate muscle glucose uptake, suggesting a role for insulin and glucose delivery as a determinant of insulin action. Thus the haemodynamic
hypothesis of insulin resistance implies that in insulin resistant states the proportion of insulin-mediated glucose uptake, which is under normal conditions facilitated by insulin-mediated vasodilatation, is diminished.

1.2.4.3. Candidates for antecedence of the insulin resistance syndrome
It appears possible that insulin resistance is the mechanism underlying all features of the insulin resistance syndrome (Fig. 1.1). However, another possibility is that insulin resistance is not the primary defect but merely a part of the syndrome and that a common antecedent underlies the insulin resistance syndrome, for example NEFA, obesity, endothelial dysfunction, oxidative stress or cytokines (Fig. 1.2). The major work compromising this thesis is exploring the relationship of NEFA and endothelial dysfunction to the insulin resistance syndrome.
Fig. 1.1: Insulin resistance is central to the Insulin resistance syndrome.

Fig. 1.2: A common antecedent is central to the Insulin resistance syndrome.
1.2.4.3.1. How insulin resistance itself may underlie the syndrome

The standard explanation of the syndrome and its name is that the primary defect is that of insulin resistance (47). This explanation suggests that failure of glucoregulation by insulin (insulin resistance in the skeletal muscle) leads directly to hyperglycaemia. The hyperglycaemia stimulates increased insulin secretion and hence hyperinsulinaemia. The hypertriglyceridaemia is likely to be the result of both an increase in NEFA-flux to the liver (due to adipocyte insulin resistance) and an insulin-related shift to the left of the NEFA concentration vs. hepatic-triglyceride synthesis relationship (113). Stern’s view is that insulin’s stimulation of VLDL synthesis and secretion “retains normal sensitivity to insulin and is overdriven by the hyperinsulinaemia” (62). Likewise hyperinsulinaemia acting on insulin-responsive systems (which are postulated to be spared from the insulin resistance within the insulin resistance syndrome) causes an increase in sympathetic activity (75;114;115) and/or sodium retention by the kidney (50;116;117) and/or changes in intracellular ionic concentrations (58) leading to hypertension. It is also suggested that increased insulin action causes atheroma (118) via a variety of mechanisms such as an increase production of PAI-1 (62) and increase in vascular cell growth (77;119).

This hypothesis clearly suggests that some elements of insulin’s action are reduced as a consequence of insulin resistance (e.g. glucoregulation) but that hyperinsulinaemia does not compensate fully for these defects (i.e. a net decrease in some of insulin’s actions). Simultaneously other actions of insulin are increased e.g. sodium retention, sympathetic stimulation, PAI-1 secretion (62). The increase in insulin action on some (responsive) tissues could also explain the increase in cardiovascular risk. For some insulin actions, it is possible that hyperinsulinaemia is acting via IGF-receptors (77;120). Likewise insulin receptors can show differential splicing within different tissues (121).

However, in general, there is no good, clear explanation as to how or why some insulin-responses are reduced and some simultaneously increased within the insulin resistance syndrome. Even for those elements where reduced insulin action is the explanation for the
features of the insulin resistance syndrome, there is uncertainty about the tissue in which the insulin resistance is occurring. For example there has been a long-running discussion about whether skeletal muscle uptake or hepatic glucose output is the prime abnormality underlying hyperglycaemia in the syndrome (6;122). Some authors have suggested that anti-lipolytic defects of insulin action need not be proportional to those on the glucoregulatory effects within any individual (123;124).

1.2.4.3.2. Obesity as an antecedent for the insulin resistance syndrome

In obesity, in the absence of hypertension or glucose intolerance, insulin resistance is global with decreased oxidative and non-oxidative glucose disposal (measured by indirect calorimetry), increased lipid oxidation, impaired suppression of lipolysis and decreased potassium uptake (125;126). In type 2 diabetes lipid oxidation and potassium uptake are relatively unaffected, but the other abnormalities are present (124;125). A study comparing 10 obese diabetic with 10 non-obese diabetic subjects reported that obesity exaggerates hepatic as well as extra-hepatic insulin resistance in type 2 diabetes. Once established, mechanisms exist that further perpetuate the insulin resistance. An important question is by which mechanism does insulin resistance associated with obesity develop and how adipose tissue might signal its mass to hepatic tissue and to peripheral skeletal muscle. The main mechanism proposed is increased circulating NEFA levels. It has been shown that in obese subjects, the enlarged fat mass results in increased NEFA serum concentrations, which aggravate resistance to insulin action on hepatic glucose and lipid metabolism and skeletal muscle glucose metabolism (124)(section 1.2.4.3.3.). Adipose tissue derived cytokines are other possible ‘culprits’ implied (127) (section 1.2.4.3.5.) and their increased action might lead to the endothelial dysfunction that has been reported in obesity independent of diabetes (128) (section 1.2.4.3.4.).

Recently, Steppan et al reported the discovery of a hormone that potentially links obesity to diabetes. They have shown that adipocytes secrete a unique signalling molecule, which they named resistin (for resistance to insulin). Circulating resistin levels were increased in diet-induced and genetic forms of obesity and decreased by rosiglitazone (an insulin
sensitizer from the group of thiazolidinediones). Treatment of normal mice with recombinant resistin impaired glucose tolerance and insulin action. Administration of anti-resistin antibody improved blood sugar and insulin action in mice with diet-induced obesity. Insulin-stimulated glucose uptake by adipocytes was enhanced by neutralization of resistin and reduced by resistin treatment (129).

1.2.4.3.3. NEFA as an antecedent for the insulin resistance syndrome

Insulin resistance, dyslipidaemia and abnormal NEFA metabolism are common features found in subjects with the metabolic syndrome (58-61;113). It has been argued that the relationship between insulin resistance, serum insulin concentrations and glucose tolerance in apparently healthy subjects is mediated to a significant degree by changes in ambient serum NEFA concentrations (47). Insulin actions include stimulation of glucose uptake via effects on GLUT-4 glucose transporter, reduction of NEFA concentrations via inhibition of hormone-sensitive lipase, and reduction of triglyceride concentrations via reduction of hepatic VLDL output and stimulation of LPL. Reduced availability of NEFA favours a decline in hepatic VLDL synthesis, and insulin-mediated activation of hepatic triglyceride lipase promotes reverse cholesterol transport by HDL lipoproteins.

The question arises if

1) Abnormalities in NEFA metabolism lead to insulin resistance or
2) Increased insulin resistance leads to abnormal NEFA metabolism (with increased levels and decreased suppressibility).

These will be examined as part of this thesis in healthy volunteers and patients with type 2 diabetes. Furthermore the role of hypertriglyceridaemia as a primary or secondary event in relationship between insulin resistance and abnormal NEFA metabolism will be examined.

To date, most studies investigating the relation of insulin resistance to lipoprotein abnormalities have focused on the relationships between lipids and glucoregulatory effects of insulin (47;68;130). Studies using the hyperinsulinaemic clamp have shown
relationships between impaired ability to stimulate glucose uptake and dyslipidaemia, mainly hypertriglyceridaemia and low HDL-cholesterol concentration (131). However, elevated NEFA levels also correlate with some indices of insulin resistance (113). A close relation between NEFA and triglyceride concentrations or turnover rates has been demonstrated (113;132;133). Recently, Mostaza et al. showed that increased NEFA flux was linked to hypertriglyceridaemia, independently of obesity and waist circumference (134). The VLDL-triglyceride synthesis rate could then be considered the ‘primary’ insulin-sensitive process determining plasma triglyceride concentrations directly, and HDL-and low-density-lipoprotein (LDL)-cholesterol concentrations by secondary effects (135). In obesity and type 2 diabetes there is a shift in the net balance of lipolysis and re-esterification of NEFA, producing a rise in their circulating concentration (124), this resulting from subnormal LPL activity (136). NEFA in turn aggravate resistance to insulin-mediated glucose disposal in skeletal muscle by competing for oxidation with glucose in mitochondria (137), shifting hepatic glucose metabolism in favour of gluconeogenesis, promoting hepatic VLDL production, raising serum triglyceride levels (138) and enhancing oxidative stress (139).

Evidence is presented showing that NEFAs are one important link between obesity, insulin resistance, and type 2 diabetes. Plasma NEFA levels are elevated in most obese subjects and physiological elevation of plasma NEFA concentrations inhibits insulin-stimulated glucose uptake into muscle (140). This peripheral insulin resistance is caused by a NEFA-induced defect, which develops 3-4 hr after raising plasma NEFA levels, in insulin-stimulated glucose transport or phosphorylation, or both. This resistance is also caused by a second defect, which develops after 4-6 hr, consisting of inhibition of glycogen synthase activity. Whether elevated plasma NEFA levels inhibit insulin action on endogenous glucose production, that is, cause central insulin resistance, is more difficult to demonstrate. On the one hand, NEFA increase gluconeogenesis, which enhances endogenous glucose production; on the other hand, short-term increase in NEFA levels leads to an increase in insulin secretion, which decreases endogenous glucose production. Interestingly, chronically elevated NEFA levels lead to a decrease in
pancreatic intra-cellular insulin stores contributing to the beta-cell dysfunction seen in type 2 diabetes (141). Basal plasma NEFAs support approximately one third of basal insulin secretion in diabetic and nondiabetic subjects and, hence, are responsible for some of the hyperinsulinaemia in obese, normoglycaemic patients. In addition, elevated plasma NEFA levels potentiate glucose-stimulated insulin secretion acutely and during prolonged exposure (48 hr). It is hypothesized that obese subjects who are genetically predisposed to develop type 2 diabetes will become partially "lipid blind," that is, unable to compensate for their NEFA - induced insulin resistance with NEFA - induced insulin oversecretion. The resulting insulin resistance/secretion deficit will then have to be compensated for with glucose-induced insulin secretion, which, because of their partial "glucose blindness," will result in hyperglycaemia and eventually in type 2 diabetes (142).

Deficits in suppression of NEFA concentrations have been demonstrated in insulin resistant states, as expected from insulin’s well-established role as the major regulator of lipolysis (47;143;144). The influence of adiposity in this relationship is important since strong associations exist between obesity and insulin resistance and between obesity and lipoprotein metabolism (49;145-147).

In summary: Several lines of evidence suggest that NEFA are an important signal in insulin resistance: 1) There is a strong correlation between NEFA levels and liver glucose output under a variety of experimental conditions. 2) If NEFA are maintained at basal concentrations during insulin administration, glucose output fails to decline. 3) If NEFA concentrations are reduced independent of insulin administration, glucose output is reduced. These three points support the concept that insulin, by regulating adipocyte lipolysis, controls liver glucose production. Thus, the adipocyte is a critical mediator between insulin and liver glucose output. Evidence that NEFA also suppress skeletal muscle glucose uptake and insulin secretion from the 6-cell supports the overall central role of the adipocyte in the regulation of glycaemia. Insulin resistance at the fat cell may be an important component of the overall regulation of glycaemia because of the
relationships between NEFA and glucose production, glucose uptake, and insulin release (148).

1.2.4.3.4. Endothelial dysfunction as an antecedent for the insulin resistance syndrome

Numerous studies have examined the relationship between endothelial dysfunction and various components of the insulin resistance syndrome as well as insulin resistance itself and complications arising from it. Most studies aimed to match for at least age, BMI and lipid profile. Some matched for “all” cardiovascular risk factors. However, numbers studied are generally small and subjects remain rather heterogeneous in their geno- and phenotype.

In a group of normotensive subjects with type 2 diabetes the correlation between insulin resistance and endothelial dysfunction remained significant after adjustment for obesity. Vascular response to acetylcholine (ACh), measured by venous occlusion plethysmography, was impaired in the diabetic group, whereas vascular response to sodium nitroprusside (SNP) was similar to the matched controls. The degree of insulin resistance, measured by an intravenous glucose tolerance test, did not independently predict greater impairment of vascular response to acetylcholine (149). In another study of patients with type 2 diabetes without evidence of atherosclerosis there was no difference in basal and ACh-stimulated NO generation and endothelium-dependent relaxation between diabetic patients and control subjects. In both groups, forearm NO₂⁻ and NO₃⁻ net balance following ACh stimulation was related to changes in the forearm blood flow. ACh-induced increase in forearm blood flow was associated with an increase in glucose uptake only in control subjects but not in diabetic patients. These results would argue against a role of impaired NO generation and blood flow regulation in determining the insulin resistance of uncomplicated diabetic patients but support an independent insulin regulation of haemodynamic and metabolic effects (150).
In a study examining differences between patients with essential hypertension or type 2 diabetes and normal controls using the hyperinsulinaemic clamp and venous occlusion plethysmography in response to insulin/glucose, N-monomethyl-L-arginine (L-NMMA), and noradrenaline no significant differences among groups were found for these parameters. However, pooled correlation analyses revealed associations between insulin resistance and vascular reactivity to insulin and L-NMMA, supporting the idea of functional coupling between insulin action (both metabolic and vascular) and basal endothelial nitric oxide production in humans (151).

Balletshofer et al reported a significant association between endothelial dysfunction and insulin resistance in normotensive and normoglycaemic first-degree relatives of patients with type 2 diabetes independent of the classic cardiovascular risk factors (152).

Insulin resistance is coupled to glucose uptake for which the mass of the skeletal muscle is largely responsible. Increased glucose uptake appears to be due in part to a NO-mediated (153;154) increase in blood flow (84) as well as to an increase in arterio-venous glucose difference (haemodynamic hypothesis 1.2.4.2.). Insulin acts as a vasodilator and in insulin resistant states this effect can be impaired. Furthermore, insulin resistant subjects have been reported to have decreased skeletal muscle and skin capillarisation (103;155;156). Thus, both reduced capillary surface area and impaired capillary endothelial function, along with a failure to vasodilate, could contribute to insulin resistance through delayed delivery of insulin to the target tissues. This suggests that cellular perfusion may be a limiting condition and raises the possibility that insulin resistance may be a consequence of inadequate tissue perfusion due to loss of NO activity. It is possible that endothelial dysfunction causes insulin resistance either indirectly or by attenuating insulin mediated endothelium-dependent vasodilatation. Alternatively, decreased insulin sensitivity may cause decreased basal production of eNOS, perhaps by limiting availability of L-arginine substrate or one of the other cofactors needed for NO synthesis.
Several mechanisms have been proposed to explain abnormalities in endothelial function in insulin resistant states. Abnormalities in endothelial function could represent the impact of subclinical disturbance of metabolism or alternatively the presence of a common cellular defect that influences both nitric oxide bioavailability and insulin mediated glucose disposal. A primary defect in the vascular action of insulin may be a key intermediate mechanism that links endothelial dysfunction with reduced insulin-mediated cellular glucose uptake in metabolic and cardiovascular disorders. Insulin has vasculo-protective effects through production of nitric oxide in the endothelial cells, while it produces atherogenic effects by stimulating proliferation and migration of vascular smooth muscle cells. The insulin-activated pathway is the phosphatidylinositol 3-kinase pathway responsible for NO production in the endothelial cells and MAP kinase pathway responsible for proliferation and migration in the vascular smooth muscle cells. Insulin resistance and hyperinsulinaemia may result in the attenuation of the endothelium-mediated action and stimulation of the vascular smooth muscle cells-mediated action. The association of insulin resistance and endothelial dysfunction may cause vicious cycle, leading to the metabolic and cardiovascular diseases. Endothelial function is discussed in more detail in section 1.3.

In summary, recent data suggest that endothelial dysfunction is intrinsic to the insulin resistance syndrome even before onset of diabetes or cardiovascular complications. Mechanisms to date are widely discussed but not very clear.

1.2.4.3.5. Cytokines and leptin as an antecedent for the insulin resistance syndrome

Cytokines as an antecedent for the insulin resistance syndrome

Cytokines are a family of soluble (secreted) regulatory proteins or glycoproteins which modulate the growth, migration and differentiation of a number of tissue cells, most significantly those of the haematopoietic system. Modulation is achieved by a local signalling system involving binding of the cytokines to cytokine receptors on target cells. These receptors are coupled to intracellular signal transducers within the cells, which effect the response.
Interleukin-6 (IL-6) and tumour necrosis factor (TNFα) are pro-inflammatory cytokines, secreted by macrophage, adipocyte and muscle cells, with important effects on lipid and glucose metabolism (157). Both have been shown to stimulate glucose uptake in cultured adipocytes and to inhibit LPL-activity (158;159). IL-6 has been reported to stimulate insulin release from hamster islet cells (160). In humans IL-6 was found to stimulate glucose and fatty acid oxidation, as well as to induce glucagon and cortisol release (161). Another study reports that a polymorphism of the IL-6 gene influences the relationship among insulin sensitivity, postload glucose levels after a glucose tolerance test, and peripheral white blood cell count (162).

Several recent studies have examined whether TNFα is associated with components of the insulin resistance syndrome. The reports are diverse and the evidence is unclear. A number of investigators examined local, adipose tissue produced TNFα concentrations rather than circulating levels (163). Only a few others have looked at circulating concentrations (164). In a study of 85 non-diabetic patients with essential hypertension circulating TNFα levels did not differ between hypertensive and normotensive subjects (165). Dasliva et al reported that in African Americans and whites, neither the TNF2 allele nor another polymorphism in the TNFα gene or a neighbouring gene with which the TNF2 allele is in linkage disequilibrium is associated with differences in the level of or increased clustering of components of the insulin resistance syndrome (166). TNF-α has been reported to inhibit the tyrosine phosphorylation of insulin receptor substrate-1 by insulin binding (167;168), suppress the action of lipoprotein lipase (LPL) (169), stimulate lipolysis (170) and impair endothelial function (171). IL-6 has similar effects (172-174). If these cytokines are the antecedent of insulin resistance, the former is probably the most likely mechanism.

Cytokines form an active part of the acute phase response. Some recent studies examined if insulin resistance or other components of the syndrome correlate with an increase in acute phase proteins. In a cross-sectional study of 107 non-diabetic subjects circulating
levels of C-reactive protein, IL-6 and TNFα correlated with measures of obesity, insulin resistance (assessed by HOMA), blood pressure, dyslipidaemia and markers of endothelial function (plasma levels of von Willebrand factor, tissue plasminogen activator, and cellular fibronectin). Levels of C-reactive protein were significantly related to those of IL-6 and TNFα. A mean standard deviation score of levels of acute phase markers correlated closely with a similar score of insulin resistance syndrome variables, this relationship being weakened only marginally by removing measures of obesity from the insulin resistance score. The authors suggest that a low-level, chronic inflammatory state, produced by adipose tissue generated cytokines, may induce insulin resistance and endothelial dysfunction and thus link the latter phenomena with obesity and cardiovascular disease (56;57). Pickup et al also investigated whether elevated acute-phase reactants and their major cytokine mediators (IL-6) are associated with type 2 diabetes and the insulin resistance syndrome. They compared diabetic subjects with 4 or more components of the insulin resistance syndrome with diabetic subjects without any other component of the insulin resistance syndrome. Serum sialic acid and C-reactive protein were used as markers of acute response. Highest levels of IL-6 and markers of acute response were found in the diabetic subjects with several components of the syndrome; lowest levels were seen in normal controls. The authors suggested that abnormalities of the innate immune system might be a contributor to the hypertriglyceridaemia, low HDL cholesterol, hypertension, glucose intolerance, insulin resistance and accelerated atherosclerosis of type 2 diabetes (175;176).

**Leptin as an antecedent for the insulin resistance syndrome**

Leptin, the circulating product of the obesity gene (ob) is a glycoprotein expressed and secreted primarily by the adipocytes. Leptin’s main actions are its feed back effect on hypothalamic energy regulation and its role in the maturation of reproductive function. Insulin has been shown in vitro and in vivo to stimulate leptin production (177;178). Leptin has been associated with both obesity and insulin resistance (179).
Some animal and human data suggest that hyperleptinaemia rather than, or synergistically
with, hyperinsulinaemia may play a central role in the genesis of the insulin resistance
syndrome. Studies in Psammomys obesus (the Israeli sand rat) suggest
hyperinsulinaemia/insulin resistance is an early metabolic lesion in the development of
obesity and type 2 diabetes. This animal also develops other features of the syndrome,
making it an excellent model to investigate aetiology. Psammomys, when placed on an ad
libitum laboratory diet, develops hyperinsulaemia, insulin resistance, impaired glucose
tolerance, diabetes, and dyslipidaemia. It also develops hyperleptinaemia and leptin
insensitivity, and hyperleptinaemia is correlated with insulin resistance independent of
changes in body weight. It is likely that a similar sequence occurs in the transition from
the prediabetic state to type 2 diabetes in humans (180). In contrast, Shimomura et al
studied a transgenic mouse model of congenital generalized lipodystrophy accompanied
by severe insulin resistance. Adipose tissue from these mice was markedly deficient in
messenger RNAs encoding several fat-specific proteins, including leptin. Insulin
resistance in these lipodystrophic mice could be overcome by a continuous systemic
infusion of low doses of recombinant leptin. Their findings thus support the idea that
leptin modulates insulin sensitivity and glucose disposal and that leptin deficiency
accounts for the insulin resistance found lipodystrophy (181).

Several groups studied the association between leptin concentrations and insulin
resistance in humans. It is known that plasma leptin concentrations correlate with the
amount of adipose tissue in the body. Shmulewitz et al consider high leptin levels to be a
component of the insulin resistance syndrome (182). De Merve et al examined the
relationship between leptin concentrations, various metabolic indices and body
composition in different groups (diabetic-lipoatrophic, white obese, black obese, normal
controls). Across the groups, there were positive linear correlations between leptin
concentrations, BMI, subcutaneous fat mass and NEFA levels (183).

Stejskal et al did not find an association between leptin concentrations and hypertension,
a component of the insulin resistance syndrome (184). Tai et al reported in a study of 21
non-diabetic women that abdominal subcutaneous fat mass was the most important
determinant of insulin resistance and plasma leptin (185).

In a study of women with preeclampsia, also an insulin-resistant state, leptin levels were
higher in preeclampsia than in controls. However, insulin sensitivity assessed by an
intravenous glucose tolerance test showed no relationship to leptin concentrations (186).
Similarly, Mohamed-Ali et al reported that insulin levels but not insulin resistance
correlated with leptin levels in type 2 diabetes (187).

A nested case-control study suggested that leptin is a risk factor for acute myocardial
infarction. Men with first-ever acute myocardial infarction had higher BMI, plasma
insulin and leptin, and diastolic blood pressure than the controls (188). Haffner et al
reported that leptin concentrations are associated with several cardiovascular risk factors
related to the insulin resistance syndrome. These associations were only partly explained
by leptin's relationship with BMI and glucose disposal rate (189). In a population-based
study of 240 subjects (59 with diabetes) leptin concentrations were positively correlated
with BMI, fasting insulin and blood pressure irrespective of glucose tolerance status.
Leptin levels were associated with insulin sensitivity (assessed by HOMA) independent
of age, BMI, waist/hip ratio, triglycerides, HDL-cholesterol and hypertension. The
association with individual components of the syndrome was equivocal after correction
for BMI and insulin resistance (190).

In summary: both, cytokines and leptin, are strongly implicated in the regulation of
energy balance, possibly contributing to several components of the insulin resistance
syndrome. However, the overall systemic effects of increasing cytokine concentrations
are unclear.

1.2.5. Factors influencing the development of the insulin resistance syndrome
Another question is why insulin resistance and other components of the syndrome
manifest in some cohorts/individuals and not in others. Environmental as well as genetic
factors appear to influence the manifestation of the syndrome in individuals. Most workers have postulated that both genetic and environmental factors contribute to the development of human insulin resistance.

1.2.5.1. Intrauterine growth retardation and the development of the insulin resistance syndrome

Intrauterine growth retardation has long term consequences for postnatal growth and development. Affected infants have a higher risk of morbidity and mortality from several neonatal diseases (191). Recent findings suggest that several components of the insulin resistance syndrome such as hypertension, type 2 diabetes or insulin resistance are more common in adults who had an abnormally low birth weight (192-195). In a bi-ethnic population in San Antonio, Texas, low birth weight was an independent risk factor for insulin resistance (196). However, some of these studies did not take into account the actual length of gestation in their low birth weight subjects. A large population based study investigating adults born small for gestational age found evidence of a correlation between intrauterine growth retardation and raised insulin and proinsulin concentrations but not any of the other components of the insulin resistance syndrome in young adults (197). Two other studies have shown small babies to become insulin resistant adults in their 50s and 60s with low birth weight predicting high blood pressure, insulin resistance, truncal obesity and high plasminogen activator inhibitor-1 activity but not the abdominal obesity or dyslipidaemia of the insulin resistance syndrome (198,199). Goodfellow et al suggested that endothelial dysfunction is a consequence of fetal malnutrition, contributing to the clinical features of the 'Small Baby Syndrome' in later adult life. In their study flow-related dilatation was impaired in low birth weight relative to normal birth weight subjects (200).

The mechanisms underlying programming are largely unknown. The ‘thrifty genotype’ theory suggested that this genotype has evolved to aid survival in subsistence conditions. This would then, in modern conditions of plentiful food supply, obesity and sedentary living, lead to insulin resistance and high rates of type 2 diabetes (201). This hypothesis
has been fully supported in a recent article by Hattersley and Tooke, who propose that genetically determined insulin resistance results in impaired insulin-mediated growth in the fetus as well as insulin resistance in adult life. They propose that low birth weight, measures of insulin resistance in adult life, and ultimately glucose intolerance, diabetes, and hypertension could all be phenotypes of the same insulin-resistant genotype. Abnormal vascular development during fetal life and early childhood, as a result of genetic insulin resistance, could also explain the increased risk of hypertension and vascular disease (202).

There is also a ‘thrifty phenotype’ theory (‘fetal origin’ hypothesis) proposing that undernutrition during sensitive periods of fetal life programmes for insulin resistance, hypertension and other metabolic changes leading to an increased cardiovascular risk (203). This fetal undernutrition may result from maternal malnutrition or from reduced transfer of nutrients to the fetus, permanently reducing the responsiveness of peripheral tissue to insulin (204). This hypothesis is supported by animal experiments showing that undernutrition of pregnant animals programmes permanent changes in the offspring including raised blood pressure and insulin resistance (205;206).

### 1.2.5.2. Lack of exercise and insulin resistance
Improving physical fitness is well known to increase insulin sensitivity (207;208). Weight reduction also results in an improvement in insulin sensitivity (209). However, physical exercise is associated with an increase in insulin sensitivity, even when training bouts are too brief to be associated with weight reduction (210). Reported improvements include changes in enzyme activity, increased responsivity of various intracellular mechanisms, reduced activation of insulin antagonistic mechanisms, changes in body composition/fat distribution and histological changes within muscle (207;210-212).

### 1.2.5.3. Cigarette smoking and insulin resistance
Insulin resistance is exaggerated by acute (213) and chronic (214) cigarette smoking. Data from the Insulin resistance and Atherosclerosis study (IRAS) did not support this
finding. The study reported that active smoking was not associated with insulin resistance
and that environmental tobacco exposure was associated with lower insulin sensitivity
(215).

1.2.5.4. Genetic models and insulin resistance
A number of studies have demonstrated insulin resistance in offspring or first degree
relatives of patients with type 2 diabetes (216-218). In a study of families of 123 Pima
Indians siblings Prochazka et al reported that insulin action at maximally stimulating
insulin concentrations had a trimodal frequency distribution, particularly among obese
individuals. As well as tight family clustering a gene on 4q contributes to in vivo insulin
action in these Pima Indian families (219).

Twin studies in pairs discordant for type 2 diabetes have reported insulin resistance in the
non-affected twin (220). Another, smaller study compared dizygotic and monozygotic
twins in their correlation for insulin sensitivity, measured by the euglycaemic clamp, as
well as first-phase and late-phase insulin secretion, measured by intravenous glucose
tolerance test. Intrapair correlations for all traits were consistently higher in monozygotic
than in dizygotic pairs. Genetic variance accounted almost 60% for the variance in
glucose-stimulated insulin secretion and almost 40% for the variance in insulin-
stimulated glucose uptake. The authors concluded that genetic variability seems to
contribute to the variance of insulin sensitivity as well as of insulin secretion (221).
However, effects of a shared intra-uterine environment could potentially influence
observations in such twin studies (222).

The Botnia study included 2152 first-degree relatives of patients with type 2 diabetes and
528 age- and weight-matched spouses without a family history of type 2 diabetes in
Western Finland and reported that abdominal obesity, insulin resistance, and decreased
resting metabolic rate are characteristic features of first-degree relatives of patients with
diabetes. A sex-specific paternal effect was observed on insulin and HDL cholesterol
concentrations suggesting a possible unprecedented maternal or paternal inheritance of different type 2 diabetes phenotypes (223).

Another recent large study examined a possible association between the single nucleotide polymorphism c.533A>C (K121Q) in the glycoprotein PC-1 gene and features of the insulin resistance syndrome in paired siblings in 922 subjects from Finland and Sweden. The investigators reported that, although the Q allele of the human glycoprotein PC-1 gene is associated with surrogate measures of insulin resistance, it might not be enough to increase the susceptibility to type 2 diabetes (224).

Insulin resistance is clearly modifiable by environmental factors, and the extent to which it is genetically determined remains controversial. The effects of a common intra-uterine environment also potentially affect twin studies (193;222). In the case of type 2 diabetes, genetic investigations are complicated by the heterogeneity of the phenotype, confusion over the primacy of insulin resistance and pancreatic beta-cell insufficiency and the arbitrary diagnostic threshold.

In humans, a few rare genetic conditions have been shown to cause severe insulin resistance. For some of these profound abnormalities of the insulin receptor have been established as the molecular defect (225;226). However, these genetic abnormalities are far more severe than those seen in the insulin resistance syndrome and the androgenisation and IGF-receptor activation is much more florid. So far, genetic variation of candidate insulin receptor and post-receptor mechanisms has not been shown to underlie the common insulin resistance syndrome in humans. The syndromes of severe insulin resistance do cause diabetes, although it is usually not like either Type I or Type II diabetes, being ketosis-resistant, insulin-requiring, but poorly insulin-responsive. It is not clear whether these syndromes cause excess vascular disease resembling that seen in the insulin resistance syndrome.
Over the last few years the ability to ‘knock out’ various genes in animal models has given different and novel insights into molecular mechanisms for insulin resistance. A relatively large number of candidate genes has been examined by these methods. In general, no mutation of the insulin receptor, or the down-stream intra-cellular insulin signalling mechanisms (including IRS-1, IRS-2, PI3-kinase, and various tyrosine kinases) has mimicked the insulin resistance syndrome. In a surprising number of instances, genetic defects of these molecules do not even produce significant insulin resistance (121). Conversely, insulin resistance and some components of the insulin resistance syndrome can be induced in animals by a wide variety of other genetic defects. Insulin resistance can be induced by genetic variation in IGF-BPl (227) and Fat/CD36 (a fatty acid transporter) (228).

A provocative finding was the suggestion that by inducing GLUT-1 (a non-insulin responsive glucose transporter) insulin resistance is induced (229), which may be relevant to the human type 2 diabetic in which ‘non-insulin mediated glucose uptake’ is relatively increased due to the mass action effect of hyperglycaemia. Knock-out methodology has also allowed the production of tissue specific knock-outs that changed our understanding of the role of insulin in various tissues. For example rodents with the insulin-receptor knocked-out of muscle cells are hypertriglyceridaemic (230). Mice with muscle-specific inactivation of the insulin receptor gene (MIRKO) are normoglycaemic but have increased fat mass. MIRKO and control mice were studied under hyperinsulinaemic-euglycaemic conditions. Insulin-stimulated muscle glucose transport and glycogen synthesis were decreased by about 80% in MIRKO-mice, whereas insulin- stimulated fat glucose transport was increased threefold in MIRKO-mice. These data demonstrate that selective insulin resistance in muscle promotes redistribution of substrates to adipose tissue thereby contributing to increased adiposity and development of the prediabetic syndrome (231). To analyse the role of the insulin signalling pathway in abnormal insulin action in muscle, adipose tissue, and liver and by altered beta-cell function mice were generated with combined heterozygous null mutations in insulin receptor (IR), insulin receptor substrate (IRS-1), and/or IRS- 2. Although combined heterozygosity for ir/irs-
1(+/−) and ir/irs-2(+/−) results in a similar number of diabetic mice, there are significant differences in the underlying metabolic abnormalities. ir/irs-1(+/−) mice develop severe insulin resistance in skeletal muscle and liver, with compensatory beta-cell hyperplasia. In contrast, ir/irs(+/−) mice develop severe insulin resistance in liver, mild insulin resistance in skeletal muscle, and modest beta-cell hyperplasia. Triple heterozygotes develop severe insulin resistance in skeletal muscle and liver and marked beta-cell hyperplasia. These data indicate tissue-specific differences in the roles of IRSs to mediate insulin action, with IRS-1 playing a prominent role in skeletal muscle and IRS-2 in liver (232). Recently Kahn’s group reported in Nature that in mice downregulation of the glucose transporter GLUT4 selectively in the adipose tissue (GLUT4 expression was preserved in skeletal muscle) leads to development of insulin resistance in muscle and liver. These mice became glucose intolerant and hyperinsulinaemic (233).

1.2.6. The epidemiology of the insulin resistance syndrome

The prevalence of insulin resistance in the apparently healthy population is unknown. This is because of the difficulties of defining insulin resistance in clinical terms and of quantifying insulin action in man. Other features of the insulin resistance syndrome can be identified in a proportion of apparently healthy individuals (69).

There is a large body of data suggesting that many of the features of the insulin resistance syndrome co-segregate (62). However, some studies have found that such co-segregation is not always present when one examines general populations, and the value of considering these factors as a syndrome has been questioned (65;66).

The population-based Bruneck Study assessed the prevalence of insulin resistance in 888 subjects with impaired glucose tolerance, type 2 diabetes, dyslipidaemia, hyperuricaemia and hypertension. The results documented that 1) the vast majority of subjects with multiple metabolic disorders are insulin resistant, 2) in isolated dyslipidaemia, hyperuricaemia or hypertension, insulin resistance is not more frequent than can be
expected by chance alone and 3) in the general population, insulin resistance can be found even in the absence of any major metabolic disorders (36).

This inconsistency of the co-segregation is especially found in studies which have tried to exclude obese and/or physically unfit subjects (considering them to suffer from the insulin resistance syndrome). Even amongst those studies which show co-segregation there are clear variations between the consistency and strength with which the individual components of the insulin resistance syndrome are associated with each other. Although the individual studies show some heterogeneity, dyslipidaemia is generally more central to the syndrome than is hypertension (234). A factor analysis approach (235) suggested that there were three or four separate strands to the insulin resistance syndrome rather than Reaven’s five (which excluded obesity) or the possible 12 shown in Table 1.5.

1.2.7. Clinical significance of the insulin resistance syndrome
Currently, there are two clinical consequences of the insulin resistance syndrome that justify the development of therapeutic strategies to treat the syndrome: cardiovascular disease and diabetes mellitus.

1.2.7.1. The insulin resistance syndrome as a cardiovascular risk factor
Perhaps the main motivation for treating the insulin resistance syndrome is concern about its role in vascular disease. The insulin resistance syndrome is recognised as a cardiovascular risk factor. It is unclear whether this is simply due to the additive effect of each of the components on cardiovascular risk (they are common and their overlap is heterogeneous) or whether there is a synergistic effect due to clustering (in concordance with the presence of a syndrome) leading to an even greater risk. This uncertainty as to whether there are synergistic effects between the individual components and how they are mediated makes rational targeting of elements of the syndrome difficult.
A closer examination of the literature shows that each of the individual components of the insulin resistance syndrome has been demonstrated to be a cardiovascular risk factor in its own right in several previous studies.

1.2.7.1.1. Hyperglycaemia as a cardiovascular risk factor

In a recent review of a number of studies glycaemia was shown to be a risk factor for cardiovascular disease in diabetic subjects (236;237). However, efforts to demonstrate a similar relationship in non-diabetic subjects, within the normoglycaemic range, have given much less consistent results (238). Gerstein and Yusuf (239) have classified ‘Dysglycaemia’ or an elevated glucose concentration within the normal range, as risk factor for CVD.

The United Kingdom Prospective Diabetes Study (UKPDS), including 3867 subjects with type 2 diabetes, failed to show any conclusive evidence of a significant reduction in macro-vascular death or myocardial infarction through glucose lowering (240). Treatment with metformin was shown to be effective in reducing the risk of cardiovascular events if used as a primary agent in obese patients. For all macrovascular events together the group allocated metformin had a 30% lower risk than the group randomised to conventional therapy. When added as secondary treatment in addition to a sulphonylurea in normal-weight patients metformin seemed to have a detrimental effect on coronary risk (241).

To test whether reversing insulin resistance and hyperglycaemia could lead to decreased vascular complications the BIGuanides and Prevention of Risks in Obesity (BIGPRO1) compared the efficiency of Metformin together with life-style modification measures (diet and exercise advise) versus placebo in non-diabetic subjects with central adiposity. A number of markers associated with cardiovascular risk improved significantly (FPG, FPI, tPA, total and LDL-cholesterol). However, no cardiovascular endpoint was used (242).
1.2.7.1.2. **Hyperinsulinaemia as a cardiovascular risk factor**

Insulin resistance is highly correlated with fasting plasma insulin concentration, so that the latter is frequently used in epidemiological studies as a surrogate measure of insulin resistance (12). Hyperinsulinaemia was most famously proposed as a cardiovascular risk factor by Stout (243), although his view has not been universally accepted (244). Epidemiological studies have been inconsistent as to whether hyperinsulinaemia is a cardiovascular risk factor (60;245). In a recent metaanalysis Ruige et al found that hyperinsulinaemia was a weak risk indicator for the occurrence of CVD and that the relationship between hyperinsulinaemia and CVD was modified by ethnic background and by the type of insulin assay involved (246).

Lehto et al recently carried out a prospective study to examine the predictive value of fasting plasma insulin and "hyperinsulinaemia cluster" with regard to the risk of CVD mortality. The predictive value of hyperinsulinaemia with regard to death from CVD was independent of conventional cardiovascular risk factors but not independent of risk factors clustering with hyperinsulinaemia (247).

1.2.7.1.3. **Insulin resistance as a cardiovascular risk factor**

There have been no studies of cardiovascular mortality and insulin resistance measured directly by either euglycaemic hyperinsulinaemic clamp or by intravenous glucose tolerance test. The Insulin Resistance Atherosclerosis Study has shown that insulin resistance as indicated by intravenous glucose tolerance test is associated with increased intima media thickness (in some ethnic groups) (248). So far no data on cardiovascular morbidity or mortality have been published.

1.2.7.1.4. **Dyslipidaemia as a cardiovascular risk factor**

Dyslipidaemia is a common component of the insulin resistance syndrome. Several abnormalities are of interest: 1) HDL-Cholesterol concentrations are reduced, 2) LDL-cholesterol particles are smaller in size and denser in structure (235;249), 3)
hypertriglyceridaemia is commonly present (250;251) and 4) postprandial lipaemia is accentuated (252). All of these components have been identified as cardiovascular risk factors.

There is evidence that hypertriglyceridaemia is associated with an increased cardiovascular risk (253-255). It has been demonstrated that hypertriglyceridaemia is associated with lower HDL-cholesterol concentrations, smaller, denser LDL-particles, and accentuated postprandial lipaemia-all of which have been identified as increasing risk of CVD (12;252;256). The Paris Prospective Study demonstrated that in men with impaired glucose tolerance or diabetes an increase in plasma triglycerides was associated with cardiovascular death (254). However, because the lipid abnormalities are strongly inter-related, it is unclear whether hypertriglyceridaemia per se increases cardiovascular risk (12;252;256;257). In a prospective study of 313 diabetic subjects high triglyceride level (>2.3 mmol/L) was associated with a two-fold increase in the risk of CVD events independently of other lipoprotein abnormalities (258). The Finnish Diabetes Prevention Study also reported that hypertriglyceridaemia is a risk factor for CVD (259;260).

Interestingly, it has been reported that isolated hypercholesterolaemia is not associated with insulin resistance (261;262).

A number of intervention trials have looked at the effect of correcting dyslipidaemia on coronary risk (263-265). However, none of these have specifically addressed this question in diabetes or other insulin resistant states. Three of these studies have included a subgroup of subjects with diabetes and these data have been analysed post hoc. The Helsinki Heart Study showed a trend in reduction of cardiovascular disease in diabetic subjects treated with gemfibrozil but the numbers were too small to reach significance (266). A post hoc analysis of the Scandinavian Simvastatin Survival Study (4S) was carried out to examine the diabetes subgroup more closely. The results strongly suggest that cholesterol lowering with simvastatin improves the prognosis of diabetic patients with CVD. The absolute clinical benefit achieved by cholesterol lowering may be greater
in diabetic than in nondiabetic patients with CVD because diabetic patients have a higher absolute risk of recurrent CVD events and other atherosclerotic events (267). The Cholesterol and Recurrent Events Trial (CARE) with pravastatin also found a reduction in coronary events in the treated diabetic subgroup (268).

Those agents targeting high levels of LDL-cholesterol might not be the most useful in treating the dyslipidaemia of the insulin resistance syndrome, as other abnormalities of the lipoprotein profile are more prominent. Although trials show benefit from drugs that lower LDL-cholesterol concentration, it is only recently that agents which specifically affect HDL, triglycerides and/or LDL size have been shown to reduce cardiovascular disease (263;265). The Veterans Affairs High-density Lipoprotein Cholesterol Intervention Group has reported that raising HDL-cholesterol and lowering triglycerides with gemfibrozil reduces the rate of coronary events without lowering LDL-cholesterol levels (269). Four studies to look at the association of diabetes and lipid lowering treatment are now under way. The Diabetes Atherosclerosis Intervention Study, for example, has now been started with the primary aim to examine whether lipid reduction in type 2 diabetes leads to reduction in cardiovascular risk (270).

1.2.7.1.5. Hypertension as a cardiovascular risk factor
Hypertension has long been recognised as a cardiovascular risk factor. A large number of studies have shown a reduction in cardiovascular morbidity and mortality with various treatments of blood pressure (271). Treating hypertension in diabetic patients has also been shown to be beneficial. In general the benefit appears to depend upon the hypotensive effect achieved rather than the type of agent used (272).

1.2.7.1.6. Central obesity as a cardiovascular risk factor
Central obesity was first described as an adverse health factor in the early 1950’s (273) and later confirmed by numerous studies (274-276). The availability of CT scanning then allowed looking at the impact of intra-abdominal fat collection separately from generalised obesity and it was proposed that only intra-abdominal fat correlated with the
other components of the insulin resistance syndrome (277). Reduction of intra-abdominal fat by caloric restriction or exercise ameliorates the insulin resistance and other components of the insulin resistance syndrome (278). The cause of increased cardiovascular risk in obesity is likely to be multifactorial. The abnormal lipid profile as well as the hypertension, insulin resistance and coagulation defects often occurring with obesity could also each be contributing to the increased cardiovascular risk.

1.2.7.1.7. Coagulation factors as a cardiovascular risk factor
Several coagulation factors have been associated with an increased cardiovascular risk. Of these three have been reported to be associated with insulin resistance: PAI-1 (279;280), fibrinogen (281;282), and von Willebrand–factor (56;281;283).

1.2.7.1.8. The effect of a combination of different components of the insulin resistance syndrome on cardiovascular risk
Thus there is evidence that most if not all of the components of the insulin resistance syndrome are associated with an increased cardiovascular risk. Data from the Whitehall II study, estimating insulin resistance from the results of an oral glucose tolerance test, suggests that the increased prevalence of the insulin resistance syndrome may contribute to the higher coronary risk found in individuals belonging to lower socio-economic groups (284). It is important to know which component(s) is/are the most dangerous.

In the Caerphilly and Speedwell studies Yarnell et al. looked at the concepts of the insulin resistance syndrome in two prospective cohorts of white men. In three different models of metabolic syndrome no predictive value for cardiovascular disease was found after adjustment for conventional and metabolic risk factors. The study did not detect any complex relation among the five variables defining the insulin resistance syndrome. Thus the excess cardiovascular risk found in certain subjects was no greater than can be explained by individual effects of the defining variables in a multiple logistic model. There was also no evidence of clustering of the single components of the insulin resistance syndrome in these cohorts (67).
Epidemiologists have pointed out the methodological difficulties in ‘factoring out’ the relative risks from individual risk factors when those risk factors are themselves highly inter-correlated (285). Thus most studies examining the influence of individual components of the insulin resistance syndrome are difficult to interpret, as (by definition of this syndrome) several putative cardiovascular risk factors occur simultaneously.

A related question is whether agents that improve multiple components of the insulin resistance syndrome are more beneficial on cardiovascular risk than those that affect only a single component. For example metformin (which improves several components of the syndrome) (286-288) was superior to insulin (which improves only glucose tolerance) in the obese subgroup of the UKPDS (241). Metformin decreased cardiovascular risk to a substantially greater degree than could be explained on the basis of an improved glycaemic control. However the parallel Hypertension in Diabetes Study gave no indication that ACE-inhibitors (which improve several components of the syndrome) are superior to beta-blockers (which improve only blood pressure and may even increase insulin resistance) (272). At present we have relatively few such data and therefore cannot say whether agents influencing several components are generally superior to more focussed treatments.

If the increased cardiovascular risk of the insulin resistance syndrome is the consequence of an antecedent (e.g. NEFA, circulating cytokines or endothelial dysfunction) then treating the insulin resistance itself may have little or no impact on cardiovascular risk.

1.2.7.2. The insulin resistance syndrome and type 2 diabetes mellitus

A number of studies have examined the relationship between the insulin resistance syndrome and the incidence of subsequent type 2 diabetes mellitus (62). It has been shown that the insulin resistance syndrome (with insulin resistance, hyperinsulinaemia, dyslipidaemia and hypertension) can antedate diabetes by several years and thus, perhaps contribute to the cardiovascular risk in the prediabetic patient (289). Both insulin resistance and impaired insulin secretion are important for the development of diabetes
In Pima Indians insulin resistance was found to be a major risk factor for the development of diabetes, predicting two thirds of the cases of diabetes (7). Only one third was predicted by a reduced insulin response. However, insulin resistance was not found to be an universal concomitant of diabetes in all epidemiological studies: In a small group of black patients with type 2 diabetes only 50% of those studied were insulin resistant (291), suggesting a heterogeneity of aetiology, particularly apparent in different ethnic groups.

Himsworth was the first to suggest that type 2 diabetic patients could be divided into ‘insulin sensitive’ and ‘insulin resistant’ on the basis of the ability to dispose of an oral glucose load by exogenous insulin (292). Subsequently a large number of studies reported that plasma insulin levels during an oral or intravenous load in patients with type 2 diabetes were normal or higher than normal, concluding that tissue insensitivity to insulin plays an important role in the glucose intolerance (27;29;293). Alford documented a blunted decline in plasma glucose concentration following intravenous insulin in type 2 diabetic patients with fasting hyperglycaemia (294). Human forearm perfusion technique studies have also shown an impairment in insulin mediated glucose uptake in type 2 diabetes (22-24). Using the quadruple infusion technique (co-infusion of epinephrine and propranolol to suppress endogenous insulin production, glucose and insulin) Reaven et al. demonstrated that diabetic patients manifest insulin resistance (25-29).

In Type 1 diabetes, those with least insulin resistance are reported to have the best cardiovascular prognosis (295). The same may also apply in type 2 diabetes (296). In diabetic subjects it is not usually clear whether insulin resistance is detrimental above and beyond the impaired glucose tolerance that it induces. Thus, review of records from the Aberdeen diabetic clinic demonstrated that weight loss in type 2 diabetes was advantageous in terms of improved life expectancy. This study could not distinguish whether better glycaemic control or improved insulin resistance was the reason for the benefit (297). Recently, the UKPDS implied that metformin as a first line treatment in
overweight diabetic patients produced a better cardiovascular outcome than sulphonylureas or insulin despite equivalent glycaemic control (241).

Analysis of the Paris Prospective Study suggests that a high plasma NEFA concentration is a risk marker for deterioration of glucose tolerance independent of the insulin resistance or the insulin secretion defect that characterizes subjects at risk for type 2 diabetes (298). This might suggest that abnormal NEFA metabolism antedates insulin resistance or the development of diabetes.

Three lines of evidence have been used to promote insulin resistance rather than insulin deficiency as the primary defect leading to the development of type 2 diabetes (as opposed to being secondary to hyperglycaemia). Firstly, insulin resistance has (at least in some populations) an inherited component (299;300). Secondly, some studies have pointed to abnormal insulin action but normal insulin secretion in patients at high risk of developing type 2 diabetes before hyperglycaemia ensues (301;302). However, it is not easy to define strictly normal insulin secretion, and subtle abnormalities such as loss of normal pulsatile insulin release have been identified in subjects with impaired glucose tolerance (303). Thirdly, insulin resistant subjects progress from normal glucose tolerance to diabetes more rapidly than their insulin sensitive counterparts (304;305).

To conclude it thus appears overwhelmingly likely that, at the minimum, insulin resistance causes diabetes to become clinically overt earlier than it would otherwise. Indeed there may be a large number of patients in whom reduction in insulin resistance may delay, prevent or reverse diabetes. Such delay, prevention or reversal of diabetes has been achieved by weight loss. Therapeutic agents which reduce insulin resistance might have the same benefits, so that a legitimate aim of treatment might be to delay or prevent diabetes mellitus in susceptible patients. In subjects already diabetic, there may be additional benefits to reducing insulin resistance in regard to long-term outcome. It would appear that the prognosis of Type 2 diabetic subjects is less determined by their
glycaemic control than whether they show other features of the insulin resistance syndrome in addition to hyperglycaemia (306).

1.3. Endothelial function

Blood vessels are lined by endothelial cells. The vascular endothelium not only maintains thromboresistance, but also has many additional functions (table 1.7). Ultrastructural and physiological studies in vivo and studies of endothelial cells in vitro have demonstrated that the endothelium acts as a highly specialised and metabolically active organ. It has a central role in modulating the tone of the underlying smooth muscle cells in response to physiological and pharmacological stimuli, thereby regulating blood flow and blood pressure in resistance vessels (307;308). It produces powerful vasodilator and vasoconstrictor agonists and inactivates many circulating vasoactive mediators (e.g. thrombin, bradykinin) (309-311).

Endothelium-dependent vasoconstriction is mainly due to the action of superoxide anions and endothelin (312).

Endothelium-dependent vasodilatation is predominantly due to the release of endothelium derived relaxing factor (EDRF), which has been confirmed to be nitric oxide (NO) (307;313-315). NO is generated from its substrate, L-arginine, by Nitric Oxide-synthase (315;316). NO stimulates smooth muscle guanylate cyclase, leading to a rise in intracellular cyclic guanosine monophosphate (cGMP) thereby inhibiting smooth muscle contractility. It thus relaxes vascular smooth muscle, acting as a vasodilator. Vallance et al showed that the forearm arterial circulation continually releases NO (317). NO release can be activated in response to a wide range of agonists (platelet release products, neurotransmitters, circulating hormones) and also by longitudinal shear stress generated by blood flow (318).

Changes/ defects in endothelial function can therefore be assessed by two main approaches: First, measuring blood flow in response to various pharmacological and
mechanical stimuli will give measures of endothelial function in resistance vessels and (if shear stress is the stimulus applied) in conduit vessels. Second, measuring circulating concentrations of various endothelial cell products will give information about capillary endothelial function, the largest contributor to endothelial volume.

### Table 1.7: Function of the vascular endothelium

<table>
<thead>
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<th>Function</th>
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<tbody>
<tr>
<td>Maintenance of thromboresistance</td>
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<tr>
<td>Maintenance of selective permeability</td>
</tr>
<tr>
<td>Integration and transduction of blood borne signals</td>
</tr>
<tr>
<td>Modulation of leukocyte interactions with tissues</td>
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<tr>
<td>Regulation of inflammatory and immune reactions</td>
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<tr>
<td>Regulation of vascular tone</td>
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<td>Regulation of vascular growth</td>
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<tr>
<td>Synthesis and secretion of peptides</td>
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#### 1.3.1. Assessment of endothelial function through measurement of forearm blood flow

Two widely accepted dynamic techniques for assessment of forearm blood flow are the measurement of response of vessel diameter and blood flow to stimuli using

a) Doppler ultrasound measurement of the brachial artery (conduit vessels) and

b) Venous occlusion plethysmography (resistance vessels).

#### 1.3.1.1. High resolution Doppler flow measurements

These are non-invasive and allow simultaneous study of large conduit vessels (assessed by measurement of brachial artery diameter) and small arteries (assessed by measurement of total flow) (319;320). However, because the cross sectional area of a vessel is proportional to its squared radius, small errors in the measurement of brachial artery diameter will result in large errors in the calculation of flow. Furthermore the difference in arterial diameter between diastole and systole presents a further problem. If the aim of a study is to measure the effects of pharmacological or physiological interventions on the
small arterioles that are the primary determinants of flow, Doppler ultrasound has no advantage over plethysmography (321).

1.3.1.2. **Venous occlusion plethysmography**

This technique has been widely used to study forearm blood flow (322). The underlying principle is that if venous return is obstructed and arterial inflow continues unimpeded, the forearm swells at a rate proportional to the rate of arterial inflow.

Under resting conditions forearm blood flow is predominantly (approx. 50–70%) blood flow through skeletal muscle. Blood flow in the hand must be excluded from circulation, as this is mainly skin blood flow with a high proportion of arterio-venous shunts (323). The rate of swelling of the forearm can be calculated from changes in forearm circumference, measured in millimetres per minute, with a mercury strain gauge placed around the forearm. Flow is expressed per unit volume of forearm, usually as millilitres per 100 ml forearm volume per minute. Arrest of the forearm venous return is achieved by inflating a cuff placed round the upper arm to 40-50 mmHg for 10 seconds. This does not affect arterial inflow or pressure. With the forearm placed at the level of the right atrium, inflation of the venous occlusion cuff for 10 seconds in every 15 seconds causes a linear increase in forearm volume and the 5 seconds deflation is usually long enough to allow emptying of the forearm veins before the next measurement. Exclusion of the hands from circulation is achieved by inflating a wrist cuff to suprasystolic pressure.

Venous occlusion plethysmography estimates total flow in the forearm from the wrist cuff to the venous occlusion cuff, and not the flow in the cross section of the arm immediately underneath it (321). The technique has the advantage that vessels are studied in their physiological environment under the influence of neuronal, circulating and local mediators. Measuring flow simultaneously in both arms can compensate for small alterations in arterial pressure or sympathetic arousal (321;324). In the absence of intervention the ratio of blood flow in the two arms approaches unity and stays constant even if systemic arterial pressure changes (324). By studying the arm on which
physiological or pharmacological interventions are performed, as well as the contralateral arm, the latter can be used as an in-built physiological control. This thereby allows adjustment for systemic physiological changes, as any change in the ratio between the flow in the two arms is a direct reflection of the local vascular tone changes.

Most researchers studying the endothelium, have chosen to measure forearm blood flow in response to acetylcholine and other muscarinic agonists by venous occlusion plethysmography (322). To avoid central effects, hormonal responses, changes in sympathetic output and alterations in blood pressure, vasoactive drugs can be infused locally. Drug administration through a cannula placed in the brachial artery allows study of the direct vascular effects of these drugs. This technique also allows infusing drug doses that produce large enough changes in local blood flow without altering systemic arterial pressure. This technique is generally considered to be safe (321).

1.3.1.2.1. Vascular response to Acetylcholine

Acetylcholine (ACh) induces vasodilatation through the release of NO (325). Local infusion of this endothelium-dependent agent, compared with endothelium-independent agents (e.g. sodium nitroprusside) can thus be used to assess vascular control in more detail (321). This approach, however, has its limitations. It is clear that only part of the vasodilator response to ACh is due to endothelial NO synthesis. A large component is resistant to N-monomethyl-L-arginine (326). Already in 1953 Duff et al reported that ACh is rapidly broken down by plasma pseudocholinesterase, so that not all of the drug infused into the brachial artery will reach the resistance vessel and influence forearm blood flow. When administered into the brachial artery more than 99% of the infused dose of ACh is destroyed before reaching the hand (327). Therefore there is a strong negative correlation between forearm length and blood flow in response to ACh (328).

1.3.1.2.2. Vascular response to Sodium Nitroprusside

Acetylcholine responses also affect production of prostacyclin, the delivery of NO to vascular smooth muscle cells and guanylate cyclase activity. As a control for these
factors, pharmacologically endothelium-independent regulators can be infused to demonstrate the integrity of post-endothelial components of the response (321). Sodium nitroprusside (SNP) is an exogenous NO-donor. It acts independently of the endothelium and can therefore be used as a measure of NO-responsive non-endothelial dependent vasodilatation, such acting as a control (329). Although unstable in blood, it is broken down less rapidly than ACh (330).

1.3.1.2.3. Vascular response to N-monomethyl-L-arginine

An alternative and more specific approach can be achieved in vivo by infusing an arginine analogue, unrecognised by eNOS as a competitive inhibitor of the enzyme. N-monomethyl-L-arginine (L-NMMA) is an arginine analogue, containing one more methyl-group (CH₃) than arginine. It thereby acts as an inhibitor of NO synthesis in vivo rather than a substrate. Inhibition of endothelial nitric oxide synthase in the forearm by local infusion of L-NMMA results in a 30-40% decrease in blood flow (317). The vasoconstrictor response to L-NMMA is an index of basal endothelial NO production, i.e. the proportion of resting vasodilator tone which can be attributed to NO production (331).

Local infusion of these endothelium-dependent (e.g. acetylcholine) and endothelium-independent agents (e.g. Sodium Nitroprusside) can thus be used to assess vascular control in resistance vessels in more detail (321).

1.3.1.2.4. Vascular response to insulin

In skeletal muscle glucose uptake can be determined by multiplying glucose extraction (a-v difference of glucose) by delivery (blood flow). Insulin stimulates glucose uptake by increasing glucose extraction (the a-v difference) (332). Insulin under certain conditions increases blood flow in skeletal muscle (85). This appears mediated via a NO-dependent mechanism (154). Insulin as a vasodilator has been discussed in detail in section 1.2.4.1.
1.3.2. Assessment of endothelial function through measurement of circulating endothelial cell products

Direct measurement of many endothelial cell products, as a measure of capillary endothelial activation, is now technically possible. This is a simple approach and can easily be applied to large groups of subjects. For example, circulating concentrations of t-PA (333;334), von Willebrand Factor (vWF) (335;336), thrombomodulin (337;338) and adhesion molecules (E-selectin, vascular cell adhesion molecule-1) (339-342) have been measured and associated with endothelial function in human studies. NO has also been directly measured (343). These origin of these circulating markers is likely to be from peripheral capillary endothelium (this has a far greater surface area), as opposed to large arterial endothelium. Thus altered levels of these and other endothelial cell products might potentially reflect endothelial dysfunction. However, the serum concentration of vascular cell adhesion molecule-1 and not E-selectin, P-selectin or thrombomodulin was associated with the extent of atherosclerosis in patients with peripheral vascular disease (341).

In both type 1 and type 2 diabetes fibrinolytic activity is decreased and PAI-1 activity increased (344). Increased concentration of von Willebrand factor has been reported in subjects with diabetes complicated by retinopathy (345) and microalbuminuria (335;346). However, the synthesis and secretion of endothelial cell products are known to be strongly influenced by a variety of cytokines, components of oxidised lipoproteins and fluid shear stress (347-349).

Endothelial dysfunction as a possible antecedent of insulin resistance is discussed in more detail in section 1.2.4.3.4.

1.3.3. Factors influencing endothelial function

A large number of factors have been demonstrated to influence endothelial function in animal studies and in men. These include elevated plasma lipids, NEFA and glucose concentration (350-356), dietary supplements (arginine, antioxidants, vitamin C) (357-
359), medication (GTN, aspirin, statins, fibrates) (351;360-362) and lifestyle (smoking, physical fitness) (363). Most of these have parallel effects on endothelial function and insulin resistance. However, fish oil appears to have a dissociated effect, with a neutral or adverse effect on insulin sensitivity and a beneficial effect on endothelial function (364-366). Of particular interest is the study by Vehkavaara et al, who showed that in type 2 diabetes insulin therapy improved endothelial function (367).

Animal studies also suggest that ageing is associated with impaired endothelium-dependent vasodilatation (368). The influence of age on coronary blood flow in humans has been demonstrated by Egashira et al (369). Lyons et al compared endothelium-dependent forearm blood flow in 12 young healthy volunteers with 12 healthy elderly subjects (matched for blood pressure, cholesterol and glucose) and found a blunted endothelium-dependent vascular response, together with a decreased urinary nitrate excretion, in advanced age (370).

1.3.3.1. Cytokines and endothelial function

Observational studies in humans have suggested that acute infection or acute systemic inflammation lead to a temporarily increased cardiovascular risk (371). High levels of interleukin-6 (IL-6) have been reported to be associated with transition from stable to unstable angina (372). It has also been shown that the exposure of endothelial cells to cytokines induces procoagulation (308), leads to the expression of adhesion molecules (373) and impairs endothelium-dependent vasodilatation (374). Vallance et al suggested that impairment of normal endothelial function by inflammatory response might provide a link between systemic inflammation and ischaemic coronary syndromes (375;376). Administration of tumor necrosis factor α (TNFα) depresses endothelium-dependent vasodilatation (377). “Endothelial stunning”, impaired endothelial relaxation for many days, can be produced in healthy volunteers by very brief exposure to cytokines (374). Plasma levels of the pro-inflammatory cytokine TNFα correlated with endothelium-dependent vascular response in patients with congestive heart failure after treatment, but not during the acute episode (378). Furthermore, Fichtlscherer and al have reported that
elevated C-reactive protein levels are associated with a profound impairment in endothelium-dependent vascular reactivity in patients with coronary heart disease. They also demonstrated that normalisation of C-reactive protein levels over time is associated with an improvement of endothelium-dependent vascular reactivity. However, in their study TNFα did not show any relationship with vascular reactivity (379).

1.3.3.2. NEFA and endothelial function

A number of studies have examined the relationship between NEFA and endothelial function demonstrating that increased NEFA concentrations can lead to endothelial dysfunction.

Saxena et al demonstrated in cultured cells that human plasma lipoproteins (chylomicrons and VLDL) and NEFA release LPL protein bound to endothelial cells and decrease LPL enzymatic activity (380). In later experiments the same group explored the role of LPL in atherogenesis. They showed that in cultured endothelial cells monocyte adhesion was increased in the presence of VLDL and LPL. This increase in adhesion correlated with the generation of NEFA from the hydrolysis of triglycerides in VLDL by LPL and by direct addition of oleic acid (381). This has been supported by Hennig et al who showed a decrease in endothelial barrier function in injured endothelial cells which correlated with the level of NEFA contained in the lipolytic remnants (382).

A study comparing toxic effects of NEFA upon cultured heart muscle and endothelial cells reported that different NEFA lead to cell injury over a different time course (383). The question has been raised whether the negative effect of NEFA on endothelial function depends on their chain length. De Kreutzenberg et al have shown that the effect is independent of chain length (384).

A study in preeclampsia, reported that serum from preeclamptic women had higher levels of NEFA and that there was an increase in vascular cell adhesion molecule-1 expression on endothelial cells exposed to preeclamptic serum compared to cells exposed to normal
serum. Interestingly, addition of NEFA to normal pregnancy serum mimicked the effect of preeclampsia resulting in increased vascular cell adhesion molecule-1 expression (385).

Steinberg et al demonstrated that elevated NEFA concentrations impair endothelium-dependent vasodilatation (355). Intravenous infusions of Intralipid in healthy subjects led to an increase in NEFA levels and impaired vascular response to methacholine by 20%. Vascular response to SNP remained unchanged from baseline. Lind et al reported that endothelial dysfunction induced by NEFA can be reversed by insulin (386). In their study intravenous infusion of Intralipid(R) resulted in an increase of NEFA concentrations and a decrease of endothelium-dependent vasodilation (to methacholine). This intervention was followed by an euglycaemic hyperinsulinaemic clamp which completely restored the vasodilation in response to methacholine to normal. Endothelium-independent vasodilatation (to SNP) remained unchanged throughout.

1.4. Endothelial function and insulin resistance

1.4.1. Endothelium-dependent vasodilatation in insulin resistant states

It has been difficult to determine if the endothelial defect observed in type 2 diabetes is due to impaired metabolic control, due to the insulin resistance per se or due to the consequences of obesity (71;84;128). Studies using venous occlusion plethysmography to measure forearm blood flow have been performed in non-diabetic subjects in insulin resistant states.

1.4.1.1. Hypertension

Hypertension, as an example, has been demonstrated to be associated with insulin resistance (117). The first two studies of endothelial function in hypertension by Linder et al and Panza et al reported a blunted vasodilator response to ACh compared with SNP in hypertensive patients (387;388). This was not confirmed in the larger study by Cockcroft et al., using the same technique, which reported preserved endothelium-dependent vasodilatation in hypertension (389). Kelm et al reported that endothelium-dependent and
endothelium-independent (SNP) vascular response were impaired in hypertension (390). The differences between these studies may perhaps be explained by the heterogeneity in these groups as a large number of factors influence endothelial function (obesity, lipids, age etc.). It has been postulated that the endothelial changes in hypertension might be a consequence of the high blood pressure rather than the cause (388).

Natali et al examined whether endothelial dysfunction and insulin resistance coexisted in patients with essential hypertension. They reported that metabolic insulin resistance in essential hypertension was not associated with abnormalities in vascular structure or endothelium-dependent (to ACh) and endothelium-independent (to SNP) vasodilatation (391). Hunter et al also reported no association between insulin resistance and insulin-mediated vasodilatation in their hypertensive study group (11 patients) or their healthy control group (eight subjects) (392). Similarly, Natali et al reported in a recent study of 9 patients with essential hypertension and insulin resistance that an acute improvement in endothelial function, obtained with pharmacological doses of vitamin C, restored insulin-mediated vasodilatation but did not improve insulin-mediated glucose uptake. They concluded that the endothelial dysfunction of essential hypertension might be related to free radical mediated mechanisms, but was unlikely to be responsible for their metabolic insulin resistance (393).

1.4.1.2. Obesity

Obesity is another insulin resistant state. Steinberg et al examined the leg blood flow responses to intra-arterial artery infusions of methacholine chloride or sodium nitroprusside (SNP) during saline infusion and euglycaemic hyperinsulinaemia in a group of lean insulin-sensitive controls, a group of obese insulin-resistant subjects and a group of subjects with type 2 diabetes mellitus. They found that obesity/insulin resistance was associated with a blunted endothelium-dependent, but normal endothelium-independent vasodilatation. They also reported a failure of euglycaemic hyperinsulinaemia to augment endothelium-dependent vasodilatation in the obese/insulin-resistant subjects. They concluded that obese/insulin-resistant subjects are characterised by endothelial
dysfunction and by endothelial resistance to insulin's effect on enhancement of endothelium-dependent vasodilatation and suggested that this endothelial dysfunction could contribute to the increased risk of atherosclerosis in obese insulin-resistant subjects (128).

1.4.2. Circulating markers of endothelial function in insulin resistance

In two epidemiological studies plasma concentrations of vWF were correlated with those of insulin- a surrogate marker for insulin resistance in population studies (281;394). Yudkin et al also showed relationships between endothelial markers and markers of insulin resistance (56).

1.4.3. Endothelial dysfunction as the cause of insulin resistance

Endothelial dysfunction as an antecedent for insulin resistance has been discussed in detail in section 1.2.4.3.4.

The idea that endothelial dysfunction and insulin resistance may be causally related is beginning to take root. This is supported by the similarities between the conditions in which insulin resistance and endothelial dysfunction are observed and the improvements produced in both abnormalities by similar interventions (72). It is possible that endothelial dysfunction underlies the insulin resistance or alternatively there may be a common antecedent for both.
1.5. Endothelial function in diabetes as evidenced by blood flow changes

Vascular disease is a major cause of morbidity and mortality in type 2 diabetes. Atherosclerosis, resulting in cardiovascular and cerebro-vascular disease, and small vessel pathology, resulting in retinopathy and nephropathy, are much more common than in the non-diabetic population. Decreased availability of endothelium-derived NO, leading to abnormalities of vasomotor function and atherogenesis, may contribute to vascular disease in diabetes.

There is substantial evidence that endothelium-dependent vasodilatation is impaired in animal models of diabetes (395-397). Johnstone et al. demonstrated that endothelium-dependent vasodilatation is impaired in patients with type 1 diabetes compared with matched healthy subjects, probably as a result of decreased availability of endothelial NO (398). Other groups supported these findings in similar studies (65;331). However, Halkin et al. and Smits et al. did not observe a defect in endothelium-dependent vasodilatation in subjects with uncomplicated type 1 diabetes (399;400).

McVeigh et al. reported that patients with type 2 diabetes exhibit attenuated vasodilatation to acetylcholine (endothelium-dependent) and Glyceryl trinitrate (endothelium-independent) (401). However, interpretation of these data is difficult, as there is a possible increased nitroglycerin tolerance in diabetes. This tolerance would not be expected if a direct NO donor (for example SNP) were given (402). Furthermore, the contribution of vasoconstrictor prostanoids, implicated as mediators of vascular dysfunction in experimental diabetes, has not been assessed in human diabetic subjects (403). Williams et al. compared 21 subjects with type 2 diabetes with 23 healthy age matched controls and reported an attenuated response to endothelium-dependent (methacholine) and endothelium-independent (SNP) vasostimulants (404). These findings confirm the presence of impaired endothelium-dependent vasodilatation in diabetes and also of impaired endothelium-independent response to exogenous NO donors. This might indicate that the vascular defect in type 2 diabetes is not solely attributable to abnormal endothelial production of NO but also to decreased responsiveness to NO. The same
group reported that NO-independent vasodilatation by verapamil was normal in the diabetic cohort (404) eliminating the possibility that generalised impairment of vascular smooth muscle underlies the vascular abnormality. This again points to the NO-pathway as an underlying defect. However, even though the diabetic group was age matched to the controls, their BMI and triglyceride levels differed significantly. Nevertheless in a subsequent regression analysis neither obesity nor hypertriglyceridaemia appeared to be responsible for the abnormal vascular findings in the diabetic group.

Laakso et al compared insulin-mediated blood flow in six obese type 2 diabetic patients with six lean and six obese non-diabetic controls. They reported reduced blood flow in the diabetic group, but could not determine if this was due to obesity / insulin resistance or the to diabetic state per se (405). Hogikyan et al reported the impaired endothelium-dependent vasodilatation in type 2 diabetes to be independent of obesity (149). Preik et al have recently demonstrated that both type 2 diabetes and essential hypertension impair endothelial function in an additive manner (406). Mather et al reported improvement of endothelial function with metformin in a group of patients with type 2 diabetes but no other cardiovascular risk factors. This observation might be due to the positive effect metformin has on insulin resistance (as assessed by HOMA), so that improved insulin resistance may have led to an improvement in insulin-dependent vasodilatation(72).

In conclusion, no consensus has been reached on the association between type 2 diabetes mellitus and endothelial dysfunction (407). Most investigators studying animal models and subjects with type 1 diabetes report an association. Subjects with type 2 diabetes are a heterogeneous group and this may contribute to the diversity of findings in these cohorts. Furthermore, no studies have investigated insulin resistance (assessed by euglycaemic hyperinsulinaemic clamp) and endothelial dysfunction (assessed by venous occlusion plethysmography) in a well-defined homogeneous cohort of subjects with type 2 diabetes mellitus.
1.6. NEFA and cytokines as a possible signal linking insulin resistance and endothelial dysfunction

It is possible that decreased basal endothelial NO production and insulin resistance are manifestations of a common genetic or environmental antecedent. Pro-inflammatory cytokines as well as NEFA have been suggested as possible candidates (408).

NEFA and cytokines as a possible antecedent for insulin resistance have each been discussed in detail in section 1.2.4.3. Equally the relationship between NEFA and endothelial dysfunction and between cytokines and endothelial function have been discussed in detail in section 1.3.3. The question remains as to whether the association described between insulin resistance and endothelial dysfunction in healthy subjects, in insulin resistant states and in type 2 diabetes mellitus (described in section 1.5.) could be linked by a common antecedent.

Yudkin et al examined the link between insulin resistance and endothelial dysfunction in 107 apparently healthy volunteers. They used a cluster of acute phase proteins (fibrinogen and C-reactive protein) and circulating levels of pro-inflammatory cytokines (IL-6 and TNF-α), a cluster of markers of endothelial dysfunction (thrombomodulin, von Willebrand factor, tPA, fibronectin and mean albumin excretion rate) and a cluster of markers of the insulin resistance syndrome (triglycerides, HDL, systolic blood pressure, insulin sensitivity and pro-insulin) (56). The same group suggest that insulin resistance and other components of the insulin resistance syndrome may be a consequence of circulating levels of pro-inflammatory cytokines originating from adipose tissue and that the same compounds may be responsible for the endothelial damage with which insulin resistance is associated (409).
1.7. Summary

Insulin resistance is common and together with other components of the insulin resistance syndrome has detrimental consequences for health and survival. Accumulating evidence suggests that insulin resistance is associated with endothelial dysfunction in non-diabetic states, but whether this is the case in type 2 diabetes is unclear. NEFA and pro-inflammatory cytokines have been implicated as possible signals linking endothelial dysfunction to insulin resistance. However, there are still uncertainties about this relationship. With the increased incidence of vascular pathology in insulin resistant states in mind it is important to understand more about these relationships in health and disease.

In particular, it is unclear to what extent

a) Insulin resistance is associated with endothelial dysfunction in type 2 diabetes mellitus (section 1.4. and 1.5.),

b) Associations between insulin resistance and endothelial dysfunction in type 2 diabetes mellitus can be explained by circulating concentrations of NEFA or cytokines (section 1.6.),

c) Association between insulin resistance and triglyceride concentration is mediated through the primary effect of insulin resistance on circulating concentrations of NEFA (section 1.2.4.3.3.).
1.8. Aims of this thesis

1) To assess the relationship between endothelial function and insulin sensitivity in subjects with uncomplicated type 2 diabetes by using the euglycaemic hyperinsulinaemic clamp for the assessment of insulin sensitivity and venous occlusion plethysmography as well as circulating endothelial cell products for the assessment of endothelial function.

2) To evaluate NEFA and cytokines as a possible signal linking insulin resistance and endothelial dysfunction in subjects with uncomplicated type 2 diabetes.

3) To test the hypothesis that the relationship between insulin resistance and triglyceride concentrations is essentially mediated through insulin’s effect on plasma NEFA by analysis of the European Group for the Study of Insulin Resistance (EGIR) database of healthy volunteers.
Chapter 2
Methodology

In this chapter, the common protocols for the clinical techniques used in two studies are described:

1) The multicentre study of insulin resistance, lipid and fatty acid concentrations in healthy volunteers and
2) The study of insulin resistance, vascular reactivity, lipids, non-esterified fatty acids and cytokines in type 2 diabetes mellitus.

Where the methods differ they are described in detail for each study separately.

2.1. Methods common to both studies

2.1.1. Clinical and morphometric measurements

Investigations comprised history taking, clinical examination (including anthropometry and blood pressure), blood sampling (for lipids, non-esterified fatty acids (NEFA), fasting plasma insulin (FPI), fasting plasma glucose (FPG) in all subjects and in addition for HbA1c, cytokines and circulating endothelial cell products in the diabetic subjects only). Diabetic subjects also underwent measurement of 24-hour ambulatory blood pressure (AMBP). Insulin sensitivity was assessed by hyperinsulinaemic euglycaemic clamp in all subjects. Venous occlusion plethysmography for forearm blood flow in response to various vasoactive agents was performed in the diabetic subjects only.

2.1.1.1. Body mass index

*Weight* was measured in normal clothing, after removal of shoes and jacket or coat, using a digital weighing machine, to the nearest 0.1 kg (different manufacturers in different centres; our centre used Seca, Marsden, London, UK). *Height* was measured to the nearest 1 cm (different manufacturers in different centres; our centre used a Harpenden stadiometer (Holtain, Crosswell, Crymych, Dyfed, UK). *Body mass index* (BMI) was calculated as the weight (kg) divided by the square of the height (m).
2.1.2. **Waist-to-hip-ratio**
To calculate waist: hip ratio (WHR), the waist circumference (cm) was measured at the narrowest part of the torso and hip circumference (cm) was measured in a horizontal plane at the level of the maximal circumference of the buttocks. Waist and hip were measured with a steel tape in triplicate and the mean recorded.

2.1.3. **Body surface area**
Body surface area was read from a nomogram using measured height and weight (410).

2.1.4. **Blood pressure and heart rate**
During all the clinical studies the technique of blood pressure and heart rate measurement was uniform. Systolic and diastolic blood pressure and heart rate were measured after 10 minutes supine rest by an oscillometric technique using a Dinamap Critikon (different manufactures in different centres; our centre used Johnson and Johnson Professional Products Ltd., U.K.) semi-automatic sphygmomanometer, maintained and calibrated at regular intervals (in our centre by the Clinical Physics Department of Whittington Hospital). Medium (31cm x 12 cm) and large (39cm x 16 cm) cuffs were available in order to comply with the recommendations of the British Hypertension Society (411).

2.1.2. **General study conditions**
Studies were performed in a quiet clinical research area with steady room temperature (20-24 °C). Patients rested supine for at least 30 minutes after insertion of cannulae before any measurements were undertaken.

2.1.3. **Baseline blood sampling**
Blood samples were collected in the fasting state for the measurement of FPG, FPI, fasting plasma triglyceride, plasma total cholesterol, plasma HDL-cholesterol and NEFA concentrations at baseline (NEFA₀). The mean of two baseline samples was taken for NEFA₀.
2.1.4. Blood sampling during the hyperinsulinaemic clamp

At timed intervals during the hyperinsulinaemic clamp blood samples for the measurement of steady state plasma glucose, steady state plasma insulin and NEFA at steady state (NEFASS) were taken. These steady state values were measured during the last 40 minutes of the hyperinsulinaemic clamp (80-120 min). NEFASS was calculated as the mean of three samples. NEFA suppressibility (NEFASSUPP) was calculated as the percentage decline in NEFA concentrations from fasting levels (NEFAG) to steady state levels (NEFASS).

2.1.5. Measurement of insulin sensitivity

2.1.5.1. The hyperinsulinaemic clamp

Insulin sensitivity was assessed using the hyperinsulinaemic euglycaemic clamp technique at a maintenance insulin dose of 40 mU/ min/ m² (43).

An insulin solution was prepared. For this 50 units of soluble insulin (different manufacturers in different centres; our centre used Human Actrapid, NovoNordisk, Basingstoke, Hants, UK) and 1 ml of the patient’s own venous blood were diluted in 48 ml of isotonic Saline (Sodium Chloride 0.9%, different manufacturers in different centres; our centre used Fresenius Ltd., Basingstoke, England). Patients’ own blood was used in order to minimise adsorption of insulin to the plastic surfaces of syringes and infusion lines. Blood pressure and heart rate were measured and baseline blood samples withdrawn before the insulin infusion was commenced. At time 0 (t=0 min) this insulin solution was infused via an infusion pump (different manufacturers in different centres; our centre used Danby, DSP, Danby Medical Ltd., Earls Cone, UK) into the antecubital vein as a prime constant infusion. The priming regime was as follows:

<table>
<thead>
<tr>
<th>Time duration</th>
<th>Insulin Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4 minutes</td>
<td>160 mU/m²/min</td>
</tr>
<tr>
<td>4-7 minutes</td>
<td>80 mU/m²/min</td>
</tr>
<tr>
<td>7 minutes to end of clamp</td>
<td>40 mU/m²/min</td>
</tr>
</tbody>
</table>
The protocols of the two studies then differ in that in the healthy volunteers plasma glucose levels were kept within 10% of the baseline value and in the diabetic subjects the plasma glucose levels were kept within ± 10% of the target of 6 mmol/l. The infusion rate was adjusted for body weight for each individual and expressed as mg glucose/kg body weight/minute (mg/kg/min). For example, the infusion rate of 20% dextrose equal to 1 mg/kg/min for a 70 kg individual would be calculated as:

\[
\frac{(70 \text{ kg} \times 60 \text{ minutes})}{200 \text{ g Glucose}} = 21 \text{ ml/hour}
\]

At 5-minute intervals, 2 ml blood samples were withdrawn from the cannulated arterial line. After centrifugation at the bedside, the serum glucose concentration was determined (Section 2.1.6) and the dextrose infusion rate was manually adjusted.

### 2.1.5.2. Calculation of insulin sensitivity using the hyperinsulinaemic clamp

Clamp-derived insulin action was expressed as the whole-body glucose disposal rate (M) during steady state. At the dose of insulin used in these clamps, in healthy volunteers hepatic glucose output can be assumed to be almost fully suppressed (40;43;412). In subjects with type 2 diabetes hepatic glucose output may only be partially suppressed, thus leading to an underestimation of glucose disposal rates. The M-value is a measure of total body glucose metabolism, and reflects the ability of insulin to enhance tissue glucose disposal assuming suppression of hepatic glucose production. During hyperinsulinaemia with steady-state plasma glucose concentrations (usually during the last 40-60 minutes of the procedure), the glucose infused is equal to that being removed from the glucose space (i.e. glucose metabolised, M). A glucose distribution volume of 0.25 l/kg is assumed (43).

Insulin sensitivity was calculated in concordance with the method used by Ferrannini and the European Group for the Study of Insulin resistance (EGIR) (471). Insulin action was expressed as the whole-body glucose disposal rate during steady-state euglycaemic conditions.
hyperinsulinaemia. Glucose disposal (M-value) was calculated from the exogenous glucose infusion rate (μmol/min) during the last 40 min of the clamp after correction for changes in glucose concentration in a total distribution volume of 250 ml/kg. Whole body glucose disposal was normalized per kg of body weight (Mbw) or per kg fat-free mass (Mffm) as calculated by the Hume formula (414). Units used for Mbw are therefore μmol/min/kg bodyweight and for Mffm are μmol/min/kg fat free mass. The ‘M’-value was calculated as an index of insulin sensitivity expressing the mean glucose infusion rate of the last 40 min (time 00+80 to time 00+120) of the study corrected for the simultaneous variation in plasma glucose pool. The infusion rate was expressed as mg glucose/ kg body weight/ minute. A correction factor (CF) was added to the glucose infusion rate of the last 40 min of the clamp. This correction factor takes into account the fact that in practice, the glucose infusion rate must be modified by two factors before it can be equated with M:

\[ M = I - UC + CF \]

where:
- \( I \) = glucose infusion rate (mg/kg/min)
- \( UC \) = correction for urinary glucose loss (Usually negligible during an euglycaemic clamp)
- \( CF \) = ‘space correction’ (mg/kg/min) (for deviations from euglycaemia)

The correction factor for ‘space correction’ is calculated as follows (43):

\[ CF \text{ mg/kg/min } = \frac{[(G_{00+80} - G_{00+120}) \text{ mg/dl} \times 0.25 \text{ l/kg}]}{40 \text{ min} \times 10 \text{ dl/l}} \]

where:
- \( G_{00+80} \) = plasma glucose at 80 minutes from reaching euglycaemia (mmol/l)
- \( G_{00+120} \) = plasma glucose at 120 minutes from reaching euglycaemia (mmol/l)
- 0.25 l/kg = glucose distribution volume

Another index of insulin sensitivity is the M-value adjusted for insulin concentration. This is calculated as follows

\[ M_i = \frac{\text{Glucose infusion rate}}{\text{Mean insulin concentration}} \]

The mean insulin concentration is calculated as the mean of plasma insulin levels at time 00 of the clamp (prior to infusion of Dextrose), 00+60, 00+80 and 00+120 mins of the clamp.
Expression of insulin resistance as M/I did not materially alter the observed correlations when compared with those where M was used without correction for clamp insulin levels.

The reproducibility of the M-value had not been previously been evaluated in our centre. Morris et al with a similar technique have reported a between-day intra-subject coefficient of variation of 6% in healthy subjects (413). M’ was also expressed per kg lean body mass (‘M_{LBM}’) (414). However as the results were almost identical, only M_{BMI} results will be presented.

2.1.5.3. Calculation of insulin sensitivity using the Homeostasis Model Assessment

FPG and FPI are simple indices that have been used previously. Insulin sensitivity was assessed from FPG and FPI concentrations using Homeostasis Model Assessment analysis (HOMA % S), a physiologically structured computer model of the glucose and insulin homeostatic feedback mechanism (55).

2.1.6. Measurement of plasma glucose concentration

During the hyperinsulinaemic clamp procedures, plasma glucose concentration was measured at the bedside by the glucose oxidase-method using a glucose analyser (different manufactures in different centres; our centre used Beckman 2 analyser, Beckman Instruments, Fullerton, CA, USA), with inter-assay coefficient of variation (CV) of 2%. For assessment of plasma glucose levels at all other times samples were collected in fluoride-oxalate tubes and analysed in the local hospital biochemistry laboratory. Results were expressed in mmol/l.

2.1.7. Measurement of plasma insulin concentration

Insulin concentration was measured using an enzyme immunoassay (different kits in different centres; our centre used DAKO insulin, DAKO Diagnostics Ltd., Angel Grove, UK). This assay is based on two monoclonal antibodies. Simultaneous incubation of sample and enzyme-labeled antibody in a microplate well coated with a specific anti-
insulin antibody forms a complex. Unbound enzyme-labelled antibody is then removed by washing. The bound conjugate is detected by reaction with a substrate. The reaction is stopped by addition of an acid. The achieved colorimetric endpoint is read spectrophotometrically. The inclusion of calibrators of known insulin concentration in the assay allows a calibration curve to be constructed and the level of patient’s insulin to be determined. In our centre the intra-assay coefficient of variation (CV) was 8.7 % and inter-assay CV 12 %. Cross reactivity with intact pro-insulin was less than 5 % (415).

2.1.8. Measurement of non-esterified fatty acid concentration
NEFA concentrations were measured in serum by an optimized enzymatic colorimetric assay (Half-micro test, Boehringer Mannheim, PA, USA). NEFA are converted by adenosine-5’-triphosphate (ATP) and co-enzyme A into acyl-coenzyme A, resulting in the production of adenosine-5’-monophosphate (AMP) and pyrophosphate. Further reactions with oxygen lead (via the production of hydrogen peroxide) to formation of a red dye. This can be measured in the visible range at 546 nm. Intra-assay CV was 5.2 %.

2.1.9. Measurement of plasma lipids concentration
Total-cholesterol, HDL-cholesterol and triglyceride concentrations were assayed as follows: Plasma triglyceride concentrations were measured using GPO-PAP kit (different kits in different centres; our centre used Boehringer Mannheim, Lewes, E. Sussex, UK). In our centre interassay CV was 1.9 %. Plasma cholesterol concentrations were measured by an enzymatic, colorimetric method using kit C system high performance CHOD-PAP method (different kits in different centres; our centre used Boehringer Mannheim, Lewes, E. Sussex, UK). In our centre interassay CV was 1.6%. HDL-cholesterol concentrations were measured in solution after precipitation of LDL and VLDL using sodium heparin and manganese chloride. Interassay CV was 5.5%. LDL-cholesterol was calculated using the Friedewald formula (416).
2.2. Methodology for the study of Insulin resistance, lipid and fatty acid concentrations in 867 healthy volunteers

In this section, the protocols for the clinical techniques used in the multicentre study of Insulin resistance, lipid and fatty acid concentrations in healthy volunteers are described. We analysed data from the European Group for the Study of Insulin resistance (EGIR) database, which has been described previously (50;417). Twenty-one clinical research centres in Europe participated in the retrospective collection of these data. Centres provided data from their euglycaemic hyperinsulinaemic clamps (between 21 and 129 cases per centre). Approval of the protocol by the local Ethics Committee and informed consent from all subjects was obtained prior to the studies at each centre.

2.2.1. Subjects

In this study 1666 healthy normoglycaemic volunteers without evidence of cardiac, renal, pulmonary, endocrine or rheumatic disease on history and clinical examination by a physician were studied. Highly physically trained individuals, vegetarians, subjects with recent change in body weight (> 10%) and those recently immigrated into Europe were excluded. Subject’s ethnicity was not reported.

2.2.2. Vascular access

Venous access was obtained by aseptic insertion of a venous catheter into an antecubital vein in the dominant arm (different manufacturers in different centres; our centre used Venflon® 2, 18 G/ 45 mm, Ohmeda, Helsingborg, Sweden). A wrist vein was cannulated retrogradely and heated at 60°C in a hot box or a heating pad for sampling of arterialised venous blood.

The skin was locally anaesthetised with 2 to maximum 5 ml of 2 % Lignocaine (different manufacturers in different centres; our centre used Lignocaine Hydrochloride 2 % w/v, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) prior to insertion. The line was kept
patent by the infusion of isotonic Saline (Sodium Chloride 0.9%, different manufacturers in different centres; our centre used Fresenius Ltd., Basingstoke, England) at a rate of 30 ml/hour.

2.2.3. Measurement of insulin sensitivity

The hyperinsulinaemic euglycaemic clamp

At time zero a primed constant infusion of insulin was commenced and continued for 120 minutes as described in section 2.1.5.1. An intravenous glucose infusion of 20 % Dextrose (20% Glucose, different manufacturers in different centres; our centre used Baxter, Healthcare Ltd., Thetford, UK) was started via infusion pump (different manufacturers in different centres, our centre used Baxter, Healthcare Ltd., Thetford, UK) at 4 minutes. The glucose infusion rate was adjusted to ensure that the plasma glucose levels remained within 10 % of the baseline value (section 2.1.5.1.)

2.2.4. Database and Statistical analysis

The SPSS statistical package version 6.1 (SPSS, Chicago, USA) was used to analyse the data by regression analysis.

Initially 1666 subjects were included in the database. Subjects with missing data on fasting glucose, fasting lipids, age, BMI or sex were excluded. This exclusion resulted in a database of 1266 subjects. Dummy variables were introduced and used throughout the regression analysis to account for inter-centre differences. Skewed variables such as plasma insulin levels were log-transformed to normalise their distribution. Residuals were then examined and subjects with data outside ±4 z-scores were excluded from further analysis. All parameters were thus normally distributed before submission to regression analysis. Multiple regression analysis was thereafter performed on 867 subjects (M: F =591: 276). The characteristics of the 867 subjects are shown in table 2.1. Data on NEFA concentrations were available in a subgroup of 541 subjects from 9 clinical centres. These subjects’ characteristics do not differ significantly from those of the whole cohort (n=1266) or the larger subgroup (n=867).
Because of the strong relationships between obesity and insulin resistance and between obesity and lipoprotein metabolism, we corrected for the influence of obesity by including the BMI in the model throughout the analysis. The model was also adjusted for age, sex and geographic location of the study centre. We sought univariate correlation between indices of insulin resistance, non-esterified fatty acid regulation and lipoprotein concentrations. Multiple regression analysis was subsequently carried out to examine whether such univariate relationships were independent of each other.

Table 2.1: Characteristics of the study cohort (867 subjects, Male : Female=591:276).

<table>
<thead>
<tr>
<th></th>
<th>Mean±SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years)</td>
<td>44.3±16.4</td>
<td>18-84</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.8±4.5</td>
<td>17.5-55.1</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.89±0.09</td>
<td>0.59-1.18</td>
</tr>
<tr>
<td>FPG (mmol/l)</td>
<td>5.1±0.5</td>
<td>3.2-6.5</td>
</tr>
<tr>
<td>FPI (pmol/l)</td>
<td>69.3±44.7</td>
<td>10.0-335.0</td>
</tr>
<tr>
<td>total cholesterol (mmol/l)</td>
<td>5.54±1.34</td>
<td>1.30-11.30</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.20±0.35</td>
<td>0.10-2.50</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.46± 1.09</td>
<td>0.20-11.70</td>
</tr>
<tr>
<td>NEFAq (µmol/l)</td>
<td>577.7+219.8</td>
<td>90.0-1473.0</td>
</tr>
<tr>
<td>NEFAss (µmol/l)</td>
<td>79.5+70.4</td>
<td>1.0-459.0</td>
</tr>
<tr>
<td>NEFAsupp (%)</td>
<td>85.8±11.1</td>
<td>25.6-99.9</td>
</tr>
<tr>
<td>M_LBM(µmol/min/kg lean body mass)</td>
<td>49.6+17.1</td>
<td>3.6-117.1</td>
</tr>
<tr>
<td>M_bw(µmol/min/kg body weight)</td>
<td>34.2+13.5</td>
<td>2.5-102.2</td>
</tr>
</tbody>
</table>

FPG fasting plasma glucose;
FPI fasting plasma insulin;
NEFAq fasting non-esterified fatty acid concentrations;
NEFAss non-esterified fatty acid concentrations at steady-state;
NEFA_SUPP percentage suppression of non-esterified fatty acid concentrations from fasting to steady state;
M_LBM mean glucose infusion rate at steady state normalised per kg lean body weight;
M_bw mean glucose infusion rate at steady state normalised per kg body weight;
2.3. Methodology for the study of insulin resistance, vascular reactivity, lipids, NEFA and cytokines in type 2 diabetes mellitus

In this section, the protocols for the clinical techniques used in the study of insulin resistance, vascular reactivity, lipids, NEFA and cytokines in type 2 diabetes mellitus are described.

2.3.1. Patients

The protocol was approved by the local Ethical Committees of the Whittington and Royal Free Hospitals. All clinical assessments were performed in the Centre for Diabetes and Cardiovascular Risk, Department of Medicine, University College London Medical School.

38 patients (31 male, 7 female) with type 2 diabetes mellitus were recruited from the diabetic outpatient departments at the Whittington Hospital and the Royal Free Hospital London. All patients fulfilled the ADA diagnostic criteria for the diagnosis of type 2 diabetes mellitus (418). Patients were either newly diagnosed with type 2 diabetes mellitus, having been stabilised on diet and exercise, or were taking oral hypoglycaemic agents and could be temporarily withdrawn from treatment. The following inclusion and exclusion criteria were used:

**Inclusion criteria for the vascular function study**
- age between 40 to 80 years
- haemoglobin $A_1c \leq 10\%$
- fasting plasma glucose of $\geq 7 \text{ mmol/l} \text{ to } < 15 \text{ mmol/l}$

**Exclusion criteria for the vascular function study**
- Body Mass Index $\geq 35 \text{ kg/m}^2$
- Clinically significant renal impairment (serum creatinine $> 134 \text{ µmol/l}$)
• Clinically significant hepatic disease (Alanine Transaminase, Aspartate Transaminase, total bilirubin or alkaline phosphatase more than 2.5 times upper limit of normal)
• Significant anaemia (haemoglobin< 11 g/dl for males or < 10 g/dl for females)
• Severity of diabetes imminently requiring treatment with insulin
• Proteinuria (urinary albumin excretion > 200 mg/24 hours)
• Diabetic complications such as
  • retinopathy (seen on direct dilated fundoscopy or patients requiring laser treatment within the last 6 months),
  • peripheral vascular disease (detected on clinical examination for absent peripheral pulses or requiring treatment within the previous 6 months),
  • peripheral neuropathy (detected on neurological examination for loss of reflexes, vibration sense and sensation to touch and pinprick)
  • recent evidence of ischaemic heart disease (acute cardiac event in within the previous 12 months, history of typical chest pains and evidence of acute or recent ischaemia on ECG)
• Hypertension defined as systolic blood pressure > 180 mmHg or diastolic blood pressure > 110 mmHg
• Treatment with nitrates, calcium channel blockers, nonsteroidal anti-inflammatory drugs, high dose aspirin (>75 mg/day) or warfarin
• Change of dose in treatment with Angiotensin-converting enzyme (ACE) inhibitors, beta-blockers or diuretic therapy less than 7 weeks prior to study
• Active alcohol or drug abuse within the last 6 month

There was a greater proportion of male subjects (31, 81.6%) than female subjects (7, 18.4%). The majority of subjects was Caucasian (24, 63.2%, 19 male, 5 female), another group was Asian (11, 29%, all male) and three subjects were Afro-Caribbean (3, 7.8%, one male, 2 female). All women participating in the study were postmenopausal. The
median duration of diabetes from date of diagnosis was 56.2 months (range 6.0 - 360.0).
A small number of subjects smoked tobacco (6, 15.7%).

2.3.2. Clinical and morphometric measurements
In addition to the measurements described in section 2.1.1. the following measurements were also taken in this study.

2.3.2.1. Skin fold thickness
Skin fold thickness over the biceps at the level of midpoint between the olecranon process and the head of humerus was measured using a pair of callipers (Holtain, Crosswell, Crymych, Dyfed, UK), in triplicate, and the mean recorded to the nearest mm (419).

2.3.2.2. Forearm volume
Forearm volume was measured to the nearest 2 cm by volume displacement method using a specially designed graduated water-filled measuring cylinder. Forearm volume was calculated by subtraction of volume displaced by insertion to the ulnar styloid from the volume displaced by insertion to the olecranon process.

2.3.2.3. Ambulatory blood pressure
Measurement of 24-hour ambulatory blood pressure was performed the day prior to the study with a Takeda TM-2420 ambulatory blood pressure monitor (A&D Company Ltd, Shibuya-ku, Tokyo, Japan) at 30-minute intervals during the day (8am to 10pm) and hourly at night (10pm to 8am). The blood pressure cuff was placed on the non-dominant arm and subjects were advised to carry out their normal daily routine, but to avoid intense physical exercise during these 24 hours.

2.3.2.4. Intra-arterial blood pressure measurements
Intra-arterial blood pressure was measured continuously during the study after insertion of the arterial cannula (see section 2.3.7.) using a physiological pressure transducer
attached to the brachial artery line via a disposable pressure dome (SensoNor 840, SensoNor, Horten, Norway).

2.3.3. General clinical protocol
Before entering the study, all patients underwent full clinical screening (personally performed) including history taking, physical examination, routine biochemistry, haematology, dip-stick urinalysis, and an electrocardiogram as a screening test for significant cardiovascular disease or end-organ damage.

2.3.4. Clinical characteristics
The physical examination was normal in all subjects. The characteristics of the subjects are shown in Table 2.2.

Table 2.2: Characteristics of subjects participating in the study of insulin resistance, vascular reactivity, lipids, non-esterified fatty acids and cytokines in type 2 diabetes mellitus

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data are shown as mean ± SD unless stated otherwise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (m:f)</td>
<td>38 (31:7)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.4 ±10.4</td>
</tr>
<tr>
<td>Waist-hip ratio -men</td>
<td>0.97±0.05</td>
</tr>
<tr>
<td>-women</td>
<td>0.84±0.06</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.8±3.8</td>
</tr>
<tr>
<td>Biceps skin fold (cm)</td>
<td>24.3±9.7</td>
</tr>
<tr>
<td>Duration of diabetes (median in months)</td>
<td>56.2 (range 6.0-360.0)</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>11.0±2.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.3±1.4</td>
</tr>
<tr>
<td>24 hour systolic blood pressure (mmHg)</td>
<td>136±19</td>
</tr>
<tr>
<td>24 hour diastolic blood pressure (mmHg)</td>
<td>86±14</td>
</tr>
</tbody>
</table>
2.3.5. Concomitant medication

The use of medication prior to and during the study is shown in table 2.3. All oral hypoglycaemic medication was stopped 4 weeks prior to the study. Due to symptomatic hyperglycaemia during the washout period 5 patients were unable to participate in further studies. These subjects did, on screening, not differ from the main cohort in their baseline characteristics. Ten patients had never received any hypoglycaemic medication. Aspirin was also stopped 4 weeks prior to the study. Antihypertensive medication and statins were not used on the day of the study. None of the subjects had ever received treatment with insulin.

Table 2.3: Use of medication prior and during the study (oral hypoglycaemics and aspirin were stopped 4 weeks prior to the study)

<table>
<thead>
<tr>
<th>Medication</th>
<th>Sulphonyl-ureas alone</th>
<th>Metformin alone</th>
<th>Sulphonyl-ureas and metformin</th>
<th>Aspirin (75 mg daily)</th>
<th>Beta blockers</th>
<th>Angiotensin converting enzyme inhibitors</th>
<th>Statins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>14 (36.8%)</td>
<td>7 (18.4%)</td>
<td>7 (18.4%)</td>
<td>2 (5.3%)</td>
<td>5 (13.2%)</td>
<td>9 (23.7%)</td>
<td>3 (7.9%)</td>
</tr>
</tbody>
</table>

2.3.6. Study visits

After informed consent was obtained patients attended for an initial screening visit. All oral hypoglycaemic agents were stopped thereafter and patients commenced a 4-week placebo run-in period. For the length of the study patients were advised to follow a diet to maintain their body weight. Patients were then studied for two consecutive days. On each day on which a clinical assessment was to be performed, patients were transported by taxi to the Centre for Diabetes and Cardiovascular Risk at 0730hrs after an overnight fast from 1930hrs. All patients were asked to avoid alcohol and caffeine in the 24 hours prior to an assessment. A light meal was provided at the end of each assessment prior to taxi transport home.
2.3.7. **Vascular access**

Venous access was obtained by aseptic insertion of a venous catheter (Venflon® 2, 18 G/45 mm, Ohmeda, Helsingborg, Sweden) into an antecubital vein in the dominant arm.

Arterial access was obtained by aseptic insertion of an arterial catheter (Radial Arterial Catherisation Set, Arrow, 20 G/4.45 cm, Arrow International Inc., Reading, USA) into the brachial artery in the non-dominant arm at the level of the antecubital fossa.

The skin was locally anaesthetised with 2 to maximum 5 ml of 2 % Lignocaine (Lignocaine Hydrochloride 2 % w/v, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) prior to insertion of each cannula. Both lines were kept patent by the infusion of isotonic Saline (Sodium Chloride 0.9%, Fresenius Ltd., Basingstoke, England) at a rate of 30 ml/hour.

2.3.8. **Measurement of insulin sensitivity**

2.3.8.1. **The hyperinsulinaemic clamp**

At time zero (t=0) a primed constant infusion of insulin was commenced and continued for 120 minutes as described in section 2.1.5.1. An intravenous glucose infusion of 20 % Dextrose (20% Glucose, Baxter Healthcare Ltd., London, UK) was started via infusion pump (Baxter, Healthcare Ltd., Thetford, UK) when the plasma glucose concentration reached 6 mmol/l (time 00). The infusion rate was expressed as mg glucose/ kg body weight/ minute. Arterial plasma was sampled every 5 minutes for immediate analysis of plasma glucose. 2 ml blood samples were withdrawn each time. After centrifugation at the bedside, the serum glucose concentration was determined and the dextrose infusion rate was manually adjusted to ensure that the plasma glucose levels remained within ± 10 % of the target of 6 mmol/l.
2.3.9. Measurement of endothelial function

2.3.9.1. Venous occlusion plethysmography

2.3.9.1.1. Clinical procedure

Forearm blood flow (FBF) was measured using venous occlusion plethysmography (322;420) (Hokanson set, PMS Instruments, Hastings, Sussex, UK). A mercury strain gauge of appropriate circumference (2 cm shorter than actual forearm circumference) was placed at 8 cm distally from each olecranon. The strain gauge was calibrated electronically on the arm to the chart recorder programme using a MacLab analyser (AD Instruments, Castle Hill, Australia). A wrist cuff (paediatric arterial occlusion cuff: HokansonSC5, PMS Instruments, Maidenhead, Berkshire) was inflated on each wrist to 20 mmHg above systolic pressure one minute prior to inflation of the venous cuff (HokansonSC10) on each upper arm above the elbow. The venous occlusion pressure was 50 mmHg. Rapid cuff inflation was achieved via an air cylinder coupled to a rapid cuff inflator (Hokanson E20). Care was taken to ensure that the limbs were relaxed by supporting them with foam blocks. Six consecutive readings of FBF were taken over 2 minutes and thereafter the wrist cuffs were deflated.

2.3.9.1.2. Calculation of forearm blood flow

Data were acquired via MacLab II chart recorder (AD Instruments, Castle Hill, Australia). FBF was derived from the slope of the plethysmography traces on the chart recorder. Each blood flow measurement was the mean of 6 sequential recordings. Slopes were calculated from data points by acquiring their co-ordinates using the software MacChart (AD instruments) and pasting them into a customised spreadsheet.

After discussion with another scientific group with much experience in plethysmography a number of criteria have been chosen in order to 'quality control' the blood flow measurements and to avoid artifacts or experimental errors (Natali 2001, personal communications):

1. Each dose step (vascular response to acetylcholine or sodium nitroprusside) must have at least 3 readable recordings.
2. The readable recordings within each dose step must have consistent values (variability < 50% from mean value).

3. Patients must have both the baseline [dose 0] and 2/3 of the post-baseline dose steps for SNP. In addition, the slope correlation coefficient for the SNP dose-response curve for the two lower doses of SNP must be >0.70.

4. Patients must have both the baseline [dose 0] and 3/5 of the post-baseline dose steps for ACh. In addition, the slope correlation coefficient for the ACh dose-response curve for the first three doses of ACh must be >0.70.

Application of these criteria led to exclusion of 6 (15.8%) out of the 38 patients who had been studied. These subjects did not differ from the whole cohort in any other aspect.

There are several ways of expressing the forearm blood flow (FBF):

1. Measurements can be used as absolute values (absolute FBF in ml/min/dl).

2. Measurements can be expressed after adjustment for forearm volume (321). This will be called FBF_{pv} and it can be calculated as follows:

\[ FBF_{pv} = \frac{FBF}{FV} \]

3. Taking into account the differences in basal FBF, FBF can also be expressed as a ratio change from baseline. This ratio will be called ΔFBF and can be calculated as follows:

\[ \Delta FBF = \frac{FBF_{(st)_{drug}}}{FBF_{(st)_{vehicle}}} \times 100\% \]

Where

FBF_{(st)_{drug}} = FBF in the study (infused) arm during infusion of vasoactive drug
FBF_{(st)_{vehicle}} = FBF in the study (infused) arm at baseline.
4. FBF can also been expressed as percentage change from baseline with respect to vascular resistance, whereby

\[ \text{Vascular resistance} = \frac{\text{mean blood pressure}}{\text{FBF}}. \]

5. Finally, vascular reactivity to an infusion of a vasoactive drug can be expressed as the slope of the respective dose-response curves (drug infusion rate vs. FBF). The slope (%FBF increments above baseline per µg/min/dl of infused drug) was computed by a linear regression model with no intercept (EXCEL1997 statistical package). For this analysis the FBF value at the highest dose of vasoactive drug was excluded because, with the doses here employed, this value consistently falls outside the linear proportion of the dose-response curve.

In our centre the intraindividual day to day variation is 13.1% (421)

2.3.9.2. Local administration of drugs into the brachial artery

Infusion of various vasoactive agents via infusion pumps (Danby, DSP, Danby Medical Ltd., Earls Cone, UK) enabled us to construct dose-response curves. All infusates were prepared in the Whittington Hospital Pharmacy Department under sterile conditions on the morning of the study. The design with timing of infusions and FBF measurements are shown in figure 2.1. Prior to infusion drug dosage was adjusted for forearm volume. However, taking into account that the forearm is a physiological reservoir, this may result in the introduction of an additional con-founding factor. It may be more useful to infuse the same dose into each patient and, if deemed necesssay, to adjust for forearm volume or length after measuring blood flow.
Figure 2.1: Study design for the study of insulin resistance, vascular reactivity, lipids, non-esterified fatty acids and cytokines in type 2 diabetes mellitus

FBF = Forearm blood flow, ACh = acetylcholine, SNP = Sodium Nitroprusside, L-NMMA = Monomethyl-arginine

Intra-arterial infusion of ACh
Intra-arterial infusion of SNP
Systemic infusion of insulin and Dextrose
2.3.9.2.1. Acetylcholine

Each 20 mg vial of Acetylcholine (ACh) (Hoffman-La Roche, Basel, Switzerland) was reconstituted in 2 ml of isotonic saline. This solution was then diluted in 98 ml of isotonic saline to achieve 100 ml of a mother solution (200 μg/ ml). For each subject a volume of mother solution (depending on forearm volume) was calculated using the following formula to allow delivery in a 50 ml syringe using standard infusion rates for all patients for the first three infusion steps:

\[ V (\text{ml}) = FV (\text{dl}) \times 0.15 (\mu\text{g/ min/ dl}) \times 50 (\text{ml}) \times 0.083^{-1} (\text{ml/ min}) \times (200^{-1} \mu\text{g/ml}) \]

- \( FV = \) forearm volume
- \( 0.15 (\mu\text{g/ min/ dl}) = \) first dose of ACh to be infused
- \( 50 \text{ ml} = \) total volume to allow delivery in a 50 ml syringe
- \( 0.083 \text{ ml/min} = \) correction to allow a standard delivery rate of 5 ml /hour
- \( 200 \mu\text{g/ml} = \) concentration of ACh in mother solution

This determined the volume of mother solution to be then further diluted in normal saline up to a total volume of 50 ml to allow delivery in a 50 ml syringe (for the first three dose steps). The remaining two dose steps were achieved by delivery of mother solution in a 50 ml syringe. ACh was infused in five 5-minute steps of 0.15, 0.45, 1.5, 4.5 and 15 μg/ min/ dl and FBF was measured after each dose as described above.

2.3.9.2.2. Sodium Nitroprusside

Each 100 mg vial of Sodium nitroprusside (SNP) (Malesci, Instituto Farmacobiologico S.p.A., Firenze, Italy) was reconstituted in 10 ml normal saline. This solution was then diluted in 90 ml of normal saline to achieve 100 ml of a mother solution (1000 μg/ ml). For each subject a volume of mother solution (depending on forearm volume) was calculated using the following formula to allow delivery in a 50 ml syringe using standard infusion rates for all patients.
\[
V(\text{ml}) = FV (\text{dl}) \times 1 (\mu\text{g/ min/ dl}) \times 50 (\text{ml}) \times 0.083^{-1} (\text{ml/ min}) \times 1000^{-1} \mu\text{g/ml}
\]

- \(FV\) = forearm volume
- 1 (\(\mu\text{g/ min/ dl}\)) = first dose of SNP to be infused
- 50 ml = total volume to allow delivery in a 50 ml syringe
- 0.083 ml/min = correction to allow a standard delivery rate of 5 ml/hour
- 1000 \(\mu\text{g/ml}\) = concentration of SNP in mother solution

This determined the volume of mother solution to be then further diluted in normal saline up to a total volume of 50 ml to allow delivery in a 50 ml syringe to allow delivery in a 50 ml syringe. Light exposure was avoided by covering the SNP solution with aluminum foil throughout preparation and use during the study. After a washout period of at least 30 minutes SNP was infused in three 5-minute steps of 1, 2 and 4 \(\mu\text{g/ min/ dl}\) and FBF was measured after each dose as described above.

### 2.3.9.2.3. N-monomethyl-L-arginine

Each 10 mg vial of L-monomethyl-N-arginine (L-NMMA) (Clinalfa, Clinalfa AG, Läufelingen, Switzerland) was diluted in 10 ml of normal saline into a 50 ml syringe. At the end of the clamp (time 00+120) minutes this solution was infused at a rate of 100 \(\mu\text{g/ min/dl}\) for 5 minutes and FBF was measured as described.

### 2.3.10. Measurement of forearm blood flow in response to systemic infusion of insulin

FBF was also measured following the continuous intra-venous infusion of insulin during the clamp as described in section 2.3.8.1. FBF was measured at times 60 and 120 minutes once euglycaemia had been established.

### 2.3.11. Measurement of circulating acute phase proteins and cytokines

Blood samples for measurement of cytokines were collected into tubes containing sodium citrate, centrifuged for 15 min at 3000g and 4°C. Samples were immediately aliquoted and the
separated plasma was stored at -70 °C until analysis. All samples were taken as part of the ‘baseline samples’ prior to any intervention.

2.3.11.1. Interleukin-6 (IL-6)
IL-6 was measured using an enzyme linked immunoabsorbent assay, specific for the total amount of IL-6 (R&D Systems, Oxford, UK). The assay had a intra-assay CV of 5.3% and an interassay CV of 9.2%.

2.3.11.2. Tumour necrosis factor α (TNFα)
TNFα was measured in serum by high sensitivity 2-site ELISA (Quantikine™ HS, R&D Systems, Minneapolis, USA). Intra-assay CV was 8.9% (n=20) and interassay CV was 10.2 % (n=20).

2.3.12. Measurement of parameters of fibrinolysis
Blood samples for measurement of fibrinolytic activity were collected into 5 ml volume tubes containing sodium citrate to achieve concentrations between 0.1 and 0.5 mmol if 4.5 ml of blood were added. These were then centrifuged for 15 mins at 3000g and 4°C and the separated plasma was stored at -70 °C until analysis. All samples were taken as part of the ‘baseline samples’ prior to any intervention.

2.3.12.1. Plasminogen activator inhibitor 1 (PAI-1)
PAI-1 antigen was measured by ELISA using the Biopool TintElize kit (TintElize, Biostat, Stockport, UK). The within assay CV was 4.5 % (n=10) and the inter-asssay CV was 7.5 % (n=12).

2.3.12.2. Tissue plasminogen activator antigen (t-PA)
Tissue plasminogen activator antigen (t-PA) was measured using the Biopool TintElize kit that quantifies human single-chain and two-chain t-PA antigen. This assay is a two-site ELISA, using quenching and normal antibodies as control for the t-PA specificity. This
ensures that t-PA antigen measurements are unbiased by the presence of other antibodies, e.g. rheumatoid factor. Intra-assay CV was 6% (n=10) and the interassay CV was 10% (n=10).

2.3.13. Glycated haemoglobin (HbA1c)
HbA1c was measured with agar gel electrophoresis (Corning, Halstead, UK). Interassay CV was 5% (n=20) and intra-assay CV was 3% (n=20).

2.3.14. Statistical analysis
Statistical analysis was performed using the SPSS statistical package 8.0 (SPSS, Chicago, USA). Data are reported as mean ± SD for normally distributed variables and geometric mean ×/÷ SD for skewed variables. Between-group differences in mean values were analysed by unpaired t-test (for continuous variables) and by chi-square test (for proportions). A 2-way ANOVA was used to test the effect of the grouping variable, the effect of the experimental procedure and their interaction. Simple and multiple regression analyses were performed by standard methods. Details of statistical analysis used are also shown in each relevant section of the results.

Measurements of bedside plasma glucose, insulin sensitivity and forearm blood flow in our centre were performed by myself. Assays for insulin and NEFA in our centre were performed by Dr Vidya Mohamed-Ali and colleagues at the Centre for Diabetes and Cardiovascular Risk, Department of Medicine, University College London Medical School. The assays as part of the EGIR study were performed in each local centre. Assays for lipids and HbA1c were performed by Chemical pathology at the Whittington Hospital. Dr Vidya Mohamed-Ali and colleagues also performed the assays of cytokines and fibrinolytic parameters.
Chapter 3

Insulin resistance, lipid and fatty acid concentrations in healthy volunteers (Results and discussion)

3.1. Introduction

Insulin resistance, dyslipidaemia and abnormal free fatty acid metabolism are common features of the insulin resistance syndrome (58-61;113). Deficits in suppression of NEFA concentrations have been demonstrated in insulin resistant states, as expected from insulin’s well-established role as the major regulator of lipolysis (47;143;144). NEFA as a possible antecedent for insulin resistance has been discussed in detail in section 1.2.4.3.3.

There is a strong correlation between NEFA levels and liver glucose output under a variety of experimental conditions. If NEFA are maintained at basal concentrations during insulin administration, glucose output fails to decline. If NEFA concentrations are reduced independent of insulin administration, glucose output is reduced. These points support the concept that insulin, by regulating adipocyte lipolysis, controls liver glucose production (148;422). The influence of adiposity in this relationship is important since strong associations exist between obesity and insulin resistance and between obesity and lipoprotein metabolism (49;145-147). Additional evidence that NEFA might act as the primary signal include reports that suppression of lipolysis with an adenosine receptor agonist lowers NEFA concentrations and glucose production without changing plasma insulin levels (422). It has been suggested that the control of NEFA concentrations in the blood could be an important approach to reduce insulin resistance and the complications of the insulin resistance syndrome (141). The question remains if (and how) NEFA metabolism is associated with insulin resistance in healthy subjects. The second question follows: is the hypertriglyceridaemia observed with increased insulin resistance mediated by abnormal NEFA metabolism?
3.2. Aims of the study

The aims of this study were firstly to examine the relationship of NEFA metabolism with insulin resistance in healthy volunteers and secondly to investigate if the hypertriglyceridaemia seen in insulin resistant states is mediated by abnormal NEFA metabolism. This was attempted by studying the relationship between insulin resistance and plasma triglyceride and NEFA concentrations, in healthy non-diabetic subjects by analysis of the European Group for the Study of Insulin Resistance (EGIR) database. Study methodology is described in chapter 2.

3.3. Results

The characteristics of the 867 subjects are shown in table 2.1. Markers measured in the fasting (fasting plasma insulin and fasting NEFA concentrations) and hyperinsulinaemic (M-value and steady-state NEFA concentrations) state were analysed. Correlations were seen between insulin resistance on the one hand and both triglycerides and NEFA concentrations in fasting and hyperinsulinaemic steady state on the other (Figures 3.1, 3.2, 3.3, 3.4). Because of the known relationships of obesity with insulin resistance and with abnormal lipid parameters, we also sought for these associations after correction for BMI, age, sex and inter-centre variation.

3.3.1. Insulin resistance and plasma lipids

There were significant relationships between concentrations of triglycerides and markers of insulin sensitivity in both the fasting and the hyperinsulinaemic state (table 3.1.). The association between HDL-cholesterol and indices of insulin resistance after correction for triglyceride levels was no longer significant.
Table 3.1: Partial correlations of indices of insulin resistance and other metabolic measures with fasting lipid concentration. Partial correlation coefficients are shown after correction for BMI, age, sex and geographical centre. Significant values are shown in bold.

<table>
<thead>
<tr>
<th>Triglycerides</th>
<th>HDL-cholesterol</th>
<th>Total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partial correlation coefficient β</td>
<td>p-value</td>
</tr>
<tr>
<td>WHR</td>
<td>+0.231</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FPI</td>
<td>+0.275</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FPG</td>
<td>+0.188</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HOMA%S</td>
<td>-0.286</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mbw</td>
<td>-0.224</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

WHR: waist:hip ratio  
FPI: fasting plasma insulin  
FPG: fasting plasma glucose  
HOMA%S: relative insulin sensitivity estimated by homeostasis model assessment  
Mbw: mean glucose infusion rate at steady state normalised per kg body weight

3.3.2. Insulin resistance and NEFA concentrations

3.3.2.1. Insulin resistance and fasting NEFA concentrations

After correction for age, sex, BMI and geographical centre the association of NEFA₀ with FPI and of NEFA₀ with HOMA%S was not significant. NEFA₀ remained significantly correlated to Mbw. (table 3.2).

3.3.2.2. Insulin resistance and steady-state NEFA concentrations

NEFAₜₜ concentrations during the hyperinsulinaemic clamp were also correlated to markers of insulin resistance in the fasting (FPI and HOMA%S) and hyperinsulinaemic (Mbw) state. (table 3.2).

3.3.2.3. NEFA suppressibility

NEFAₜₜ was significantly correlated to FPI and HOMA%S, but not to Mbw. (table 3.2).
Table 3.2: Partial correlations of indices of insulin resistance and other metabolic measures with non-esterified fatty acid concentrations.

Partial correlation coefficients are shown after correction for BMI, age, sex and geographical centre. Significant values are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>NEFA₀</th>
<th>NEFA₅₅</th>
<th>NEFA_SUPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial correlation coefficient β</td>
<td>p-value</td>
<td>Partial correlation coefficient β</td>
<td>p-value</td>
</tr>
<tr>
<td>WHR</td>
<td>-0.1657</td>
<td>&lt;0.05</td>
<td>+0.1658</td>
</tr>
<tr>
<td>FPI</td>
<td>+0.0282</td>
<td>NS</td>
<td>+0.1583</td>
</tr>
<tr>
<td>FPG</td>
<td>-0.0340</td>
<td>NS</td>
<td>+0.0943</td>
</tr>
<tr>
<td>HOMA%S</td>
<td>-0.023</td>
<td>NS</td>
<td>-0.1076</td>
</tr>
<tr>
<td>Mbw</td>
<td>-0.2454</td>
<td>&lt;0.0001</td>
<td>-0.1609</td>
</tr>
</tbody>
</table>

NEFA₀ fasting non-esterified fatty acid concentrations;
NEFA₅₅ non-esterified fatty acid concentrations at steady-state;
NEFA_SUPP percentage suppression of non-esterified fatty acid concentrations from fasting to steady state;
WHR waist:hip ratio;
FPI fasting plasma insulin;
FPG fasting plasma glucose;
HOMA%S relative insulin sensitivity estimated by homeostasis model assessment;
Mbw mean glucose infusion rate at steady state normalised per kg body weight;

3.3.3. Triglycerides, NEFA and insulin resistance

The correlations of fasting triglyceride concentrations with other plasma lipoprotein concentrations, NEFA concentrations and indices of insulin resistance are shown in tables 3.1 and 3.3.

Significant associations were found between fasting triglyceride concentrations and NEFA₅₅ and between fasting triglyceride concentrations and NEFA_SUPP, but not with NEFA₀. Fasting triglyceride concentrations related both to Mbw and to NEFA₅₅ (table 3.1 and 3.3). Associations between triglyceride and NEFA concentrations and between triglyceride concentrations and insulin resistance were independent of each other: Partial correlation of fasting triglyceride concentrations and Mbw as a measure of insulin resistance remained significant after correction for NEFA levels at steady-state (NEFA₅₅): β=−0.276, p<0.0001.
Interestingly, partial correlation of fasting triglyceride concentrations and NEFA$_{SS}$ remained significant (but at a lower level) even after correction for $M_{bw}:\beta=0.109$, $p=0.03$.

Table 3.3: Partial correlations of triglyceride concentrations with indices of insulin resistance, NEFA levels and other lipid concentrations. Partial correlation coefficients are shown after correction for BMI, age, sex and geographical centre.

<table>
<thead>
<tr>
<th></th>
<th>Partial correlation coefficient $\beta$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides vs. total cholesterol</td>
<td>+0.322</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. HDL-cholesterol</td>
<td>-0.343</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. LDL-cholesterol</td>
<td>+0.143</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Triglycerides vs. NEFA$_0$</td>
<td>+0.036</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides vs. NEFA$_{SS}$</td>
<td>+0.211</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. NEFA$_{SUPP}$</td>
<td>-0.257</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. FPI</td>
<td>+0.275</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. FPG</td>
<td>+0.188</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. HOMA%S</td>
<td>-0.286</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. WHR</td>
<td>+0.231</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides vs. $M_{bw}$</td>
<td>-0.224</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NEFA$_0$ fasting non-esterified fatty acid concentrations;  
NEFA$_{SS}$ non-esterified fatty acid concentrations at steady-state;  
NEFA$_{SUPP}$ percentage suppression of non-esterified fatty acid concentrations from fasting to steady state;  
WHR waist:hip ratio;  
FPI fasting plasma insulin;  
FPG fasting plasma glucose;  
HOMA%S relative insulin sensitivity estimated by homeostasis model assessment;  
$M_{bw}$ mean glucose infusion rate at steady state normalised per kg body weight;
3.4. Discussion

The results of this study, using a large cohort of apparently healthy volunteers, confirm the existence of significant relationships between indices of insulin resistance and of non-esterified fatty acid regulation. They also demonstrate significant associations between insulin resistance, triglyceride concentrations and NEFA metabolism. These relationships were independent of age, sex, BMI and geographic locus within Europe. This study was based on data from 21 geographical centres, but has reproduced previous correlations reported in single site work, for example the relationship between triglyceride and HDL (235). This study population is the largest cohort ever studied with the hyperinsulinaemic clamp methodology. The method used has several advantages for the measurement of insulin resistance. It allows accurate and precise assessment of insulin sensitivity, and is considered the gold standard (37). The use of such a large study cohort increases the confidence with which one can determine whether or not statistical associations between different parameters exist. Such associations may imply the existence of underlying physiological mechanism(s) between insulin action and lipoprotein concentrations.

3.4.1. The association between NEFA concentrations and insulin resistance

Both fasting and steady state NEFA concentrations were significantly associated with the markers of insulin resistance. However, there was some inconsistency between the association of different indices of insulin resistance and the indices of fatty acid regulation at fasting and in the hyperinsulinaemic state (table 3.2). Different strengths of correlations have been found in the fasting and in the hyperinsulinaemic state:

3.4.1.1. Fasting state

a) Markers of insulin resistance measured in the fasting state

FPI and HOMA%S were most strongly related to NEFA suppressibility and also significantly correlated with steady-state NEFA concentrations. Neither of these measures of insulin resistance correlated with fasting NEFA levels.
b) **Fasting NEFA concentrations**

Fasting NEFA concentrations correlated significantly only with \( M_{bw} \) and not with insulin resistance values derived from the fasting state.

### 3.4.1.2. Hyperinsulinaemic state

a) **Markers of insulin resistance measured in the hyperinsulinaemic (clamp) state:**

\( M_{bw} \) was most strongly related to fasting NEFA concentrations and also significantly correlated with NEFA concentration at steady state. \( M_{bw} \) was not associated with NEFA suppressibility.

b) **NEFA concentrations during the hyperinsulinaemic (clamp) state**

NEFA concentrations measured during the hyperinsulinaemic clamp/ at steady-state were significantly associated with all measures of insulin resistance, eg. at fasting and at hyperinsulinaemic state.

### 3.4.1.3. NEFA suppressibility

NEFA suppressibility, an index derived from NEFA concentrations measured both during fasting and during hyperinsulinaemia, was only significantly related with the measures of insulin resistance derived from fasting (FPI and HOMA%S).

Thus, NEFA concentrations measured during the steady-state phase of the hyperinsulinaemic clamp appear as the one parameter of NEFA metabolism most consistently associated with any measure of insulin resistance. This finding may reflect the fact that regulation of fasting NEFA concentrations depends upon a host of factors including sympathetic tone and previous diet (423). These latter factors probably have less effect on non-esterified fatty acid concentrations under the relatively standardised hyperinsulinaemic conditions of an euglycaemic clamp (‘\( \text{NEFA}_{ss} \)’). We were able to clearly show that the association between insulin resistance and NEFA-regulation is independent of obesity.

Different mechanism for the relationship between NEFA concentrations and resistance to insulin’s glucoregulatory action have been proposed but the evidence so far is not clear
Different tissues have been implicated in this relationship. Randle et al. suggested that an increase in NEFA supply, by inhibiting glucose uptake and oxidation in muscle cells, causes insulin resistance by substrate competition (137). Magnetic resonance spectroscopic studies have confirmed that this competition does occur and indicated the intracellular mechanisms involved (424). In contrast, it has been shown that insulin resistance within adipose tissue (perhaps especially visceral adipose tissue) causes failure of NEFA suppression to occur in subjects resistant to insulin's glucoregulatory action (49;143;425). Interaction between NEFAs and glucose at a hepatic level is another proposed mechanism, as an increase in the delivery rate of NEFAs to the liver leads to stimulation of gluconeogenesis (49;422;426-428). A 'toxic' effect of high NEFA concentration on pancreatic cells has also been implicated as contributing to the development of insulin resistance (429). The relative importance of each of these suggested mechanisms for the relationship between NEFA concentrations and insulin resistance is not clear.

3.4.2. The association between triglyceride concentrations and NEFA concentrations

Our results show that triglyceride concentrations were correlated with indices of free fatty acid suppressibility (NEFASS and NEFA{SUPP}) but not with fasting NEFA concentrations. Again this may reflect the large day-to-day variation of fasting NEFA concentrations. Normal subjects spend much of their usual days in the postprandial rather than the fasted state (138;430). Thus the area under the fatty acid concentration versus time curve during a typical 24-hour period probably depends more upon the NEFA suppressibility by insulin than on the fasted situation. This would explain why the correlation of triglyceride concentrations with those of fatty acids is more apparent with the NEFA indices derived from the hyperinsulinaemic state.

The results of the multivariate analysis suggest that triglyceride concentrations depend upon both insulin's glucoregulation (as reflected by M_{iso}) and its antilipolytic effects (as reflected by NEFASS). These relationships were present after adjustment for BMI, age and sex, and were independent of WHR. This conclusion agrees with that of Mostaza et al. who also found relationships between NEFA flux and triglycerides to be independent of obesity (134). They
drew the inference that multiple sites of insulin resistance underlie the hypertriglyceridaemia of non-diabetic subjects, and above results support that view.

3.4.3. The association between triglyceride concentrations and insulin resistance
All indices of insulin resistance were significantly related to triglyceride concentrations, but how insulin resistance changes triglyceride kinetics is not certain. Lithell et al studied a small group of healthy volunteers who were given a short-term carbohydrate rich dietary regimen. An increase in plasma triglyceride concentrations was noted to accompany the increase in insulin levels, possibly due to decreased muscle LPL activity (431). In a study comparing lean subjects with normal glucose tolerance with obese subjects with different severity of glucose intolerance a positive correlation was found between the glucose infusion rate during a clamp and LPL activity in skeletal muscle (432). A mechanism implicated in elevated triglyceride levels is the reduced clearance by impaired LPL activation (433).

3.4.4. Could the association between hypertriglyceridaemia and insulin resistance be mediated by NEFA?
Beyond confirming previous associations, the study examined whether the link between triglyceridaemia and insulin resistance was mediated by the suppressibility of fatty acids. It had previously been suggested that this was the principal (or even the sole) mechanism for the association (49;130;434). The performed analysis was designed to test this hypothesis. However, above data give evidence that there is another mechanism. The multiple regression analysis suggests that both glucoregulatory defects in insulin action and poor suppression of NEFA during hyperinsulinaemia have independent contributions to the triglyceride concentration (even after correction for BMI, age, gender and geographical centre).

3.5. Summary
The described analyses are based upon correlations and were not designed to study the specific mechanisms for the associations seen. However, insulin regulation in several different tissues can account for the observed relationships. Insulin resistance in the adipose tissue determines the plasma NEFA availability throughout the day and hence the substrate
supply for hepatic triglyceride stores, which are in turn the substrate for triglyceride secretion as VLDL (130;132;435). Insulin sensitive mechanisms within the liver also influence the VLDL secretion (436) and insulin action on lipoprotein lipase affects VLDL clearance. This study has not examined these liver or LPL actions of insulin directly, but has measured glucoregulatory actions of insulin. The correlations between triglyceride concentrations and clamp-derived indices need not necessarily imply that insulin acting on skeletal muscle affects triglyceride concentrations directly, but could simply reflect the possibility that those subjects who are peripherally insulin resistant (for glucose regulation) are also insulin resistant at hepatic and adipose tissue sites. This is reflected by the multiple regression analysis result showing that both insulin's antilipolytic action and its glucoregulatory action have independent effects on the triglyceride concentration.

Our observation is supported by recent reports of experiments with muscle-specific insulin receptor knockout (MIRKO) mice exhibiting more than 95% reduction in receptor content. These mice displayed elevated fat mass, serum triglyceride concentrations and free fatty acid levels. However, blood glucose, serum insulin, and glucose tolerance remained normal, suggesting insulin's lipo- and gluco-regulation to be more independent of each other than previously thought (230). A comparison of insulin action in specific tissues in these MIRKO mice in comparison with normal controls was then performed under hyperinsulinaemic euglycaemic conditions. It was observed that insulin-stimulated muscle glucose transport and glycogen synthesis were decreased by about 80% in MIRKO-mice. However, insulin-stimulated fat glucose transport was actually increased threefold in MIRKO-mice. The authors suggested that this demonstrated that selective insulin resistance in muscle promoted redistribution of substrates to adipose tissue, thereby increasing adiposity and leading to development of a pre-diabetic syndrome (231).
3.6. Conclusion

The main aim of this study was to firstly to examine the relationship of NEFA metabolism with insulin resistance in healthy subjects. Our study showed the strong associations between both, and found these correlations to differ between the fasting and the hyperinsulinaemic state. This may point towards a so far undeclared mechanism or just represent the variability of fasting NEFA under the conditions used. The hypothesis is that abnormal NEFA metabolism precedes insulin resistance. Our study design enabled us to observe a strong association between insulin resistance and NEFA metabolism in healthy volunteers. However, we were unable to confirm or refute this hypothesis. Our understanding of the relationship between NEFA and insulin action remains simple and further studies are needed to examine this in more detail. It appears that NEFA act as a signal as well as a metabolic substrate. Knowledge gained from animal models and in particular from knock-out experiments will advance studies in humans. Such studies could involve isotope studies, NMR spectroscopy or “clamping” of NEFA at low/normal levels in order to observe the effect on muscle and/or whole body insulin sensitivity.
**Figure 3.1:** Fasting plasma triglyceride concentrations for each quintile of insulin sensitivity in 867 healthy subjects. Note: triglycerides are log-transformed.

*mbw* = mean glucose infusion rate at steady-state normalised per kg body weight

**Figure 3.2:** Fasting plasma non-esterified fatty acid (NEFA) concentrations for each quintile of insulin sensitivity in 541 healthy subjects.

*mbw* = mean glucose infusion rate at steady-state normalised per kg body weight
Figure 3.3: Plasma non-esterified fatty acid concentrations (NEFA) at steady state for each quintile of insulin sensitivity in 541 healthy subjects.

mbw = mean glucose infusion rate at steady-state normalised per kg body weight

Figure 3.4: Plasma non-esterified fatty acid (NEFA) suppressibility for each quintile of insulin sensitivity in 541 healthy subjects.

mbw = mean glucose infusion rate at steady-state normalised per kg body weight
Chapter 4
Insulin resistance, lipids and non-esterified fatty acids in type 2 diabetes mellitus (Results and discussion)

4.1. Introduction
Insulin resistance rather than insulin deficiency has been implicated as the primary defect leading to the development of type 2 diabetes using the following findings as support: Firstly, some studies have pointed to abnormal insulin action but normal insulin secretion in patients at high risk of developing type 2 diabetes before hyperglycaemia ensues (301;302). Secondly, insulin resistant subjects progress from normal glucose tolerance to diabetes more rapidly than their insulin sensitive counterparts (304;305).

There appears to be a considerable epidemiological overlap between type 2 diabetes and the insulin resistance syndrome with dyslipidaemia and abnormal non-esterified fatty acid (NEFA) metabolism as common features (58-61;113). Deficits in suppression of NEFA concentrations have been demonstrated in insulin resistant states (47; 143; 144). The relationship between insulin resistance and diabetes has been discussed in section 2.7. and NEFA as a possible antecedent for insulin resistance has been discussed in detail in section 1.2.4.3.3. Analysis of the Paris Prospective Study suggests that a high plasma NEFA concentration is a risk marker for deterioration of glucose tolerance independent of the insulin resistance or the insulin secretion defect that characterizes subjects at risk for type 2 diabetes (298). This might suggest that abnormal NEFA metabolism antedates insulin resistance or/and the development of diabetes.

4.2. Aims of the study
The aims of this study were: Firstly, to examine the relationship of NEFA metabolism with insulin resistance in subjects with type 2 diabetes and Secondly, to investigate if the hypertriglyceridaemia seen in insulin resistant states is mediated by abnormal NEFA metabolism. This was attempted by studying the relationship between insulin resistance and plasma triglyceride and NEFA concentrations, in subjects with uncomplicated type 2 diabetes mellitus.
4.3. Results

Insulin resistance was measured in 38 patients with type 2 diabetes mellitus by the use of the euglycaemic hyperinsulinaemic clamp (sections 2.1.5.1. and 2.3.8.1.), the HOMA model (section 2.1.5.3.) as well as by measuring fasting plasma insulin (section 2.1.7.). Plasma lipid and NEFA concentrations were both measured at fasting and NEFA levels also at steady-state (section sections 2.1.8. and 2.1.9.). The characteristics of all patients are shown in table 2.2. However, clamp data are only available in 37 patients as one patient developed symptomatic hypoglycaemia during the clamp study and therefore no analysable data were available on him.

4.3.1. Insulin sensitivity

The $M$-value as an index of insulin sensitivity in the whole cohort was $2.85 \pm 1.47$ mg/kg/min. M-value was significantly negatively correlated with FPI (Spearman's $r = -0.64$, $p=0.001$) (figures 4.2), but not with FPG. This association was no longer significant after correction for BMI. Insulin sensitivity estimated by the HOMA model correlated highly with the M-value ($r=-0.505$, $p=0.002$). Thereafter, the M-value as a measure of insulin sensitivity measured by the hyperinsulinaemic euglycaemic clamp will be used for analysis. The associations for M-value corrected for lean body weight ($M_b$) were almost identical to those with the M-value. Therefore only data for the M-value as an index of insulin sensitivity are shown.

The study cohort was divided into tertiles of insulin sensitivity ($n=12-13-12$). The upper, insulin sensitive (IS) subgroup had an M-value of $4.56 \pm 0.99$ mg/kg/min, the middle subgroup had an M-value of $2.61 \pm 0.47$ mg/kg/min and the lower, insulin resistant (IR) subgroup group had an M-value of $1.40 \pm 0.53$ mg/kg/min (figure 4.1). The two extreme groups were well matched on a host of characteristics (table 4.1). FPI was significantly higher in the IR subgroup than the IS subgroup (figure 4.3).
Table 4.1: Characteristics of the two subgroups: insulin resistant (IR) and insulin sensitive (IS). (unpaired t-test for continuous variables, chi-square test for proportions). Variables are shown as mean ±SD apart from FPI and urinary albumin excretion rate which are shown as median (range).

<table>
<thead>
<tr>
<th></th>
<th>IR subgroup</th>
<th>IS subgroup</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Male: female</td>
<td>9:3</td>
<td>10:2</td>
<td>0.619</td>
</tr>
<tr>
<td>Ethnicity (n):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian/ Asian/ Afro-Caribbean</td>
<td>11/1/0</td>
<td>5/6/1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.1±11.1</td>
<td>60.3±8.2</td>
<td>0.262</td>
</tr>
<tr>
<td>Duration of disease (months)</td>
<td>70.6±28.5</td>
<td>54.4±53.2</td>
<td>0.373</td>
</tr>
<tr>
<td>HbAlc (%)</td>
<td>8.7±1.2</td>
<td>8.2±1.7</td>
<td>0.847</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>101.3±13.8</td>
<td>100.1±13.7</td>
<td>0.690</td>
</tr>
<tr>
<td>Systolic Blood pressure (mmHg)</td>
<td>137.7±26.2</td>
<td>132.2±14.1</td>
<td>0.732</td>
</tr>
<tr>
<td>Diastolic Blood pressure (mmHg)</td>
<td>83.2±11.2</td>
<td>84.0±17.8</td>
<td>0.865</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>11.9±2.1</td>
<td>10.3±3.1</td>
<td>0.837</td>
</tr>
<tr>
<td>Urinary albumin excretion rate (mg/24 hours)</td>
<td>0.16 (0.06-1.50)</td>
<td>0.13 (0.06-0.54)</td>
<td>0.430</td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/l)</td>
<td>68.9 (16.6-521.7)</td>
<td>30.4 (14.3-243.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Antihypertensive Treatment (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>4/12</td>
<td>4/12</td>
<td>1.0</td>
</tr>
<tr>
<td>ACE-Inhibitor</td>
<td>5/12</td>
<td>0/12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Treatment with a statin (n)</td>
<td>1/12</td>
<td>1/12</td>
<td>1.0</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>3/12</td>
<td>3/12</td>
<td>1.0</td>
</tr>
</tbody>
</table>

4.3.2. The influence of gender, age and ethnic origin on insulin sensitivity

Insulin sensitivity (M-value) did not differ significantly between men and women (2.97±1.48 mmol/kg/min vs. 2.55±1.32 mmol/kg/min, respectively; t=0.69, p=0.49) or according to ethnic origin (F(2,34)=1.92, p=0.163) (table 4.2). Insulin sensitivity did not vary significantly with age (data not shown).
Table 4.2: M-values in the subjects in relation to ethnic origin

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>N</th>
<th>M-value (mg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afro-Caribbean</td>
<td>3</td>
<td>3.19±1.51</td>
</tr>
<tr>
<td>Asian</td>
<td>11</td>
<td>3.53±1.13</td>
</tr>
<tr>
<td>Caucasian</td>
<td>23</td>
<td>2.55±1.51</td>
</tr>
</tbody>
</table>

4.3.3. Insulin sensitivity and duration of diabetes

There was no significant correlation between insulin sensitivity (M-value or FPI) and duration of diabetes ($r=-0.04$, $p=0.824$ and $r=0.09$, $p=0.597$ respectively).

4.3.4. Insulin sensitivity and glycaemic control

FPG and glycosylated haemoglobin (HbA1c) were used as markers of glycaemic control. FPG and HbA1c correlated significantly ($r=0.591$, $p<0.001$). There was no significant correlation between the M-value and FPG ($r=-0.17$, $p=0.322$) or between the M-value and HbA1c ($r=-0.76$, $p=0.655$). FPI was also not associated with FPG or HbA1c.

4.3.5. Insulin sensitivity and 24-hour ambulatory blood pressure

There was no difference in heart rate, systolic or diastolic blood pressure between the IR and IS subgroups (table 4.1). There was no correlation between the M-value and 24-hour ambulatory heart rate, mean blood pressure, systolic or diastolic blood pressure ($r=0.04$, $p=0.825$; $r=-0.15$, $p=0.383$; $r=-0.31$, $p=0.064$; $r=-0.07$, $p=0.682$). BMI and FPI were also not significantly associated with these ambulatory measurements. Five patients (13.2%) were receiving antihypertensive treatment with a beta-blocker and nine patients (23.7%) with an ACE-inhibitor. One patient received both treatments. Antihypertensive treatment was not taken on the day of the study. Exclusion of patients on an ACE-inhibitor, a beta-blocker or both from the analysis did not alter the above finding.

4.3.6. Insulin sensitivity and markers of obesity

Body mass index (BMI), waist circumference, hip circumference, waist:hip ratio (WHR) and biceps skinfold thickness were used as markers of obesity. Markers of obesity did differ
significantly between the IR and the IS subgroup (table 4.3.) The associations of BMI with other markers of obesity are shown in table 4.4.

M-value and BMI were negatively correlated (figure 4.4.). M-value was also negatively correlated with skinfold thickness, waist circumference and hip circumference, but not with waist:hip ratio. FPI was correlated with hip and waist measurements, but not with WHR or BMI. These correlations are shown in table 4.5.

4.3.7. Insulin sensitivity and non-esterified free fatty acids

M-value was negatively correlated to $\text{NEFA}_q$, $\text{NEFA}_{SS}$ and $\text{NEFA}_{SUPP}$ (figures 4.5-4.7. and table 4.6.). The association between M-value and all measures of NEFA metabolism remained significant after correction for BMI, but were less strong. Exclusion of patients treated with a HMG CoA reductase inhibitor strengthened these correlations (data not shown).

FPI was also significantly correlated with $\text{NEFA}_q$, $\text{NEFA}_{SS}$ and $\text{NEFA}_{SUPP}$. The association between FPI and $\text{NEFA}_q$ was no longer significant after correction for BMI. The association between FPI and $\text{NEFA}_{SS}$ and $\text{NEFA}_{SUPP}$ remained significant after correction for BMI. These associations are shown in table 4.6. Exclusion of patients treated with a HMG CoA reductase inhibitor did not alter these correlations (data not shown).

Table 4.7. and figures 4.8. and 4.9 show NEFA concentrations and suppressibility in the two extreme subgroups of insulin resistance, where there were significant differences. These correlations remained significant after Bonferroni correction (p<0.015).

4.3.8. Insulin sensitivity and plasma lipid concentrations

There were significant relationships of BMI and M-value with triglyceride levels (table 4.8. and figures 4.10 and 4.11). After correction for BMI the association between M-value and triglycerides was no longer significant (r= -0.146, p=0.411). Triglyceride concentrations were significantly higher in the IR subgroup than in the IS subgroup (table 4.9. and figure 4.12.).
Similarly, significant relations were found between the M-value and HDL-cholesterol. This association was again dependent on BMI but independent of triglycerides. The relationship of M-value and LDL-cholesterol showed no significant correlation. These correlations are shown in table 4.8. Cholesterol concentrations did not differ between the IR and IS subgroup (table 4.9).

Three patients had received treatment with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor. Exclusion of these patients from the analysis did not alter the correlation between the M-value and plasma lipids (data not shown).

4.3.9. The association of serum lipid concentrations with serum NEFA concentrations
Fasting NEFA concentrations did not correlate with fasting triglyceride levels. Steady state NEFA concentrations and NEFA suppressibility did correlate significantly with triglyceride concentrations after correction for obesity (BMI). The correlations of fasting triglyceride levels with other lipoproteins and NEFA concentrations are shown in table 4.10. Exclusion of patients on a HMG CoA reductase inhibitor did not alter these correlations.

4.3.10. Does the relationship between insulin resistance and triglyceride concentration depend on the confounding effect of NEFA metabolism?
The association between M-value and triglyceride concentration was independent of fasting NEFA concentrations ($\beta=-0.426, p=0.013$). The association between fasting NEFA concentrations and triglyceride concentrations was independent of insulin resistance ($\beta=-0.369, p=0.034$).

After correction for NEFA concentrations in the hyperinsulinaemic state ($\beta=0.201, p=0.263$) and NEFA suppressibility ($\beta=-0.152, p=0.399$) the correlation between M-value and triglyceride concentrations was no longer significant. The independent associations are shown in figures 4.13a and 4.13b.
Table 4.3: Markers of obesity in the insulin resistant (IR) and insulin sensitive (IS) subgroups (independent t-test). Significant values are represented in bold.

<table>
<thead>
<tr>
<th>marker</th>
<th>IR</th>
<th>IS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (cm)</td>
<td>102.5±6.7</td>
<td>93.5±8.4</td>
<td>0.009</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>109.0±7.5</td>
<td>100.5±7.6</td>
<td>0.012</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.93±0.08</td>
<td>0.92±0.05</td>
<td>0.608</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.2±3.3</td>
<td>25.7±3.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Biceps skinfold thickness (mm)</td>
<td>25.3±10.2</td>
<td>20.4±6.7</td>
<td>0.207</td>
</tr>
</tbody>
</table>

Table 4.4: The associations of body mass index with other markers of obesity in uncomplicated type 2 diabetes (Spearman’s correlation coefficient r is shown)

<table>
<thead>
<tr>
<th></th>
<th>Waist circumference</th>
<th>Hip circumference</th>
<th>Waist:hip ratio</th>
<th>Biceps skinfold thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>0.69</td>
<td>0.87</td>
<td>-0.07</td>
<td>0.54</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 4.5: Association of insulin sensitivity and markers of obesity in uncomplicated type 2 diabetes (Spearman’s correlation coefficient r is shown)

<table>
<thead>
<tr>
<th></th>
<th>Waist circumference</th>
<th>Hip circumference</th>
<th>WHR</th>
<th>BMI</th>
<th>Biceps skinfold thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-value</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.008</td>
<td>NS</td>
<td>0.007</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting plasma insulin</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.024</td>
<td>NS</td>
<td>0.025</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.6: Correlation of insulin sensitivity and Body mass index (BMI) with non-esterified free fatty acid concentrations in uncomplicated type 2 diabetes. Markers of insulin resistance were also analysed after correction for obesity. The crude correlation coefficient is shown above the diagonal and the correlation coefficient corrected for BMI below the diagonal (Spearman’s correlation coefficient r is shown for uncorrected and partial correlation coefficient beta for corrected values).

<table>
<thead>
<tr>
<th></th>
<th>NEFA₀</th>
<th>NEFAₛ</th>
<th>NEFAₚₛₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body mass index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/β</td>
<td>0.34</td>
<td>0.19</td>
<td>0.35</td>
</tr>
<tr>
<td>p-value</td>
<td>0.04</td>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Fasting plasma insulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/β</td>
<td>0.35</td>
<td>0.44</td>
<td>0.66</td>
</tr>
<tr>
<td>p-value</td>
<td>0.045</td>
<td>0.008</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>M-value</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/β</td>
<td>-0.53</td>
<td>-0.62</td>
<td>-0.55</td>
</tr>
<tr>
<td>p-value</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

| **NEFA₀**                    | fasting non-esterified fatty acid concentrations; |
| **NEFAₛ**                    | non-esterified fatty acid concentrations at steady-state; |
| **NEFAₚₛₚ**                  | percentage suppression of non-esterified fatty acid concentrations from fasting to steady state; |

Table 4.7: Plasma NEFA concentrations in the insulin resistant (IR) and insulin sensitive (IS) subgroup (independent t-test)

<table>
<thead>
<tr>
<th></th>
<th>IR</th>
<th>IS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NEFA₀ (mmol/l)</strong></td>
<td>0.61±0.27</td>
<td>0.30±0.13</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>NEFAₛ (mmol/l)</strong></td>
<td>0.15±0.11</td>
<td>0.02±0.02</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>NEFAₚₛₚ (%)</strong></td>
<td>75.6±13.5</td>
<td>92.6±3.4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

| **NEFA₀** | fasting non-esterified fatty acid concentrations; |
| **NEFAₛ** | non-esterified fatty acid concentrations at steady-state; |
| **NEFAₚₛₚ** | percentage suppression of non-esterified fatty acid concentrations from fasting to steady state; |
Table 4.8: Partial correlations of insulin sensitivity and BMI with plasma lipid concentrations in uncomplicated type 2 diabetes. Correlations were also analysed after correction for obesity. The crude correlation coefficient is shown above the diagonal and the correlation coefficient corrected for BMI below the diagonal (Spearman’s correlation coefficient r is shown for uncorrected and partial correlation coefficient beta for corrected values).

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides</th>
<th>HDL-cholesterol</th>
<th>LDL-cholesterol</th>
<th>Total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body mass index</strong></td>
<td>r/β</td>
<td>-0.23</td>
<td>0.01</td>
<td>-0.06</td>
</tr>
<tr>
<td>p-value</td>
<td>0.041</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>M-value</strong></td>
<td>r/β</td>
<td>-0.46</td>
<td>0.32</td>
<td>-0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.45</td>
<td>0.22</td>
<td>-0.18</td>
</tr>
<tr>
<td>p-value</td>
<td>0.005</td>
<td>0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Fasting plasma insulin</strong></td>
<td>r/β</td>
<td>0.26</td>
<td>-0.01</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.55</td>
<td>-0.012</td>
<td>0.49</td>
</tr>
<tr>
<td>p-value</td>
<td>NS</td>
<td>NS</td>
<td>0.018</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.9: Plasma lipid concentrations in the insulin resistant (IR) and insulin sensitive (IS) subgroup (independent t-test). Statistically significant values are represented in bold.

<table>
<thead>
<tr>
<th></th>
<th>IR</th>
<th>IS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>3.40±2.24</td>
<td>1.47±0.78</td>
<td>0.013</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.10±0.25</td>
<td>1.40±0.41</td>
<td>0.14</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.42±0.47</td>
<td>3.26±0.87</td>
<td>0.59</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.26±1.39</td>
<td>5.27±0.95</td>
<td>0.77</td>
</tr>
</tbody>
</table>
Table 4.10: Partial correlations of fasting triglyceride level with other lipoproteins and NEFA concentrations in uncomplicated type 2 diabetes.

Correlations were also analysed after correction for obesity. The crude correlation coefficient is shown above the diagonal and the correlation coefficient corrected for BMI below the diagonal. Spearman’s correlation coefficient r is shown for uncorrected and partial correlation coefficient β for corrected values.

<table>
<thead>
<tr>
<th>Partial correlation coefficient β</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides vs. total cholesterol</td>
<td>0.295 0.005 NS NS</td>
</tr>
<tr>
<td>Triglycerides vs. HDL-cholesterol</td>
<td>0.093 -0.311 NS NS</td>
</tr>
<tr>
<td>Triglycerides vs. LDL-cholesterol</td>
<td>0.255 0.299 NS NS</td>
</tr>
<tr>
<td>Triglycerides vs. NEFAₙ</td>
<td>-0.157 -0.005 NS NS</td>
</tr>
<tr>
<td>Triglycerides vs. NEFAₚ</td>
<td>0.093 0.383 NS 0.044</td>
</tr>
<tr>
<td>Triglycerides vs. NEFASUPP</td>
<td>0.206 0.402 NS 0.034</td>
</tr>
</tbody>
</table>

NEFAₙ-fasting non-esterified fatty acid concentrations; NEFAₚ non-esterified fatty acid concentrations at steady-state; NEFASUPP-% suppression of non-esterified fatty acid concentrations from fasting to steady state;

4.3.11. Summary of results

Insulin resistance was correlated with fasting plasma insulin, markers of obesity, plasma triglyceride concentrations and NEFA concentrations. The insulin resistant subgroup had significantly higher levels of FPI, plasma triglycerides, fasting and steady state NEFA concentrations than the insulin sensitive subgroup. NEFA suppressibility was significantly lower in the IR subgroup. BMI, biceps skinfold thickness, waist and hip circumference, but not WHR were also higher in this subgroup.
Figure 4.13: The association between insulin sensitivity, triglyceride concentrations and NEFA metabolism. 4.13 a: in the fasting state (top) and 4.13b: in the hyperinsulinaemic state (bottom) (independent correlation coefficient beta is shown)
4.4. Discussion

In this study of patients with uncomplicated type 2 diabetes mellitus insulin resistance and BMI were significantly correlated. Insulin resistance correlated significantly with serum lipid concentrations and serum NEFA levels. These relationships were independent of age, sex, ethnic origin or duration of disease. In fact, insulin resistance itself was not associated with age, gender, ethnic origin or duration of disease. Correction for BMI made the association between insulin resistance and triglyceride concentrations non-significant, but did not alter the association between insulin resistance and NEFA concentrations.

Division of our study group into tertiles enabled us to look at two extreme subgroups: an insulin resistant and insulin sensitive one. These groups did not differ with regard to age, gender distribution, height, smoking habits or duration of disease. Glycaemic control was similar in the two subgroups. Ethnic origin was different in the two subgroups in that the insulin sensitive group contained 6 Asian, 1 Afro-Caribbean and 6 Caucasian patients and the insulin resistant subgroup contained 11 Caucasian and two Asian patients. Use of medication was different in the two subgroups: ACE-inhibitors were used more frequently in the insulin resistant subgroup, but use of beta-blocker and statins was similar in both subgroups. This observation may reflect higher blood pressure readings in the insulin resistant patients prior to entering the study. Previously it has been demonstrated that chronic ACE inhibitor therapy increases insulin sensitivity (437) or has a neutral effect (438).

The two subgroups differed significantly in their BMI and other markers of obesity. Insulin resistant subjects had a higher BMI, waist and hip circumference, but WHR was not significantly different. They also had a more detrimental metabolic profile (hyperinsulinaemia, hypertriglyceridaemia).

Our cohort is small due to the complexity of the study protocol. However, we used the euglycaemic hyperinsulinaemic clamp (‘the gold standard’) for the measurement of insulin resistance, thus achieving accurate and precise assessment of insulin (37) and also measured markers of insulin resistance in the fasting state. We measured several markers of NEFA
metabolism (fasting, steady state and suppressibility). In addition, we have data on lipid profile as well as detailed demographic information on our patients.

Our data allow us to examine if the relationship between insulin sensitivity and triglyceride concentration depend on the confounding effect of NEFA metabolism.

4.4.1. Insulin sensitivity and triglyceride concentrations

Triglyceride concentrations were significantly higher in the insulin resistant subgroup. The M-value, as a marker of insulin resistance was significantly related to triglyceride concentrations. This association was less strong after correction for BMI suggesting obesity as at least a confounding factor. Interestingly, the correlation between fasting plasma insulin and triglycerides was significant only after correction for BMI. The mechanism is not clear. An increase in hepatic triglyceride secretion has been shown to lead to hypertriglyceridaemia in insulin resistant states (431). Another mechanism implicated in elevated triglyceride levels is the reduced clearance by impaired LPL activation (433). A recent case report of a surgical ‘cure’ of insulin resistance and ensuing diabetes (inducing lipid malabsorption by a modified biliopancreatic diversion) in 2 sisters with familial hypertriglyceridaemia provides evidence that insulin-resistant diabetes can be caused by extremely high levels of triglycerides (439). The correlations between triglycerides and clamp-derived indices need not necessarily imply that insulin acting on skeletal muscle affects triglyceride concentrations directly, but could simply reflect that those subjects who are peripherally insulin resistant (for glucose regulation) are also insulin resistant at hepatic and adipose tissue sites. In a recent study comparing lipolysis in skeletal muscle and adipose tissue Jacob et al reported a difference in suppression of lipolysis depending on whether the insulin dosage used was physiological or supra-physiological. They suggested that inadequate suppression of intramuscular lipolysis, resulting in increased availability of non-esterified fatty acids, could represent a potential mechanism involved in the pathogenesis of impaired glucose disposal, i.e. muscle insulin resistance (440).
4.4.2. NEFA and triglycerides

The results in these patients with diabetes show that triglyceride concentrations were correlated with indices of free fatty acid suppressibility (NEFA$_{SS}$ and NEFA$_{SUPP}$) but not with fasting NEFA concentrations. This may again reflect the large day-to-day variation of fasting NEFA concentrations. This would explain why the correlation of triglycerides with fatty acids is more apparent with the NEFA indices derived from the hyperinsulinaemic state.

Our findings in this cohort of patients with type 2 diabetes reflect reports that the relationships between NEFA flux and triglycerides are independent of obesity. In non-diabetic hypertriglyceridaemic men Mosatza et al reported similar findings, with the inference that multiple sites of insulin resistance underlie the hypertriglyceridaemia of non-diabetic subjects (134). Our results suggest this also to be the case in diabetic subjects.

4.4.3. The relationship between insulin sensitivity, NEFA concentrations and triglyceride concentrations in type 2 diabetes

Beyond confirming previous associations, our study in type 2 diabetes examined whether the link between triglyceridaemia and insulin resistance was mediated by the suppressibility of fatty acids, as previously suggested (49;130;434). The relationship of NEFA with triglyceride concentrations and that of insulin resistance with triglyceride concentrations were independent of each other in the fasting state. In the hyperinsulinaemic (steady) state our results show that the relationship between insulin sensitivity and triglyceride concentrations is dependent on NEFA concentrations and NEFA suppressibility.

Considering that NEFA levels measured in the steady state are probably a more reliable and consistent measure of NEFA metabolism than fasting levels (which show a large day to day variation) this finding supports the hypothesis that the relationship between insulin resistance and triglyceride concentrations are dependent on the confounding influence of NEFA.
4.4.4. The relationship between insulin sensitivity, triglyceride and NEFA concentrations in patients with type 2 diabetes and healthy volunteers

The relationship between insulin sensitivity, triglyceride and NEFA metabolism has been discussed in detail in healthy subjects as part of the EGIR study (sections 3.2.3. and 3.3.4.) and in patients with type 2 diabetes (section 4.3.1.3.). Findings were almost identical in both studies.

In both cohorts significant associations were found between fasting triglyceride concentrations and steady-state NEFA concentrations and between fasting triglyceride concentrations and NEFA suppressibility, but not with fasting NEFA concentrations. In the same way, fasting triglyceride concentrations were significantly associated with the M-value as a marker of insulin resistance in both cohorts.

Finally, the relationship between triglyceride concentrations and NEFA metabolism and between triglyceride concentration and insulin resistance, were independent of each other in the EGIR study of healthy volunteers and in the study in patients with type 2 diabetes, suggesting that health and type 2 diabetes are different parts of a continuum, rather than separate entities.

In the diabetic study FPI and the M-value as indices of insulin resistance were significantly related to NEFA\text{ss}, NEFA\text{supp} and NEFA\text{supp}. After correction for obesity (BMI) the M-value (a measure of insulin sensitivity in the hyperinsulinaemic state) remained significantly associated with all measures of NEFA metabolism. FPI (as a measure of insulin sensitivity in the fasting state) was significantly correlated only with NEFA concentrations at steady state and NEFA suppressibility after correction for BMI. Interestingly, in the healthy cohort NEFA suppressibility, after correction for BMI, was not significantly correlated with insulin sensitivity. Otherwise the findings were similar. We were able to clearly show that in health and type 2 diabetes the association between insulin resistance and NEFA-regulation is independent of obesity.
Different mechanisms in underlying the relationship between NEFA concentrations and insulin resistance have been discussed in section 3.3.1. The relative importance of each of these suggested mechanisms for the relationship between NEFA concentrations and insulin resistance is not clear.

4.5. Conclusion
Despite the fact that our cohort is small and heterogeneous, in many aspects our results confirm the findings of many and larger studies in health and type 2 diabetes mellitus. We found no evidence that the heterogeneity in respect of ethnicity, age and medication influenced any of the findings. To our knowledge no study has collected data in such a group of patients with type 2 diabetes and studied their metabolic profile in the fasting (FPI and fasting NEFA) and hyperinsulinaemic state ( hyperinsulinaemic euglycaemic clamp and steady state NEFA plus NEFA suppressibility). Data on lipid profile were also collected.

Levels of triglyceride and fasting and steady state non-esterified free fatty acid concentrations were significantly higher in the insulin resistant subgroup. Insulin resistance and hypertriglyceridaemia are strongly associated. This association depends on NEFA in the hyperinsulinaemic state and NEFA suppressibility, possibly suggesting a causative relationship. The association between insulin resistance and NEFA-regulation was independent of obesity. It was also shown that both insulin's antilipolytic action and its glucoregulatory action have independent effects on the triglyceride concentration.
Figure 4.1. Subgroups of the cohort with uncomplicated type 2 diabetes divided into tertiles of insulin sensitivity
IR (insulin resistant, n=12) and IS (insulin sensitive, n=12) show the two extreme subgroups of insulin sensitivity. Mdl represents the middle subgroup (n=13).
Figure 4.2: Correlation between insulin sensitivity (M-value) and fasting plasma insulin concentrations (log-transformed) in subjects with uncomplicated type 2 diabetes.

Figure 4.3. Fasting plasma insulin concentrations in the three subgroups of the cohort. IR (insulin resistant, n=12) and IS (insulin sensitive, n=12) show the two extreme subgroups of insulin sensitivity. MdI represents the middle subgroup (n=13).
Figure 4.4. Correlation between insulin sensitivity and BMI in subjects with type 2 diabetes

![Graph showing the correlation between insulin sensitivity and BMI in subjects with type 2 diabetes.]

Figure 4.5. Correlation between insulin sensitivity and fasting non-esterified fatty acid concentrations in subjects with type 2 diabetes

![Graph showing the correlation between insulin sensitivity and fasting non-esterified fatty acid concentrations in subjects with type 2 diabetes.]
Figure 4.6. Correlation between measurement of insulin sensitivity and steady-state non-esterified fatty acid concentrations (log-transformed) in subjects with type 2 diabetes

Figure 4.7. Correlation between measurement of insulin sensitivity and non-esterified fatty acid suppressibility (log-transformed) in subjects with type 2 diabetes
Figure 4.8. Fasting and steady-state non-esterified fatty acid concentrations in the three subgroups of the cohort. IR (insulin resistant, n=12) and IS (insulin sensitive, n=12) show the two extreme subgroups. Mdl=middle subgroup (n=13).

Figure 4.9. Non-esterified fatty acid suppressibility in the three subgroups of the cohort. IR (insulin resistant, n=12) and IS (insulin sensitive, n=12) show the two extreme subgroups. Mdl = middle subgroup (n=13).
Figure 4.10. Correlation between obesity (BMI) and fasting triglyceride concentration (log-transformed) in subjects with type 2 diabetes

Figure 4.11. Correlation between insulin sensitivity and fasting triglyceride concentration (log-transformed) in subjects with type 2 diabetes
Figure 4.12. Fasting triglyceride concentrations in the three subgroups of the cohort. IR (insulin resistant, n=12) and IS (insulin sensitive, n=12) show the two extreme subgroups. Mdl = middle subgroup (n=13).
Figure 4.14: Steady state insulin concentrations during the last 40 minutes of the euglycaemic clamp in subjects with type 2 diabetes (n=35)
Chapter 5

Vascular reactivity in type 2 diabetes mellitus

(Results and discussion)

5.1. Introduction

Vascular disease is a major cause of morbidity and mortality in type 2 diabetes and is much more common in diabetes than in the non-diabetic population (5;441). Endothelial dysfunction, leading to lack of protection from atherosclerosis, has been implicated in the pathogenesis of diabetic vascular disease (407). Many mechanisms by which hyperglycaemia can lead to endothelial dysfunction have been identified. However, robust human physiological data on diabetes and endothelial function are scarce. Venous occlusion plethysmography is most commonly used to assess endothelial function in vivo (section 1.3.1.2.). Circulating ‘markers’ of endothelial function can also be measured (section 1.3.2.). The majority of studies demonstrate impaired endothelium-dependent vasodilatation in diabetes (72;149;405;406). The main mechanisms implied are abnormal endothelial production of NO and decreased responsiveness to NO.

However, no real consensus has been reached on the association between type 2 diabetes mellitus and endothelial dysfunction. The complexity of the study methodology as well as the frequent presence of associated factors, independently associated with endothelial function, have complicated the assessment of endothelial function in diabetes. Hypertension, dyslipidaemia and obesity are each associated with endothelial dysfunction (117;128;391;393;406). Subjects with type 2 diabetes are a heterogeneous group and this may contribute to the diversity of findings in these cohorts. To our knowledge, no study has investigated insulin resistance (assessed by euglycaemic hyperinsulinaemic clamp) and endothelial dysfunction (assessed by venous occlusion plethysmography) in a well-defined, homogeneous cohort of subjects with type 2 diabetes mellitus.
5.2. Aims
The aims of this study were: Firstly, to test the hypothesis that endothelial function is impaired in association with insulin resistance in type 2 diabetes in a well-defined cohort with uncomplicated diabetes and Secondly, to examine the influence of associated factors in this relationship (hyperglycaemia, hypertension, obesity and dyslipidaemia). This was attempted by the use of the euglycaemic hyperinsulinaemic clamp technique to assess insulin sensitivity and venous occlusion plethysmography to measure forearm blood flow, as a marker of endothelial function.

5.3. Results
FBF was measured in 32 patients using venous occlusion plethysmography (section 2.3.9.1). It was measured
- in the basal state,
- in response to local, intra-arterial infusion of Acetylcholine (section 2.3.9.2.1.),
- in response to local, intra-arterial infusion of Sodium Nitroprusside (section 2.3.9.2.2.),
- in response to systemic, intravenous infusion of insulin (section 2.3.10.) and
- in response to local, intra-arterial infusion of N-monomethyl-L-arginine (section 2.3.9.2.3.).

The study design is shown in Figure 2.1.

FBF was expressed either as absolute values, as percentage change above baseline ($\Delta$ FBF), or as the blood flow ratio of the test (infused) arm to the control (contra-lateral) arm (FBF ratio). Forearm volume was adjusted for in some of these analyses (Forearm blood flow adjusted for forearm volume- FBF$_{pv}$). Vascular resistance was calculated. The dose-response curve slope was also used to express vascular reactivity (section 2.3.9.1.2.). A summary of correlations of FBF (basal and in response to stimulation) with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity is shown in table 5.1. Results are also reported in detail in the following sections.
Table 5.1: Summary of correlations of FBF (basal and the slope in response to various stimuli) with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity. Spearman’s correlation coefficient \( r \) is shown. Statistical significant values are shown in bold and as follows: * \( p<0.05 \), ** \( p < 0.01 \), *** \( p < 0.0001 \)

<table>
<thead>
<tr>
<th>Demography</th>
<th>Basal FBF</th>
<th>FBF in response to ACh</th>
<th>FBF in response to SNP</th>
<th>FBF in response to insulin</th>
<th>FBF in response to L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>( r )</td>
<td>0.242</td>
<td>-0.175</td>
<td>0.034</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>( p )-value</td>
<td>0.898</td>
<td>0.183</td>
<td>0.339</td>
<td>0.863</td>
</tr>
<tr>
<td>Duration of Diabetes</td>
<td>( r )</td>
<td>0.111</td>
<td>0.343</td>
<td>0.034</td>
<td>-0.137</td>
</tr>
<tr>
<td></td>
<td>( p )-value</td>
<td>0.545</td>
<td>0.055</td>
<td>0.854</td>
<td>0.480</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycaemic control</th>
<th>FBF in response to ACh</th>
<th>FBF in response to SNP</th>
<th>FBF in response to insulin</th>
<th>FBF in response to L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG</td>
<td>( r )</td>
<td>-0.015</td>
<td>0.117</td>
<td>-0.043</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.936</td>
<td>0.044</td>
<td>0.522</td>
<td>0.824</td>
</tr>
<tr>
<td>HbA1C</td>
<td>( r )</td>
<td>-0.237</td>
<td>0.063</td>
<td>-0.008</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.192</td>
<td>0.005</td>
<td>0.731</td>
<td>0.967</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasma lipids</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>LDL cholesterol</th>
<th>Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )-value</td>
<td>0.389</td>
<td>-0.036</td>
<td>0.133</td>
<td>0.140</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.041</td>
<td>0.181</td>
<td>0.066</td>
<td>0.460</td>
</tr>
<tr>
<td>( r )-value</td>
<td>-0.036</td>
<td>0.848</td>
<td>0.527</td>
<td>0.460</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.481</td>
<td>0.172</td>
<td>0.281</td>
<td>0.656</td>
</tr>
<tr>
<td>( r )-value</td>
<td>0.048</td>
<td>0.527</td>
<td>0.075</td>
<td>0.695</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.470</td>
<td>0.045</td>
<td>0.356</td>
<td>0.741</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NEFA metabolism</th>
<th>Fasting NEFA</th>
<th>Steady-state NEFA</th>
<th>NEFA suppressibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )-value</td>
<td>-0.052</td>
<td>-0.074</td>
<td>-0.155</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.783</td>
<td>0.696</td>
<td>0.414</td>
</tr>
<tr>
<td>( r )-value</td>
<td>-0.137</td>
<td>-0.038</td>
<td>-0.113</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.181</td>
<td>0.627</td>
<td>0.945</td>
</tr>
<tr>
<td>( r )-value</td>
<td>-0.247</td>
<td>0.093</td>
<td>0.013</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.470</td>
<td>0.741</td>
<td>0.741</td>
</tr>
<tr>
<td>( r )-value</td>
<td>-0.013</td>
<td>-0.065</td>
<td>-0.065</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.949</td>
<td>0.741</td>
<td>0.849</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers of obesity</th>
<th>Skinfold thickness</th>
<th>Waist circumference</th>
<th>Hip circumference</th>
<th>WHR</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )-value</td>
<td>0.012</td>
<td>0.177</td>
<td>0.047</td>
<td>0.230</td>
<td>0.086</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.950</td>
<td>0.586</td>
<td>0.156</td>
<td>0.341</td>
<td>0.638</td>
</tr>
<tr>
<td><strong>0.009</strong></td>
<td>0.875</td>
<td>0.520</td>
<td>0.010</td>
<td>0.553</td>
<td>0.869</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>24 hour blood pressure measurements</th>
<th>Heart rate</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
<th>Mean BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )-value</td>
<td>-0.042</td>
<td>-0.024</td>
<td>-0.067</td>
<td>-0.029</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.821</td>
<td>0.896</td>
<td>0.714</td>
<td>0.877</td>
</tr>
<tr>
<td><strong>0.003</strong></td>
<td>0.380</td>
<td>0.895</td>
<td>0.757</td>
<td>0.968</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.220</td>
<td>0.503</td>
<td>0.224</td>
<td>0.363</td>
</tr>
<tr>
<td><strong>0.041</strong></td>
<td>0.905</td>
<td>0.063</td>
<td>0.853</td>
<td>0.062</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers of insulin sensitivity</th>
<th>FPI</th>
<th>M-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )-value</td>
<td>0.206</td>
<td>0.007</td>
</tr>
<tr>
<td>( p )-value</td>
<td>0.821</td>
<td>0.970</td>
</tr>
<tr>
<td><strong>0.037</strong></td>
<td>0.380</td>
<td>0.563</td>
</tr>
</tbody>
</table>

ACh= Acetylcholine, BMI= Body mass index, BP= Blood pressure, FBF= forearm blood flow, FPG= Fasting plasma glucose, FPI= fasting plasma insulin, HbA1C = glycosylated Haemoglobin, L-NMMA= N-monomethyl-L-arginine, SNP= Sodium Nitroprusside, WHR= waist:hip ratio
5.3.1. Basal forearm blood flow

Baseline FBF was not different in the study and the control arm (2.40 ±1.19 vs. 2.24±1.24 ml/dl/min respectively, t=-1.19, df=31, p=0.243).

FBF returned to baseline values after the 30 minutes rest period between each infusion period. This does not apply to the FBF before the infusion of L-NMMA, as this followed directly after the systemic infusion of insulin as part of the euglycaemic hyperinsulinaemic clamp. The actual comparison is shown in table 5.2. Adjustment for forearm volume did not alter these findings. Thereafter only FBF in the study arm will be reported.

Table 5.2: Comparison of baseline values in study and control arm at time points prior to each infusion period (Paired sample t-test was used for analysis).

<table>
<thead>
<tr>
<th></th>
<th>FBF at baseline (=FBF before ACh infusion) (ml/dl/min)</th>
<th>FBF before SNP infusion (ml/dl/min)</th>
<th>T</th>
<th>Df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study arm</strong></td>
<td>2.22±1.30</td>
<td>2.41±1.21</td>
<td>-1.81</td>
<td>31</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Control arm</strong></td>
<td>2.24±1.24</td>
<td>2.13±1.07</td>
<td>1.01</td>
<td>31</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>1.19</td>
<td>1.84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Df</strong></td>
<td>31</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>0.243</td>
<td>0.750</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FBF= Forearm blood flow, ACh= Acetylcholine, SNP= Sodium Nitroprusside

5.3.1.1. Gender and ethnic origin and basal blood flow

Basal FBF in men and women did not differ significantly (2.33±1.40 vs 1.60±0.66 ml/dl/min; independent samples t test: t=1.14, dF=30, p=0.264). Basal FBF in accordance to ethnicity did also not differ significantly (Caucasian/ Indian/ Afro-Carribean = 2.41±1.56 / 1.91±0.82/ 1.80±0.85 ml/dl/min; One way ANOVA F=1.16. dF=2, p=0.329). Adjustment for forearm volume (FBF_{fv}) did not alter these findings.
5.3.1.2. Duration of diabetes, glycaemic control and basal blood flow
There was no significant correlation between basal absolute FBF and HbA1c or FPG. Duration of diabetes was not associated with basal absolute FBF. Analysis of FBF\textsubscript{RV} did not differ significantly from unadjusted analysis.

5.3.1.3. The association of plasma lipid and NEFA concentrations with basal blood flow
Basal absolute FBF and total cholesterol concentrations were positively correlated (Spearman’s \( r = 0.389, p=0.041 \)). There was no significant correlation between basal FBF and plasma levels of triglyceride or HDL-cholesterol. Three patients had received treatment with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor. Exclusion of these patients from the analysis did not alter the above findings.

There was no significant correlation between any measure of NEFA concentration and basal FBF.

5.3.1.4. Insulin resistance and basal blood flow
There was no significant correlation between measures of insulin resistance (M-value, M\textsubscript{p}, FPI) and basal FBF. Analysis of FBF\textsubscript{RV} did not differ from unadjusted analysis. No difference in basal FBF was noted in the three subgroups of insulin resistance (IR group, Middle group, IS group: 2.58\(\pm\)2.11, 2.23\(\pm\)0.89, 1.77\(\pm\)0.61 ml/dl/min, respectively; \( F(2,28)=0.90, p=0.42 \)).

5.3.1.5. 24-hour ambulatory blood pressure and basal blood flow
There was no significant correlation between ambulatory heart rate, systolic, diastolic or mean blood pressure and basal absolute FBF. Analysis of FBF\textsubscript{RV} did not differ significantly from unadjusted analysis.

5.3.1.6. Markers of obesity and basal FBF
There was no significant correlation between markers of obesity (BMI, WHR, waist, hip circumference and skin fold) and basal absolute FBF. Analysis of FBF\textsubscript{RV} did not differ significantly from unadjusted analysis.
5.3.1.7. The influence of confounding factors on basal blood flow

The only significant correlation observed was the one between basal FBF and total cholesterol (p=0.050). No other significant association was found between basal blood flow and the above possible confounding factors.

5.3.2. Forearm blood flow in response to intra-arterial infusion of SNP

Only 30 subjects had an intra-arterial infusion of the highest dose of SNP as the other two did not tolerate this and developed severe symptomatic hypotension. The dose-response curve to SNP is shown in figure 5.1. The flow during each SNP infusion period is expressed as absolute FBF, FBF ratio, ΔFBF and vascular resistance (table 5.3). Vascular resistance was a useful measure as intra-arterial blood pressure changed significantly during the SNP infusion (table 5.4). Statistical analysis was performed using the slope of the dose-response curve. For this analysis the FBF value at the highest dose of SNP was excluded because, with the doses employed here, this value consistently falls outside the linear proportion of the dose-response curve. Correlations are shown for the dose-response curve slope unless they differ from maximum FBF, in which case both are shown separately. Table 5.1 summarises the correlations between FBF in response to SNP with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity.

5.3.2.1. The association of vasodilatory response to SNP with gender, age and ethnicity

Vascular response to SNP did not differ between men and women (4.20±2.10 vs 2.3±2.04 ml/dl/min; independent samples t test: t=2.31. dF=30, p=0.797). Basal FBF in accordance to ethnicity did also not differ (Caucasian/ Asian/ Afro-Caribbean: 4.08±2.29/ 3.96± 2.07/ 1.98±1.23 ml/dl/min; one way ANOVA F=2.072. dF=2, p=0.144). Adjustment for FOREARM VOLUME did not alter these findings. Vascular reactivity to SNP was also not significantly correlated with age (Spearman’s r=-0.175, p=0.339).
Table 5.3: FBF in response to intra-arterial infusion of SNP (mean and SD). Repeated measurements ANOVA was used for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Baseline before intra-arterial infusion of SNP</th>
<th>Intra-arterial infusion of SNP at a dose of 1 µg/min/dl</th>
<th>2 µg/min/dl</th>
<th>4 µg/min/dl</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute FBF (ml/dl/min)</td>
<td>2.41 ±1.21</td>
<td>5.18±3.30</td>
<td>10.23±5.13</td>
<td>12.18±5.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>δFBF (%)</td>
<td>100</td>
<td>231.9+130.0</td>
<td>441.1+149.7</td>
<td>535.0+155.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vascular resistance (mmHg/ml/dl/min)</td>
<td>48.4±23.4</td>
<td>23.9±12.8</td>
<td>11.1±7.7</td>
<td>7.8±4.2</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

FBF= forearm blood flow; SNP= Sodium Nitroprusside

Table 5.4: Intra-arterial blood pressure during infusion of Sodium Nitroprusside (mean and SD). Repeated measurements ANOVA was used for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Baseline before intra-arterial infusion of SNP</th>
<th>Intra-arterial infusion of SNP at a dose of 1 µg/min/dl</th>
<th>2 µg/min/dl</th>
<th>4 µg/min/dl</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP (mmHg)</td>
<td>95.8 ±10.2</td>
<td>91.3±11.1</td>
<td>83.5±13.1</td>
<td>76.2±13.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

MBP= Mean blood pressure, SNP= Sodium Nitroprusside
**Figure 5.1: Dose-response curve in response to Sodium Nitroprusside**

Boxplots are shown as summary plots based on the median, quartiles, and extreme values. Each box represents the interquartile range which contains the 50% of values. The whiskers are lines that extend from the box to the highest and lowest. A line across the box indicates the median.

- T - test arm
- C - control arm,
- BL - baseline,
1- Sodium Nitroprusside dose of 1μg/dl/min
2- Sodium Nitroprusside dose of 2μg/dl/min
3- Sodium Nitroprusside dose of 4μg/dl/min
5.3.2.2. The association of vasodilatory response to SNP with duration of diabetes and glycaemic control
There was no correlation of vascular response to SNP and duration of diabetes, FPG or HbA1c.

5.3.2.3. Vasodilatory response to SNP and plasma lipid concentrations
There was no significant correlation between vascular response to SNP and plasma lipid concentrations. Exclusion from the analysis of patients treated with an HMG CoA reductase inhibitor did not alter these findings.

5.3.2.4. Vasodilatory response to SNP and plasma NEFA concentrations
There was no significant correlation between vascular response to SNP and NEFA concentrations or NEFA suppressibility. Exclusion of patients on an HMG CoA reductase inhibitor from the analysis did not alter this finding.

5.3.2.5. Vasodilatory response to SNP and 24-hour ambulatory heart rate and blood pressure
Systolic blood pressure was significantly negatively associated with the slope of vascular reactivity to SNP. Five patients (13.2%) were receiving antihypertensive treatment with a beta-blocker and nine patients (23.7%) with an ACE-inhibitor. One patient received both treatments. Antihypertensive treatment was not taken on the day of the study. Exclusion of patients on antihypertensive treatment from the analysis did not alter these results.

5.3.2.6. Vasodilatory response to SNP and obesity
Significant negative correlations were noted between the slope of vascular reactivity to SNP and BMI and between the slope of vascular reactivity to SNP and biceps skinfold thickness, but not with waist circumference, hip circumference or WHR.
5.3.2.7. Vasodilatory response to SNP and insulin resistance

A significant positive correlation was noted between the slope of vascular reactivity to SNP and the M-value. There was no correlation between vascular response to SNP and FPI. Vascular response to did not differ significantly between the IR and the IS subgroups (data not shown).

5.3.2.8. The influence of confounding factors on the relationship between vasodilatory response to SNP with insulin resistance and obesity

Partial correlation coefficient $\beta$ of vascular response to SNP with BMI after correction for a number of variables is shown in table 5.5. The relationship between vascular response to SNP and BMI remained significant when triglyceride concentrations and cytokines were included into the model, but not when NEFA concentrations or insulin sensitivity were adjusted for. The relationship between cytokines and vascular reactivity is reported and discussed in chapter 6.

Partial correlation coefficient $\beta$ of vascular response to SNP with the M-value (as a measure of insulin resistance) after correction for a number of variables is shown in table 5.5. The relationship between vascular response to SNP and M-value remained significant when triglyceride concentrations and TNF alpha were included into the model, but not when NEFA concentrations, IL-6 or obesity were adjusted for.
Table 5.5: Partial correlation of the slope of vascular response to Sodium Nitroprusside with Body Mass Index after correction for different variables. Significant values at a level of p<0.05 are shown in bold.

<table>
<thead>
<tr>
<th>Partial correlation coefficient beta</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>-0.379</td>
</tr>
<tr>
<td>M-value</td>
<td>-0.296</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.349</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>-0.414</td>
</tr>
<tr>
<td>NEFA_q</td>
<td>-0.316</td>
</tr>
<tr>
<td>NEFA_{ss}</td>
<td>-0.336</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>-0.453</td>
</tr>
<tr>
<td>Tumour necrosis factor alpha</td>
<td>-0.511</td>
</tr>
</tbody>
</table>

NEFA_q: fasting non-esterified fatty acid concentrations,
NEFA_{ss}: non-esterified fatty acid concentrations at steady-state.

Table 5.6: Partial correlation of the slope of vascular response to Sodium Nitroprusside with insulin resistance after correction for different variables. Significant values at a level of p<0.05 are shown in bold.

<table>
<thead>
<tr>
<th>Partial correlation coefficient beta</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>0.376</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.344</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.348</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.395</td>
</tr>
<tr>
<td>NEFA_q</td>
<td>0.350</td>
</tr>
<tr>
<td>NEFA_{ss}</td>
<td>0.345</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>0.316</td>
</tr>
<tr>
<td>Tumour necrosis factor alpha</td>
<td>0.452</td>
</tr>
</tbody>
</table>

NEFA_q: fasting non-esterified fatty acid concentrations,
NEFA_{ss}: non-esterified fatty acid concentrations at steady-state.
5.3.3. Forearm blood flow in response to local infusion of Acetylcholine

32 subjects had a full dose-response curve to intra-arterial infusion of Ach (figure 5.2). FBF during each ACh infusion period is shown in table 5.7 as absolute FBF, FBF ratio and δFBF. Vascular resistance was a particularly useful measure as during the ACh infusion intra-arterial blood pressure changed significantly (table 5.8). Statistical analysis was performed using the slope of the dose-response curve and maximum vascular response. Correlations are shown for the dose-response curve slope unless they differ from maximum FBF, in which case both are shown separately. There was no statistical difference between the FBF values at baseline before ACh and before SNP in either arm (table 5.2). Table 5.1 summarises the correlations of FBF in response to ACh with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity.

5.3.3.1. The association of vascular response to ACh with gender, ethnicity and age

Vascular response to ACh in men and women did not differ (73.35+52.61 vs 95.37+58.27 ml/dl/min, respectively; independent samples t test: t=−0.847, dF=30, p=0.766). FBF did not differ by ethnicity (Caucasian/ Asian/ Afro-Caribbean: 79.92+46.79/ 77.19+69.41/ 43.49+14.49 ml/dl/min, respectively; One way ANOVA F=0.411. dF=2, p=0.667). Adjustment for Forearm volume did not alter these findings. Age was also not associated with vascular response to ACh.

5.3.3.2. The association of vascular response to ACh with duration of diabetes and glycaemic control

There was a significant negative correlation between the slope of vascular response to ACh and glycaemic control. Duration of diabetes did not correlate with vascular response to ACh.

5.3.3.3. Vascular response to ACh and plasma lipid concentrations

There was no significant correlation between vascular response to ACh and plasma lipid concentrations. Exclusion from the analysis of patients treated with an HMG CoA reductase inhibitor did not alter this finding.
Figure 5.2: Dose-response curve in response to Acetylcholine

Boxplots are shown as summary plot based on the median, quartiles, and extreme values. Each box represents the interquartile range which contains the 50% of values. The whiskers are lines that extend from the box to the highest and lowest values. A line across the box indicates the median.

- T: test arm,
- C: control arm,
- BL: baseline,
1. Acetylcholine dose of 0.15 μg/dl/min
2. Acetylcholine dose of 0.45 μg/dl/min
3. Acetylcholine dose of 1.5 μg/dl/min
4. Acetylcholine dose of 4.5 μg/dl/min
5. Acetylcholine dose of 15 μg/dl/min
Table 5.7: FBF in response to intra-arterial infusion of Acetylcholine (ACh) (mean and SD). Repeated measurements ANOVA was used for comparison.

<table>
<thead>
<tr>
<th>Before ACh</th>
<th>Intra-arterial infusion of ACh at a dose of</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 µg/min/dl</td>
<td>0.45 µg/min/dl</td>
<td>1.5 µg/min/dl</td>
</tr>
<tr>
<td>Absolute FBF (ml/min/dl)</td>
<td>2.21±1.3</td>
<td>2.40±1.19</td>
</tr>
<tr>
<td>δFBF (%)</td>
<td>100</td>
<td>111.43±22.06</td>
</tr>
<tr>
<td>Vascular resistance</td>
<td>48.4±22.1</td>
<td>39.8±19.4</td>
</tr>
</tbody>
</table>

FBF- Forearm blood flow; ACh- Acetylcholine

Table 5.8: Intra-arterial blood pressure during Acetylcholine infusion (Mean and SD)

<table>
<thead>
<tr>
<th>Before ACh</th>
<th>Intra-arterial infusion of ACh at a dose of</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 µg/min/dl</td>
<td>0.45 µg/min/dl</td>
<td>1.5 µg/min/dl</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>88.5±17.4</td>
<td>83.5±17.8</td>
</tr>
</tbody>
</table>

MBP- mean blood pressure, ACh- Acetylcholine

5.3.3.4. Vascular response to ACh and plasma NEFA concentrations

There was no significant correlation between vascular response to ACh and NEFA concentrations or NEFA suppressibility. Exclusion of patients on an HMG CoA reductase inhibitor from the analysis did not alter this finding.

5.3.3.5. Vascular response to ACh and 24-hour ambulatory heart rate and blood pressure

There was no correlation between vascular response to ACh and ambulatory heart rate, systolic, diastolic or mean blood pressure. Exclusion of patients on an antihypertensive treatment from the analyses did not alter these results.
5.3.3.6. Vascular response to ACh and obesity

WHR was negatively correlated with the slope of vascular response to ACh. No significant correlations were noted between vascular reactivity to ACh and BMI, waist circumference, hip circumference or skin fold thickness.

5.3.3.7. Vascular response to ACh and insulin resistance

There was no association between vascular response to ACh and the M-value or FPI. Correction for BMI did not alter this. Vascular response to ACh did not differ between the IR and the IS subgroups.

5.3.3.8. The influence of confounding factors on the relationship between vascular response to ACh and obesity

WHR but not BMI as marker of obesity was associated with vascular response to ACh (section 5.3.3.6.). Partial correlation coefficient β of vascular response to ACh with WHR after correction for a number of variables is shown in table 5.9. Confounding factors made the association between WHR and response to ACh non-significant for all variables included into the model.

Table 5.9: Partial correlation of vascular response to ACh with WHR after correction for different variables

<table>
<thead>
<tr>
<th>Partial correlation coefficient beta</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>-0.384</td>
</tr>
<tr>
<td>M-value</td>
<td>0.092</td>
</tr>
<tr>
<td>Total cholesterol concentrations</td>
<td>0.063</td>
</tr>
<tr>
<td>Triglyceride concentrations</td>
<td>0.079</td>
</tr>
<tr>
<td>NEFAs concentrations</td>
<td>0.094</td>
</tr>
<tr>
<td>NEFAs concentrations</td>
<td>0.345</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>-0.389</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha</td>
<td>-0.294</td>
</tr>
</tbody>
</table>

NEFAs, fasting non-esterified fatty acid concentrations, NEFAs, non-esterified fatty acid concentrations at steady-state,
5.3.4. Forearm blood flow in response to systemic infusion of insulin

The FBF at baseline, 60 minutes and 120 minutes after intra-venous (‘systemic’) infusion of insulin is shown in table 5.10.

As a reminder: insulin was infused intravenously into the previously called ‘control’ arm. To assess a possible local effect, FBF in the ‘insulin infused’ arm vs. FBF in the contra-lateral (previously ‘study’) arm was also analysed (Table 5.10). There was no significant difference between the infused and contra-lateral arm after infusion of insulin at any time before or during the insulin infusion. For correlation analysis maximum vascular response to insulin is being used. Table 5.1 summarises the correlations of FBF in response to insulin with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity.

5.3.4.1. The association of vascular response to insulin with gender, ethnicity and age

There was no association between vascular response to insulin and age, gender (2.07±1.14 vs. 2.47±1.31 ml/min/dl, respectively; independent t-test t=-0.635, p=0.560) and ethnic origin (Caucasians/ Asians/ Afro-Caribbean: 2.75±1.23/ 1.70±0.73/ 3.05±0.07 ml/min/dl, respectively; One way ANOVA F=3.49, dF=2, p=0.055).

5.3.4.2. The association of vascular response to insulin with duration of diabetes or glycaemic control

There was no association between vascular response to insulin and duration of diabetes.

5.3.4.3. Vascular response to insulin and plasma lipid concentrations

There was no association between vascular response to insulin and plasma lipid concentrations. Exclusion of patients on an HMG CoA reductase inhibitor from the analysis did not alter these results.
Table 5.10: Comparison of forearm blood flow (FBF) in study and control arm in response to systemic infusion of insulin and local infusion of L-NMMA

FBF in response to insulin was compared to baseline, FBF in response to L-NMMA was compared to maximum response to insulin (Paired sample t-test and repeated measurements ANOVA were used for analysis)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Time after reaching euglycaemia</th>
<th>F</th>
<th>p-value</th>
<th>Post L-NMMA</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min post insulin</td>
<td>120 min post insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBF in study arm (ml/dl/min)</td>
<td>2.31±1.2 6</td>
<td>2.40±1.08</td>
<td>3.52</td>
<td>0.064</td>
<td>2.23±1.2 2</td>
<td>3.39</td>
<td>0.002</td>
</tr>
<tr>
<td>FBF in control arm (ml/dl/min)</td>
<td>2.25±1.2 3</td>
<td>2.28±1.08</td>
<td>0.81</td>
<td>0.452</td>
<td>2.44±1.2 7</td>
<td>-0.239</td>
<td>0.813</td>
</tr>
<tr>
<td>p-value</td>
<td>0.056</td>
<td>0.371</td>
<td>0.192</td>
<td></td>
<td>0.197</td>
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<td></td>
</tr>
</tbody>
</table>

5.3.4.4. Vascular response to insulin and plasma NEFA concentrations

There was no association between vascular response to insulin and plasma NEFA concentrations. Exclusion of patients on a HMG CoA reductase inhibitor or correction for triglyceride concentrations did not alter this finding.

5.3.4.5. Vascular response to insulin and 24-hour ambulatory heart rate and blood pressure

There was no association between vascular response to insulin and 24-hour ambulatory blood pressure or heart rate. Exclusion of patients receiving antihypertensive treatment did not alter this finding.

5.3.4.6. Vascular response to insulin and obesity

No significant correlations were noted between vascular reactivity to insulin and BMI, WHR, waist circumference, hip circumference or skin fold thickness.
5.3.4.7. Vascular response to insulin and insulin resistance
There was no association between vascular response to insulin and M-value. Vascular response to insulin did not differ between the IR and the IS group (F=0.471, p=0.63).

5.3.5. Forearm blood flow in response to local infusion of L-NMMA (post insulin)
The flow after infusion of L-NMMA decreased significantly (2.68±1.42 vs. 2.23±1.22, F (1,28)=11.47, p=0.002) (table 5.10).
FBF in response to L-NMMA is expressed as the ratio of FBF after L-NMMA infusion to FBF immediately before (after 120 min of euglycaemia) and then used for analysis.
Table 5.1 summarises the correlations of FBF in response to L-NMMA with indices of glycaemic control, lipid and NEFA metabolism, 24 hour blood pressure measurements, obesity and insulin sensitivity.

5.3.5.1. The association of vascular response to L-NMMA with gender, ethnicity and age
There was no association between vascular response to L-NMMA and gender (independent t-test t=1.8, p=0.179), age or ethnicity (One way ANOVA F=0.009, dF=2, p=0.991).

5.3.5.2. The association of vascular response to L-NMMA with duration of diabetes and glycaemic control
There was no association between vascular response to L-NMMA and duration of disease or glycaemic control.

5.3.5.3. Vascular response to L-NMMA and plasma lipid concentrations
Vascular response to L-NMMA was significantly negatively associated with fasting plasma triglyceride concentrations (r= -0.402, p=0.038). After correction for BMI this was no longer significant (β = -0.219, p=0.299). There was no association between vascular response to L-NMMA and any other plasma lipid concentrations. These findings did not change after adjustment for BMI or exclusion of patients on treatment with an HMG CoA reductase inhibitor from the analysis.
5.3.5.4. Vascular response to L-NMMA and plasma NEFA concentrations
There was no association between vascular response to L-NMMA and plasma NEFA concentrations. This did not change after adjustment for BMI or triglyceride levels or after exclusion of patients on treatment with an HMG CoA reductase inhibitor from the analysis.

5.3.5.5. Vascular response to L-NMMA and 24-hour ambulatory heart rate and blood pressure
There was no association between vascular response to L-NMMA and 24-hour ambulatory blood pressure or heart rate. Exclusion of patients receiving antihypertensive treatment did not alter this finding.

5.3.5.6. Vascular response to L-NMMA and obesity
No significant correlations were noted between vascular reactivity to insulin and BMI, WHR, waist circumference, hip circumference or skin fold thickness.

5.3.5.7. Vascular response to L-NMMA and insulin resistance
There was no association between vascular response to L-NMMA and M-value. Vascular response to L-NMMA did not differ between the IR and the IS group (F=0.436, p=0.404).
5.4. Summary

_Basil FBF_ did not differ between the control and the test arm. There was no significant relation of FBF with gender, age or ethnicity. Total cholesterol concentration was positively correlated with basal FBF. This association was no longer significant after including triglyceride concentrations, BMI and insulin sensitivity in the model. There was no significant correlation between markers of obesity and basal absolute FBF. Basal blood flow did not differ between the insulin resistant and insulin sensitive subgroups.

_Vascular response to SNP_ and insulin sensitivity were significantly associated. This was dependent on BMI, cholesterol levels, NEFA levels and IL-6 but not triglycerides. BMI and skin fold thickness, but none of the other measures of obesity were significantly negatively correlated with the slope of vascular reactivity to SNP. Systolic blood pressure was significantly negatively correlated with the slope of response to SNP.

_Vascular response to ACh_ was not significantly correlated with insulin resistance. It did not differ between the insulin resistant and the insulin sensitive subgroups. Glycaemic control was significantly negatively associated with the slope of vascular response to ACh. Only WHR as a measure of obesity was significantly and negatively correlated with the slope of vascular response to ACh. This was dependent on insulin resistance, lipid, NEFA and cytokine levels.

_Vascular response to insulin or L-NMMA_ did not differ between the insulin resistant and the insulin sensitive subgroups. No association between vascular response to systemic infusion of insulin and insulin resistance or obesity was observed in our cohort. No significant vasodilatory response to insulin was observed across the whole cohort. However, a trend was noted (p=0.064). Vasoconstriction after local infusion of L-NMMA was observed. This was not associated with insulin resistance or obesity.
5.5. Discussion

To date, no real consensus has been reached on the association between type 2 diabetes mellitus and endothelial dysfunction. The majority of studies demonstrate impaired endothelium-dependent vasodilatation in diabetes (72;149;405;406). However, on closer observation of the literature a number of groups report impaired endothelium-independent vascular reactivity (i.e. to SNP or GTN) (367;401;404). All of these studies compared a group of patients with type 2 diabetes with healthy controls. The complexity of the study methodology as well as the frequent presence of associated factors, independently associated with endothelial function, have complicated the assessment of endothelial function in diabetes.

The first aim of our study was to test the hypothesis that endothelial function is impaired in the insulin resistance of type 2 diabetes. We investigated this by using the postulation that vascular reactivity in response to intra-arterially infused acetylcholine is endothelium dependent and that to sodium nitroprusside endothelium independent. We were thus comparing change in forearm blood flow in response to these agents, administered intra-arterially, as a surrogate means of identifying endothelial dysfunction. We did not study a control group. No final conclusion on this first aim could therefore be drawn. However, we were able to examine relationships within these subjects, grouping them by severity of insulin resistance. Our secondary aim was to examine the influence of associated factors in this relationship (hyperglycaemia, hypertension, obesity, and dyslipidaemia).

Our cohort was not homogenous, but we were able to demonstrate that age, gender, ethnic origin, duration of diabetes, glycaemic control or medication were not associated with blood flow and there was no significant difference between the insulin resistant and the insulin sensitive subgroup in these parameters.

*Endothelium-dependent vascular reactivity*

Hyperglycaemia has previously been suggested as an obvious candidate mechanism for causing endothelial dysfunction in diabetes (403). In our cohort a negative association
between fasting plasma glucose or HbA1c (a marker of chronic exposure to hyperglycaemia) and endothelium-dependent vascular response was observed. Another mechanisms implied for endothelial dysfunction in diabetes are increased circulating low-density lipoprotein concentrations. These decreased together with improvement of endothelial –dependent vascular response after treatment with insulin in a cohort with type 2 diabetes (367). In our study plasma concentrations of lipids were not associated with vascular response to Acetylcholine.

Several studies have shown that insulin resistance is associated with endothelial dysfunction independent of hyperglycaemia (152;442). It has been suggested that insulin’s effect to promote glucose disposal in muscle and adipose tissue might share downstream post-receptor effects with its effect on NO production and that any defect of the post receptor insulin signalling pathway would affect both processes in parallel (151;407;443). Evidence for this is a shared pathway for glucose uptake and NO-production via PI-3 kinase (407). No association between insulin resistance and impairment in vascular response to Acetylcholine, an endothelium-dependent measure, was observed in our cohort. This might be explained by the fact that we did not study a healthy control group in comparison to our diabetic cohort. It is plausible that in our cohort endothelium-dependent vascular response was generally impaired. However, comparing two extreme subgroups of insulin resistance did also not show a difference in vascular response to Acetylcholine. Insulin resistance presents as a continuous rather than a categorical phenomenon. The fairly narrow range of insulin resistance in our cohort might have reduced the strength of correlations. Our study cohort had uncomplicated diabetes and thus might present differently from the ‘usual’ diabetes cohort, exhibiting resistance to insulin’s action on glucose uptake but not on NO-regulation.

Waist-hip ratio, but not BMI was the only marker of obesity associated with endothelium-dependent vascular reactivity. This is an interesting finding considering the work of Baron’s group suggesting endothelial-dependent vasodilatation to be due to obesity (84;128). Furthermore Despres’ group has reported waist circumference to be greatly associated with cardio-vascular risk factors and this to be more predictive than BMI or WHR (444;445). In
our cohort no association between waist circumference and endothelium-dependent
vasodilatation was observed. Furthermore, if central obesity correlates with endothelial
function the question of possible signals arises. Correction of the relationship between
vascular response to ACh and WHR for lipid, NEFA or cytokine levels shows that each of
these indices could act as a signal between obesity and endothelial function (table 5.9).

**Endothelium-independent vascular reactivity**
We studied vascular response to Sodium Nitroprusside, an endothelium-independent
vasodilator, as a control when examining if insulin resistance is associated with endothelial
dysfunction. Surprisingly, in our cohort vascular response to Sodium Nitroprusside, as a
marker of endothelium-independent vascular reactivity, was significantly correlated with
insulin resistance as well as with obesity (BMI and biceps skin fold thickness). These
associations were independent of triglyceride concentrations, but depended on NEFA and
cytokine levels. The associations of vascular response to SNP with obesity and insulin
resistance were mutually dependent.

Several mechanisms have been postulated for endothelial dysfunction. Increased inactivation
of NO through increased superoxide, high levels of oxygen-derived free radicals,
glycosylation end-products and transport barriers such as thickened basement membranes has
been previously implicated as a cause. Another mechanism suggested is decreased
responsiveness of the NO-guanylate cyclase pathway at the level of vascular smooth muscle
(395;397). Increased release of vasoconstrictor prostanoids that counteract the vasodilatation
by NO has also been reported. Steinberg suggested that NO production in response to the
different stimuli might be mediated via different signalling pathways (446). It is unclear why
in our cohort only endothelium-independent vasodilatation was associated with insulin
resistance and obesity. The fact that exogenous NO response (to SNP) but not endogenous
NO response (to ACh) is affected makes neutralisation of NO or defects of smooth muscle
cells improbable mechanisms.
Confounding factors

Hypertension, dyslipidaemia and obesity are each associated with endothelial dysfunction (117,128;391;393;406). Subjects with type 2 diabetes are generally a heterogeneous group and this may contribute to the diversity of findings in these cohorts. Our study set also out to examine the influence of associated factors in the relationship between endothelial function and insulin resistance (hyperglycaemia, hypertension, obesity and dyslipidaemia). No association was observed between endothelium-dependent vascular response (to ACh) and hyperinsulinaemia, plasma triglycerides or NEFA concentrations. Ambulatory blood pressure measurements were not associated with vascular response to ACh. Waist-hip ratio was the only marker of obesity associated with vascular response to ACh in our cohort.

A number of confounding factors were in themselves strongly negatively associated with vascular response to SNP (systolic blood pressure, BMI and biceps skin fold thickness). The association between vascular response to SNP and insulin resistance was dependent on several confounding factors (NEFA and cytokine concentrations and markers of obesity).

In conclusion, we found no evidence for a strong association between insulin resistance and endothelial dysfunction on blood flow measurements. This is somehow surprising, considering that several studies in the literature point to the NO-pathway as a defect in insulin resistance. We studied a small cohort with uncomplicated diabetes with little evidence of vascular damage. Choosing a cohort with evidence of more progressive atherosclerosis might elicit the presence of endothelial damage. However, the presence of several abnormalities at once might prevent a clear view on underlying/confounding factors.
Figure 5.3: Vascular reactivity to Sodium Nitroprusside (slope percentage over baseline) in three subgroups according to their insulin sensitivity.

Patients from the two extreme tertiles were identified as insulin resistant (IR) and insulin sensitive (IS). Mdl shows the middle group. Boxplots are shown as summary plot based on the median, quartiles, and extreme values. Each box represents the interquartile range which contains the 50% of values. The whiskers are lines that extend from the box to the highest and lowest values. A line across the box indicates the median.
Figure 5.4: Vascular reactivity to Acetylcholine (slope percentage over baseline) in three subgroups according to their insulin sensitivity.

Patients from the two extreme tertiles were identified as insulin resistant (IR) and insulin sensitive (IS). Mdl shows the middle group. Boxplots are shown as summary plot based on the median, quartiles, and extreme values. Each box represents the interquartile range which contains the 50% of values. The whiskers are lines that extend from the box to the highest and lowest values. A line across the box indicates the median.
Chapter 6
Cytokines in type 2 diabetes mellitus
(Results and discussion)

6.1. Introduction
Although the main physiological abnormalities in type 2 diabetes are thought to be insulin resistance and impaired insulin secretion (6;7;8;290), an accumulating body of evidence suggests that inflammation may play a role in its pathogenesis. Experimental evidence and more recent cross-sectional data suggests that circulating levels of cytokines are associated with hyperglycaemia, insulin resistance and type 2 diabetes: Interleukin-6 (IL-6) and tumour necrosis factor (TNFα) are such pro-inflammatory cytokines with important effects on lipid and glucose metabolism (157-159;161). Both cytokines have been shown to stimulate glucose uptake in cultured adipocytes and to inhibit LPL-activity (158;159;169). IL-6 stimulates insulin release from Hamster islet cells (160) and in humans stimulates glucose and NEFA oxidation, as well as induces glucagon and cortisol release (161). Fernandez-Real et al recently reported that a polymorphism of the IL-6 gene influences the relationship among insulin sensitivity and peripheral white cell count (162). TNFα has been reported to inhibit the tyrosine phosphorylation of insulin receptor substrate-1 by insulin binding (167;168), suppress the action of lipoprotein lipase (LPL) (169) and stimulate lipolysis (170). Again, IL-6 has similar effects (172-174).

It has been put forward that pro-inflammatory cytokines form part of the insulin resistance syndrome and they have been implicated as a common antecedent underlying the syndrome (Fig. 1.2) (56;57;127;175;176). The relationship between cytokines and insulin resistance has been discussed in more detail in section 1.2.4.3.5. Only a few investigators have looked at circulating concentrations of cytokines (163-166;175;176;187). Studying acute phase response in three groups of subjects (non-diabetic, type 2 diabetic without syndrome X and type 2 diabetic with syndrome X) highest levels of IL-6 and markers of acute phase response were found in the diabetic subjects with several components of the insulin resistance
syndrome; lowest levels were seen in normal controls (175). Yudkin et al studied a group of 107 non-diabetic subjects and related levels of C-reactive protein and interleukin-6 to markers of the insulin resistance syndrome and of endothelial dysfunction. Levels of acute phase markers correlated closely with insulin resistance syndrome variables, this relationship being weakened only marginally by adjusting for measures of obesity. Following analysis of a recent large prospective study in healthy middle-aged women Pradhan et al reported that IL-6 (and CRP) levels were predictors for the development of type 2 diabetes independently of family history of diabetes and obesity (447).

Observational studies in humans have suggested that acute infection or acute systemic inflammation lead to an increase in cardio-vascular risk (448;449). Cytokines, as part of the acute response, leading to endothelial damage have been implicated as one possible mechanism (374-376). The relationship between cytokines and endothelial function has been discussed in more detail in section 1.3.3.1.

In summary, cytokines are strongly implicated in the regulation of energy balance, possibly contributing to several components of the insulin resistance syndrome. They are also implemented in causing endothelial dysfunction. It might be plausible that these pro-inflammatory cytokines are the link between these two pathologies.

6.2. Aims

The aim of this study was to evaluate cytokines as a possible signal linking insulin resistance and endothelial dysfunction in subjects with uncomplicated type 2 diabetes.
6.3. Results

Of the 38 patients participating in the study of insulin resistance, vascular reactivity, lipids, non-esterified fatty acids and cytokines in type 2 diabetes mellitus, cytokine measurements were available in 34 subjects (TNFα) and 30 subjects (IL-6). These subgroups did not differ from the patients in whom measurements were unavailable.

Levels of TNFα and IL-6 concentrations correlated strongly with each other (Spearman’s r=0.615, p<0.0001) (Fig. 6.1). One subject was noted to have a raised C-reactive protein (16.2 mg/l) in comparison with the whole cohort (mean 4.44, 95% CI 3.85- 5.04). As this suggested a sub-acute inflammatory response this patient was excluded from this analysis.

6.3.1. TNFα

TNFα was measured in 34 patients (mean 2.70±0.92 SD pg/ml). Data on lipid and NEFA concentrations and insulin sensitivity measurements were available in all 34. Data on vascular reactivity were available in 29 out of the 34 patients (TNFα mean 2.65±0.91 SD pg/ml; this was not significantly different from TNFα in the group of 5 patients without these measures).

There was no correlation between TNFα levels and measures of glycaemic control (for FPG r=0.203, p=0.250 and for HbA1c r=-0.012, p=0.945). Measures of obesity did not correlate significantly with TNFα concentrations (for BMI r=-0.035, p=0.843, for WHR r=-0.118, p=0.514).

6.3.1.1. TNFα and insulin resistance

TNFα concentrations did not correlate with the M-value (Spearman’s r= -0.232, p=0.202) or FPI (r=0.033, p=0.854) (Figure 6.2). TNFα did not differ between the IR and the IS subgroup (2.96±0.70 pg/ml vs. 2.82±1.21 pg/ml respectively; t=-0.781, p=0.454) (Fig 6.3).
There were significant correlations between TNFα concentrations and plasma total cholesterol as well as calculated LDL-cholesterol levels. There was no significant correlation between TNFα and triglyceride concentrations or any measure of NEFA concentrations. These associations are shown in table 6.1.

Table 6.1: Correlations between circulating concentrations of cytokines and plasma lipid and NEFA concentrations. Spearman’s correlation coefficient r is shown, p-values significant at < 0.05 level are shown in bold

<table>
<thead>
<tr>
<th></th>
<th>TNFα</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.164</td>
<td>0.217</td>
</tr>
<tr>
<td>p-value</td>
<td>NS (0.371)</td>
<td>NS (0.268)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.389</td>
<td>0.383</td>
</tr>
<tr>
<td>p-value</td>
<td>0.031</td>
<td>0.049</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>-0.219</td>
<td>0.066</td>
</tr>
<tr>
<td>p-value</td>
<td>NS (0.220)</td>
<td>NS (0.734)</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.409</td>
<td>0.360</td>
</tr>
<tr>
<td>p-value</td>
<td><strong>0.028</strong></td>
<td>NS (0.077)</td>
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<tr>
<td>NEFA_fast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.187</td>
<td>0.372</td>
</tr>
<tr>
<td>p-value</td>
<td>NS (0.296)</td>
<td>NS (0.051)</td>
</tr>
<tr>
<td>NEFA_SS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.125</td>
<td>0.272</td>
</tr>
<tr>
<td>p-value</td>
<td>NS (0.496)</td>
<td>NS (0.162)</td>
</tr>
<tr>
<td>NEFA_supp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.031</td>
<td>0.100</td>
</tr>
<tr>
<td>p-value</td>
<td>NS (0.867)</td>
<td>NS (0.608)</td>
</tr>
</tbody>
</table>

**Abbreviations**

- TNFα: tumor necrosis factor alpha
- IL-6: Interleukin-6
- NEFA_fast: fasting non-esterified fatty acid concentrations
- NEFA_SS: non-esterified fatty acid concentrations at steady-state
- NEFA_supp: percentage suppression of non-esterified fatty acid concentrations from fasting to steady state
6.3.1.3. TNFα and vascular reactivity

There was no correlation between TNFα concentrations and basal FBF or between TNFα and vascular reactivity to SNP, ACh, insulin or L-NMMA (Table 6.2).

Table 6.2: Correlations between circulating concentrations of cytokines and vascular reactivity. Spearman’s correlation coefficient r is shown, p-values significant at < 0.05 level are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>TNFα</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal forearm blood flow</td>
<td>r</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.869)</td>
</tr>
<tr>
<td>Maximum vascular response to ACh</td>
<td>r</td>
<td>0.131</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.500)</td>
</tr>
<tr>
<td>Slope of vascular response to ACh</td>
<td>r</td>
<td>0.242</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.201)</td>
</tr>
<tr>
<td>Maximum vascular response to SNP</td>
<td>r</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.669)</td>
</tr>
<tr>
<td>Slope of vascular response to SNP</td>
<td>r</td>
<td>-0.075</td>
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<tr>
<td></td>
<td>p-value</td>
<td>NS (0.581)</td>
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<tr>
<td>Vascular response to insulin</td>
<td>r</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.747)</td>
</tr>
<tr>
<td>Vascular response to L-NMMA</td>
<td>r</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>NS (0.739)</td>
</tr>
</tbody>
</table>

ACh Acetylcholine
SNP Sodium Nitroprusside
L-NMMA N-monomethyl-L-arginine
IL-6 Interleukin-6
TNFα Tumor necrosis factor alpha

6.3.2. Interleukin-6 (IL-6)

IL-6 was measured in 30 patients (mean 2.47±1.35 SD pg/ml). Data on lipid and NEFA concentrations, insulin sensitivity and vascular reactivity were available in all 30 patients. There was no correlation between IL-6 concentrations and glycaemic control (for FPG r=0.213, p=0.258 and for HbA1c r=−0.179, p=0.399). Measures of obesity also did not correlate with IL-6 concentrations (for BMI r=0.095, p=0.616 and for WHR r=0.076, p=0.691).
6.3.2.1. IL-6 and insulin resistance

IL-6 concentrations differed between the IR and IS subgroup (3.32±1.40 pg/ml vs. 1.93±0.973 pg/ml, t=2.458, p=0.026) (Fig 6.4). In the pooled analysis there was no significant correlation between IL-6 concentrations and insulin resistance (for M-value r=-0.349, p=0.063 and for FPI r=0.244, p=0.195) (Fig. 6.5).

6.3.2.2. IL-6 and NEFA and plasma lipid concentrations

There were significant correlations between IL-6 concentrations and total plasma cholesterol levels. There was no significant correlation between IL-6 and triglyceride or HDL concentration or any measure of NEFA concentrations. These associations are shown in table 6.1.

6.3.2.3. IL-6 and vascular reactivity

The associations between IL-6 and vascular reactivity are shown in table 6.2. IL-6 concentrations were positively correlated with basal FBF (r=0.419, p=0.033). IL-6 concentrations were not correlated with maximum vasodilatation in response to SNP (r=-0.376, p=0.064), but correlated negatively with the SNP dose-response curve slope (r=-0.489, p=0.011). IL-6 concentrations were significantly positively correlated with maximum vasodilatation in response to ACh (r=0.400, p=0.043) but not with the ACh dose-response curve slope (r=0.171, p=0.405). However, after Bonferroni correction the association between IL-6 levels and vascular reactivity was no longer significant. There was no correlation between IL-6 concentrations and vascular response to insulin or L-NMMA.
6.4. Summary
Circulating levels of TNFα and IL-6 correlate strongly with each other. Both concentrations of circulating cytokines correlated significantly with plasma cholesterol concentrations but not with triglyceride or NEFA levels.

IL-6 levels but not TNFα levels differed significantly between the two extreme subgroups of insulin resistance. In the pooled analysis no significant association between insulin resistance and circulating cytokine levels were observed.

No correlation between TNFα and vascular reactivity was observed. The correlation between IL-6 and vascular reactivity to SNP and ACh did not remain significant after Bonferroni correction.

6.5. Discussion
To date, no study has measured circulating cytokine concentrations in a cohort which had at the same time measurements of insulin resistance (by euglycaemic, hyperinsulinaemic clamp) and endothelial function (by venous occlusion plethysmography in response to vasoactive stimulants). Our study group is small but fairly homogenous without any evidence of vascular complications.

Few reports are available on the relationship between vascular reactivity and circulating cytokine levels. In contrast to our study, most other groups examining the relationship between vascular reactivity and cytokines have studied a response to infused cytokines rather than circulating cytokine levels (376;450). Holm et al recently reported increased levels of TNFα and IL-6 in recipients of a heart-lung transplant. In addition they reported a negative correlation between TNFα levels and vascular response to ACh (451). A larger number of studies report on the relationship between systemic cytokine levels and insulin resistance.
Circulating levels of TNFα were observed to correlate with insulin resistance in subjects with cancer and chronic sepsis, but infusion of TNFα antibodies to subjects with type 2 diabetes did not change their sensitivity to insulin’s effect on glucose or lipid metabolism.

Our aim was to examine if cytokines could be a possible link between insulin resistance and endothelial dysfunction in type 2 diabetes. We also studied the influence of obesity on these relationships. We firstly examined the relationship between cytokine concentrations and insulin resistance. In our cohort circulating levels of IL-6 were significantly higher in the insulin resistant subgroup than in the insulin sensitive one. We did not observe a link between circulating levels of TNFα and insulin resistance. We secondly examined the relationship between cytokine concentrations and endothelial function. Circulating levels of IL-6 correlated significantly and positively with measures of endothelium-dependent (to Acetylcholine) vascular reactivity and negatively with measures of endothelium-independent (to Sodium Nitroprusside) vascular reactivity. These results may have been the consequence of a Type 1 error. No correlation between circulating levels of TNFα and vascular reactivity was observed. We thirdly examined the relationship between cytokine concentrations and obesity. Circulating levels of cytokines were not associated with measures of obesity. This finding is unlike that of Mohamed-Ali et al, who reported a significant correlation between circulating IL-6 (but not TNFα) levels and adiposity indexes (BMI and %body fat) in non-diabetic subjects (163). Their cohort was larger and the subjects more obese (median BMI 32.9, interquartile range 20.0 kg/m²) than ours (BMI 28.8, interquartile range 5.7 kg/m²). Their study did not include diabetic subjects. Pickup et al suggested that type 2 diabetes is associated with an elevated acute-phase response, particularly in those with features of the insulin resistance syndrome and this may explain different findings in subjects with or without diabetes (175).

We then sought to establish if cytokines could act as a confounding factor in the relationship between insulin resistance and endothelial dysfunction. In our cohort no evidence of an association between endothelial dysfunction (assessed by vascular response to Acetylcholine)
and insulin resistance (assessed by the clamp) was observed (section 5.3.3.7) and this has
been discussed in section 5.5. However, there was a significant correlation between
endothelium-independent vascular response (to SNP) and insulin resistance (section 5.3.2.6).
Adjustment for circulating concentrations of IL-6 attenuated the relationship of response to
SNP with insulin resistance (M-value). The partial correlation coefficient decreased from
0.376 to 0.316 with IL-6 forced into the model (it increased from 0.376 to 0.452 with TNFα
forced in). These observations implicate IL-6 as a possible signal between endothelium-
independent vasodilatation and insulin resistance.

We also observed a correlation between endothelium-independent vascular response (to SNP)
and obesity (section 5.3.2.7). Adjustment for circulating IL-6 concentrations increased rather
than attenuated the strength of the association between obesity (BMI) and vascular response
to SNP, the partial correlation coefficient increased from -0.379 to -0.453 with IL-6 forced
into the model. This might suggest that the impaired vascular response to SNP observed in
obesity is not mediated via circulating IL-6. Interestingly, it has been shown that in non-
diabetic subjects only 10-30% of circulating levels of IL-6 is attributable to adipose tissue
(454). It is possible that cytokines of non-adipose tissue origin play, at least in part, a
different role from those produced by the adipose tissue, especially in diabetes.

Our findings suggest that the pro-inflammatory cytokine interleukin-6 might have a role in
linking insulin resistance and obesity with vascular function. More studies into these
questions are needed, however, to advance the development of therapeutic interventions for
these patients at high risk of cardiovascular disease.
Figure 6.1: Correlation between circulating levels of Tumour necrosis factor α (TNFα) and circulating levels of interleukin-6 (IL-6)

Figure 6.2: Correlation between insulin resistance and circulating Tumour necrosis factor α (TNFα) concentrations
Figure 6.3. Circulating TNFα concentrations in the three subgroups of the cohort divided into tertiles of insulin resistance: IR (insulin resistant, n=12) and IS (insulin sensitive, n=10) show the two extreme subgroups of insulin sensitivity. Mdl is the middle subgroup (n=11).

Figure 6.4. Circulating IL-6 concentrations in the three subgroups of the cohort divided into tertiles of insulin sensitivity. IR (insulin resistant, n=11) and IS (insulin sensitive, n=10) show the two extreme subgroups of insulin sensitivity. Mdl is the middle subgroup (n=9).
Figure 6.5.: Correlation between insulin resistance and circulating interleukin-6 (IL-6) concentrations.
Chapter 7
Circulating endothelial cell products in type 2 diabetes mellitus
(Results and discussion)

7.1. Introduction
Circulating concentrations of tissue plasminogen activator, von Willebrand Factor, thrombomodulin and cellular fibronectin have been measured and shown to be of endothelial cell origin in human studies (333-338). The origin of these circulating markers is likely to be from peripheral capillary endothelium as opposed to large arterial endothelium. Thus altered levels of these and other endothelial cell products might potentially reflect endothelial activation. This enables us to draw not only on vascular reactivity but also on these circulating endothelial cell products when assessing endothelial function.

*Von Willebrand Factor* (vWF) is synthesised and secreted mainly by endothelial cells. An increase in circulating levels of vWF has been reported in smokers, subjects with essential hypertension, in first degree relatives of patients with diabetes and in patients with diabetes (336;455-457). These increased concentrations were associated with albuminuria, raised fibrinogen levels, retinopathy and obesity (335;345;346;456) and were predictive of acute coronary events (283). In population studies vWF concentrations were associated with plasma insulin levels (281;394;458). Furthermore associations between the insulin resistance syndrome and vWF levels have been shown (459). Metformin decreases vWF levels (460). In contrast, Nilsson et al observed no correlation between vWF and lipid, anthropometric, or glucose metabolism variables in a group of healthy volunteers (461). However, vWF is also synthesised by megakaryocytes and released by platelets (462). vWF levels are increased in a number of clinical conditions not necessarily associated with endothelial damage (462). Therefore, vWF levels may be a less specific marker of endothelial dysfunction than others.

Circulating *tissue plasminogen activator* levels have also been shown to be elevated in glucose intolerant states, in the hyperinsulinaemia observed in HIV-positive patients and in diabetes (458;463;464). Similarly to vWF concentrations, tPA levels are lowered by
treatment with Metformin (460;464). Several groups reported that tPA levels are independently related with markers of insulin resistance (56;281;283;458;465). However, evidence is strong that tPA antigen levels correlate with plasminogen activator inhibitor (PAI-1) activity, a major regulator of the fibrinolytic system promoting thrombotic events. tPA is bound to and inhibited by PAI-1. High levels of active PAI-1 convert more tPA into the tPA/PAI-1 complex, thus slowing the clearance of total tPA antigen (466). PAI-1 itself is strongly related to hyperinsulinaemia and insulin resistance (279;280;467) and it may be, at least in part, be responsible for any correlations seen between tPA and insulin resistance.

Much less data is available on the association of thrombomodulin and fibronectin with insulin resistance. Thrombomodulin (TM) is a thrombin-binding membrane protein expressed on the endothelial cell surface of various tissues. The soluble form of TM has recently been demonstrated as a marker of endothelial function and thus a surrogate for cardiovascular risk. Thrombomodulin concentrations are raised in several disorders, including diabetes, preeclampsia, renal failure, hypercholesterolaemia, septicaemia and acute myocardial infarction (337;463;468;469). Seigneur at al also observed a positive correlation between plasma TM levels and t-PA. However, a significant positive correlation was found between the plasma TM levels and PAI-1 only for patients with peripheral vascular disease (337). In a cohort of patients with diabetes circulating thrombomodulin levels correlated with insulin resistance (assessed by HOMA), FPI and total number of insulin resistance syndrome variables (457). Finally, cellular fibronectin is a further circulating endothelial cell product which might represent endothelial damage in insulin resistant states.

In summary, an increase in circulating endothelial cell products (as a marker of endothelial activation) is strongly implicated in the insulin resistance syndrome. Once again, cytokines or abnormal NEFA metabolism might link these two pathological processes.
7.2. Aims

The aim of this study was to assess the relationship between endothelial function and insulin sensitivity in subjects with uncomplicated type 2 diabetes by using circulating endothelial cell products for the assessment of endothelial function and the euglycaemic hyperinsulinaemic clamp for the assessment of insulin sensitivity. Furthermore we sought to assess whether circulating levels of cytokines or NEFA could link the two.

7.3. Results

Circulating endothelial cell products were measured in patients with uncomplicated type 2 diabetes mellitus (n=30-34). In consequence, this study had the power only to detect correlations >0.40 and in fact after Bonferroni correction this reduced further. Data are shown in table 7.1. The inter-correlations between measured markers are shown in table 7.2. Data on lipid and NEFA concentrations and insulin sensitivity measurements were available in all 33 patients (table 7.3). Data on vascular reactivity were available in 30 patients (table 7.4). Correlation between markers of insulin sensitivity and circulating endothelial cell products are shown in table 7.5. Taking into consideration that tPA is bound to PAI-1 we also measured PAI-1 antigen levels in this cohort.

<table>
<thead>
<tr>
<th>Table 7.1.: Circulating concentrations of endothelial cell products in a cohort with uncomplicated type 2 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mean (range)</strong></td>
</tr>
<tr>
<td>Tissue plasminogen activator antigen (ng/ml) (n=33)</td>
</tr>
<tr>
<td>von Willebrand factor (IU/ml) (n=30)</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml) (n=30)</td>
</tr>
<tr>
<td>Cellular fibronectin (ng/ml) (n=30)</td>
</tr>
</tbody>
</table>
Table 7.2.: The inter-correlation between circulating endothelial cell products in a cohort with uncomplicated type 2 diabetes. (Spearman's correlation coefficient $r$ and $p$-value are shown. $P$-values with a significance <0.05 are printed in bold)

<table>
<thead>
<tr>
<th></th>
<th>Tissue plasminogen activator antigen</th>
<th>von Willebrand Factor</th>
<th>Thrombo-modulin</th>
<th>Cellular Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrand Factor</td>
<td>$r$ 0.162</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$p$-value NS (0.391)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombo-modulin</td>
<td>$r$ 0.319</td>
<td>-0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$p$-value NS (0.086)</td>
<td>NS (0.536)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular Fibronectin</td>
<td>$r$ -0.141</td>
<td>0.445</td>
<td>-0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$p$-value NS (0.459)</td>
<td><strong>0.014</strong></td>
<td>NS (0.635)</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3: Correlations between circulating concentrations of endothelial cell products and plasma lipid and NEFA concentrations in a cohort with uncomplicated type 2 diabetes (Spearman's correlation coefficient $r$ is shown, p-values significant at < 0.05 level are shown in bold)

<table>
<thead>
<tr>
<th></th>
<th>Tissue plasminogen activator antigen</th>
<th>Von Willebrand Factor</th>
<th>Thrombo-modulin</th>
<th>Cellular Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td>$r$ 0.115</td>
<td>0.170</td>
<td>-0.184</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td>$p$-value 0.536</td>
<td>0.387</td>
<td>0.350</td>
<td>0.350</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>$r$ 0.111</td>
<td>0.458</td>
<td>-0.025</td>
<td>0.237</td>
</tr>
<tr>
<td></td>
<td>$p$-value 0.559</td>
<td><strong>0.016</strong></td>
<td>0.901</td>
<td>0.234</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>$r$ -0.247</td>
<td>0.216</td>
<td>-0.078</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>$p$-value 0.173</td>
<td>0.260</td>
<td>0.689</td>
<td>0.742</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>$r$ 0.106</td>
<td>0.323</td>
<td>-0.007</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>$p$-value 0.583</td>
<td>0.108</td>
<td>0.974</td>
<td>0.509</td>
</tr>
<tr>
<td>NEFA&lt;sub&gt;9&lt;/sub&gt;</td>
<td>$r$ 0.487</td>
<td>0.212</td>
<td>0.001</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>$p$-value <strong>0.005</strong></td>
<td>0.270</td>
<td>0.994</td>
<td>0.774</td>
</tr>
<tr>
<td>NEFA&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>$r$ 0.474</td>
<td>0.179</td>
<td>0.105</td>
<td>0.113</td>
</tr>
<tr>
<td></td>
<td>$p$-value <strong>0.006</strong></td>
<td>0.352</td>
<td>0.588</td>
<td>0.560</td>
</tr>
<tr>
<td>NEFA&lt;sub&gt;supp&lt;/sub&gt;</td>
<td>$r$ 0.353</td>
<td>0.180</td>
<td>0.047</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>$p$-value <strong>0.047</strong></td>
<td>0.356</td>
<td>0.807</td>
<td>0.586</td>
</tr>
</tbody>
</table>

$\text{NEFA}_9$: fasting non-esterified fatty acid concentrations, $\text{NEFA}_{ss}$ non-esterified fatty acid concentrations at steady-state, $\text{NEFA}_{supp}$ percentage suppression of non-esterified fatty acid concentrations from fasting to steady state.
Table 7.4: Correlations between circulating concentrations of endothelial cell products and vascular reactivity in a cohort with uncomplicated type 2 diabetes (Spearman's correlation coefficient $r$ is shown, p-values significant at < 0.05 level are shown in bold)

<table>
<thead>
<tr>
<th>Tissue plasminogen activator</th>
<th>Von Willebrand Factor</th>
<th>Thrombomodulin</th>
<th>Cellular Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal FBF</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.021</td>
<td>0.419</td>
<td>-0.130</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.915</td>
<td><strong>0.033</strong></td>
<td>0.570</td>
</tr>
<tr>
<td><strong>FBF$_{max}$ in response to ACh</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.211</td>
<td>0.012</td>
<td>-0.035</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.281</td>
<td><strong>0.954</strong></td>
<td><strong>0.0867</strong></td>
</tr>
<tr>
<td><strong>Slope of vascular response to ACh</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.167</td>
<td>0.012</td>
<td>-0.018</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.396</td>
<td><strong>0.396</strong></td>
<td><strong>0.931</strong></td>
</tr>
<tr>
<td><strong>FBF$_{max}$ in response to SNP</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.046</td>
<td>0.087</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.818</td>
<td><strong>0.679</strong></td>
<td><strong>0.956</strong></td>
</tr>
<tr>
<td><strong>Slope of vascular response to SNP</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.049</td>
<td>0.128</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.803</td>
<td><strong>0.533</strong></td>
<td>0.602</td>
</tr>
<tr>
<td><strong>FBF in response to insulin</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.021</td>
<td>0.077</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.918</td>
<td><strong>0.722</strong></td>
<td><strong>0.735</strong></td>
</tr>
<tr>
<td><strong>FBF in response to L-NMMA</strong></td>
<td><strong>r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.124</td>
<td>0.174</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td><strong>p-value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.545</td>
<td><strong>0.415</strong></td>
<td><strong>0.086</strong></td>
</tr>
</tbody>
</table>

ACh Acetylcholine
FBF forearm blood flow
FBF$_{max}$ maximum forearm blood flow
L-NMMA N-nomethyl-L-arginine
SNP Sodium Nitroprusside
Table 7.5: Correlations between circulating concentrations of endothelial cell products and markers of insulin resistance in cohort with uncomplicated type 2 diabetes (Spearman’s correlation coefficient r is shown, p-values significant at < 0.05 level are shown in bold)

<table>
<thead>
<tr>
<th>Tissue plasminogen activator antigen (n=33)</th>
<th>Von Willebrand Factor (n=30)</th>
<th>Thrombomodulin (n=30)</th>
<th>Cellular Fibronectin (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPI r</td>
<td>0.273</td>
<td>-0.015</td>
<td>0.070</td>
</tr>
<tr>
<td>p-value</td>
<td>0.124</td>
<td>0.939</td>
<td>0.713</td>
</tr>
<tr>
<td>HOMA% r</td>
<td>0.342</td>
<td>0.125</td>
<td>0.065</td>
</tr>
<tr>
<td>p-value</td>
<td>0.052</td>
<td>0.509</td>
<td>0.735</td>
</tr>
<tr>
<td>M-value r</td>
<td>-0.418</td>
<td>0.145</td>
<td>0.167</td>
</tr>
<tr>
<td>p-value</td>
<td>0.016</td>
<td>0.446</td>
<td>0.379</td>
</tr>
</tbody>
</table>

FPI = Fasting plasma insulin
HOMA%S = insulin sensitivity assessed by Homeostasis Model Assessment
M-value = insulin sensitivity assessed by the hyperinsulinaemic clamp technique

Table 7.6: Correlations between circulating concentrations of endothelial cell products and levels of cytokines in a cohort with uncomplicated type 2 diabetes (Spearman’s correlation coefficient r is shown, p-values significant at < 0.05 level are shown in bold)

<table>
<thead>
<tr>
<th>Tissue plasminogen activator antigen (n=30)</th>
<th>Von Willebrand Factor (n=30)</th>
<th>Thrombomodulin (n=30)</th>
<th>Cellular Fibronectin (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 r</td>
<td>0.230</td>
<td>0.028</td>
<td>-0.071</td>
</tr>
<tr>
<td>p-value</td>
<td>0.222</td>
<td>0.885</td>
<td>0.709</td>
</tr>
<tr>
<td>TNFα r</td>
<td>0.221</td>
<td>0.278</td>
<td>-0.069</td>
</tr>
<tr>
<td>p-value</td>
<td>0.213</td>
<td>0.140</td>
<td>0.717</td>
</tr>
</tbody>
</table>

IL-6 = interleukin 6
TNFα = tumour necrosis factor α
7.3.1. tPA antigen

tPA antigen was measured in 33 patients. tPA antigen correlated negatively with the M-value (r = -0.418, p = 0.016) but did not correlate with FPI (figure 7.1 and table 7.5). tPA antigen differed significantly between the IR and IS subgroup (10.1 ± 7.2 ng/ml vs. 7.6 ± 7.4 ng/ml respectively, F = 5.5, p = 0.028) (figure 7.2). Table 7.7 shows the correlation between tPA antigen levels and M-value after adjustment for cytokines and NEFA levels. Adjustment for NEFA concentrations but not cytokines removed significance of the relationship.

tPA antigen and vascular response to ACh, SNP, insulin or L-NMMA were not correlated (table 7.4).

There was a significant correlation between tPA antigen and NEFA concentrations (for NEFA₀ r = 0.487, p = 0.005 and for NEFAₜₐₙ r = 0.474, p = 0.006). tPA antigen did not correlate with markers of obesity, lipid concentrations or cytokine levels (table 7.3 and 7.6).

The association between tPA and insulin resistance was no longer significant after inclusion of PAI-1 into the model (β = -0.308, p = 0.098). Equally the association between tPA and NEFA levels was no longer significant after inclusion of PAI-1 into the model (for NEFA₀ β = 0.188, p = 0.328 and for NEFAₜₐₙ β = 0.304, p = 0.109).

**Table 7.7**: Partial correlation of insulin sensitivity (M-value) with tPA antigen concentrations after correction for cytokine and NEFA concentrations in a cohort with uncomplicated type 2 diabetes. Significant values at a level of p < 0.05 are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>Partial correlation coefficient β</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>-0.418</td>
<td>0.016</td>
</tr>
<tr>
<td>NEFA₀ concentration</td>
<td>-0.199</td>
<td>0.294</td>
</tr>
<tr>
<td>NEFAₜₐₙ concentration</td>
<td>-0.185</td>
<td>0.320</td>
</tr>
<tr>
<td>NEFA suppressibility</td>
<td>-0.318</td>
<td>0.081</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>-0.473</td>
<td>0.010</td>
</tr>
<tr>
<td>Tumour necrosis factor α</td>
<td>-0.376</td>
<td>0.034</td>
</tr>
</tbody>
</table>

NEFA₀: fasting non-esterified fatty acid concentrations, NEFAₜₐₙ: non-esterified fatty acid concentrations at steady-state, NEFA_sup: percentage suppression of non-esterified fatty acid concentrations from fasting to steady state.

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7.3.2. Von Willebrand factor

Von Willebrand factor (vWF) was measured in 30 patients. vWF did not correlate with M-value \((r=-0.265, p=0.150)\) but correlated with FPI \((r=0.533, p=0.002)\) (table 7.5). vWF did not differ significantly between the IR and IS subgroup \((31.6\pm9.6 \text{ IU/ml vs. } 24.4\pm10.9 \text{ IU/ml respectively, } F=2.48, p=0.113)\). Table 7.8 shows the correlation between vWF levels and FPI after adjustment for cytokines and NEFA levels. Adjustment for either NEFA concentrations or levels of cytokines removed the significance of this relationship.

vWF and vascular response to ACh, SNP, insulin or L-NMMA were not correlated (table 7.4).

There was a significant correlation between vWF and total cholesterol concentrations \((r=0.458, p=0.016)\). vWF did not correlate with markers of obesity, NEFA concentrations or other lipid concentrations (table 7.3). Circulating levels of the cytokine IL-6 and vWF were positively correlated \((r=0.534, p=0.002)\), but levels of TNFa were not correlated with vWF (table 7.6).

### Table 7.8: Partial correlation of insulin resistance (FPI) with von Willebrand factor concentrations after correction for cytokine and NEFA concentrations in a cohort with uncomplicated type 2 diabetes. Significant values at a level of \(p<0.05\) are shown in bold.

<table>
<thead>
<tr>
<th>Partial correlation coefficient (\beta)</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>0.533</td>
</tr>
<tr>
<td>(\text{NEFA}_a) concentration</td>
<td>0.167</td>
</tr>
<tr>
<td>(\text{NEFA}_{ss}) concentration</td>
<td>0.062</td>
</tr>
<tr>
<td>NEFA suppressibility</td>
<td>0.258</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>0.246</td>
</tr>
<tr>
<td>Tumour necrosis factor (\alpha)</td>
<td>0.231</td>
</tr>
</tbody>
</table>

\(\text{NEFA}_a\) fasting non-esterified fatty acid concentrations, \(\text{NEFA}_{ss}\) non-esterified fatty acid concentrations at steady-state, \(\text{NEFA}_{SSP}^p\) percentage suppression of non-esterified
7.3.3. Soluble thrombomodulin

Soluble thrombomodulin (TM) was measured in 30 patients. TM levels did not correlate with M-value (table 7.5) and did not differ between the IR and IS subgroup (4.35\pm0.26 ng/ml vs. 4.64\pm0.90 ng/ml respectively, F=5.41, p=0.331).

TM levels and vascular response to ACh, SNP, insulin or L-NMMA were not correlated (table 7.4).

Markers of obesity correlated with levels of TM (for BMI r=-0.372, p=0.043 and for hip circumference r=-0.373, p=0.042). This was no longer significant after Bonferroni correction. TM levels did not correlate with NEFA concentrations, plasma lipid or cytokine levels (table 7.3 and table 7.6).

7.3.4. Cellular fibronectin

Cellular fibronectin was measured in 30 patients. Fibronectin levels did not correlate with M-value or with FPI (table 7.5) and did not differ between the IR and IS subgroup (192.4\pm112.3 ng/ml vs. 302.3\pm178.2 ng/ml respectively, F=17.0, p=0.122).

Fibronectin levels and vascular response to ACh, SNP, insulin or L-NMMA were not correlated (table 7.5).

Fibronectin levels did not correlate with NEFA concentrations, plasma lipid or cytokine levels (table 7.3 and table 7.6). Markers of obesity did also not correlate with fibronectin levels.

7.3.5. PAI-1 antigen

PAI-1 antigen was measured in 31 patients (mean 24.4\pm15.9 ng/ml). PAI-1 antigen correlated negatively with insulin sensitivity (M-value r=-0.542, p=0.002 and FPI r=0.360, p=0.046).

PAI-antigen differed significantly between the IR and IS subgroup (35.5\pm10.4 ng/ml vs. 13.3\pm13.8 ng/ml respectively, F=19.1, p<0.001)
There was a significant correlation between PAI-1 antigen and NEFA concentrations (for NEFA$_o$ $r=0.513$, $p=0.004$ and for NEFA$_{ss}$ $r=0.477$, $p=0.008$). PAI-1 antigen did not correlate with any marker of vascular reactivity. PAI-1 antigen did not correlate with markers of obesity, lipid concentrations or cytokine concentrations.

7.4. Summary

Plasma concentrations of fibronectin and vWF were significantly correlated. No other significant correlation between these circulating endothelial cell products was observed. There was no significant relationship between levels of these endothelial cell products and any measure of vascular reactivity (except vWF and basal FBF).

tPA levels were negatively correlated with insulin sensitivity (M-value) and were significantly higher in the insulin resistant subgroup when compared with the insulin sensitive subgroup. This association was dependent on PAI-1 activity. vWF correlated with fasting plasma insulin levels and insulin sensitivity assessed by HOMA but not with M-value and no difference in vWF levels was observed between the two extreme subgroups of insulin resistance.

Markers of NEFA metabolism correlated with levels of tPA, but not with any other measured endothelial cell product. Again, this association depended on PAI-1 activity. The pro-inflammatory cytokine IL-6 correlated only with vWF.
7.5. Discussion

The aim of this study was to assess the relationship between endothelial dysfunction (assessed by measurements of circulating endothelial cell products) and insulin sensitivity (assessed by the euglycaemic hyperinsulinaemic clamp) in subjects with uncomplicated type 2 diabetes. The hypothesis was that insulin resistance and endothelial dysfunction are associated. This is supported by previous reports that insulin resistance itself and components of the insulin resistance syndrome are associated with abnormalities of endothelial function (56;455;457;459;470). These studies reported that concentrations of the endothelial cell derived protein vWF and fibronectin are elevated in insulin resistant states. Aso et al in a recent study of 53 patients with type 2 diabetes reported that plasma thrombomodulin might be a better marker of endothelial damage than plasma vWF (TM and vWF correlated highly with insulin resistance assessed by HOMA, whereas only TM correlated also with FPI, systolic blood pressure and HDL-cholesterol). Interestingly, in their cohort no correlation between triglyceride levels, usually associated with insulin resistance and TM levels was observed.

In the diabetic cohort studied here, higher levels of tPA and vWF were associated with insulin resistance. tPA levels were higher in the insulin resistant group. The relationship between these markers of endothelial dysfunction and insulin resistance was mediated by NEFA, but not by cytokine levels. The correlation between tPA and M-value can only in part be explained by the fact that tPA is bound to PAI-1 (inclusion of PAI-1 into the model decreased the correlation coefficient from -0.418 with p=0.016 to -0.308 with a p-value of 0.098). No correlation was observed between insulin resistance and thrombomodulin or fibronectin levels.

This study was thus unable to demonstrate a clear association between insulin resistance and most of the measured endothelial cell markers. Most of the measured endothelial cell markers did not correlate with each other. Furthermore circulating endothelial cell products did not correlate with vascular response to acetylcholine or insulin, markers of endothelial function at
the level of resistance vessels. However, plasma levels of circulating products of endothelial cells may only in part be representative of the severity of endothelial damage sustained. Increased synthesis of these products may also result in increased plasma levels. Thus, an increase in these circulating levels may be seen as a sign of endothelial-cell damage ('dysfunction') or an increase in production of these markers as a preventative mechanism ('activation'). It is also unclear how reliable a single measurement of any of these markers is and to what degree a biological variability exists even in healthy subjects. Serial sampling might provide further information on this. Furthermore lifestyle and genetic make-up could influence any of these measures, in parallel to endothelial dysfunction we were aiming to measure. The specificity of these products as markers of endothelial dysfunction remains to be established. Further study is required to establish whether these markers predict vascular risk and diabetes.
Figure 7.1: Correlation between tissue plasminogen activator (tPA) antigen concentrations and M-value as a measure of insulin sensitivity in 33 patients with type 2 diabetes.

Figure 7.2: Plasma tissue plasminogen activator (tPA) antigen concentrations in the three subgroups of the cohort divided into tertiles of insulin sensitivity. IR (insulin resistant, n=11) and IS (insulin sensitive, n=12) show the two extreme subgroups of insulin sensitivity. Mdl is the middle subgroup (n=10).
Chapter 8
Conclusion

This work has explored the relationship between non-esterified fatty acid concentrations and insulin sensitivity in a large cohort of healthy subjects as well as in a smaller group of patients with uncomplicated type 2 diabetes. In the diabetic cohort endothelial function was also measured. NEFAs and cytokines have previously been implicated as possible signals linking endothelial function and insulin resistance. A series of studies is described in which insulin sensitivity was assessed by the hyperinsulinaemic clamp technique and fasting insulin concentrations, and endothelial function was assessed by forearm venous occlusion plethysmography with local intra-arterial infusions as well as by measurements of circulating endothelial cell products.

Chapter 1 summarises and critically explores the evidence available on these issues. The insulin resistance syndrome is common and detrimental to health and life expectancy on an epidemiological scale. Subjects with components of the insulin resistance syndrome suffer from premature accelerated atherosclerosis. The large body of scientific evidence on causative mechanisms is not conclusive and the ‘culprit’ linking insulin resistance and endothelial dysfunction (and thus possibly leading to increased cardiovascular morbidity and mortality in insulin resistant subjects) remains to be sought. Understanding the underlying mechanisms responsible for the increased incidence of cardiovascular disease in these subjects is of importance in devising prevention strategies.

Chapter 2 describes the methodologies utilised. The euglycaemic hyperinsulinaemic clamp technique as well as fasting insulin concentrations were used for assessment of insulin sensitivity in the fasting and hyperinsulinaemic state. Venous occlusion plethysmography in response to different vasoactive infusions as well as measurement of circulating endothelial cell products were used for assessment of endothelial function. Plasma NEFA and cytokine levels were also measured.
Chapter 3 explores the relationship between insulin resistance, plasma lipids and NEFA concentrations by use of a large European database of healthy non-diabetic volunteers. The main aim of this study was to examine the relationship of NEFA metabolism with insulin resistance in healthy subjects. This study showed the strong associations between both and found these correlations to differ between the fasting and the hyperinsulinaemic state. This may point towards a so far unidentified mechanism or just represent the variability of fasting NEFA under the conditions used. Understanding of the relationship between NEFA and insulin action remains simplistic and further studies are needed to examine this in more detail. It appears that NEFA act as a signal as well as a metabolic substrate. Knowledge gained from animal models and in particular from knock-out experiments will advance studies in humans. Such studies could involve isotope techniques, NMR spectroscopy or “clamping” of NEFA at low/normal levels in order to observe the effect on muscle and/or whole body insulin sensitivity. In addition, our results clearly show that the association between insulin resistance and NEFA-regulation is independent of obesity. We have tested the hypothesis that the relationship between insulin resistance and triglyceride concentrations is essentially mediated through insulin’s effect on plasma NEFA concentrations and our observations do not support this. Instead, our results suggest that insulin resistance and hypertriglyceridaemia are strongly associated independently of fatty acid concentration suppression. The analysis might suggest that both glucoregulatory defects in insulin action and impaired NEFA suppressibility make independent contributions to hypertriglyceridaemia.

In chapter 4 similar correlations were observed examining insulin resistance and concentrations of lipids and non-esterified fatty acids in diabetic subjects. The cohort was small and included only patients with uncomplicated type 2 diabetes mellitus. It was divided into tertiles of insulin resistance and NEFA and lipid concentrations were examined in the two extreme subgroups. These groups did not differ in their epidemiological characteristics, but the insulin resistant subgroup had a metabolic profile characteristic of the insulin resistance syndrome with central obesity, hypertriglyceridaemia, hyperinsulinaemia and high levels of NEFA. Insulin resistance was correlated with fasting plasma insulin, markers of obesity, plasma triglyceride concentrations and fasting and steady state NEFA concentrations.
The association between insulin resistance and NEFA-regulation was independent of obesity. The association between insulin resistance and triglyceride concentrations in diabetes was dependent on NEFA concentrations during hyperinsulinaemia and on NEFA suppressibility. This finding differs from that in the healthy cohort, where the relationship between insulin resistance and triglyceride concentration was independent of NEFA suppressibility. It supports the hypothesis of NEFA being essential and possibly causative in this relationship.

Thus having explored the associations between insulin resistance, triglyceride and NEFA concentrations (and having generally made similar observations in health and type 2 diabetes), chapter 5 proceeds to examine vascular reactivity as a measure of endothelial function in type 2 diabetes and its correlations with insulin resistance. Vascular response to acetylcholine was used as an endothelium-dependent marker whereas response to sodium nitroprusside was used as the endothelium-independent control. Each patient’s contralateral arm also served as an in-built physiological control. Basal blood flow did not differ between the insulin resistant and insulin sensitive subgroups.

Endothelium-dependent vasodilatation did not correlate with insulin resistance. As outlined in section 5.3 this finding is in contrast with some but not all studies. However, some groups have also previously reported this observation. The subjects studied here were a selected group of patients with type 2 diabetes without evidence of complications, thus being on the ‘healthy’ part of the spectrum and this might possibly account for these findings. Waist-hip ratio was the only marker of obesity associated with endothelium-dependent vasodilatation. Interestingly, endothelium-independent vascular response to the nitric oxide donor SNP correlated positively with the M-value as a measure of insulin resistance, showing impaired vascular reactivity to SNP in insulin resistant subjects. This association remained significant after adjustment for triglyceride and TNFα concentrations but not after adjustment for obesity, NEFA levels or IL-6. Possible mechanisms for this are defects in smooth-muscle cell structure or function or increased destruction of exogenous nitric oxide in insulin resistant states.
No association between endothelium-dependent vascular reactivity and insulin resistance was observed. Nevertheless, the secondary aim was to examine the influence of cytokines on this relationship. Chapter 6 examines the association between pro-inflammatory cytokine levels and insulin resistance as well as between cytokine levels and endothelial function. IL-6 and TNFα levels correlated strongly with each other. IL-6 concentrations were significantly higher in the insulin resistant subgroup. IL-6 concentrations were also significantly positively correlated with maximum vascular response to acetylcholine (endothelium-dependent) and negatively with the slope of vascular reactivity to sodium nitroprusside (endothelium-independent). No association between TNFα levels and insulin resistance or TNFα levels and vascular reactivity was observed. Circulating cytokine levels were not associated with obesity. The hypothesis was that cytokines are the signal linking insulin resistance with endothelial dysfunction. In the cohort studied IL-6 concentrations were associated with both insulin resistance and one of the two measures of endothelium-dependent vascular reactivity. This at first appears to represent an important link between these two abnormalities. However, the association between vascular response to acetylcholine and IL-6 was positive, i.e. subjects with high levels of IL-6 had more marked vascular reactivity to acetylcholine. Furthermore the correlation was no longer significant after Bonferroni correction.

In this study two techniques were used to assess endothelial integrity: venous occlusion plethysmography and measurements of circulating endothelial cell products. Chapter 7 examines the association between insulin resistance and circulating endothelial cell products and also looks at the correlation between these two different measures of endothelial function. Most previous studies have used either one or the other (56;72;149;281;331;394;398;402;404;405). Findings on endothelial function in this cohort appear discordant: no association between endothelium-dependent vascular response and insulin resistance was observed, but a significant association between tPA, an endothelial cell product, and insulin resistance was found. tPA antigen levels were negatively correlated with insulin resistance (M-value) and were significantly higher in the insulin resistant subgroup when compared with the insulin sensitive subgroup. However, the association between insulin resistance and tPA was dependent on PAI-1 antigen levels. The correlation between
tPA and M-value can in part be explained by the fact that tPA is bound to PAI-1, and it is plausible that tPA antigen levels do not represent a specific marker of endothelial dysfunction in this cohort. vWF concentrations correlated with fasting plasma insulin levels and insulin sensitivity calculated by HOMA model but not with M-value. No difference in vWF levels was observed between the two extreme subgroups of insulin resistance. Soluble thrombomodulin, another marker of endothelial dysfunction was also not associated with insulin resistance. Thus, comparing the two methods used to assess endothelial function (plethysmography and measurement of circulating endothelial cell markers), neither of them was associated with insulin resistance in this cohort with uncomplicated type 2 diabetes.

Despite the fact that the present cohort was small, in many aspects our results confirm the findings of many and larger studies in health and in type 2 diabetes mellitus. To my knowledge no study has collected data in such a phenotypically homogeneous group of patients with type 2 diabetes and studied their metabolic profile in the fasting (FPI and fasting NEFA) and hyperinsulinaemic state (hyperinsulinaemic euglycaemic clamp and steady state NEFA plus NEFA suppressibility). Uniquely, measurements were made of circulating endothelial markers as well as blood flow in response to different agents. Further studies to explore these associations are needed ideally including a healthy control group. Defining the subgroups in the diabetic population with the most detrimental metabolic risk factor profile may allow better targeting of preventive measures.

The aims of this work were

1) To assess the relationship between endothelial function and insulin sensitivity in subjects with uncomplicated type 2 diabetes by using the euglycaemic hyperinsulinaemic clamp for the assessment of insulin sensitivity and venous occlusion plethysmography as well as circulating endothelial cell products for the assessment of endothelial function,

2) To evaluate NEFA and cytokines as possible signals linking insulin resistance and endothelial dysfunction in subjects with uncomplicated type 2 diabetes and
3) To test the hypothesis that the relationship between insulin resistance and triglyceride concentrations is essentially mediated through insulin’s effect on plasma NEFA by analysis of the European Group for the Study of Insulin Resistance (EGIR) database of healthy volunteers.

In conclusion, we studied insulin resistance and NEFA metabolism in healthy volunteers and in subjects with uncomplicated type 2 diabetes. We also investigated the association between insulin resistance and endothelial dysfunction in type 2 diabetes. We evaluated the role of cytokines and non-esterified fatty acids as a confounding factor in this relationship. We found a strong association between NEFA and insulin resistance in health and diabetes and observed different links in the fasting and hyperinsulinaemic states. The insulin resistant subgroup had evidence of several components of the insulin resistance syndrome with central obesity, hypertriglyceridaemia, hyperinsulinaemia, high levels of NEFA, elevated tPA and IL-6 concentrations.

No evidence for a relationship between insulin resistance and endothelial dysfunction was observed on plethysmography. Interestingly, insulin resistance was associated with endothelium-independent vascular response and the mechanism remains to be explored. However, measurement of circulating endothelial cell products showed a significant association between the tPA antigen levels and insulin resistance. This relationship was dependent on PAI-1 antigen levels and might not represent endothelial damage in this cohort. The association between insulin resistance and tPA was strongly influenced by measures of NEFA metabolism and to a much lesser extent by cytokine levels. This might suggest that NEFA rather than cytokines affect this association. Our hypothesis was that a common antecedent exists, linking insulin resistance with endothelial dysfunction, and cytokines and NEFA were likely candidates. Our findings point towards NEFA as a putative signal.
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PAPERS:


ABSTRACTS:


ORAL PRESENTATIONS

- Baldeweg SE, Coppack SW, Yudkin JS. Endothelial function and insulin resistance in type 2 diabetes mellitus. Annual meeting of the Anglo-Danish-Dutch Diabetes Group, Vaals, The Netherland, May 1999

- Baldeweg SE, Coppack SW, Yudkin JS, Insulin resistance and vascular reactivity in type 2 diabetes mellitus, The 4th Scientific Meeting of the Hypertension in Diabetes EASD Study Group, Katowice, Poland, April 1999