Modification of Postprandial Lipid Profiles in People With Diabetes

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A thesis submitted to the Faculty of Clinical Medicine at the University of Oxford for the degree of Doctor of Philosophy

Hilary Term 2003

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I dedicate this work to my family and loving partner who have always supported me, and who are always in my heart despite being so far away.

Acknowledgements

Thank you to Professor Rury Holman for undertaking the supervision of this work, overseeing the clinical studies, and having confidence in me. I am grateful for the opportunity he has provided me with, and his generosity will always be remembered. I would like to express my gratitude to Lynne Tucker not only for administrative support, but someone who has always been there for guidance, encouragement and friendship. I acknowledge Dr Joanne Shaw whose own experiences inspired my interest in diabetes. Her personal recommendation to Professor Robert Turner was the foundation for embarking on this work.

To everyone who participated in the clinical studies - their time and help is greatly appreciated. Thank you to the nursing staff, lead by Beryl Barrow, for their assistance and humor during the many study days which we enjoyed over the years. I acknowledge the Biochemistry Laboratory and Diabetes Research Laboratory for the processing of samples and the IT staff for technical support.

Thank you to Dr Andrew Neil for helpful discussions and guidance, and Dr Fredrik Karpe for advice and interpretation of the data as well as enthusiasm for the work. I am grateful to Dr Paul Harrison from the Haemophilia Unit, Churchill Hospital, Oxford for our collaboration in platelet function. Thank you also to Irene Stratton and Richard Morris for statistical teaching, and Pamela Dyson for dietary advice. I appreciate the support from the Foundation of Young Australians, Centenary Scholarship.

There are others not mentioned who in many ways contributed to this thesis, who I take this opportunity to thank for their kindness and assistance.

Memorandum

The studies, analysis and interpretation presented in this thesis are original work of the author, carried out under the supervision of Professor Rury Holman in the Diabetes Trials Unit, Nuffield Department of Clinical Medicine at the University of Oxford. The processing of samples was done by the Diabetes Trials Unit Biochemistry Laboratory, Diabetes Research Laboratory, and Haemophilia Unit, University of Oxford.

I hereby state that no part of this thesis has been submitted for any other degree at this or any other universities, although most parts of the work have been published in abstract form.

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Abstract

Background: Abnormalities in postprandial lipid metabolism may explain, in part, the 2 to 4 fold increased risk of coronary heart disease (CHD) in diabetic individuals compared with the general population. **Aims**: To develop an Oral Triglyceride Tolerance Test (OTTT) to evaluate reliably post challenge metabolism and to determine whether more physiologic insulin profiles can improve lipid responses in diabetic subjects. **Methods**: Post 50g fat and 50g carbohydrate challenge triglyceride and glucose profiles were measured 2 hourly for 8 hours on 2 occasions to establish test reproducibility and to characterise responses in type 2 diabetic and non diabetic subjects. The potential effects on post challenge lipid and glucose metabolism of modifying endogenous insulin delivery via the portal system or augmenting post challenge systemic insulin levels were assessed using the OTTT. **Results**: Reproducible post challenge triglyceride, NEFA, glycerol, glucose, insulin and C peptide responses were obtained in non diabetic and type 2 diabetic subjects. In type 2 diabetic subjects both ultra rapid endogenous and exogenous insulin delivery decreased post challenge hyperglycaemia and improved NEFA-glycerol suppression, relative to placebo and soluble insulin respectively. Enhancing portal insulin secretion had no effect on triglyceride levels. Increasing peripheral insulin levels showed no difference in triglyceride profiles between insulins tested although 6 hour triglyceride excursions were less than endogenous enhancement. Type 1 diabetic subjects displayed triglyceride profiles similar to non diabetic subjects, and reduced triglyceride excursion was seen with soluble than analogue insulin replacement. **Conclusion**: Increasing the peripheral to portal insulin ratio may relieve hepatic exposure to chronic hyperinsulinaemia characteristic of type 2 diabetes thus reducing post challenge triglyceride excursions. Improving postprandial metabolism, by delaying atherogenesis, may help decrease the risk of CHD.

Glossary

ADA – American Diabetes Association
ANOVA – Analysis of Variance
apo- - apolipoprotein
AUC – area under the curve
BDA (now DUK) – British Diabetic Association (Diabetes UK)
CADP – collagen adenosine diphosphate
CARE – Cholesterol And Recurrent Events Study
CEPI – collagen epinephrine
CHD – Coronary Heart Disease
Cmax – maximum concentration achieved
Cmin – minimum concentration achieved
CRU – Clinical Research Unit
CV – coefficient of variation
DECODE – Diabetes Epidemiology Collaborative Analysis of Diagnostic Criteria in Europe
DTU – Diabetes Trials Unit
FFA – free fatty acids
HDL – high density lipoprotein
HbA_{1c} - haemoglobin A_{1c}
HL – hepatic lipase
HMG-CoA – hydroxymethylglutaryl-coenzyme A
HOPE – Heart Outcomes Prevention Evaluation Study
HPS – Heart Protection Study
HSL – hormone sensitive lipase
IDL – intermediate density lipoprotein
IQR – interquartile range
IAUC – incremental area under the curve
K-ATP – potassium adenosine triphospate
LDL – low density lipoprotein
LPL – lipoprotein lipase
NEFA – non esterified fatty acids
NHS –National Health Service
OGTT – Oral Glucose Tolerance Test
OTTT – Oral Triglyceride Tolerance Test
SD – standard deviation
4S – Scandinavian Simvastatin Survival Study
Tmax – time to achieve maximum concentration
Tmin – time to achieve minimum concentration
UKPDS – United Kingdom Prospective Diabetes Study
VLDL – very low density lipoprotein
VWF – von Willebrand's Factor
WHO – World Health Organisation

1 Chapter 1 Introduction and Aims

1.1 Background

1.1.1 Definition, Diagnosis and Classification of Diabetes

Diabetes mellitus is a common disease of multiple aetiology in which relative or absolute insulin deficiency leads to abnormalities in carbohydrate, fat and protein metabolism, characterised by hyperglycaemia and glycosuria. Diabetes is a disorder affecting many different organs of the body, and patients are at risk of complications involving the heart and blood vessels, eyes, kidneys and nerves.

Diabetes signs and symptoms are largely secondary to hyperglycaemia. The diagnosis is confirmed by a fasting venous plasma glucose reading of $\geq$ 7 mmol/l, verified by repeat testing or by the Oral Glucose Tolerance Test (OGTT), where after a 75g oral carbohydrate challenge, the 2 hour post load venous plasma glucose reading is $\geq$ 11 mmol/l (WHO 1999).

Diabetes is classified into primary and secondary, with primary cases divided into autoimmune: mainly type 1 diabetes, and non autoimmune: predominately type 2 diabetes. Approximately 85 to 90% of diabetic patients have type 2 diabetes, which is associated with defects in insulin secretion and usually, resistance to the action of insulin. Type 1 diabetes is characterised by absent (or very low) endogenous insulin secretion and patients require exogenous insulin therapy as essential treatment.

1.1.2 Epidemiology of Diabetes

The prevalence of diabetes is increasing rapidly worldwide in developing and developed countries. It was estimated that in 1995 the worldwide prevalence was 4.0% which is to rise to 5.4% in 2025, making the number of adults with diabetes in the world rise from 135 million to 300 million mainly in developing countries (King, Aubert et al. 1998). Bagust et al (Bagust A, Hopkinson P K et al. 2002) have projected that there will be approximately 20% more cases of type 2 diabetes in 2036 than 2000 due to the ageing population. In the UK, an increase in the number of cases from 1 863 000 in 2000 to 2 880 000 in 2010 is predicted for type 2 diabetes whereas the prevalence for type 1 diabetes will decrease from 189 000 in 2000 to 183 000 in 2010 (Amos, McCarty et al. 1997). In Australia, the prevalence of diabetes is now 7.4%, one of the highest yet reported for a country with predominately Caucasian background (Dunstan, Zimmet et al. 2002).

With the increase in the number of cases of diabetes comes a rise in diabetes-related complications. People with diabetes have a 2 -4 fold increased risk of coronary heart disease (CHD), and approximately 66% of deaths are due to cardiovascular disease (Zimmet and Alberti 1997). Morgan et al (Morgan, Currie et al. 2000) looked at the prevalence of CHD, cerebrovascular disease, diabetic foot complications, retinopathy and nephropathy within the UK district health authority population. They showed, of the 10 709 patients identified having diabetes (prevalence of 2.47%), almost half had at least one complication. The increase in risk is particularly evident in younger age groups and

in women, with diabetic patients having a 50% greater in hospital mortality. The United Kingdom Prospective Diabetes Study (UKPDS) (UKPDS Group 1990) showed that approximately 50% of all diabetic patients had complications at diagnosis. There is also a 2 fold increased rate of death within 2 years of surviving a myocardial infarction (Ginsberg and Tuck 2001).

During the year 1998 the direct medical costs per patient with type 2 diabetes in the UK were €866 for ambulatory costs and €769 for hospitalisation, making a 3.4% total cost as a proportion of health care expenditure (Passa 2002). It has been estimated that 5% of total National Health Service (NHS) resources and up to 10% of hospital inpatient resources are used to care for people with diabetes (Department of Health 2001). It has been predicted that diabetes-related complications will peak at 20-30% above present levels between 2035 and 2045, with the cost of health care for type 2 diabetic patients rising by 25% generating a relative economic burden increase of 40-50% due to reductions in economically active age groups (Bagust A, Hopkinson P K et al. 2002).

1.1.3 Traditional Risk factors for CHD

Traditional risk factors for atherosclerosis include hypertension, smoking, age, and total cholesterol (Garcia, McNamara et al. 1974). It is well recognised that diabetic individuals are at increased risk of CHD. The Multiple Risk Factor Intervention Trial (MRFIT) showed total cholesterol as well as smoking and blood pressure predicted the development of cardiovascular disease in diabetic as well as non–diabetic subjects (Vaccaro, Stamler et al. 1998) (Haffner, Miettinen et al. 1996). The UKPDS confirmed

coronary artery disease was significantly associated with elevated HbA_{1c} levels in addition to the deadly quartet of an increased concentration of LDL cholesterol, decreased concentration of HDL cholesterol, systolic blood pressure, and smoking (UKPDS Group 1998).

1.1.4 Novel risk Factors for CHD

The UKPDS showed that treatment of hyperglycaemia was not adequate to prevent CHD and altered glucose metabolism did not entirely account for accelerated atherosclerosis (UKPDS Group 2000). It has been suggested that the excessive risk may be related to non traditional risk factors such as markers of inflammation such as C reactive protein, white cell count and homocysteine, prothrombotic factors such as plasminogen activator inhibitor-1 , fibrinogen, D dimer, and platelet abnormalities as well as endothelial dysfunction including nitrotyrosine and advanced glycosylated endproducts (Danesh, Collins et al. 1998) (Hayden and Reaven 2000). Postprandial glucose and triglyceride levels may also be potentially modifiable risk factors for CHD.

1.1.5 Postprandial metabolism and CHD risk

Several studies have highlighted the importance of the postprandial period by proposing that the development of atherosclerosis may be linked more to postprandial glucose/lipid responses, rather than the fasting levels. This assumption has gained credibility with epidemiologic studies, such as DECODE, which showed that two-hour post challenge glucose levels were more predictive of all-cause mortality and cardiovascular mortality

than fasting glucose levels (The Decode Study Group 1999). The Diabetes Intervention Study, which examined type 2 diabetic subjects over 11 years, found a significant relationship between cardiovascular risk and postprandial glucose, but not with fasting plasma glucose (Hanefeld, Fischer et al. 1996). The association of increased risk of CHD with post challenge glucose levels was again demonstrated in the Paris Prospective Study (Eschwege and Balkau 2001). This study of 6629 subjects, showed men with diabetes had an all cause mortality rate twice as high as those without diabetes, regardless of whether the 2 hour glucose was above or below 11.1 mmol/l. However, for those with fasting glucose <7.0 mmol/l and 2 hour glucose levels ≥ 11.1 mmol/l, all cause mortality was three fold higher than for men without diabetes.

Numerous novel risk factors are being investigated, including alterations in lipid handling. Despite fasting normocholesterolaemia, people with type 2 diabetes exhibit a characteristic dyslipidaemia, with low HDL cholesterol levels and elevated triglyceride levels relative to individuals without diabetes (Steiner 1994). The UKPDS demonstrated that triglyceride concentration was a CHD risk factor when adjusted for age and sex but not an independent risk factor (UKPDS Group 1998). Hokanson et al (Hokanson and Austin 1996) however, found after adjusting for HDL cholesterol, that fasting triglyceride concentration was a statistically significant risk factor for CHD in a meta analysis of 17 prospective studies in the non diabetic population. The UKPDS authors concluded possible reasons for their finding, to be the greater biological variability of triglyceride measurements and the metabolic relationship between triglyceride and HDL particles. It

has been proposed that postprandial triglyceride concentration may play an important role in atherogenesis.

Although there are numerous studies providing clinical evidence to establish a relationship between postprandial lipoproteins and CHD they are primarily case control and cross sectional in design, with no prospective studies conducted to date. Studies comparing people with and without CHD have found conflicting results, with some groups finding increased post challenge triglyceride levels in CHD cases, and others not (Karpe 1999). Reasons for this include that when adjusted for baseline, the increase in triglyceride levels was no longer statistically significant, or that the CHD cases had higher fasting triglyceride levels at baseline. A more recent study by Karpe et al (Karpe, de Faire et al. 1998) looked at 30 healthy normo- and hypertriglyceridaemic men and measured carotid intima media thickness using ultrasound of the common carotid artery after an oral fat challenge. Thickening of the intima media is a valid and reproducible marker of early atherosclerotic disease. The study showed late postprandial plasma triglyceride (in particular 6 hours post challenge) to positively correlate with intima media thickness, independent of both LDL cholesterol and fasting triglyceride concentration.

Less information is available with respect to cardiovascular risk with postprandial triglyceride levels in diabetic subjects. Syvänne et al (Syvänne, Hilden et al. 1994) compared postprandial lipaemia responses in 4 groups: type 2 diabetic subjects with CHD, type 2 diabetic subjects without CHD, non diabetic subjects with CHD, and non

diabetic subjects without CHD. No difference was found in postprandial responses between the diabetic group with or without CHD or when comparing to the non diabetic groups. Previous studies have compared postprandial lipid profiles between non diabetic individuals and type 2 diabetic patients (Lewis, O'Meara et al. 1991; Cavallero, Dachet et al. 1994; Curtin, Deegan et al. 1994; Tan, Cooper et al. 1995; Cooper, Tan et al. 1996). Significant differences in postprandial lipid levels were found primarily in the diabetic subjects who had fasting hypertriglyceridaemia. Reznik (Reznik, Pousse et al. 1996) however, found higher peak postprandial triglyceride levels in normotriglyceridaemic type 2 diabetic individuals when comparing them to non diabetic subjects. A possible explanation for the inconsistency between clinical studies is that there is no consensus on test meal constituents and test conditions for postprandial lipaemia studies. Also, no standardised oral fat tolerance test has been proposed and assessed.

1.2 The Postprandial Period

The physiological alterations in metabolism that occur after the ingestion of a meal are referred to as the postprandial period. These perturbations may last up to 10 hours after eating, and with most individuals having at least 2 - 3 meals a day, the major proportion of a day although difficult to quantify, is spent in this state. People with diabetes exhibit magnified and extended postprandial responses in glucose and lipid handling which may be due to loss of first phase and extended second phase insulin secretion.

1.2.1 Loss of first phase insulin secretion

Carbohydrate and fat metabolism are intimately related primarily by insulin, a hormone secreted into the portal circulation by the pancreas. In the fasting state, lipid energy stores are utilized and have specific effects on glucose pathways. In the postprandial state, glucose stimulated insulin release influences lipoprotein dynamics.

Insulin is coded for on chromosome 11 and synthesized in the pancreatic islet β cells. Pre-pro-insulin is manufactured in the ribosomes from insulin mRNA. The hydrophobic "pre" portion is enzymatically cleaved off in the Golgi apparatus (Braunwald 1997). The pro-insulin is subsequently packaged into secretory granules which, once mature, pass towards the cell membrane where they are stored before release. C peptide ("connecting" peptide) is the biochemically inert peptide fragment which is split off from pro-insulin in the secretory process so that equimolar quantities of insulin and C peptide are released into the circulation. The β cell can also secrete a small amount of insulin directly into the circulation bypassing the secretory granules. The insulin molecule consists of A and B chains linked by disulphide bonds.

Insulin secretion is regulated by glucose. Glucose entering the β cell generates ATP (adenosine triphosphate) which closes the potassium channels in the cell membrane thereby causing depolarisation. This allows calcium ions to enter via calcium selective channels in the membrane which triggers activation of calcium dependent phospholipid protein kinase. Through a series of intermediary phosphorylation steps there is fusion of

After secretion, insulin enters the portal circulation and reaches its target organ, the liver. Approximately 50% of the insulin secreted is extracted and degraded by the liver, with the remainder broken down by the kidneys. Therefore insulin is present in the portal circulation at higher concentrations than in the systemic circulation. C peptide provides a useful index of the rate of insulin secretion as it is only partially extracted by the liver and is mainly degraded by the kidneys.

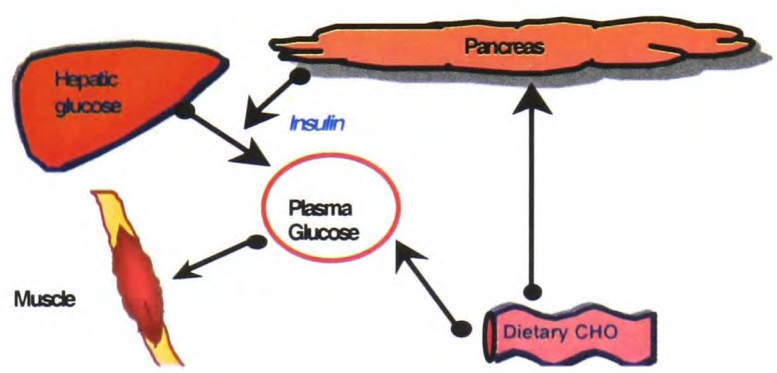


Figure 1 Plasma glucose concentration is dependent upon hepatic glucose production, dietary glucose delivery, and peripheral tissue glucose uptake.

Glucose homeostasis is dependent upon the coordination of insulin secretion, tissue glucose uptake and hepatic glucose production (Porte 2001) (Figure 1). Fasting plasma glucose levels are maintained at a constant level by glucose production from the liver. During the fasting state, insulin is released as discrete pulses every 10-14 minutes, and superimposed on this is a slower cycle of 105-120 minutes (Polonsky, Given et al. 1988). In the postprandial state, glucose is the primary regulator of the pancreatic β cell producing the insulin response which follows a 2 phase pattern (Figure 2). Glucose initiates an immediate insulin secretion, the first phase peaking at 3-5 minutes and is over by 10 minutes, and this represents the release of a small pool of preformed and readily accessible secretory vesicles. The second phase of insulin secretion, requiring both de

by 10 minutes, and this represents the release of a small pool of preformed and readily accessible secretory vesicles. The second phase of insulin secretion, requiring both de novo insulin synthesis as well as release from stored secretory granules, is gradual, with a much lower peak, and persists as long as glucose levels are elevated. Insulin exerts an inhibitory effect of hepatic glucose production by suppressing glycogenolysis and stimulating glucose uptake and glycogen synthesis levels. This allows glucose flux from the gut to replace endogenous production of glucose to nearly exactly balance glucose use by the central nervous system, with only minimal extra glucose remaining to be taken up and stored by peripheral tissues (Sindelar, Chu et al. 1998).

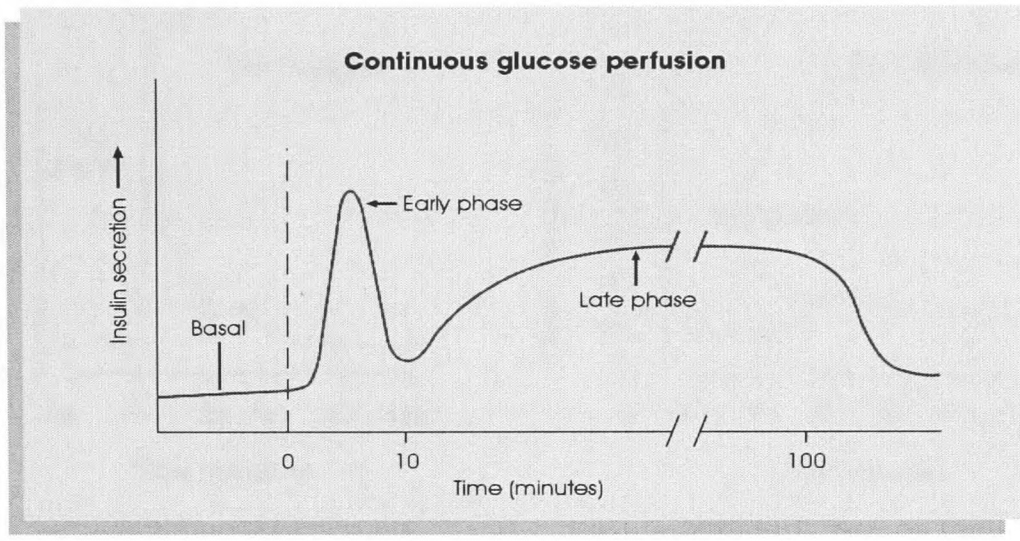


Figure 2 Two phase secretory pattern of endogenous insulin secretion. Adapted from (Howell 1991).

To maintain normoglycaemia, the β cell is capable of increasing its function to completely compensate for quite severe insulin resistance. Studies show that 24 hour insulin secretion is three fold higher in glucose tolerant obese than non-obese controls, with some obese individuals unable to sustain the required level of hyperinsulinaemia. Persistent overstimulation of the β cell may exhaust its secretory capacity, particularly in the presence of a genetic predisposition to β cell dysfunction (Ferrannini, Galvan et al. 1999). Hyperinsulinaemia downregulates insulin action at the receptor as well as post receptor level.

Loss of the early insulin response to exogenous glucose is one of the first defects noted in patients with type 2 diabetes (Figure 3). Alterations in pulse mass, pulse frequency and reduction preformed insulin vesicles have previously been recorded in these individuals. Loss of first phase insulin secretion causes failure to suppress hepatic glucose production as well as regulation of postprandial hyperglycaemia and second phase insulin secretion is prolonged.

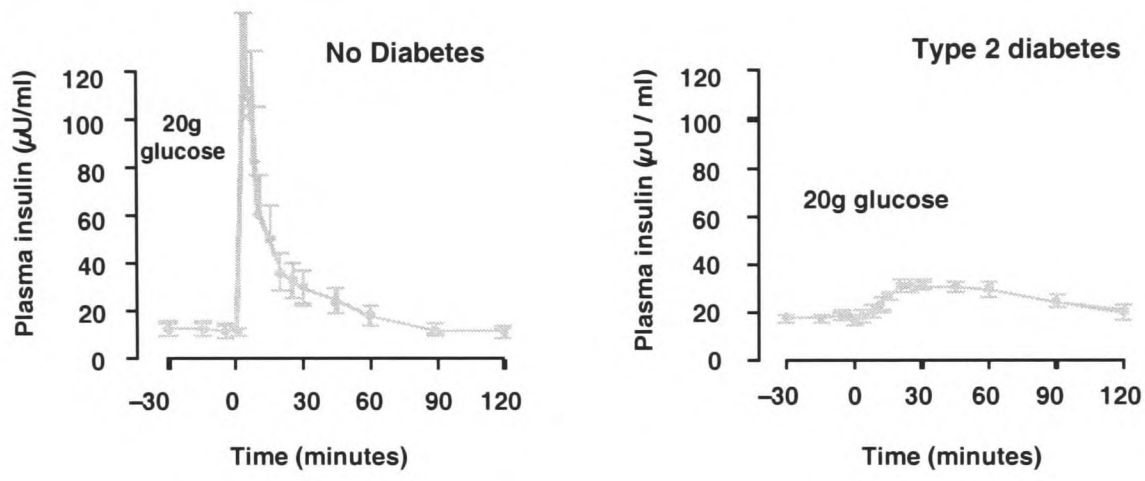


Figure 3 Loss of first phase insulin secretion after glucose load (adapted from (Ward, Beard et al. 1984))

Compromised β cell function is a prerequisite to type 2 diabetes, although insulin resistance can unmask a coexisting defect (Evans and Krentz 2001). The UKPDS demonstrated how, once type 2 diabetes has developed, progressive glucose control deterioration is dictated by further loss of β cell function whilst insulin resistance did not change (UKPDS Group 1995). They showed that at the time of diagnosis, patients with type 2 diabetes only had approximately 50% of the β cell function of normal individuals and 6 years later it had reduced to 25%.

1.2.2 Downstream consequences of chronic hyperinsulinaemia

There is increasing evidence that insulin resistance and compensatory hyperinsulinaemia are related to an elevated CHD risk. The mechanism may actually be mediated through abnormalities in lipid handling, with studies showing resistance to insulin mediated glucose disposal and compensatory hyperinsulinaemia being strongly correlated with triglyceride levels and VLDL secretion rate (Reaven and Laws 1994).

Exogenous Lipid Metabolism

The daily caloric intake is 30 -40% fat consumption which equates to between 60-120g in the average adult (Ros 2000). During absorption the lipids solubilized in micelles dissociate from them which occurs adjacent to the enterocyte. There is rapid diffusion of fatty acid monomers across the microvillous membrane. Almost 95% of fatty acids and monoglycerides are absorbed leaving the faecal fat content to be usually below 5%. Within the enterocyte endoplasmic reticulum, the fatty acids are re-esterified and converted back to triglyceride to be incorporated into chylomicrons (Figure 4).

Chylomicrons consist mainly of triglyceride packaged together with apo B-48, phospholipid, unesterified cholesterol as well as several A apolipoproteins. They are secreted into lacteals and transported via the thoracic duct into the blood, where they acquire apolipoprotein C and E from HDL. Lipoprotein lipase (LPL) is an extracellular lipase attached by heparin sulphate chains to the endothelium of muscle and adipose tissue capillaries. LPL hydrolyses the triglyceride within the chylomicron core. The

apoproteins, therefore reduces in size. Cholesterol ester is transferred from mature HDL to the particles in exchange for triglyceride by cholesterol ester transfer protein. The apo E on the surface of the chylomicron remnant is recognized by receptors on the hepatic parenchymal cells and then rapidly removed from the blood stream.

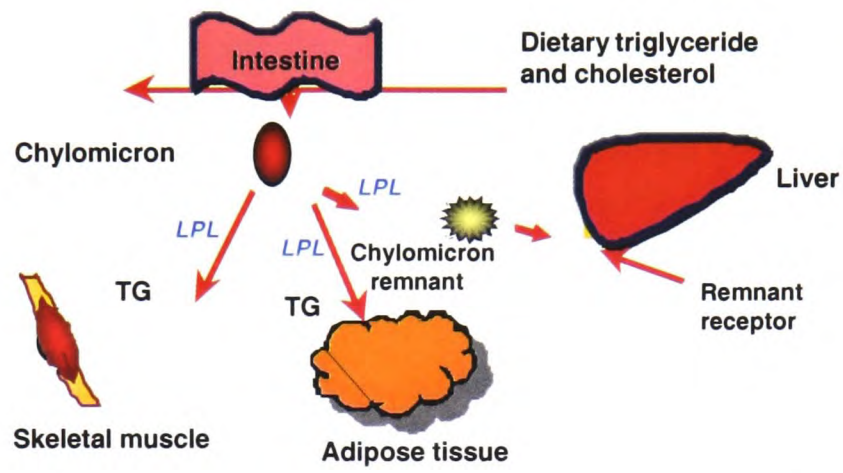


Figure 4 Diagram of the exogenous lipid pathway. Dietary triglyceride (TG) is absorbed in the gut and assembled into chylomicrons. Lipoprotein lipase (LPL) lipolyses the triglyceride core of these particles to release triglyceride for uptake into peripheral tissues.

Endogenous Lipid Metabolism

In the fasting state, non esterified fatty acids (NEFA) are released into the circulation by hydrolysis of triglyceride stored in adipocytes by hormone sensitive lipase (Frayn 1998). This enzyme is suppressed by insulin in the postprandial state. After a meal, chylomicron and VLDL triglyceride compete for hydrolysis by LPL present on the capillaries of adipose tissue. The fatty acids produced flow into the adipocyte for esterification and storage as triglyceride. However a proportion of fatty acids do circulate to venous plasma. NEFA are important substrates of triglyceride synthesis in the liver (Coppack, Jensen et al. 1994) that is, VLDL synthesis (Figure 5).

For visceral adipose tissue stores, which are only 10% of total body fat, there is a high rate of fatty acid turnover (triglyceride uptake and lipolysis). As the portal vein directly links visceral adipose tissue to the liver, this increased turnover causes high fatty acid concentrations to be delivered to the liver. This increases gluconeogenesis, increases hepatic triglyceride synthesis, and inhibits clearance of insulin (Arner 2001). In subcutaneous fat which makes up 80% of total body fat, the rate of fatty acid turnover is slowest. Also there is no direct contact with the portal vein circulation, and therefore mobilization of lipid into peripheral circulation exposes the pancreas and skeletal muscle to elevated NEFA levels, causing decreased insulin secretion and decreased glucose uptake respectively.

Surplus fatty acids in the liver are esterified to form triglyceride. This is packaged together with cholesterol, cholesterol ester, phospholipids, apo B-100, and again apo C and E from HDL to form VLDL which is secreted into the bloodstream. LPL then acts to hydrolyse the triglyceride to form IDL. These particles can either be taken up by LDL receptors on the liver, or undergo further delipidation by hepatic lipase (HL) to form LDL. LDL particles are removed from the circulation by LDL receptors on the liver and to a lesser extent, extrahepatic tissues.

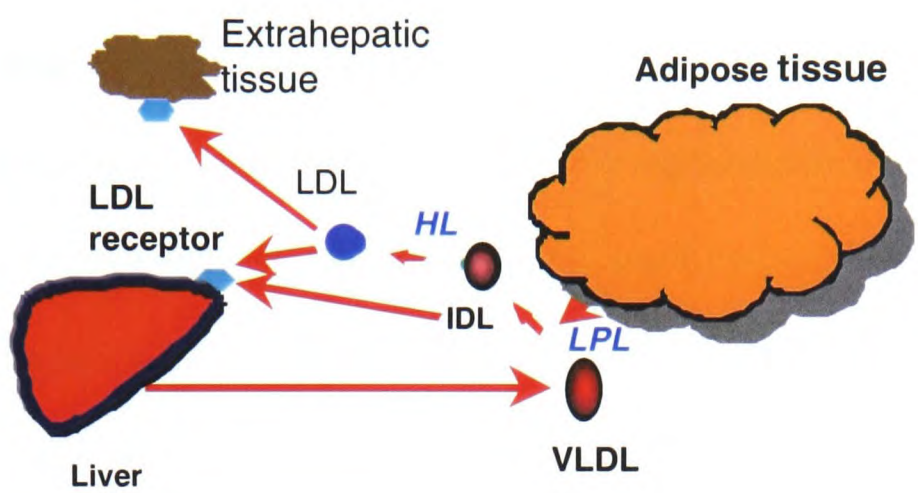


Figure 5 Endogenous lipid pathway. Hepatic triglyceride (VLDL) is hydrolyses by LPL and hepatic lipase (HL) to form IDL and LDL respectively, which are taken up by the liver and peripheral tissues.

The main triglyceride containing lipoproteins are therefore chylomicrons and VLDL. In the postprandial state, endogenous apo B 100 containing particles constitute a large proportion of the total triglyceride rich lipoprotein pool (Karpe 1999). Endogenous triglyceride rich lipoproteins make up 96-97% in the fasting state and this is reduced to 91-96% in the postprandial period. Therefore VLDL production and turnover are important factors determining the overall lipaemic response to an oral fat challenge.

In non diabetic individuals, subtle fluctuations in plasma insulin levels regulate free fatty acid release from adipose tissue, with mealtime increases in plasma insulin inhibiting lipolysis and fatty acid release facilitated by increased glucose uptake. Deficient insulin action on adipose tissue impedes the suppression of fatty acid release. This elevation in fatty acid levels enhances hepatic gluconeogenesis and inhibits glucose uptake and utilization in insulin sensitive tissues. Therefore, insulin may indirectly regulate VLDL

utilization in insulin sensitive tissues. Therefore, insulin may indirectly regulate VLDL production by reducing plasma NEFA levels as a consequence of its inhibitory effect on HSL in adipose tissue (Coppack, Jensen et al. 1994). Chen et al (Chen, Golay et al. 1987) showed that the ability of insulin to suppress plasma NEFA concentrations as well as stimulate glucose uptake was significantly reduced in subjects with type 2 diabetes both obese and non obese. The reduction in NEFA suppression was more prominent at lower insulin concentrations, whereas resistance to insulin stimulated glucose uptake was more evident at higher insulin levels. Riemans et al (Riemens 2000) showed that fasting plasma NEFA levels were increased and less suppressible by insulin in obese type 2 diabetic subjects. They propose that the high triglyceride concentration causes a higher escape of NEFA uptake from tissues after intravascular triglyceride lipolysis.

Studies have shown that subjects with high insulin and triglyceride levels have low HDL levels (Laws and Reaven 1992). Increased triglyceride concentration enhances transfer of cholesteryl esters from HDL to triglyceride rich lipoproteins in exchange for triglyceride and therefore HDL concentration decreases. Thus elevated triglyceride levels are the driving force for reduced HDL cholesterol values (Patsch, Prasad et al. 1984). Hypertriglyceridaemia is also associated with increase small dense LDL particles. Non diabetic subjects with increased small dense LDL particles tend to be relatively insulin resistant, glucose intolerant and hyperinsulinaemic as well as having low HDL concentrations and elevated triglyceride levels (Reaven, D et al. 1993).

As previously stated, people with type 2 diabetes often have low HDL cholesterol levels and elevated triglyceride levels. The median triglyceride level is usually less than 2.3 mmol/l with 85- 95% of patients having levels below 4.5 mmol/l (American-Diabetes-Association 2002). More detailed studies have shown that in the fasting state, as only small amounts of apo B-48 are normally found, the elevated triglyceride seen in type 2 diabetes consists mainly of VLDL triglyceride (de Man, Castro Cabezas et al. 1996). These VLDL particles appear to be larger and more triglyceride enriched as compared with normal VLDL. Insulin resistance increases the supply of substrate to the liver, particularly glucose and fatty acids, which may be the cause of VLDL triglyceride overproduction. An in vitro study in cultured liver cells showed newly synthesized apo B-100 may be degraded before secretion which could be inhibited by free fatty acid uptake by the liver (Ginsberg and Illingworth 2001). If this occurred in vivo, the high free fatty acid flux in type 2 diabetes may drive high secretion rates of VLDL. Also, the normal insulin suppression of VLDL apoB is impaired in type 2 diabetic individuals which results in inappropriate release of VLDL particles in the fasting and postprandial phase (Mero, Malmström et al. 2000). These VLDL particles are preferentially removed directly from the circulation rather than be converted to IDL, showing impairment of the normal elimination pathway. After an oral fat challenge, the increased levels of triglyceride rich lipoproteins (including VLDL particles) presents an increased burden on the lipolytic pathway and impaired elimination of chylomicron remnants compared with non diabetic individuals. Elevated postprandial triglyceride levels are explained to a large extent by the accumulation of endogenous triglyceride rich lipoproteins (Karpe, Hellénius et al. 1999).

Other studies have demonstrated that normotriglyceridaemic type 2 diabetic subjects have a significant increase in IDL cholesterol and remnant lipoprotein like particles (RLP) (Yoshino, Hirano et al. 2002). These IDL particles undergo further metabolism to LDL, the lifetime of which appears to be mainly determined by the availability of LDL receptors that recognize apo B and account for approximately 60 – 70% of LDL catabolism. LDL apo B is significantly increased in normolipidaemic type 2 diabetic individuals. The remainder of the LDL particles are taken up by non receptor or alternative receptor pathways which may recognise glycosylated and/ or oxidatively modified lipoproteins often present in increased amounts in diabetes (Ginsberg and Illingworth 2001). People with type 2 diabetes do not normally have elevated absolute LDL concentrations, however there is a preponderance of smaller denser LDL particles (Steiner 1994). LDL particles from these patients appear to be abnormally modified on electrophoresis (Reaven 2002). The normal bimodal distribution, Pattern A, represents almost three quarters of the general population, where LDL migrating in the peak represent large particles. Most patients with type 2 diabetes exhibit Pattern B where the majority of LDL shifts to the peak containing smaller and denser particles. These particles are associated with hyperinsulinaemia, hypertriglyceridaemia, and low HDL concentrations and are more easily oxidised thereby enhancing atherogenesis. As well as being more susceptible to oxidation, small dense LDL has decreased binding capacity to the LDL receptor (Yoshino, Hirano et al. 2002).

Another potential mechanism for lipoprotein abnormalities seen in type 2 diabetes is a delayed clearance of VLDL triglyceride due to decreased LPL activity. The role of LPL, present on the endothelium of blood vessels, is the hydrolysis of triglyceride rich lipoproteins, with the amount of enzyme available being the rate limiting factor. Other functions of LPL include involvement in the binding of chylomicron remnants to the LDL receptor, bridging of lipoproteins to proteoglycans and the synthesis of LDL from VLDL. As alluded to above, both apoB-48 and apoB-100 containing triglyceride rich lipoprotein particles compete for this limited LPL activity and uptake by common hepatic receptors also might saturate the lipolytic capacity for hours (Mero, Malmström et al. 2000). Impaired lipolysis may lead to the accumulation of fatty acids. Even in non obese type 2 diabetic patients, after ingestion of a meal, fatty acids levels are persistently elevated. These higher free fatty acid levels contribute to peripheral insulin insensitivity and further inhibit lipolysis.

Hyperglycaemia, inevitably present in type 2 diabetes, has lead to some investigators proposing that it is the increased glycation of apolipoproteins that may adversely influence lipoprotein metabolism (de Man, Castro Cabezas et al. 1996). This has been confirmed by several in vitro studies. The action of apo E, responsible for the binding of triglyceride rich remnants to the LDL receptor, may be impaired due to glycation. The binding of IDL too to the LDL receptor, mediated by apo B-100 may be affected by its glycation. The hydrolysis of triglyceride rich lipoproteins by LPL, as discussed earlier, requires apo C-II whose function can be reduced by glycation. Furthermore, direct

glycation of LDL itself, slows LDL catabolism, and in vitro has been shown to reduce fibroblast degradation.

1.2.3 Mechanism of dyslipidaemia leading to atherosclerosis

The mechanisms of postprandial dyslipidaemia leading to CHD has been discussed in the literature (Anderson, Jones et al. 2001). It was Zilversmit over 20 years ago who proposed atherogenesis may occur during the postprandial period (Zilversmit 1979). It was described that in humans, chylomicron remnants as well as low-density lipoproteins were taken up by arterial cells and that lipoprotein studies after administration of a test meal containing fat and cholesterol were urgently needed. Chylomicrons and VLDL/IDL exchange their triglyceride for cholesterol ester from LDL and HDL, mediated by cholesterol ester transfer protein to become chylomicron and VLDL remnants. It has been postulated that increased transfer of cholesteryl ester from HDL to triglyceride rich lipoproteins occurs in those with hypertriglyceridaemia and low plasma HDL levels. The cholesterol enriched particles are taken up by macrophages and deposited in the developing atheromatous plaque. Lipoproteins function to redistribute and recycle cholesterol in healthy people. Primary lipoprotein disorders such as familial combined hyperlipidaemia, eventuate from alterations in lipid metabolism (Knopp 1999). The triglyceride enriched LDL particles are hydrolysed by hepatic lipase to become small dense LDL. These particles have poor receptor affinity and therefore circulate longer. They can penetrate the vascular intima, and are more susceptible to oxidation and macrophage uptake (Karpe 1999). Normally, HDL carries cholesterol away from the arterial wall but triglyceride enriched HDL particles also undergo delipidation by hepatic

lipase to become small dense HDL. These particles are rapidly removed from the circulation and are therefore less cardio protective.

Postprandial lipaemia causes an increase in the activation of factor VII which may through subsequent coagulation events, potentially generate thrombin production upon plaque rupture (Karpe 1999). Another mechanism is that FFA formed from lipolysis may enhance the endothelial penetration of LDL and remnant lipoproteins. Other studies have proposed that postprandial lipaemia causes endothelial dysfunction by reducing nitric oxide production. An animal study showed that a decrease in coronary blood flow reserve during hyperlipidaemia in dogs is secondary to an increase in blood viscosity (Rim, Leong-Poi et al. 2001). Other studies have looked at the direct effect of a high fat meal, and showed that this does not impair endothelium dependent vasodilation in resistance arteries unless diabetic patients have impaired renal function (Shand, Scott et al. 2001).

1.3 Treatment strategies in diabetes

Multiple risk factor intervention is essential in the management of this complex disease. Treatment of individual risk factor in diabetes has been shown to be of benefit, with large studies showing the risk reduction with intensive blood glucose control (UKPDS Group 1998), blood pressure lowering (Heart Outcomes Prevention Evaluation Study Investigators 2000), and cholesterol treatment (Heart Protection Study) (HPS Collaborative Group 2002). A recent study by Gaede et al (Gæde, Vedel et al. 2003)

demonstrated the benefits of a target driven, long term, intensified intervention aimed at multiple risk factors in patients with type 2 diabetes and microalbuminuria which reduces the risk of cardiovascular and microvascular events by approximately 50%.

1.3.1 Cholesterol lowering strategies

Published guidelines for the treatment of dyslipidaemia in diabetes state that target levels should be less than 2.6 mmol/l for LDL cholesterol and less than 2.3 mmol/l for triglyceride (American Diabetes Association 2002). In practice, treatment is mainly recommended for patients with total cholesterol levels greater than 5 mmol/l and a 10 year CHD risk of over 20% (UK) and 15% (USA), however there is emerging epidemiological evidence for CHD risk reduction at lower LDL cholesterol concentrations (HPS-Collaborative-Group 2002).

LDL lowering agents

The first line mainstay of cholesterol lowering treatment are the drugs belonging to class statins. These agents are competitive inhibitors to hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase which regulates the final step in cholesterol synthesis. By upregulating LDL receptor activity and reducing LDL entry into the circulation, these drugs primarily lower LDL concentrations. In the 4S study, simvastatin reduced the 5 year cumulative incidence of coronary events in diabetic subjects with high LDL levels and previous CHD from 45% in the placebo group (n=97) to 23% in the treated group (n=105) (p=0.002) (Scandinavian Simvastatin Survival Study Group 1994). In the CARE study, the 5 year cumulative incidence was 37% in the placebo group (n=304) compared

with 29% in the diabetic group with average LDL levels and previous CHD treated with pravastatin (n=282) (p=0.05) (Sacks, Pfeffer et al. 1996). More recently the Heart Protection Study showed the addition of simvastatin 40mg to existing treatments in high risk patients irrespective of initial cholesterol concentration to reduce the rate or myocardial infarction, stroke and revascularisation by approximately 25% (HPS-Collaborative-Group 2002).

Bile acid binding resins decrease LDL cholesterol by 10 -20% by binding bile acids in the intestine and interrupting enterohepatic circulation thus increasing the conversion of cholesterol into bile acids in the liver. However, hepatic synthesis of cholesterol is increased, so there is increased secretion of VLDL and raised triglyceride concentrations. These days combination with a statin to further reduce LDL cholesterol levels (with a small increase in HDL concentration) is its primary indication for hypercholesterolaemic conditions and not hypertriglyceridaemia. (Knopp 1999).

HDL raising agents

Nicotinic acid reduces the hepatic synthesis of triglyceride and secretion of VLDL by inhibiting the mobilization of free fatty acids from peripheral tissues, and also decreases the conversion of VLDL to LDL. Nicotinic acid can increase HDL concentrations by up to 30% and can shift small dense LDL particles to large, buoyant particles as well as lowering Lp (a) lipoprotein by 30%. The drug can be used in patients with familial combined hyperlipidaemia often in combination with statin therapy. Studies using

nicotinic acid have shown the drug to reduce CHD when used in combination with fibrate therapy or bile acid binding resins (Knopp 1999).

1.3.2 Triglyceride lowering strategies

The current treatment of hypertriglyceridaemia in diabetes is based upon behavioural therapy of weight loss, increased physical activity, modest alcohol consumption as well as dietary fat restriction. Uncontrolled diabetes can lead to hypertriglyceridaemia, and therefore optimizing blood glucose control is important (Simpson, Mann et al. 1979). Treatment of triglyceride levels between 2.3 – 4.5 mmol/l are dependent upon clinical judgement, however levels above these, pharmacological agents are considered.

Fibrate or fibric acid drugs partly resemble short chain fatty acids, and increase the oxidation of fatty acids in liver and muscle. In liver there is decreased secretion of triglyceride rich lipoproteins and in muscle there is an increase in lipoprotein lipase activity and uptake of fatty acids. These agents act by activating peroxisome proliferators-activated receptor α (PPARα) a nuclear transcription factor, as well as up regulating the expression of LDL cholesterol and apolipoprotein AI genes, and down regulating the expression of the apolipoprotein CII genes. Fibrates primarily lower triglyceride levels. The effects on LDL cholesterol are dependent on their level in association with hypertriglyceridaemia that is, at high triglyceride levels associated with low LDL cholesterol levels, LDL cholesterol may increase with fibrate use, whilst if the LDL cholesterol concentration is high, fibrates can lower LDL levels. These lipid lowering agents can also beneficially increase the buoyancy of LDL particles. The

primary indication for fibrate therapy is for triglyceride levels > 11.5 mmol/l. The Helsinki Heart Study (HHS) and the Veteran's Affairs HDL intervention trial (VA-HIT) have shown that treatment with gemfibrozil in primary and secondary preventions trials respectively reduced the frequency of heart disease (Robins, Collins et al. 2001). In the HHS the 5 year cumulative incidence of coronary heart disease in the gemfibrozil treated diabetic men (n=59) was 3.4% compared with 10.5% in the matched placebo group (n=76) (p=0.19). This equated to a 24% reduction in cardiovascular events in diabetic subjects with prior CHD (Knopp 1999).

The n-3 fatty acids contained in fish oil are effective in decreasing plasma triglyceride concentrations up to 40% at high dose (Burnett and Watts 2001). Fish oil decreases VLDL secretion by the liver, and increase its conversion to LDL. There is also an increase in chylomicron clearance and a reduction in postprandial triglyceride response. Their mechanism of action remains unclear.

Table 1 Summary of current lipid lowering agents and degree of LDL and triglyceride reduction

Treatment	**LDL cholesterol reduction**	**Triglyceride reduction**
Diet	8-15%	20% low carbohydrate, 4% low fat diet
Statins	24 – 60%	16 – 29%
Fibrates	Little effect	33 – 47%
Bile acid resins	10 – 20%	Increase triglyceride levels
Nicotinic acid	Lp(a) lipoprotein by 30%	Increase HDL 30% and lowers triglyceride
Fish oil		40%

Summarised from (Knopp 1999), (Robins, Collins et al. 2001) and (Samaha, Iqbal et al. 2003).

Kanters et al (Kanters, Algra et al. 1999) assessed the feasibility of an intensive lipid lowering strategy to target plasma lipid levels in diabetic subjects. Of the subjects eligible to participate, only a minority volunteered. The authors concluded that there was

potentially a large compliance problem for a general lipid lowering program to be initiated in a diabetes clinic. However the study was able to demonstrate that intensive lipid lowering with drug combinations was attainable in 66% of subjects, with a plasma lipoprotein composition shift towards a less atherogenic profile.

1.3.3 Could restoring first phase insulin secretion improve postprandial metabolism?

There are several mechanisms proposed in the literature by which restoration of the first phase insulin response may contribute to the modification of post challenge lipid profiles. Acute hyperinsulinaemia produced in clamp technique experiments to overcome insulin resistance has been shown to reduce hepatic secretion of VLDL apoB100 in type 2 diabetic patients (Cummings M H, Watts G F et al. 1995; Malmström R, Packard C J et al. 1997). In vitro animal studies using rat hepatocytes have demonstrated the acute inhibitory effect of insulin on the secretion of very low density lipoprotein triacylglycerol and apolipoprotein B is not altered in states of chronic exogenous hyperinsulinaemia (Bourgeois, Wiggins et al. 1996).

The early rise in insulin concentration, provides a more physiologic exposure of insulin to the liver, faster suppression of endogenous glucose production and thus preventing hyperglycaemia and persistent hyperinsulinaemia in the postprandial period (Del Prato 2003). Without this, post meal hyperglycaemia maintains a non-physiologic stimulatory stress on the β cell. The rapid insulin induced glucose uptake would reduce the supply of glucose to the liver and may suppress endogenous triglyceride production (de Man, Castro Cabezas et al. 1996). This would improve the catabolism of postprandial

triglyceride rich lipoproteins and lower the fasting triglyceride level. Improved glycaemic control should reduce the glycation of lipoproteins thereby enhancing catabolism and preventing oxidative modification. Insulin induced stimulation of glucose metabolism in fat cells increases the availability of alpha glycerol phosphate to re esterify NEFA (from intracellular depots or transferred from the plasma), causing a decline in NEFA concentration (Ferrannini, Galvan et al. 1999). The activity of HSL in adipose tissue is inhibited by insulin. Therefore acute adminstration of insulin post meal should cause rapid NEFA and glycerol suppression and release from adipose tissue, thereby providing the liver with less substrate for VLDL triglyceride synthesis (Figure 6).

Insulin itself is a stimulator of LPL activity in adipose tissue, and thus increasing LPL activity would improve chylomicron and VLDL triglyceride clearance (Taskinen, Beltz et al. 1986). Insulin also inhibits HL, therefore curtailing intracellular lipolysis (Ferrannini, Galvan et al. 1999). Another possible mechanism, although not confirmed, is that insulin causes a small increase in CETP activity ie reverse cholesterol transport causing a rise in HDL (Geltner, Lechleitner et al. 2002). Therefore the catabolic rate of HDL is slowed by insulin whilst de novo triglyceride synthesis is stimulated. Studies using insulin infusions have also shown an increase in PAI 1 concentrations (Ferrannini, Galvan et al. 1999).

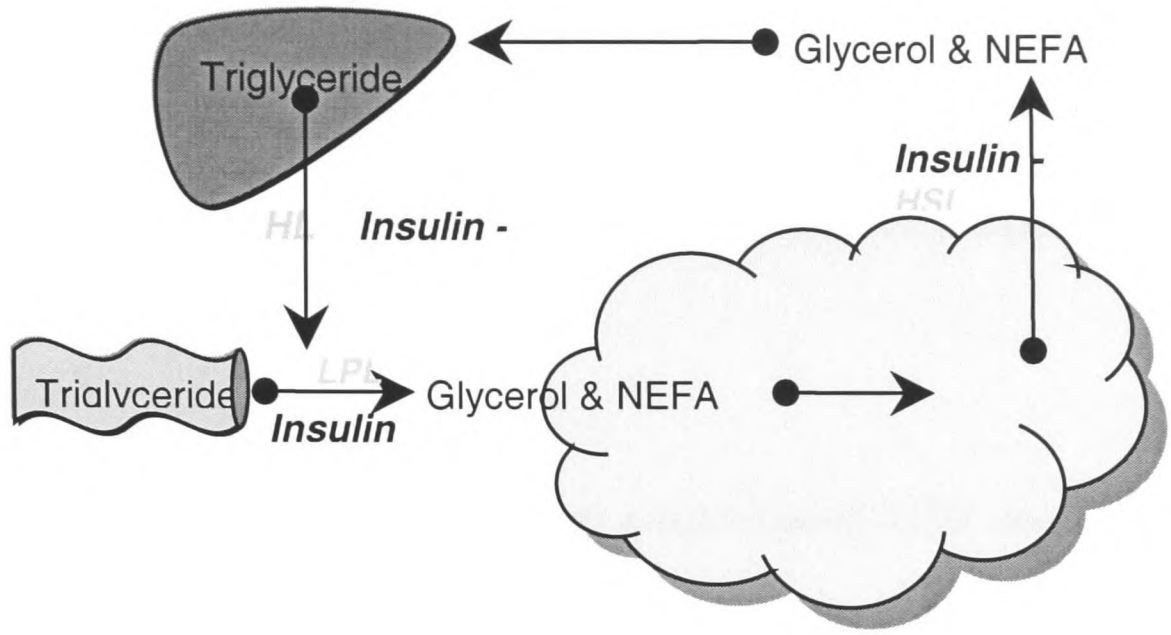


Figure 6 Insulin effects on lipid metabolism. Insulin stimulates the activity of LPL promoting lipolysis of triglyceride rich lipoproteins (chylomicrons and VLDL). Insulin inhibits HSL thereby reducing NEFA and glycerol supply from adipose tissue to the liver, which is required for VLDL triglyceride synthesis.

1.3.4 Glycaemic treatment strategies targeting the postprandial period

The UKPDS showed that intensive blood glucose control either with sulphonylureas or insulin, substantially decreases the risk of microvascular complications by 25%. The evidence was inconclusive for macrovascular complications (16% reduction, p=0.052) (UKPDS Group 1998). Therapeutic decisions are based on fasting parameters, however perhaps the focus should be towards targeting the postprandial period as most of our time is spent in this state.

Agents that stimulate early insulin secretion and have a more rapid onset of action have been developed to target post meal glucose excursions. Gliclazide is a short acting sulphonylurea which may have some effect on first phase insulin secretion as well as second phase, although does not normalise it (Hosker, Rudenski et al. 1989). More recently rapidly acting insulin secretagogues have been developed such as repaglinide

and nateglinide. These agents rapidly increase insulin secretion in a glucose dependent manner in response meals (Malaisse 1995). The early rise in insulin concentrations seen with the non sulphonylurea insulin secretatagogues is more comparative to the first phase insulin response.

Exogenous insulin and insulin analogue administration is the mainstay of treatment for many individuals with type 2 diabetes as well as type 1 diabetic patients. A study by (Bruce D G, Chisholm D J et al. 1988) compared glucose profiles after giving no insulin, early insulin delivery, delayed insulin infusion, and constant instant infusion in type 2 diabetic subjects after a standard mixed meal. High levels of blood glucose were found when subjects had no insulin, delayed insulin infusion, and constant insulin infusion, whilst the glucose profile improved with early insulin delivery (soluble insulin). Similar effects may be seen with the use of fast acting synthetic insulin analogues. Insulin lispro and insulin aspart give a rapid rise in plasma insulin levels, and less prolonged stimulation compared with administration of an equivalent amount of soluble insulin which produces a longer lasting hyperinsulinaemic tail (Bruttomesso D B, Pianta A et al. 1999).

There are a variety of other agents that also target post meal glucose excursions. The alpha glucosidase inhibitor, acarbose regulates glucose absorption from the gut and overall reduces HbA1c by 0.3% (UKPDS Group 1999). Amylin and amylin analogues such as pramlintide slow gastric emptying and may also have an effect by suppressing glucagon production (Del Prato and Tiengo 2001). Glucagon-like peptide-1 (GLP-1) is

an insulinotropic intestinal hormone released in response to a meal. In type 2 diabetic patients, GLP-1 infusion is associated with a prompt and sustained acute increase in mealtime insulin secretion by potentiating β cell function. There is also suppression of plasma glucagon concentration. Currently, analogues of GLP-1 such as exendin, are being tested, however their clinical use is still uncertain due to the rapid proteolytic degradation of GLP-1 (Del Prato and Tiengo 2001).

1.4 Hypothesis

Traditional and novel risk factors contributing to CHD in type 2 diabetes have been described in the literature, however the degree to which the remaining risk is explained is yet to be determined. There are many factors contributing to excessive mealtime glucose excursions seen in type 2 diabetes. Peripheral insulin resistance (muscle and adipose tissue), sustained endogenous glucose production during mealtime, inadequate suppression of mealtime plasma glucagon concentrations, persistence of elevated free fatty acids, and altered gastric emptying all may be of importance (Del Prato and Tiengo 2001). It is possible that these abnormalities may in part be due to loss of first phase insulin secretion and restoration may improve lipid alterations seen in type 2 diabetes. Therefore, prandial treatment strategies for type 2 diabetes which more closely mimic physiological glucose-stimulated insulin release would not only be important to enable glucose utilisation, but also prevent triglyceride rich lipoprotein formation. By enhancing portal insulin levels the liver would be presented with rapid and early insulin delivery, decreasing hepatic glucose production, and potentially having a direct effect on reducing hepatic triacylglycerol production. Another method of augmenting first phase insulin

secretion in type 2 diabetes would be to provide prandial insulin therapy and examine the effects of rapid and short acting systemic insulin delivery on the glucose and lipid handling.

1.5 Aims

This DPhil thesis seeks to explore more closely post challenge lipid handling in diabetes and to undertake a series of intervention studies seeking to determine whether more physiologic insulin profiles may beneficially modify post challenge lipid profiles. The main aims are:

1. Formulate a standardised oral test meal to assess reliably postchallenge responses. The test meal would preferably be inexpensive, simple to manufacture and easy to use in clinical practice.

2. To evaluate short term reproducibility of post challenge biochemical factors derived from this standardised postprandial test to enable use in subsequent clinical studies.

3. To assess the use of capillary fingerprick sampling as an alternative to venipuncture for the OTTT.

4. Characterise OTTT glucose and triglyceride responses in non diabetic and diabetic subjects.

5. To investigate potential benefits of enhanced endogenous insulin secretion in type 2 diabetic subjects on OTTT responses and differences in rapid versus less rapid delivery of insulin directly to the liver through the portal circulation as well as increasing peripheral insulin levels.

6. To investigate the potential benefits of increasing systemic insulin levels in type 2 diabetic subjects on OTTT responses and differences in rapid versus less rapid delivery of insulin.

7. Exploring and characterising post challenge lipid handling in type 1 diabetic patients by providing exogenous insulin replacement and contrast OTTT responses with type 2 diabetic patients.

8. To investigate the effects of acute post challenge hyperinsulinaemia and hyperlipidaemia on platelet function in order to elucidate potential mechanistic causes of atherosclerosis development.

2 Chapter 2 Methodology

2.1 Ethical Issues

All studies were conducted adhering to the principles stated in the Declaration of Helsinki (World Medical Association 2000) and the International Conference of Harmonisation Good Clinical Practice guidelines (GCP) (Medical Research Council 1998). Appropriate Local Research Ethics Committee (LREC) approval was sought prior to the start of each study. The permission of the relevant healthcare professional, such as General Practitioner and/or Diabetologist, was sought prior to contacting potential subjects. All information provided to subjects received LREC approval. Subjects were given a subject information letter which clearly outlined the aims and procedures involved in a study. Written informed consent was obtained prior to the subject's participation in any study. Blood samples collected for apo E genotyping were not used for any other purpose and were not stored after analysis.

2.2 Data Collection

Clinical Research Unit

Studies were conducted in the Clinical Research Unit (CRU). This is a 6 bed research facility staffed by 5 research nurses. The CRU functions to provide an environment for clinical research following GCP guidelines.

Data Management

Data was collected and recorded on pre-printed Case Report Forms (CRF) which were double-keyed by the Diabetes Trials Unit (DTU) data entry staff. Data was held in the DTU Data File Repository - a password protected database system providing a secure location for projects with automatic daily backup.

DTU laboratory

The DTU Laboratory is a containment 2 level laboratory and following Good Laboratory Practice guidelines. Performance was monitored by an 'in house' quality control program 'QSTAT' using tri-level quality control sera with application of Westgard rules for acceptance of results.

Assays results from the DTU Laboratory were transferred electronically directly to the Data File Repository.

2.3 Subject Selection

Detailed recruitment criteria for participating subjects are outlined in the Methodology section for each study (Chapter 3, 4, 5 and 6). In general, Caucasian males and females who were not taking lipid lowering therapy and had no history of established cardiovascular disease were asked to participate. Subjects with diabetes had been diagnosed according to WHO criteria (WHO 1999) for at least one year. Subjects were excluded if they had a plasma triglyceride level (>5 mmol/l), poorly controlled diabetes (HbA_{1c} > 10%), a major lipid abnormality (eg familial hypercholesterolaemia or

hyperlipidaemia), gastrointestinal problems that might influence lipid absorption (eg. delayed gastric emptying, chronic inflammatory bowel disease), any serious underlying medical conditions, a history of alcohol abuse, were pregnant or lactating females.

2.4 Standard Measurements

Subjects were weighed using Avery scales (serviced by Avery Company Ltd, UK annually) and blood pressure recorded as the mean of 3 automatic sphygmometer readings (Takeda UA-751, A&D Company Ltd, UK) (quality control monthly with mercury sphygmometer) (UKPDS Group 1998). Waist circumference was measured using a tape measure according to World Health Organisation (WHO) criteria (WHO 1999). Subcutaneous fat was estimated by measuring triceps skin fold thickness (mean of 3 readings) using Harpenden callipers (John Bull, British Indicator Ltd, UK).

2.5 Safety Measurements

All subjects were underwent a check of fitness assessed by a physician prior to further participation. Haematological and biochemical checks prior to any study included full blood count, alanine amino transferase (ALT), and creatinine. Subjects were excluded from a study if their ALT was > 2 x the upper limit of normal (75 IU/L for males and 49 IU/L for females), their creatinine was > 150 μmol/L or they were anaemic (haemoglobin < 14 g/dL for males, <12 g/dL for females).

2.6 Oral Triglyceride Tolerance Test (OTTT) Drink Composition

Several formulations were considered with the ingredients chosen discussed in Chapter 3. The 200 mL test drink was produced by adding 50g of carbohydrate as spray dried maltodextrin gluten free powder (Polycal, Nutricia, Cuijk, Holland) to 100 mL of a commercially produced strawberry flavoured fat emulsion containing 50g long chain triglyceride (Calogen, SHS, Cheshire, UK). The test drink was prepared on the morning of the OTTT. Nutrient information for the test drink, which had an energy content of 2715kJ (656kcal) is summarised in Table 2.

Table 2 - Test drink nutrient composition

Component		Per 200 ml
Fat		50g
	Saturated	19.0%
	Monosaturated	59.5%
	Polyunsaturated	21.5%
Fatty acid profile		g/50g fatty acid
	C16:0	5
	C18:0	1.5
	C18:1	29
	C18:2	10
	C18:3	<1
	C20:0	0.5
	C20:1	0.5
	C22:0	1.5
	C24:0	0.5
Carbohydrate		50g
	Glucose	1.6g
	Maltose	2.7g
	Polysaccharides	45.7g
Protein		0g
Fibre		0g
Water		92.5g
Sodium		<10 mg
Energy		2715 kJ
		656 kcal

2.7 OTTT Operating Procedure

On the day and night prior to the test, subjects were asked not to consume alcohol, or undertake any exercise outside of their normal practice. All routine non-diabetic medication was continued as prescribed. Antidiabetic therapy was adjusted according to specific protocol of the study the subject was undertaking (refer to Methodology section of Chapter 3, 4, 5 and 6).

Subjects were admitted to the DTU CRU on the morning of the test following a 10 hour overnight fast (water permitted). A 20G venous cannula was inserted into a forearm vein and baseline blood samples collected. The test drink was served chilled for ingestion within a maximum of ten minutes. Blood samples were then drawn at 2, 4, 6, and 8 hours for measurements. All samples were collected from subjects resting for a minimum of 15 minutes recumbent at 45°. After each sample the cannula was flushed with 5 ml normal saline (not heparin). Before each sample was taken, 2 ml of blood was obtained using a syringe and discarded to avoid potential dilution with saline.

Plasma glucose levels were monitored using a glucose meter (One Touch II Blood Glucose Monitoring System, Lifescan, Johnson and Johnson Company, UK) at each timepoint, and at any other time thought appropriate, as a safety measure to monitor for hypoglycaemia. To minimise this risk, any routinely prescribed lunchtime dose of insulin or oral hypoglycaemic agent was omitted. Subjects fasted for the duration of the 8 hour study period and refrained from smoking. During the test, subjects were allowed water to drink and could move around freely within the CRU. Subjects were given magazines and

newpapers to read and TV/video was also available. At the end of the 8 hours, a meal was provided before subjects returned home. Blood glucose was again re checked and if there was any concern about low blood glucose levels at the end of the test subjects were given extra snacks.

For all the OTTT studies fasting and post challenge samples were collected in EDTA, fluoride, and lithium heparin tubes. Capillary blood samples using an Autolancet (Medisense, Abbot Laboratories Company, UK) were placed in microvette capillary tubes containing EDTA at 0 and 6 hours for triglyceride measurement (Chapter 9). Immediately after collection blood samples were centrifuged at 3000 rpm for 10 minutes at 4˚C and the plasma separated for assay. An additional whole blood sample for apo E genotyping was taken in a 10mL EDTA tube only once for each subject. Samples were transferred to the DTU laboratory (on the same floor) after centrifugation, kept at 4˚C, for assay within 24 hours. After assaying, samples were held in storage at –70°C.

2.8 Food Diary

The 3 day food diary was used as a general indicator of the daily dietary intake completed once by subjects recruited for the OTTT study (Chapter 3 and 4). It was emphasised to subjects that filling in the food diary completely and accurately was important, and that their normal diet should not be altered in any way. Subjects were instructed to complete the food diary over 3 days including all food and drink consumed including alcohol, snacks and nibbles between meals. The first day was to be a Sunday, then Monday and Tuesday, and were not to include the day of the OTTT. The format of the diary

incorporated documentation of type of food/drink, portion size and frequency as well as a general questionnaire. Food diaries were issued at the initial recruitment visit to be completed then collected before the first OTTT. Food diaries were coded by a specialist dietician and nutrient content determined using Dietplan 5 (Forestfield Software 1992). Nutrient information was recorded on CRF and double-entered into the Data File Repository.

2.9 Adverse Events

Serious side effects from the OTTT were not expected. Potential side effects were thought to be indigestion, nausea or "stomach upset". The 8 hour test period, equated to a missed lunch time meal, was not expected to adversely affect overall diabetic control but could cause temporary problems such as hypoglycaemia.

Any adverse events were recorded on study specific case report forms. Subjects were asked during and at the end of an OTTT about possible symptoms such as nausea, abdominal pain, headache, or any other symptoms. The symptoms and relevant history were recorded in detail in the subject notes. Serious adverse events would have required LREC notification but none occurred.

2.10 Biochemical Measurements

Triglyceride

Plasma triglyceride was measured in EDTA plasma by enzymatic, colorimetric endpoint method using Beckman Synchron CX Delta Systems' Triglycerides GPO reagent and Multicalibrator referenced to Centre for Disease Control, Atlanta, with no correction for free glycerol (approximately 0.11 mmol/l). Intra-assay variation (CV) for the mid control was 2% for triglyceride 1.4 mmol/l. The assay range was 0.1 – 11.3 mmol/l and fasting normal range defined by the DTU laboratory was 0.4 – 1.8 mmol/l.

Cholesterol

Cholesterol was measured in EDTA plasma using the Beckman Synchron CX Delta Systems' CHOL kit and Multicalibrator. Intra-assay variation (CV) for the mid control was 1% for cholesterol 5.5 mmol/l. The assay range was 0.13 – 19.40 mmol/l and 3.6 – 8.0 mmol/l was defined by the DTU laboratory as the fasting normal range.

LDL cholesterol

LDL cholesterol was directly measured in EDTA plasma using the Genzyme N-Geneous LDL-cholesterol kit and calibrator (Biostat Ltd, Stockport, Lancs) traceable to the National Reference System for Cholesterol (NRS/CHOL). Intra-assay variation (CV) for the mid control was 1.6% for LDL cholesterol 3.9 mmol/l. The assay range was 0.17 – 25.65 mmol/l and fasting normal range defined by the DTU laboratory to be 1.5 – 5.3 mmol/l.

HDL cholesterol

HDL cholesterol was directly measured in EDTA plasma using the Genzyme N-Geneous HDL-cholesterol kit and calibrator (Biostat Ltd, Stockport, Lancs) traceable to HDL cholesterol reference method, Centre for Disease Control, Atlanta, with ultracentrifugation and chemical precipitation of HDL cholesterol, and cholesterol measurement by Abell Kendall method. Intra-assay variation (CV) for the mid control 2% for HDL cholesterol was 1.31 mmol/l. The assay range was 0.05 – 5.17 mmol/l and a fasting normal range of 0.91 - 1.69 mmol/l was defined by the DTU laboratory.

VLDL cholesterol

VLDL cholesterol was calculated by subtracting HDL and LDL cholesterol values from total cholesterol.

NEFA

NEFA was measured in EDTA plasma by an enzymatic, colorimetric, endpoint method using the Wako NEFA-C kit (Alpha Laboratories, Eastleigh, Hants) on a Cobas MIRA discrete analyser (ABX Diagnostics, Shefford, Bedfordshire). CV was 4.6% at 581 umol/L for NEFA. The assay range was 0 – 2000 umol/l. Normal ranges defined by the DTU laboratory were 400 – 800 umol/l for fasting levels, and 20 –50 umol/l for non fasting levels.

Glycerol

Free glycerol was measured in EDTA plasma using the GPO Trinder method for triglyceride with no lipase to break down triglyceride to its constituents, Triglyceride Reagent A and standard, 250 mg/dL equivalent triglyceride concentration (Sigma-Aldrich Company Ltd, Poole, Dorset) on a Cobas FARA centrifugal analyser (BM Browne (UK) Ltd Reading, Berks). CV for glycerol was 4.9% at 2.27 umol/l equivalent triglyceride concentration. The assay range was 0 – 20.4 umol/l and the normal reference range, as defined by the DTU laboratory to be 0.33 – 1.85 umol/l.

HbA_{1c}

HbA_{1c} was measured in EDTA whole blood by high performance liquid chromatography (Variant™ Haemoglobin Testing System, Biorad Laboratories Ltd, Hemel Hempstead, UK). Intra-assay variation (CV) for the mid control was <2% for HbA_{1c}. The normal range defined by the DTU laboratory was 4.5 – 6.2%.

Plasma Glucose

Plasma glucose was measured in fluoride/oxalate plasma by the hexokinase method on a Beckman Synchron CX4 Delta analyser. Intra-assay variation (CV) for the mid control was 2% for glucose at 6.7 mmol/l. The assay range was 0.3 – 38.8 mmol/l and fasting normal range 3.9- 5.8 mmol/l defined by the DTU laboratory.

Insulin

Immunoreactive insulin was measured in heparin plasma by double antibody radioimmunoassay with Sepharose attached to the second antibody for separation by decanting (PhRIA100; Pharmacia & Upjohn Ltd, Milton Keynes, Bucks), CV 7.1% at 314 pmol/L. The CV for low control 5.6% at 96.2 pmol/L, mid control 5.5% at 324.2 pmol/l and 5.9% at 820.8 pmol/l for high control. The assay range was 14.8 – 1781 pmol/l and fasting normal range 21.5 -115.0 pmol/l.

C peptide

C-Peptide was measured in heparin plasma by radioimmunoassay using guinea pig anti-human C-Peptide antibody and a second antibody with polyethylene glycol for precipitation (Biogenesis LTD, Poole, UK). The CVs for low, mid and high control were 4.2% at 0.55 nmol/l, 5.6% at 2.14 nmol/l and 7.0% at 3.66 nmol/l respectively. The assay range was 0.02 – 3.3 nmol/l and assay sensitivity 0.02 nmol/l. The fasting normal range was defined as 0.17 – 0.5 nmol/l by the DTU laboratory.

Apo E Genotype

DNA was extracted from EDTA whole blood using the Nucleon^{TM} II DNA extraction kit (Scotlab Ltd, Coatbridge, Strathcyde UK). DNA yield and purity was quantified spectrophotometrically at 260 nm and 280 nm, and deionised water used for suspension of DNA. Genotyping for apo E was carried out by PCR amplification of a 246 bp fragment, followed by mapping of restriction fragments obtained by digestion with CfoI, on 4% metaphore agarose gels (Wenham, Price et al. 1991).

2.11 Statistical methods

2.11.1 Sample Size Calculations

For the initial evaluation of the OTTT, repeat testing in 30 type 2 diabetic subjects and 20 non-diabetic subjects was thought to be sufficient to characterise the post challenge lipid profiles obtained. Sample size calculations were then performed for each study based on the assumption that a 35% reduction in the 6 hour post challenge triglyceride level would be of clinical interest. Previous intervention studies have shown beneficial effects in reduction of fasting triglyceride levels by 35- 47% using fibrate therapy (Robins, Collins et al. 2001).

2.11.2 Data Validation

Data was assessed for missing values and high or low values outside pre defined laboratory ranges. These were then accounted for through verification by laboratory records and clinical notes (eg hypoglycaemia causing early cessation of the OTTT). Data, examined in Microsoft ® Excel 2000-2002 (Copyright © Microsoft Corporation 1985 - 2002) and C Stat for Windows © Oxtech Ltd 1993 (Cherwell Scientific Publishing Limited, Oxford UK), was then described as normally or non normally distributed according to frequency histograms and Shapiro- Wilk W test. Non normally distributed data was log transformed for analysis.

2.11.3 Data Analysis

Using Microsoft Excel and Cstat packages, data are presented as mean and standard deviation (SD), or median and inter-quartile range (IQR) according to their distribution with geometric mean (1SD range) for triglyceride, NEFA, insulin and C peptide values. Actual and change from baseline biochemical profiles are described graphically using Delta Graph ® 4.5 (©SPSS Inc and Pantone Inc ®, 1998) and values tabulated.

Comparison Diabetic and Non Diabetic Subjects

Subject characteristics and biochemistry data were compared between the non-diabetic and diabetic groups using the 2 sample t-test, Mann Whitney U test, chi-squared test (χ^2) or Fisher's exact test. The diabetic subgroups in the initial OTTT study were compared using Analysis of Variance (ANOVA) or Kruskal-Wallis test. Food diary results were compared between the non-diabetic and diabetic groups using the 2 sample t-test.

Intervention Studies

Paired t-tests and Wilcoxon matched pairs signed rank sum test were used to compare biochemistry data between the different treatments assessed within the cross over study. In addition to fasting measurements, time points of interest were selected for each biochemical profile prior to conducting statistical testing. The timepoint chosen to depict the elevation of triglyceride was 6 hours from previous postprandial studies (Karpe, de Faire et al. 1998) (Patsch, Miesenbock et al. 1992). To assess the degree of post challenge NEFA and glycerol suppression due to insulin, the 2 hour timepoint was chosen. The 8 hour timepoint was then chosen to depict the effects of insulin subsiding.

The 2 hour timepoint was chosen to depict peak glucose, insulin and C peptide levels. Pearson correlation coefficients were used to demonstrate associations between fasting/post challenge triglyceride levels with glucose and insulin levels, as well as demographic characteristics such as age, BMI, waist circumference, triceps skin fold thickness and blood pressure.

2.11.4 Derived Variables

Reproducibility

Reproducibility of measurements (Chapter 3 and 5) was assessed by analysing paired data from the same individual (intra individual variation) using the formula CV = SD/mean, where CV is the coefficient of variation, SD is the standard deviation and "mean" is the average of the 2 tests. The median (IQR) CV for non normally distributed data, or mean (SD) CV for normally distributed data was then calculated to give the CV of the analyte for all subjects. Difference plots were used to graphically depict the differences between the 2 tests against the mean for each subject using Deltagraph.

Area Under the Curve

For all studies, the area under the curve (AUC) for biochemistry 8 hour profiles was derived from trapezoidal integration (Altman 1991). All 8 hour profiles were divided into 4 trapeziums with the area of each trapezium (A) was defined as $A_1 = 2 \times ((y_1 + y_2)/2)$, where y is the value at baseline (y_1), 2, 4, 6 and 8 hours. The AUC was calculated as the sum of the 4 trapeziums.

Incremental area under the curve

Incremental area under the curve (IAUC) was calculated by subtracting the baseline area over 8 hours from AUC, that is, IAUC = AUC – (y_1 x 8).

Maximum and minimum concentration

Peak values (Cmax) were calculated using Microsoft Excel where MAX (y_1, y_2, y_3, y_4, y_5) returns the largest value in a set of values over the 8 hour period. Nadir values (Cmin) were also calculated using Microsoft Excel where MIN (y_1, y_2, y_3, y_4, y_5) returns the lowest value in a set of values over the 8 hour period.

Time to peak and nadir

Time to peak (Tmax) over the 8 hour period was calculated by determining which timepoint was equivalent to Cmax, using the formula:

IF(y_1=Cmax,0,IF(y_2= Cmax,2,IF(y_3= Cmax,4,IF(y_4= Cmax,6,8)))).

Time to reach the lowest concentration (Tmin) over the 8 hour period was calculated by determining which timepoint was equivalent to Cmin, using the formula:

IF(y_1=Cmin,0,IF(y_2= Cmin,2,IF(y_3= Cmin,4,IF(y_4= Cmin,6,8)))).

3 Chapter 3 The Oral Triglyceride Tolerance Test (OTTT)

3.1 Introduction

As early as in the 1950s, a relationship between postprandial lipaemia and CHD had been suggested(Moreton 1950) and the need for a standardised challenge test proposed (Zilversmit 1979), yet there is still a lack of clinical studies providing evidence for reproducible post-challenge glycaemic and lipid profiles. One reason may be the high variation in triglyceride measurements with studies reporting fasting triglyceride coefficients of variation (CV) of 22% in non-diabetic subjects (Sebastian-Gambaro, Liron-Hernandez et al. 1997; Widjaja, Morris et al. 1999). In comparison, the well-established oral glucose tolerance test (OGTT) has a reported 2 hour CV ranging from 15 to 46% (Levy, Morris et al. 1999), and is considered the "gold standard" test incorporating post challenge plasma glucose values for the diagnosis of diabetes mellitus (WHO 1999). Previous studies have shown the intra-individual variation of fasting and post-challenge triglyceride levels to be influenced by smoking, alcohol and exercise (Weintraub, Rosen et al. 1989; Superko 1992; Hartung, Lawrence et al. 1993; Sethi, Isherwood et al. 1994; Axelsen, Eliasson et al. 1995), which must therefore be considered in the test design.

A standardised test to assess triglyceride tolerance is required if reliable assessment of postprandial lipid metabolism in clinical trials and routine practice is to be undertaken. Studies of post challenge triglyceride levels subjects have used different test meals or drinks consisting of "everyday" foods such as dairy products, bacon, margarine, and eggs

or liquid formulations. These have contained varying percentages of total energy (amount in grams) of fat 33 - 66 % (up to 110g), carbohydrate 14 - 43% (up to 75g) and protein 16 - 20% (up to 46g) and occasionally added vitamin A (Lewis, O'Meara et al. 1991; Cavallero, Dachet et al. 1994; Curtin, Deegan et al. 1994; Tan, Cooper et al. 1995; Cooper, Tan et al. 1996). There is however, no "off the shelf" product available in countries around the world that can be used in order to prove a challenge, which is an essential prerequisite if the OTTT is to be accepted for use in the future. Review of the literature, shows the relationship between postprandial lipid levels and CHD in type 2 diabetes is still unclear. One of the major reasons for this is despite the increasing interest in the area of postprandial lipid metabolism, there is no standardised methodology for measuring post challenge lipid handling (Karpe 1997) making comparisons among studies difficult to conduct.

3.2 Aims

In order to investigate the potential impact of different therapies on post challenge lipid handling it was necessary first to devise a practical and reproducible oral triglyceride challenge. The aims therefore of this study were to:

1. formulate a standardised oral fat and carbohydrate challenge to facilitate reliable assessment of oral triglyceride tolerance.
2. evaluate the acceptability and reproducibility of this Oral Triglyceride Tolerance Test (OTTT) in subjects with type 2 diabetes and in non-diabetic subjects
3. explore and define post challenge in metabolic responses (Chaper 4).

4. investigate the use of capillary fingerprick sampling as a possible simpler alternative to venepuncture for testing in the community and in large scale clinical trials. (Chapter 8)

3.3 Methods

3.3.1 Study design

Non diabetic and diabetic subjects underwent an oral fat and carbohydrate challenge on 2 separate occasions at least one week apart. Metabolic responses were assessed over an 8 hour test period.

3.3.2 Study subjects

A total of 23 non diabetic university employees, laboratory staff and members of the general public volunteered after seeing an advertisement for the study. The first 21 were recruited. A total of 180 diabetic identified through general practice and diabetes centre clinics were approached. Of these, only 87 met the inclusion criteria (outlined in Chapter 2) of which 30 were recruited. Subjects selected were, aged 35-65 years, did not have diabetes (n=21) or had been diagnosed with diabetes for at least one year. The diabetic subjects were selected according to treatment diet alone (n=10), sulphonylurea (n=10) or metformin (n=10), to assess whether therapy influenced post challenge responses (Chapter 4).

3.3.3 Study procedure

Subjects were asked to complete a three day food diary in the week prior to the first OTTT and then underwent the OTTT on two occasions, at least one week apart. All routine medication was continued as prescribed, except in the case of subjects taking oral hypoglycaemic agents three times a day, in whom the midday dose was omitted on test days. Samples were collected and analysed according to the OTTT protocol (Chapter 2).

3.3.4 OTTT test drink

Fat Content

The amount of fat is the foundation of the test meals used in postprandial lipid studies and must be balanced with palatability as well as provide a sufficient lipid challenge to the subject. Jeppesen et al (Jeppesen, Chen et al. 1995) showed that the ingestion of fat leads to an increase in the postprandial concentration of triacylglycerol-rich lipoproteins of intestinal origin and that these concentrations will rise in approximately a linear fashion as the oral fat load is increased. However, the degree of postprandial lipaemia is accentuated with increasing fat loads in those with high fasting triglyceride levels. Again Dubois et al (Dubois, Beaumier et al. 1998) demonstrated a stepwise increase in postprandial rise of chylomicron and serum triacylglycerols with progressive increases of fat. They suggest this is due to an elevation in gastric inhibitory polypeptide and post heparin LPL activity or insulin response. They conclude that 15g of fat per meal is a threshold dietary level that does not overpass the clearance capacity and thus does not alter postprandially the lipid composition of circulating lipoproteins such as LDL cholesterol and HDL cholesterol in healthy normolipidemic subjects. When examining

the fat intake of 106 healthy adult males observed over 7 days, Shishehbor et al (Shishehbor, Roche et al. 1999) found that 26% of occasions fat ingestion is below 5g, 41% between 5-20g, and 33% of occasions has fat intake above 20g. This is consistent with other studies of daily fat intake which report 12-30g of fat is involved in most eating situations (De Castro 1987). Shishehbor and colleagues showed that fat intakes of 14-15g significantly increased plasma triacylglycerol concentrations but were small and short lived with no significant differences in NEFA or insulin levels shown in high or low fat meals. On the basis of the above mentioned studies, 50g of triglyceride emulsion for the OTTT test drink was thought to be sufficient to elicit a triglyceride response. To standardise and simplify preparation, this was not adjusted for body weight.

Fatty Acid Composition

The fatty acid composition of the test meal influencing the postprandial response is supported by some studies and not others. Jackson et al (Jackson, Zampelas et al. 1999)stated that substitution of a meal high in saturated fatty acid (SFA) meal with monounsaturated fatty acid (MUFA) offers no advantage in terms of attenuating the total lipaemic response or reducing the circulating levels of intestinally derived lipoproteins. They showed that as MUFA content increases there is a tendancy for the monophasic response typically seen after a single meal following an overnight fast to be replaced by a biphasic response with both early and late peaks for triacylglycerol, apoB48 and retinyl ester palmitate. Mero et al (Mero, Syvänne et al. 1998) compared 3 fatty meals (mixed meal, liquid cream and soybean oil), and found that when the amount of fat is similar (63g), postprandial responses of triglyceride, apoE, apoB48, apoB100 and HDL

cholesterol were comparable. This was despite differences in fatty acid composition and energy content. A review by Williams (Williams 1998) explored how polyunsaturated fatty acids (PUFA) result in an attenuated postprandial triglyceride response compared with meals rich in saturated fatty acids. When examining the effect of fatty acid composition in type 2 diabetic subjects, differences in test meal fatty acid composition are less clear with some studies showing a linoleic acid diet to increase circulating lipid particles (Madigan, Ryan et al. 2000) whilst others showing oleic acid to increase the formation of chylomicron remnants (Higashi, Shige et al. 2001). The fatty acid composition of the OTTT test drink was predominately the monounsaturated fatty acid, oleic acid.

Amount of Carbohydrate

The addition of carbohydrate to a test meal can influence the post challenge lipaemic response. Jeppesen et al (Jeppesen, Chen et al. 1995) showed how the ingestion of carbohydrate in the form of fructose can accentuate the magnitude of lipaemia. Although a 5 gram fat load did not change post challenge triglyceride levels, the addition of 50 g of fructose led to a significant increase. Westphal et al (Westphal, Leodolter et al. 2002) added 75g of glucose to an oral fat load which resulted in a delay in the chylomicron response and a marked suppression of the postprandial increase in VLDL by 40%. Postprandial triglyceride levels could be increased after a meal containing 25% fat and 60% carbohydrate (Chen, Swami et al. 1993) studies in type 2 diabetic subjects on such low fat high carbohydrate diets also show increased levels of glucose, insulin and postprandial triglyceride levels (Chen, Coulston et al. 1995). The type and amount of

carbohydrate is particularly important when attempting to design a test meal for diabetic subjects in order to avoid hyperglycaemia and hypoglycaemia with the prolonged periods of fasting involved in postprandial studies. Unflavoured maltodextrin powder 50g was chosen for the OTTT test drink.

Vitamin A

Vitamin A has been added to most test meals in the past to investigate postprandial lipaemia. Retinyl palmitate is incorporated into the chylomicron core and can therefore be used as a marker of postprandial chylomicronaemia (Havel 1994). However, this may not be as accurate a measure as time progresses given that retinyl palmitate is transferred from chylomicrons to other lipoproteins in the circulation (Krasinski, Cohn et al. 1990) and larger chylomicrons carry more than smaller particles (Karpe 1999). For this reason, the OTTT test drink did not incorporate Vitamin A, although this could be added by other investigators if so desired. Novel methods for the assessment of apo B48 containing particles and chylomicron remnants are currently being developed to explore endogenous and exogenous lipid pathways separately in a new area of lipid research.

Solid or Liquid

Gastroparesis and delayed gastric emptying need to be considered when designing a test meal for diabetic individuals as hyperglycaemia may influence the absorption rate. Delayed gastric emptying may be present in up to 30 – 50% of people with type 1 or type 2 diabetes (Horowitz, Wishart et al. 1996). Kong et al (Kong, King et al. 1999) found that solid and liquid gastric emptying were similarly affected in type 2 diabetic

individuals with autonomic neuropathy (and had no association with increased risk of death). However in a review by Horowitz (Horowitz M, O'Donovan D et al. 2002) discussed that overall patterns of gastric emptying were critically dependent on the physical and chemical composition of the meal. Solids, nutrient liquids and non nutrient liquids all empty from the stomach at different rates. A study by Robertson et al (Robertson, Jackson et al. 2002) showed, in non diabetic subjects, that meals containing PUFAs increase gastric emptying compared with saturated and monosaturated fatty acids. A small volume liquid formulation was chosen to ensure similar gastric delivery and digestive handling in all groups.

Ingredients

The precise combination of ingredients to formulate a standardised oral fat challenge is a topic for debate and often requires specialist or dietician preparation. Karpe (Karpe 1997) designed a meal using normal food constituents (eg chicken and pasta) tolerated by most people, with 64g fat (long-chain triglyceride), 67g of carbohydrate and 29g of protein. The OTTT was formulated to contain ingredients suitable for those with dietary or religious restrictions such as vegetarian/vegans, lactose intolerant and coeliac disease. Commercially available products are shown in Table 3.

Table 3 Commercially available products in the UK (taken from the British National Formulary (British-Medical-Association. 1999)).

	Liquigen	Scandishake	Pro-Cal	Vita-Savoury	QuickCal	Thickshake
Volume	100ml	100g	100g	100ml	100g	444ml
Energy kJ/kcal	1850/450	2153/514	2788/667	870/210	780/321	560kcal
Fat (g)	50	24.7	56.2	18	77	16
Carbohydrate (g)	0	68.2	26.8	8	17	89
Protein (g)	0	4.7	13.5	4	4.6	14
Comments	coconut oil ketogenic	mix with milk	bland powder lactose	chicken/ tomato soup	bland powder lactose	palatable

We chose Calogen, as 100ml provided 50g of long chain triglyceride emulsion that was a palatable strawberry flavour. Other flavours are available, however butterscotch was felt to be too sweet. Unflavoured carbohydrate (50g) was then added to this preparation (Table 2, Chapter 2).

3.3.5 Statistical methodology

Statistical methodology is outlined in Chapter 2.

Sample size

Repeat testing in 30 type 2 diabetic subjects and 20 non-diabetic subjects was thought to be sufficient to characterise the post challenge lipid profiles obtained.

Reproducibility

Repeatability was determined by analysing the difference between paired data from the same individual studied more than once. The statistical methodology used in this chapter calculated the coefficient of variation (CV), ie the standard deviation of the differences between measurements divided by the mean and converted to a percentage. This method has been used in most studies investigating biological and analytical variation. One concern however is whether this statistical representation was accurate for comparing non normally distributed data sets. Although the standard deviation is a good measure of precision, it is influenced by the magnitude of the variable (ie units). However by "normalising" or "standardising" the standard deviation, by taking into account the mean and presenting the result as a percentage, accounts for this. Also, Levy et al (Levy,

Morris et al. 1999) suggested the coefficient of variation which relates imprecision to the midpoint of the range is often inappropriate as it does not take account of the dynamic range of a test and is not always comparable between different scales of measurement. Cooper et al (Cooper, Smith et al. 1994) have proposed a technique based on relative range to qualitatively estimate the biological variation for total cholesterol and improve determination of the mean value of the individual.

Mean difference plots were used to graphically depict systematic differences between the first and second OTTT (Bland and Altman 1986). To statistically show differences between the first and second test, paired T tests (for normally distributed data) and Wilcoxon matched pairs signed rank sum test (for non parametric analysis) were used to compare data within non diabetic and diabetic groups.

To determine whether CVs obtained from the diabetic group were similar to the non diabetic group, T tests (for normally distributed data) and Mann Whitney tests (for non parametric analysis) were used to highlight statistically significant differences.

3.4 Results

3.4.1 Subjects

A total of 50 subjects completed two tests, with one non diabetic subject, who withdrew, due to a migraine, being replaced. Diabetic subjects had significantly higher BMI and waist circumference and systolic blood pressure, and had moderately well controlled

glycaemia. Subject characteristics are tabulated in Table 4 and differences between non diabetic and diabetic subjects are reported in Chapter 4. The median (IQR) time between tests was 16.5 (14.0 – 27.6) days.

Table 4 Subject Characteristics of non diabetic and diabetic subjects

	No diabetes	Diabetes	*p*
n	20	30	
Sex (M/F) *	10/10	15/15	
Age (years)	52.1 (9.0)	55.5 (7.4)	*0.15*
Duration of diabetes (years) †	n/a	4.5 (2.0-6.0)	*n/a*
BMI (kg/m^2)	27.9 (5.6)	32.7 (6.2)	*0.0078*
Waist (cm)	92.9 (17.0)	103.1 (14.5)	*0.026*
Triceps skinfold thickness (mm)	15.9 (6.3)	18.8 (10.4)	*0.22*
BP (mm Hg)			
Systolic	129 (13.3)	143 (20.8)	*0.0049*
Diastolic	81 (8.9)	84 (8.4)	*0.23*
Smoking (%)			
Non smoker (n=25)	45	54	
Ex smoker (n=20)	50	33	
Smoker (n=5)	5	13	*0.77*
Hormone use (%) (n=6)	10	33	*0.42*
Post menopausal (%) (n=12)	40	53	*0.84*
Nutrient Intake (%)			
Fat	36	36	*0.64*
Carbohydrate	43	44	*0.56*
Alcohol	5.4	3.2	*0.21*
Apo E genotype *			*0.626*
3/3	6	22	
4/3	1	5	
2/3	2	2	
2/4	1	1	
HbA1c (%)	5.7 (0.4)	7.6 (1.2)	*n/a*
Fasting plasma glucose (mmol/l) **	5.4 (0.59)	9.0 (2.5)	*n/a*
Fasting total cholesterol (mmol/l) **	5.0 (1.00)	5.0 (0.98)	*0.8692*
Fasting LDL cholesterol (mmol/l) **	3.11 (0.880)	3.25 (0.8236)	*0.004*
Fasting HDL cholesterol (mmol/l) **	1.51 (0.399)	1.20 (0.309)	*0.5697*
Fasting VLDL cholesterol (mmol/l) **	0.38 (0.221)	0.50 (0.221)	*0.05*
Fasting triglyceride (mmol/l) ‡**	0.77 (0.46-1.29)	1.35 (0.86-2.14)	*0.0002*

Data are mean (SD) except * number of subjects, † median (IQR), and ‡ geometric mean (SD range), and **results from first OTTT.

3.4.2 Test Drink

The test drink was consumed in a median (IQR) of 2.0 (1.0 to 2.8) minutes. All subjects reported the test drink to be agreeable in flavour, of palatable texture and the amount not excessive. It was well tolerated with no nausea, vomiting or abdominal discomfort after consumption. A single non-diabetic subject suffered an episode of loose stools four hours after the end of the first test, and one diabetic subject on sulphonylurea therapy had mild hypoglycaemic symptoms at the 4 hour timepoint. The test was discontinued but the subject returned to complete two further tests.

Fasting lipid profile

The mean CV for fasting total cholesterol was 5.5 (4.3)% for the non-diabetic group and 4.1(3.9)% for the diabetic group. Fasting mean CVs for diabetic and non-diabetic groups respectively were 5.1 (3.7)% and 6.3(4.9)% for HDL cholesterol, 5.0 (3.9)% and 7.4(5.7)% for LDL cholesterol, and 21.3(14.1) and 17.5 (11.7)% for VLDL cholesterol levels. There were no significant differences in CVs for the diabetic and non-diabetic group.

3.4.3 Triglyceride and Glucose Response

The maximum triglyceride rise ranged from 0.27 to 0.97 mmol/l in the non diabetic group and 0.31 to 1.31 mmol/l in the diabetic group for the first test and 0.18 to 1.08 mmol/l and 0.44 to 1.45 mmol/l respectively for the second test. The maximum rise was seen at *circa* 5 hours in the non diabetic group (Tmax) and 6 hours in the diabetic group (Chapter

4, Table 7). The maximum glucose rise ranged from 0 to 1.4 mmol/l in the non diabetic group and 4.0 to 6.3 mmol/l in the diabetic group for the first test and 0 to 3.6 mmol/l and 1.1 to 6.8 mmol/l respectively for the second test.

3.4.4 Reproducibility of post challenge values

Triglyceride

The median (IQR) triglyceride CV at the 6 hour timepoint was 17.5 (7.0 to 29.0)% and 17.0 (10.0 to 28.0)% for the non diabetic and diabetic groups respectively (Table 5). This was similar to the triglyceride CV at baseline which was 14.5 (9.5 to 23.0)% and 12.0 (5.0 to 24.0)% for the non diabetic and diabetic groups respectively. The geometric mean fasting triglyceride value between the first and second tests was statistically different ($p=0.021$) with the diabetic group having a 0.2 mmol/l higher fasting triglyceride level in the second OTTT. Mean difference plots for all timepoints and IAUC (Figure 7) showed no systematic difference in triglyceride responses within individuals between the first and second test.

NEFA

The 2 hour post challenge CV for NEFA was similar to baseline CV, 22.4 (13.15 – 44.55)% and 19.5 (6.25 – 51.55)% for the non diabetic group respectively and 18.1 (11.5 – 29.53)% and 15.2 (9.5 – 34.0)% for the diabetic group respectively (Table 5). For both non diabetic and diabetic groups, fasting and 2 hour NEFA levels were significantly lower for the second OTTT ($p<0.0001$ for both groups at fasting and 2 hours). Mean

difference plots for all timepoints and IAUC for the first and second OTTT NEFA responses are shown in Figure 8.

Glycerol

The mean glycerol CV decreased 2 hours post challenge in the non diabetic group 30.7 (25.0)% to 18.1 (15.0)% whilst glycerol CVs increased from 16.6 (12.8)% at baseline to 26.2 (26.3)% 2 hours post challenge in the diabetic group (Table 5). Mean difference plots for glycerol at all timepoints and IAUC are highlighted in Figure 9 and showed no systematic differences.

Glucose

The mean (SD) CV for glucose increased 2 hours post challenge in the non diabetic group 3.3 (2.5)% to 15.3(12.7)% (Table 5). Fasting and post challenge glucose CVs were similar in the diabetic group, 6.6 (5.7)% and 8.3 (9.3)% respectively. There was no statistically significant difference between plasma glucose values at fasting or at 2 hours between the first and second test in non diabetic or diabetic groups. Mean difference plots for plasma glucose at all timepoints and IAUC are shown in Figure 10.

Insulin

The 2 hour post challenge CV for insulin increased in the non diabetic group, 19.1 (2.6 – 25.9)% to 33.2 (17.5 – 43.0)% (Table 5). The insulin CV was similar at fasting and 2 hours post challenge in the diabetic group, 17.9 (8.8 – 29.1)% and 16.7 (8.2 – 26.1)%. Although statistically significant differences were found in insulin response between first

and second tests in both groups, these differences were relatively small. For example, the 2 hour post challenge insulin level was *circa* 181 pmol/l for the non diabetic group in test 1 (Chapter 4, Table 7) and *circa* 190 pmol/l for test 2 (Appendix 1). Mean difference plots for all timepoints and IAUC are highlighted in Figure 11 and show no systematic differences in insulin responses within individuals between the first and second test.

C peptide

In concordance with insulin CVs the 2 hour post challenge median CVs for C peptide increased in the non diabetic group 13.0 (8.9 – 22.3)% to 20.6 (8 – 27.1)% whilst post challenge CVs were similar to baseline in the diabetic group, 11.1 (4.6 – 25.7)% to 9.5 (4.3 – 15.3)% at 2 hours. Subtle but statistically significant differences were also found between first and second tests for both groups. Mean difference plots for C peptide responses between the first and second test at all timepoints and IAUC are highlighted in Figure 12 and show no systematic differences within individuals.

Differences in variability between non diabetic and diabetic subjects

CVs for triglyceride and NEFA levels were similar for non diabetic and diabetic subjects. Fasting mean glycerol CV were significantly greater in the non-diabetic group compared to the diabetic group (p=0.0289) (Table 5).

The CV for fasting plasma glucose was significantly higher in the diabetic group compared to the non diabetic group (p = 0.0081), however by 2 hours post challenge, the glucose CV in the non diabetic group was significantly higher (p = 0.0307) (Table 5).

This increase in 2 hour post challenge CV in the non diabetic group was also seen with insulin levels.

Table 5 Coefficient of Variation (CV) for analytes for non diabetic and diabetic subjects

	No Diabetes			*p*	Diabetes			*p*	*p CV*
Triglyceride (mmol/l)									
0 hour	14.5	9.50	23.00	*ns*	12.0	5.00	24.00	*0.021*	*ns*
2 hours	14.6	6.00	21.50		11.9	8.00	27.00		
4 hours	15.6	8.50	25.50		15.5	8.00	31.00		
6 hours	17.5	7.00	29.00	*ns*	17.0	10.00	28.00	*ns*	*ns*
8 hours	15.0	10.00	26.00		10.8	6.00	20.00		
$IAUC_{0\text{-}8hours}$	32.5	10.76	70.01		30.0	16.70	75.35		
6-0 hours	30.8	-80.36	147.06	*ns*	58.7	21.22	173.64	*ns*	
NEFA (umol/l)									
0 hour	19.5	6.25	51.55	*<0.0001*	15.2	9.50	34.00	*<0.0001*	*ns*
2 hours	22.4	13.15	44.55	*<0.0001*	18.0	11.50	29.53	*<0.0001*	*ns*
4 hours	20.4	15.00	36.55		27.6	10.78	44.55		
6 hours	15.6	10.60	38.85		12.7	5.60	19.20		
8 hours	12.6	5.20	22.80		11.0	5.30	16.60		
$IAUC_{0\text{-}8hours}$	-18.4	-76.86	31.28		-36.1	76.71	-7.87		
2-0 hours	-32.1	-67.44	-17.76		-25.1	50.75	-14.61		
Glycerol (mmol/l)									
0 hour	30.7	7.80	52.20	*ns*	17.3	5.37	24.34	*ns*	*0.0289*
2 hours	18.1	7.46	22.61	*ns*	25.4	8.45	31.75	*ns*	*ns*
4 hours	22.1	8.46	36.82		26.9	11.34	29.57		
6 hours	21.2	9.58	31.19		25.0	8.51	37.03		
8 hours	23.7	9.97	30.44		18.5	8.98	25.70		
$IAUC_{0\text{-}8hours}$	17.6	142.36	94.19		4.7	93.71	97.17		
2-0 hours	-65.5	108.61	-25.37		-53.4	80.57	-26.92		

Data are median CV (IQR) %
p = paired T test from first and second OTTT
p CV = T test and Mann Whitney test comparison of CVs between non diabetic and diabetic groups

Continued Table 5 CV for analytes for non diabetic and diabetic subjects

	No Diabetes				*p*	Diabetes				*p*	*p CV*
Glucose (mmol/l)											
0 hour	3.3	1.33	-	5.69	*ns*	6.6	2.87	-	9.51	*ns*	*0.0081*
2 hours	15.3	6.73	-	22.47	*ns*	8.3	2.01	-	12.21	*ns*	*0.0307*
4 hours	7.4	1.70	-	11.59		8.4	2.73	-	11.27		
6 hours	2.2	0.97	-	3.09		8.8	2.43	-	12.31		
8 hours	2.7	1.48	-	3.35		8.5	3.93	-	12.85		
$IAUC_{0\text{-}8hours}$	-41.7	68.22	-	23.70		-19.8	54.79	-	11.91		
2-0 hours	-10.6	28.28	-	0.00	*ns*	-20.0	24.66	-	-8.20	*ns*	*ns*
Insulin (pmol/l)											
0 hour	19.1	2.60	-	25.90	*<0.0001*	17.9	8.80	-	29.10	*<0.0001*	*ns*
2 hours	33.2	17.50	-	43.00	*<0.0001*	16.7	8.20	-	26.10	*<0.0001*	*0.0109*
4 hours	12.6	3.40	-	27.90		20.2	10.90	-	30.30		
6 hours	13.5	10.00	-	22.70		12.3	10.40	-	22.80		
8 hours	21.9	10.10	-	35.00		17.7	9.40	-	33.60		
$IAUC_{0\text{-}8hours}$	40.1	8.01	-	66.55		20.3	6.96	-	42.65		
2-0 hours	58.1	27.68	-	93.20	*ns*	25.1	8.09	-	41.55	*0.046*	*ns*
C peptide (nmol/l)											
0 hour	13.0	8.90	-	22.30	*0.0013*	11.1	4.60	-	25.70	*<0.0001*	*ns*
2 hours	20.6	8.00	-	27.10	*<0.0001*	9.5	4.30	-	15.30	*<0.0001*	*ns*
4 hours	12.9	6.30	-	20.20		15.2	6.50	-	27.00		
6 hours	10.8	6.10	-	16.00		12.4	6.70	-	21.80		
8 hours	14.1	7.70	-	21.50		10.9	7.30	-	16.60		
$IAUC_{0\text{-}8hours}$	18.5	1.63	-	89.08		20.1	6.86	-	45.10		
2-0 hours	29.4	12.34	-	44.80	*ns*	21.4	7.09	-	32.46	*ns*	

Data are median CV (IQR) %
p = paired T test from first and second OTTT
p CV = T test and Mann Whitney test comparison of CVs between non diabetic and diabetic groups

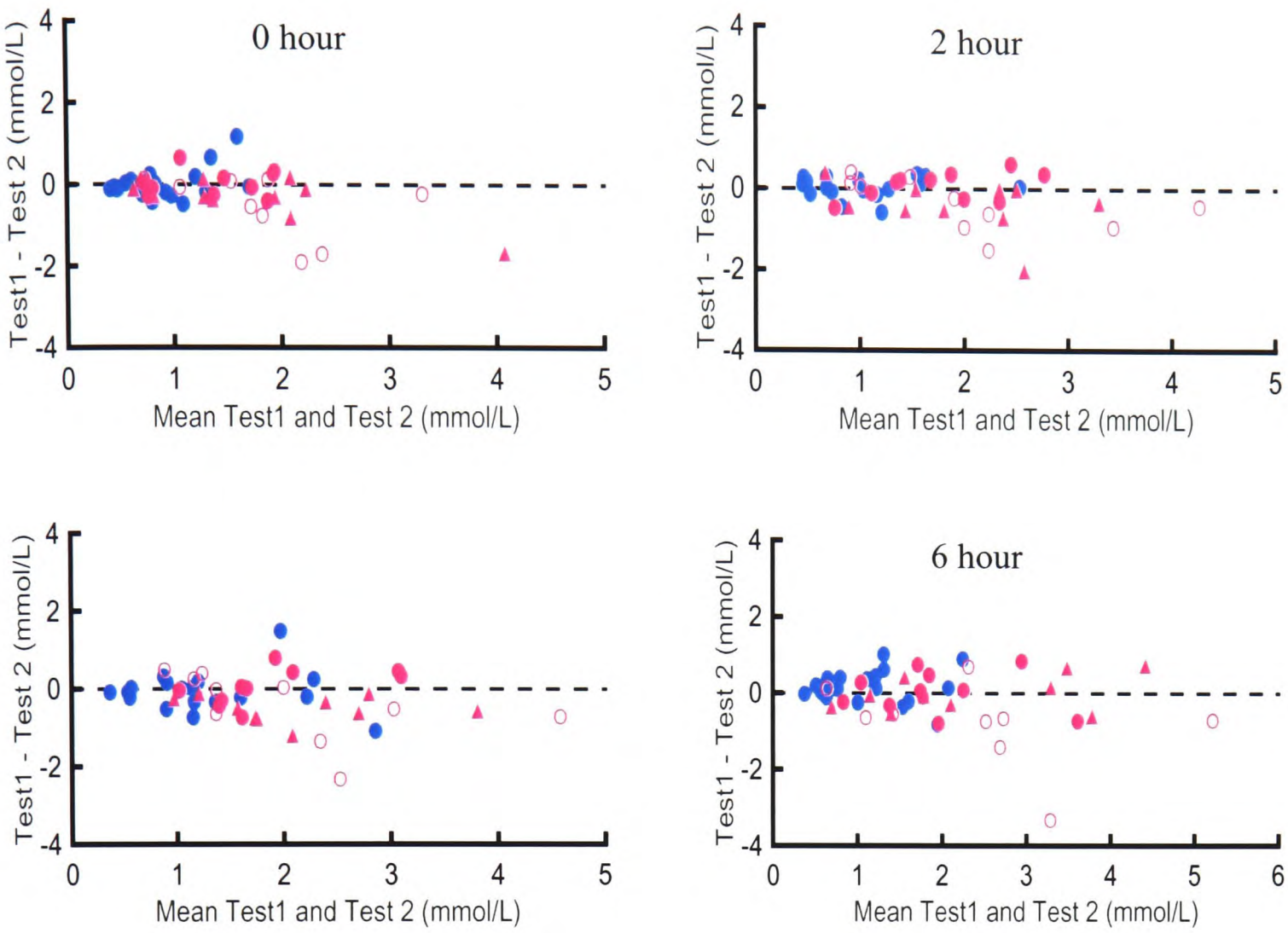
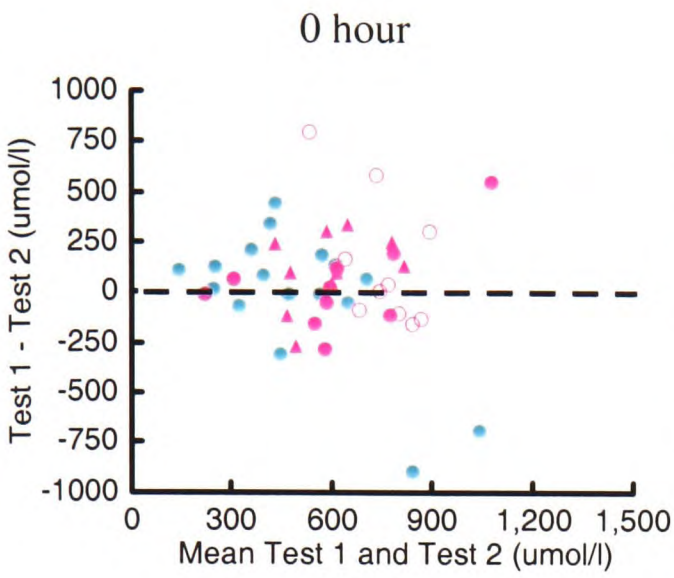
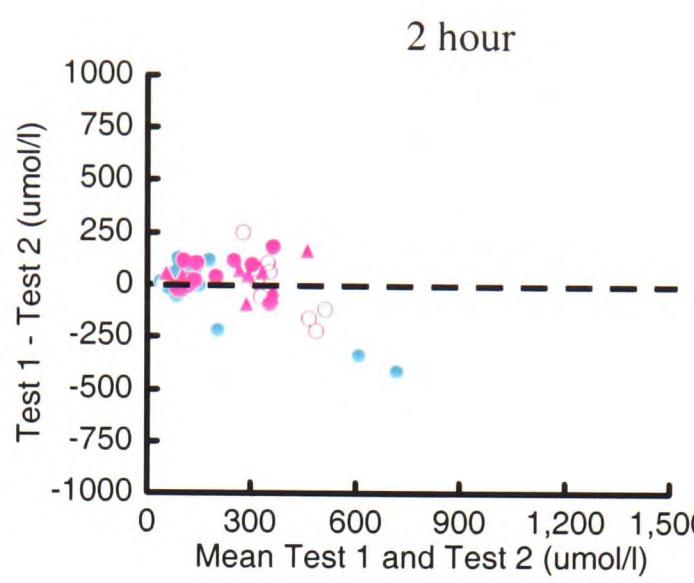
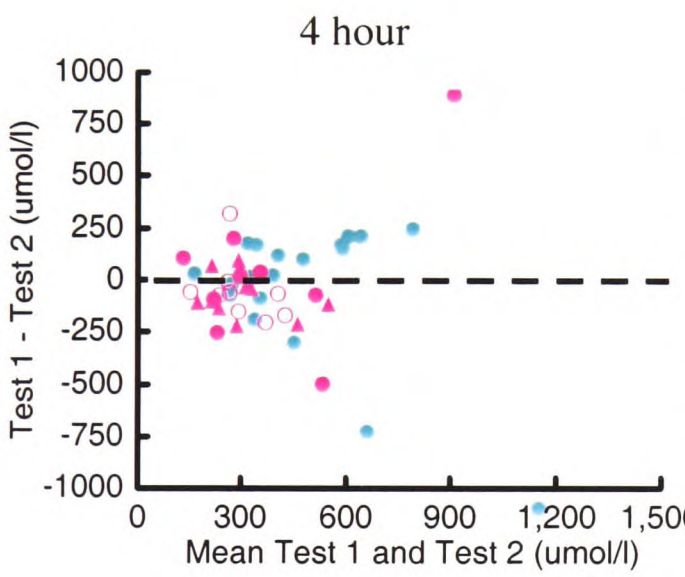
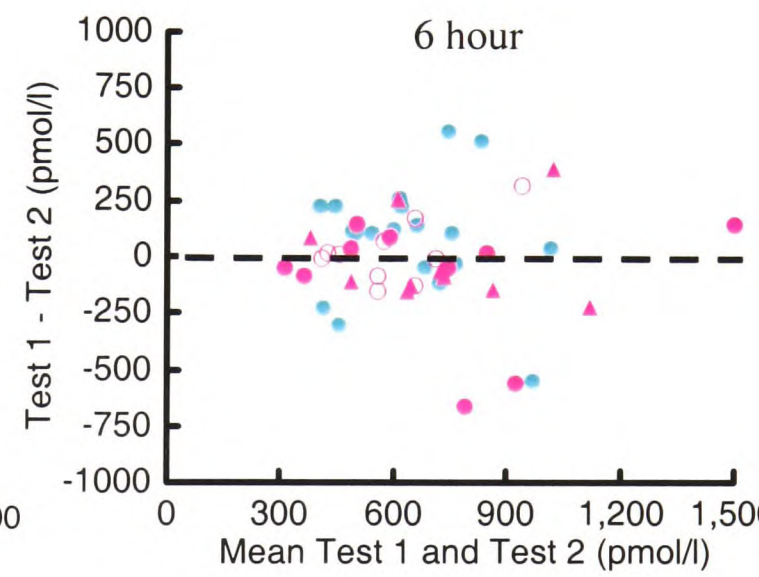


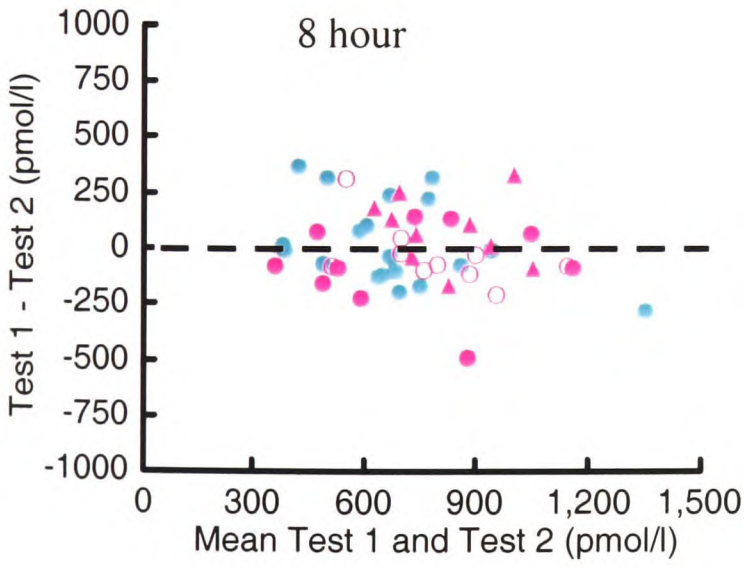
Figure 7 Mean difference plots for triglyceride for test 1 and test 2. Non diabetic subjects are shown as ● , and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.











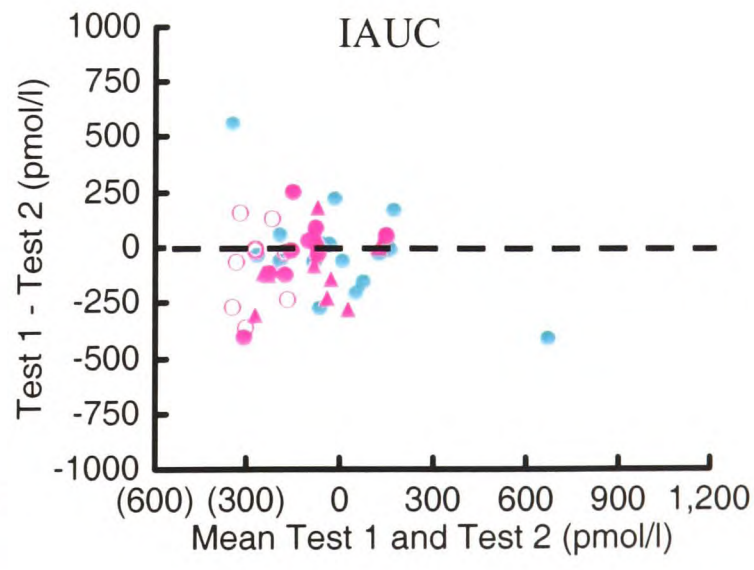


Figure 8 Mean difference plots for NEFA for test 1 and test 2. Non diabetic subjects are shown as ● , and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.

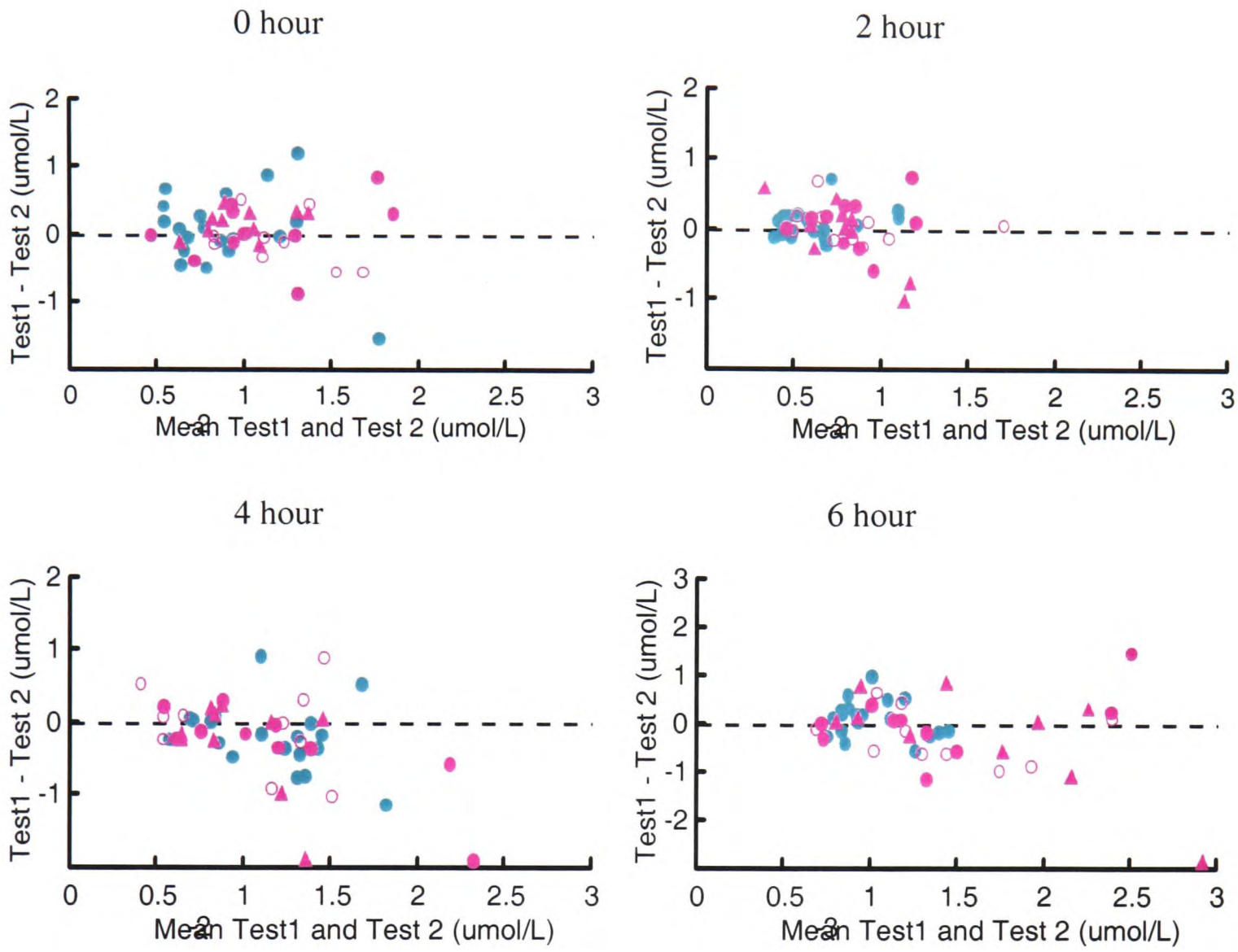


Figure 9 Mean difference plots for glycerol for test 1 and test 2. Non diabetic subjects are shown as ● , and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.

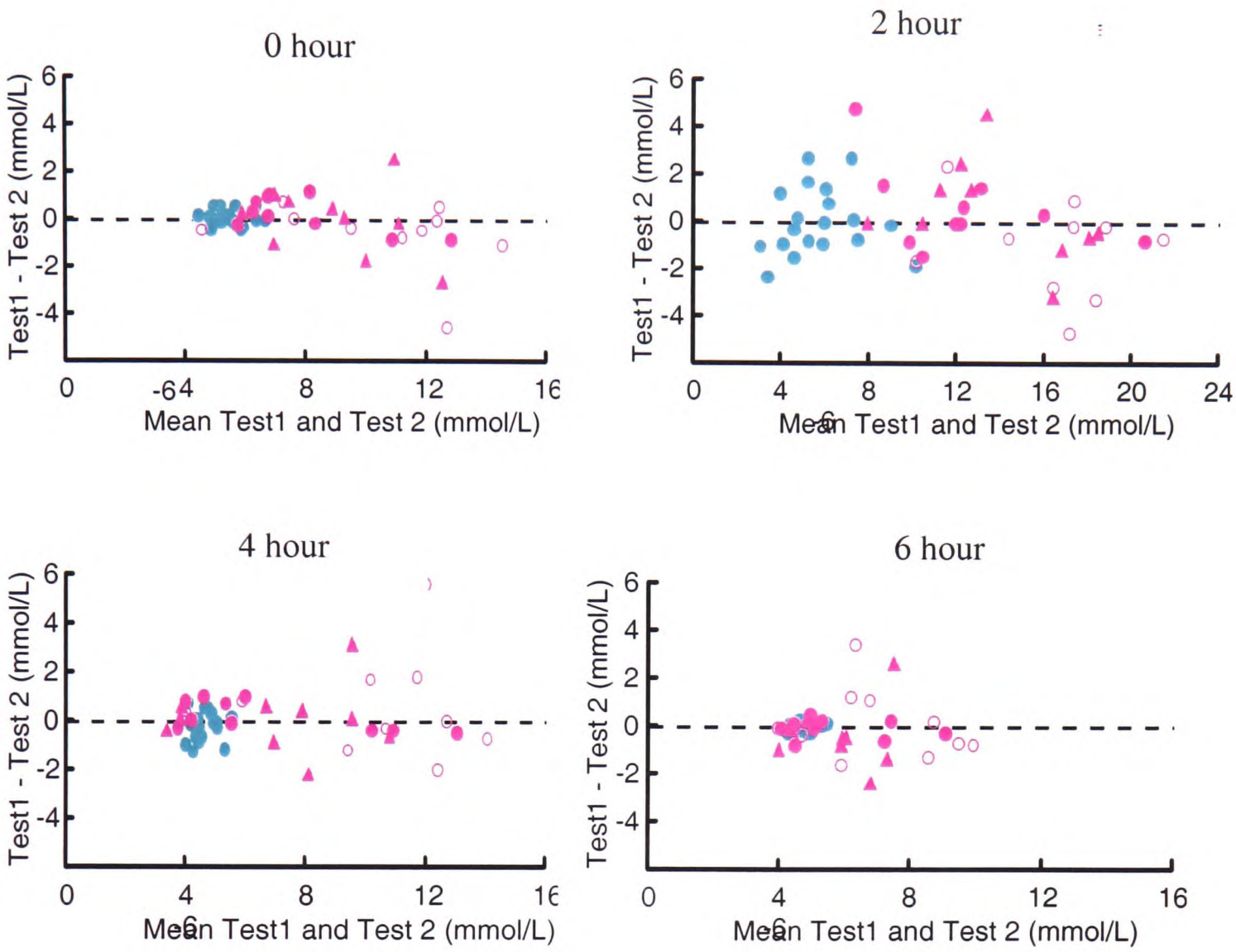


Figure10 Mean difference plots for glucose for test 1 and test 2. Non diabetic subjects are shown as ●, and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.

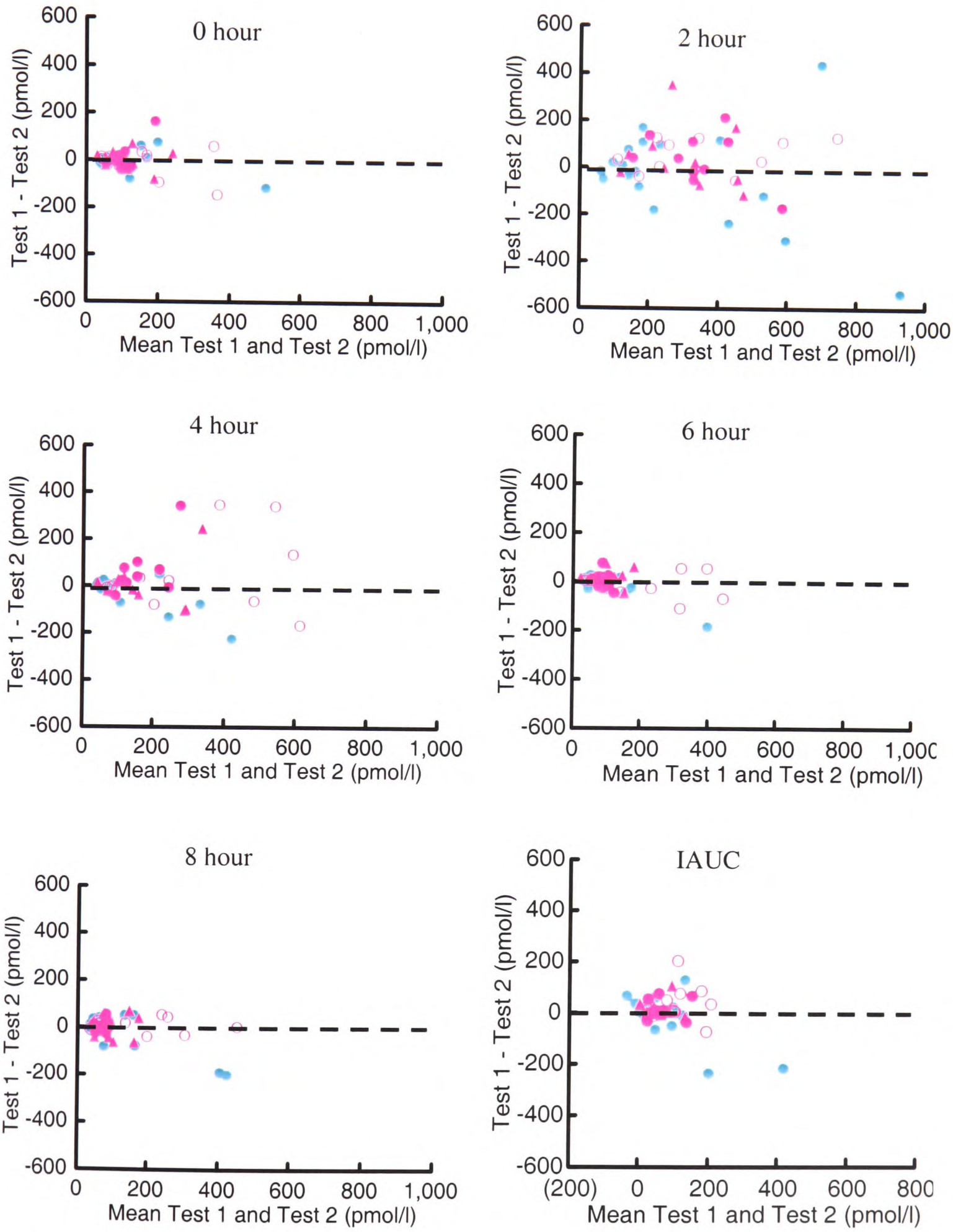


Figure 11 Mean difference plots for insulin for test 1 and test 2. Non diabetic subjects are shown as ● , and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.

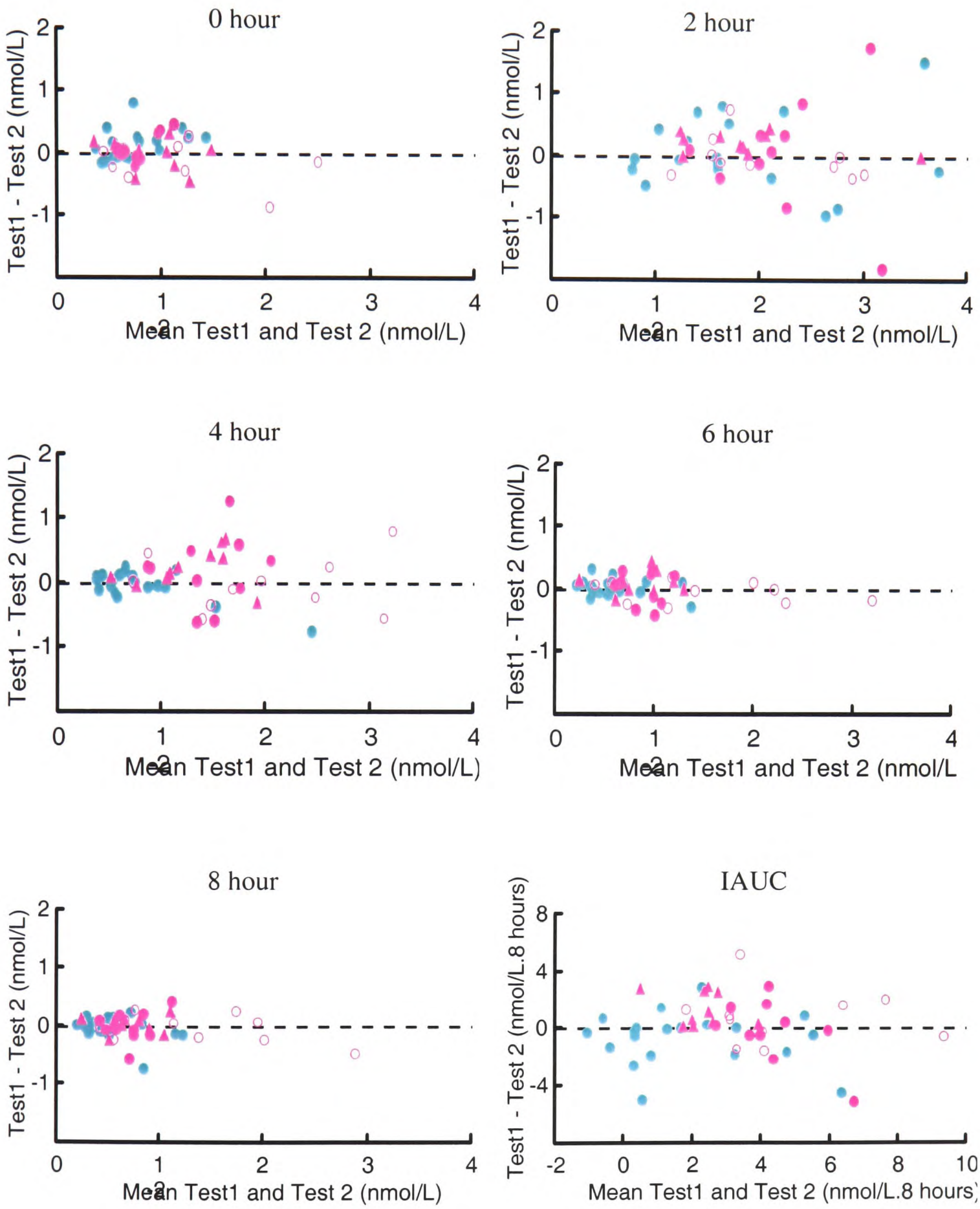


Figure 12 Mean difference plots for C peptide for test 1 and test 2. Non diabetic subjects are shown as ●, and diabetic subjects as ● on diet alone, ▲ metformin, and ○ sulphonylurea.

3.5 Discussion

The OTTT evaluated in this study utilizes a standardised fat and carbohydrate test drink designed to clinically produce meaningful post challenge triglyceride and glucose responses under acceptable test conditions. The results showed that the OTTT achieved reproducible post challenge triglyceride and glucose profiles. The median (IQR) triglyceride CV at the 6 hour timepoint was 17.5 (7.0 to 29.0)% and 17.0 (10.0 to 28.0)% for the non diabetic and diabetic groups respectively. Overall, the variability of post challenge lipid measurements was not different between diabetic and non diabetic subjects. The mean (SD) CV for glucose 2 hours post challenge in the non diabetic and diabetic group was 15.3(12.7)% and 8.3 (9.3)% respectively.

3.5.1 OTTT acceptability

The OTTT test drink was simple to prepare, within 5 minutes on the day of the scheduled test, within the CRU. Previous studies using everyday ingredients such as double cream, eggs, cheese and bacon, not only exclude subjects with various dietary constraints, but require a longer preparation time than the OTTT test drink. The OTTT was found to be tolerable with 50 subjects agreeing to return for further testing. Migraine causing one non diabetic subject to withdraw was most likely unrelated to the OTTT as this subject had a positive history for recurrent migraines (no trigger elucidated) and no other subjects participating in the study suffered from migraine or headache. Glycaemic control in individuals with type 2 diabetes was not detrimentally affected with maximum and minimum glucose changes by diabetic subjects during the 8 hour study period of

approximately *circa* 4.8 mmol/l and – 3.4 mmol/l respectively (Chapter 4, Table 7). Only one diabetic subject, who was taking sulphonylurea therapy, had any hypoglycaemic symptoms that occurred at 4 hours post challenge, and returned to complete two further tests without problems. In terms of safety, this confirmed that the OTTT could be used for future studies in diabetic subjects.

3.5.2 Triglyceride response

Our results show that in general, type 2 diabetic subjects tend to have a higher maximum triglyceride response than non diabetic subjects utilizing the OTTT. In diabetic subjects, the increase of 0.6 - 0.8 mmol/l occurred later at 6 hours post challenge than in non diabetic subjects. The increase in post challenge triglyceride levels observed from the OTTT was found to be similar to studies of postprandial lipaemia, but lower than seen in some other studies. Cavellero et al (Cavallero, Dachet et al. 1994) produced a similar triglyceride response of *circa* 0.5 mmol/l in non diabetic subjects and 0.8 mmol/l in normotriglyceridaemic type 2 diabetic subjects using a test meal (900-1200kcal) containing 45-55% lipids (32g lipids/m^2 body surface area) and 30-40% carbohydrate. Greater triglyceride responses up to *circa* 1.5 – 2.0 mmol/l have been produced in other studies (Lewis, O'Meara et al. 1990; Cooper, Tan et al. 1996) using greater amounts of fat (60-70g) and adjusting for body surface area. In agreement with our findings, previous studies also showed a delay in peak triglyceride response. The post challenge triglyceride increment and time to peak was greater and further delayed respectively in studies of hypertriglyceridaemic type 2 diabetic subjects (Lewis, O'Meara et al. 1990; Cooper, Tan et al. 1996, Cavallero, 1994 #2803). The balance of producing a triglyceride response

that can be interpreted without causing the subject undue harm by potential risk of pancreatitis and/or gastrointestinal discomfort must be considered when formulating a test meal.

3.5.3 Reproducibility

Variation of lipid parameters

This study showed for the first time that post challenge triglyceride responses using the OTTT were more reproducible than those seen for glucose after an OGTT. As there is a paucity of studies examining the repeatability of post challenge measurements, the duplicate OTTTs provide new insight into post challenge variability. If post challenge triglyceride responses are to be proposed as a potentially modifiable risk factor for CHD, reproducibility is important. The degree of association of a biochemical measurement as a risk factor for CHD may be influenced by the variability of the measurement, where high variability could lead to uncertain estimates (Marcovina, Gaur et al. 1994). The results show that post challenge triglyceride CVs were similar to fasting CVs and that the variability of postprandial responses was not greater in the diabetic population.

Previous studies have predominately been conducted in non-diabetic individuals to examine the variability of *fasting* triglyceride measurements. The OTTT study triglyceride CVs of *circa* 12-14.5% at fasting are comparable, if not lower, to previous studies. Marcovina et al (Marcovina, Gaur et al. 1994) showed in 20 healthy adults having blood collected 4 times over 2 weeks the CV for triglyceride was 28.2%. In a review of 30 studies from 1970 to 1992 Smith et al (Smith, Cooper et al. 1993) found the

best composite estimates CVs of the average intraindividual biological variability in triglyceride to be 22.4-22.9%. This included a study by Jacobs et al (Jacobs and Barrett-Connor 1983) who found CVs of about 25% for triglyceride and showed that two thirds of individuals in the upper quintile of cholesterol and/ or triglyceride at first measurement remain in this rank and one third lower on retest. Another study of 35 subjects having 4 samples taken weekly showed the total test variability (ie biological and analytical) for triglyceride to be 20.1% (Schectman, Patsches et al. 1996). A more recent larger study examining the reproducibility of duplicate samples from 379 subjects found plasma CVs for triglyceride of 23% (Despres, 1999).

The repeatability of a test is important when interpreting the results of postprandial studies. The CV of postprandial triglyceride measurements using the OTTT was *circa* 17% at 6 hours. Castro et al (Castro Cabezas, Halkes et al. 2001) found the variability of diurnal triglyceride sampling to be better than fasting. However, both fasting and postprandial CVs from the OTTT were lower than shown in this study. One possible reason for this is that subjects in study by Castro et al conducted their own capillary triglyceride sampling (Chapter 9) at various times during their normal day, whilst the OTTT had preset and defined test conditions and sampling timepoints. A small study of 10 non diabetic subjects showed that 9 hour post lipid challenge triglyceride measurements from 2 visits 10 days apart correlated stronger than fasting levels (Brown, E et al. 1992). Subjects participating in our study returned within a short time for repeat testing, minimising dietary and lifestyle changes which could influence the reproducibility of triglyceride measurements (see latter discussion). A higher triglyceride

CV of 29% was demonstrated by Rössner et al (Rossner 1982) who administered the intravenous fat tolerance test in a group of healthy volunteers 5 years after the first test.

Repeat testing undertaken in the OTTT study also provided the opportunity to assess the variation of more routine clinical biochemical measurements. The fasting lipid profile is an important blood test used not only in research but in clinical practice and determines the prescribing of a lipid lowering agent for non-diabetic and diabetic individuals. The OTTT study results of fasting total cholesterol CVs for the diabetic group 4.1(3.9)% and the non-diabetic group 5.5 (4.3)% are similar to previous studies in non-diabetic individuals (CVs 5.4-7.8%) (Smith, Cooper et al. 1993; Choudhury, Lyons-Wall et al. 1994; Marcovina, Gaur et al. 1994; Schectman, Patsches et al. 1996). Also, the fasting mean CVs for all lipid subfractions in the OTTT study where consistent with other studies where CVs ranged from 7.0-9.6% for LDL cholesterol, 6.2-7.5% for HDL cholesterol, and up to 31% for VLDL cholesterol (Choudhury, 1994; Marcovina, 1994; Schectman, 1996; Smith, 1993; Despres, 1999). These results show that diabetes itself does not affect the biological variability of lipid measurements.

Variation of glycaemic parameters

Glucose results from the OTTT study were reproducible with CVs *circa* 3-6% fasting and 8-15% at 2 hours. The reproducibility of post challenge glycaemic parameters has been investigated in studies using the OGTT. Sievenpiper et al (Sievenpiper, Jenkins et al. 2001) showed in 35 normoglycaemic individuals that dilution of the 75g OGTT (300ml, 600ml, 900ml) affected the reproducibility of post challenge results (CVs at 45 minutes

approximately 10%, 18% and 20% respectively) and confirmed the high intra subject variability. Previous studies had suggested that gastric emptying resulting from the OGTT's high osmolality may increase variability, but this study does not support this. Ko et al (Ko, Chan et al. 1998) studied the reproducibility and usefulness of the OGTT in the Chinese population. Only 65.6% of OGTTs were reproducible, and fasting and 2 hour concentrations of the first OGTT were generally higher than those measured 6 weeks later. A recent study by Wolever et al (Wolever, Chiasson et al. 1998) showed that 50g of carbohydrate load administered as a standardised test meal (including 10g fat and 12g protein) has a 47% lower CV than the 75g OGTT (9% vs 16% respectively). The authors explained the difference to be due to gastric emptying, with the standardised meal due the physiological mix of nutrients stimulating more consistent gastric emptying. There is some evidence showing that an intragastric infusion of fat stimulates gastrduodenal motor activity more so than glucose (White, 1983).

A higher 2 hour post challenge glucose CV was particularly evident in the non diabetic group who underwent the OTTT. Consistent with these results, the 2 hour insulin CV in the non diabetic group was higher than the diabetic group. This may be explained by non diabetic subjects producing variable insulin responses to an oral lipid and glucose challenge, whereas individuals with diabetes are unable to do so. This variation in 2 hour insulin levels seen with repeat testing, may influence NEFA suppression, as higher insulin levels with enhance 2 hour NEFA suppression. Overall the biological variation of insulin was higher in fasting and post challenge levels. This may partly be due to the

analytical variation of the insulin radioimmunoassay used being higher than other laboratory assays (up to 6% for the high control).

3.5.4 Sources of biochemical variation

Sources influencing biological variability have previously been discussed in the literature, and must be adhered to when conducting clinical trials of lipid metabolism. The analytical variability of a measurement should be considered in the interpretation of results. Smith et al review of 30 studies found the analytical method used was an important factor contributing to the biological variation of lipid levels. The analytical variation for assays used in the OTTT study is unlikely to be the main factor contributing to the overall variation. The LDL cholesterol results are supported by other studies which demonstrate that the direct LDL assay does not reduce the variability compared with conventional LDL calculation (Friedewald equation) (Schectman, Patsches et al. 1996). Also, the improvements in methods, instruments and calibration materials have appreciably decreased the analytical variation of measurements (Cooper, Smith et al. 1994).

Study design

The design of the study may also influence the variability of lipid measurements. In the OTTT study, subjects were asked to repeat the second test a minimum of 1 week later with most subjects returning between 14 to 26 days. Choudhury et al (Choudhury, Lyons-Wall et al. 1994) showed that the variability in total cholesterol increased as the interval between sampling increased from 1 to 12 days and HDL 1 to 7 days, and

proposed that the minimal interval for collecting a second blood sample for analysis to be approximately 2 weeks. Smith et al (Smith, Cooper et al. 1993) found important factors influencing biological variation were the number of subjects, the sampling interval, and the number of measurements per subject. Other investigators have suggested that the effect of biological variation can be minimized by increasing the number of collected specimens (Cooper, Smith et al. 1994) and that multiple measurements should be within 2 months, at least 1 week apart before making a medical decision about further action (Rifai 1997).

Sample collection

Common principles can be applied to minimize the variability of lipid measurements in research and clinical practice. From the outset, the lipid profile should be measured in an individual in a steady metabolic state. It is well established that lipid derangements are present after acute myocardial infarction and in diabetic patients with poor glycaemic control (Simpson, Mann et al. 1979). The OTTT study recruited subjects who had no history of established cardiovascular disease and excluded those with poorly controlled diabetes defined as $HbA_{1c} > 10\%$. For lipid profiles, fasting specimens should be collected and individuals should maintain their usual diet and weight for at least 2 weeks prior. Subjects participating in the OTTT study fasted for 10 hours the night prior to the test and were not given any dietary information or blood test results (such as cholesterol) until completion of the study to avoid potential confounding dietary and lifestyle changes.

Blood collection methods, sample conditions and processing, as well as specimen storage are important elements to consider when conducting biochemical measurements (Rifai 1997) (Cooper, Smith et al. 1994). The individual should be seated for at least 5 minutes before specimen collection and the tourniquet should not be kept on more than 1 minute during venipuncture. During the 8 hour OTTT study, subjects were not confined to bed and were free to move around the CRU. However, 15 minutes prior to each 2 hourly specimen collection, subjects were asked to return to their allocated bed (if not already) and lie at 45° supine for drawing of the blood from a cannula sited in the antecubital region. It is also stipulated that if EDTA is used as the anticoagulant, plasma should be immediately cooled to 2-4°C to prevent changes in composition and specimens stored frozen at –70 °C or lower for triglyceride and lipoprotein testing. All samples in the OTTT study were immediately centrifuged at 3000rpm for 10 minutes at 4°C, plasma pipetted into a separate sarstedt tubes for analysis within 24 hours and storage at –70°C.

Gastrointestinal transit

Other factors influencing post challenge lipid levels are more difficult to elucidate. Physiological interference with exogenous fat absorption in the intestine due to increased velocity of intestinal transit causing decreased exposure time to digestion and absorption may contribute to differences. This may occur in some chronic disease states such as pancreatic enzyme deficiency and inflammatory bowel disease, however such individuals were excluded from participation in the OTTT study. There were no acute gastrointestinal disturbances causing symptoms of diarrhoea, nausea and/or vomiting documented during the OTTT. Those individuals taking drugs that interfere with fat

absorption, such as Orlistat, and/or drugs affecting gastric motility such as metoclopramide, were also excluded from the study. Other physiological states that have been suggested to interfere lipid absorption are increased bile acid production which act as weak detergents thereby decrease micellar solubilization, and obesity where increased cholesterol competes with dietary cholesterol for micellar solubilization (Ros 2000).

Diet

Postprandial studies have specifically investigated the effect of factors such as diet and lifestyle of post challenge triglyceride variability. The demonstration of chylomicrons appearing in the bloodstream after a meal resembling those produced by the previous meal (Fielding, Callow et al. 1996) has led to some investigators giving a standard meal prior to the actual test challenge. Other dietary elements such as increased plant and marine sterols and soluble fibre may also influence postprandial triglyceride levels (Ros 2000). A standard meal prior to the OTTT was not incorporated as part of the pre test instructions, for reasons of simplicity and feasibility if using the test in the future for large scale studies. This may therefore have influenced the reproducibility of the OTTT.

Alcohol

Van Tol et al (van Tol A, van der Gaag M S et al. 1998) showed daily consumption of 40g of alcohol with dinner resulted in increased postprandial plasma triglyceride levels (5 hours post meal) and decreased LDL cholesterol concentrations. In contrast, HDL was raised at all timepoints. Subjects recruited to OTTT studies were asked not to consume alcohol the day prior to the test, in order to minimise this effect. People with increased

alcohol intake or a past history of alcohol abuse were excluded from the study. The increase in postprandial lipaemia seen with alcohol intake is due to delay in the removal of postprandial triglycerides due to competition between chylomicron and VLDL particle removal, which is worse after a meal containing saturated fatty acids or in subjects with fasting hypertriglyceridaemia (Pownall 1994).

Smoking

Cigarette smoking can acutely impair insulin sensitivity and smokers are insulin resistant and lipid intolerant compared to non smokers (Axelsen, Eliasson et al. 1995). Impaired insulin sensitivity may be caused by increased levels of counter regulatory hormones and increased sympathetic nervous system activity. Cessation of smoking can improve insulin sensitivity determined by euglycaemic hyperinsulinaemic clamp and improve the lipoprotein profile (Idzior-Walus, Mattock et al. 2001). Studies of postprandial lipaemia show that as well as low HDL levels, smokers have elevated post challenge triglyceride profiles due to defective clearance of chylomicrons and their remnants (Mero N, Syvänne M et al. 1997) The OTTT study did not exclude subjects who smoke from participating, but no smoking was permitted during the OTTT. Sharrett et al (Sharrett A R, Heiss G et al. 2001) investigated the determinants of postprandial lipaemia independent of fasting triglyceride values in subjects without CHD and found that smoking was a major influence.

Exercise

Numerous studies have demonstrated the effect of physical exercise on reducing fasting and postprandial triglyceride levels, which is why subjects were asked to avoid exercise prior to (and during) the OTTT (Weintraub, Rosen et al. 1989). Koutsari et al (Koutsari, Karpe et al. 2001) has demonstrated that just 60 minutes of brisk walking daily can abolish carbohydrate induced increases in circulating triglyceride rich particles. It is thought that mechanisms other than increased triglyceride clearance (Gill J M R, Mees G P et al. 2001) or increased insulin sensitivity (Gill J M R, Herd S L et al. 2002) are responsible for this effect. It has been proposed that increased lipid deposition to muscle thereby promoting lipid oxidation, as well as higher LPL activity in exercised skeletal muscle (Hardman 1998) may be important.

4 Chapter 4 Post triglyceride and glucose challenge metabolic assessment in diabetic and non diabetic individuals

4.1 Introduction

Formal investigation of postprandial lipaemia in diabetic and non diabetic subjects has been undertaken in relatively few clinical studies, with variable results. Elevated and prolonged postprandial lipid levels in hypertriglyceridaemic diabetic subjects were found in previous studies (Lewis, O'Meara et al. 1991; Cavallero, Dachet et al. 1994; Curtin, Deegan et al. 1994; Tan, Cooper et al. 1995; Cooper, Tan et al. 1996). A study by Syvänne et al (Syvänne, Hilden et al. 1994) compared post challenge responses in 4 groups: type 2 diabetes with CHD, type 2 diabetes without CHD, no diabetes with CHD, and no diabetes, no CHD. No difference was found in postprandial responses in the diabetes subjects with or without CHD compared with those without diabetes. Reznik (Reznik, Pousse et al. 1996) found higher postprandial triglyceride peaks in obese normotriglyceridaemic type 2 diabetic subjects compared with obese non diabetic individuals. De Man et al (de Man, Castro Cabezas et al. 1996) concluded that normotriglyceridaemic type 2 diabetic individuals may also show disturbances in postprandial lipid metabolism, as well as those individuals who have hypertriglyceridaemia at baseline.

4.2 Aims

1. To explore and define post triglyceride and carbohydrate challenge alterations in lipid metabolism in diabetic and non diabetic subjects.

2. To assess whether there are systematic differences between diabetic patients treated with diet alone, sulphonylurea and metformin therapies.

4.3 Methods

Methodology is described in Chapter 2 and 3. This chapter further analyses the data from Chapter 3 with specific reference to differences in non diabetic and diabetic subjects, and differences with diabetic therapies.

4.3.1 Study design

Non diabetic and diabetic subjects underwent an oral fat and carbohydrate challenge on 2 separate occasions at least one week apart. Data from the first test is presented in this Chapter. Data from the second test is in Appendix 1. Metabolic responses were assessed over an 8 hour test period.

4.3.2 Study subjects

Subject selection is outlined in Chapter 3 (Methodology, Study Subjects). Subjects selected were, aged 35-65 years, did not have diabetes (n=21) or had been diagnosed with

diabetes for at least one year. The diabetic subjects were selected according to treatment diet alone (n=10), sulphonylurea (n=10) or metformin (n=10), to assess whether therapy influenced post challenge responses.

4.3.3 Study procedure

Subjects were asked to complete a three day food diary in the week prior to the first OTTT and then underwent the OTTT on two occasions, at least one week apart. All routine medication was continued as prescribed, except in the case of subjects taking oral hypoglycaemic agents three times a day, in whom the midday dose was omitted on test days. Samples were collected and analysed according to the OTTT protocol (Chapter 2).

Between subject CVs for the non diabetic and diabetic group, were calculated by dividing the SD by the mean for each group. For data from a single OTTT, the results from the first OTTT are highlighted in tables and graphs in the Chapters 3 and 4, and results of the second OTTT in the Appendix.

4.4 Results

4.4.1 Subjects

Diabetic and non-diabetic groups did not differ significantly with respect to baseline characteristics except that the diabetic group had a higher mean BMI, waist circumference and systolic blood pressure (Chapter 3, Table 4). There were no significant differences in the number of smokers, hormone users or menopausal women between diabetic and non-diabetic groups. Analysis of food diary results showed no differences in fat, carbohydrate or alcohol intakes between groups. The apo E 3/3 genotype was predominant with no subjects having the 2/2 genotype.

Both diabetic and non-diabetic groups had similar fasting mean (SD) total cholesterol levels, 5.0 (1.00) mmol/l and 5.0 (0.98) mmol/l respectively (p= 0.8692), and LDL cholesterol levels. However, although within the normal range for fasting values, the diabetic group had lower HDL cholesterol levels compared with the non-diabetic group 1.21 (0.31) mmol/l vs 1.45 (0.31) respectively (p = 0.004), and higher VLDL cholesterol levels (p = 0.05). Fasting plasma triglyceride levels were higher in diabetic subjects 1.35 (0.86 – 2.14) mmol/l compared to non diabetic subjects 0.77 (0.46 – 1.29) mmol/l, although within the normal range.

The mean (SD) HbA1c within the diabetic subgroups was 6.6 (0.73)% for diet treated subjects, 7.9(1.03)% for metformin treated subjects, and 8.2 (1.21)% for sulphonylurea treated subjects, with the diet treated group having significantly better glycaemic control (p=0.0046) (Table 6).

Table 6 Subject characteristics of diabetic subjects treated by diet, metformin and sulphonylurea therapy

	Diet	Metformin	Sulphonylurea	*p*
n	10	10	10	
Sex (M/F) *	5M/5F	5M/5F	5M/5F	
Age (years)	54.5 (8.3)	57.0 (8.2)	55.1 (6.1)	*0.7571*
Duration of diabetes (years) †	3.5 (2.25 - 5.75)	4.5 (3.25 - 8.0)	5.0 (2.5 - 5.0)	*0.5249*
BMI (kg/m^2)	33.5 (4.6)	34.1 (8.5)	30.5 (4.9)	*0.408*
Waist (cm)	106.9 (8.46)	105.6 (19.20)	96.9 (13.19)	*0.2526*
Triceps skinfold thickness (mm)	19.6 (6.30)	18.1 (12.70)	18.9 (9.76)	*0.9534*
BP (mm Hg)				
Systolic	136.9 (9.79)	153.1 (30.25)	139.1 (14.42)	*0.1676*
Diastolic	80.9 (5.38)	87.4 (10.52)	84.7 (8.14)	*0.2286*
HbA1c (%)	6.6 (0.73)	7.9 (1.03)	8.2 (1.21)	*0.0046*
Fasting plasma glucose (mmol/l) **	8.0 (2.06)	9.0 (2.07)	10.1 (2.91)	*0.164*
Fasting total cholesterol (mmol/l) **	5.0(1.19)	5.0 (0.76)	4.8 (1.03)	*0.8754*
Fasting LDL cholesterol (mmol/l) **	3.34 (0.95)	3.22 (0.66)	3.19 (0.92)	*0.9157*
Fasting HDL cholesterol (mmol/l) **	1.24 (0.35)	1.24 (0.34)	1.15 (0.25)	*0.7937*
Fasting VLDL cholesterol (mmol/l) **	0.48 (0.21)	0.52 (0.26)	0.49 (0.21)	*0.9276*
Fasting triglyceride (mmol/l) ‡**	1.28 (0.823 - 1.991)	1.41 (0.827 - 2.417)	1.37 (0.896 - 2.104)	*0.8888*

Data are mean (SD) except * number of subjects, † median (IQR), and ‡ geometric mean (SD range), and **results from first OTTT.

4.4.2 Post challenge profiles

Triglyceride

The geometric mean (1 SD range) triglyceride level differed between diabetic and non diabetic groups at the 6 hour timepoint 1.82 (1.865-3.225) mmol/l and 1.11 (0.655-1.106) mmol/l respectively (p=0.003) (Table 7). The results remained significant when adjusted for BMI. (Figure 13). AUC triglyceride was significantly greater in the diabetic group compared with the non-diabetic group (p=0.0003). The IAUC triglyceride was greater in diabetic compared to non diabetic subjects. Cmax was significantly higher in the diabetic group, 2.10 (1.343-3.293) mmol/l vs 1.34 (0.823 – 2.180) mmol/l for the non diabetic group (p<0.0015) with median Tmax at 6 hours and 5 hours respectively. Although 60%

of non diabetic subjects attained Tmax at 4 hours or less post challenge, 60% of diabetic subjects had maximal triglyceride levels at 6 or 8 hours post challenge period.

There were no significant differences in triglyceride 6 hour post challenge levels, IAUC or Cmax found between diet, metformin and sulphonylurea treated diabetic subgroups (Table 8, Figure 15). The geometric mean incremental rise in triglyceride was 0.68 mmol/l for diet only, 0.59 mmol/l for metformin, and 0.65 mmol/l for sulphonylurea treated diabetic subjects ($p = 0.7937$).

NEFA

Diabetic subjects had significantly elevated geometric mean (1 SD range) NEFA (μmol/L) levels at baseline 653.6 (446.42-956.81) compared with non-diabetic subjects 468.6 (313.95-699.28) ($p = 0.0047$), with significantly reduced NEFA suppression at 2 hours ($p < 0.0001$) (Table 7, Figure 13). IAUC NEFA to 8 hours was significantly greater in the diabetic group ($p = 0.0075$) compared with the non-diabetic group. The Cmin NEFA was also significantly higher in the diabetic group (<0.0001).

Within the diabetic subgroups, IAUC NEFA differed ($p = 0.0268$) (Table 8, Figure 15) with sulphonylurea treated subjects having lower IAUC NEFA levels.

Glycerol

Glycerol levels were significantly higher in diabetic than non-diabetic subjects at baseline, 1.09 (0.89-1.25) mmol/l vs 0.8 (0.65-1.05) mmol/l respectively ($p = 0.024$) (Table 7, Figure 13). Diabetic subjects did display reduced glycerol suppression at 2 hours ($p = 0.002$). No differences were found in AUC, or IAUC to 4 and 8 hours. Cmin and Cmax glycerol was also significantly higher in the diabetic group ($p = 0.0067$ and $p = 0.0333$ respectively).

There were no significant differences in glycerol levels amongst the diabetic subgroups (Table 8, Figure 15).

Mean total cholesterol, LDL cholesterol, HDL cholesterol and VLDL cholesterol levels did not change significantly in any group over the timepoints

Glucose

Fasting mean (SD) glucose levels were 9.2 (3.0) mmol/l and 5.5 (0.6) mmol/l for diabetic and non diabetic groups respectively and increased to 14.1 (4.4) mmol/l and 5.8 (1.9) mmol/l by 2 hours respectively. Glucose levels peaked at 14.1 (3.8) mmol/l (Cmax) at the 2 hour timepoint (Tmax) and were lowest 5.7 (1.1) mmol/l (Cmin) at the 6 hour timepoint (Tmin) for diabetic subjects (Table 7, Figure 14). Tmin glucose in the non diabetic group was 4 hours which was significantly shorter than the diabetic group ($p<0.0001$).

Within the diabetic group, fasting glucose levels were 7.8 (2.5) mmol/l for diet treated, 9.1 (2.5) mmol/l for metformin treated and 10.7 (3.4) mmol/l for sulphonylurea treated subjects (p = 0.164) (Table 8). This increased to 12.0 (4.3) mmol/l, 13.6 (4.1) mmol/l and 10.7 (3.4) mmol/l respectively by 2 hours (p = 0.1144) (Figure 16).

Insulin

Baseline geometric mean (1 SD range) insulin levels were higher in diabetic subjects 100.8 (54.60-186.13) pmol/l compared to non-diabetic subjects 66.2 (36.65-119.64) pmol/l (p=0.022) (Table 7, Figure 14). The 2 hour insulin increment was greater in the diabetic group 229.2 (159.78-306.05) pmol/l vs 87.9 (58.10-257.05) in the non diabetic group (p = 0.0267). The diabetic group had significantly greater AUC (p = 0.001) and IAUC to 8 hours (p=0.0034) for insulin. Post challenge insulin levels were highest in diabetic subjects, Cmax 341.1 (205.29-566.63) pmol/l compared with non-diabetic group 189.0 (84.74-421.40) pmol/l (p = 0.0066).

There was no significant difference in insulin levels when comparing diabetic subgroups although IAUC insulin was higher in the sulphonylurea treated group (Table 8, Figure 16).

C peptide

There were no differences in C peptide levels at baseline between the 2 groups and although 2 hour C peptide levels were higher in diabetic compared with non diabetic

subjects, this was not statistically significant (Table 7, Figure 14). AUC and IAUC C peptide profiles were significantly greater in the diabetic group ($p = 0.0008$ and $p = 0.0004$ respectively) compared with the non-diabetic group.

C peptide profiles were similar in diet, metformin and sulphonylurea treated groups (Table 8, Figure 16).

Table 7 Fasting and post challenge OTTT responses for non diabetic & diabetic subjects

	No Diabetes			Diabetes			*p*
Triglyceride (mmol/l)							
0 hour	0.77	0.457	-1.291	1.35	0.858	-2.138	*0.0002*
2 hours	0.90	0.557	-1.462	1.63	1.026	-2.598	
4 hours	1.06	0.589	-1.897	1.70	1.117	-2.590	
6 hours	1.11	0.655	-1.106	1.82	1.865	-3.225	*0.003*
8 hours	0.78	0.468	-1.290	1.40	0.787	-2.493	
AUC^{+}	7.87	4.878	-12.701	13.30	8.654	-19.572	*0.0003*
$IAUC_{0\text{-}6hours}{}^{*+}$	1.2	0.750	-1.873	1.8	0.718	-2.528	*0.3123*
$IAUC_{0\text{-}8hours}{}^{*+}$	1.3	0.963	-2.230	2.1	1.323	-3.828	*0.1109*
Cmax	1.34	0.823	-2.180	2.10	1.343	-3.293	*0.0015*
deltaCmax	0.51	0.270	-0.970	0.64	0.310	-1.310	*0.2535*
Tmax*±	5	4	-6	6	4	-6	*0.2937*
6-0 hours*	0.23	-0.025	-0.550	0.42	0.170	-0.890	*0.1373*
NEFA (umol/l)							
0 hour	468.6	313.95	-699.28	653.6	446.42	-956.81	*0.0047*
2 hours	101.2	60.65	-168.84	249.5	143.99	-432.37	*<0.0001*
4 hours	395.9	249.03	-629.36	276.9	162.76	-470.99	
6 hours	624.0	428.72	-908.29	612.9	421.11	-892.08	
8 hours	624.1	471.34	-826.36	731.0	534.52	-999.62	*0.0693*
AUC^{+}	3456.2	2619.82	-4559.64	3880.9	3063.19	-4916.91	*0.1337*
$IAUC_{0\text{-}4hours}{}^{*+}$	-862.5	-1274.25	- -360.75	-1119	-1734.25	- -799.50	*0.079*
$IAUC_{0\text{-}8hours}{}^{*+}$	-367.5	-1380.75	-180.00	-1398.0	-2356.25	-720.00	*0.0075*
Cmin	101.0	60.68	-168.02	196.4	119.68	-322.36	*<0.0001*
Cmax	732.2	572.54	-936.28	841.6	631.97	-1120.77	*0.0815*
Tmax*±	6	2	-6	6	0	-8	*0.8320*
2-0 hours*	-394.0	-556.75	- -220.50	-361.5	-561.50	- -234.5	*0.8130*
8-0 hours*	143	-21.75	-299.75	36.5	-88.00	-263.00	*0.2231*
Glycerol (mmol/l)*							
0 hour	0.8	0.65	-1.05	1.09	0.89	-1.25	*0.0240*
2 hours	0.6	0.46	-0.65	0.77	0.65	-0.96	*0.0020*
4 hours	1.0	0.79	-1.25	0.92	0.68	-1.19	
6 hours	1.0	0.92	-1.24	1.22	1.02	-1.49	
8 hours	0.9	0.71	-1.03	1.08	0.79	-1.28	*0.4875*
AUC^{+}	7.3	6.45	-8.25	7.70	7.09	-9.61	*0.0685*
$IAUC_{0\text{-}4hours}{}^{+}$	-0.4	-0.86	-0.26	-0.51	-1.87	-0.07	*0.3597*
$IAUC_{0\text{-}8hours}{}^{+}$	0.2	-0.88	-1.60	-0.19	-2.18	-1.22	*0.5127*
Cmin	0.5	0.42	-0.62	0.66	0.57	-0.77	*0.0067*
Cmax	1.2	1.06	-1.50	1.46	1.22	-1.83	*0.0333*
Tmax±	4	4	-6	6	2	-6	*0.3704*
2-0 hours	-0.3	-0.57	-0.07	-0.23	-0.63	-0.05	*0.9186*
8-0 hours	0.04	-0.42	-0.26	-0.03	-0.21	-0.28	*0.8952*

Data are geometric mean (1 SD range) except * median (IQR).
Units are as tabulated except + units.hour and ± hours

Continued Table 7 Fasting and post challenge OTTT responses for non diabetic and diabetic subjects

	No Diabetes			Diabetes			*p*
Glucose (mmol/l)†							
0 hour	5.4	0.59		9.0	2.46		*n/a*
2 hours	5.8	2.00		14.1	3.51		*n/a*
4 hours	4.4	0.54		8.2	3.45		
6 hours	4.8	0.32		6.1	1.77		
8 hours	4.8	0.28		5.8	1.04		
AUC^{+}	40.2	4.70		71.5	19.44		*n/a*
$IAUC_{0\text{-}4hours}$*$^{+}$	-0.5	-1.83	-1.93	9.55	5.83	-12.23	*0.0001*
$IAUC_{0\text{-}8hours}$*$^{+}$	-4.5	-5.80	- -0.53	-2.9	-6.58	-3.05	*0.0879*
Cmax	6.3	1.40		14.1	3.51		*n/a*
Cmin	4.1	0.77		5.4	1.37		*n/a*
Tmax*±	2	2	-2	2	2	-2	*0.1374*
Tmin*±	4	3	-4	6	4	-8	*0.0001*
2-0 hours*	0.4	0.58		5.1	1.66		*n/a*
Insulin (pmol/l)							
0 hour	66.2	36.65	-119.64	100.8	54.60	-186.13	*0.022*
2 hours	181.3	76.06	-432.26	322.5	199.46	-521.60	*0.0118*
4 hours	69.2	39.03	-122.55	185.8	87.40	-394.92	
6 hours	57.0	34.82	-93.34	106.7	55.42	-205.31	
8 hours	57.7	33.81	-98.48	93.0	46.78	-184.73	
AUC^{+}	809.6	432.29	-1516.21	1490.7	864.46	-2570.48	*0.001*
$IAUC_{0\text{-}4hours}$*$^{+}$	170.7	91.28	-546.78	540	379.60	-546.78	*0.0715*
$IAUC_{0\text{-}8hours}$*$^{+}$	169.2	64.68	-569.98	578.3	368.60	-986.00	*0.0034*
Cmax	189.0	84.74	-421.40	341.1	205.29	-566.63	*0.0066*
Tmax*±	2	2	-2	2	2	-2	*0.076*
2-0 hours*	87.9	58.10	-257.05	229.2	159.78	-306.05	*0.0267*
C peptide (nmol/l)							
0 hour	0.7	0.460	-1.117	0.8	0.544	-1.295	*0.2198*
2 hours	1.61	0.927	-2.802	1.99	1.463	-2.714	*0.1304*
4 hours	0.70	0.442	-1.112	1.49	0.966	-2.288	
6 hours	0.56	0.354	-0.885	0.97	0.588	-1.595	
8 hours	0.46	0.285	-0.757	0.80	0.468	-1.358	
AUC^{+}	7.06	4.449	-11.199	10.73	7.431	-15.492	*0.0008*
$IAUC_{0\text{-}4hours}$*$^{+}$	2.075	0.510	-3.025	2.9	2.125	-3.485	*0.2299*
$IAUC_{0\text{-}8hours}$*$^{+}$	1.44	-0.218	-3.380	3.68	2.860	-4.705	*0.0004*
Cmax	1.61	0.931	-2.799	2.02	1.456	-2.806	*0.1114*
Tmax*±	2	2	-2	2	2	-2	*0.2937*
2-0 hours*	1.11	0.320	-1.410	1.10	0.870	-1.270	*0.5127*

Data are geometric mean (1 SD range) except † mean (SD) and * median (IQR).
Units are as tabulated except + units.hour and ± hours

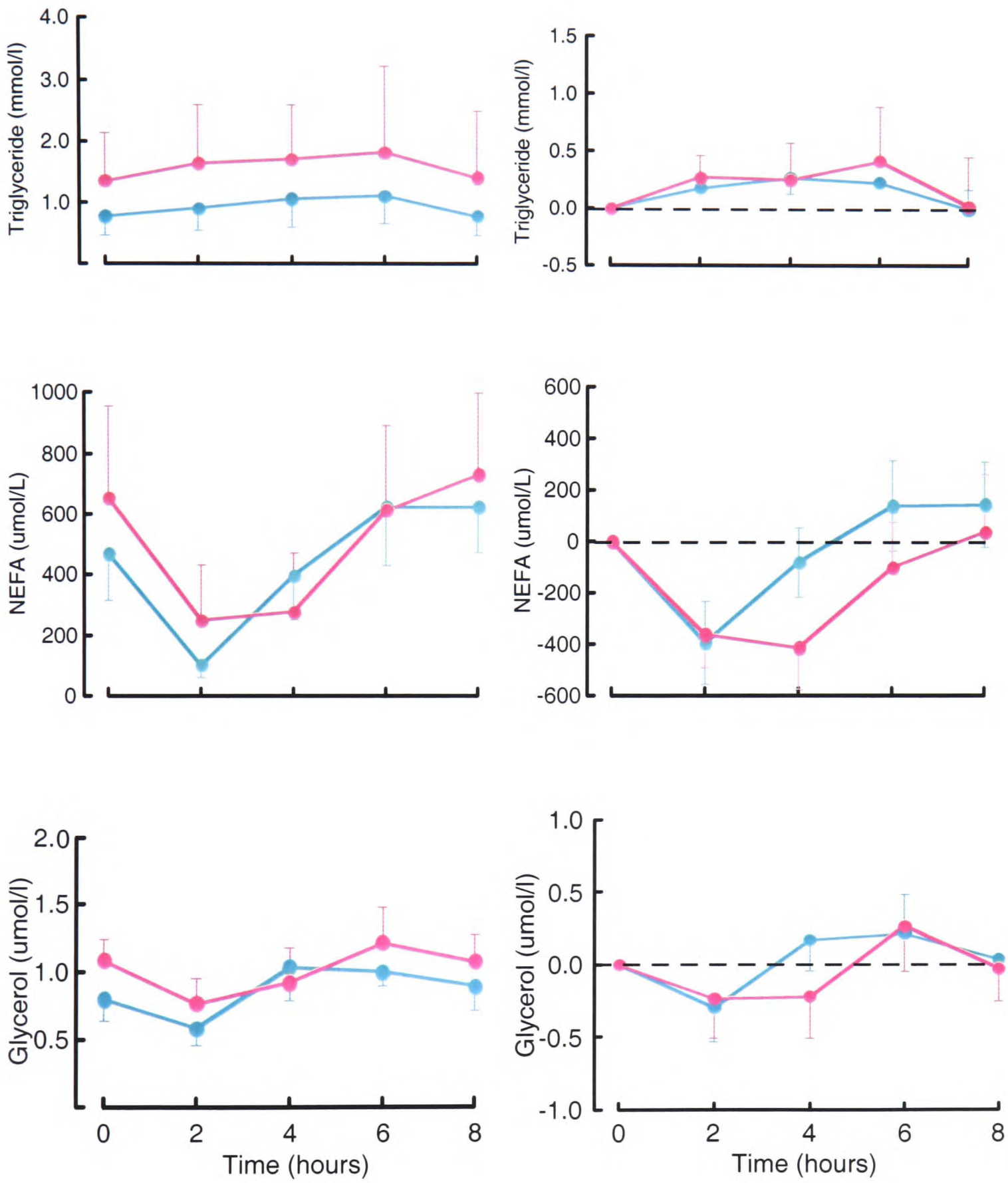


Figure 13 Triglyceride, NEFA and glycerol profiles for non diabetic ● and diabetic ● subjects. Absolute values are depicted on the left, and change from baseline (delta) on the right.

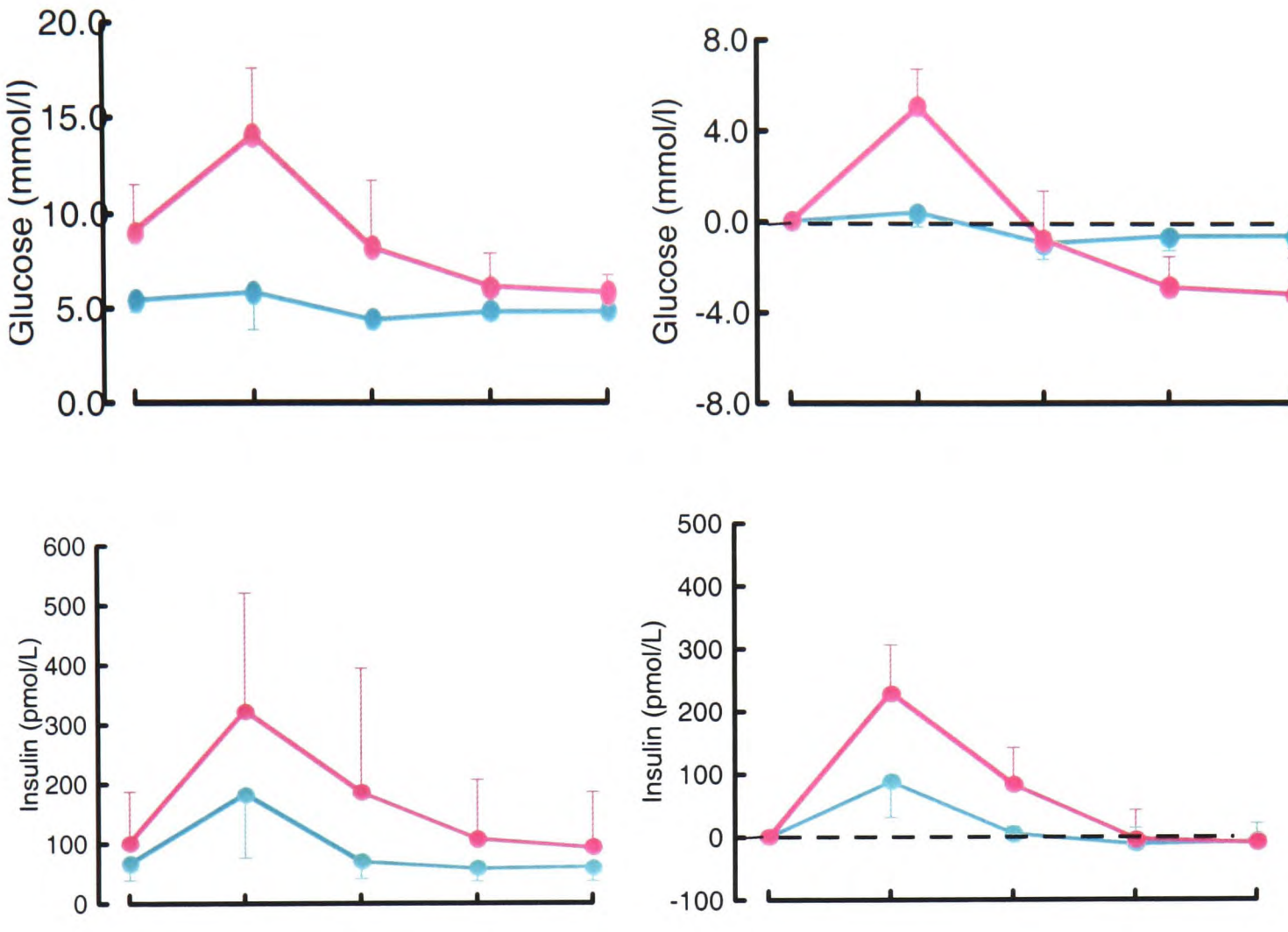


Figure 14 Glucose, insulin and C peptide profiles for non diabetic ● and diabetic ● subjects. Absolute values are depicted on the left, and change from baseline (delta) on the right.

Table 8 Fasting and post challenge levels for diabetic subgroups.

	Diet			Metf	ormin		Sulphonylurea			*p*
Triglyceride (mmol/l)										
0 hour	1.28	0.823	-1.991	1.41	0.827	-2.417	1.37	0.896	-2.104	*0.8888*
2 hours	1.65	0.993	-2.739	1.57	0.974	-2.546	1.68	1.067	-2.634	
4 hours	1.76	1.150	-2.701	1.68	1.075	-2.617	1.66	1.078	-2.567	
6 hours	1.78	0.981	-2.863	1.96	0.965	-3.918	1.73	1.027	-3.094	*0.8823*
8 hours	1.12	0.736	-2.620	1.62	0.798	-3.291	1.51	0.868	-2.621	
AUC^{+}	13.01	7.962	-23.383	13.64	8.470	-20.746	13.26	8.464	-20.906	*0.9744*
$IAUC_{0\text{-}6hours}$*$^{+}$	2.29	1.520	-2.700	1.78	0.740	-1.960	1.395	0.220	-2.350	*0.4137*
$IAUC_{0\text{-}8hours}$*$^{+}$	2.22	2.018	-2.888	2.18	1.323	-4.803	1.83	1.163	-2.833	*0.907*
Cmax	2.05	1.380	-3.059	2.16	1.267	-3.694	2.09	1.337	-3.278	*0.9685*
deltaCmax	0.68	0.361	-1.280	0.59	0.258	-1.355	0.65	0.304	-1.394	*0.7937*
Tmax*±	4	4	-6	6	6	-6	6	4	-8	*0.0853*
6-0 hours*	0.39	0.253	-0.558	0.54	0.310	-0.128	0.47	1.455	-0.698	*0.7801*
NEFA (umol/l)										
0 hour	558.1	335.16	-929.35	616.8	449.69	-846.12	810.9	692.04	-950.17	*0.0703*
2 hours	207.4	120.83	-356.02	232.9	121.59	-446.06	321.6	219.81	-470.51	*0.1826*
4 hours	335.4	161.15	-697.88	245.4	158.37	-380.16	257.9	182.89	-363.75	
6 hours	586.7	361.68	-951.75	677.4	484.21	-947.79	579.3	428.93	-782.39	
8 hours	616.8	401.82	-946.67	840.7	710.30	-995.09	753.2	603.86	-939.58	*0.075*
AUC^{+}	3714.6	2607.59	-5291.69	3936.1	3233.27	-4791.70	3997.8	3564.41	-4483.78	*0.7775*
$IAUC_{0\text{-}4hours}$*$^{+}$	-885.0	1448.00	- -450.75	-849.5	-1446.00	- - 803.25	-1476.0	-1824.25	-1257.50	*0.0761*
$IAUC_{0\text{-}8hours}$*$^{+}$	-721.0	1937.00	- -300.00	-948.0	-2367.00	- -421.00	-2288.5	-3190.00	- -1966.00	*0.0268*
Cmin	181.4	107.27	- -306.79	174.4	102.13	- -297.84	239.5	160.15	- -358.19	*0.3049*
Cmax	758.0	492.52	-1166.55	905.8	753.57	-1088.69	868.2	740.17	-1018.46	*0.3597*
Tmax*±	3	0	-6	8	6	-8	0	0	-8	*0.0596*
2-0 hours*	-328.0	-497.00	- -152.00	-296.5	-533.00	- -233.00	-452.5	-621.00	- -360.00	*0.246*
8-0 hours*	-32.0	-122.00	-194.00	237.0	57.00	-301.00	-32.0	-260.00	-70.00	*0.0642*
Glycerol (mmol/l)*										
0 hour	1.0	0.87	-1.25	1.1	0.94	-1.18	1.1	0.96	-1.25	*0.9107*
2 hours	0.8	0.68	-1.00	0.8	0.62	-0.86	0.8	0.67	-0.98	*0.5436*
4 hours	1.0	0.75	-1.19	0.8	0.60	-0.98	0.9	0.68	-1.21	
6 hours	1.2	0.85	-1.22	1.5	1.16	-1.81	1.2	1.01	-1.38	
8 hours	0.8	0.71	-1.05	1.3	0.98	-1.36	1.1	0.81	-1.21	*0.4336*
AUC^{+}	7.6	7.02	-8.67	8.3	7.10	-9.85	8.3	7.20	-9.61	*0.8764*
$IAUC_{0\text{-}4hours}^{+}$	0.0	-1.78	-0.25	-0.6	-1.72	- -0.12	-0.6	-1.74	- -1.67	*0.7896*
$IAUC_{0\text{-}8hours}^{+}$	-0.7	-1.80	-0.68	0.1	-0.82	-1.17	-1.9	-2.33	-1.95	*0.7646*
Cmin	0.7	0.50	-0.76	0.6	0.57	-0.79	0.7	0.61	-0.75	*0.9958*
Cmax	1.2	1.21	-1.50	1.6	1.37	-1.96	1.5	1.25	-1.69	*0.5167*
Tmax±	6.0	4	-6	6.0	6	-8	4	0	-6	*0.0629*
2-0 hours	-0.2	-0.63	- -0.17	-0.2	-0.54	- -0.11	-0.4	-0.59	- -0.07	*0.8986*
8-0 hours	-0.03	-0.26	-0.08	0.2	-0.07	-0.36	-0.2	-0.32	-0.18	*0.1792*

Data are geometric mean (1 SD range) except * median (IQR).
Units are as tabulated except + units.hour and ± hours

Continued Table 8 Fasting and post challenge levels for diabetic subgroups

	Diet			Metformin			Sulphonylurea			*p*
Glucose (mmol/l)†										
0 hour	8.0	2.06		9.0	2.07		10.1	2.91		*0.164*
2 hours	12.5	3.49		13.9	3.24		15.7	3.33		*0.1144*
4 hours	6.9	3.16		7.1	2.77		10.6	3.32		
6 hours	5.7	1.60		5.6	1.42		7.1	1.98		
8 hours	5.6	0.92		5.6	1.01		6.0	1.21		
AUC^{+}	63.6	18.60		67.9	16.14		82.9	19.53		*0.0584*
$IAUC_{0\text{-}4hours}$*$^{+}$	8.5	4.40	-10.68	6.6	4.58	-11.55	11.4	9.88	-14.95	*0.1224*
$IAUC_{0\text{-}8hours}$*$^{+}$	2.0	-4.23	-3.05	-5.2	-7.58	-3.28	-0.5	-4.88	-11.80	*0.1925*
Cmax	12.5	3.49		13.9	3.24		15.7	3.33		*0.1144*
Cmin	5.1	1.27		5.2	1.46		5.9	1.36		*0.323*
Tmax*±	2	2	-2	2	2	-2	2	2	-2	*>0.1*
Tmin*±	6	4	-6	6	4	-8	8	8	-8	*0.0464*
2-0 hours*	-2.4	1.27		-3.3	1.19		-4.0	2.07		*0.072*
Insulin (pmol/l)										
0 hour	97.2	62.48	-151.25	96.2	52.96	-174.58	109.6	48.99	-245.27	*0.8767*
2 hours	342.9	245.00	-480.05	292.6	176.76	-484.22	334.5	183.37	-610.01	*0.7426*
4 hours	174.4	102.92	-295.43	134.0	67.69	-265.32	272.7	113.42	-655.55	
6 hours	88.4	70.67	-110.70	94.7	52.87	-169.70	144.8	58.16	-360.75	
8 hours	72.1	51.42	-101.13	83.6	45.47	-153.55	133.3	54.31	-327.23	
AUC^{+}	1438.8	1112.65	-1860.61	1256.0	743.82	-2120.80	1826.5	893.79	-3732.34	*0.7251*
$IAUC_{0\text{-}4hours}$*$^{+}$	489.0	403.80	-774.90	420.1	363.40	-582.20	695.4	548.10	-1004.45	*0.2266*
$IAUC_{0\text{-}8hours}$*$^{+}$	555.6	413.10	-733.40	433.7	296.38	-591.65	1026.7	816.88	-1564.58	*0.0612*
Cmax	350.6	248.52	-494.57	293.5	176.80	-487.09	385.6	202.87	-732.98	*0.4897*
Tmax*±	2	2	-2	2	2	-2	2	2	-4	*0.4565*
2-0 hours*	222.2	175.88	-358.48	211.0	154.85	-279.13	233.9	177.25	-320.50	*0.7796*
C peptide (nmol/l)										
0 hour	0.79	0.582	-1.076	0.84	0.573	-1.235	0.89	0.489	-1.616	*0.8444*
2 hours	2.13	1.570	-2.901	1.88	1.397	-2.536	1.97	1.402	-2.768	*0.6698*
4 hours	1.46	1.056	-2.030	1.29	0.843	-1.981	1.74	1.041	-2.894	
6 hours	0.88	0.689	-1.126	0.83	0.529	-1.298	1.25	0.646	-2.403	
8 hours	0.67	0.466	-0.960	0.67	0.445	-1.021	1.12	0.590	-2.139	
AUC^{+}	10.59	8.303	-13.498	9.64	6.861	-13.542	12.11	7.485	-19.576	*0.3911*
$IAUC_{0\text{-}4hours}$*$^{+}$	3.31	2.863	-3.995	2.48	2.118	-3.085	2.79	1.875	-4.040	*0.2022*
$IAUC_{0\text{-}8hours}$*$^{+}$	4.00	3.473	-4.983	2.92	2.133	-3.848	3.73	3.320	-6.898	*0.0626*
Cmax	2.14	1.573	-2.908	1.88	1.397	-2.536	2.05	1.386	-3.038	*0.6876*
Tmax*±	2	2	-2	2	2	-2	2	2	-4	*0.1352*
2-0 hours*	1.24	1.048	-1.528	0.97	0.865	1.140	1.08	0.710	1.200	*0.1959*

Data are geometric mean (1 SD range) except † mean (SD) and * median (IQR).
Units are as tabulated except + units.hour and ± hours

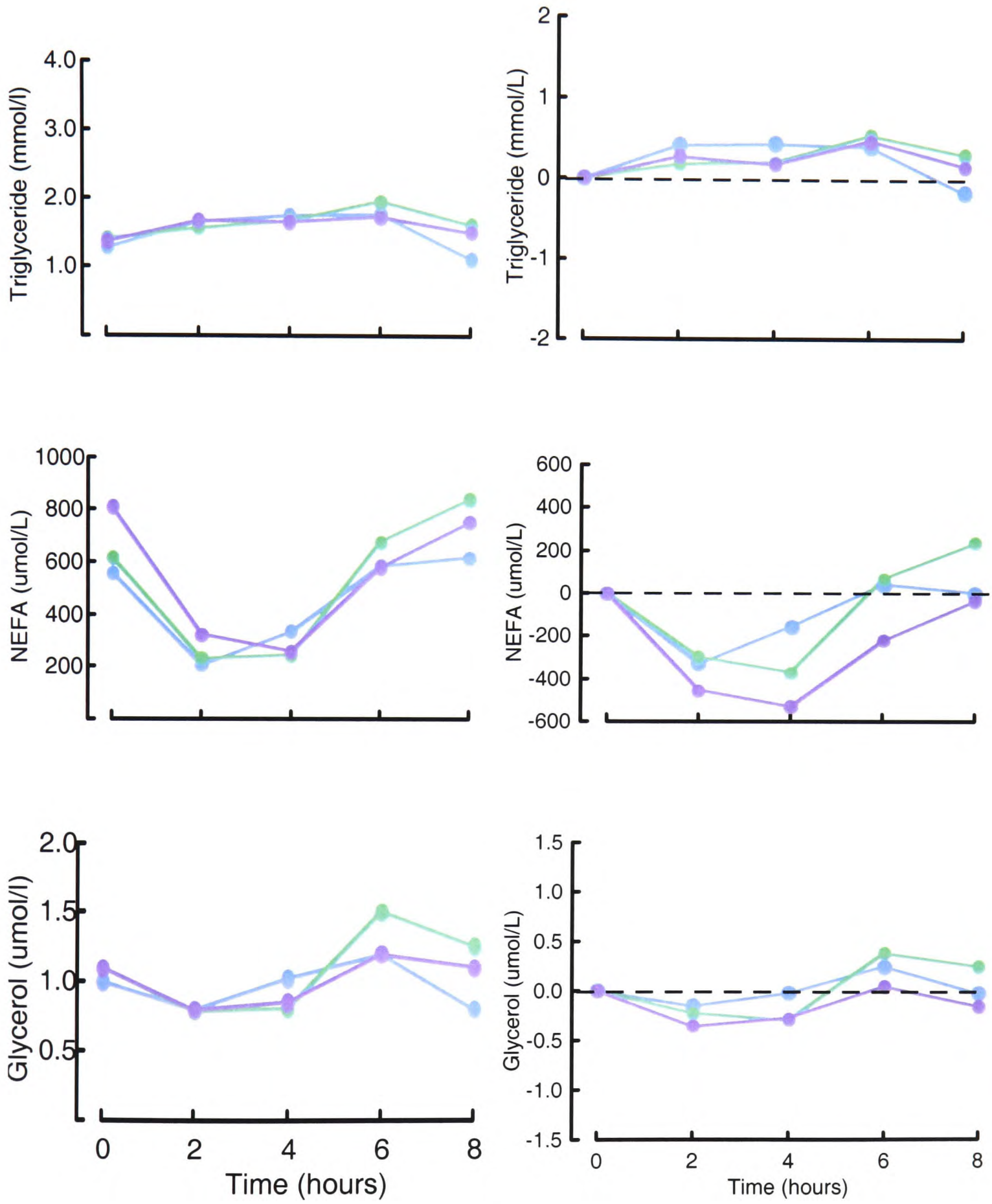


Figure 15 Triglyceride, NEFA, and glycerol profiles profiles for the diabetic subgroups, ● diet alone, ● metformin and ● sulphonylurea treatment. Absolute values are depicted on the left, and change from baseline (delta) on the right.

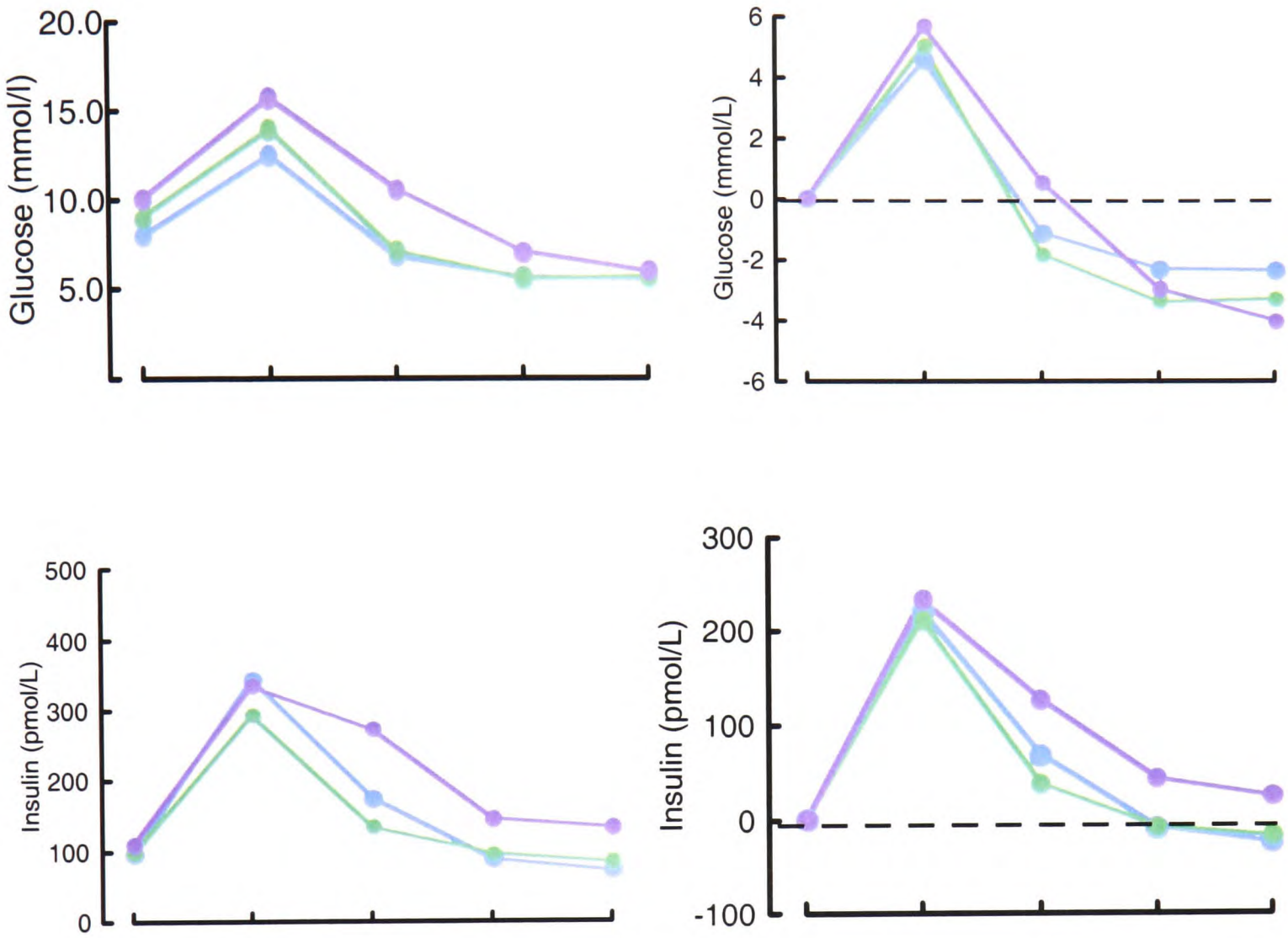


Figure 16 Glucose, insulin and C peptide profiles profiles for the diabetic subgroups, ● diet alone, ● metformin and ● sulphonylurea treatment. Absolute values are depicted on the left, and change from baseline (delta) on the right.

4.4.3 Inter individual variability

The inter individual variation was between 45 – 55% for triglyceride. The between subject variability for glucose was 10.8% and 27.3% at baseline for non diabetic and diabetic subjects, and 34.4% and 25% at 2 hours post challenge respectively.

Table 9 Summary of inter individual CVs for analytes for non diabetic and diabetic subjects

	No Diabetes CV(%)	Diabetes CV(%)
Triglyceride (mmol/l)		
0 hour	56.6	44.9
6 hours	48.6	54.4
NEFA (umol/l)		
0 hour	36.2	34.0
2 hours	47.9	44.2
Glycerol (mmol/l)		
0 hour	41.6	34.2
2 hours	42.2	34.2
Glucose (mmol/l)		
0 hour	10.8	27.3
2 hours	34.4	25.0
Insulin (pmol/l)		
0 hour	89.0	44.2
2 hours	82.0	76.8
C peptide (nmol/l)		
0 hour	45.4	47.7
2 hours	53.7	31.8

4.4.4 Correlations

Pearson correlation coefficients for each timepoint with baseline triglyceride concentration showed a strong correlation between post challenge and fasting triglyceride values for both diabetic and non diabetic groups (Table 10).

Table 10 Correlation coefficients for fasting with post challenge triglyceride levels

	No diabetes		Diabetes	
	r	*p*	r	*p*
2 hour	0.8709	*<0.0001*	0.887	*<0.0001*
4 hour	0.8575	*<0.0001*	0.8231	*<0.0001*
6 hour	0.7174	*0.0004*	0.9089	*<0.0001*
8 hour	0.6553	*0.0017*	0.9032	*<0.0001*

Fasting triglyceride concentration correlated with fasting plasma glucose and insulin levels (r = 0.5955, p = 0.0005 and r = 0.5863, p = 0.0007) in diabetic subjects, and fasting plasma glucose in non diabetic subjects (r = 0.5199, p = 0.0188).

The delta 6 hour triglyceride level showed a positive correlation with delta 2 hour post challenge insulin levels (r = 0.5086, p = 0.0262), IAUC insulin to 4 hours (r= 0.5069, p = 0.0267) and IAUC insulin to 8 hours (r = 0.4708, p = 0.0419) in the non diabetic but not diabetic group. For non diabetic subjects only, the delta 6 hour triglyceride concentration also correlated with the delta 2 hour post challenge glucose levels (r = 0.5086, p = 0.0262), IAUC glucose to 4 hours (r= 0.5069, r = 0.0267) and IAUC to glucose to 8 hours (r = 0.4708, p =0.0419). The delta 6 hour triglyceride level correlated with delta 2 hour glucose level in diabetic subjects (r = 0.4286, p = 0.0181).

The incremental AUC triglyceride correlated with fasting insulin (r = 0.5178, p = 0.0232) in non diabetic subjects. In diabetic subjects, Tmax triglyceride correlated with IAUC glucose and insulin (r = 0.3964, p = 0.0333 and r = 0.3948, p = 0.0309 respectively).

4.5 Discussion

The metabolic abnormalities seen in type 2 diabetic subjects are not always present in the fasting state and may only become apparent in postprandial period after provocation by a fat and carbohydrate test challenge. The results show that patients with moderately well-controlled type 2 diabetes, display exaggerated fasting and post challenge lipid and glycaemic responses compared with non diabetic subjects . Post challenge triglyceride levels were strongly associated with fasting triglyceride levels in both diabetic and non diabetic groups. Correlations were found between post challenge triglyceride levels and 2 hour glucose and insulin excursions in non diabetic subjects . In diabetic subjects, time to reach maximum triglyceride concentration was associated with glucose and insulin increment.

Triglyceride profiles

In this study the increase in post challenge triglyceride levels seen in diabetic subjects is consistent with other studies of postprandial lipaemia (Lewis, O'Meara et al. 1991; Cavallero, Dachet et al. 1994; Curtin, Deegan et al. 1994; Tan, Cooper et al. 1995; Cooper, Tan et al. 1996). We showed the elevation in triglyceride levels to 6 hours (delta 6 hour) in diabetic subjects to be 80% greater than non diabetic subjects. In the 8 hour post challenge period, triglyceride IAUC was 60% greater in diabetic subjects compared to non diabetic subjects. Although 60% of non diabetic subjects attained Tmax at 4 hours or less post challenge, 60% of diabetic subjects had maximal triglyceride levels at 6 or 8 hours post challenge period. Further illustration of the differences between the diabetic and non-diabetic groups may have been evident if the fat challenge was increased for

body surface area or weight as done in some previous studies of postprandial lipaemia, as the diabetic group had a significantly higher BMI.

One possible explanation for postprandial elevation of triglyceride levels is chylomicron competition with VLDL for triglyceride removal by LPL, an enzyme which has been previously shown to have limited function in type 2 diabetic individuals (Mero et al). Also, the elevation in postprandial triglyceride levels is promoted by an accumulation of exogenous triglyceride rich lipoproteins (Karpe 1999). Patsch et al (Patsch, Miesenbock et al. 1992) showed that single postprandial triglyceride levels at 6 hours post challenge were highly discriminatory and displayed a high accuracy of predicting the presence of CHD in a study of non diabetic individuals. Another study by Karpe et al (Karpe, de Faire et al. 1998) again demonstrated that it was 6 hour post challenge triglyceride values, above other timepoints, that correlated positively with intima media thickness, independently of both LDL cholesterol and fasting triglyceride concentration. In our study, all post challenge triglyceride values correlated strongly with fasting levels in both diabetic and non diabetic subjects. Karpe et al (Karpe, de Faire et al. 1998) too showed postprandial triglyceride values to correlate strongly with fasting triglyceride levels.

NEFA and glycerol profiles

Our results show that diabetic compared with non diabetic subjects have elevated fasting NEFA and glycerol levels, a finding that has previously been observed (Reaven 1988). It has been postulated that elevation of plasma NEFA concentration is an important factor contributing to hypertriglyceridaemia and may be associated with the increased

cardiovascular risk for subjects with abnormal glucose tolerance (Byrne, Wareham et al. 1997). Elevation of plasma NEFA and glycerol levels may increase the risk of developing diabetes (Paolisso, Tataranni et al. 1995) by promoting gluconeogenesis and insulin resistance (Ferrannini, Galvan et al. 1999; Bergman and Ader 2000).

We found the degree of NEFA suppression (delta 2 hour) to be similar between diabetic and non diabetic subjects although previous studies have found differences (Reaven 1988). The normal suppression of fatty acid release is dependent upon the action of insulin in adipose tissue, an action which is impaired in insulin resistant states (Mero, Malmström et al. 2000). We found diabetic subjects treated with sulphonylurea therapy to have lower IAUC NEFA which may be a consequence of higher circulating insulin levels. Failure to suppress post challenge NEFA release further increases fatty acid substrate input to the liver required for VLDL triglyceride formation. Normotriglyceridaemic subjects with abnormal glucose tolerance have shown elevation of NEFA levels after administering the 75g OGTT to compared to non-diabetic subjects (Byrne, Wareham et al. 1997). Previous studies have demonstrated that hypertriglyceridaemic type 2 diabetic subjects have significantly higher NEFA concentrations during the postprandial period after a fatty meal compared with non-diabetic subjects, however normotriglyceridaemic type 2 diabetic subjects do not (Lewis, O'Meara et al. 1990; Cooper, Tan et al. 1996). Interference of endothelium-dependent vasodilatation has been shown with elevated postprandial NEFA levels (Steinberg, Tarshoby et al. 1997), which may be a possible mechanism for the development of CHD.

Glucose profiles

After a 50g carbohydrate and 50g lipid challenge, our diabetic subjects produced a 56% elevation in plasma glucose levels from 9.0 mmol/l (baseline) to 14.1 mmol/l at 2 hours. The rise in 2 hour glucose levels in the diabetic subjects was similar whether treated by diet alone, metformin or sulphonylurea therapy. The glucose rise seems proportionate to the amount of carbohydrate given, as Ko et al (Ko, Chan et al. 1998) found an 85% increase in glucose with 75g OGTT in a study screening 87 untreated Chinese subjects who where then diagnosed with type 2 diabetes according to WHO criteria. In type 2 diabetes there is failure to suppress hepatic glucose production as well as regulation of mealtime hyperglycaemia. The OTTT provokes a significant increase in 2 hour glucose levels similar to the OGTT although the glucose excursion is approximately one third less. The most likely reason for this is the lower carbohydrate content of the OTTT test drink. Wolever et al (Wolever, Chiasson et al. 1998) compared the glucose response after 75g OGTT and 50g carbohydrate biscuit test meal (combined with 10g fat and 12 g protein) in 10 diet treated type 2 diabetic subjects. The 2 hour glucose response for the standard meal was similar to our findings (57%) and the increase to the 75g OGTT was over 2 fold compared to fasting plasma glucose concentrations. Other possible reasons for a reduced glucose excursion seen with the OTTT compared with the OGTT could be the triglyceride content of the test drink slowing gastric emptying and absorption of carbohydrate (Westphal, Leodolter et al. 2002). Although studies directly comparing the OTTT and OGTT in more subjects would enable further conclusions to be drawn, the OTTT may not only provide information regarding post challenge triglyceride handling, but could potentially provide information on an individual's glycaemic tolerance.

Insulin and C peptide profiles

The insulin response seen in subjects participating in the OTTT was significantly greater in diabetic individuals even when adjusted for baseline hyperinsulinaemia. Fasting insulin levels were circa 50% higher in diabetic subjects, and increased at 2 hours by 3 fold (2 fold increase in C peptide levels). Theses changes in insulin and C peptide levels were similar across diet, metformin and sulphonylurea treated diabetic groups. For the 8 hour study duration, insulin IAUC was 2 to 3 fold greater in the diabetic group, with sulphonylurea treated subjects having the higher IAUC insulin values than the other subgroups. IAUC C peptide levels were also increased over the 8 hour time period, indicating continued insulin production. Loss of the early insulin response to exogenous glucose is one of the first defects noted in patients with type 2 diabetes. Hyperglycaemia combined with insulin resistance causes sustained late phase insulin secretion leading to hyperinsulinaemia, and this has been noted in some previous studies (Reaven 1988) (Cooper, Tan et al. 1996) but not other postprandial lipaemia studies (Lewis, O'Meara et al. 1990; Curtin, Deegan et al. 1994). However although despite this chronic hyperinsulinaemia, total insulin secretion is still deficient relative to the degree of hyperglycaemia.

Correlations

The metabolic relationship of insulin, insulin resistance and triglyceride in type 2 diabetes has been debated in the literature by numerous authors (Lewis, Uffelman et al.

1993; Couillard, Bergeron et al. 1999). We found significant relationships between post challenge but not fasting triglyceride levels and glucose- insulin responses despite post challenge triglyceride levels being highly correlated with fasting values. The delta 6 hour post challenge triglyceride levels were associated with the 2 hour glucose increase, in diabetic subjects. Glucose IAUC was also significantly correlated with time to reach maximum triglyceride in diabetic but not non diabetic subjects. This may indicate an interaction between hyperglycaemia and post challenge triglyceride levels.

The results of the OTTT showed non diabetic subjects to have significant associations between delta 6 hour triglyceride levels and post challenge insulin response as well as IAUC. Boquist et al (2000) found a strong association between basal plasma insulin and postprandial triglyceridaemia, with an exaggerated and prolonged triglyceride response in hyperinsulinaemic subjects without diabetes. Studies of diabetic subjects have also demonstrated this association (Castro Cabezas, Halkes et al. 2001). Other studies have highlighted insulin resistance to be associated with lipid and lipoprotein abnormalities rather than hyperinsulinemia (Yki-Järvinen, 1988; Laakso, Sarlund et al. 1990). Baynes et al (Baynes, Henderson et al. 1991), however, concluded that it was defects in glucoregulatory and antilipolytic actions of insulin that contributed to mild fasting hypertriglyceridemia in NIDDM and that these defects cannot be attributed solely to obesity.

Between subject variability

The inter individual variability of a chosen parameter in the OTTT study was found to be higher that the intra individual variability. This is a consistent finding in clinical studies including a study by Marcovina et al (Marcovina, Gaur et al. 1994) found each lipid analyte to have considerable variation between individuals. In general, this between subject variability is not as important as intra individual variability and can be removed in studies of a paired design (Altman 1991). Inter individual variability may be due to age, sex, ethnicity, genetic factors, obesity, as well as medications.

Age

The effect of age on postprandial triglyceride levels has been investigated. Increased fasting plasma glucose, impairment of glucose tolerance and elevated fasting and postprandial triglyceride rich lipoprotein levels is documented in the elderly population (Cohn J S, McNamara J R et al. 1988; Krasinski S D, Cohn J S et al. 1990). Cassader et al (Cassader, Gambino et al. 1996) demonstrated that healthy normotriglyceridaemic elderly subjects, mean (SD) age 74.4 (6.5) years, tended to have prolonged postprandial hypertriglyceridaemia peaking at 6 hours post oral fat challenge. This was most likely due to a combination of reduced LPL activity and delayed metabolism of triglyceride rich particles rather than diminution in intestinal absorption. Therefore, it is possible that elderly subjects with type 2 diabetes may be more likely to display abnormalities of the postprandial profile. Subjects with type 2 diabetes who participate in clinical studies such these tend to be older, as type 2 diabetes is usually found in the older population (although earlier and younger diagnosis is becoming more prevalent today). Also,

because of the nature and duration of the OTTT and other studies of the postprandial period, the subjects recruited are commonly of retirement age. To date, no studies have specifically looked at the effect of age on postprandial lipaemia in diabetic subjects.

Gender

Review of the literature reveals few studies that specifically investigate male - female differences in postprandial lipaemic response. A study by Halkes et al(Halkes, Castro Cabezas et al. 2001), determined the postprandial triglyceride response in male and female obese and non obese subjects. Diurnal triglyceride levels were lower in lean females than in lean males, but this difference was not found between overweight females and males. The authors concluded that due to a significant positive association between diurnal triglyceridaemia and waist circumference, females who were overweight had less favourable postprandial profiles due to abdominal fat accumulation and insulin resistance. Previously Couillard et al (Couillard, Bergeron et al. 1999) had also found the gender difference in postprandial plasma triglyceride response to be eliminated when men and women were matched for visceral adipose tissue accumulation. Wing et al (Wing R R and W 1995) showed that modest weight loss (10 – 15% of initial body weight) lead to a 25% reduction in fasting plasma triglyceride concentration. In this study, men had greater improvements in cardiovascular risk factors with weight loss than women. It has been documented that post menopausal women lose their protective sex effect against the development of cardiovascular disease and their prevalence of atherosclerosis approaches or equals that of men (Westerveld, 1998). In women with diabetes, once diagnosed with CHD, their prognosis is worse (Perez, Wagner et al. 2000).

A deficiency of endogenous oestrogens has been associated with a higher incidence of CHD, and menopause causes an elevated and prolonged postprandial response (van Beek A P, de Ruijter-Heijstek F C et al. 1999). Oestrogen has been shown to modify the postprandial lipid profile by improving chylomicron remnant clearance (Westerveld 1998). Therefore women taking oestrogen, either for replacement therapy or contraception may not demonstrate as clearly an abnormality in their postprandial triglyceride profile. Clear characterisation of subjects according to gender including menopausal and/or hormonal status is therefore important for interpretation of postprandial lipid profiles.

Ethnicity

Recruitment to most studies exploring modifiers of postprandial lipaemia has mainly been restricted to the Caucasian population. It is well documented that certain ethnic groups who migrate to Western countries have increased CHD morbidity and mortality (McKeigue P M, Shah B et al. 1991; Howard B V, Mayer-Davis E J et al. 1998). Variation in triglyceride response as well as insulin resistance have been associated with this increased CHD risk (Whincup P H, Gilg J A et al. 2002) (Zampelas, Roche et al. 1998). Genetic factors can also modulate postprandial lipaemia in type 2 diabetes. Individuals with apo E genotypes E2/3 and E3/4 show slower clearance than E3/3 (Reznik, 1996).

Obesity

Increased triglyceride levels and decreased HDL concentrations are frequently observed in persons with central obesity and are associated with the insulin resistance syndrome which increases the risk of coronary artery disease (Idzior-Walus, Mattock et al. 2001). Studies of obesity have shown both male and female normolipidaemic obese subjects to display elevated postprandial triglyceride levels associated with hyperinsulinaemia or insulin resistance (Lewis, O'Meara et al. 1990). Abdominal obesity results in high levels of free fatty acids which contribute to dyslipidaemia, beta cell dysfunction and hepatic and peripheral insulin resistance (Carey 1998). This may influence the interpretation of results between individuals. People with type 2 diabetes typically are obese, with a higher BMI and waist circumference. Subjects underwent sequential OTTTs within one month from recruitment into the study, to ensure weight remained stable. This is because even modest weight loss of approximately 1.5 kg in overweight well controlled type 2 diabetic subjects has been shown to reduce postprandial glycaemic excursions and postprandial lipaemia (Ybarra, James et al. 2001).

Medication

There are certain medications apart from lipid lowering drugs and oestrogen that may influence lipid levels. The oral hypoglycaemic agent, acarbose has been shown to reduce postprandial triglyceride levels (Hanefeld 1998). No subjects participating in the OTTT were taking acarbose.Metoprolol enhances fasting and postprandial triglyceridaemia in middle aged men by increasing the hepatic production of VLDL (Boquist S, Ruotolo G et al. 1998). Although subjects were not excluded if they were taking ß blockers, no medication changes occurred during participation in the study.

The OTTT highlights differences in carbohydrate and lipid handling in diabetic and non diabetic subjects. Firstly, in diabetic subjects, there is loss of first phase glucose mediated insulin secretion. This causes early post challenge hyperglycaemia and subsequent late phase hyperinsulinaemia. The elevation in insulin secretion is mismatched for the glucose response provoked by the OTTT test drink, in that the onset of insulin release does not parallel the rise in plasma glucose in the postprandial state. Therefore hepatic glucose production is not immediately suppressed and glucose uptake is delayed. This "relative" insulin deficiency during the early post challenge period reduces NEFA suppression. Increased circulating NEFA and glycerol concentrations as well as chronic hyperinsulinaemia presents the liver with greater substrate and formation of VLDL triglyceride through the endogenous lipid pathway. The increase in VLDL particles competes with chylomicrons, which are part of the exogenous lipid pathway, augmented by the triglyceride content of the OTTT test drink. These triglyceride rich particles cause elevation and prolongation of post challenge triglyceride profiles.

5 Chapter 5 Does enhancing endogenous insulin secretion improve post challenge lipid handling in people with type 2 diabetes?

5.1 Introduction

As previously highlighted, defects in glucose mediated insulin secretion seen in type 2 diabetes may play a role in alterations in lipid handling apparent in these individuals. In type 2 diabetes, the pancreatic β cell initially increases insulin production to counteract insulin resistance, but with time, is unable to sustain such an output. There is loss of first phase insulin secretion with inevitable hyperglycaemia. Total insulin secretion relative to the degree of hyperglycaemia is mismatched. In type 2 diabetic subjects the effect of insulin to suppress the production of triglyceride rich VLDL in the liver is defective (Malmström R, Packard C J et al. 1997). Studies have demonstrated that this contributes to the postprandial hypertriglyceridaemia observed in these individuals. Insulin is also required in the immediate postprandial period for lipid handling in peripheral tissues, with diabetic subjects showing reduced inhibition of lipolysis and NEFA release from adipose tissue (Porte 2001). Restoration of first phase insulin secretion may therefore have beneficial consequences on lipid handling. This would require investigating the effect of antidiabetic agents which enhance endogenous insulin secretion directly into the portal circulation in a timeframe which is rapid and of short duration.

Augmentation of pancreatic β cell insulin secretion enhances insulin delivery to the liver via the portal circulation suppressing hepatic glucose production and thus reducing postprandial hyperglycaemia. Enhancing insulin delivery within the portal vein may

have direct consequences upon the endogenous lipid pathway and subsequently exogenous lipid metabolism. As insulin has a direct inhibitory effect on VLDL secretion by the liver (Geltner, Lechleitner et al. 2002) this could reduce VLDL release. There is therefore less VLDL to compete with chylomicrons for LPL thus potentially decreasing circulating triglyceride levels.

The restoration of first phase insulin secretion is also dependent on delivering insulin rapidly in response to a meal, with a more "physiological" short duration of action, similar to that seen in non diabetic individuals. Mimicking this early rise in plasma insulin concentrations may have beneficial effects by inhibiting the action of HSL in adipose tissue. This would improve the suppression of NEFA and glycerol which should occur immediately after meals. Substrate delivery to the liver would thereby be minimised, thus reducing VLDL triglyceride production. The downstream consequences of decreased VLDL levels suggests reduced competition for LPL with the exogenous lipid pathway, minimising circulating triglyceride. The duration of insulin secretion may also be fundamental in influencing lipid metabolism. Chronic prolonged insulin exposure to the liver may have adverse effects on VLDL production and secretion (Reaven and Laws 1994) (Gibbons, Brown et al. 2002), emphasizing the importance of minimising late phase insulin secretion and hyperinsulinaemia.

5.2 Aims

1. To determine whether increasing endogenous insulin levels after an oral lipid and carbohydrate challenge can improve lipid handling.

2. To determine whether earlier rather than late augmentation of endogenous insulin secretion after an oral lipid and carbohydrate challenge may be more effective.

5.3 Methods

5.3.1 Study Design

To examine the effect of enhancing endogenous insulin secretion, 2 oral insulin secretatogues licensed in the UK were chosen to compare with placebo. Single doses of these agents were evaluated post oral lipid and carbohydrate challenge (OTTT) in diet treated type 2 diabetic subjects. This three way study was double blinded to minimise bias and of crossover design to reduce the possibility of a carry over of treatment effect or a period effects (Altman 1991). As the antidiabetic agents were of short duration of action and type 2 diabetic subjects were treated by diet alone, a "washout" period was not necessary, however, repeated OTTTs were conducted a minimum of 1 week apart. The study was funded by an unrestricted educational grant from Novartis Pharmaceutical UK Ltd who played no part in the conduct, analysis or interpretation of the study other than to supply medication.

5.3.2 Study Subjects

Following approval by the Oxford Research Ethics Committee (OXREC) and Aylesbury Vale Local Research Ethics Committee (AVLREC), a total of 87 diet treated diabetic patients were identified and approached through general practice and diabetes centre clinics. Twenty one subjects with diet controlled type 2 diabetes were recruited and gave written informed consent. They were male and female, Caucasian, aged 35-75 years, not taking lipid lowering therapy and had no history of established cardiovascular disease. Subjects with type 2 diabetes had been diagnosed according to WHO criteria (WHO 1999) for at least 3 months. Diet only treated diabetic subjects were chosen to ensure the effects seen would be due to the study drug. Subjects were excluded if they had a fasting plasma triglyceride level >5 mmol/l, poorly controlled diabetes (HbA_{1c} > 10%), a major lipid abnormality, gastrointestinal problems eg. delayed gastric emptying, chronic inflammatory bowel disease, any serious underlying medical conditions, a history of alcohol abuse, were pregnant or lactating females. Clinical, anthropometric and safety measurements were taken as outlined in Chapter 2.

5.3.3 Study Medication

Currently available oral hypoglycaemic agents in the UK are insulin secretagogues, insulin sensitizers (biguanides and thiazolidinediones) and α-glucosidase inhibitors. These pharmacological agents have different modes of action on the glycaemic pathways.

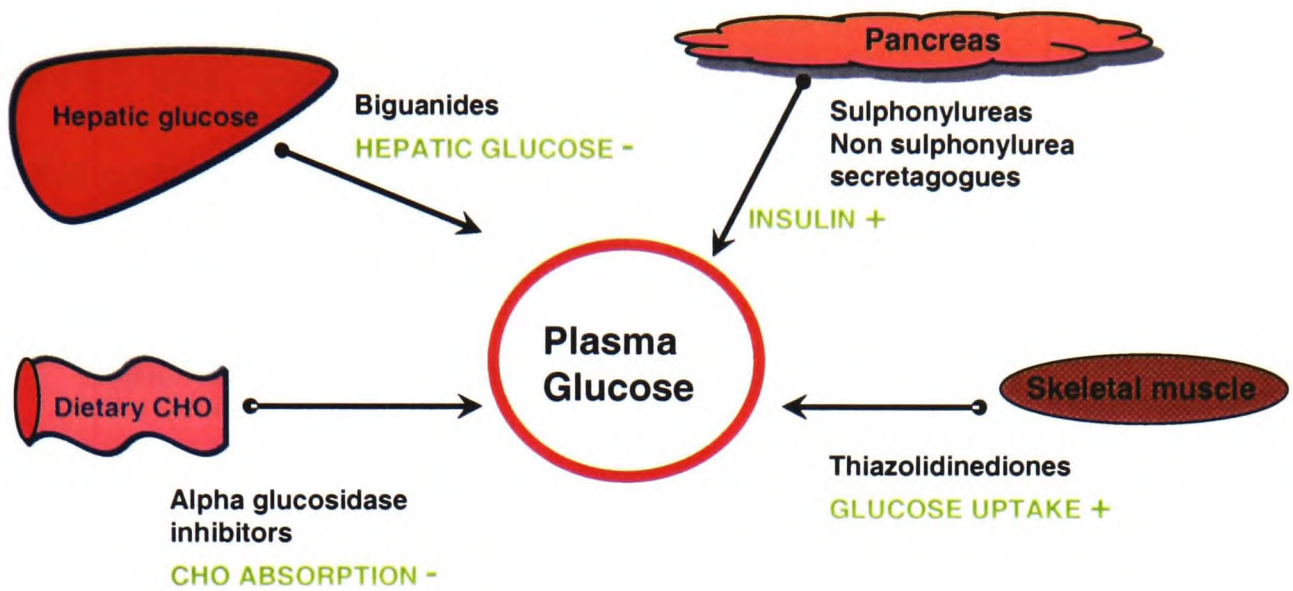


Figure 17 Overview of the main mechanisms of glucose lowering with various pharmacological agents used in the treatment of type 2 diabetes. Adapted from (Inzucchi 2002)

To examine the metabolic effects of enhanced endogenous insulin secretion, sulphonylureas and non sulphonylurea insulin secretagogues were chosen. A relatively short acting sulphonylurea would augment insulin secretion over an 8 hour time period, also covering the late postprandial phase (6 to 8 hours). In comparison, the non sulphonylurea insulin secretagogues were selected to predominately enhance early insulin secretion.

Few studies have been conducted comparing the insulin exposure and glucose lowering effects of various insulin secretagogues. Kahn et al (Kahn, Montgomery et al. 2001) showed that compared with glyburide (glibenclamide) which enhances later phase insulin secretion, nateglinide increased the early insulin response which was associated with an improvement in glucose tolerance. Also the glucose concentration took less time to return to the level prevailing before glucose administration. Glyburide resulted in continued insulin release despite glucose levels returning to baseline, thus further

lowering glucose levels. A larger study by Hollander et al (Hollander, Schwartz et al. 2001) also compared the long term effects of these therapies in type 2 diabetic subjects. They demonstrated nateglinide to provide better mealtime glucose control with less insulin exposure than glyburide. No head to head studies have been conducted comparing the postprandial effects of nateglinide with shorter acting sulphonylureas in diabetic subjects. One animal study using glipizide maintains that nateglinide increases early phase insulin release, compared with which this relatively short acting sulphonylurea, which still increased total insulin release, following a glucose load (de Souza, Gagen et al. 2001).

Sulphonylureas

Sulphonylurea therapy has been the mainstay of glucose lowering treatment for many years. Gliclazide, a commonly used short acting sulphonylurea, is prescribed once or twice daily, and has a peak onset of action from 6 to 10 hours. Gliclazide enhances first phase insulin secretion and also second phase secretion. The total daily dose is between 40-320mg (single dose 160mg). A single dose of 80mg gliclazide was chosen for the study as the typical starting dose for this agent is usually 40 to 80mg. Sulphonylureas enhance the release of insulin containing secretory granules from the pancreatic β cell. They bind to the sulphonylurea receptor thereby closing the ATP sensitive K^+ channels. This increases the ATP:ADP ratio and calcium concentration, with membrane depolarisation and exocytosis of insulin containing granules. Gliclazide is well absorbed with a half life between 10 -12 hours. It is extensively metabolised by the liver, with less than 5% excreted unchanged in the urine. Although much of the mealtime

hyperglycaemia-limiting effect of sulphonylureas is related primarily to the impact of lowering fasting hyperglycaemia, gliclazide appears to blunt late post-meal hyperglycaemia rather than early mealtime glucose excursions. The side effect profile of sulphonylureas includes weight gain, gastrointestinal disturbance, and hypoglycaemia (Landgraf, Bilo et al. 1999; Nattrass 2000).

Non sulphonylurea secretagogues

The non-sulphonylurea secretagogues have been developed to target postprandial hyperglycaemia. To enhance endogenous insulin secretion, these agents bind to the sulphonylurea receptor on the pancreatic β cell and block K-ATP channels. Binding to the K-ATP channels with reduction in membrane K+ permeability, causes membrane depolarization. This activates calcium (Ca2+) influx through voltage dependent Ca2+ channels thereby increasing cytoplasmic calcium. Exocytosis is triggered and insulin secretion occurs similar to the process seen with sulphonylureas. These agents differ from sulphonylurea in that their effect is more rapid and duration of action shorter. Because of this drug induced -hypoglycaemia is less common with non-sulphonylurea secretagogues than with the sulphonylureas (Landgraf, Bilo et al. 1999) (Floyd 1999).

Nateglinide (N-(trans-4-isopropylcyclohexyl-carbonyl)-D-phenylalanine, A-4166 or SDZ DJN 608A-4166), is a new non-sulphonylurea secretagogue which is an amino acid derivative. Nateglinide is rapidly absorbed and has been shown to stimulate primarily first phase insulin secretion within 10 minutes, by synergistic interaction with elevated mealtime plasma glucose concentrations (Bouter, Deijns et al. 1998; Karara, Dunning et

al. 1999; Keilson, Mather et al. 2000). This agent is usually prescribed 60mg to 180mg within 30 minutes prior to main meals up to a maximum 3 times a day. A single dose of 120mg nateglinide was chosen for the study. When administered before meals, the insulin response is rapid, peaking by 2 hours, and is greatest when the glucose concentrations are the highest (Keilson, Mather et al. 2000). This action, although faster than repaglinide, is rapidly reversible. Nateglinide has a half life of 1 – 1.8 hours and time to maximum concentration is 0.5 – 1 hour with greatest effects seen between 1.5 – 4 hours. Nateglinide is extensively metabolised by the liver (cytochrome P450, mixed function oxidase system), with 10% secreted unchanged by the kidney. It is not known to exhibit enterohepatic recycling. Nateglinide and its metabolites are completely and rapidly eliminated. Nateglinide is well tolerated and mild hypoglycaemic symptoms are the most common drug related adverse event.

Randomization and masking of the nateglinide, gliclazide and placebo was performed by Novartis Drug Supply Management using a validated system that automates the randomisation codes for each subject. The randomization scheme was reviewed by the Quality Management Biostatistics Group in Novartis Medical Information Processing and Statistics Department and locked by them after approval. Randomization data was kept strictly confidential, accessible only to authorized persons, until the time of unblinding.

5.3.4 Study Procedure

Subjects underwent the OTTT on 3 separate occasions at least one week apart. On the morning of the first OTTT, subjects were allocated a randomisation code in serial order.

Methodology for the OTTT is outlined in Chapter 2. After baseline blood sampling, the study medication was taken with 50 mL of water. Subjects then waited 15 minutes to enable the study medication to digest/ absorb before consuming the OTTT test drink within a maximum of 10 minutes. All routine medication was continued as prescribed. Samples collected at fasting, 2, 4, 6 and 8 hours were analysed for glucose, insulin, C peptide, lipid profile, NEFA and glycerol according to biochemical methodology highlighted in Chapter 2.

5.3.5 Statistical methodology

Sample size calculations

In the OTTT study (Chapter 4), the mean of all logarithmically transformed 6 hour triglyceride values from subjects undergoing to OTTT was found to be 0.658. The standard deviation of the differences was 0.339. A sample size of 20 subjects for each study would be required to achieve a power of 80% at the 5% significance level. This would be to detect a clinically meaningful 35% relative reduction in 6 hour post challenge triglyceride levels (Robins, Collins et al. 2001).

Data Analysis

Statistical methodology regarding data checking and analysis as well as derived variables is summarised in Chapter 2. IAUC to 4 hours (as well as IAUC to 8 hours) was calculated in order to enable assessment of differences between study medications in the early postprandial period.

The analysis plan was to compare nateglinide with placebo administration and gliclazide with placebo administration. Paired t tests and Wilcoxon matched pairs signed rank sum test were used to compare parametric and non parametric data respectively from placebo with nateglinide and with gliclazide.

5.4 Results

5.4.1 Subjects

A total of 20 subjects completed both tests. One subject who withdrew, due to a change in social circumstances, was replaced. Mean (SD) age was 59.4 (10.7) years, BMI 32.2 (5.5) kg/m^2, and HbA1c 6.9 (1.0)%. Subject characteristics are outlined in Table 12. Mild hypoglycaemia occurred in 3 subjects at mean (SD) 3.4 (0.5) hours after taking nateglinide. In each case the OTTT was stopped and subjects given a meal. Data presented are with these subjects excluded although there was no substantial difference when these subjects were included in the analysis. The apo E 3/3 genotype was predominant with no subjects having the 2/2 genotype. The fasting lipid profile was within the normal range for a non diabetic population. No significant differences were found comparing baseline triglyceride, NEFA, glycerol, glucose, insulin or C peptide levels prior to placebo, nateglinide or gliclazide administration.

Table 11 Subject Characteristics of type 2 diabetic subjects

n	20
Sex (M/F)*	13/7
Age (years)	59.4 (10.7)
Duration of diabetes (years) †	1.0 (0.25 – 2.25)
BMI (kg/m^2)	32.2 (5.5)
Waist (cm)	103.3 (10.6)
Triceps skinfold thickness (mm)	16.3 (9.8)
BP (mm Hg)	
Systolic	130 (18.8)
Diastolic	81.3 (9.4)
Smoking (%)	
Non smoker (n=11)	55
Ex smoker (n=8)	40
Smoker (n=1)	5
Hormone use (%) (n=2)	10
Post menopausal (%) (n=3)	43
Apo E genotype *	
3/3	15
2/3	3
2/4	2
HbA1c (%)	6.9 (1.0)
Fasting plasma glucose (mmol/l)	8.0 (2.1)
Fasting total cholesterol (mmol/l)	4.5 (0.9)
Fasting LDL cholesterol (mmol/l)	3.0 (0.6)
Fasting HDL cholesterol (mmol/l)	1.13 (0.22)
Fasting VLDL cholesterol (mmol/l)	0.50 (0.34)
Fasting triglyceride (mmol/l)*	1.30 (0.69-2.46)

Data are mean (SD) except †median (IQR) and * geometric mean (1 SD range)

5.4.2 Post challenge profiles

Triglyceride

There was no significant difference in 6 hour post challenge geometric mean (1 SD range) triglyceride levels between placebo 1.95 (1.11 – 3.40) with both nateglinide 2.16 (1.35 – 3.47) mmol/l (p = 0.5429) or gliclazide 1.91 (1.13 – 3.23) mmol/l (p = 0.7809) (Table 12, Figure 18). Also no difference was found in IAUC or delta Cmax when

comparing nateglinide and gliclazide with placebo. Tmax for triglyceride was 6 hours for all study medications.

NEFA

The 2 hour post challenge NEFA concentrations did not differ for placebo compared with both nateglinide and gliclazide (Table 12, Figure 18). However, the IAUC NEFA to 4 hours was significantly reduced with nateglinide ($p = 0.0245$) compared with placebo. The 8 hour post challenge NEFA concentration was also significantly higher with nateglinide compared with placebo, with Tmax at 8 hours and 6 hours respectively. No significant differences were noted between placebo and gliclazide NEFA results.

Glycerol

No significant differences were found comparing median (IQR) glycerol levels (mmol/l) between placebo with nateglinide or gliclazide at 2 hours 0.73 (0.478 – 0.983), 0.74 (0.532 – 0.923) and 0.71 (0.429 – 1.086) respectively (Table 12, Figure 18). However the delta 2 hour glycerol level and IAUC to 4 hours was reduced with nateglinide ($p = 0.0495$ and $p = 0.0442$ respectively). Glycerol results for placebo and gliclazide were similar.

Glucose

Nateglinide significantly reduced 2 hour post challenge glucose concentrations compared with placebo, median (IQR) delta 2 hour mmol/l 2.8 (1.03 – 4.75) vs 5.35 (2.85 – 6.35) respectively ($p=0.0004$) (Figure 19). IAUC and Cmax were also significantly lowered

with nateglinide therapy. No significant differences in glucose concentrations were found between gliclazide and placebo.

Insulin

The 2 hour geometric mean (1 SD range) insulin levels (pmol/l) placebo 312.7 (154.9 – 631.5), nateglinide 398 (199.5 – 797.3) and gliclazide 307.4 (159.0 – 594.1) also were not significantly different. However, IAUC insulin for the first 4 hours was significantly increased compared with placebo by nateglinide ($p = 0.0299$) but not gliclazide. No significant differences were found in Cmax or Tmax when comparing both nateglinide and gliclazide with baseline (Table 12, Figure 19).

C peptide

C peptide levels were higher with nateglinide administration compared with placebo at 2 hours (median (IQR) delta 2 hour nmol/l 1.80 (1.03 – 2.35) vs 1.16 (1.02 – 1.71) respectively) ($p = 0.0031$), but no increase was seen with gliclazide (median (IQR) delta 2 hour C peptide level 1.33 (1.13 – 2.07) nmol/l) ($p = 0.1074$) (Table 12, Figure 19). IAUC and Cmax for C peptide were also significantly greater comparing nateglinide with placebo. There were no significant differences in C peptide levels between gliclazide and placebo.

Table 12 Fasting & post challenge OTTT responses to placebo, nateglinide & gliclazide.

	Placebo		Nateg linide		*p*	Glicl azide		*p*
Triglyceride (mmol/l)								
0 hour	1.30	0.685 -2.456	1.52	0.987 -2.349	*0.377*	1.33	0.788 -2.256	*0.7519*
2 hours	1.67	0.899 -3.106	1.89	1.263 -2.825		1.70	1.025 -2.817	
4 hours	1.73	0.928 -3.215	2.02	1.288 -3.177		1.74	1.053 -2.859	
6 hours	1.95	1.114 -3.404	2.16	1.349 -3.473	*0.9644*	1.91	1.126 -3.234	*0.7809*
8 hours	1.39	0.796 -2.418	1.72	1.049 -2.808		1.43	0.800 -2.540	
AUC^{+}	13.59	7.705 -23.968	15.63	10.421 -23.442	*0.4309*	13.63	8.377 -22.186	*0.9659*
$IAUC_{0\text{-}6hours}$*$^{+}$	2.36	1.530 -3.690	2.29	1.883 -3.550	*0.7928*	2.29	1.725 -2.845	*0.3901*
$IAUC_{0\text{-}8hours}$*$^{+}$	3.20	1.835 -4.280	3.21	2.108 -4.650	*0.6518*	3.13	2.090 -3.925	*0.5746*
Cmax	2.14	1.246 -3.686	2.47	1.682 -3.616	*0.989*	2.15	1.346 -3.434	*0.9112*
deltaCmax	0.77	0.455 -1.311	0.87	0.529 -1.442	*0.5004*	0.74	0.406 -1.337	*0.7414*
Tmax*±	6	4 -6	6	4 -6	*0.7879*	6	4 -6	*0.7715*
6-0 hours*	0.63	0.335 -0.780	0.67	0.360 -1.170	*0.9152*	0.61	0.190 -0.875	*0.9985*
NEFA (umol/l)								
0 hour	485.5	282.85 -833.21	569.6	378.95 -856.05	*0.069*	525.1	315.99 -872.69	*0.5331*
2 hours	168.8	93.05 -306.19	152.6	73.36 -317.50	*0.3538*	176.2	84.56 -366.94	*0.7259*
4 hours	249.6	138.22 -450.63	275.7	134.64 -564.70		253.5	150.98 -425.80	
6 hours	600.0	445.85 -807.37	667.7	497.69 -895.87		590.4	386.44 -902.02	
8 hours	666.2	485.99 -913.36	776.3	609.05 -989.43	*0.0059*	620.4	414.94 -927.49	*0.364*
AUC^{+}	3372.8	2612.98 -4353.60	3798.5	183310.98 283365.24	*0.072*	3395.2	2527.75 -4560.22	*0.7188*
$IAUC_{0\text{-}4hours}$*$^{+}$	-764.5	-1370.00 - -338.5	-950.0	-1430.25 - -638.25	*0.0245*	-799.0	-1218.00 - -593.50	*0.3314*
$IAUC_{0\text{-}8hours}$*$^{+}$	-473.0	-1511.00 -167.00	-577.0	-1878.50 -167.25	*0.4483*	-746.5	-2183.75 - -64.25	*0.3132*
Cmin	153.9	86.94 -272.59	137.9	1.15 -276.40	*0.3928*	157.9	78.90 -316.16	*0.8429*
Cmax	731.6	537.20 -996.24	849.5	646.99 -1115.49	*0.0232*	748.8	562.61 -996.52	*0.4462*
Tmax*±	6	6 -8	8	6 -8	*0.4144*	6	0 -8	*0.304*
2-0 hours*	-256.5	-483.00 - -162.0	-384.0	-468.00 - -306.5	*0.0615*	-305.0	-453.50 - -185.50	*0.3901*
8-0 hours*	99	-106.25 -195.50	212	65.00 -351.00	*0.4625*	65.5	26.00 -357.25	*0.411*
Glycerol (mmol/l)*								
0 hour	0.78	0.576 -1.162	0.85	0.673 -1.227	*0.1476*	0.83	0.586 -1.127	*0.7484*
2 hours	0.73	0.478 -0.983	0.74	0.532 -0.923	*0.2761*	0.71	0.429 -1.086	*0.6481*
4 hours	0.87	0.706 -1.108	0.86	0.597 -1.194		0.78	0.627 -1.023	
6 hours	1.07	0.899 -1.480	1.26	1.064 -1.477		1.10	0.847 -1.463	
8 hours	0.90	0.744 -1.213	0.96	0.793 -1.129	*0.6861*	1.03	0.706 -1.173	*0.7158*
AUC^{+}	7.16	6.382 -9.502	7.78	6.494 -8.839	*0.4937*	7.10	5.671 -9.819	*0.5347*
$IAUC_{0\text{-}4hours}$$^{+}$	0.02	-0.808 -0.928	-0.29	-1.343 -0.388	*0.0442*	-0.15	-1.173 -0.320	*0.7006*
$IAUC_{0\text{-}8hours}$$^{+}$	0.80	-0.022 -2.297	0.57	-1.905 -1.887	*0.1075*	0.60	-0.977 -1.743	*0.3132*
Cmin	0.56	0.424 -0.744	0.60	0.391 -0.814	*0.9152*	0.58	0.429 -0.755	*0.9914*
Cmax	1.28	1.129 -1.927	1.48	1.260 -1.781	*0.5853*	1.26	1.072 -1.955	*0.8669*
Tmax±	6	3 -6	6	4 -6	*0.8784*	6	4 -6	*0.8763*
2-0 hours	-0.07	-0.280 -0.180	-0.18	-0.620 -0.060	*0.0495*	-0.16	-0.445 -0.065	*0.9368*
8-0 hours	0.04	-0.212 -0.269	0.08	-0.163 -0.293	*0.3435*	0.18	-0.035 -0.356	*0.2683*

Continued Table 12 Fasting & post challenge OTTT responses to placebo, nateglinide & gliclazide

	Placebo		Nateg linide		*p*	Glic lazide		*p*
Glucose (mmol/l)								
0 hour	8.0	2.07	8.3	1.98	*0.8843*	7.8	1.84	*0.1356*
2 hours	12.7	3.41	11.0	3.24	*0.0004*	12.3	2.64	*0.4135*
4 hours	6.6	2.75	6.5	2.64		6.5	2.48	
6 hours	5.5	1.62	5.4	1.45		5.4	1.49	
8 hours	5.6	1.07	5.8	1.25		5.4	1.03	
AUC^{+}	63.1	16.55	60.0	14.81	*0.0033*	61.5	14.42	*0.2878*
$IAUC_{0\text{-}4hours}$*$^{+}$	9.1	3.65 -11.35	3.2	1.68 -7.15	*0.0004*	7.9	5.80 -11.50	*0.9438*
$IAUC_{0\text{-}8hours}$*$^{+}$	-2.1	-4.35 -2.40	-7.8	-10.18 - -2.38	*0.0016*	-0.2	-4.05 -3.50	*0.6659*
Cmax	12.7	3.39	11.3	2.91	*0.0011*	12.3	2.64	*0.3918*
Cmin	5.3	1.35	5.0	1.39	*0.0742*	5.0	1.21	*0.1874*
Tmax*±	2	2 -2	2	2 -2	*0.5795*	2	2 -2	*0.3299*
Tmin*±	6	4 -6	6	4 -6	*0.8976*	6	4 -6	*0.7624*
2-0 hours*	5.4	2.85 -6.35	2.8	1.03 -4.75	*0.0004*	4.4	3.40 -6.10	*0.7623*
Insulin (pmol/l)								
0 hour	112.5	67.50 -187.51	117.2	70.02 -196.15	*0.6277*	98.5	57.36 -169.01	*0.3555*
2 hours	312.7	154.88 -631.48	398.8	199.49 -797.28	*0.1497*	307.4	159.05 -594.12	*0.9004*
4 hours	168.0	88.74 -317.94	226.6	102.94 -498.67		149.9	73.50 -305.70	
6 hours	107.1	59.86 -191.64	117.5	67.28 -205.06		103.5	57.26 -186.99	
8 hours	89.9	52.67 -153.59	99.1	60.92 -161.17		79.3	47.51 -32.34	
AUC^{+}	1439.2	825.82 -2508.27	1778.4	966.80 -3271.27	*0.1347*	1342.1	749.28 -2404.00	*0.5107*
$IAUC_{0\text{-}4hours}$*$^{+}$	598.9	221.20 -918.90	610.5	370.20 -1227.90	*0.0299*	482.3	236.85 -938.45	*0.9333*
$IAUC_{0\text{-}8hours}$*$^{+}$	422.8	168.23 -1019.43	819.6	413.90 -1519.80	*0.0557*	534.2	211.55 -981.80	*0.9015*
Cmax	323.4	170.64 -612.76	7.0	212.62 -818.87	*0.1943*	307.4	159.05 -594.12	*0.742*
Tmax*±	2	2 -2	2	2 -2	*0.1881*	2	2 -2	*0.1628*
2-0 hours*	271.7	117.20 -387.40	278.3	112.30 -553.40	*0.0879*	217.6	102.75 -385.85	*0.8385*
C peptide (nmol/l)								
0 hour	0.93	0.611 -1.418	0.99	0.703 -1.384	*0.3595*	0.88	0.616 -1.259	*0.1761*
2 hours	2.26	1.695 -3.007	2.72	1.846 -3.995	*0.0018*	2.35	1.675 -3.311	*0.232*
4 hours	1.52	0.946 -2.425	1.77	1.008 -3.095		1.54	0.927 -2.571	
6 hours	0.98	0.648 -1.472	1.09	0.678 -1.746		1.05	0.681 -1.616	
8 hours	0.83	0.570 -1.212	0.86	0.560 -1.323		0.82	0.543 -1.225	
AUC^{+}	11.44	8.174 -15.997	13.24	8.841 -19.827	*0.0688*	12.02	8.332 -17.341	*0.6453*
$IAUC_{0\text{-}4hours}$*$^{+}$	2.78	2.335 -4.265	4.86	2.930 -6.285	*0.0019*	3.41	2.760 -5.235	*0.1258*
$IAUC_{0\text{-}8hours}$*$^{+}$	3.29	2.585 -4.820	6.09	3.910 -7.923	*0.0056*	5.14	3.448 -6.538	*0.2597*
Cmax	2.29	1.704 -3.077	2.77	1.899 -4.028	*0.0021*	2.35	1.675 -3.311	*0.5918*
Tmax*±	2	2 -2	2	2 -2	*0.1985*	2	2 -2	*0.1625*
2-0 hours*	1.16	1.020 -1.710	1.80	1.030 -2.350	*0.0031*	1.33	1.125 -2.065	*0.1074*

Data are geometric mean (1 SD range) except † mean (SD) and * median (IQR).
Units are as tabulated except + units.hour and ± hours

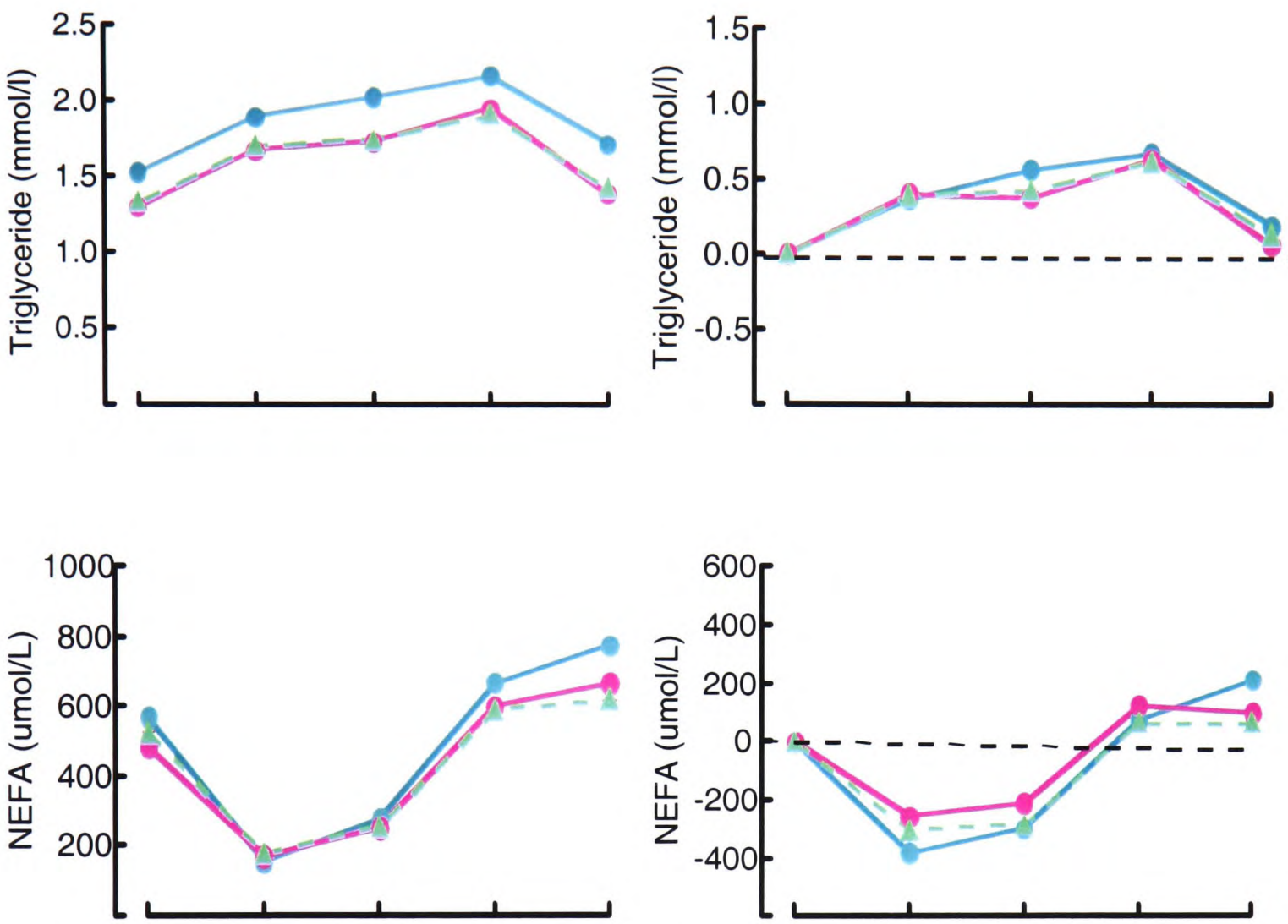


Figure 18 Triglyceride, NEFA and glycerol profiles for placebo ●, nateglinide ● and gliclazide ▲. Absolute values are depicted on the left, and change from baseline (delta) on the right.

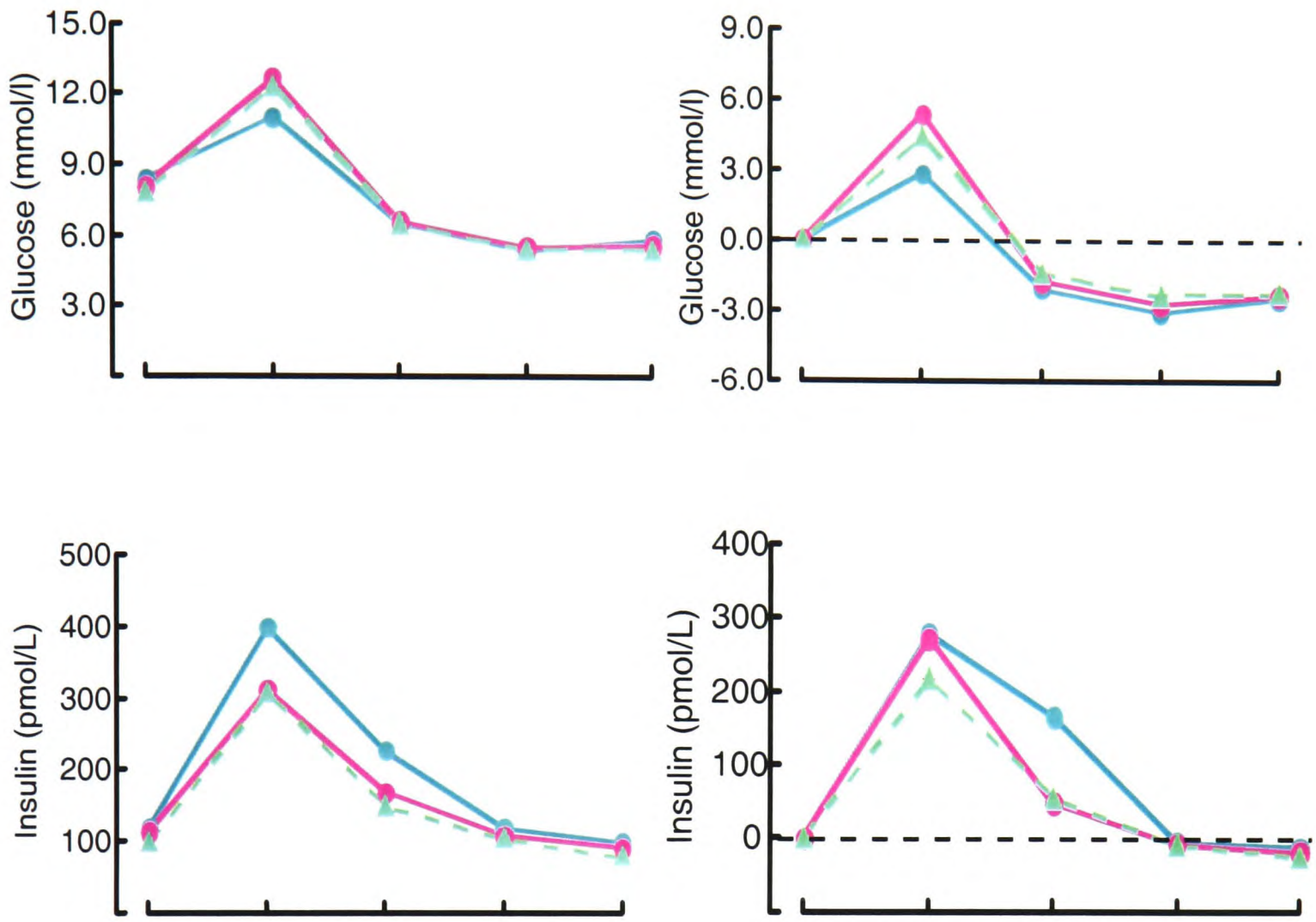


Figure 19 Glucose, insulin and C peptide profiles for placebo ●, nateglinide ● and gliclazide ▲. Absolute values are depicted on the left, and change from baseline (delta) on the right.

5.4.3 Gliclazide Results

Post challenge insulin and glucose levels were not influenced by gliclazide, with this drug displaying almost identical profiles to the placebo arm. Also no downstream differential effects on lipid metabolism compared with placebo were observed. Various steps were taken to further investigate the similarity between gliclazide and placebo results for post challenge glucose and insulin levels in view that correct procedural issues had been undertaken regarding the randomisation and unblinding of the study. This can also be concluded from the glucose lowering effect and insulin response seen with nateglinide administration in our study. Review of the literature reveals only one study by Schweizer et al (Schweizer, Ball et al. 2002), comparing the effects of one week's therapy using nateglinide, gliclazide and placebo on postprandial glucose and insulin profiles in 23 diet treated type 2 diabetic subjects. This group demonstrated that after a meal containing 73.6% carbohydrate, 11.6% protein and 14.8% fat, post meal glucose was reduced by both nateglinide and gliclazide compared with placebo. Nateglinide however was more effective than gliclazide in controlling postprandial hyperglycaemia.

Although both nateglinide and gliclazide are considered to be relatively short acting in comparison to other oral hypoglycaemic agents, their pattern of effect on glucose levels during the postprandial period is distinct. Schweizer et al (Schweizer, Ball et al. 2002) found significant differences in postprandial glucose levels at 2 hours with each therapy compared with placebo: nateglinide 5.83 ± 1.895 mmol/l, gliclazide 7.21 ± 2.255 mmol/l and placebo 8.09 ± 2.432 mmol/l. Also, gliclazide continued to decrease glucose levels up to 7 hours post meal, whilst glucose levels in those taking nateglinide, although

reduced at 2 hours, became comparable to placebo by 5 hours post meal. In our study, 2 hour glucose levels were statistically significant between nateglinide and placebo arms with profiles becoming similar by the 4 hour timepoint. Glucose levels from the gliclazide arm in our study, showed no reduction at 2 hours, but more significantly, showed no difference to placebo even at the 4 and 6 hour timepoints, as would be expected. Davis et al (Davis, Daly et al. 2000) too, showed that the maximal hypoglycaemic effect of gliclazide, taken by type 2 diabetic subjects for at least 4 weeks prior to the test meal challenge, was evident at 2 to 3 hours post meal (Tmax glucose 2.2 ± 0.3 hours). Studies investigating the acute effect of gliclazide in drug naïve type 2 diabetic subjects have also shown the maximal glucose lowering effect should be evident at 2 hours post challenge with significant lower incremental area above baseline to 4 hours (Riccio, Lisato et al. 1996).

The corresponding glucose stimulated insulin secretory pattern was also evaluated in the above mentioned studies. The study by Schweizer et al (Schweizer, Ball et al. 2002) showed that nateglinide resulted in an enhanced and earlier response compared to placebo (net integral concentrations pmol/l/hour 1081.2 ± 735.25 vs 632.3 ± 471.73 respectively), while gliclazide only had a very small effect on early insulin release (845.2 ± 571.71). They concluded that gliclazide mainly affects the second phase insulin response, which could be one possible explanation for the similarity between placebo and gliclazide insulin profiles seen in our study. Yet Davis et al (Davis, Daly et al. 2000) did find a corresponding increase in insulin levels, Tmax 2.4± 0.5 hours, after a minimum of 4 weeks gliclazide administration.

To further explore the mechanisms of acute and chronic hypoglycaemic action of gliclazide in type 2 diabetic subjects an article by da Tos et al (da Tos, Maran et al. 2000) is highlighted. To study the acute effects they gave 160 mg of gliclazide or placebo after a 4 hour intravenous glucose infusion with (3-^{3}H)glucose to 7 type 2 diabetic subjects. Gliclazide treatment 80 mg three times a day was then reassessed after 2 months. After acute administration of gliclazide, endogenous glucose production was suppressed despite no comparable increase in plasma insulin and C peptide levels. It was only after 2 months of therapy that the effect of insulin on the suppression of endogenous glucose production became more evident, because of the concomitant improvement of insulin secretion. Although double the dose of gliclazide was used in the "acute" study compared with our study, we would still have expected to see a reduction of glucose in a drug naïve type 2 diabetic population.

Clamp studies have also been conducted in type 2 diabetic subjects before and after a 3 to 6 week course of gliclazide 80mg (median daily dose) in order to study the effect of sulphonylurea therapy on the glucose-insulin stimulus-response curve (Hosker, Rudenski et al. 1989). Before gliclazide therapy, diabetic subjects displayed decreased first and second phase insulin and C peptide responses. The authors found C peptide levels to increase to near normal after gliclazide therapy for both first and second phases. The first phase insulin response was approximately doubled by gliclazide, with little difference in second phase compared with pre treatment, which showed only a modest non significant

increase. Again our study, demonstrated no difference in C peptide levels between placebo and gliclazide groups.

A possible explanation for the lack of glucose reduction seen in our chosen timepoints, was the timing of gliclazide administration. Batch et al (Batch, Ma et al. 1990) looked at the relationship between ingestion time of gliclazide to meals on plasma glucose, insulin and C peptide levels. Gliclazide was given 30 minutes before, at the start of, 30 minutes after breakfast, (or omitted altogether) in 10 type 2 diabetic subjects. No significant difference was noted in plasma glucose, insulin or C peptide patterns with any of the gliclazide administration protocols, and these authors concluded that in clinical practice, the timing of gliclazide ingestion in relation to meals is not critical. Therefore, the administration of gliclazide 15 minutes prior to giving the OTTT test drink should not have interfered with its effect.

Gliclazide assays, although not part of the original protocol, were required to quantify drug absorption. Studies describing the efficacy of gliclazide have shown that a serum gliclazide concentration of 1.5 mg/l represents the threshold for maximal hypoglycaemic effect (Campbell, Adrianessens et al. 1980). To determine which timepoint best reflects maximum gliclazide concentrations achieved for analysis, the pharmacokinetics and pharmacodynamics of gliclazide were investigated. Gliclazide kinetics in Caucasian type 2 diabetic subjects was assessed as part of a study comparing this population to Australian Aboriginal type 2 diabetic individuals (Davis, Daly et al. 2000). The Caucasian type 2 diabetic subjects (n=9) were included in the study if they had been

taking gliclazide 80mg twice daily as the sole treatment for their diabetes for at least 4 weeks before recruitment. Samples were collected before and after a standard breakfast (66g carbohydrate, 21g fat and 19g protein, 2228kJ). The time to reach maximum (Tmax) for gliclazide was approximately 2 hours. When the authors compared this result with previous studies using 80mg dose of gliclazide, and proposed that pharmacokinetic properties such as Tmax, were not concentration dependent. The influence of gastric emptying was also investigated, as gender and BMI differences have been demonstrated (Brogna, Ferrara et al. 1998). No association between Tmax and gender or BMI was found. Hyperinsulinaemia, however, was shown to be a significant positive predictor of Tmax, which is consistent with the literature. As the subjects in our study acted as their own control and with no significant differences in baseline insulin levels detected between the treatment groups, it was expected the Tmax gliclazide concentration to be at 2 hours. Post challenge 2 hour EDTA reserve samples from the gliclazide arm were sent aliased for analysis in an independent laboratory and showed no gliclazide to be detected.

It is therefore possible that the OTTT test drink, having a high fat content, may actually inhibit gliclazide absorption. Fat content modifying the absorption of nutrients has been shown in previous studies (Westphal, Leodolter et al. 2002). To demonstrate that the OTTT test drink may interfere with the absorption of gliclazide, the gliclazide arm of the study was replicated in a non-diabetic subject. Gliclazide was obtained by prescription from the hospital pharmacy. Again samples were sent aliased to the laboratory for gliclazide analysis (Table 13, Figure 20).

Table 13 Table of fasting and post challenge results from a non diabetic subject taking a single dose of gliclazide

Time (hours)	0	2	4	6	8
Triglyceride (mmol/l)	0.81	1.07	0.92	0.71	
Glucose (mmol/l)	4.8	4.5	3.6	4	
Insulin (pmol/l)	15	311.6	53.4	47.4	
Gliclazide (mg/L)*	0	1.4	7.3	6.8	4.4

* Limit of detection for gliclazide assay is 0.1 mg/L, Guy's Hospital, London.

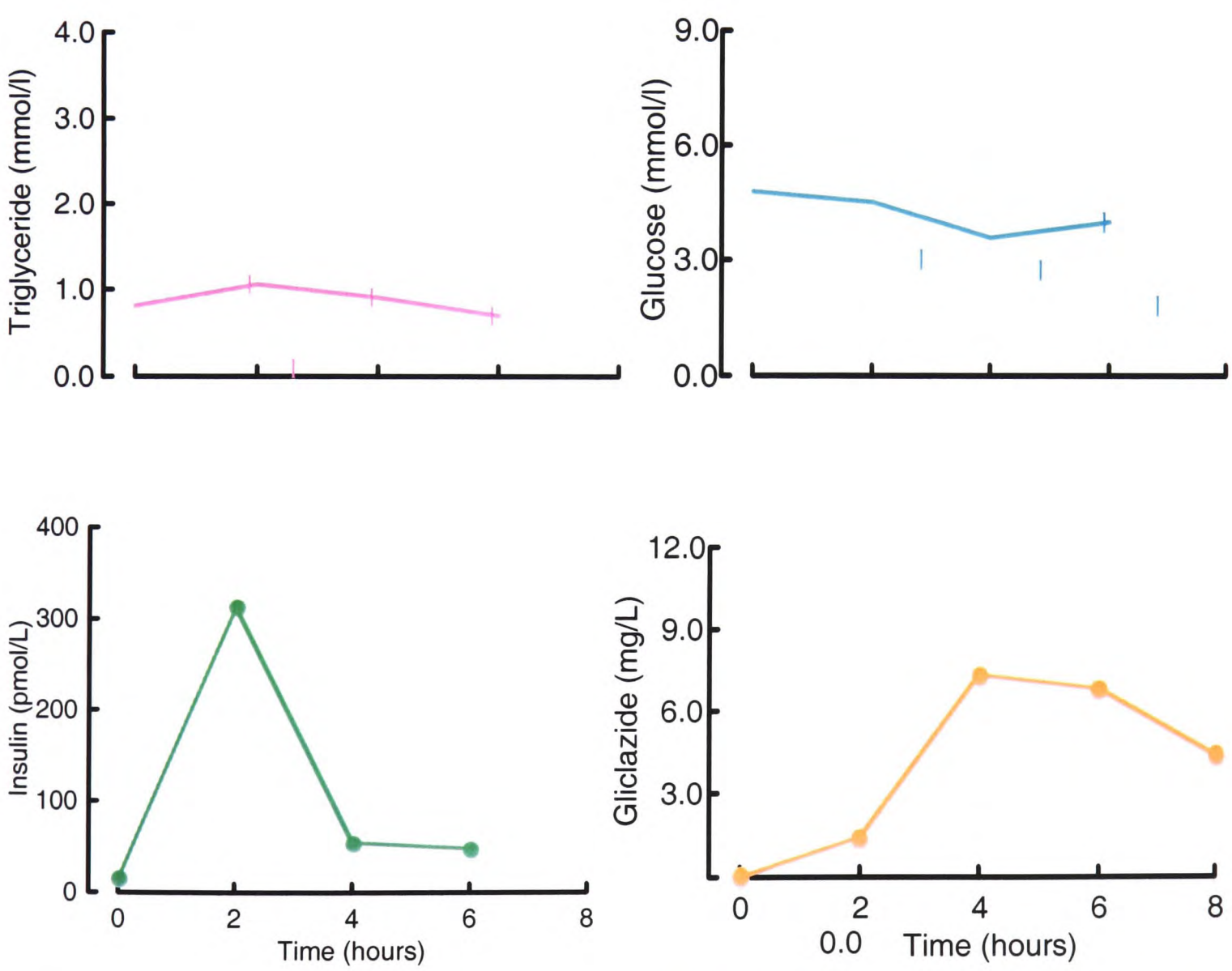


Figure 20 Graphs of post challenge triglyceride ●, glucose ●, insulin ● and gliclazide ● results after taking gliclazide.

The results showed that gliclazide was detected and the OTTT test drink did not materially interfere with drug absorption. To date there is no explanation for the absence of gliclazide in our study, as packaging and labelling procedures had been checked rigorously by the company. As the gliclazide was "inactive" we have the opportunity to investigate the reproducibility of post challenge profiles. The remainder of the discussion and thesis will therefore be based upon results from nateglinide and placebo arms only.

The median (IQR) CV (%) for triglyceride at 6 hours was 10.5 (5.7-19.5) and the 2 hour CV for mean (SD) glucose was 9.2 (7.7)% (Table 14).

Table 14 Coefficient of Variation between gliclazide and placebo profiles

		CV (%)		
Triglyceride	0 hour	15.1	5.0-	29.4
	6 hour	10.5	5.7-	19.5
Glucose*	0 hour	5.6	4.5	
	2 hour	9.2	7.7	

Data are median CV (IQR)% except * which is mean CV (SD)%

5.4.4 Correlations

Similar to previous studies, in this study postprandial triglyceride concentrations were highly correlated with fasting triglyceride levels for both nateglinide and placebo (Table 15).

Table 15 Correlation coefficients for fasting with post challenge triglyceride levels

	Placebo		Nateglinide	
	r	p	r	p
2 hour	0.9229	<0.0001	0.9053	<0.0001
4 hour	0.9099	<0.0001	0.9046	<0.0001
6 hour	0.9326	<0.0001	0.7934	<0.0001
8 hour	0.9012	<0.0001	0.7673	0.0003

No significant correlations were found between fasting or delta 6 hour post challenge triglyceride levels with fasting or delta 2 hour post challenge insulin/glucose levels for placebo or nateglinide arms. The delta 6 hour but not fasting triglyceride concentration correlated with systolic blood pressure ($r = 0.6292$, $p = 0.003$) for placebo.

5.5 Discussion

The results show that nateglinide reduces postprandial glucose excursions in type 2 diabetic subjects through enhancement of early prandial insulin secretion with possible antilipolytic effects. The apparent failure to provide active gliclazide meant that the gliclazide and placebo arms of the study where essentially identical. It is not therefore possible to examine the potential effects of a longer acting insulin secretagogue here.

It is important to compare our results with previous studies using nateglinide to assess the degree of endogenous insulin increase and glucose reduction, in order to interpret downstream antilipolytic effects. We demonstrated that nateglinide reduced the 2 hour post challenge glucose excursions by 48%, which equated to approximately a 2 mmol/l decrease in 2 hour glucose levels. Previous studies have demonstrated the glucose lowering effect of nateglinide. Gribble et al (Gribble, Manley et al. 2001) showed that a single dose of nateglinide 120mg administered to type 2 diabetic subjects with fasting hyperglycaemia increased insulin secretion and reduced postprandial glucose excursions. The mean increase in plasma glucose after placebo (2.9 (2.3 -3.6)) mmol/l was reduced

by 1.8 mmol/l with nateglinide comparable to our study. Although overall glucose IAUC was significantly decreased with nateglinide, this reduction was seen primarily during the early post challenge period (0 to 4 hours), with little difference in glucose levels from placebo from 4 to 8 hours. Subsequently, 3 subjects experienced mild hypoglycaemia during the early post challenge period. Hanefeld et al (Hanefeld, Bouter et al. 2000) confirmed hypoglycaemia in 3 patients in a study of 289 diet controlled diabetes patients randomised to nateglinide. Other studies of nateglinide have not experienced any hypoglycaemic episodes (Keilson, Mather et al. 2000).

In our study, insulin IAUC in the first 4 hours post challenge appeared to be greater with nateglinide than placebo although no statistical difference was found at the 2 hour timepoint (2.8 fold increase in insulin concentration with placebo and 3.4 fold with nateglinide). This probably reflects the fact that peak insulin levels after nateglinide occur earlier, and would not therefore be apparent using the two hourly sampling interval used for our OTTT. Gribble et al (Gribble, Manley et al. 2001) demonstrated an increase in insulin by 5.1 fold with a single dose of nateglinide compared with 3.3 fold with placebo seen in the first 90 minutes, however by 3 hours insulin concentrations decrease similar to placebo. Other studies have also shown nateglinide to significantly increase insulin secretion in type 2 diabetic patients in particular restoring the first phase insulin response (Walter, Spratt et al. 2000; Whitelaw, Clark et al. 2000) (Hu, Boettcher et al. 2003). We would hypothesize a significant difference would have been reached should we have measured between the 0 to 2 hour timepoint. Our results are in agreement with Kalbag et al (Kalbag, Walter et al. 2001) who also showed a 3 fold increase in the rate of

insulin secretion despite 2 hour post meal insulin concentrations being similar. Keilson et al (Keilson, Mather et al. 2000) however found the maximum change in insulin levels from baseline after 7 days administration of 120mg nateglinide to be at the 2 hour timepoint.

We demonstrated a single dose of nateglinide enhances NEFA and glycerol suppression. The relationship between fatty acid and glucose metabolism has been previously investigated in few studies. Hollander et al (Hollander, Schwartz et al. 2001) showed in a 8 week study in patients with type 2 diabetes, nateglinide reduced mealtime glucose excursions particularly with breakfast and conclude this may relate to the influence of fatty acids on glucose metabolism. Elevated NEFA levels have been shown to enhance hepatic gluconeogenesis and inhibit glucose uptake and utilization in insulin sensitive tissues (Boden and Shulman 2002). At breakfast time, fatty acid levels are higher than any other time of the day. Our results show that nateglinide decreases NEFA and glycerol IAUC during the first 4 hours post challenge, most likely due to the rapid effect of insulin in suppressing HSL activity in adipose tissue. This enzyme is responsible for lipolysis in the fasting state, maintaining a high level of NEFA in the circulation. The endogenous enhancement of insulin secretion increases NEFA suppression which thus aids in diminishing hepatic glucose production and also improves peripheral glucose uptake and utilization.

The decrease in NEFA and glycerol concentrations during the early post challenge period reduces the supply of substrate for VLDL production in the liver. However no difference

was seen in post challenge triglyceride profiles comparing nateglinide with placebo. Hanefeld et al (Hanefeld, Bouter et al. 2000) also demonstrated that after a liquid meal challenge, nateglinide had no effect on triglyceride levels. However, the test meal contained less fat than the OTTT test drink and post challenge levels were only measured to 4 hours. A recently published study comparing the effects of nateglinide and glibenclamide on postprandial lipaemia, also demonstrated improved NEFA suppression with nateglinide but not with glibenclamide (Vakkilainen, Mero et al. 2002). However, no effect on 8-hour post challenge triglyceride excursions (IAUC) was demonstrated. The authors proposed that the recommended doses of oral insulinotropic agents did not sufficiently reduce NEFA levels to attenuate hepatic VLDL production.

After nateglinide administration, NEFA levels were significantly higher than placebo at 8 hours. This most likely reflects the action of insulin diminishing, with uninhibited lipolysis generating a rise in NEFA concentration. Glucose stimulated insulin secretion in type 2 diabetic subjects is thought to be partially dependent upon this late elevation (Boden G, Chen X et al. 1995). It has been demonstrated in fasting obese subjects that reduced glucose stimulated insulin secretion occurred if their NEFA levels were lowered (by nicotinic acid), whilst no effect was found in non obese subjects (Dobbins R L, Chester M W et al. 1998).

It is possible that a single dose of nateglinide may not be enough to demonstrate antilipolytic effects. However, since completing the study, Vakkilainen et al (Vakkilainen, Mero et al. 2002) published a 12 week study with the drug and found

similar results although the study design and subject inclusion criteria were different. It is possible that further influences of nateglinide on lipid metabolism may be demonstrated in other tissues which we did not measure. Dimitriadis et al (Dimitriadis, Boutati et al. 2002) showed NEFA flux in anterior abdominal subcutaneous adipose tissue to be lower post challenge, after nateglinide than glibenclamide.

We found total insulin IAUC, although mainly in the first 4 hours post challenge, to be higher with nateglinide for the 8-hour study duration. The elevated 8-hour C peptide profile confirms the overall enhancement in endogenous insulin production compared with placebo. Most previous studies have demonstrated insulin concentrations with nateglinide or placebo to return to baseline by 4 to 5 hours post challenge (Kahn, Montgomery et al. 2001). Our results show insulin levels for both nateglinide and placebo did not return to baseline until 6 hours. Walter et al (Walter, Spratt et al. 2000) found 120mg of nateglinide administered with a bedtime snack actually maintained insulin raising and glucose lowering effects overnight. These findings of prolonged insulin secretion may relate to the high fat content of the OTTT test drink perhaps slowing the absorption of carbohydrate and its release (Westphal, Leodolter et al. 2002) stimulating continued insulin secretion. In agreement, Vakkilainen et al (Vakkilainen, Mero et al. 2002) also used a high fat test meal containing 63 g fat and 25g carbohydrate and found insulin concentrations to return to baseline by 6 hours post challenge. For our results, overall, although there is early and rapid enhancement of endogenous insulin secretion, overall insulin exposure is increased with nateglinide and this relative hyperinsulinaemia may be one possible explanation for the absence of observing

triglyceride lowering effects despite NEFA and glycerol reduction with nateglinide. As previously highlighted, chronic hyperinsulinaemia may have detrimental effects on hepatic VLDL triglyceride production and secretion (Gibbons, Brown et al. 2002).

The results of the study confirm the relationship between post challenge and fasting triglyceride levels. However previously established correlations between post challenge triglyceride with glucose/ insulin levels (Chapter 4) were not reproduced. This may be due to smaller numbers of subjects in this study, or a specific effect of nateglinide although unlikely. An interesting correlation between systolic BP and triglyceride IAUC has previously been established in postprandial lipaemia studies (Castro Cabezas, Halkes et al. 2001). It has been suggested that increased arterial stiffness occurs commonly in type 2 diabetes (Lehmann, 1992) and triglyceride rich lipoproteins result in endothelial dysfunction by oxidative stress and (Anderson, Evans et al. 2001). However a recent study found no evidence for an association between fasting or postprandial triglyceridaemia and arterial stiffness (Van-Dijk, 2003).

6 Chapter 6 Does prandial exogenous insulin delivery improve post challenge lipid handling in type 2 diabetes?

6.1 Introduction

Studies in type 2 diabetic subjects have shown deficient early post challenge insulin release although total insulin secretion may be normal or elevated. In Chapter 4 we demonstrated elevated insulin concentrations in type 2 diabetic subjects compared to non diabetic individuals. We found insulin - glucose excursions to significantly correlate with post challenge triglyceride levels, which are also elevated in these individuals. It is possible that restoration of the early post challenge insulin response using various pharmacological agents may have downstream effects on lipid handling. Studies in obese non diabetic subjects as well as type 2 diabetic subjects have demonstrated acute hyperinsulinaemia to decrease VLDL secretion (Lewis, Uffelman et al. 1993; Cummings M H, Watts G F et al. 1995). Intensive insulin therapy in type 2 diabetic subjects has been shown to decrease VLDL and triglyceride levels as well as increase HDL levels (Taskinen, Kuusi et al. 1988).

Subcutaneous insulin is absorbed in the peripheral circulation and circulates in turn to the liver at concentrations similar to systemic levels. This is in contrast to non diabetic individuals where portal insulin levels are substantially higher than systemic (approximately 50-70% hepatic extraction) (Shah et al, 2002 and Braunwald, 1997). High levels of insulin in the peripheral circulation may have additional benefits in lipid lowering not just in reducing hyperglycaemia. Type 2 diabetic subjects have reduced

function of enzymes which act primarily in peripheral tissues such as adipose tissue, important in triglyceride metabolism. Insulin therapy may be advantageous in improving LPL action, the function of which is sensitive to insulin and is decreased in type 2 diabetic individuals. LPL, present in adipose tissue, is required for lipolysis of both endogenous and exogenously produced triglyceride rich lipoproteins in the postprandial state. Improved LPL action increases the lipolysis of chylomicrons and VLDL, decreasing plasma triglyceride concentration.

In the postprandial state, insulin inhibits the action of HSL in non diabetic individuals. The main role of HSL is to hydrolyse triglyceride stores in adipose tissue in the fasting state, to produce NEFA and glycerol substrate to the liver. This function is not required in the postprandial state, however diabetic subjects tend to have elevated post challenge NEFA and glycerol levels (Chapter 4). Enhancing early prandial insulin provision may therefore promote the inhibition of HSL, improving the transition from fasting to postprandial state, reducing NEFA and glycerol delivery to the liver, subsequently decreasing VLDL triglyceride formation and plasma triglyceride levels. The duration of action of postprandial insulin therapy may also be important in modifying lipid handling.

6.2 Aims

1. To determine whether increasing circulating insulin levels by giving exogenous insulin with an oral lipid and carbohydrate challenge can improve lipid handling in type 2 diabetic subjects.

2. To determine whether rapid acting exogenous insulin differentially effects post challenge lipid handling compared with soluble insulin in type 2 diabetic subjects.

6.3 Methods

6.3.1 Study Design

To examine the effect of exogenous insulin delivery, 2 injectable insulin preparations with different action profiles licensed in the UK were chosen to compare. Single injections of these insulins were evaluated post oral lipid and carbohydrate challenge (OTTT) in type 2 diabetic subjects. This two way study was double blinded to minimise bias and of crossover design to reduce the possibility of a carry over of treatment effect or a period effects (Altman 1991). Although the chosen insulins were of short duration of action, subjects underwent the OTTT a minimum of 1 week apart. The study was funded by an unrestricted educational grant from Lilly Pharmaceuticals Ltd who played no part in the conduct, analysis or interpretation of the study.

6.3.2 Study Subjects

Following approval by the Oxford Research Ethics Committee (OXREC), a total of 26 type 2 diabetic subjects treated by diet alone, sulphonylurea and/or metformin, soluble/analogue and/or basal insulin (but not insulin mixtures) were identified and

approached through general practice and diabetes centre clinics. Subjects on these various antidiabetic regimens could be included as exogenous insulin therapy does not rely on β cell functioning to lower plasma glucose. Twenty subjects were recruited and gave written informed consent. They were male and female, Caucasian, aged 35-75 years, not taking lipid lowering therapy and had no history of established cardiovascular disease. Subjects with type 2 diabetes had been diagnosed according to WHO criteria (WHO 1999) for at least 6 months. Subjects taking insulin mixtures were excluded as the effects of both short and long acting insulins in these preparations may effect post challenge profiles. No subjects were routinely taking both short and long acting insulin as separate injections in the morning. Subjects were also excluded if they had a fasting plasma triglyceride level >5 mmol/l, poorly controlled diabetes (HbA_{1c} > 10%), a major lipid abnormality, gastrointestinal problems eg. delayed gastric emptying, chronic inflammatory bowel disease, any serious underlying medical conditions, a history of alcohol abuse, were pregnant or lactating females. Clinical, anthropometric and safety measurements were taken as outlined in Chapter 2.

6.3.3 Study medication

Currently available prandial insulin therapy consists of soluble insulin preparations and the newer insulin analogues. The subcutaneous tissue absorption and kinetics of currently available soluble insulin preparations are too slow to mimic the normal physiological postprandial insulin release. Insulin analogues have been designed using recombinant DNA techniques to reduce intermolecular binding properties responsible for

hexameric self association which influences the rate of absorption in subcutaneous tissue. This enables rapid delivery of insulin into the peripheral circulation.

Soluble insulin

Soluble insulin requires subcutaneous administration 30 minutes prior to meals. It has a duration of action of 8 hours, however peak action is between 2 and 4 hours. The main side effect is hypoglycaemia.

Insulin lispro

Insulin lispro is a rapid-acting insulin analogue developed by substituting lysine for proline at position 28 site of the insulin ß chain. When given by injection immediately before or after a meal it has a faster absorption rate, causing increased peak serum insulin levels with a shorter duration of action. Insulin lispro has a more predictable action profile with less variability and twice as fast absorption rate from different injection sites than soluble human insulin (Ter-Braak, R et al. 1996).

Studies comparing insulin lispro with soluble insulin, show that this analogue has the same glucose lowering potency when injected intravenously. Clamp studies have demonstrated earlier and higher peak serum insulin concentrations with insulin lispro enabling greater glucose to be infused during the first 2 hours compared with soluble insulin (Heinemann, Starke et al. 1990; Milicevic Z, Profozic J et al. 2001). Also type 2 diabetic patients on insulin lispro therapy may have a decreased rate of mild

hypoglycaemic episodes (Anderson, Brunelle et al. 1997). Long term studies of insulin lispro in insulin naïve type 2 diabetic subjects have demonstrated similar reductions in HbA1c as with soluble insulin (Ross, Zinman et al. 2001).

The study medication was ordered from the local hospital pharmacy. The treatment randomisation codes were generated and managed by a Statistician who was independent to the study. The masking and allocation of study medication into syringes was performed by members of the CRU who had no direct involvement in the study. Treatment codes were kept in the strictest confidence.

6.3.4 Study Procedure

Subjects underwent the OTTT on 2 separate occasions at least one week apart, and received soluble or lispro insulin on either visit. On the morning of the first OTTT, subjects were allocated a treatment randomisation code in serial order. At the end of the study, all subjects had received soluble and lispro insulin.

Methodology for the OTTT is outlined in Chapter 2. After baseline blood sampling the study medication was given by subcutaneous injection into the abdomen. Due to insulin lispro being prescribed immediately before meals and soluble insulin ideally given 30 minutes prior to meals, subjects were given soluble insulin at -30 minutes and saline placebo immediately prior to the triglyceride and carbohydrate challenge, or saline placebo at –30 minutes and insulin lispro immediately prior to consuming the test drink

within a maximum of 10 minutes. To avoid repeated injections in the same subcutaneous region which could potentially influence absorption, the first injection was always given in the lower right quadrant and the second injection the lower left quadrant of the abdomen.

The insulin dose was initially calculated at 0.2U/kg insulin based on the energy content of the test drink. However 3 subjects suffered a mild hypoglycaemic episode approximately 3 to 4 hours post challenge and the test was discontinued. A protocol amendment was made with the dose of insulin changed to 0.15U/kg of insulin lispro (Humalog) and soluble insulin (Humulin S). All three subjects returned to complete two further tests. For subjects routinely prescribed insulin and/or oral hypoglycaemic therapy at breakfast and/or lunchtime, these doses were omitted, to avoid hypoglycaemia.

6.3.5 Statistical methodology

Sample size calculations

Again sample size calculations were based on the initial OTTT study (Chapter 4). The mean of all logarithmically transformed 6 hour triglyceride values from subjects undergoing to OTTT was found to be 0.658. The standard deviation of the differences was 0.339. This would be to detect a clinically meaningful 35% relative reduction in 6 hour post challenge triglyceride levels (Robins, Collins et al. 2001). A sample size of 20 subjects for each study would be required to achieve a power of 80% at the 5% significance level.

Data Analysis

Statistical methodology regarding data checking and analysis as well as derived variables is summarised in Chapter 2. Paired t tests and Wilcoxon matched pairs signed rank sum test were used to compare parametric and non parametric data respectively for soluble and lispro insulin.

6.4 Results

6.4.1 Subjects

A total of 13 male and 7 female subjects completed both tests. Subjects were routinely treated by diet alone (n=7), sulphonylurea alone (n=1), metformin alone (n=3), sulphonylurea and metformin (n=5), night time basal insulin alone (n=1), night time basal insulin and metformin (n=1), soluble and basal insulin (n=1) or analogue and basal insulin (n=1). There were more men than women recruited into the study and mean (SD) age was 65.1 (7.0) years. The subjects were obese, BMI 30.9 (5.0) kg/m^2 and had moderately good glycaemic control, HbA1c 7.7 (1.0)%. They had systolic hypertension, mean (SD) systolic blood pressure of 135.8 (13.9) mm Hg. Subject characteristics are outlined in Table 16.

Baseline lipid profile was within normal limits (Chapter 2) with mean (SD) mmol/l total cholesterol level 5.0 (0.50), LDL cholesterol 3.12 (0.63), HDL cholesterol 1.20 (0.24) and geometric mean (1 SD range) triglyceride level 1.8 (1.04 – 3.03) mmol/l. No

significant differences were found comparing baseline triglyceride, NEFA, glycerol, glucose, insulin or C peptide levels prior to soluble or lispro insulin administration.

The mean (SD) insulin dose administered was 13.5 (2.5)U. No hypoglycaemic events occurred at the dose of 0.15 U/kg.

Table 16 Subject Characteristics of type 2 diabetic subjects

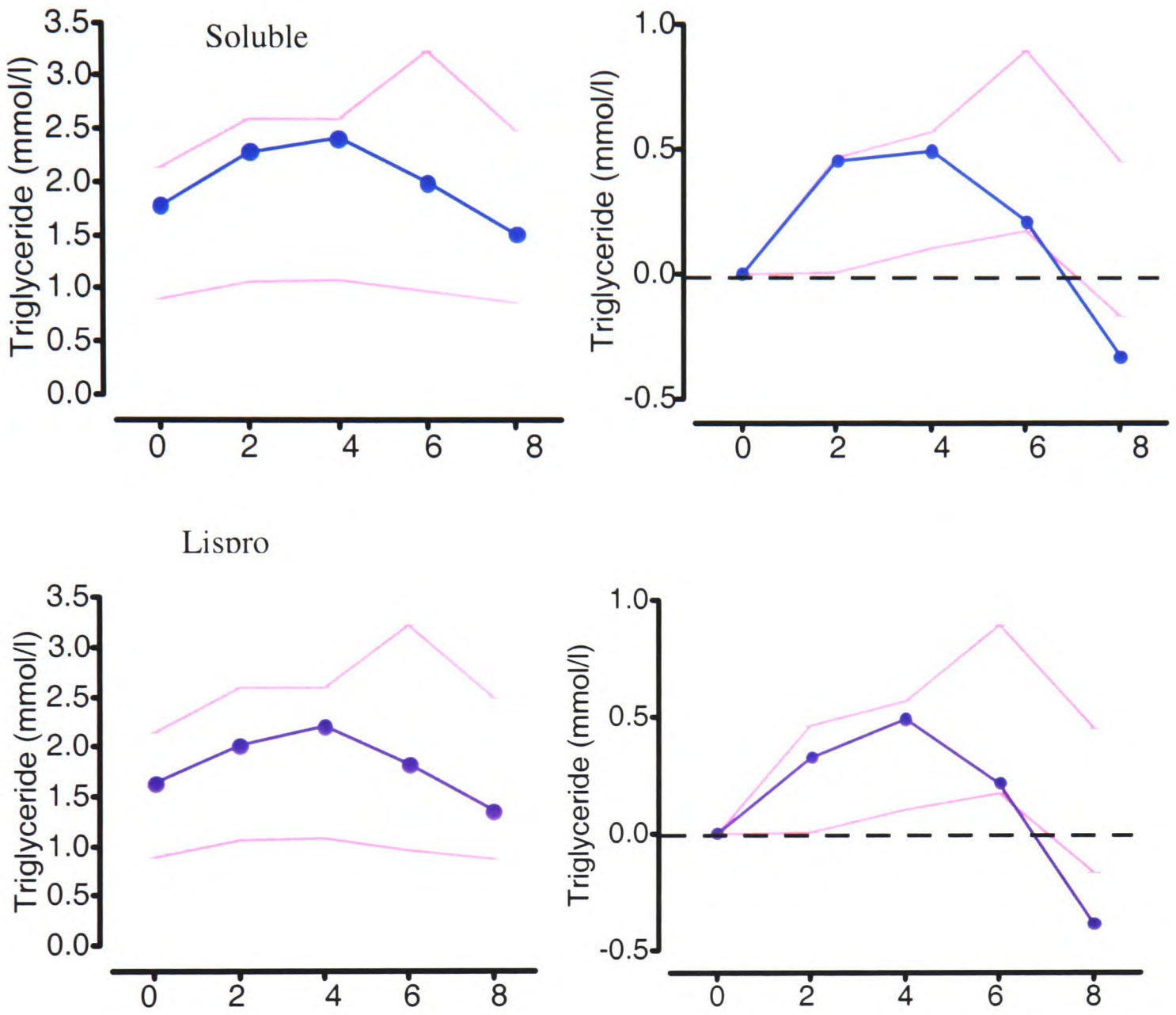
n	20
Sex *	13M /7F
Age (years)	65.1 (7.0)
Duration of diabetes (years) †	6.5 (4.0-17.0)
BMI (kg/m2)	30.9 (5.0)
Waist (cm)	102.3 (11.6)
Triceps skinfold thickness (mm)	15.9 (12.4)
BP (mm Hg)	
Systolic	135.8 (13.9)
Diastolic	76.5 (7.6)
Smoking (n)	
Non smoker	8
Ex smoker	11
Smoker	1
Hormone use (n)	7
Menopausal (n)	1
HbA1c (%)	7.7 (1.0)
Fasting plasma glucose (mmol/l)	9.0 (2.3)
Fasting total cholesterol (mmol/l)	5.0 (0.5)
Fasting LDL cholesterol (mmol/l)	3.12 (0.63)
Fasting HDL cholesterol (mmol/l)	1.20 (0.24)
Fasting VLDL cholesterol (mmol/l)	0.88 (1.10)
Triglyceride (mmol/l) *	1.8 (1.04 - 3.03)

Data are mean (SD) except †median (IQR) and * geometric mean (1 SD range)

6.4.2 Postchallenge Profiles

Triglyceride

For both soluble and lispro insulin, although the delta Cmax and IAUC were similar to those seen in Chapter 4, the delta 6 hour triglyceride level was lower. The Tmax for both insulins was 4 hours, which is earlier than previously established.



Graphs showing soluble (top) and lispro (bottom) triglyceride profiles and 1 SD range from type 2 diabetic subjects (Chapter 4). Absolute values are depicted on the left, and change from baseline (delta) on the right.

Comparing triglyceride responses between insulins, no statistically significant difference was found at the 6 hour timepoint or with Cmax triglyceride values (Table 17). Although the triglyceride IAUC profile from baseline to 6 hours was greater with soluble insulin than lispro, this was not statistically significant (p = 0.6801) and IAUC to 8 hours showed no difference between insulins.

NEFA

The geometric mean (1 SD range) 2 hour NEFA suppression (μmol/l) was greater with lispro insulin than soluble insulin, 146 (87.7 – 242.4) vs 200.9 (120.4 – 335.1) respectively (p = 0.0113) with minimum concentration of NEFA lower for lispro insulin (Cmin: p = 0.0511)) (Table 17, Figure 21). Significantly higher peak NEFA levels were evident with insulin lispro 771.5 (605.1 – 983.7) μmol/l, compared with soluble insulin 652.7 (486.8 – 875.2) μmol/l (Cmax: p=0.0039). The NEFA AUC was significantly greater lispro insulin compared with soluble insulin (p = 0.0351) but not IAUC NEFA (p = 0.8351).

Glycerol

The median (IQR) 2 hour glycerol suppression (mmol/l) was greater with insulin lispro 0.5 (0.364 – 0.763) vs 0.83 (0.586 – 1.113) for soluble insulin (p = 0.0032)) (Table 17, Figure 21). There were no significant differences in glycerol IAUC.

Mean total cholesterol, LDL cholesterol, HDL cholesterol and VLDL cholesterol levels did not change significantly in any group over the timepoints.

Glucose

Post challenge mean glucose (SD) levels (mmol/l) differed between insulins at the 2 hour timepoint with concentrations of 12.6 (5.01) and 10.7 (4.09) (p = 0.0128), delta 3.6 (4.20) and 1.8 (4.09) (p = 0.0163) for soluble and lispro insulin respectively (Table 17, Figure 22). Glucose AUC profiles were lower with insulin lispro (p = 0.0458), as were IAUC levels for 0 to 4 hours (0.0197).

Insulin

Geometric mean (1 SD range) peak insulin levels (pmol/l) were higher with insulin lispro at 2 hours than soluble insulin (432.1 (221.5-842.9) vs 379.0 (195.2-736.0) respectively) however this was not statistically significant (0.0789) (Table 17, Figure 22). IAUC insulin was greater with soluble than lispro insulin, but this did not reach statistical significance (p = 0.6009). Although the 4, 6 and 8 hour insulin values were lower with insulin lispro than soluble insulin, the IAUC for 4 to 8 hours failed to reach statistical significance (p = 0.27).

C peptide

No significant difference was found at 2 hours between the insulins. C peptide IAUC was greater with soluble insulin although this was not statistically significant however, median (IQR) IAUC from 4 to 8 hours was significantly lower with soluble than lispro

insulin –0.89 (-77.85 - -28.35) nmol/l.hour and –0.50 (-56.25 - -14.70) nmol/l.hour respectively (p = 0.0206) (Table 17, Figure 22).

Table 17 Fasting and post challenge OTTT responses to soluble and lispro insulin.

	Soluble		Lispro		*p*
Triglyceride (mmol/l)					
0 hour	1.8	1.04 - 3.03	1.6	0.99 - 2.69	*0.2434*
2 hours	2.3	1.42 - 3.67	2.0	1.26 - 3.22	
4 hours	2.4	1.47 - 3.97	2.2	1.31 - 3.71	
6 hours	2.0	1.17 - 3.39	1.8	1.10 - 3.04	*0.3885*
8 hours	1.5	0.88 - 2.60	1.4	0.79 - 2.33	
AUC^{+}	17.0	10.61 - 27.08	15.3	9.55 - 24.55	*0.1263*
$IAUC_{0\text{-}6hours}{}^{*+}$	2.4	0.95 - 3.97	2.0	0.68 - 3.55	*0.6801*
$IAUC_{0\text{-}8hours}{}^{*+}$	2.0	0.60 - 4.74	2.0	0.82 - 3.75	*0.3699*
Cmax	2.7	1.66 - 4.28	2.4	1.49 - 3.89	*0.5848*
deltaCmax	0.8	0.39 - 1.60	0.7	0.34 - 1.49	*0.6238*
Tmax*±	4	4 - 6	4	4 - 6	*0.7747*
6-0 hours*	0.2	-0.19 - 0.70	0.2	0.02 - 0.49	*0.8791*
NEFA (umol/l)					
0 hour	534.2	357.15 - 799.02	554.0	376.29 - 815.75	*0.5697*
2 hours	200.9	120.41 - 335.06	146.0	87.87 - 242.42	*0.0113*
4 hours	251.2	153.59 - 410.76	302.6	186.77 - 490.42	
6 hours	466.6	324.28 - 671.43	517.7	376.73 - 711.33	
8 hours	564.1	380.38 - 836.50	676.8	474.04 - 966.17	*0.1175*
AUC^{+}	3113.6	2505.92 - 3868.74	3329.7	2760.51 - 4016.16	*0.0351*
$IAUC_{0\text{-}4hours}{}^{*+}$	761.6	487.60 - 930.15	672.6	567.925 - 1152.35	*0.4326*
$IAUC_{0\text{-}8hours}{}^{*+}$	-1152.5	-2344 - -553.25	-1229.5	-1884 - -443.75	*0.8351*
Cmin	169.1	108.05 - 264.53	143.8	87.70 - 235.84	*0.0511*
Cmax	652.7	486.78 - 875.20	771.5	605.08 - 983.66	*0.0039*
Tmax*±	6.0	0 - 8	8.0	6 - 8	*<0.02*
2-0 hours*	-353.5	-485.75 - -212.00	-426.5	-515.50 - -288.50	*0.1169*
8-0 hours*	-5.5	-91.00 - 148.00	184.5	40.00 - 269.25	*0.0304*
Glycerol (mmol/l)					
0 hour	0.68	0.497 - 1.135	0.85	0.597 - 1.034	*0.9923*
2 hours	0.83	0.586 - 1.113	0.58	0.364 - 0.763	*0.0032*
4 hours	0.74	0.581 - 1.132	0.67	0.505 - 1.338	
6 hours	0.77	0.527 - 1.040	0.78	0.671 - 1.004	
8 hours	0.77	0.489 - 1.029	0.71	0.592 - 0.901	*0.6801*
AUC^{+}	6.20	5.473 - 6.200	6.38	5.028 - 6.380	*0.4361*
$IAUC_{0\text{-}4hours}{}^{*+}$	0.47	-0.193 - 1.240633	-0.63	-1.241 - 0.424	*0.0675*
$IAUC_{0\text{-}8hours}{}^{*+}$	0.91	-0.375 - 1.696717	-0.24	-2.001 - 1.879	*0.4578*
Cmin	0.49	0.342 - 0.595	0.48	0.301 - 0.589	*0.1689*
Cmax	1.17	0.947 - 1.466	1.19	0.874 - 1.876	*0.5602*
Tmax*±	4	2 - 6	4	3 - 6	*0.8004*
2-0 hours*	0.15	-0.190 - 0.421	-0.25	-0.551 - 0.062	*0.0271*
8-0 hours*	0.03	-0.147 - 0.130	-0.11	-0.312 - 0.263	*0.6665*

Data are geometric mean (1 SD range) except * median (IQR).
Units are as tabulated except + units.hour and ± hours

Table 17 Continued Fasting & post challenge OTTT responses to soluble & lispro insulin

	Soluble		Lispro		*p*
Glucose (mmol/l)†					
0 hour	9.0	2.32	8.9	2.35	*0.4858*
2 hours	12.6	5.01	10.7	4.09	*0.0128*
4 hours	7.3	3.35	6.9	3.36	
6 hours	5.5	1.53	5.8	1.55	
8 hours	5.7	1.16	5.8	1.36	
AUC^{+}	65.7	19.77	61.6	17.39	*0.0458*
$IAUC_{0\text{-}4hours}$*$^{+}$	4.7	-2.98 - 13.05	1.7	-5.43 - 8.50	*0.0197*
$IAUC_{0\text{-}8hours}$*$^{+}$	-9.8	-21.03 - 7.63	-10.2	-16.30 - -1.55	*0.1222*
Cmax	13.1	4.51	11.8	3.24	*0.0603*
Cmin	5.0	1.26	5.2	1.57	*0.3277*
Tmax*±	2	2 - 2	2	2 - 2	*0.2585*
Tmin*±	6	4 - 8	6	4 - 8	*ns*
2-0 hours*	3.9	1.00 - 6.4	2.2	-1.65 - 4.63	*0.0163*
Insulin (pmol/l)					
0 hour	109.8	54.74 - 220.04	104.9	49.85 - 220.93	*0.9506*
2 hours	379.0	195.19 - 735.96	432.1	221.54 - 842.85	*0.1736*
4 hours	250.9	138.53 - 454.36	204.2	113.55 - 367.18	
6 hours	156.1	88.31 - 275.82	123.0	66.73 - 226.61	
8 hours	119.9	61.91 - 232.23	92.7	47.15 - 182.09	
AUC^{+}	1892.4	1049.01 - 3414.01	1772.2	986.62 - 3183.28	*0.6252*
$IAUC_{0\text{-}4hours}$*$^{+}$	762	487.60 - 930.15	673	567.93 - 1152.35	*0.2121*
$IAUC_{0\text{-}8hours}$*$^{+}$	1042	676.65 - 1191.55	723	650.00 - 1298.45	*0.6009*
Cmax	387.5	205.44 - 730.97	448.9	245.43 - 13.68	*0.0789*
Tmax*±	2	2 - 2	2	2 - 2	*ns*
2-0 hours*	272.4	149.58 - 346.43	301.2	245.53 - 433.55	*0.1559*
C peptide (nmol/l)					
0 hour	0.76	0.468 - 1.246	0.71	0.445 - 1.122	*0.0623*
2 hours	1.32	0.723 - 2.399	1.19	0.712 - 1.996	*0.1204*
4 hours	0.86	0.514 - 1.442	0.72	0.394 - 1.304	
6 hours	0.55	0.327 - 0.909	0.52	0.283 - 0.936	
8 hours	0.50	0.286 - 0.859	0.51	0.292 - 0.887	
AUC^{+}	6.97	4.360 - 11.142	6.32	3.966 - 10.063	*0.0896*
$IAUC_{0\text{-}4hours}$*$^{+}$	1.03	0.435 - 2.030	0.80	0.398 - 1.895	*0.3799*
$IAUC_{0\text{-}8hours}$*$^{+}$	1.10	-0.325 - 1.913	0.26	-0.105 - 1.585	*0.6746*
Cmax	1.35	0.764 - 2.397	1.23	0.741 - 2.039	*0.088*
Tmax*±	2	2 - 2	2	2 - 2	*ns*
2-0 hours*	0.50	0.183 - 0.805	0.40	0.218 - 0.688	*0.3503*

Data are geometric mean (1 SD range) except † mean (SD) and * median (IQR).
Units are as tabulated except + units.hour and ± hours

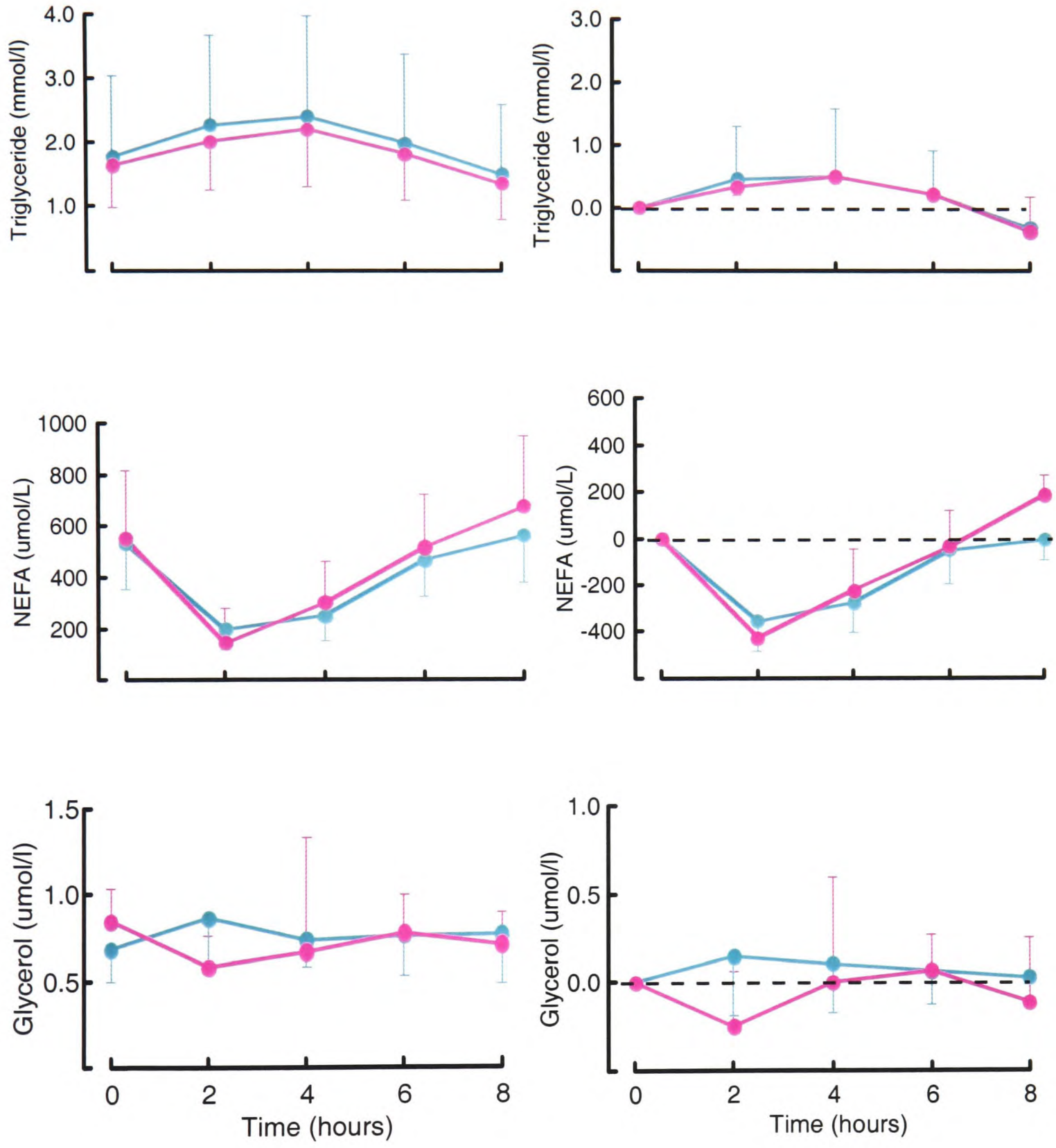


Figure 21 Triglyceride, NEFA and glycerol profiles for lispro ● and soluble ● insulin. Absolute values are depicted on the left, and change from baseline (delta) on the right.

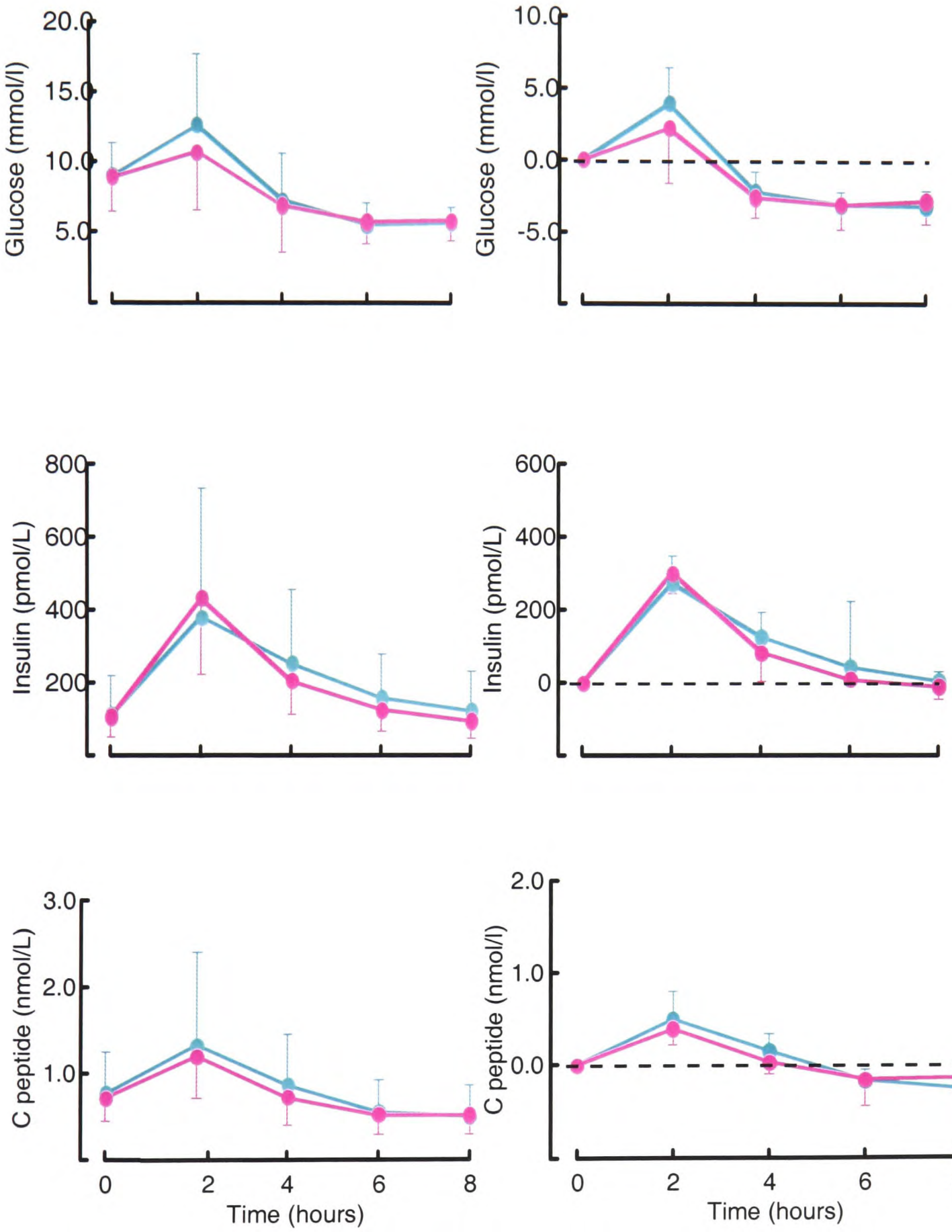


Figure 22 Glucose, insulin and C peptide profiles for lispro ● and soluble ● insulin. Absolute values are depicted on the left, and change from baseline (delta) on the right.

6.4.3 Correlations

Pearson correlation coefficients for each timepoint showed a strong correlation between postprandial and fasting triglyceride values for both insulins as previously demonstrated (Table.18).

Table 18 Correlation coefficients for fasting triglyceride level with post challenge triglyceride levels

	Soluble Insulin		Insulin lispro	
Triglyceride	r	p	r	p
2 hour	0.9111	<0.0001	0.8836	<0.0001
4 hour	0.8771	<0.0001	0.8562	<0.0001
6 hour	0.7703	<0.0001	0.8816	<0.0001
8 hour	0.8373	<0.0001	0.8575	<0.0001

For both soluble and lispro insulin, the fasting triglyceride level correlated with fasting insulin values ($r = 6056$, $p = 0.0047$ and $r = 0.4505$, $p = 0.0462$) respectively. However, the delta 6 hour triglyceride concentration correlated with insulin IAUC for soluble insulin ($r = 0.5875$, $p = 0.0082$) and delta 2 hour glucose level ($r = 0.487$, $p = 0.0294$). Also, triglyceride IAUC correlated with glucose IAUC ($r = 0.514$, $p = 0.0204$) for soluble insulin only.

6.5 Discussion

This study comparing soluble and lispro insulin demonstrated post challenge changes to glucose and lipid metabolism in type 2 diabetic subjects. A single injection of insulin lispro reduces post-challenge glucose excursions and appears to have acute antilipolytic effects compared with soluble insulin, possibly through enhancing post meal insulin mediated NEFA and glycerol suppression, and triglyceride clearance.

The degree of insulinaemia and reduction in post challenge glycaemia achieved in this study is firstly discussed in comparison with other studies using short acting insulins before lipid results are interpreted.

Insulin Profiles

For the 8 hour study duration, insulin IAUC was 30% less with lispro than soluble insulin although this was not statistically significant. Our results are comparable to a study by Bruttomesso et al (Bruttomesso D B, Pianta A et al. 1999) which also aimed to restore the early rise in plasma insulin levels, but after a 50g oral glucose load. In their crossover study, subjects (previously treated with sulphonylurea therapy prior to 7 day washout) were given regular or lispro insulin by subcutaneous injection (0.075U/kg). They showed that over the 5 hour study duration, IAUC insulin was similar between insulins. Overall, our incremental rise in 2 hour insulin concentration after both insulins was greater than in the study by Bruttomesso et al. This may be due to the increased dose of insulin given (0.15U/kg), test drink constituents as well as subject characteristics. Most subjects in our

study were treated by diet or oral hypoglycaemic therapy, indicating the ability to produce endogenous insulin. Our subjects did not undergo a washout period, however morning or lunchtime prescribed doses of antidiabetic medication on the day of the OTTT were omitted.

It is possible that changes in the insulin profile rather than in the absolute amount of circulating insulin affect glucose tolerance in patients with type 2 diabetes (Bruttomesso D B, Pianta A et al. 1999). Further sampling between the 2 hourly timepoints in our study may have also demonstrated an earlier peak rise in insulin levels with insulin lispro. The 2 hour insulin concentration in our study was higher with insulin lispro than with soluble insulin although not statistically significant. Studies have demonstrated that peak insulin concentrations were found at approximately 1 hour after administration with insulin lispro, compared with a peak at approximately 2 hours with regular insulin (Bruttomesso D B, Pianta A et al. 1999) (Milicevic Z, Profozic J et al. 2001).

Late postprandial hyperinsulinaemia has been documented with soluble insulin with plasma insulin concentrations 3 to 5 hours post challenge, higher than those seen with insulin lispro (Bruttomesso D B, Pianta A et al. 1999). Although the 4, 6 and 8 hour insulin values in our study were lower with insulin lispro than soluble insulin, the IAUC for 4 to 8 hours failed to reach statistical significance. Again, additional sampling between the 2 hourly timepoints may have supported this finding.

C peptide Profiles

In our study C peptide results reflected insulin findings with no differences between the tested insulins seen in IAUC or peak concentrations. Previous studies have showed lower IAUC C peptide levels with insulin lispro (Bruttomesso D B, Pianta A et al. 1999) and despite its short duration of action, a compensatory increase in endogenous insulin secretion is not required in the late post challenge phase. Insulin lispro may actually have a sparing effect on the β cell confirmed by plasma C peptide levels remaining low during the postprandial period. Our results showed C peptide IAUC were lower with lispro than soluble insulin although not statistically significant indicating persistent endogenous insulin production with soluble insulin.

Glucose Profiles

Compared with soluble insulin, insulin lispro reduced mean glucose IAUC to 4 hours by *circa* 30% in our study. Other studies have showed reductions of up to 46% (Bruttomesso D B, Pianta A et al. 1999), with plasma glucose levels remaining lower until the end of the study (5 hours duration) despite plasma insulin concentrations that, from the 2 hour timepoint onwards, were lower compared with regular insulin. Our study showed that delta 2 hour glucose values were significantly lower with insulin lispro. Also, despite the short duration of action of insulin lispro, glucose levels remained constant and low throughout the study duration, and were comparable to concentrations seen with soluble insulin. Some authors have suggested that insulin lispro may actually promote greater suppression of endogenous glucose production thereby significantly

improving glucose tolerance, also ameliorating late hyperglycaemia and hyperinsulinaemia (Bruttomesso D B, Pianta A et al. 1999).

Associations between early post challenge insulin levels and glucose concentrations have previously been established. Mitrakou et al (Mitrakou, Kelley et al. 1992) observed that in subjects with impaired glucose tolerance, the 30 minute plasma insulin concentration after an oral glucose challenge (which is indicative of early β cell function), inversely correlated with the 2 hour glucose concentration, proposing that the early β cell response is a major determinant of prandial glucose tolerance. If prandial hyperglycaemia persists this propagates increased insulin secretion and hyperinsulinaemia. A clamp study simulating chronic physiologic hyperinsulinaemia created by exogenous insulin infusion given to healthy subjects, lead to the development of insulin resistance (Del Prato, Leonetti et al. 1994). As well as affecting glucose metabolism, hyperinsulinaemia may have downstream effects on lipid handling (Gibbons, Brown et al. 2002).

Triglyceride Profiles

Our results show that type 2 diabetic subjects given single doses of prandial insulin, have peak post challenge triglyceride concentrations achieved by 4 hours which return to baseline by 6 hours. This is different to our earlier studies showing type 2 diabetic subjects to achieve triglyceride concentrations 6 hours post challenge, whilst non diabetic subjects do so at 4 hours. The few studies which have examined the effects of prandial insulin on lipid metabolism mostly using soluble insulin. Evans et al (Evans, Anderson et al. 2003) showed after 6 weeks of insulin lispro therapy in type 2 diabetic subjects

previously on oral hypoglycaemic agents, fasting and postprandial triglyceride levels were significantly reduced. Improved glycaemic control from continuous subcutaneous insulin infusion also reduced triglyceride levels after an oral fat load after 12 days of therapy (Georgopoulos A, Margolis S et al. 1988). Short term hyperinsulinaemia can inhibit VLDL production but does not reduce plasma triglyceride levels to the same extent in obese compared to non obese subjects (Lewis, Uffelman et al. 1993). In type 2 diabetic subjects, a reduction in triglyceride concentration was observed during a hyperinsulinaemic euglycaemic clamp study compared with saline infusion (Cummings M H, Watts G F et al. 1995).

Our study is the first to compare the effect on lipid metabolism of both soluble and lispro insulin. The results showed no statistical differences in triglyceride profiles after soluble or lispro insulin administration. However both triglyceride IAUC to 6 hours and maximum concentration of triglyceride achieved were lower with insulin lispro compared to soluble insulin. It is possible that changes in NEFA (and glycerol) levels may better reflect subtle differences between the 2 insulins examined, as NEFA is a substrate for hepatic triglyceride synthesis.

NEFA and glycerol profiles

It has been previously demonstrated that early insulin administration with soluble or lispro can result in free fatty acid levels falling more rapidly in diabetic subjects (Bruce D G, Chisholm D J et al. 1988) (Evans, Anderson et al. 2003). An acute decrease in NEFA and glycerol levels was detected at the 2 hour timepoint in our study with insulin lispro

thereby giving a significantly greater suppression than soluble insulin. This could indicate that insulin, in particular, rapidly acting insulin, may alleviate the failed suppression of NEFA and glycerol previously demonstrated in type 2 diabetic subjects through HSL inhibition. Bruttomesso et al (Bruttomesso D B, Pianta A et al. 1999) however, found no difference in 2 hour NEFA levels between soluble and lispro insulin in subjects who had baseline NEFA concentrations similar to our subjects. A possible reason for the difference in suppression is that Bruttomesso et al used a 50g carbohydrate rather than mixed meal challenge (Westphal, Leodolter et al. 2002).

Whether these changes to NEFA and glycerol suppression with insulin lispro can be translated into downstream effects on other lipoproteins is yet to be investigated. Malmström et al (Malmström R, Packard C J et al. 1997) showed that despite significant reduction in plasma NEFA levels with the acute administration of insulin to 6 type 2 diabetic subjects previously treated by diet and/or oral hypoglycaemic agents, there was persistent failure to induce a suppression of VLDL apo B production as found in healthy subjects. A possible reason for the lack of effect seen in the study by Malmström et al was that subjects were normotriglyceridaemic at baseline (as with our study), where as a more profound fall in VLDL apo B secretion is observed in hypertriglyceridaemic type 2 diabetic subjects (Cummings M H, Watts G F et al. 1995). It is possible that a lowering of triglyceride profiles with insulin lispro may have been seen if our study had included subjects who were hypertriglyceridaemic at baseline.

Our results showed that late post challenge NEFA levels were higher with lispro compared with soluble insulin. In accordance with our findings, Bruttomesso et al (Bruttomesso D B, Pianta A et al. 1999) also demonstrated this elevation. This is most likely related to the short duration of action seen with insulin lispro, where after 2 hours, NEFA levels rise as circulating insulin concentrations diminish. In comparison, soluble insulin has a longer duration of action, consequently NEFA levels take longer to rise. This elevation of late postprandial NEFA levels may be more important for instigating glucose stimulated insulin secretion for type 2 diabetic subjects than non diabetic individuals (Boden G, Chen X et al. 1995). Dobbins et al (Dobbins R L, Chester M W et al. 1998) demonstrated that after overnight fasting, obese subjects had reduced glucose stimulated insulin secretion if their NEFA levels were lowered (by nicotinic acid), whilst in non obese subjects this produced no effect.

Correlations

The elevation of plasma triglyceride levels seen in individuals with type 2 diabetes most likely results from an overproduction of VLDL triglyceride (Taskinen 1995). This may either be a direct effect of hyperinsulinaemia or a consequence of defective suppression of VLDL production by insulin. Correlations from our study demonstrated positive associations between post challenge triglyceride excursions and post challenge insulin - glucose levels with soluble insulin however less associations were evident with insulin lispro. This suggests that postprandial triglyceridaemia may be related to insulin exposure.

7 Chapter 7 Does prandial exogenous insulin replacement modify post challenge lipid handling in type 1 diabetes?

7.1 Introduction

7.1.1 Aetiology of Type 1 Diabetes

Both genetic and environmental factors contribute to the development of type 1 diabetes, yet the aetiology and natural history is still unclear. The HLA effect confers 70 -75% susceptibility to the disease, however it may actually be an environmental trigger such as viral infections that initiates the process leading to autoimmune β cell destruction. Type 1 diabetes usually presents in childhood or adolescence with weight loss, polyuria and polydipsia and sometimes ketoacidosis. This catabolic state resulting from the complete lack of insulin causes hyperglycaemia from increased hepatic glucose production and reduced utilization. Glucose is not used as the oxidative fuel, but is replaced by fatty acids which form ketone bodies, thereby producing the acidosis. This is unique to type 1 diabetes and is not a feature of type 2 diabetes.

7.1.2 Epidemiology of Type 1 Diabetes

In an analysis of data on published incidence trends in type 1 diabetes by Onkamo et al (Onkamo, Väänänen et al. 1999), the mean incidence of the disease among the study populations varied from 0.5 to 30.3 per 100 000 a year during the observation period. They determined that type 1 diabetes was increasing globally by 3.0% per year, and

global estimations predict that the incidence will increase by 40% over the period between 1998 to 2010 (Gale 2002).

7.1.3 Morbidity and Mortality in Type 1 Diabetes

Although the prognosis of type 1 diabetes has improved considerably in the last 50 years these individuals still have an increased mortality compared with the general population (Pyorala 1990). In younger type 1 diabetic patients, the main causes of death are acute complications such as hypoglycaemia and ketoacidosis. In the older population, a 3 to 4 fold increase in CHD is a main contributor alongside renal disease (Laing, Swerdlow et al. 1999) and renal failure present in approximately 35% of patients who develop diabetic nephropathy (Rossing, Hougaard et al. 1996). In their study, potentially modifiable CHD risk factors in type 1 diabetes included microalbuminuria, hypertension, age, smoking, social class and poor glycaemic control. A 1% increase in HbA1c was associated with an 11% increase in the risk of dying. The EURODIAB complications study showed the overall prevalence of CHD to be 10% in 15 - 29 year old patients, increasing to 25% in 45 – 59 year old patients and with duration of diabetes (Koivisto, Stevens et al. 1996). Moss et al (Moss, Klein et al. 1994) found an association between glycaemic control and mortality due to ischaemic heart disease and stroke. Other studies have shown abnormal lipoprotein metabolism alongside duration of diabetes, retinopathy and renal impairment predict cardiovascular disease or death in type 1 diabetic patients (Weis, Turner et al. 2001). Plasma lipid concentrations were independently associated with an increase in mortality and coronary artery disease although within the accepted reference range.

7.1.4 Lipid handling in type 1 diabetes

Individuals with type 1 diabetes usually have normal fasting lipid levels, including triglyceride and HDL cholesterol (Sosenko, Breslow et al. 1980; Lopes-Virella, Wohltmann et al. 1981; Dunn 1992). Fasting triglyceride levels are frequently raised in type 1 diabetes patients with poor glycaemic control (Sosenko, Breslow et al. 1980), and past studies have shown lowering of plasma triglyceride levels upon improvement of blood glucose profiles (Pietri A O, Dunn F L et al. 1983; Gonen, White et al. 1985). Also, hypercholesterolaemia is a frequent finding in type 1 diabetic patients with nephropathy. Yet other studies have found total cholesterol and triglyceride concentrations were not different from age matched non-diabetic controls, despite poor glycaemic control (Perez, Wagner et al. 2000) however LDL cholesterol was higher and HDL lower. More recently, the EURODIAB Complications study assessed the determinants and prevalence of hyperlipidaemia as defined by the American Diabetes Association criteria (American-Diabetes-Association 2002) in type 1 diabetic patients (Idzior-Walus, Mattock et al. 2001). Hyperlipidaemia was observed in approximately 50% in of the studied population (n=3159). Elevated LDL cholesterol levels (> 3.35 mmol/l) were seen in 45% of subjects. The prevalence of hypertriglyceridaemia (> 1.7 mmol/l) and decreased HDL levels (< 0.9 mmol/l) was low although more common in men than in women. An increased HbA1c level indicating poor metabolic control was positively associated with cholesterol, triglyceride and LDL cholesterol, and negatively with HDL cholesterol. Smokers had poorer glycaemic control, higher prevalence of microalbuminuria, higher triglyceride levels, and lower HDL cholesterol.

Studies of postprandial lipaemia have demonstrated that when compared with non-diabetic age- and sex- matched control subjects, type 1 diabetic individuals have alterations in postprandial triglyceride metabolism (Georgopoulos, Bantle et al. 1998). These abnormalities include decreased clearance and abnormal composition of the lipoproteins.

7.1.5 Mechanism of dyslipidaemia causing atherosclerosis

Few studies propose specific mechanisms by which dyslipidaemia may cause atherosclerosis in type 1 diabetic subjects. Prior glycaemic control was not associated with carotid intima media thickness in the DCCT follow up study (Epidemiology of Diabetes Interventions and Complications Research Group 1999). However decompensated type 1 diabetic patients may have hypertriglyceridaemia and low plasma HDL levels due to increased transfer of cholesteryl ester from HDL to triglyceride rich lipoproteins (Ginsberg and Tuck 2001). A study of type 1 diabetic subjects by Valabhji et al (Valabhji, Donovan et al. 2002) also showed that increased triglyceride concentration promoted esterified cholesterol enrichment of apo B containing lipoproteins, yet HDL cholesterol levels were maintained. This may explain the high CHD incidence seen in type 1 diabetic subjects despite normal or even raised HDL levels.

Some studies suggest a defect in lipoprotein clearance to be the main abnormality. Noutsou et al (Noutsou and Georgopoulos 1999) demonstrated that normolipaemic type 1

diabetic subjects displayed alterations in triglyceride rich lipoprotein composition and reduced clearance. Yet a study by Van Waard et al (Van Waarde W M, Odink R J et al. 2001) showed normotriglyceridaemic teenagers (with cholesterol levels greater than 5.2 mmol/l) to have elevated lipid concentrations that were not due to delayed chylomicron clearance

7.1.6 Lipid lowering strategies in type 1 diabetes

As reviewed in the introduction (Chapter 1) primary and secondary intervention trials have demonstrated that LDL cholesterol lowering therapy can reduce cardiovascular morbidity and mortality, even when cholesterol levels are in the normal population range. Intensive lipid lowering strategy in 36 type 1 patients was achieved in 66% of patients with significantly decreased cholesterol-triglyceride ratio in VLDL (Kanters, Algra et al. 1999). Orchard et al (Orchard, Forrest et al. 2001) proposed that because of the higher occurrence of microvascular complications in type 1 diabetes, goal setting for treatment was more complex. They suggested goal levels from the Pittsburgh Epidemiology of Diabetes Complications Study 10 year incidence data are: LDL cholesterol < 2.6 mmol/l (or 3.3 mmol/l for complications), HDL cholesterol >1.1 mmol/l, and triglyceride < 1.7 mmol/l. Age, sex and glycaemic control had little influence on these goals. Lifestyle intervention is also important in the management of lipids in type 1 diabetes. An intervention study in type 1 patients showed an increase in physical activity was associated with an elevation in HDL, a decrease in LDL cholesterol and an improved in insulin sensitivity (Idzior-Walus, Mattock et al. 2001).

7.1.7 Can glycaemic control improve lipid handling in type 1 diabetes?

Changes in glycaemic control have been shown to effect lipid handling in type 1 diabetic subjects. In a study by Perez et al (Perez, Wagner et al. 2000) 334 type 1 diabetic subjects (compared with 803 non diabetic controls) underwent 3 to 6 months of intensive insulin therapy using multiple doses, to assess the effect of glycaemic optimisation on the prevalence of dyslipidaemia. The increased LDL levels seen at baseline improved with glycaemic therapy, but low HDL levels did not rise.

In type 1 diabetes, triglyceride levels correlate closely with glycaemic control and marked hyperlipidaemia can be found in ketotic diabetic patients (Ginsberg and Tuck 2001). The elevated plasma lipid levels seen in uncontrolled type 1 diabetes with ketoacidosis consist of an accumulation of triglyceride rich lipoproteins (Dunn 1992). The acute deficiency of insulin causes increased fatty acid mobilization from adipose tissue, leading to increased VLDL secretion. The fatty acids can be converted to ketone bodies by the liver, which reduces VLDL secretion. LPL activity is also impaired, thereby decreasing the clearance of triglyceride rich lipoproteins from plasma. These abnormalities are restored to normal levels in most patients within 24 hours by insulin treatment.

It has been proposed that the LDL receptor may be partially regulated by insulin and severe insulin deficiency may lead to reduced LDL catabolism. Other abnormalities of

LDL metabolism that have been postulated include non enzymatic glucosylation of LDL and decreased LDL receptor binding (Dunn 1992). Insulin therapy has been shown to improve LDL receptor binding and decrease LDL apo B production.

Changes in HDL levels by improving glycaemic control have been noted in some studies and not others. Continuous subcutaneous insulin therapy decreases triglyceride concentration and increases HDL levels due to reduced rates of VLDL production but also higher peripheral insulin concentrations activating adipose tissue LPL (Dunn 1992). Feinglos et al (Feinglos, Thacker et al. 1997) showed insulin lispro to significantly reduce fasting total cholesterol and increase HDL.

7.1.8 The effects of insulin replacement on lipid handling

Studies of postprandial lipaemia in type 1 diabetic subjects are few, and give conflicting results. Georgopoulos et al (Georgopoulos A, Margolis S et al. 1988) demonstrated that improving glycaemic control in type 1 diabetes patients over a 12 day period lowered post challenge plasma triglyceride levels assessed using a test meal containing 50g of saturated fat. Lewis et al (Lewis, O'Meara et al. 1991) however, found no alteration in postprandial triglyceride responses with short-term deterioration in glycaemic control or level of postprandial insulin replacement. Improving glycaemic control using continuous subcutaneous insulin infusion has been shown to decrease fasting and mean 24 hour integrated cholesterol and triglyceride concentrations (Hershcopf R, Plotnick L P et al. 1982).

Caixas et al (Caixas A, Perez A et al. 1998) conducted a randomised crossover study of soluble and lispro insulin over a six month period to investigate the effects of rapid insulin replacement on lipid metabolism. After a breakfast containing approximately 67% carbohydrate, 23% protein and 10% fat, postprandial triglyceride levels were reduced with soluble insulin. However, levels were only measured 2 hours post meal.

7.2 Aims

1. To characterise lipid handling after an oral lipid and carbohydrate challenge in type 1 diabetic subjects taking exogenous prandial insulin.
2. To determine whether rapid acting exogenous insulin replacement after an oral lipid and carbohydrate challenge compared to soluble insulin will improve lipid handling in type 1 diabetic subjects.

7.3 Methods

7.3.1 Study Design

Two short acting injectable insulin preparations with different action profiles licensed in the UK were chosen to compare. Single injections of these insulins were evaluated post oral lipid and carbohydrate challenge (OTTT) in type 1 diabetic subjects. This two way

double blinded crossover study was similar design to the study in type 2 diabetic subjects (Chapter 6). The study was funded by an unrestricted educational grant from Lilly Pharmaceuticals Ltd who played no part in the conduct, analysis or interpretation of the study.

Procedural, biochemical and statistical methodology are outlined in Chapter 2. Twenty type 1 diabetic subjects treated by were 0.1U/kg doses of insulin lispro (Humalog) and soluble insulin (Humulin S). Subjects had been diagnosed with type 1 diabetes for at least 6 months.

7.3.2 Study Subjects

Following approval by the Oxford Research Ethics Committee (OXREC), a total of 34 type 1 diabetic subjects treated by prandial and basal insulin (but not insulin mixtures) were identified and approached through general practice and diabetes centre clinics. Twenty subjects were recruited and gave written informed consent. They were male and female, Caucasian, aged 21-75 years, not taking lipid lowering therapy and had no history of established cardiovascular disease. Subjects with type 1 diabetes had been diagnosed according to WHO criteria (WHO 1999) for at least 6 months. Subjects were excluded if they had a fasting plasma triglyceride level >5 mmol/l, poorly controlled diabetes (HbA_{1c} > 10%), a major lipid abnormality, gastrointestinal problems eg. delayed gastric emptying, chronic inflammatory bowel disease, any serious underlying medical conditions, a history of alcohol abuse, were pregnant or lactating females. Clinical, anthropometric and safety measurements were taken as outlined in Chapter 2.

7.3.3 Study medication

As reviewed in Chapter 6, soluble and lispro insulin were compared. A previous study has compared insulin lispro and regular human insulin in 33 type 1 diabetic patients with hypoglycaemia unawareness, and found no difference in glycaemic control between the 2 therapies as determined by the HbA1c after each period (Ferguson, Strachan et al. 2001). However insulin lispro significantly reduces meal related glucose excursions (Holleman F, Schmitt H et al. 1997; Garg S K, Anderson J H et al. 1999; Gale and Group 2000). Fasting plasma glucose levels and pre prandial glucose levels tend to be higher in those treated with insulin lispro (Gale and Group 2000). Type 1 diabetic patients on insulin lispro therapy may have a decreased rate of mild hypoglycaemic episodes (Ferguson, Strachan et al. 2001).

The study medication was ordered from the local hospital pharmacy. The treatment randomisation codes were generated and managed by a Statistician who was independent to the study. The masking and allocation of study medication into syringes was performed by members of the CRU who had no direct involvement in the study. Treatment codes were kept in the strictest confidence.

7.3.4 Study procedure

Subjects underwent the OTTT on 2 separate occasions at least one week apart, and received soluble or lispro insulin on either visit. On the morning of the first OTTT, subjects were allocated a treatment randomisation code in serial order. At the end of the study, all subjects had received soluble and lispro insulin.

Methodology for the OTTT is outlined in Chapter 2. After baseline blood sampling the study medication was given by subcutaneous injection into the abdomen. Due to insulin lispro being prescribed immediately before meals and soluble insulin ideally given 30 minutes prior to meals, subjects were given soluble insulin at -30 minutes and saline placebo immediately prior to the triglyceride and carbohydrate challenge, or saline placebo at –30 minutes and insulin lispro immediately prior to consuming the test drink within a maximum of 10 minutes. To avoid repeated injections in the same subcutaneous region which could potentially influence absorption, the first injection was always given in the lower right quadrant and the second injection the lower left quadrant of the abdomen.

The insulin dose was initially calculated at 0.15U/kg insulin based on the energy content of the test drink. However 2 subjects suffered a mild hypoglycaemic episode approximately 3 hours post challenge, and the test was discontinued. One of these subjects continued with the next visit (taking the other insulin at 0.15U/kg) but still suffered a hypoglycaemic event. Therefore the dose was changed to 0.1U/kg of insulin lispro (Humalog) and soluble insulin (Humulin S). Both subjects returned to complete 2

further tests on the lower dose of insulin. Routinely prescribed prandial insulin at breakfast and/or lunchtime were omitted, to avoid hypoglycaemia. Basal insulin was taken the night before the OTTT as routinely prescribed, and for subjects who also take long acting insulin in the morning, this was also given on the morning of the OTTT.

Samples collected at fasting, 2, 4, 6 and 8 hours were analysed for glucose, insulin, C peptide, lipid profile, NEFA and glycerol according to biochemical methodology highlighted in Chapter 2.

7.3.5 Statistical methodology

Sample size calculations

Sample size calculations were based on the initial OTTT study (Chapter 4 and 6, Statistical methodology). After review of previous studies, it was predicted that type 1 diabetes subjects would display similar triglyceride profiles to type 2 diabetes subjects thus calculations were based on detecting a 35% relative reduction in 6 hour post challenge triglyceride levels.

Data Analysis

Statistical methodology regarding data checking and analysis as well as derived variables is summarised in Chapter 2. IAUC to 4 hours (as well as IAUC to 8 hours) was

calculated in order to enable assessment of differences between study medications in the early postprandial period.

Paired t tests and Wilcoxon matched pairs signed rank sum test were used to compare parametric and non parametric data respectively for soluble and lispro insulin.

7.4 Results

7.4.1 Subjects

There were more males than females participating in the study. Subjects were routinely treated by soluble and basal insulin (n=11) or analogue and basal insulin (n=9). Subjects were younger than our previous studies of type 2 diabetic subjects, mean (SD) age 43.0 (12.7) years, and were slightly overweight BMI 26.0 (4.4) kg/m^2. Blood glucose control was not as well controlled as seen in our type 2 diabetic subjects from previous studies with HbA1c 8.4 (0.9)%. The subjects were not hypertensive, mean (SD) systolic blood pressure of 118.0 (14.8) mm Hg and diastolic blood pressure of 67.8 (10.1) mm Hg. Subject characteristics are outlined in Table 19.

Baseline lipid profile was within normal limits (Chapter 2) with mean (SD) mmol/l total cholesterol level 4.6 (1.10), LDL cholesterol 2.83 (0.82), HDL cholesterol 1.46 (0.42) and geometric mean (1 SD range) triglyceride level 1.1 (0.7 - 1.7) mmol/l. No significant differences were found comparing baseline triglyceride, NEFA, glycerol, glucose, insulin or C peptide levels prior to soluble or lispro insulin administration.

The mean (SD) insulin dose administered was 7.9 (1.83)U. Mild hypoglycaemia occurred in 4 subjects after the 6 hour timepoint with both insulin lispro (n=2) and soluble insulin (n=2). In each case the OTTT was stopped and subjects given a meal. Data presented are with these subjects included although there was no substantial difference when these subjects were excluded in the analysis.

Table 19 Subject characteristics of type 1 diabetic subjects

n	20
Sex *	14M /6F
Age (years)	43.0 (12.7)
Duration of diabetes (years) †	16.5 (11.5 - 27.0)
BMI (kg/m2)	26.0 (4.4)
Waist (cm)	82.3 (15.4)
Triceps skinfold thickness (mm)	15.7 (9.0)
BP (mm Hg)	
Systolic	118.0 (14.8)
Diastolic	67.8 (10.1)
Smoking (n)	
Non smoker	9
Ex smoker	5
Smoker	6
Hormone use (n)	3
Menopausal (n)	3
HbA1c (%)	8.4 (0.9)
Fasting plasma glucose (mmol/l)	13.1 (5.2)
Fasting total cholesterol (mmol/l)	4.6 (1.1)
Fasting LDL cholesterol (mmol/l)	2.83 (0.82)
Fasting HDL cholesterol (mmol/l)	1.46 (0.42)
Fasting VLDL cholesterol (mmol/l)	0.36 (0.19)
Triglyceride (mmol/l) *	1.1 (0.7 - 1.7)

Data are mean (SD) except † median (IQR) and * geometric mean (1SD range).

7.4.2 Post challenge profiles

Triglyceride

A statistically significant difference was found 6 hours post challenge triglyceride levels, 1.2 (0.61-2.38) mmol/l and 1.4 (0.79-2.44) mmol/l respectively (p=0.037). The triglyceride IAUC was also greater with insulin lispro compared with soluble insulin (p = 0.044) (Table 20, Figure 23).

NEFA

NEFA levels were lower at 2 hours post challenge with insulin lispro compared with soluble insulin, this was not statistically significant (p = 0.2754) (Table 20, Figure 23). No difference in IAUC NEFA between insulins was found (p = 0.7984).

Glycerol

At 2 hours post challenge there was no significant difference in median (IQR) glycerol levels (mmol/l) between soluble and lispro insulin, 0.48 (0.337 – 0.711) and 0.66 (0.353 – 0.774) respectively (Table 20, Figure 23). The AUC glycerol was significantly greater with insulin lispro (p = 0.034) although IAUC showed no difference.

Mean total cholesterol, LDL cholesterol, HDL cholesterol and VLDL cholesterol levels did not change significantly in any group over the timepoints

Glucose

Post challenge glucose levels did not differ between insulins with values of 16.5 (5.93) mmol/l and 16.0 (4.32) mmol/l at 2 hours and mean (SD) Cmax glucose levels (mmol/l) of 17.3 (5.54) and 16.6 (4.33) ($p = 0.622$) respectively (Table 20, Figure 24). Glucose values were lowest at 6 hours (Tmin) 6.9 (2.94) mmol/l and 7.0 (3.36) mmol/l for soluble and lispro insulins respectively ($p = 0.9302$). There was no significant difference in glucose total IAUC or IAUC to 4 hours between the 2 insulins, although values were lower with soluble insulin. IAUC glucose from 4 to 8 hours was lower with soluble than lispro insulin ($p = 0.0429$).

Insulin

The 2 hour post challenge geometric mean (1 SD range) insulin levels between soluble and lispro insulin, 239.2 (135.60 – 421.86) and 211.3 (97.91 – 4555.91) respectively did not differ significantly (Table 20, Figure 24). The IAUC insulin from baseline to 4 hours and baseline to 8 hours appeared greater with soluble insulin than with lispro insulin, but this was not statistically significant ($p = 0.5495$ and $p = 0\ 2936$ respectively).

C peptide levels measured at baseline and post challenge were undetectable.

Table 20 Fasting & post challenge responses to soluble & lispro insulin in type 1 diabetic subjects

	Soluble		Lispro		*p*
Triglyceride (mmol/l)					
0 hour	1.1	0.68- 1.74	1.1	0.68- 1.68	*0.5858*
2 hours	1.3	0.80- 2.06	1.3	0.81- 2.09	
4 hours	1.3	0.75- 2.31	1.3	0.77- 2.31	
6 hours	1.2	0.61- 2.38	1.4	0.79- 2.44	*0.0368*
8 hours	0.9	0.52- 1.45	1.0	0.66- 1.60	
AUC^{+}	10.1	6.00- 17.03	10.4	6.51- 16.48	*0.1639*
$IAUC_{0\text{-}6hours}{}^{*+}$	1.0	0.53- 2.74	1.8	0.56- 2.50	*0.1535*
$IAUC_{0\text{-}8hours}{}^{*+}$	1.1	0.36- 2.68	1.8	0.63- 2.41	*0.0437*
Cmax	1.6	0.95- 2.74	1.7	1.07- 2.73	*0.2865*
deltaCmax	0.47	0.19- 1.19	0.6	0.31- 1.22	*0.0232*
Tmax*±	4	3- 6	4	4- 6	*ns*
6-0 hours*	0.1	-0.11- 0.61	0.3	0.08- 0.48	*0.1403*
NEFA (umol/l)					
0 hour	445.8	253.95- 782.74	477.6	289.31- 788.35	*0.6694*
2 hours	170.5	92.95- 312.63	147.1	90.15- 239.90	*0.2754*
4 hours	234.1	125.21- 437.86	257.1	162.11- 407.60	
6 hours	371.7	214.96- 642.73	488.6	314.48- 759.05	
8 hours	438.0	248.97- 770.44	510.0	302.51- 859.81	*0.8769*
AUC^{+}	2721.0	2179.53- 3396.91	2993.5	2193.19- 4085.89	*0.0962*
$IAUC_{0\text{-}4hours}{}^{*+}$	-907.2	-1264.25- -471.50	-890.5	-1314.00- -396.75	*0.7636*
$IAUC_{0\text{-}8hours}{}^{*+}$	-1638.5	-2278.00- -964.00	-1154.5	-2319.00- -171.00	*0.7984*
Cmin	134.9	74.86- 243.15	137.5	90.88- 207.98	*0.8915*
Cmax	640.0	520.90- 786.40	654.9	457.21- 938.18	*0.498*
Tmax*±	0	0- 8	6	0- 8	*0.2212*
2-0 hours*	-312.0	-420.00- -201.00	-361.0	-488.00- -201.00	*0.5495*
8-0 hours*	-88.5	-172.25- 138.50	43.5	-152.25- 173.75	*0.7942*
Glycerol (mmol/l)					
0 hour	0.53	0.383- 0.893	0.71	0.437- 0.855	*0.9565*
2 hours	0.48	0..337- 0.711	0.66	0.353- 0.774	*0.2121*
4 hours	0.54	0.412- 0.836	0.67	0..462- 0.787	
6 hours	0.56	0..323- 0.844	0.75	0.532- 1.042	
8 hours	0.51	0..385- 0.635	0.64	0.412- 0.801	*0.9152*
AUC^{+}	4.94	3.64- 6.04	5.24	4.30- 6.80	*0.0342*
$IAUC_{0\text{-}4hours}{}^{*+}$	-0.19	-0.630- 0.380	-0.24	-0.624- 0.304	*0.5495*
$IAUC_{0\text{-}8hours}{}^{*+}$	-0.09	-1.987- 0.510	-0.06	-1.520- 1.205	*0.3517*
Cmin	0.27	0.20- 0.39	0.35	0.21- 0.49	*0.1503*
Cmax	0.89	0.77- 1.21	1.00	0.76- 1.27	*0.8216*
Tmax*±	4	0- 6	4	0- 6	*0.6739*
2-0 hours*	-0.13	-0.277- 0.046	-0.14	-0.323- 0.081	*0.6278*
8-0 hours*	-0.15	-0.402- -0.027	-0.01	-0.277- 0.147	*0.9152*

Continued Table 20 Fasting and post challenge OTTT responses to soluble and lispro insulin in type 1 diabetic subjects

	Soluble		Lispro		*p*
Glucose (mmol/l)					
0 hour	13.0	5.22	11.6	5.14	*0.4282*
2 hours	16.5	5.93	16.0	4.32	*0.7297*
4 hours	11.1	4.73	10.2	4.09	
6 hours	7.9	3.46	8.4	4.25	
8 hours	8.5	2.94	9.3	4.42	
AUC^{+}	97.0	27.16	94.1	27.64	*0.9149*
$IAUC_{0\text{-}4hours}$*$^{+}$	3.2	-4.05 - 15.1	9.5	-2.40 - 20	*0.4931*
$IAUC_{0\text{-}8hours}$*$^{+}$	-13.8	-23.8 - 7.6	5.2	-12.2 - 23.4	*0.1337*
Cmax	17.3	5.54	16.6	4.33	*0.6216*
Cmin	6.9	2.94	7.0	3.36	*0.9302*
Tmax*±	2	2 - 2	2	2 - 2	*0.562*
Tmin*±	6	6 - 8	6	4 - 8	*0.6514*
2-0 hours*	2.4	1.00 - 7.18	5.0	1.14 - 8.25	*0.3621*
Insulin (pmol/l)					
0 hour	110.2	44.49 - 272.76	110.7	41.58 - 294.70	*0.6251*
2 hours	239.2	135.60 - 421.86	211.3	97.91 - 455.91	*0.2585*
4 hours	170.2	82.59 - 350.71	136.1	50.67 - 365.77	
6 hours	122.0	51.27 - 290.36	98.8	35.90 - 271.69	
8 hours	95.7	36.32 - 252.38	76.4	28.32 - 205.90	
AUC^{+}	1289.1	612.81 - 2711.95	1006.5	442.11 - 2291.40	*0.1077*
$IAUC_{0\text{-}4hours}$*$^{+}$	243.0	191.08 - 360.80	237.9	165.58 - 351.03	*0.5495*
$IAUC_{0\text{-}8hours}$*$^{+}$	322	255.10 - 474.20	200	27.55 - 356.93	*0.2936*
Cmax	246.8	139.56 - 436.62	223.8	106.41 - 470.76	*0.2415*
Tmax*±	2	2 - 2	2	2 - 2	
2-0 hours*	103.1	87.95 - 144.23	95.7	71.08 - 135.05	*0.5746*

Data are geometric mean (1 SD range) except † mean (SD) and * median (IQR).
Units are as tabulated except + units.hour and ± hours

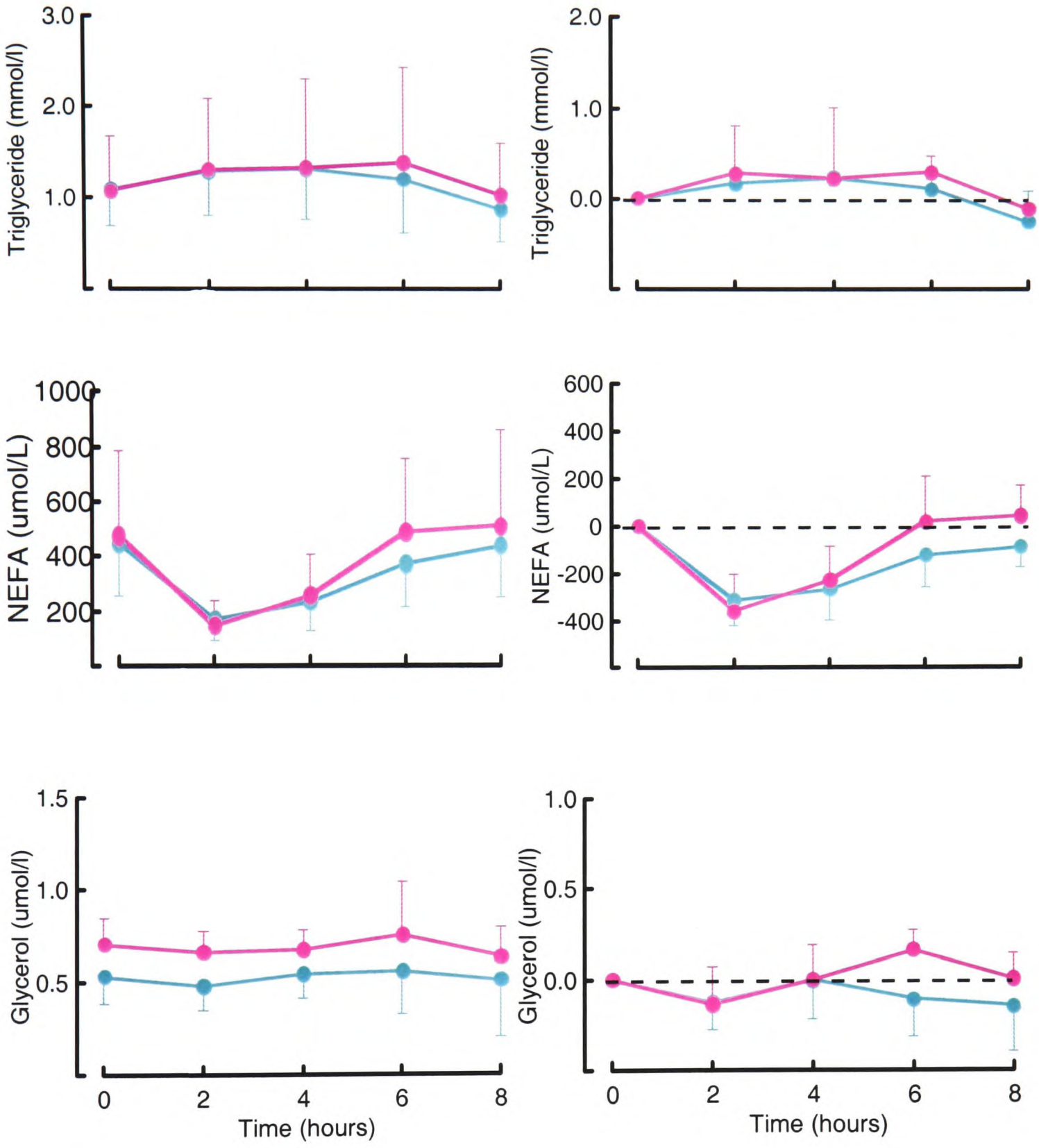


Figure 23 Triglyceride, NEFA and glycerol profiles for lispro ● and soluble ● insulin in type 1 diabetic subjects. Absolute values are depicted on the left, and change from baseline (delta) on the right.

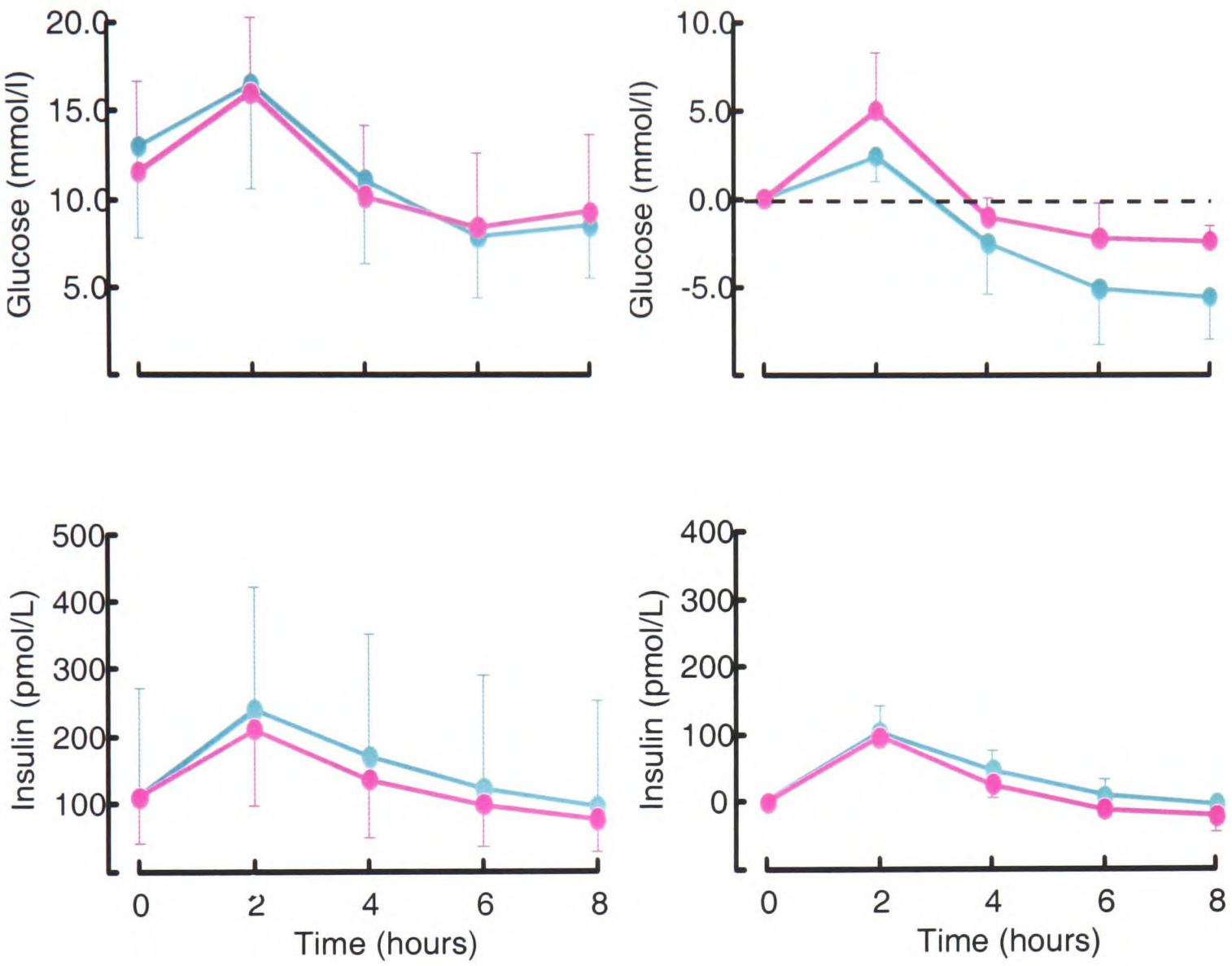


Figure 24 Glucose and insulin profiles for lispro ● and soluble ● insulin in type 1 diabetic subjects. Absolute values are depicted on the left, and change from baseline (delta) on the right.

7.4.3 Correlations

Pearson correlation coefficients showed a strong association between post challenge and fasting triglyceride values for both insulins (Table 21)

Table 21 Correlation coefficients for fasting triglyceride concentration with postprandial triglyceride levels.

	Soluble Insulin		Insulin lispro	
	r	p	r	p
2 hour	0.7972	<0.0001	0.6946	0.0007
4 hour	0.7764	<0.0001	0.7927	<0.0001
6 hour	0.8408	<0.0001	0.821	<0.0001
8 hour	0.9136	<0.0001	0.8384	<0.0001

Fasting triglyceride concentration correlated with fasting insulin concentration ($r = 0.4608$, $p = 0.0409$) for lispro but not soluble insulin. IAUC TG correlated with IAUC glucose ($r = 0.484$, $p = 0.042$) for soluble insulin.

7.5 Discussion

This study showed that post challenge triglyceride profiles over 8 hours (IAUC) were lower after soluble than lispro insulin. Although insulin IAUC was reduced over the 8 hour study period with insulin lispro, this was not statistically significant. Late post challenge glucose levels were higher with lispro insulin.

Our findings of similar 2 hour post challenge insulin and glucose profiles after administration of soluble or lispro insulin is consistent with some studies but not others. Ferguson et al (Ferguson, Strachan et al. 2001) too found no difference in 2 hour post

breakfast glucose levels between the two insulins tested or in HbA1c after 12 months of therapy. Other studies have found insulin lispro to significantly reduce 2 hour post breakfast glucose excursions (Holleman F, Schmitt H et al. 1997; Garg S K, Anderson J H et al. 1999; Gale and Group 2000). In the study by Garg et al (Garg S K, Anderson J H et al. 1999), serum insulin levels at 30 and 60 minutes were significantly higher with insulin lispro compared to soluble insulin, but levels were similar by 2 hours. Our results too may have demonstrated a significant increase in early post challenge insulin concentrations if levels were assessed between the 0 to 2 hour timepoint.

C peptide levels were undetectable in all of the type 1 diabetic subjects participating in the study, confirming the absolute deficiency of endogenous insulin production. The average insulin dose chosen to administer to type 1 diabetic subjects of 8U was adequate to lower post challenge glucose levels by the end of the study period. By 2 hours post challenge, insulin levels approximately doubled for both insulins. This increase in insulin is similar to that seen in previous studies (Garg S K, Anderson J H et al. 1999), with comparable doses of insulin prescribed (mean insulin dose of 8.5 (3.0)U). By 3 hours in the study by Garg et al, insulin levels were higher after soluble insulin compared with lispro insulin, as found in our study at 4 hours.

Previous studies have found less hypoglycaemic episodes with insulin lispro compared with soluble insulin (Holleman F, Schmitt H et al. 1997; Gale and Group 2000). Reduced frequency of severe hypoglycaemia was found as well as nocturnal hypoglycaemia. Our study showed the number of hypoglycaemic events was the same with soluble and lispro

insulin administration. These mild episodes occurred during the late postprandial phase (ie after 6 hours). No reason for subjects having hypoglycaemia with one insulin over the other could be established.

The results of our study show that normotriglyceridaemic type 1 diabetic subjects after a 50g fat and 50g carbohydrate challenge, produce a maximum triglyceride increase (delta Cmax) that is on average, 0.5 mmol/l, with median time to achieve this being 4 hours. This is similar to previous postprandial lipid studies in type 1 diabetic subjects which show maximum triglyceride concentrations between 3 to 5 hours post oral fat load, similar to non diabetic subjects (Lewis, O'Meara et al. 1991). A "placebo arm", that is type 1 diabetic subjects not receiving prandial insulin, was not incorporated into the study due to the possibility of hyperglycaemia influencing lipid handling. Previous authors have proposed this post challenge triglyceride excursion may actually reflect lipoprotein particle abnormalities. A study by Valabhji et al (Valabhji, Donovan et al. 2002) highlighted the correlation between triglyceride concentration and rates of cholesterol esterification and transfer to and from HDL and apolipoprotein B containing lipoproteins. Their study showed that in normotriglyceridaemic type 1 diabetic subjects compared with non diabetic individuals, due to the slope of the association, small increases in triglyceride concentrations could result in more marked increases in the rates of esterified ester net mass transfer, resulting in greater cholesterol enrichment of apo B containing lipoproteins which could contribute to a higher CHD risk, despite normal HDL levels. This finding was also noted in an earlier study showing abnormally high cholesterol to triglyceride ratio in VLDL suggesting VLDL remnants or beta VLDL which may

enhance atherosclerosis (Pietri A O, Dunn F L et al. 1983). More detailed VLDL analysis would have examined this issue in our subjects.

Soluble insulin lowered 6 hour triglyceride levels as well as reducing the increase to maximal triglyceride concentration compared with insulin lispro in our study. Overall triglyceride IAUC for the post challenge duration was reduced by 40% with soluble insulin compared with insulin lispro. This reduction is greater than the 33% reduction in triglyceride AUC with the use of simvastatin in 8 normotriglyceridaemic type 1 diabetic subjects with elevated LDL levels (Noutsou and Georgopoulos 1999). The type and amount of routinely prescribed prandial insulin therapy used by their subjects in the study was not described. Simvastatin had no effect on the fasting triglyceride level, but yet decreased the number and therefore triglyceride and apo B content, of circulating intestinal and hepatic triglyceride rich lipoprotein particles. The authors proposed that the mechanism could be either improved lipoprotein and VLDL clearance by the upregulation of LDL receptors or decreased production of VLDL.

Our findings are consistent with a study by Caixas et al (Caixas A, Perez A et al. 1998) who showed that intensive insulin therapy with regular insulin decreased cholesterol and triglyceride concentrations more so than insulin lispro. A 13% reduction in 2 hour post challenge triglyceride levels was found with soluble insulin compared with lispro insulin. A possible explanation for our findings may be related to LPL activity which has been shown to increase in response to insulin and glucose stimuli (Yki-Järvinen, Taskinen et al. 1984). The postprandial hyperinsulinaemia induced by soluble insulin rather than the

rapid insulin release of lispro, may be required to induce maximum stimulation of LPL activity. However abnormalities of LPL causing hypertriglyceridaemia are reported only in severely hyperglycaemic and ketotic patients and not necessarily poorly controlled type 1 diabetic patients (Pietri A O, Dunn F L et al. 1983).

The influence of various insulin replacement regimens on triglyceride metabolism has been investigated in one study. Lewis et al (Lewis, O'Meara et al. 1991) reduced the dose of insulin over a 2 week period in 7 type 1 diabetic patients with previously good metabolic control. Identical triglyceride responses were found in subjects who had raised pre meal glucose levels (14 -17 mmol/l), good glycaemic control (5-6 mmol/l) and/or insulin infusion during the test meal to ensure postprandial glucose levels did not rise above 10 mmol/l. The authors concluded that during periods of poor control, the hepatic response to the increased availability of substrate may take some time to manifest. Conversely, during periods of good glycaemic control there is rapid inhibition of lipolysis causing an immediate reduction in the amount of substrate available for VLDL triglyceride synthesis. More detailed lipid analysis to include lipoprotein particles and fractions in our study may have demonstrated further differences between soluble and lispro insulin. Caixas et al (Caixas A, Perez A et al. 1998) demonstrated in type 1 diabetic subjects, 2 hour postprandial VLDL particles had a high lipid to protein ratio compared with non diabetic subjects, which improved with both insulins, but was only corrected with insulin lispro.

To further elucidate subtle differences in triglyceride metabolism seen with insulin lispro and soluble insulin, NEFA profiles were examined as both glucose and NEFA are substrates for hepatic triglyceride synthesis. Elevated fasting NEFA levels and decreased postprandial suppression are characteristic lipid abnormalities found in type 2 diabetic individuals. Our subjects had fasting NEFA levels that were, although within the normal range, higher than our non diabetic subjects in the OTTT study but lower than levels seen in the type 2 diabetic subjects. Elevated NEFA levels have been noted in type 1 diabetic subjects during conventional insulin treatment (Greenfield, Kolterman et al. 1980). Studies show NEFA levels in type 1 diabetic subjects to be affected by acute variations in insulin levels (Lewis, O'Meara et al. 1991). The effect of insulin lispro and soluble insulin on NEFA levels in our study was similar during the immediate post challenge period. NEFA concentrations decreased at the 2 hour timepoint and then increased 6 to 8 hours post challenge. This latter elevation was particularly evident with insulin lispro, whilst NEFA levels with soluble insulin were highest at baseline. Post challenge glycerol profiles also portrayed a similar pattern, with mean AUC being greater with insulin lispro. As plasma NEFA levels are a reflection of the rate of chylomicron triglyceride lipolysis by LPL and re esterification into adipose tissue, the rise in NEFA concentration may relate directly to the degree of mealtime insulinization. Lewis et al (Lewis, O'Meara et al. 1991) demonstrated that when insulin infusion was used in type 1 diabetic subjects to simulate the physiological postprandial increase of insulin, NEFA concentrations decreased, but when the infusion was held constant, the NEFA levels increased.

The type 1 diabetic subjects recruited to this study were on short acting prandial insulin replacement, and basal insulin injected at bedtime. All subjects injected basal insulin (isophane or lente) the night before the study with only 2 subjects injecting twice daily. One possibility for the lower triglyceride excursion seen with soluble insulin, is related to the action of basal insulin diminishing by the last timepoint of the study. This is reflected by IAUC for glucose during the last 4 hours of the study being higher with insulin lispro. The inadequate insulinization and late postprandial hyperglycaemia then contribute to elevated triglyceride levels. This is highlighted by the suppression of NEFA and glycerol in the last 4 hours of the study, being greater with soluble insulin than insulin lispro although not statistically significant. The close relationship of post challenge glucose and triglyceride is emphasized by significant associations of glucose and triglyceride IAUC. Therefore the extended action profile of soluble insulin may be important in itself or it may be compensating for inadequate maintenance of basal insulin delivery. However in the study by Caixas et al (Caixas A, Perez A et al. 1998), subjects were given either soluble or lispro insulin as well as ultralente insulin prior to the test meal containing 10% fat. Their results showed that the peripheral hyperinsulinism induced by soluble insulin caused triglyceride levels to appear lower with this therapy than with lispro insulin. They also found LDL particles were cholesterol enriched and proteins depleted, with neither insulin correcting this, although mass decreased with soluble insulin. Larger more detailed studies would be required to confirm whether the extended action profile of soluble insulin is important in itself or could be compensating for inadequate maintenance of basal insulin delivery.

Although a triglyceride response was provoked in all subjects in this study, a higher fat load may have posed as a greater metabolic challenge, and differences between the two insulins could have been further established. A randomised crossover study comparing high carbohydrate (24% fat, 61% carbohydrate and 15% protein) and high mono-fat (40% fat, 45% carbohydrate and 15% protein) diets in type 1 diabetic subjects showed the latter to produce higher postprandial triglyceride levels, although still in the normal range (Georgopoulos, Bantle et al. 1998). Maximal triglyceride concentrations produced in our study were lower than Georgopoulos et al and Lewis et al (Lewis, O'Meara et al. 1991), despite a slightly higher baseline triglyceride concentration noted in our subjects. Overall it is unlikely that the higher triglyceride excursion seen with insulin lispro would be disadvantageous as clinically there was 0.2 mmol/l difference in triglyceride between the two insulins tested.

The effect of prandial insulin administration in hypertriglyceridaemic type 1 diabetic subjects would also assist in providing evidence for a triglyceride lowering effect. Our subjects had a similar fasting lipid profile to the 3159 type 1 diabetic subjects in the EURODIAB IDDM Complications Study (Idzior-Walus, Mattock et al. 2001). Normal fasting cholesterol levels in our subjects were slightly lower than the EURODIAB study mainly due to lower LDL levels, but HDL and triglyceride concentrations were comparable. Although studies in hypertriglyceridaemic subjects may have produced higher triglyceride excursions to perhaps enable greater modification by insulin, our subjects were more representative of the type 1 diabetic population.

8 Chapter 8 Platelet Function in Diabetes

8.1 Introduction

The increase CHD risk seen in patients with diabetes may be contributed to, in part, by non traditional risk factors such as markers of inflammation, thrombosis and endothelial dysfunction. Eighty percent of diabetes mellitus related deaths are due to alterations in thrombosis, with 75% of these deaths due to cardiovascular complications, and the remainder due to cerebrovascular events and peripheral vascular complications (Carr 2001). Abnormalities of atherothrombosis have been described in diabetes, although the mechanisms are poorly understood. Growing evidence indicates that diabetic patients may display increased platelet adhesion and aggregation. Hypercoagulability has been demonstrated in individuals with type 1 or type 2 diabetes mediated by alterations in platelet function (Tschoepe and Menart-Houtermans 2002). Atherosclerotic lesions are susceptible to arterial thrombosis, with platelets integral to the process of thrombogenesis. Platelet emboli can also cause microvascular occlusions and complications. Studies have demonstrated insulin to have direct effects on insulin receptors present on the platelet membrane. In addition, recent work has suggested that diabetic dyslipidaemia may possibly play a modulatory role in platelet function.

8.1.1 Overview of platelet function

Platelets are anucleated blood cells derived from bone marrow megakaryocytes. Their physiological function is to arrest haemorrhage from tissue trauma. The main trigger

after vascular injury for thrombus formation, is the loss of integrity of the endothelium. In the process of haemostasis, platelets are intimately involved with the vessel wall, coagulation factors and fibinolysis. Platelet responses are divided into three successive phases, adhesion, activation and aggregation.

Platelet adhesion is dependent upon many constituents present in the extracellular matrix such as collagen and von Willebrand factor (vWF), and the plasma. Collagen is a unique agonist by simultaneously being an adhesive surface and a potent activator for platelets (Sixma, van-Zanten et al. 1997). Within seconds, platelets, upon interaction with these factors, form a monolayer over the lesion. Propagation of the thrombus by platelets adhering to collagen receptors in conditions of high blood flow cannot occur without plasma vWF binding to collagen (Savage, Ginsberg et al. 1999).

The activation of platelets is stimulated by the initial adhesion phase and is delicately controlled by inhibitors and inducers of the process. Prostacyclin and nitric oxide limit thrombus generation to within the lesion (Ruggeri 2002). Collagen, vWF, α thrombin, ADP, epinephrine and thromboxane A_2 all induce platelet activation. Activation of platelets also provides the interface for cofactors from the coagulation cascade. The coagulation cascade consists of two independent pathways which converge onto a common pathway with thrombin generation to form a fibrin clot as the end point. The deposition of fibrin contributes to the stability of the thrombus. Also once activated, platelets secrete various components from storage granules which enhance further activation and lead to platelet aggregation within minutes.

The function of platelets is to arrest bleeding, however in atherosclerotic arteries they may cause occlusion of the vessel lumen, impeding blood flow, therefore causing ischaemic injury to tissues. In large vessels this may be demonstrated in myocardial infarction or acute coronary syndrome, whilst in smaller arteries it may be lead to microangiopathy present in diabetes related complications such as retinopathy.

8.1.2 The role of insulin in platelet function

Human platelets have insulin receptors that participate in the regulation of platelet activity (Falcon, Pfliegler et al. 1988). Insulin induced hypoglycaemia has been shown to cause platelet hyperaggregability which is attributable to epinephrine (Trovati and Anfossi 2002). Also, insulin has been shown to induce a significant reduction in the aggregating response to many agonists, including adenosine diphosphate (ADP), platelet activating factor, collagen, sodium arachidonate, and epinephrine.

It has also been observed that in a hyperinsulinaemic state (without hypoglycaemia and therefore without counter-regulatory hormone activation) there is a significant decrease of platelet aggregability (Trovati and Anfossi 2002). The mechanism was unclear as insulin did not reduce thromboxane A2 production and calcium influx into platelets was not a key feature. However, insulin did increase platelet concentrations of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) which are involved in calcium flux and are nitric oxide mediated. In summary, insulin exerts opposite effects on platelet function at physiological and supraphysiological

concentrations, that is, at supraphysiological concentrations, insulin has an antiaggregatory effect on platelets.

8.1.3 Platelet function in diabetes

In vivo studies by Westerbacka et al (Westerbacka, Yki-Järvinen et al. 2002) demonstrated that normal insulin action inhibited platelet interaction with collagen under conditions mimicking thrombus formation and reduced aggregation to several agonists. In healthy non obese subjects, in vivo insulin reduced platelet deposition to collagen in flowing whole blood and impaired the platelet-related primary hemostasis under high shear rate conditions. In obese insulin-resistant subjects, insulin completely failed to inhibit platelet-collagen interaction.

As previously discussed, hyperinsulinaemia is often present in type 2 diabetes (Chapter 1). From the review above, it would be expected that if normal platelet sensitivity to insulin were present, people with type 2 diabetes would have an increased insulin-induced attenuation of platelet aggregation, resulting in protective effects against thrombosis and atherogenesis. Few studies have investigated platelet function in diabetic subjects. A study comparing non diabetic and diabetic subjects (Trovati, Mularoni et al. 1995) demonstrated how insulin concentrations in insulin resistant states, although elevated, were not able to physiologically modulate platelet function. Also insulin action on cAMP, was severely impaired in insulin-resistant patients, and therefore human platelets were a site of insulin resistance and platelet function could therefore potentially contribute to the pathogenesis of atherosclerosis. Platelet responses in a study of obese

non-diabetic and obese type 2 diabetic subjects were comparable, showing that when metabolic control was good, impairment was less likely (Anfossi, Mularoni et al. 1998). This study also highlighted that lean type 2 diabetic subjects had normal responses to the insulin effects of platelet aggregation and on cGMP. Other haematological disturbances such as the vasodilating effects of insulin are impaired in obesity and type 2 diabetes (Laasko, Edelman et al. 1992) which may also contribute to the atherothrombosis seen in these states.

Type 1 diabetic subjects have increased platelet aggregation responses particularly when metabolic control is poor, as demonstrated by an elevation of HbA_{1c} (Valles, Santos et al. 1997). Also, acute elevations of blood glucose to levels of approximately 15 mmol/l in type 1 diabetic patients causes the number of platelets to significantly decrease, compared with non diabetic subjects (Khodabandehlou, Zhao et al. 1998). Physiological concentrations of insulin have been shown to enhance platelet fibrinogen binding in both type 1 diabetic subjects as well as non diabetic individuals (Hu, Li et al. 2002). Intensive insulin therapy can also reduce platelet aggregation in type 1 diabetic patients (Turk, Flego et al. 1996).

Table 22 Insulin effects on platelets in diabetes. Summarised from (Trovati and Anfossi 2002)

- Increase adhesion
- Increase aggregation in response to agonists
- Increase thromboxane production
- Increase platelet specific protein release
- Increase calcium mobilization
- Increased adhesion molecule expression
- Decrease of sensitivity to anti aggregating agents
- Changes in platelet volume, shape, life span, turnover, membrane fluidity

The plasma levels of many clotting factors including fibrinogen, factor VII, factor VIII, factor XI, factor XII, kallikrein, and vWF are elevated in diabetes. vWF, a multimeric glycoprotein synthesized mainly by endothelial cells, is a marker for endothelial dysfunction. It is involved in platelet adhesion and aggregation and has an important role in the process of angiogenesis. Increased levels of vWF, reflecting activation or damage of endothelial cells, have been described in association with proliferative diabetic retinopathy (Beranek, Kankova et al. 2002).

8.1.4 Postprandial lipaemia and thrombosis

Several studies have indicated that dietary fat intake may induce detrimental changes in the postprandial period related to haemostasis, endothelial dysfunction and oxidative stress contributing to the pathogenesis of vascular disease in both diabetic and non diabetic subjects (Miller 1998; Anderson, Evans et al. 2001). Significant correlations have been demonstrated between platelet sensitivity to ADP and LDL cholesterol (Turk, Flego et al. 1996). Watanabe et al (Watanabe, Wohltmann et al. 1988) postulated that the increased degree of glycosylation of LDL seen in diabetic patients may alter the structure of platelet membrane thereby enhancing platelet reactivity to aggregating agents. Other authors believe that fluctuations in plasma NEFA levels may interfere with platelet aggregation (Myrup, Bregengaard et al. 1991). Studies of diabetic subjects show platelet adhesion to correlate with triglyceride levels and inversely to HDL levels (Knobler, Savion et al. 1998).

8.2 Aims

1. To characterise post triglyceride and carbohydrate challenge platelet responses in type 1 and type 2 diabetic subjects.

2. To examine differences in platelet function in type 1 and type 2 diabetic subjects after rapid and less rapid exogenous insulin administration.

8.3 Methods

8.3.1 Study Design

Platelet function was measured in the studies investigating the effects of exogenous insulin administration (Chapter 6 and Chapter 7) at baseline, 2 and 6 hours post lipid and carbohydrate challenge. These timepoints were chosen to best characterise platelet function when insulin (2 hours) and triglyceride (6 hours) levels would be highest.

8.3.2 Subjects

The collaboration to investigate platelet function in diabetes was initiated during the intervention study examining the metabolic effects of exogenous insulin therapy in type 2 diabetic subjects (Chapter 6). Therefore 9 of the 20 subjects were able to be recruited to the platelet study. All 20 subjects participating in the intervention study examining the metabolic effects of exogenous insulin replacement in type 1 diabetes participated in the platelet study (Chapter 7).

8.3.3 Study Procedure

Study medication and procedures were outlined in Chapters 2, 6 and 7. Subjects were asked to refrain from taking aspirin for 3 days prior to the OTTT. At the 0, 2, and 6 hour timepoints, 5 ml of blood was collected from the forearm venous cannula in sodium citrate tubes at room temperature for immediate platelet function analysis.

8.3.4 Biochemical Methods

Platelet function

The PFA-100 or platelet function analyser is a simple and rapid in vitro test designed to measure platelet related primary haemostasis under shear rate conditions. The system utilizes two cartridges, collagen/ADP (CADP) and collagen/epinephrine (CEPI), which are made up of ADP or epinephrine incorporated into a collagen fibril mesh. Citrated whole blood encounters this membrane at a shear rate of 5000-6000/s within a capillary. The fall in flow rate as the platelets form a haemostatic plug within an aperture (150 μm) within the centre of the membrane is measured. The normal range was defined for CADP as 55 – 112 seconds and for CEPI 79 – 164 seconds.

vWF

Plasma levels of VWF:Ag were measured from sodium citrated plasma using a fully automated immunoturbidometric assay (Sta-Liatest® VWF kit, Diagnostic Stago & Roche Diagnostics) on an MDA-180™ analyser (Biomerieux, Basingstoke, Hampshire).

The assay was performed according to the manufacturer's instructions and calibrated using the 8th British Reference Plasma (98/734, NIBSC, South Mimms, Herts). The normal range was defined as 50 – 200% activity.

Haematocrit and platelet count

Haematocrit, and platelet count were measured from sodium citrated plasma using the Sysmex KX21 Haematology Impedance Analyser. Normal ranges were defined as haematocrit >35% and platelet count 150 -450 x 10^9 /L.

8.3.5 Statistical methods

Statistical methodology regarding data checking and analysis is summarised in Chapter 2. The CV was calculated for both baseline CADP and CEPI. Paired t tests and Wilcoxon matched pairs signed rank sum test were used to compare parametric and non parametric data respectively for soluble and lispro insulin. T tests and Mann Whitney tests were used to compare parametric and non parametric data respectively for type 1 and type 2 diabetic subjects.

8.4 Results

Subject characteristics are outlined in Table 23. In the study of type 2 diabetic subjects, 9 subjects completed both tests. In the study of type 1 diabetic subjects, 20 subjects completed both tests. A total of 174 PFA tests were conducted, with one baseline sample from a type 1 diabetic subject aggregating prior to analysis and one 6 hour sample from a

type 1 diabetic subject unable to be analysed due to underfilling of the sample tube. Data presented are with these subjects included although there was no substantial difference when these subjects were excluded in the analysis.

Table 23 Subject characteristics of type 1 and type 2 diabetic subjects participating in study of platelet function.

	Type 2 diabetes	Type 1 Diabetes
n	9	20
Sex *	4M /5F	14M /6F
Age (years)	65.1 (7.0)	43.0 (12.7)
Duration of diabetes (years) †	4.0 (2.5-13.0)	16.5 (11.5 - 27.0)
BMI (kg/m^2)	30.6 (4.8)	26.0 (4.4)
Waist (cm)	99.0 (10.6)	82.3 (15.4)
Triceps skinfold thickness (mm)	14.51(8.6)	15.7 (9.0)
BP (mm Hg)		
Systolic	132.8 (12.9)	118.0 (14.8)
Diastolic	74.5 (5.3)	67.8 (10.1)
HbA1c (%)	7.7 (1.0)	8.4 (0.9)
Fasting plasma glucose (mmol/l)	8.6 (2.2)	13.1 (5.2)
Fasting total cholesterol (mmol/l)	4.8 (0.4)	4.6 (1.1)
Fasting LDL cholesterol (mmol/l)	2.87 (0.74)	2.83 (0.82)
Fasting HDL cholesterol (mmol/l)	1.23 (0.26)	1.46 (0.42)
Fasting VLDL cholesterol (mmol/l)	0.65 (0.34)	0.36 (0.19)
Triglyceride (mmol/l) ‡	1.7 (0.69 - 2.72)	1.1 (0.7 - 1.7)

Data are mean (SD) except † median (IQR) and ‡ geometric mean (1 SD range)

8.4.1 Reproducibility of closure times

The median CV (IQR) % for fasting CADP closure time in type 1 diabetic subjects 12.6 (3.6 – 14.3) did not differ to type 2 diabetic subjects 11.6 (7.9 – 16.0) ($p > 0.1$). There was also no significant difference in fasting CEPI CVs 14.5 (8.0 -17.1)% and 15.5 (8.6 – 32.1)% respectively ($p > 0.1$).

8.4.2 Type 2 Diabetes results

Median (IQR) CADP (seconds) was 97 and 93 for soluble and lispro insulin respectively at baseline ($p > 0.05$). At 2 hours, CADP increased to 107 seconds and 102 seconds for soluble and lispro insulin respectively, and decreased to 87 seconds and 82 seconds at 6 hours respectively (Table 24, Figure 25). There were no statistically significant differences found between insulins at 2 or 6 hours.

For soluble and lispro insulin, no difference was found in fasting median (IQR) CEPI (seconds) 126 and 132 respectively. CEPI after soluble insulin was 132 seconds and 115 seconds at 2 and 6 hours respectively. After insulin lispro, CEPI was 130 seconds and 126 seconds at 6 hours (Table 24, Figure 26).

Table 24 Comparison of soluble and lispro insulin on platelet function in type 2 diabetic subjects

	Soluble		Lispro		*p*
CADP (sec)					
0 hour	97	89.5 - 116.8	93	74.0 - 109.3	*>0.05*
2 hours	107	86.5 - 113.5	102	89.0 - 122	*>0.1*
6 hours	87	75.0 - 99.3	82	72.5 - 90	*>0.05*
2-0 hours	-3	-16.3 - 8.3	8	3.8 - 25.5	*<0.01*
CEPI (sec)					
0 hour	126	121.3 - 165.5	132	113.5 - 147.5	*>0.05*
2 hours	132	125.0 - 166.3	130	105.5 - 150.5	*<0.001*
6 hours	115	103.0 - 127.0	126	97.0 - 142	*>0.05*
2-0 hours	5	-19.0 - 10.3	-19	-25.3 - 5.3	*<0.02*
Haematocrit (%)					
0 hour	37	35.5 - 36.6	36	34.2 - 37.6	*>0.05*
2 hours	35	32.4 - 35.9	35	33.2 - 36.4	*0.2038*
6 hours	35	33.8 - 35.9	36	34.1 - 36.6	*>0.1*
Platelet Ct (x10^9/L)					
0 hour	193	146.0 - 223	196	115.0 - 220	*>0.05*
2 hours	182	163.0 - 228	162	111.0 - 223	*>0.05*
6 hours	181	114.0 - 244	156	86.0 - 228	*>0.05*
vWF (%)*					
0 hour	118.65	34.287	130.02	37.334	*0.2746*
2 hours	106.75	40.595	119.80	40.518	*0.1162*
6 hours	119.13	36.840	114.58	28.498	*0.3835*

Data are median (IQR) except * mean (SD)

8.4.3 Type 1 Diabetes results

Median (IQR) CADP (seconds) was 80 and 79 for soluble and lispro insulin respectively at baseline (p = 0.7612). At 2 hours, CADP was 76 seconds and 85 seconds for soluble and lispro insulin respectively, and decreased to 73 seconds and 77 seconds at 6 hours respectively (Table 25, Figure 25). There were no statistically significant differences between insulins found at 2 or 6 hours.

For soluble and lispro insulin, no difference was found in fasting median (IQR) CEPI (seconds) 113 and 123 respectively. CEPI after soluble insulin was 115 seconds and 122 seconds at 2 and 6 hours respectively. After insulin lispro, CEPI was 106 seconds and 112 seconds at 6 hours (Table 25, Figure 26).

Table 25 Comparison of soluble and lispro insulin on platelet function in type 1 diabetic subjects

	Soluble			**Lispro**			***p***
CADP (sec)							
0 hour	80	71.3	- 92.8	79	69.5	- 91.5	*0.7612*
2 hours	76	68.0	- 89.5	85	68.5	- 98.5	*0.2761*
6 hours	73	65.0	- 87.0	77	67.5	- 86.3	*0.499*
2-0 hours	-4	-8.8	- 7.0	6	-3.5	- 16.5	*0.0559*
CEPI (sec)							
0 hour	113	96.3	- 125.0	123	107.5	- 134.8	*0.2483*
2 hours	115	103.5	- 128.0	122	102.5	- 137.3	*0.7009*
6 hours	106	92.0	- 129.3	112	96.3	- 137.3	*0.4625*
2-0 hours	1	-20.3	- 17.3	-3	-18.3	- 20.3	*0.9015*
Haematocrit (%)							
0 hour	35	33.6	- 36.25	36	34.2	- 36.7	*0.1811*
2 hours	35	32.95	- 36.4	36	34.05	- 36.5	*0.3339*
6 hours	36	32.95	- 36.75	36	33.6	- 37.3	*0.1362*
Platelet Ct ($x10^9/L$)							
0 hour	206	176.00	- 240	208	162	- 243	*0.8542*
2 hours	204	154.00	- 239	195	162	- 255.5	*0.4321*
6 hours	213	174.50	- 227.5	217	180.5	- 241.5	*0.5532*
vWF (%)*							
0 hour	125.46	35.307		116.37	38.651		*0.5316*
2 hours	120.77	34.388		111.61	34.196		*0.6939*
6 hours	116.20	37.109		114.80	37.353		*0.732*

Data are median (IQR) except * mean (SD)

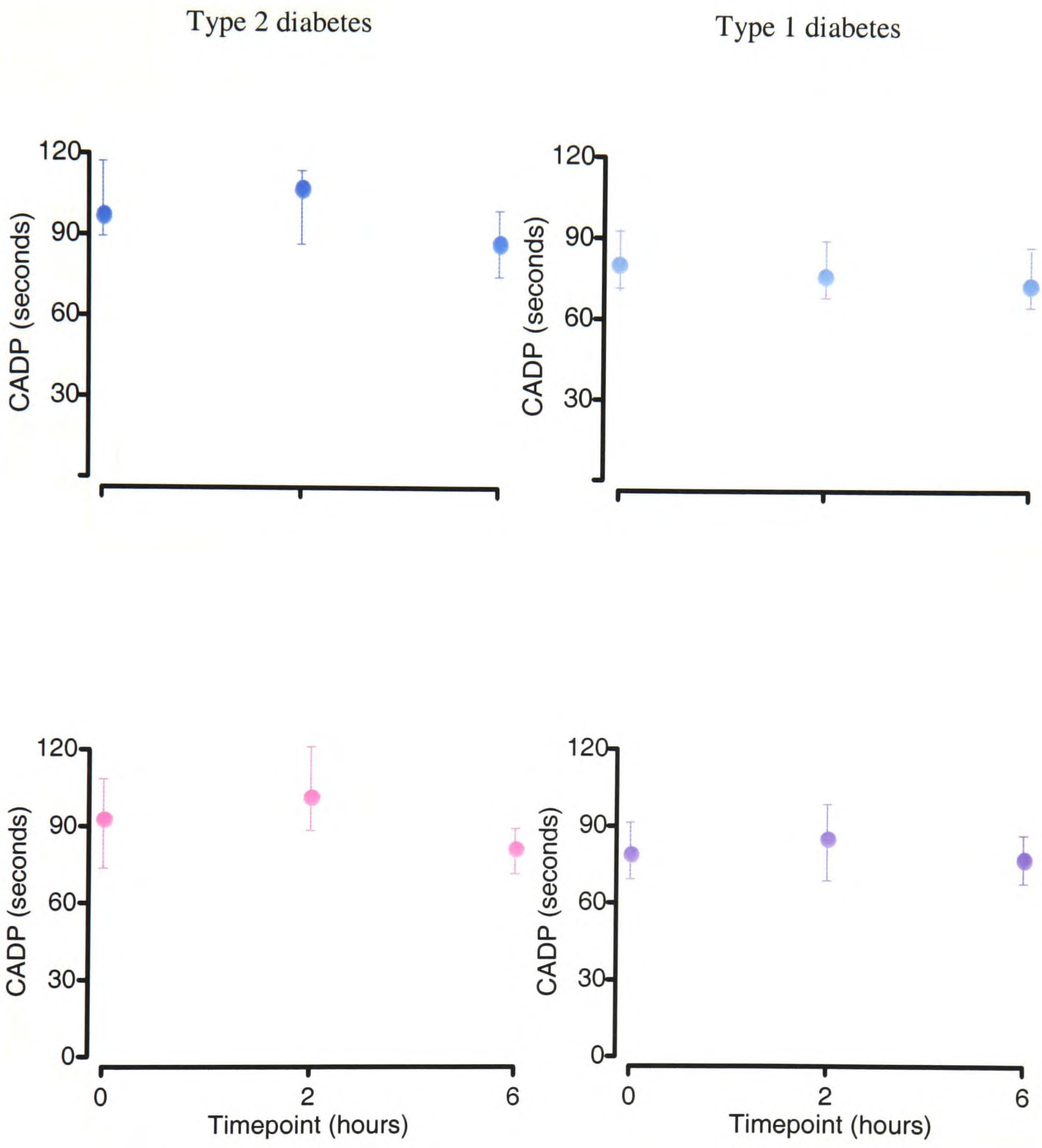


Figure 25 CADP results for type 2 and type 1 diabetic subjects on soluble and lispro insulin. Graphs are type 2 ● and type 1 ● diabetic subjects after soluble insulin (upper panels) and type 2 ● and type 1 ● diabetic subjects after insulin lispro (lower panels).

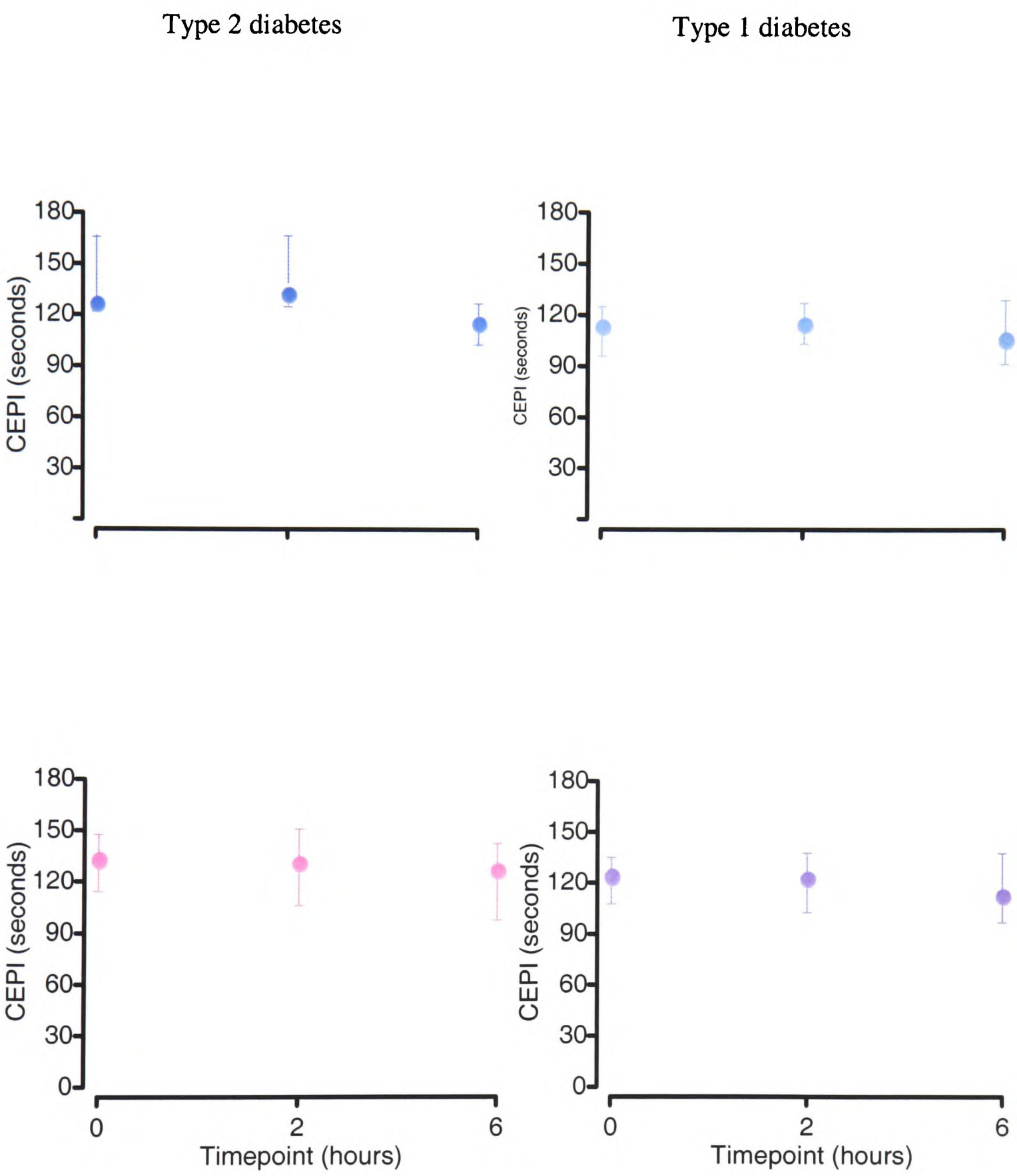


Figure 26 CEPI results for type 2 and type 1 diabetic subjects on soluble and lispro insulin. Graphs are type 2 ● and type 1 ● diabetic subjects after soluble insulin (upper panels) and type 2 ● and type 1 ● diabetic subjects after insulin lispro (lower panels).

8.4.4 Differences between type 1 and type 2 diabetic subjects

CADP and CEPI closure times were significantly greater at baseline and 2 hours in subjects with type 2 compared with type 1 diabetes with soluble insulin (Table 26). CADP closure time at 2 hours was significantly greater in type 2 diabetic subjects compared with type 1 diabetic subjects with insulin lispro (Table 27). For both soluble and lispro insulin, no significant differences in haematocrit, platelet count or vWF were found between type 1 and type 2 diabetic subjects.

Table 26 Comparison of platelet function in type 2 and type 1 diabetic subjects after soluble insulin

	Type 2 Diabetes		**Type 1 Diabetes**		*p*
CADP (sec)					
0 hour	97	89.5 - 116.8	80	71.3 - 92.8	*<0.001*
2 hours	107	86.5 - 113.5	76	68.0 - 89.5	*<0.001*
6 hours	87	75.0 - 99.3	73	65.0 - 87.0	*>0.1*
2-0 hours	-3	-16.3 - 8.3	-4	-8.8 - 7.0	*>0.1*
CEPI (sec)					
0 hour	126	121.3 - 165.5	113	96.3 - 125.0	*<0.001*
2 hours	132	125.0 - 166.3	115	103.5 - 128.0	*<0.001*
6 hours	115	103.0 - 127.0	106	92.0 - 129.3	*>0.1*
2-0 hours	5	-19.0 - 10.3	1	-20.3 - 17.3	*>0.1*
Haematocrit (%)					
0 hour	37	35.5 - 36.6	35	33.6 - 36.25	*>0.1*
2 hours	35	32.4 - 35.9	35	32.95 - 36.4	*>0.1*
6 hours	35	33.8 - 35.9	36	32.95 - 36.75	*>0.1*
Platelet Ct ($x10^9$/L)					
0 hour	193	146.0 - 223	206	176.00 - 240	*>0.1*
2 hours	182	163.0 - 228	204	154.00 - 239	*>0.1*
6 hours	181	114.0 - 244	213	174.50 - 227.5	*>0.1*
vWF (%)*					
0 hour	118.65	34.287	125.46	35.307	*0.6336*
2 hours	106.75	40.595	120.77	34.388	*0.3559*
6 hours	119.13	36.840	116.20	37.109	*0.8481*

Data are median (IQR) except * mean (SD)

Table 27 Comparison of platelet function in type 2 and type 1 diabetic subjects after lispro insulin

	Type 2 Diabetes		**Type 1 Diabetes**		*p*
CADP (sec)					
0 hour	93	74.0 - 109	79	69.5 - 91.5	*>0.01*
2 hours	102	89.0 - 122	85	68.5 - 98.5	*<0.001*
6 hours	82	72.5 - 90	77	67.5 - 86.3	*>0.1*
2-0 hours	8	3.8 - 25.5	6	-3.5 - 16.5	*>0.1*
CEPI (sec)					
0 hour	132	113.5 - 148	123	107.5 - 134.8	*>0.1*
2 hours	130	105.5 - 151	122	102.5 - 137.3	*>0.1*
6 hours	126	97.0 - 142	112	96.3 - 137.3	*>0.1*
2-0 hours	-19	-25.3 - 5.3	-3	-18.3 - 20.3	*>0.1*
Haematocrit (%)					
0 hour	36	34.2 - 37.6	36	34.2 - 36.7	*>0.1*
2 hours	35	33.2 - 36.4	36	34.05 - 36.5	*>0.1*
6 hours	36	34.1 - 36.6	36	33.6 - 37.3	*>0.1*
Platelet Ct (x10^9/L)					
0 hour	196	115.0 - 220	208	162 - 243	*>0.1*
2 hours	162	111.0 - 223	195	162 - 255.5	*>0.1*
6 hours	156	86.0 - 228	217	180.5 - 241.5	*>0.1*
vWF (%)*					
0 hour	130.02	37.334	116.37	38.651	*0.3902*
2 hours	119.80	40.518	111.61	34.196	*0.5814*
6 hours	114.58	28.498	114.80	37.353	*0.9875*

Data are median (IQR) except * mean (SD)

8.4.5 Correlations

For both soluble and lispro insulin, fasting CADP was strongly correlated with CADP at 2 (r= 0.8171, p<0.0001 and r= 0.6157, p= 0.005 respectively) and 6 hours (r= 0.6282, p=0.0052 and r=0.5934, p=0.0074 respectively). Baseline CEPI correlated with 2 hour CEPI for both soluble and lispro insulin (r =0.567, p = 0.0114 and r=0.4558 and p=0.0499 respectively). Significant correlations are shown in Table 28.

Table 28 Correlation of CADP to CEPI in type 1 diabetic subjects

		Soluble		Lispro	
		r	***p***	**r**	***p***
CADP 0 hours	CADP 2 hours	0.8171	*<0.0001*	0.6157	*0.005*
	CADP 6 hours	0.6282	*0.0052*	0.5934	*0.0074*
	CEPI 0 hours	0.4851	*0.0353*	0.6981	*0.0009*
	CEPI 2 hours	0.47	*0.0423*		
CADP 2 hours	CADP 6 hours	0.5503	*0.018*	0.7495	*0.0002*
	CEPI 0 hours			0.6166	*0.0049*
	CEPI 2 hours	0.6652	*0.0019*	0.5242	*0.0212*
	CEPI 6 hours			0.5143	*0.0243*
CADP 6 hours	CEPI 6 hours	0.5781	*0.0151*	0.47	*0.0423*
CEPI 0 hours	CEPI 2 hours	0.567	*0.0114*	0.4558	*0.0499*
CEPI 2 hours	CEPI 6 hours			0.5677	*0.0112*

For insulin lispro, CADP at 2 and 6 hours but not fasting showed a strong negative association with IAUC insulin (r = -0.6514, p=0.0063 and r= -0.5827 and p = 0.0179 respectively) and delta 2 hour insulin (r = -0.6198, p= 0.0046 and r = 0.45, p = 0.05).

The delta 6 hour triglyceride level significantly correlated with 6 hour CADP for insulin lispro (r= 0.4779, p= 0.0386) and CEPI for soluble insulin (r= 0.6065, p = 0.0098). IAUC triglyceride was also associated with 6 hour CADP (r = 0.481, p= 0.05) and CEPI (r = 0.7466, p = 0.0009) for soluble insulin. There were no significant associations with delta 6 hour or IAUC triglyceride levels and baseline or 2 hours CADP or CEPI times.

8.5 Discussion

Platelet function results show that both type 1 and type 2 diabetic subjects have PFA times within the normal limits. It appears however, that type 1 diabetic subjects lie in the "lower range" of normal and have PFA times that are shorter than type 2 diabetic subjects. These differences are not explained by variations in haematocrit, platelet count or vWF levels. No differences in PFA times were noted after soluble or lispro insulin in diabetic subjects. Significant associations between post challenge insulin and triglyceride responses and closure times were demonstrated.

Our study found fasting and post challenge CADP and CEPI times to be within the non diabetic range for both type 1 and type 2 diabetic subjects. In a study of 206 non diabetic subjects with no platelet abnormalities, mean closure time was 93 seconds for CADP and 132 seconds for CEPI (Mammen, Comp et al. 1998). The PFA test had a clinical sensitivity of 94.9% and a specificity of 88.8% with previous studies showing CADP cartridges to detect thrombocytopathies and CEPI cartridge to detect qualitative platelet defects including aspirin use. Our results indicate that the PFA test has acceptable reproducibility, with no systematic differences in type 1 and type 2 diabetic subjects. The interaction of platelets with collagen under flowing whole blood conditions mimics the early steps of arterial thrombus formation in vivo and enables studies of adhesive platelet receptors and their activation routes to be conducted (Westerbacka, Yki-Järvinen et al. 2002).

Platelet function in type 2 diabetes

After both soluble and lispro insulin, 2 hour CADP increased in type 2 diabetic subjects but had no effect of CEPI, with a 2 hour insulin concentration of approximately 380 and 430 pmol/l respectively. This is an unexpected finding as hyperinsulinaemia (460 – 600 pmol/l) in obese subjects produced by a 3 hour insulin infusion, actually shortened CADP and CEPI closure times by approximately 12-13 seconds (Westerbacka, Yki-Järvinen et al. 2002). Previous studies in obese type 2 diabetic subjects (BMI 32.1 +/- 1.5) similar to our subjects (BMI of approximately 30 kg/m^2) also show the platelet anti aggregating effect of insulin to be impaired (Trovati, Mularoni et al. 1995). Other tests of platelet function including aggregation studies and cGMP concentrations may have explained why we were unable to reproduce findings by Westerbacka et al. Other studies have shown that the reduced response seen in type 2 diabetic subjects to the anti aggregating effects of insulin at physiological concentrations can be overcome with supraphysiological concentrations (1920 pmol/l) compared with 240 -480 pmol/l in healthy subjects (Trovati, Mularoni et al. 1995).

It is possible that the increase in closure times seen in type 2 diabetic subjects may be explained by haematocrit, platelet count and vWF levels. Increased haematocrit, platelet count and vWF levels can all reduce closure times. However our results show no significant differences in these factors between the groups. A previous study in type 2 diabetic subjects also found plasma levels of vWF to be similar to non diabetic subjects and were not altered by hyperglycemia (Yngen, Ostenson et al. 2001).

By 6 hours post challenge, CADP and CEPI closure times had shortened compared to baseline after both soluble and lispro insulin administration in type 2 diabetic subjects. Insulin levels at 6 hours had decreased to approximately 150 pmol/l for soluble insulin and 120 pmol/l for insulin lispro but had not quite returned to baseline. This may be due to the duration of action of the insulin diminishing by 6 hours post challenge however there is difficulty interpreting our findings due to small subject numbers and study design. In the study of type 2 diabetic subjects, there was no placebo arm, that is, PFA analysis was not conducted without exogenous insulin administration. Also no PFA testing was conducted with non diabetic subjects undergoing the OTTT. Therefore it may be possible that any differences seen between diabetic and non diabetic subjects could be restored with insulin therapy. Acutely increasing insulin concentrations may increase closure times (upper normal range) and reduce thrombosis. Further studies in this area would clarify this hypothesis.

Platelet function in Type 1 diabetes

At 2 hours, no effect of insulin on post challenge closure times was seen in type 1 diabetic subjects. Insulin concentrations at this time were lower that those achieved in type 2 diabetic subjects, but higher than previous studies in the non diabetic population (Chapter 4). By 6 hours post challenge, CADP and CEPI closure times had shortened compared to baseline after both soluble and lispro insulin in type 1 diabetic subjects. It may be that type 1 diabetic subjects also display impaired platelet response to insulin, with enhancement of thrombus generation rather than attenuation. Previous studies in type 1 diabetic subjects showed that intensive insulin therapy by continuous

subcutaneous insulin delivery did reduce platelet aggregation (Turk, Flego et al. 1996) yet other studies found no effect on platelet function when glycaemic control was improved (Blann and Lip 1998).

A negative correlation was demonstrated between 2 and 6 hour post challenge CADP closure times and insulin IAUC with insulin lispro. This may indicate that the greater the insulin excursion, the shorter the closure time. This is consistent with findings by Westerbacka et al (Westerbacka, Yki-Järvinen et al. 2002) who found insulin induced change in platelet deposition to collagen was inversely correlated with both CADP and CEPI closure times. Yet, elevated platelet aggregation seen in uncontrolled type 1 diabetes returned to normal after a 24 hour insulin infusion in an earlier study by Juhan et al (Juhan, Vague et al. 1982). The relationship of insulin to platelets although not obvious when fasting, may be important in the postprandial state.

Relationship of post challenge triglyceridaemia and platelet function

A significant association between platelet function and triglyceride IAUC was established in type 1 diabetic subjects. The results showed total triglyceride response (IAUC) and delta 6 hour triglyceride increment to positively correlate with both 6 hour post challenge CADP and CEPI times. Westerbacka et al (Westerbacka, Yki-Järvinen et al. 2002) showed triglyceride concentration was positively associated with insulin induced change in platelet deposition to collagen. We found no significant associations at fasting or 2 hours post challenge between triglyceride and platelet function. The relationship was only evident during the late post challenge period. This may illustrate a possible

mechanism for postprandial hypertriglyceridaemia contributing to CHD. The triglyceride response may have a direct effect on platelet function. Studies of dyslipidaemia in non diabetic subjects have demonstrated that LDL, VLDL and HDL bind to specific binding sites on platelets thereby modifying platelet function (Brook and Aviram 1988; Surya and Akkerman 1993), with LDL and VLDL both increasing platelet aggregation.

Exaggerated postprandial lipaemia may contribute to atherothrombotic vascular disease through mechanisms of platelet and endothelial dysfunction. Therapy with lipid lowering agents such as fibrates improve fasting and postprandial endothelial function in type 2 diabetes possibly through the attenuation of postprandial lipaemia and oxidative stress (Evans, Anderson et al. 2000). Our results show platelet function to differ in type 1 and type 2 diabetic subjects with greater resistance of platelets to the action of prandial insulin in type 1 diabetic subjects. Further studies in diabetic subjects are required in order to clarify the relationship between lipid handling, the antiaggregatory effects of insulin, and platelet function in this at risk population.

9 Chapter 9 Capillary Triglyceride Sampling

9.1 Introduction

To facilitate and simplify postprandial testing in an outpatient setting, capillary fingerpick sampling as an alternative to venepuncture would be advantageous. Only a small amount of blood would be required without the need for cannulation. However relatively few studies have investigated the accuracy and reproducibility of post challenge capillary triglyceride sampling. A study in diabetic patients by Stewart et al (Stewart, Albers et al. 1996) demonstrated that self monitored capillary triglyceride measurements using the Reflotron analyser were similar to laboratory measured venous samples. This study assessed the variability of triglyceride concentrations in 24 type 2 diabetic patients under everyday ambulatory conditions for 6 months including more than 1600 triglyceride measurements. No studies have investigated capillary triglyceride levels after a standardised oral fat and carbohydrate challenge in diabetic and/or non diabetic individuals.

9.2 Aims

1. To assess whether fasting and post lipid and carbohydrate challenge capillary triglyceride levels are comparable to simultaneous venous measurements.

2. To examine the reproducibility of fasting and post challenge capillary triglyceride measurements.

9.3 Methods

9.3.1 Study design and subjects

Capillary triglyceride sampling was conducted as part of the initial OTTT reproducibility study where non diabetic and diabetic subjects underwent a standardised oral fat and carbohydrate challenge on 2 separate occasions at least one week apart (Chapter 3 and 4).

9.3.2 Study procedure

Samples were collected as per the OTTT protocol outlined in Methodology Chapter 2, 3 and 4. Additional 150 ul capillary blood samples for triglyceride measurement were collected at 0 and 6 hours using an Autolancet (Medisense, Abbot Laboratories Company, UK) and placed in microvette capillary tubes containing EDTA.

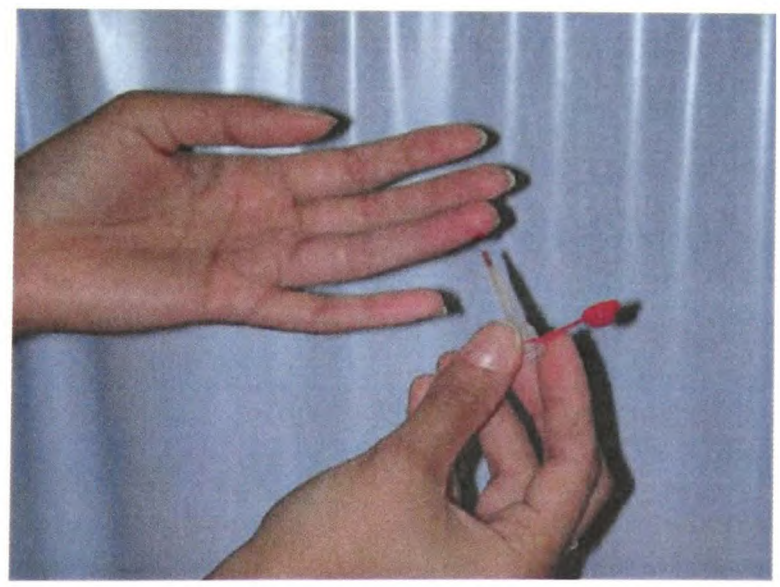


Figure 27 Example of microvette tube used for capillary triglyceride sampling.

9.3.3 Biochemical methods

Immediately after collection, microvette tubes were centrifuged at 3000 rpm for 10 minutes at 4°C. Capillary plasma volume for triglyceride measurement was determined by weighing the sample on a 3 place Sartorius digital balance. Plasma triglyceride was measured by enzymatic, colorimetric endpoint method using the Beckman Synchron CX4 Delta analyser (Beckman Instruments Ltd, High Wycombe, UK) (Chapter 2).

9.3.4 Statistical methods

Capillary vs venous triglyceride levels

Venous and capillary results were graphically depicted using mean difference plots and compared using paired t tests. Pearson correlation coefficients were used to determine associations between capillary and cannula triglyceride values.

Reproducibility of capillary triglyceride levels

The coefficient of variation was calculated to assess the reproducibility of capillary sampling between the first and second tests for 0 and 6 hour timepoints.

9.4 Results

9.4.1 Subjects

All 50 subjects participated in capillary fingerprick sampling. Subjects characteristics are highlighted in table 4 Chapter 3. A total of 200 capillary samples were collected from

subjects over 2 visits. Of these, 6 samples were unable to be assayed due to inadequate amount of blood placed in the microvette tube.

9.4.2 Capillary Triglyceride

Capillary vs venous cannula sampling

Comparisons of venous cannula and capillary triglyceride values at 0 and 6 hours are shown as a mean difference plots (Figure 28) and tabulated (Table 29). Capillary values were approximately 10% higher than cannula values, but this difference was not statistically significant.

Reproducibility of capillary measurements

The repeatability of capillary triglyceride testing was similar for both diabetic and non diabetic groups, with median (IQR) CV (%) of 12.2 (7.74 - 24.50) and 15.3 (6.64 – 21.29) respectively at baseline, and 14.2 (8.29 – 21.36) and 16.4 (10.3 – 27.64) at 6 hours respectively (Figure 29).

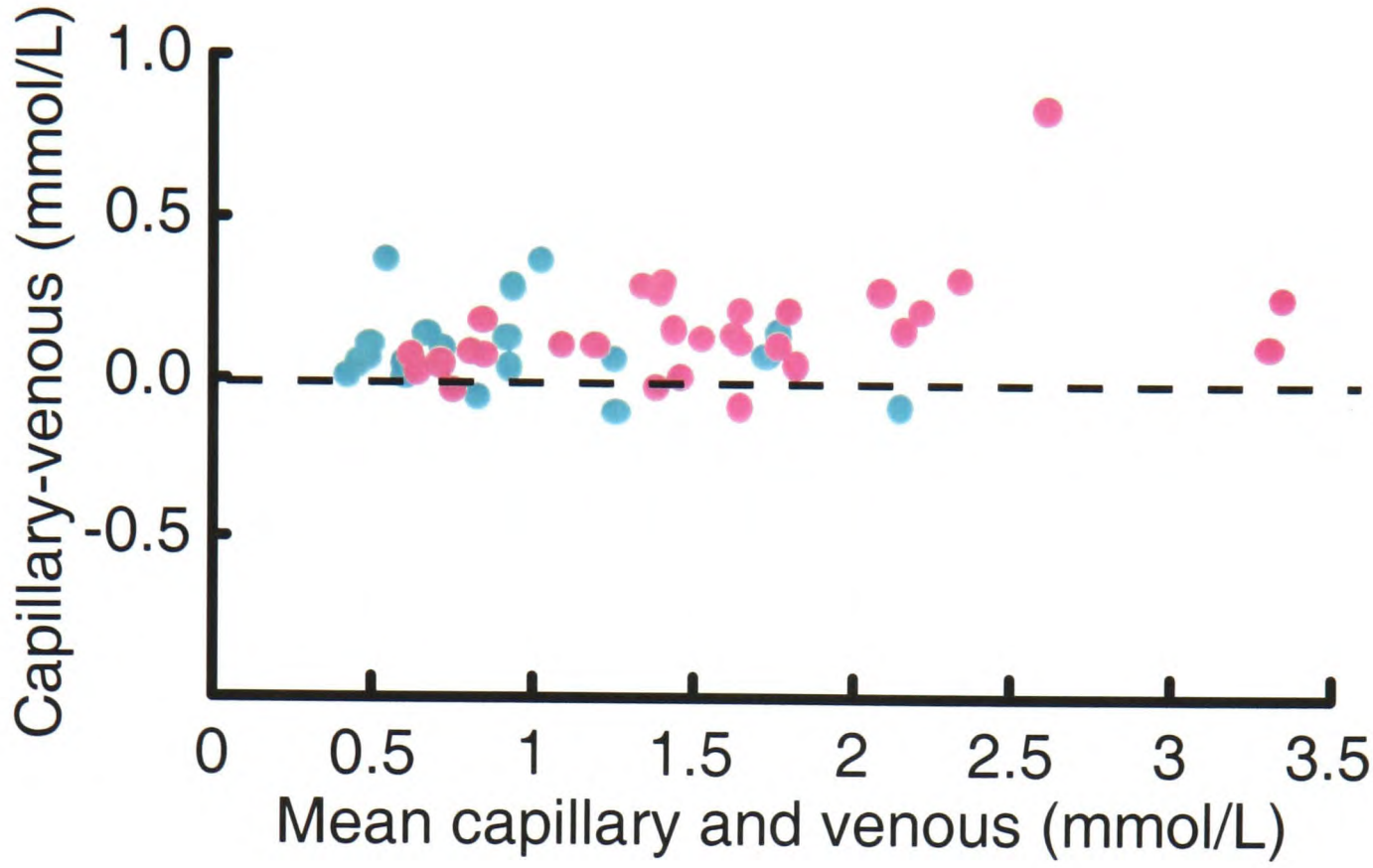
Difference between diabetic and non diabetic subjects

The capillary geometric mean (1 SD range) triglyceride values (mmol/l) at fasting were 1.71 (1.04 – 2.81) and 0.97 (0.67 – 1.40) for diabetic and non-diabetic groups respectively ($p = 0.0001$) and at 6 hours 2.23 (1.34 – 3.72) and 1.16 (0.67 – 2.01) respectively ($P = 0.0002$).

Table 29 Table comparing capillary and venous cannula triglyceride sampling in non diabetic and diabetic subjects.

	No Diabetes		Diabetes	
Triglyceride (mmol/l)*				
Capillary				
0 hour	0.97	0.67 - 1.40	1.71	1.04 - 2.81†
6 hour	1.16	0.67 - 2.01	2.23	1.34 - 3.72‡
Venous				
0 hour	0.82	0.55 - 1.22	1.55	0.91 - 2.62
6 hour	0.96	0.55 - 1.69	2.05	1.19 - 3.53
CVcapillary vs venous (%)				
0 hour	10.8	4.61 - 14.87	4.5	3.69 - 6.60
6 hour	10.5	7.87 - 15.69	6.4	3.87 - 9.99
Difference capillary – cannula (%)				
0 hour	15		9	
6 hour	17		8	
p (capillary vs venous)				
0 hour	*0.0032*		*<0.0001*	
6 hour	*0.0032*		*<0.0001*	
CV test 1 vs 2 (%)				
0 hour	15.3	6.64 - 21.29	12.2	7.74 - 24.50
6 hour	16.4	10.3 - 27.64	14.2	8.29 - 21.36

CV are median (IQR) and * geometric mean (1 SD range)
† $p = 0.0001$ and ‡ $p = 0.0002$ comparing non diabetic and diabetic subjects



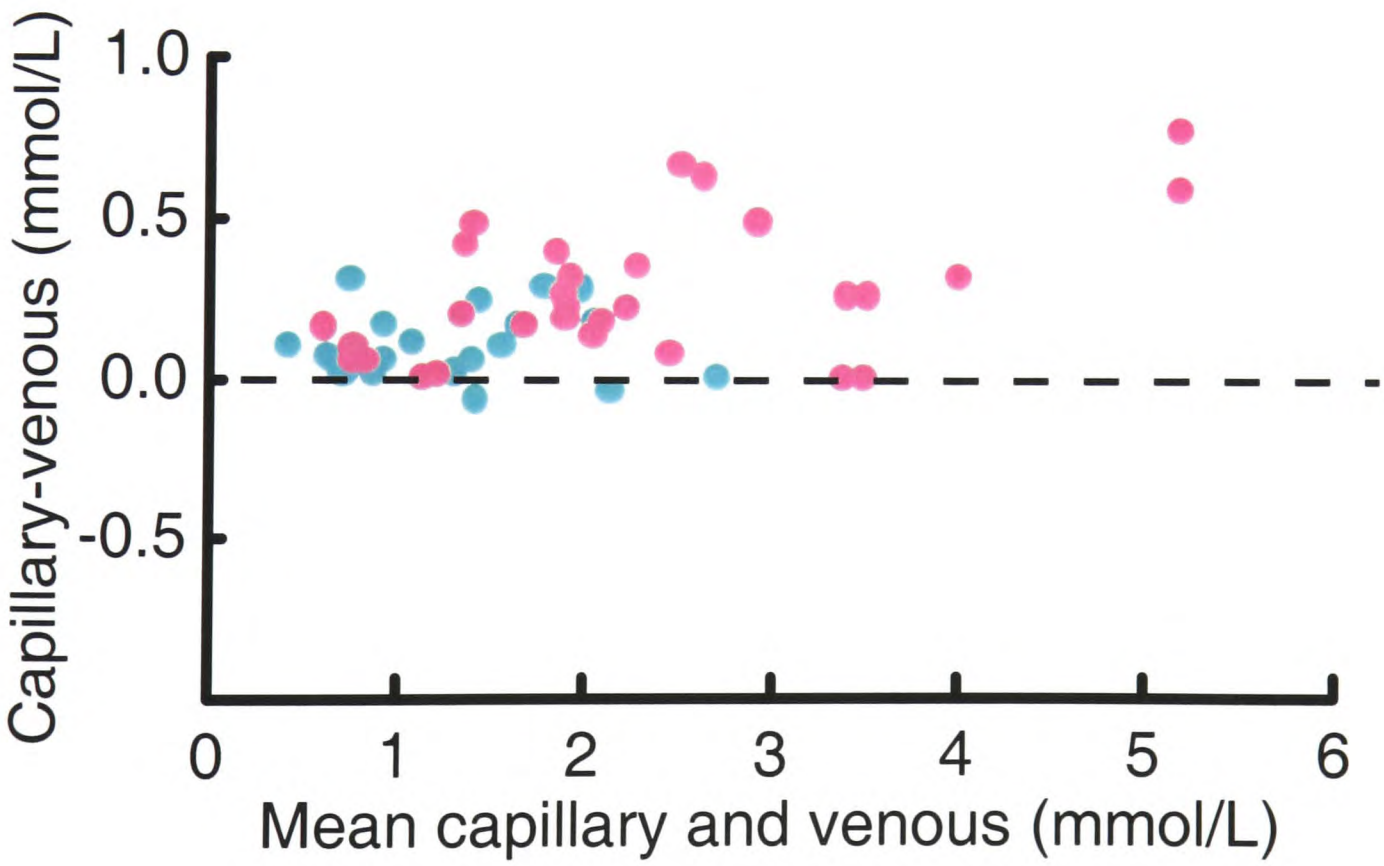
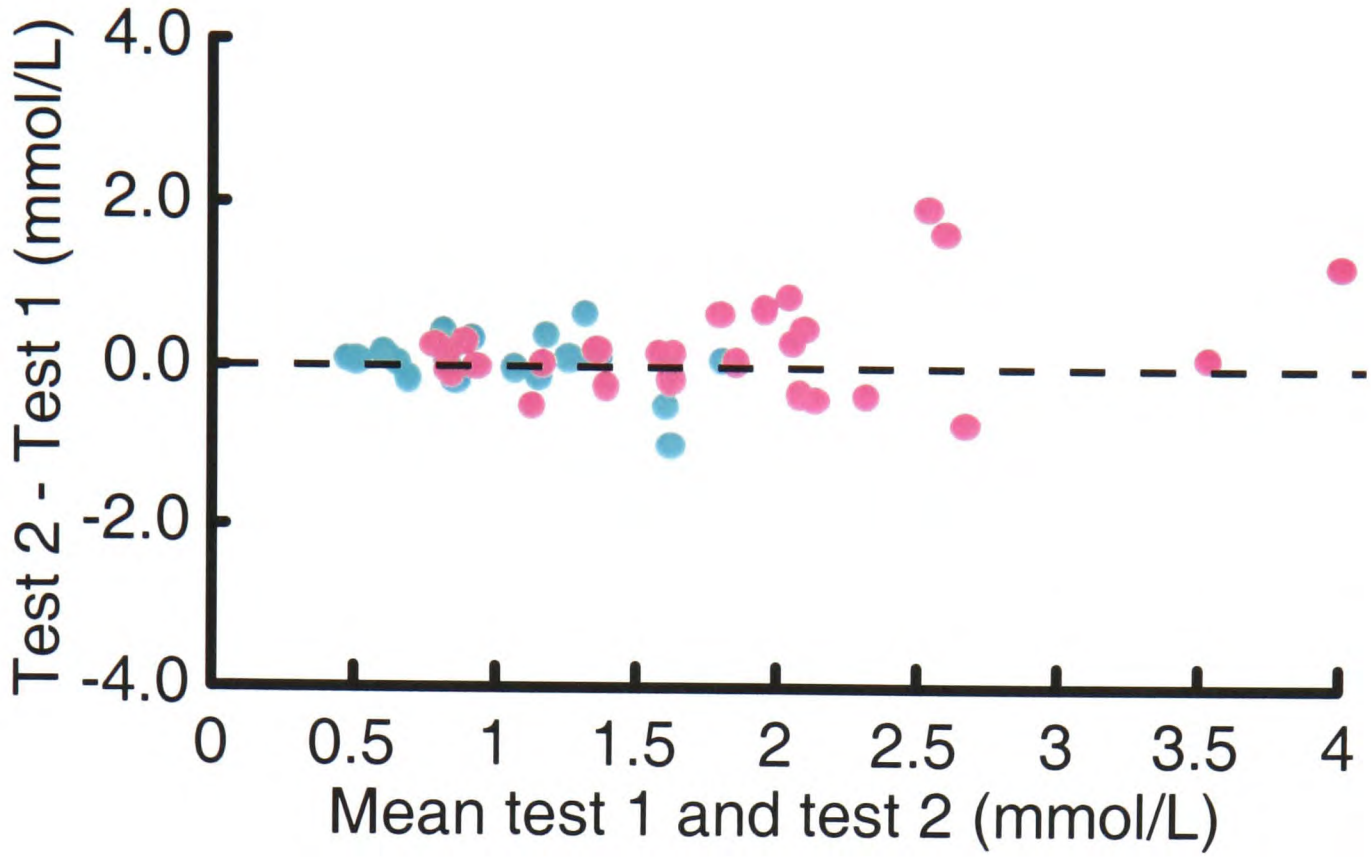


Figure 28 Mean difference plots comparing capillary and venous cannula triglyceride measurements at fasting (top) and at 6 hours (bottom). Non diabetic subjects are shown as ● and diabetic subjects as ●.



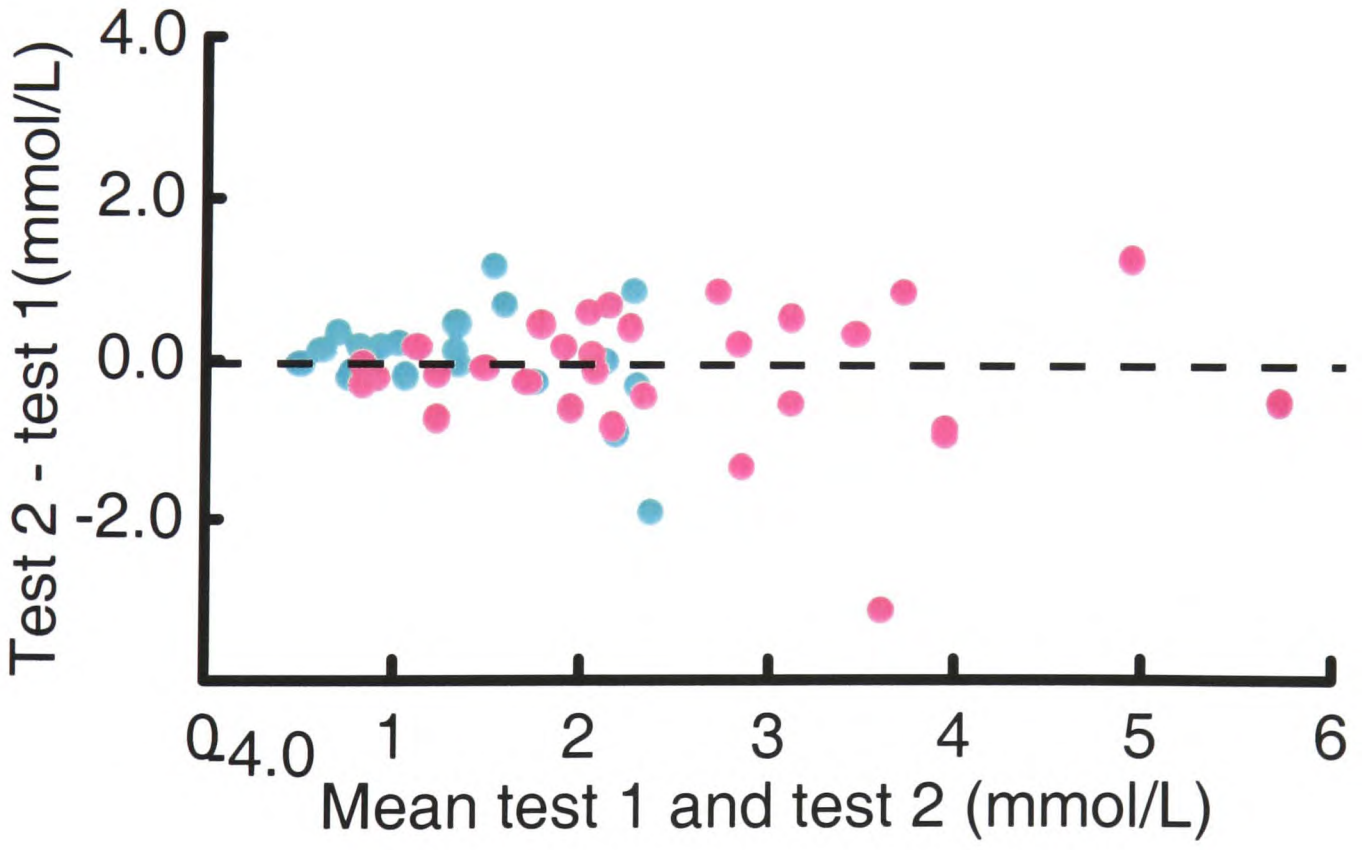


Figure 29 Mean difference plots comparing capillary triglyceride measurements between the first and second test at fasting (top) and at 6 hours (bottom). Non diabetic subjects are shown as ● and diabetic subjects as ●.

9.4.3 Correlations

Capillary triglyceride measurements were strongly associated with venous triglyceride levels, fasting ($r = 0.9225$, $p < 0.0001$ and $r = 0.9939$, $p < 0.0001$) and at the 6 hour timepoint ($r = 0.9664$, $p < 0.0001$ and $r = 0.9652$, $p < 0.0001$) for diabetic and non diabetic groups respectively.

The 6 hour and fasting capillary triglyceride levels were highly correlated in both diabetic and non diabetic groups ($r = 0.8384$, $p < 0.0001$ and $r = 0.8065$, $p < 0.0001$) respectively.

9.4.4 Venous cannula vs venous microvette triglyceride levels

A possible explanation for triglyceride measurements being consistently higher than venous values could be the sampling tube used. The microvette tube compared with the 5 mL EDTA vacucontainer may influence the triglyceride measurement. To explore this, further triglyceride capillary sampling at 0 and 6 hours was incorporated into the study of the intervention study manipulating endogenous insulin secretion using nateglinide (Chapter 5). In addition to this, venous blood collected from the cannula by syringe at 0 and 6 hours was filled into microvette tubes, also for triglyceride analysis (table 30).

Table 30 Capillary, venous cannula and venous microvette triglyceride measurements from modification of endogenous insulin secretion (Chapter 5)

	Placebo			Nateglinide		
Triglyceride (mmol/l)*						
Capillary						
0 hour	1.50	0.84 - 2.68		1.74	1.16 - 2.62	
6 hour	2.31	1.41 - 3.81		2.43	1.53 - 3.88	
Cannula						
0 hour	1.30	0.69 - 2.46		1.52	0.99 - 2.35	
6 hour	1.95	1.11 - 3.40		2.16	1.35 - 3.47	
Venous						
0 hour	1.35	0.72 - 2.53		1.56	1.02 - 2.38	
6 hour	2.05	1.18 - 3.55		2.25	1.39 - 3.66	
Difference venous – cannula (%)						
0 hour	4			3		
6 hour	5			4		
Difference capillary – cannula (%)						
0 hour	13			13		
6 hour	16			11		
Difference capillary – venous (%)						
0 hour	10			10		
6 hour	11			7		
CV capillary vs cannula (%)						
0 hour	4.1	2.39 - 8.66		7.7	3.54 - 12.23	
6 hour	8.1	5.05 - 11.23		8.6	4.27 - 12.86	
CV capillary vs venous (%)						
0 hour	2.9	1.86 - 5.96		6.5	2.83 - 11.50	
6 hour	3.9	1.74 - 7.78		4.9	1.01 - 10.48	

CV are median (IQR) and * geometric mean (1 SD range)

Capillary, venous cannula and venous microvette triglyceride measurements were similar, although capillary levels were still at least 10% higher than venous cannula values. A small difference, *circa* 5%, was found between venous triglyceride levels measured from cannula and microvette tubes (Table 30).

9.5 Discussion

The results of this study show that capillary sampling is a possible alternative method of triglyceride assessment giving both reproducible and similar results to venous cannula levels.

Our results show capillary triglyceride levels are similar to venous cannula values (Chapter 3) and highlight the differences in post challenge triglyceride concentrations between diabetic and non diabetic groups. We demonstrated strong correlations between the 2 sampling techniques. There is a paucity of studies directly comparing capillary and venous triglyceride sampling measurements. Stewart et al (Stewart, Laker et al. 1993) showed capillary triglycerides correlated with laboratory measured triglyceride concentrations ($r = 0.88$, $p<0.001$) and other studies have shown associations between capillary plasma triglyceride levels and oral fat loading venous levels ($r=0.771$, $p<0.001$) (Castro Cabezas, Halkes et al. 2001).

We found capillary triglyceride measurements to be significantly 10% higher than venous values. A possible explanation for our findings could be the sampling tube used, that is, the microvette tube compared with the 5 mL EDTA vacucontainer may influence the triglyceride measurement. To explore this possibility, further capillary triglyceride measurement were collected in the intervention study enhancing endogenous insulin secretion using nateglinide (Chapter 5) and microvette tubes filled with venous blood were also analysed for triglyceride. The results confirm a greater than 10% difference between capillary and venous cannula triglyceride measurements, again with capillary

measurements giving higher triglyceride concentrations. There is only a small difference (*circa* 5%) between venous triglyceride levels collected via the cannula and the microvette tube. This suggests the difference between capillary and venous triglyceride measurements is unlikely due entirely to the microvette tube, but rather due to differences in venous and capillary triglyceride concentrations in the circulation. Kafonek et al (Kafonek, Donovan et al. 1996) demonstrated a 12% increase in triglyceride levels measured on the capillary desktop analyser compared to the laboratory. One possible explanation for the consistent higher triglyceride values seen with capillary samples, may be due to the process of greater extraction of triglyceride as blood circulates from the capillary to venous vasculature. Triglyceride carried in triglyceride rich lipoproteins is hydrolysed by LPL to release fatty acids for uptake into other tissues for re esterification or oxidation. LPL is found in a number of tissues outside the liver and acts on particles as they pass through capillaries (Frayn 1996). Venous levels may therefore reflect slightly lower triglyceride concentrations due to lipolytic breakdown.

Other collection "techniques" could possibly contribute to the increase in capillary triglyceride concentrations. Producing a droplet of blood on the fingertip requires pressure to be applied to the area. This may increase the extrusion of extra cellular material, lowering the cell count thus increasing the proportion of plasma per droplet. This may have significant consequences when dealing with small quantities of sample. Studies of glucose monitoring have shown capillary glucose levels to be significantly higher than venous glucose levels after the OGTT (Eriksson, Fex et al. 1983). A more recent study by Ellison et al (Ellison, Stegmann et al. 2002) found that glucose values

measure at the forearm lag behind values obtained from the finger when glucose levels are changing rapidly. Forearm sites were rubbed before testing (and sometimes a warming pad was used), the difference was not entirely mitigated which has previously been suggested in order to minimise the difference. As our capillary fingerprick samples were centrifuged and plasma separated for laboratory analysis, this is unlikely to effect triglyceride values.

We found fasting capillary triglyceride CVs of *circa* 15% in the diabetic population which were similar to non diabetic individuals, with no increase in variation at 6 hours post challenge. Previous studies of capillary triglyceride sampling have demonstrated a wide variation in both fasting and postprandial triglyceride levels in type 2 diabetic subjects. Stewart et al (Stewart, Albers et al. 1996) monitored type 2 diabetic subjects over 6 months in day to day life and demonstrated that there was a large intra- individual variation in triglyceride concentrations. The most likely explanation for this is that our study was conducted using a standardised oral fat and carbohydrate challenge and specific test conditions, whilst this previous study observed subjects in "everyday" conditions, therefore introducing confounders such as diet, exercise and alcohol which all influence triglyceride levels. The Reflotron machines (Boehringer Mannheim, Germany) used in their study have shown good reproducibility (Stewart, Laker et al. 1993). Postprandial capillary triglyceride levels were assessed in 18 healthy subjects in an outpatient setting (Castro Cabezas, Halkes et al. 2001). Subjects were asked to take capillary triglyceride measurements for 3 weekdays fasting, before and 3 hours after lunch and dinner, and at bedtime. They found fasting capillary CVs of 24.87 (1.44-

72.71)% but lower variability of integrated area under the curve postprandial values 15.12 (0.60-45.90)%.

We found strong correlations between fasting and post challenge capillary triglyceride concentrations. Pre prandial and bedtime capillary triglyceride levels have been highly associated in previous studies of normotriglyceridaemic type 2 diabetic subjects (Stewart, Laker et al. 1993). Stewart et al (Stewart, Albers et al. 1996) showed a strong correlation when comparing an individual's mean fasting and mean postprandial concentrations ($r=0.925$, $p<0.001$), but this did not apply when comparing the fasting triglyceride with the subsequent postprandial value. The authors concluded that this latter finding was most likely due to the influence of diet, exercise and alcohol on triglyceride metabolism.

Our results show non diabetic and diabetic subjects to have capillary triglyceride levels of circa 1 mmol/l and 1.7 mmol/l at baseline respectively and 1.2 mmol/l and 2.2 mmol/l at 6 hours post challenge respectively. Three serial (monthly) fasting capillary measurements were collected from 83 healthy subjects in a study by Kafonek et al (Kafonek, Donovan et al. 1996). They found mean (SD) triglyceride concentrations to be 1.26 (0.60) mmol/l which is slightly higher than found in our non diabetic population. Another study in non diabetic individuals confirmed mean (SD) fasting capillary triglyceride levels to be 1.05 (0.33) mmol/l for females and 1.18 (0.36) mmol/l for males (Castro Cabezas, Halkes et al. 2001) similar to our results. Stewart et al (Stewart, Albers et al. 1996) found median (IQR) fasting triglyceride concentration to be 1.95 (0.8-6.7) mmol/l, and postprandial triglyceride concentration 2.68 (0.8 – 6.8) mmol/l in type 2

diabetic subjects. Our results show similar median capillary triglyceride concentrations but with narrower interquartile ranges. The most likely explanation for this again is due to their study being conducted in a non clinical setting without using a standardised oral fat and carbohydrate challenge or specific test conditions.

It have previously been proposed that postprandial studies in a metabolic ward setting are time consuming and cannot be applied in clinical practice or to large populations (Castro Cabezas, Halkes et al. 2001). A study by Stewart et al (Stewart, Laker et al. 1993) was conducted with 12 normolipidaemic type 2 diabetic subjects over 6 months, to determine whether self monitoring of plasma triglyceride concentrations improved lipid concentrations and/ or glucose concentrations indirectly. They found that, compared with controls, the mean plasma triglyceride concentration of patients who monitored themselves before meals and before going to bed twice a week fell significantly by 1.1 (-0.1 – 2.3) mmol/l as did their plasma cholesterol concentration. However there was no difference in HbA1c concentration or body weight. Interestingly three months after completion of the study triglyceride concentrations had risen in the patients who had self monitored from 1.6 to 2.2 mmol/l, although this was a non significant statistical increase. Simplifying the OTTT to 2 measurements at fasting and 6 hours using capillary sampling (adjusting for the 10% difference compared with venous values), may assist in directly increasing patients' self awareness of CHD risk and provoke lifestyle changes, although this remains to be evaluated.

10 Chapter 10 General Discussion

The elevated fasting and post-challenge triglyceride levels seen in diabetic individuals, secondary in part to disordered insulin secretion and insulin resistance, may contribute to their substantially increased risk of cardiovascular disease. The main aim of this thesis was to determine whether attempting to achieve more physiological post-challenge insulin profiles would impact favourably on lipid pathways in addition to improving plasma glucose concentrations.

We used currently available antidiabetic preparations to enhance preferentially endogenous or circulating insulin levels following an oral fat and carbohydrate challenge in patients with type 2 diabetes. We investigated also whether earlier, rather than later, insulin delivery was more beneficial in these patients. A group of patients with type 1 diabetes were studied in addition to examine the effect of earlier, rather than later, insulin provision in the absence of endogenous insulin delivery to the liver.

As a prelude to these studies, it was necessary to formulate and evaluate a palatable standardised oral fat challenge, as no universally accepted test meal was available. The Oral Triglyceride Tolerance Test (OTTT) developed permits comparative post-challenge testing in people with and without diabetes.

10.1 OTTT Development

We have developed an acceptable oral fat and carbohydrate challenge that can be used for post challenge assessment of triglyceride and glucose responses. We have used this

OTTT to establish normal and "usual" type 2 diabetic ranges. A reproducible mean rise in triglyceride of 0.51 mmol/l (CV 17.6%) is seen in non diabetic subjects and 0.64 mmol/l (CV 17.4%) in individuals with type 2 diabetes. A reproducible mean rise in plasma glucose of 0.9 mmol/l (CV 15.3%) is seen in non diabetic subjects and 5.1 mmol/l (CV 8.3%) in individuals with type 2 diabetes. The OTTT provides a standardised method for assessing post challenge triglyceride and glucose responses for clinical and epidemiological studies and for evaluating potential therapeutic responses in intervention trials.

The composition the OTTT test drink is important when establishing a standardised test for triglyceride assessment. The 50g fat and 50g carbohydrate components were chosen after review of the literature to act as a "metabolic challenge" that would be palatable, safe in people with diabetes, and easy to prepare. It could be debated that the test drink should contain more fat to act as a greater challenge, or less fat to recreate a more realistic everyday life scenario. However increasing the fat content may make the drink less palatable and tolerable for participating subjects. Likewise, although decreasing the fat content may enhance palatability, differences between non diabetic and diabetic subjects may have been more difficult to discriminate.

The OTTT has potential to be used to establish glucose and triglyceride tolerance simultaneously. Our results show better reproducibility than the 75g OGTT and proportional increases in 2 hour glucose values. However some authors believe postprandial studies in a metabolic ward setting are time consuming and can not be

applied in clinical practice or to large populations (Castro Cabezas, Halkes et al. 2001). Castro Cabezas showed no significant differences between ward conducted postprandial studies and their method of six timepoint daily profiles to represent daily triglyceridaemia (Chapter 9). Guerci et al (Guerci, Paul et al. 2001) too proposed simplying the AUC over 8 hours to 3 timepoints (0, 4 and 8 hours). Our OTTT could be simplified to capillary measurements taken only at baseline and 6 hours in diabetic subjects, enabling the test to be conducted in an outpatient setting or large scale.

10.2 Differences between diabetic and non diabetic subjects

Our results confirm higher fasting triglyceride in type 2 diabetic subjects compared to non diabetic individuals with geometric mean triglyceride levels of 1.35 mmol/l. We used these established OTTT ranges to compare our subsequent intervention study results for changes in shape and timing of the profile. However, triglyceride concentrations are often much higher than this in type 2 diabetic individuals, which may have produced greater differences when comparing to non diabetic values. We had selected our subjects based on fasting triglyceride levels less than 5 mmol/l. Our studies have been conducted in a normotriglyceridaemic subjects. Previous studies have shown that hypertriglyceridaemic individuals have prolonged and exaggerated triglyceride excursions (Karpe 1999) and OTTT studies in this population would be of great interest to compare with normotriglyceridaemic individuals. Also, we have not differentiated the contribution of hepatic from intestinal lipoproteins. However although this may be scientifically of interest clinically an individual's CHD risk would be assessed by the degree of triglyceridaemia as an addition to other well established CHD risk factors. The

advantage of the OTTT is that it could be used as described or adapted to further investigate postprandial abnormalities in more detailed studies. The Heart Protection Study has demonstrated lowering LDL cholesterol from 2.5 to 1.7 mmol/l produces CHD risk reduction as great as that seen when lowering high LDL cholesterol levels (HPS-Collaborative-Group 2002). This could potentially be important for triglyceride reduction as well.

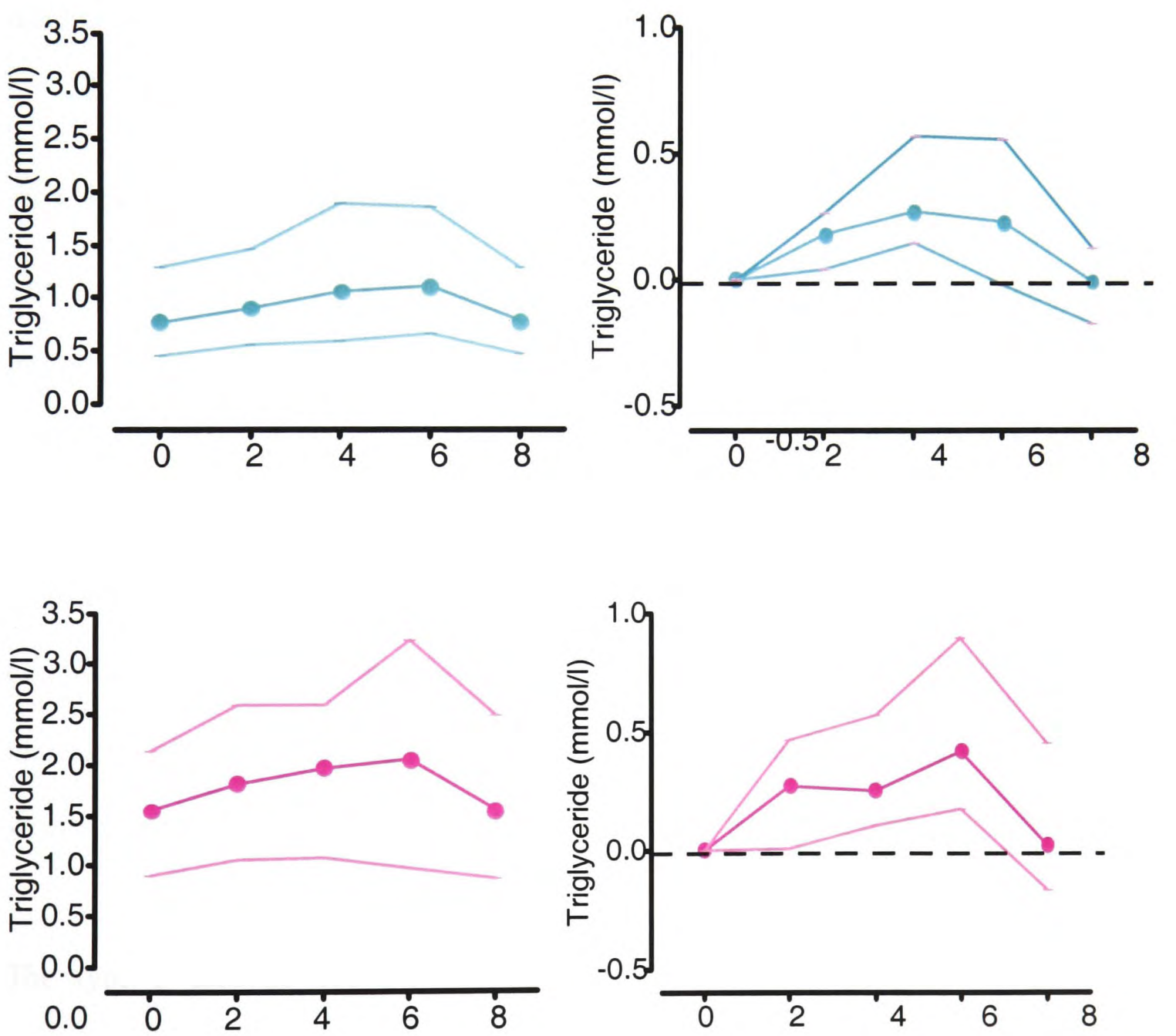


Figure 30 Triglyceride geometric mean and 1 SD ranges for non diabetic ● and diabetic ● subjects on diet or oral hypoglycaemic therapy (from Chapter 4). Absolute values are depicted on the left, and change from baseline (delta) on the right.

Diabetic subjects appeared to have increased triglyceride IAUC, with a higher proportion of subjects achieving maximum triglyceride concentrations at 6 hours, compared with non diabetic subjects. As previously discussed (Chapter 4), this may partly be explained by defects in the regulation of triglyceride handling by insulin. We found that delta 6 hour triglyceride levels correlated with the 2 hour insulin increment in non diabetic subjects. In type 2 diabetic subjects we demonstrated a relationship between time of maximum triglyceride response and IAUC insulin and glucose IAUC. In our study of type 2 diabetic subjects taking soluble insulin, we found the delta 6 hour triglyceride level to correlate with IAUC insulin as well as the 2 hour glucose increment.

10.3 Modifying triglyceride levels by providing early insulin augmentation

The results of the OTTT study (Chapter 4) show that type 2 diabetic subjects achieve higher peak insulin concentrations compared with non diabetic subjects in response to a triglyceride and carbohydrate test challenge in an attempt to achieve normoglycaemia. Previous studies in non diabetic obese individuals with varying degrees of "pre diabetic" glucose tolerance have also demonstrated this phenomenon (Axelsen, Smith et al. 1999). Our series of intervention studies aim to examine the effects on lipid handling of firstly, increasing circulating insulin levels in the early post challenge period, and secondly whether more rapid provision of insulin during this period offers further benefit.

The type 2 diabetic subjects participating in the intervention studies had similar demographic characteristics and mainly represent one end of the spectrum of diabetes, that is, they still had remaining ß cell function and relied on diet modification and/or oral

hypoglycaemic agents to reduce plasma glucose levels. Two modes of increasing circulating insulin concentrations were examined, firstly enhancement of endogenous insulin secretion, and secondly, exogenous insulin delivery.

When augmenting endogenous insulin secretion (nateglinide) (Chapter 5), insulin IAUC throughout the 8 hour study increased compared with placebo, particularly in the first 4 hours post challenge. Significantly greater circulating C peptide concentrations and consequent glucose reduction, support this finding. Despite increasing peripheral insulin levels, endogenous insulin enhancement failed to modify the post challenge triglyceride IAUC (Figure 31) or 6 hour increment (Figure 32). Although type 2 diabetic subjects participating in the exogenous insulin study (Chapter 6) did not undergo a "baseline" OTTT to compare with profiles produced from exogenous insulin supplementation, total insulin exposure was increased relative to previously characterised insulin profiles. Soluble insulin produced a greater insulin response than insulin lispro over the 8 hour study period in type 1 and type 2 diabetic subjects (although not statistically significant). Overall insulin exposure was less in type 1 diabetic subjects compared with type 2 diabetic individuals, but was relatively greater than normally produced in non diabetic subjects. Exogenous insulin supplementation although not changing the IAUC of the profile or maximum rise in triglyceride when comparing to subjects treated routinely by diet or oral hypoglycaemic therapy (Figure 31), caused a reduction in 6 hour triglyceride increment by approximately 50% (Figure 32).

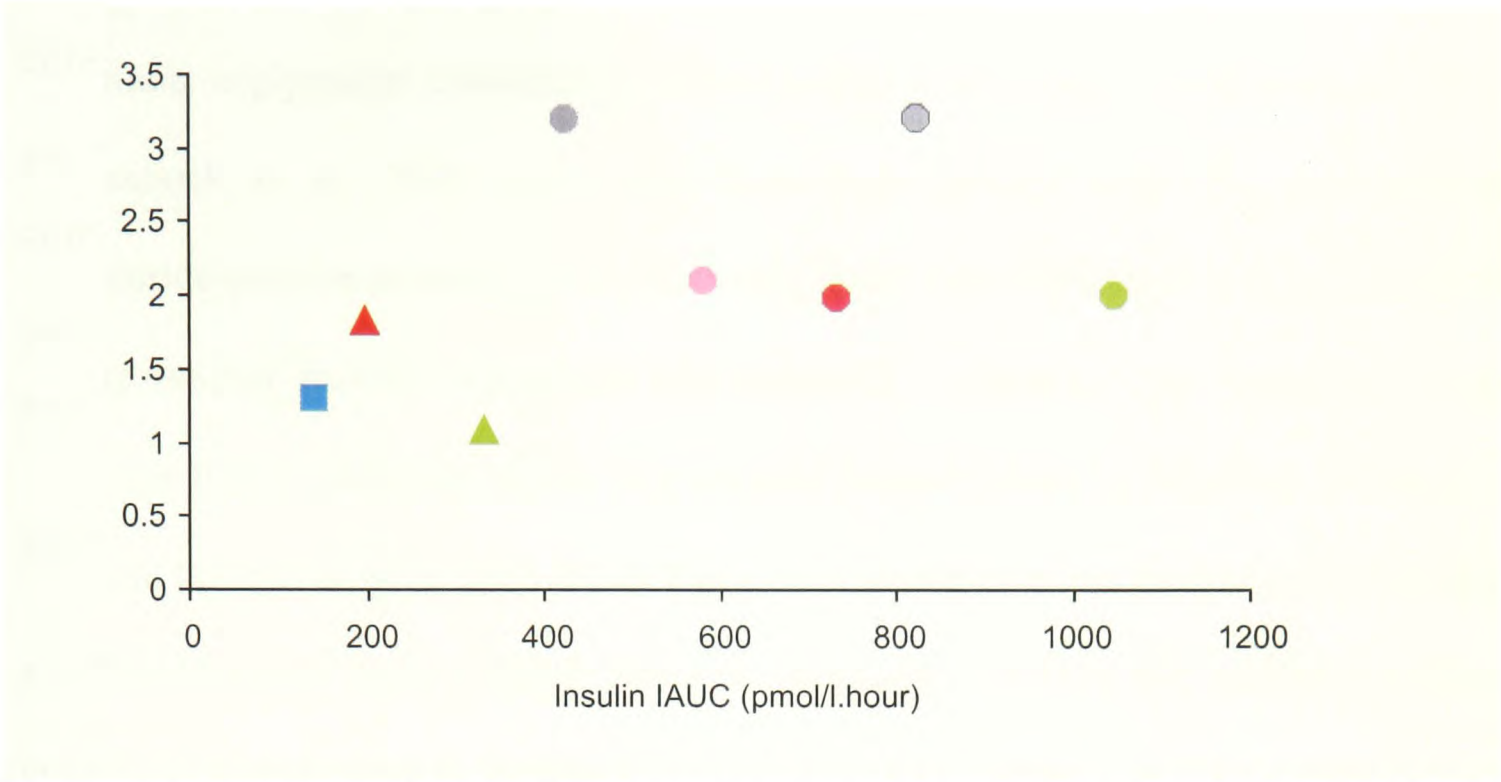


Figure 31 Summary of triglyceride and insulin exposure (IAUC). Subjects with no diabetes ■ and type 2 diabetes routinely treated by diet or oral hypoglycaemic therapy ●, diet only ● after nateglinide ☽, soluble ● and insulin lispro ● insulin. Type 1 diabetic subjects after soluble ▲ and ▲ lispro insulin.

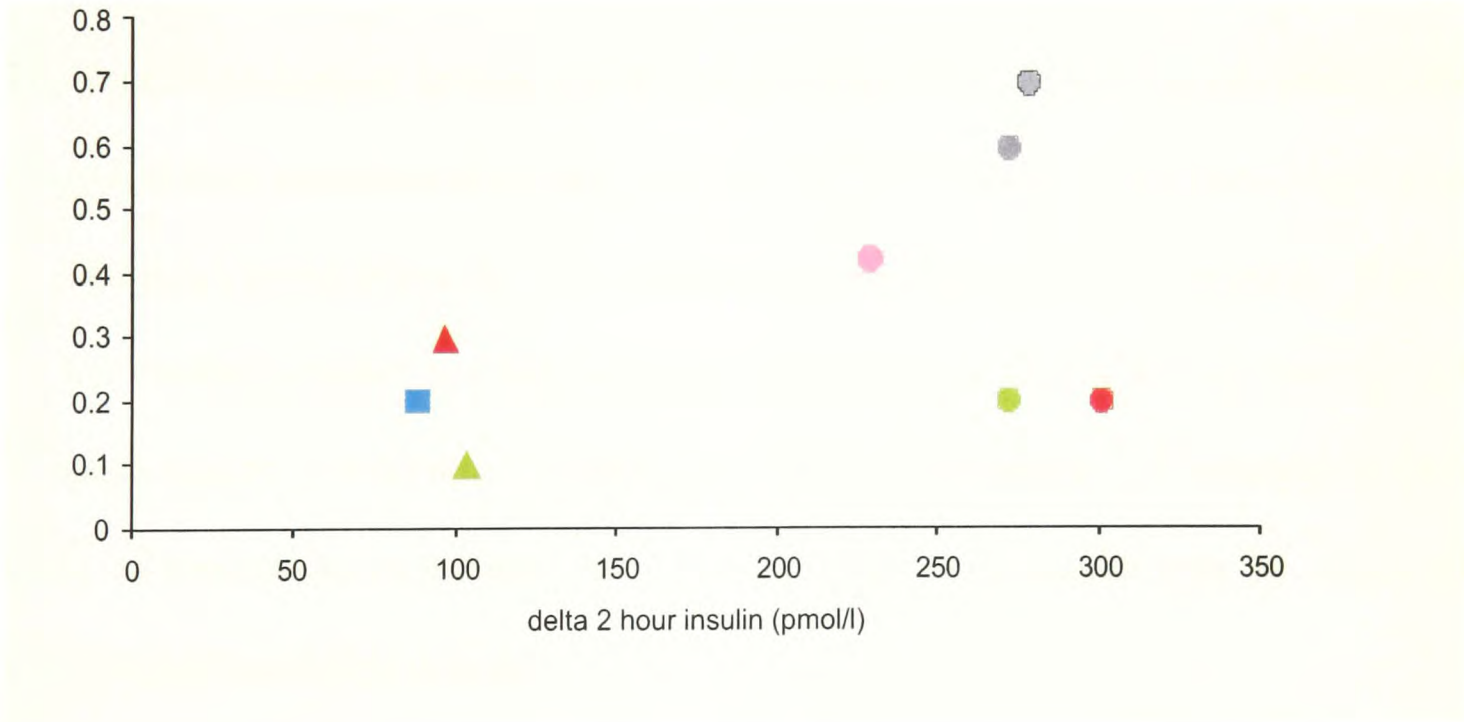


Figure 32 Summary of delta 6 hour triglyceride and delta 2 hour insulin levels. Subjects with no diabetes ■ and type 2 diabetes routinely treated by diet or oral hypoglycaemic therapy ●, diet only ● after nateglinide ☽, soluble ● and insulin lispro ● insulin. Type 1 diabetic subjects after soluble ▲ and ▲ lispro insulin.

Exogenous and not endogenous insulin augmentation shifted the time to achieve maximum triglyceride concentration from 6 hours to 4 hours. Patsch et al (Patsch, Miesenbock et al. 1992) found that individuals without CHD had post challenge triglyceride profiles peaking at 4 hours, whilst those with CHD peaked at 6 hours despite having normal fasting triglyceride concentrations. Although the magnitude of post challenge triglyceridaemia cannot be compared between study by Patsch et al and ours as different test meals were used, the similarities in the shape of the profile between diabetic and CHD subjects again suggests that alterations lipid handling seen in diabetic subjects potentially contributing to increased risk CHD. Type 1 diabetic subjects may also be at risk when glycaemic control is not optimal with triglyceride excursions peaking at 6 hours only when late post challenge hyperglycaemia persists.

The higher circulating insulin concentrations seen with soluble insulin administration compared with endogenous enhancement of insulin secretion (nateglinide) may suggest that greater insulin exposure will ultimately lower triglyceride excursions. However, similar insulin exposure was seen using insulin lispro and nateglinide, yet the triglyceride response was only altered by exogenous insulin supplementation. It is proposed that the mode of insulin delivery rather than absolute quantity of insulin may be important in modifying postprandial lipaemia.

10.4 Endogenous versus exogenous insulin delivery

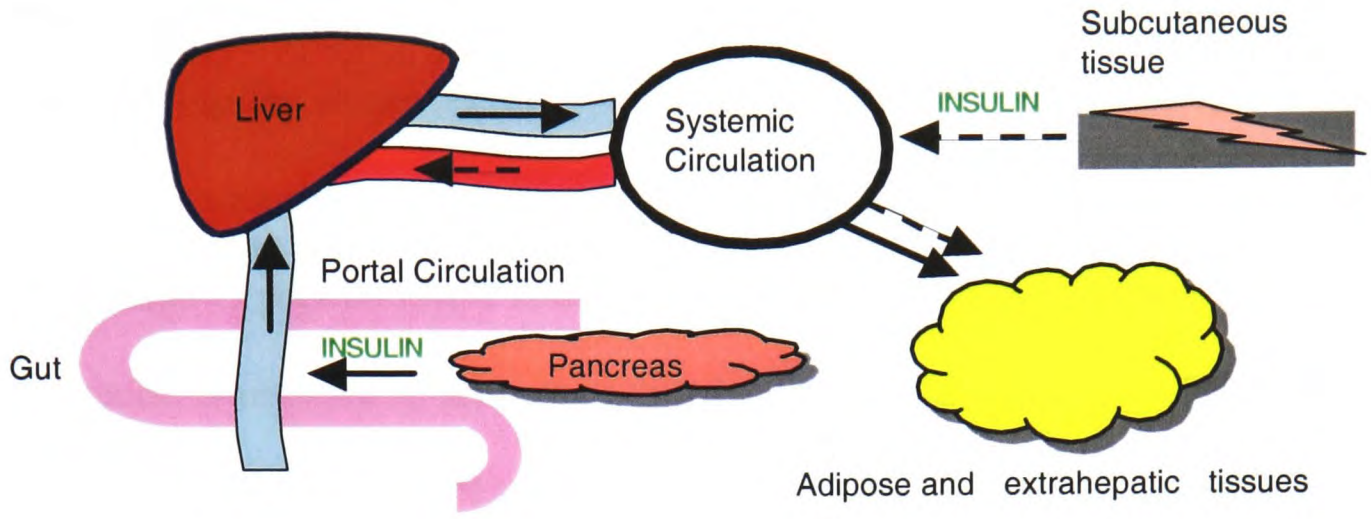


Figure 33 Diagram showing circulatory pathways of endogenous (full arrow) and exogenous (dashed arrow) insulin delivery.

Normally after secretion, insulin enters the portal circulation to reach the liver where approximately 50-70% is extracted and degraded (Shah et al 2002). Therefore insulin is present in the portal circulation at higher concentrations than in the systemic circulation. Enhancement of endogenous insulin secretion was thought to provide a more rapid and therefore more physiological insulin response directly to the liver. Insulin has a direct inhibitory effect on the VLDL secretion by the liver (Geltner, Lechleitner et al. 2002) and could potentially decrease circulating triglyceride levels by reducing VLDL competition with chylomicrons for LPL. Although insulin analogues aim to exogenously provide a peripheral insulin concentration that mimics the rise in early phase secretion, they neither restore physiological functioning of the β cell nor deliver insulin directly to the portal circulation. Because they are administered subcutaneously and absorbed into the systemic circulation, it may be difficult to provide sufficient insulin to the liver without exposing extrahepatic tissues to excessively high concentrations.

C peptide provides a useful index of the rate of endogenous insulin secretion as it is only partially extracted by the liver and is mainly degraded by the kidneys. Comparison of C peptide and insulin responses in type 2 diabetic subjects, highlights the differences among interventions (Table 31, Figure 34). Type 2 diabetic subjects display increased post challenge C peptide levels compared with non diabetic subjects, as greater insulin concentration is required to achieve normoglycaemia. As expected, endogenous insulin enhancement increased C peptide production. In comparison, exogenous insulin supplementation decreased C peptide levels, indicating insulin secretion by the pancreas to be lower than non diabetic individuals but not completely absent as in type 1 diabetic subjects. In fact, it appears insulin lispro suppresses endogenous insulin production to a greater extent than soluble insulin, and although the results were not statistically significant this has been demonstrated in previous studies (Bruttomesso D B, Pianta A et al. 1999).

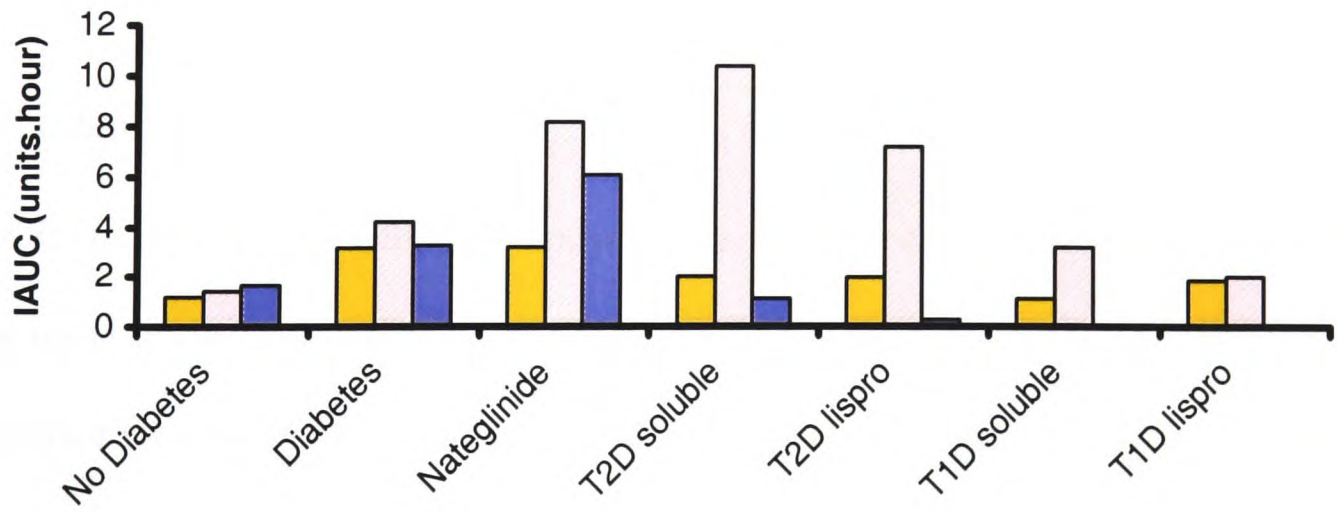


Figure 34 Summary of triglyceride ■ insulin ■, and C peptide ■ IAUC for all studies. (IAUC insulin is divided by 100). T2D is type 2 diabetes and T1D is type 1 diabetes.

Table 31 Insulin to C peptide ratios for all studies

Subjects	Insulin: C peptide ratio
No Diabetes	1:1
Diabetes	1.5:1
Nateglinide	1.5:1
T2D soluble	20:1
T2D lispro	16:1

Exogenous insulin administration exhibits the converse of normal insulin physiology ie relatively higher systemic versus portal insulin concentrations, although portal levels were not measured directly. The higher peripheral insulin concentrations produced by exogenous insulin delivery may exert differential effects on extrahepatic tissues compared to the liver to cause differences in postprandial lipaemia. Potential mechanisms for acute peripheral hyperinsulinaemia decreasing post challenge triglyceride excursions include:

1. inhibition of HSL activity
2. increase in LPL activity
3. relief of chronic portal hyperinsulinaemia.

10.4.1 Inhibition of HSL activity

Insulin is an inhibitor of HSL in adipose tissue, the key enzyme responsible for NEFA release into the circulation (Frayn 1996). The effect of insulin on HSL is rapid, occurring 1- 2 hours after a meal. The OTTT study demonstrated diminished 2 hour post challenge NEFA suppression in type 2 diabetic subjects compared with non diabetic subjects, a characteristic lipid abnormality documented in previous studies. This "relative" increase in supply of fatty acids to the liver increases the rate of hepatic VLDL triglyceride

production (Lewis, Uffelman et al. 1995) thereby contributing to elevation in the triglyceride excursion. Some studies of postprandial lipaemia show that type 2 diabetic subjects are resistant to the insulin suppression of NEFA concentrations (Chen Y D, Golay A et al. 1987; Riemens S C, Sluiter W J et al. 2000). Yet, the series of intervention studies demonstrate improvement of early post challenge NEFA suppression with endogenous- (nateglinide vs placebo) and exogenously (lispro vs soluble) increased insulin concentration in type 2 diabetic subjects (Figure 35). Prompt suppression of NEFA in the postprandial period using rapidly acting agents such as nateglinide and lispro may potentially reduce triglyceride levels more so than less rapid agents (gliclazide and soluble insulin).

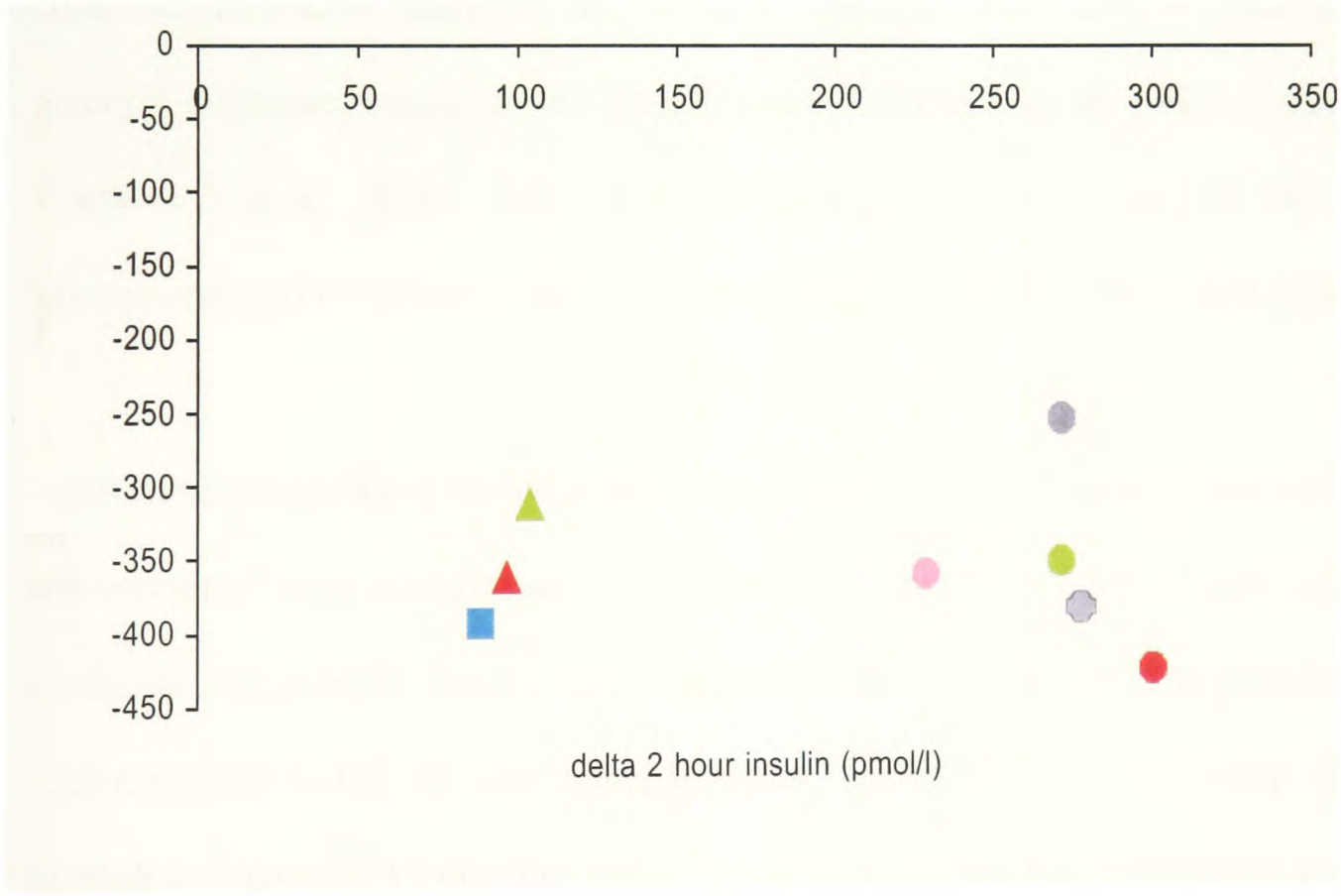


Figure 35 Summary of delta 2 hour NEFA and delta 2 hour insulin levels. Subjects with no diabetes ■ and type 2 diabetes routinely treated by diet or oral hypoglycaemic therapy ●, diet only ● after nateglinide ○ , soluble ● and insulin lispro ● insulin. Type 1 diabetic subjects after soluble ▲ and ▲ lispro insulin.

Failure to suppress HSL activity in the postprandial state has previously been demonstrated in type 2 diabetic subjects. Clamp studies have shown acute increased peripheral circulating insulin levels to inhibit HSL activity efficiently suppressing NEFA and glycerol release from adipose tissue (Lewis, Uffelman et al. 1993; Cummings M H, Watts G F et al. 1995; Malmström R, Packard C J et al. 1997). Despite decreased delivery of substrate to the liver, intravenous insulin was unable to acutely inhibit the release of hepatic VLDL (Malmström R, Packard C J et al. 1997). The authors suggested that it may take a period of time before liver stores of triglyceride are depleted enough to sufficiently reduce VLDL secretion. Other studies however had demonstrated that intravenous insulin given to type 2 diabetic as well as non diabetic and obese subjects could reduce VLDL secretion and that this was most likely due to decrease NEFA and glycerol substrate supply to the liver (Lewis, Uffelman et al. 1993; Cummings M H, Watts G F et al. 1995). Our results do not appear to be explained by this as both endogenous and exogenous insulin delivery similarly reduced NEFA and glycerol levels.

Vakkilainen et al (Vakkilainen, Mero et al. 2002) too found endogenous insulin secretion enhancement with nateglinide to significantly improve NEFA suppression, yet post challenge triglyceride and apo B100 responses did not change. They proposed that the recommended doses of oral hypoglycaemic agents may not decrease NEFA levels enough to attenuate VLDL production in the liver. However, our results show a similar degree of NEFA suppression was achieved with endogenous and exogenous insulin delivery in our studies despite a relative increase in peripheral insulin concentration seen with exogenous insulin therapy. A possible explanation for the similar NEFA

suppression obtained with all interventions, may be due to maximal inhibition of HSL by peripheral hyperinsulinaemia, with additional increases in postprandial insulin levels unable to inhibit lipolytic activity further (Lewis, O'Meara et al. 1991).

Recent work suggests that the defects in lipid metabolism seen in type 2 diabetes may not necessarily be due to reduced NEFA suppression, but escape of NEFA uptake after intravascular triglyceride lipolysis caused by high triglyceride levels (Riemens S C, Sluiter W J et al. 2000). Furthermore, McLaughlin et al (McLaughlin, Abbrasi et al. 2000) demonstrated prolonged insulinaemia produced by high carbohydrate diet, increased plasma triglyceride level despite lowering free fatty acid concentrations by 60%. The authors proposed that although exogenous hyperinsulinaemia may acutely inhibit hepatic triglyceride secretion this effect is very different from lipid changes seen with elevation of endogenous insulin secretion.

10.4.2 Increase LPL activity

Within 3 – 4 hours post challenge, insulin stimulates LPL activity in adipose tissue. This enzyme is responsible for the hydrolysis of chylomicron and VLDL triglyceride, leading to the production of fatty acids, most of which are re esterified for storage with adipose tissue. Some studies in type 2 diabetic subjects have shown decreased LPL activity (de Man, Castro Cabezas et al. 1996) which may also contribute to postprandial hypertriglyceridaemia seen in these subjects. Harbis et al (Harbis A, Defoort C et al. 2001) measured LPL activity at 6 hours in non diabetic subjects who consumed test meals of varying glycaemic indices to induce endogenous insulin secretion as well as

participating in clamp studies where insulin was infused over 3 hours. They found no difference in activity relative to the mode of insulin delivery and concluded that transient and moderate hyperinsulinaemia did not modify LPL activity. However this may not be accurate as LPL activity commences by 3 hours post meal, so differences at 6 hours post challenge when the insulin infusion had ceased, may not be evident. Also this study was in non diabetic subjects who do not demonstrate reduced LPL activity compared with diabetic subjects. Yet, a recent study by Eriksson et al (Eriksson, Burén et al. 2003) showed although LPL activity and mass tended to be lower in diabetic subjects, no significant differences in meal related increases in LPL activity in subcutaneous adipose tissue were found. The 8 diabetic subjects participating in this study were routinely treated by diet, sulphonylurea and metformin therapy and therefore LPL activity after exogenous insulin therapy was not assessed. Intensive insulin therapy in type 2 diabetic subjects has been shown to decrease VLDL and triglyceride levels as well as increase HDL levels possibly through the stimulation of LPL activity (Taskinen, Kuusi et al. 1988). Geltner et al (Geltner, Lechleitner et al. 2002) demonstrated a reduction in postprandial triglyceride levels independent of fasting triglyceride levels, when sulphonylurea treated diabetic patients were changed to a mixed insulin preparation (Mixtard 30/70). Peripheral insulin concentrations were the same for both interventions, and C peptide levels were lower with insulin therapy. They showed LPL activity to be significantly increased with exogenous insulin therapy. Although we did not measure LPL activity, it is thought that this is a potential mechanism to explain differences between exogenous and endogenous insulin delivery.

10.4.3 Relief of chronic portal hyperinsulinaemia

In insulin resistant states, peripheral hyperinsulinaemia is caused by both insulin hypersecretion and reduced hepatic extraction of insulin (Lewis, Carpentier et al. 2002). Normally 50% of insulin secreted by the pancreas is removed on first pass by the liver before reaching the peripheral circulation. Elevated free fatty acid levels typical in type 2 diabetic subjects, also reduce hepatic insulin extraction. It is possible then that endogenous insulin enhancement is more likely to expose the liver to extreme concentrations of insulin through the portal circulation, more so than exogenous insulin therapy. When insulin is infused through the system circulation, the impact on the liver of the resultant peripheral insulin level is less than with physiological portal insulin delivery (Lewis, Carpentier et al. 2002). Del Prato et al (Del Prato, Leonetti et al. 1994) studied the effects of chronic hyperinsulinaemia similar to that seen in type 2 diabetes over 72 - 96 hours in normotriglyceridaemic non diabetic subjects in a hyperglycaemic hyperinsulinaemic clamp study. After glucose infusion an elevation in triglyceride levels was observed, and the authors concluded that chronic physiologic hyperinsulinaemia was able to stimulate net lipid synthesis in the presence of concomitant hyperglycaemia. A mechanism for this, although not proposed by the authors, may relate to glucose mediated endogenous insulin secretion. Enhanced endogenous insulin secretion, confirmed by high C peptide levels relative to total insulin exposure, may infer that the portal circulation and therefore the liver, encounters extreme insulin concentrations. McLaughlin et al (McLaughlin, Abbrasi et al. 2000) too produced higher day long plasma insulin levels using a 60% carbohydrate diet for 2 weeks in non diabetic subjects and found this compared with a 40% carbohydrate diet, resulted in higher fasting and day

long plasma triglyceride concentrations. Increases in portal blood flow after meal ingestion exacerbate this, although it is not known whether portal blood flow differs between diabetic and non diabetic subjects (Bruce D G, Chisholm D J et al. 1988). Conversely, with exogenous insulin therapy, higher insulin concentrations circulate mainly in the systemic circulation, with a relative reduction in pancreatic insulin production evidenced by the decrease in C peptide concentrations. Therefore relieving this relative "portal hyperinsulinaemia" by supplying exogenous insulin therapy, may result in improvements in lipid metabolism by providing the liver with a more physiologic insulin concentration exposure.

There is evidence in vitro and in vivo that prolonged hyperinsulinaemia may have negative consequences on lipid metabolism through direct effects on the liver. Relieving chronic portal hyperinsulinaemia may affect lipid handling as hyperinsulinism has been shown to directly contribute to the development of VLDL triglyceride overproduction when hyperglycaemia is present (Pietri A O, Dunn F L et al. 1983). There is a scarcity of studies comparing the effects of endogenous and exogenous insulin delivery on lipid parameters in diabetic subjects to support this hypothesis. The effects of insulin and sulphonylurea therapy on fasting triglyceride concentration at the same level of blood glucose control has been assessed in type 2 diabetic subjects (Rivellese, Patti et al. 2000). After 2 months of subcutaneous insulin therapy, plasma triglyceride levels decreased compared with sulphonylurea treatment with authors concluding a reduction in large VLDL particles to explain the change in triglyceride excursions. No comment or comparison of insulin or C peptide levels were described in this study, which may have

suggested a potential mechanism being insulin therapy reducing endogenous insulin secretion, relieving portal hyperinsulinaemia thereby reducing VLDL overproduction. Romano et al (Romano, Patti et al. 1997) compared the effects of insulin and sulphonylurea therapy in type 2 diabetic subjects on lipoprotein metabolism over 2 months. Although glucose control was similar at the end of the study period, triglyceride, VLDL, and HDL were all significantly lower with insulin therapy. The authors suggested that, the higher fasting insulin to C peptide ratio indicated relative hepatic hypoinsulinization during subcutaneous insulin therapy and this may have a direct effect in reducing VLDL synthesis.

In non diabetic subjects, acute portal and peripheral insulin delivery decreases NEFA, triglyceride and VLDL production (Lewis, Zinman et al. 1994). However exogenous insulin was infused at a rate to match peripheral C peptide levels calculated from the pancreatic insulin secretory rate of each subject. This differs from our studies where we found C peptide levels to be lower after exogenous insulin delivery compared with endogenous insulin enhancement. The integration of hepatic and adipose tissue lipid metabolism after a 75 g oral glucose load and hyperinsulinaemic euglycaemic clamp was investigated in non diabetic subjects (Bülow, Simonson et al. 1999). The circulating insulin level aimed for in the clamp study, equivalent to peak portal vein concentration reached in the glucose experiment, was estimated after considering hepatic extraction of insulin. The authors found no inhibition of splanchnic VLDL triglyceride when plasma insulin levels were increased after glucose similar to our results. Yet studies stimulating pancreatic insulin secretion (tolbutamide) in non diabetic subjects to increase portal

insulin levels did reduce VLDL output (Vogelberg, Gries et al. 1980; Lewis, Zinman et al. 1994; Gibbons, Brown et al. 2002) although this effect was not produced in insulin deficient diabetic subjects (Lewis, Zinman et al. 1994).

Bülow et al (Bülow, Simonson et al. 1999) clamp experiments did however produce marked suppression of splanchnic VLDL output in support of our findings. Acute hyperinsulinaemia produced by euglycaemic hyperinsulinaemic clamp reducing large triglyceride rich VLDL particles production in non diabetic and to some degree in type 2 diabetic subjects has been demonstrated in certain studies (Lewis, Uffelman et al. 1993; Cummings M H, Watts G F et al. 1995; Malmström R, Packard C J et al. 1997; Bioletto, A et al. 2000) but not others (Bioletto, A et al. 2000). However, these studies do not distinguish between the direct effect of insulin on the liver and the reduction in NEFA delivery to the liver both of which contribute to decreased VLDL secretion. Bülow et al (Bülow, Simonson et al. 1999) found greater NEFA suppression with insulin infusion, and thus reduced NEFA supply to the liver explained the decrease in VLDL triglyceride concentration seen. This does not however explain our results, as both endogenous enhancement and exogenous supplementation of insulin increased NEFA suppression to a similar degree. Other studies have shown that even when hepatic NEFA supply was not limited, VLDL output could be suppressed during clamp studies of non diabetic subjects (Lewis, Uffelman et al. 1995). Bülow et al (Bülow, Simonson et al. 1999) also argued another possible reason for their results was that acute exogenous hyperglycaemia itself increases splanchnic output of VLDL triglyceride in comparison to the euglycaemic hyperinsulinaemic clamp experiment. Our studies used a standardised 50g carbohydrate

(and 50g fat) load in both endogenous and exogenous insulin studies, making this explanation less likely .

A potential reason for exogenous insulin administration having different post challenge consequences than endogenous insulin enhancement, is because of the difference in subjects recruited. Although type 2 diabetic subjects in both studies were of similar age and BMI, some subjects in the exogenous insulin intervention study had previously been on antidiabetic therapy, that is, oral hypoglycaemic agents and/or insulin. No washout period was undertaken prior to conducting the OTTT, and therefore effects could be attributed to the agent they were routinely prescribed even though these were not taken on the day of the test. Examination of the subgroups in the OTTT study showed subjects on long term metformin and sulphonylurea therapy who took their morning dose of medication on the day of the OTTT, had no significant difference in triglyceride IAUC profiles from diet only treated subjects.

10.5 Exogenous insulin therapy in type 1 and type 2 diabetic subjects

The metabolic effects of exogenous insulin delivery in type 1 diabetic subjects may exhibit differences distinct from type 2 diabetic subjects. Individuals with type 1 diabetes have complete deficiency of endogenous insulin secretion and therefore solely rely upon peripheral insulin delivery to maintain normoglycaemia. With lower post challenge insulin excursions but similar degrees of NEFA suppression compared with type 2 diabetic subjects, differences in post challenge triglyceride levels were demonstrated in type 1 diabetic subjects.

Soluble insulin produced triglyceride profiles similar to those described in non diabetic individuals. Yet the overall insulin response was higher than seen in non diabetic subjects but lower than observed in diet treated type 2 diabetic subjects (placebo), again suggesting peripheral insulin administration may have a beneficial effects on triglyceride metabolism compared with portal delivery, rather than absolute quantity of insulin. Again the most likely reason for this is the effect of high systemic concentrations of insulin stimulating LPL activity in extrahepatic tissues. A previous study of type 1 diabetic subjects showed no difference in triglyceride response compared with non diabetic subjects (Lewis, O'Meara et al. 1991).

The post challenge triglyceride response was statistically lower with soluble than lispro insulin in type 1 diabetic subjects. Subcutaneous soluble insulin therapy causing persistent elevation of peripheral insulin levels has been shown to normalise plasma glucose levels, as well as decrease triglyceride and VLDL levels (Bagdade and L 1992). As discussed in Chapter 7 the extended action profile of soluble insulin may itself be important but is more likely compensating for inadequate maintenance of basal insulin delivery. This is supported by increased glucose exposure seen during the late postprandial period with insulin lispro due to its short duration of action. It has been suggested that hyperglycaemia can increase hepatic VLDL triglyceride production (de Man, Castro Cabezas et al. 1996).

Further explanation of the results may relate to the residual endogenous insulin production by the pancreas in type 2 diabetic subjects. The ability to do this in these subjects may assist in maintaining normoglycaemia during the late postprandial period after the effects of insulin lispro have begun to diminish. In the type 2 diabetes study, although lispro insulin significantly minimised the glucose excursion during the first 4 hours of the study relative to soluble insulin, the remainder of the study showed no difference in glucose levels between insulins, despite lispro being of shorter duration of action. C peptide IAUC from 4 to 8 hours was also higher with lispro than soluble insulin, indicating endogenous insulin production. Yet in type 1 diabetic subjects taking insulin lispro, late post challenge hyperglycaemia relative to soluble insulin was evident. With these subjects relying solely upon the action of exogenous insulin to suppress hepatic glucose production and increase glucose uptake by tissues, prandial insulin replacement must be supplemented by long acting basal insulin therapy between meals.

It is unlikely that the reduced dose of insulin (0.1U/kg vs 0.15U/kg) given to type 1 compared with type 2 diabetic subjects could explain differences in the effects of insulin lispro between the two studies. In type 2 diabetes higher doses of insulin are required to combat insulin resistance. Type 1 diabetic subjects experienced more episodes of hypoglycaemia during the study, and increasing the dose of insulin would risk further events.

In a miniature pig model of human type 1 diabetes, portal and peripheral insulin delivery had a similar effect on fasting lipid profiles and subfractions (Canavan, Flecknell et al.

1997). In type 1 diabetic subjects, intra peritoneal insulin therapy has been shown to normalise plasma glucose and insulin profiles and restore the normal portal peripheral gradient similar to non diabetic individuals (Bagdade and Dunn 1992). The results of the effect of insulin delivery via this route on lipid metabolism are variable. Some studies have found no change in cholesterol, apoB or triglyceride synthesis by the liver, and others have found it to normalise triglyceride levels. Intraperitoneal insulin delivery is thought to increase hepatic lipase activity, decreasing the triglyceride to apo B ratio in VLDL as well as improve cholesterol ester transfer and HDL composition towards normal with no effect on LPL activity (Ruotolo, Parlavecchia et al. 1994). However these changes may be due to better glycaemic control and lower NEFA levels, which have also been shown to improve although not completely normalise with intraperitoneal insulin delivery (Ruotolo, Micossi et al. 1990).

10.6 Fasting versus postprandial triglyceride levels

The strong association between fasting and postprandial triglyceride concentrations may suggest no further information can be gained by assessing post challenge profiles. This series of intervention studies demonstrate the mechanistic effect of insulin is not due to improvement of fasting triglyceride level, but is a postprandial phenomenon. A novel feature about the study design implemented was the use of single, "one off" doses of antidiabetic medication tested rather than long term therapy. Post challenge changes were not due a shifting baseline and may be interpreted as a real effect relatively independent of the fasting level. It has been proposed that pronounced postprandial

lipaemia encourages the formation of small dense LDL and loss of HDL, which subsequently alters the fasting lipid profile, and therefore greater improvements in post challenge than fasting triglyceride profiles are seen after insulin therapy (Geltner, Lechleitner et al. 2002). Assessment of post challenge profiles can offer further insight into metabolic abnormalities present in diabetes.

Previous studies have found relationships between postprandial triglyceride levels and insulin exposure. Castro Cabezas (Castro Cabezas, Halkes et al. 2001) found a significant correlation between insulin and absolute triglyceride AUC and Geltner et al (Geltner, Lechleitner et al. 2002) showed the occurrence of small dense LDL was related to insulin levels. In hyperinsulinaemic non diabetic subjects, triglyceride IAUC was significantly related to the quartile distribution of fasting plasma insulin concentration independently of fasting triglyceride level, BMI, waist hip ratio and blood glucose concentration (Boquist S, Hamsten A et al. 2000). In our studies we found strong relationship between fasting and post challenge triglyceride levels in non diabetic and diabetic subjects, with most correlations between fasting triglyceride concentration and insulin-glucose values being maintained when testing 6 hour post challenge triglyceride levels. However the converse did not hold true, that is, we found certain post challenge but not necessarily fasting triglyceride values to be associated with post challenge insulin levels. This as well as the paucity of postprandial studies in diabetic subjects, highlights the complexity in assessing the CHD risk attributable to serum triglycerides from one or two triglyceride measurements in any one individual. Although there is a strong

correlation between the mean fasting and postprandial triglyceride concentrations the fasting triglycerides may still not be an adequate measure of an individual's risk.

Studies in non diabetic individuals have found postprandial triglyceride levels to correlate with CHD. Patsch et al (Patsch, Miesenbock et al. 1992) showed postprandial but not fasting triglyceride levels exhibited an association with CHD that was statistically independent and stronger than that of HDL cholesterol. Karpe et al (Karpe, de Faire et al. 1998) showed the 6 hour postprandial triglyceride concentration correlated with intima media thickness of the common carotid artery independently of fasting plasma triglyceride concentrations and LDL.

Patsch et al (Patsch, Miesenbock et al. 1992) found that individuals without CHD had post challenge triglyceride profiles peaking at 4 hours, whilst those with CHD peaked at 6 hours despite having normal fasting triglyceride concentrations. Type 2 diabetic subjects participating in the OTTT studies also peaked at 6 hours, except when taking prandial insulin when triglyceride concentrations peaked at 4 hours. Although the magnitude of post challenge triglyceridaemia cannot be compared between the study by Patsch et al and ours as different test meals were used, the similarities in the shape of the profile between diabetic and CHD subjects again suggests that alterations lipid handling seen in diabetic subjects potentially contributing to increased risk CHD. Type 1 diabetic subjects may also be at risk when glycaemic control is not optimal with triglyceride excursions peaking at 6 hours only when late post challenge hyperglycaemia persists.

Although fasting triglyceride has been shown to be an independent risk factor for CHD in the non diabetic population (Hokanson and Austin 1996) studies in type 2 diabetic subjects have not supported this (UKPDS Group 1998). An important issue surrounding both fasting and postprandial triglyceride levels and their association with CHD, is related to HDL cholesterol. HDL is a strong independent risk factor for CHD and insulin resistance has been shown to be inversely correlated with plasma HDL (Rashid, Uffelman et al. 2002). However, although low HDL can be present in some individuals without hypertriglyceridaemia, the absence of low HDL with hypertriglyceridaemia is rare. Studies by Lewis et al (Lewis, O'Meara et al. 1990) showed the magnitude of postprandial triglyceride response to be inversely associated with HDL concentration which is in concordance with previous studies. Although we found no significant correlations between post challenge triglyceride concentration and HDL in our studies. Elevated levels of chylomicrons and VLDL enable increased translocation of cholesteryl esters from HDL to triglyceride rich lipoproteins in exchange for triglycerides and therefore HDL concentration decreases. Thus elevated triglyceride levels are the driving force for reduced HDL cholesterol values (Patsch, Miesenbock et al. 1992). It is often seen in the literature that triglyceride is eliminated by HDL in statistical analyses of CHD risk factor studies. As the half life of HDL greatly exceeds that of triglyceride rich lipoproteins, HDL could serve as a marker of triglyceride metabolic capacity, perhaps better than fasting triglyceride levels (Patsch, Miesenbock et al. 1992).

Our platelet studies (Chapter 8) have attempted to elucidate whether post challenge triglyceride excursions may incite a prothrombotic environment. These initial studies in

type 1 and type 2 diabetic subjects do not demonstrate an effect between rapid and short acting insulin therapy on platelet function. However, we found significant correlations between platelet function and post challenge triglyceride levels but not fasting. More recent studies are investigating the direct effects of a fatty meal on the vasculature. Alterations in platelet function, vascular endothelium and hypercoagulability have all been demonstrated post lipid challenge (Anderson, Jones et al. 2001). The OTTT could be used for further studies in this area in order to clarify the mechanism of postprandial lipaemia accelerating atherosclerosis. Highlighting potentially additional risk factors such as platelet abnormalities in the postprandial period may be advantageous in the management of dyslipidaemic patients.

10.7 Potential implications for therapeutic decision making in type 2 diabetes.

Current management of the diabetic individual involves detailed assessment of potential CHD risk factors. Our results show that despite normal fasting triglyceride values, type 2 diabetic subjects display elevated post challenge triglyceride excursions compared with non diabetic individuals. It is possible that this may potentially place them at increased risk of CHD. The instigation of lipid lowering therapy has been suggested in diabetic subjects before the signs of macrovascular disease (Mero, Malmström et al. 2000). The Heart Protection Study provided epidemiologic evidence that simvastatin reduces CHD morbidity and mortality in individuals with normal fasting lipid profiles (HPS-Collaborative-Group 2002).

We found a single subcutaneous injection of prandial insulin decreased the 6 hour triglyceride increment by *circa* 50% in type 2 diabetic subjects with normal fasting lipid profiles compared with subjects routinely treated by diet or oral hypoglycaemic therapy. We believe this insulin effect to be maximal as higher doses of insulin caused hypoglycaemia in our subjects. However, we have not explored the effects of insulin therapy in diabetic subjects with elevated fasting triglyceride levels. It is possible that hypertriglyceridaemic individuals may show an even greater modification of the triglyceride response with insulin therapy.

We have not investigated the effects on lipid handling of long term insulin therapy in type 2 diabetic subjects. Smaller long term studies have shown insulin to decrease fasting triglyceride levels by 30 - 40% (Rivellese, Patti et al. 2000) through modification of VLDL kinetics (Taskinen, Packard et al. 1990). Over time, decrease in 6 hour triglyceride increment we demonstrated with subcutaneous insulin therapy may relieve competition on endogenous- and exogenously produced triglyceride rich lipoproteins and reduce fasting triglyceride levels. This would then further modify the postprandial triglyceride response as the postprandial triglyceride excursion is dependent upon the fasting triglyceride level.

This additional effect of insulin may be important in minimising CHD risk in type 2 diabetes patients, particularly if compliance or feasibility are of concern (Kanters, Algra et al. 1999). These issues are often raised when considering therapy individuals who have normal fasting lipid profiles, who do not "qualify" for lipid lowering treatment

following normal practicing guidelines (Ansell, Watson et al. 1999). The progression of ß cell failure in type 2 diabetes and current practice encouraging intensive blood glucose control to minimise diabetic complications, more diabetic patients are making the transition from oral hypoglycaemic therapy to insulin. They may potentially be benefiting from an improvement in lipid status as well as glucose control thus reducing their CHD risk. A large scale trial investigating CHD outcomes of insulin therapy targeting the postprandial period incorporating post challenge metabolic assessment may not only determine additional benefits of insulin therapy on lipid handling, but provide greater clarification into the relationship of fasting triglyceride and CHD risk.

Other current alternatives to modifying postprandial triglyceride levels include lifestyle modification such as increased physical activity, dietary changes, and reducing alcohol intake. Lipid lowering therapies also modify the postprandial triglyceride excursion, although it is difficult to determine whether this is due to changes in fasting triglyceride levels, as single dose studies have not been conducted. Fibrate therapy has been shown to reduce the 6 hour postprandial triglyceride increment by approximately 50 - 60% (Attia, Durlach et al. 1997; Evans, Anderson et al. 2000). Statin therapy too reduces triglyceride AUC by approximately 30 - 50% in diabetic subjects (Noutsou and Georgopoulos 1999; Battula, Fitzsimons et al. 2000; Sheu, Jeng et al. 2001). Other agents such as fish oils, orlistat and acarbose, may have effects on postprandial lipid metabolism although not fully investigated (Hanefeld, 1991; Nattrass, 2000; Burnett and Watts 2001).

10.8 Contribution to clinical medicine - summary

We have formulated a standardised oral test meal that is inexpensive and simple to manufacture and can be administered under conditions that are safe and acceptable to diabetic and non diabetic subjects. This fat and carbohydrate test meal can be used as a challenge to produce postprandial responses which are reproducible. The design of the test enables implementation in clinical practice, and the use of capillary fingerprick sampling as an alternative to venipuncture would allows assessment in an outpatient and/or large scale setting. A test such as this has for many years been requested for studies of postprandial lipaemia by investigators in this field (Karpe 1997).

We have used the OTTT as a standardised tool for postprandial assessment to explore differences in metabolism in non diabetic, type 2 and type 1 diabetic subjects. Our results highlighted normotriglyceridaemic type 2 diabetic subjects have exaggerated post challenge triglyceride, NEFA, glycerol, glucose, insulin and C peptide responses to an oral fat and carbohydrate load compared with non diabetic subjects. Our results also confirm that single doses of prandial antidiabetic agents which aim to produce an early rapid increase in insulin concentration (nateglinide and lispro) significantly lower blood glucose levels in type 2 diabetic subjects.

We found enhancing portal insulin secretion in type 2 diabetic subjects did not modify post challenge triglyceride handling. Although secreting early and rapid insulin into the portal circulation may be a more "physiological" mode of insulin delivery, it appears the beneficial effects of insulin on lipid handling, as previously described in the literature, are

not evident. However, acute systemic administration of insulin reduces triglyceride exposure to a comparable degree as seen with statin therapy, in the post challenge period. It is proposed that the triglyceride lowering effect of subcutaneous insulin is due to restoration of portal insulin concentrations (inferred from C peptide levels) to levels similar to non diabetic individuals, thus relieving the liver of the detrimental effects of chronic hyperinsulinaemia on lipid handling.

Our studies in type 1 diabetic subjects show that post challenge triglyceride responses are similar to non diabetic individuals. However ultra rapid insulin replacement may unmask inadequate basal insulinisation evidenced by late post challenge hyperglycaemia which cause an elevation in triglyceride excursions.

We are the first to conduct fasting and post oral lipid and carbohydrate challenge platelet function analyses in type 1 and type 2 diabetic subjects. Our results indicate no immediate abnormalities in platelet function in diabetic subjects as a potential mechanistic cause of atherosclerosis development. However we have established definite relationships between platelet function and insulin, as well as post challenge triglyceride levels.

11 Conclusions

To suggest that abnormalities in the postprandial period are risk factors for cardiovascular morbidity and mortality implies certain assumptions have been made. Firstly that there is a reliable method for assessing various measurements in the postprandial state, secondly that there is epidemiological evidence for the association, thirdly, that a plausible pathophysiological mechanism by which atherosclerosis may develop can be established, and finally that correcting the variation will lower the incidence of disease. This DPhil thesis has attempted to address many of these issues which have been unresolved in the field of postprandial lipid research. We have produced a simple and reliable method for post challenge metabolic assessment, the OTTT. The OTTT could now become the platform for post challenge testing in large scale epidemiologic studies to provide evidence linking postprandial triglyceridaemia to CHD morbidity and mortality. The OTTT could also be used for more detailed postprandial studies to explore potential mechanisms accelerating atherogenesis. We have confirmed that type 2 diabetic subjects display alterations in both lipid and glucose handling compared with non diabetic individuals, and it is these abnormalities that may contribute, through accelerated atherogenesis and decreased vasodilatation, to their increased risk of CHD seen in this population. The intervention studies have sequentially investigated potential treatments for the metabolic abnormalities, aiming to restore loss of first phase insulin secretion the primary defect in type 2 diabetes, which may be the underlying pathophysiology causing early atherogenesis. Subcutaneous insulin delivery was shown to improve, although not completely normalise, elevated post challenge triglyceride levels. This again supports

aggressive antidiabetic treatment for CHD prevention, and may have potential implications for prescribing in the future.

12 Appendices

Table of triglyceride, NEFA & glycerol responses from second OTTT (Chapter 3 and 4)

	No Diabetes			Diabetes			*p*
Triglyceride (mmol/l)							
0 hour	0.82	0.551	-1.215	1.55	0.914	-2.621	*<0.0001*
2 hours	0.87	0.503	-1.495	1.81	1.065	-3.073	
4 hours	1.15	0.690	-1.926	1.97	1.232	-3.138	
6 hours	0.96	0.549	-1.694	2.05	1.185	-3.534	*<0.0001*
8 hours	0.80	0.499	-1.288	1.54	0.867	-2.728	
AUC+	7.8	4.900	-12.360	15.0	9.350	-24.153	*<0.0001*
$IAUC_{0\text{-}6hours}$*+	1.0	0.410	-2.075	2.0	0.960	-3.180	*0.0376*
$IAUC_{0\text{-}8hours}$*+	1.2	0.430	-2.225	2.2	0.970	-3.850	*0.0747*
Cmax	1.33	0.829	-2.147	2.42	1.529	-3.837	*<0.0001*
deltaCmax	0.45	0.184	-1.081	0.8	0.443	-1.450	
Tmax*±	4	4	-6	6	4	-6	*0.8027*
6-0 hours*	0.03	-0.045	-0.400	0.52	0.110	-0.980	*0.0962*
NEFA (umol/l)							
0 hour	395.4	216.8	-721.1	556.3	358.9	-862.1	*0.0244*
2 hours	92.8	54.0	-159.6	206.9	97.8	-438.1	*0.0002*
4 hours	417.5	262.5	-663.8	316.5	186.9	-536.1	
6 hours	555.9	410.8	-752.4	638.7	436.1	-935.6	
8 hours	598.7	419.5	-854.6	739.6	542.9	-1007.5	
AUC+	3288.8	2536.8	-4263.6	3864.2	2951.3	-5059.4	*0.0421*
$IAUC_{0\text{-}4hours}$*+	-686.5	-1010.5	--208.5	-1093.0	-1333.5	--539.8	*0.0613*
$IAUC_{0\text{-}8hours}$*+	-272.5	-904.0	-884.5	-981.0	-1621.8	--422.3	*0.0227*
Cmin	91.6	54.5	-153.8	175.9	91.5	-338.1	
Cmax	717.3	540.8	-951.2	805.5	600.3	-1080.9	*0.1713*
Tmax*±	7	6	-8	6	6	-8	*0.8333*
2-0 hours*	-341.5	-444	--166	-375.0	-576.3	--164	*0.6607*
Glycerol (umol/l)*							
0 hour	0.85	0.48		1.07	0.37		*0.0699*
2 hours	0.57	0.21		0.81	0.38		*0.0055*
4 hours	1.22	0.48		1.18	0.67		
6 hours	0.96	0.31		1.56	0.80		
8 hours	1.02	0.31		1.17	0.54		
AUC+	7.35	1.54		9.33	3.17		*0.0052*
$IAUC_{0\text{-}4hours}$+	0.04	-0.87	-0.94	-0.5	-0.93	-0.44	*0.5768*
$IAUC_{0\text{-}8hours}$+	1.6	-0.87	-2.53	0.6	-0.74	-2.70	*0.8218*
Cmin	0.51	0.16		0.68	0.28		*0.006*
Cmax	1.49	0.39		1.84	0.82		*0.0508*
Tmax±	4	4	-6	6	4	-6	*0.8494*
2-0 hours	-0.28	0.52		-0.26	0.38		*0.8834*

Data are geometric mean (1 SD range) except * median (IQR). Units are as tabulated except + units.hour and ± hours

Table of Glucose, Insulin and C peptide responses in non diabetic and diabetic subjects (second OTTT, Chapter 3 and 4)

	No Diabetes			Diabetes			*p*
Glucose (mmol/l)†							
0 hour	5.5	0.6		9.2	3.0		*n/a*
2 hours	5.8	1.9		14.1	4.4		*n/a*
4 hours	4.7	0.5		7.9	3.4		
6 hours	4.8	0.3		6.2	1.8		
8 hours	4.8	0.3		5.7	1.3		
AUC+	40.9	4.0		71.4	22.1		*n/a*
$IAUC_{0\text{-}4hours}$*+	-0.4	-1.7	-1.6	8.1	5.1	-13.1	*<0.0001*
$IAUC_{0\text{-}8hours}$*+	-3.0	-3.9	- -1.2	-3.0	-7.3	-1.8	*0.6693*
Cmin	4.4	0.5		5.4	1.5		*n/a*
Cmax	6.3	1.6		14.2	4.3		*n/a*
Tmax*±	2	2	-2	2	2	-2	*0.6249*
2-0 hours*	-0.8	-1.0	- -0.3	-3.2	-5.3	- -1.8	*n/a*
Insulin (pmol/l)							
0 hour	73.2	44.9	-119.5	97.5	49.3	-192.9	*0.119*
2 hours	190.2	90.8	-398.5	268.2	150.5	-470.3	*0.0718*
4 hours	78.1	39.8	-153.1	157.6	78.1	-318.2	
6 hours	57.9	33.2	-101.0	99.2	46.6	-211.5	
8 hours	58.9	34.2	-101.5	91.0	47.1	-175.6	
AUC+	818.3	444.0	- 1508.0	1314.2	747.4	- 2311.0	*0.0084*
$IAUC_{0\text{-}4hours}$*+	182.2	32.4	-555.0	426.4	226.7	-559.7	*0.071*
$IAUC_{0\text{-}8hours}$*+	139.3	-4.1	-602.1	398.4	234.1	-787.7	*0.0512*
Cmax	191.4	91.8	-399.0	281.1	165.5	-467.7	*0.0366*
Tmax*±	2	2	-2	2	2	-2	*0.5191*
2-0 hours*	95.7	21.9	-232.4	163.8	97.7	-257.5	*0.1007*
C peptide (nmol/l)							
0 hour	0.63	0.42	-0.93	0.85	0.53	-1.37	*0.0219*
2 hours	1.58	0.97	-2.58	1.91	1.36	-2.68	*0.1437*
4 hours	0.70	0.41	-1.20	1.35	0.84	-2.15	
6 hours	0.54	0.32	-0.91	0.92	0.52	-1.64	
8 hours	0.50	0.29	-0.86	0.80	0.46	-1.39	
AUC+	6.90	4.37	-10.90	10.25	6.89	-15.24	*0.0021*
$IAUC_{0\text{-}4hours}$*+	1.79	1.07	-3.84	2.51	1.77	-3.13	*0.2121*
$IAUC_{0\text{-}8hours}$*+	1.66	0.42	-3.75	2.79	1.73	-4.49	*0.0434*
Cmax	1.61	1.01	-2.58	1.97	1.41	-2.73	*0.1117*
Tmax*±	2	2	-2	2	2	-2	*0.0701*
2-0 hours*	0.92	0.52	-1.68	0.91	0.65	-1.29	*0.8627*

Data are geometric mean (1 SD range) except * median (IQR). Units are as tabulated except + units.hour and ± hours

Table of triglyceride, NEFA and glycerol responses in diabetic subgroups (second OTTT, Chapter 3 and 4)

	Diet			Metformin			Sulphonylurea			*p*
Triglyceride (mmol/l)										
0 hour	1.27	0.85	-1.90	1.71	0.99	-2.96	1.71	0.93	-3.12	*0.3581*
2 hours	1.66	1.19	-2.32	1.90	1.04	-3.46	1.88	0.98	-3.59	
4 hours	1.80	1.33	-2.44	2.22	1.49	-3.31	1.90	0.99	-3.66	
6 hours	1.76	1.12	-2.76	2.10	1.24	-3.58	2.31	1.20	-4.47	*0.5395*
8 hours	1.24	0.76	-2.02	1.54	0.85	-2.78	1.90	1.04	-3.49	
AUC^{+}	13.22	575.43	-1093.73	15.94	9.78	-25.97	16.11	8.86	-29.26	*0.5946*
$IAUC_{0\text{-}6hours}$*$^{+}$	2.44	94.60	-195.00	2.25	0.96	-3.19	1.40	0.92	-2.66	*0.5182*
$IAUC_{0\text{-}8hours}$*$^{+}$	2.40	98.40	-292.80	1.78	0.95	-3.85	2.16	0.57	-3.73	*0.7917*
Cmax	2.20	1.57	-3.08	2.50	1.54	-4.04	2.59	1.47	-4.55	*0.7209*
deltaCmax	0.87	0.55	-1.37	0.72	0.36	-1.46	0.81	0.42	-1.57	*0.6874*
Tmax*±	5	4	-6	4	4	-6	6	4	-6	*0.0776*
6-0 hours*	0.54	0.11	-0.98	0.22	-0.08	-0.87	0.69	0.16	-1.42	*0.711*
NEFA (umol/l)										
0 hour	548.5	352.3	-854.0	516.0	397.9	-669.1	608.2	339.6	-1089.2	*0.712*
2 hours	150.1	80.5	-279.9	195.5	82.2	-465.2	314.8	173.3	-571.9	*0.0722*
4 hours	337.0	169.5	-670.0	321.3	217.9	-473.8	290.4	174.9	-482.1	
6 hours	667.9	400.5	-1114.1	686.3	477.9	-985.5	568.5	450.8	-716.8	
8 hours	696.8	478.1	-1015.7	754.2	596.9	-952.8	769.8	556.6	-1064.7	
AUC^{+}	3750.8	2538.5	-5541.9	3880.3	3215.6	-4682.3	3975.8	3247.0	-4868.3	*0.9369*
$IAUC_{0\text{-}4hours}$*$^{+}$	-1108	-1270.25	- -653.5	-643.5	-1180.5	- -34.5	-1194	-1359	- -923.0	*0.3115*
$IAUC_{0\text{-}8hours}$*$^{+}$	-944.5	-1211.0	- -471.0	-530.5	-1296.0	-583.0	-2143.0	-2326.3	- -1238.3	*0.0111*
Cmin	142.4	80.6	-251.5	168.3	76.7	-369.3	227.1	130.1	-396.6	*0.2774*
Cmax	769.5	515.5	-1148.6	805.0	617.1	-1050.3	843.8	686.2	-1037.5	*0.7939*
Tmax*±	8	6	-8	5	4	-6	8	6	-8	*0.0682*
2-0 hours*	-447.5	-590.0	- -227.0	-235.0	-416.0	- -145.0	-395.0	-473.8	- -258.5	*0.344*
Glycerol (mmol/l)*										
0 hour	1.09	0.42		0.90	0.21		1.21	0.40		*0.1743*
2 hours	0.80	0.27		0.82	0.47		0.82	0.40		*0.9907*
4 hours	1.37	0.88		1.13	0.53		1.04	0.57		
6 hours	1.37	0.53		1.77	1.14		1.52	0.63		
8 hours	1.17	0.78		1.05	0.40		1.28	0.38		
AUC^{+}	9.34	3.72		9.40	3.26		9.25	2.82		*0.9951*
$IAUC_{0\text{-}4hours}$$^{+}$	-0.57	-0.77	- -0.07	0.39	-0.81	-0.61	-1.06	-1.75	-0.16	*0.0998*
$IAUC_{0\text{-}8hours}$$^{+}$	0.91	-0.25	-1.54	3.06	-0.63	-4.28	-0.22	-2.26	-2.44	*0.1459*
Cmin	0.71	0.24		0.66	0.28		0.69	0.33		*0.9209*
Cmax	1.80	0.89		1.92	1.05		1.79	0.50		*0.9296*
Tmax±	6	4	-6	6	4	-6	6	4	-6	*0.7915*
2-0 hours	-0.30	-0.49	- -0.25	-0.11	-0.35	-0.18	-0.40	-0.77	- -0.03	*0.1756*

Data are geometric mean (1 SD range) except * median (IQR). Units are as tabulated except + units.hour and ± hours

Table of glucose, insulin and C peptide responses in diabetic subgroups (second OTTT, Chapter 3 and 4)

	Diet			Met formin			Sulph onylurea			*p*
Glucose (mmol/l)†										
0 hour	7.8	2.5		9.1	2.5		10.7	3.4		*0.0845*
2 hours	12.0	4.3		13.6	4.1		16.8	3.8		*0.0353*
4 hours	6.7	3.5		7.0	2.7		10.0	3.3		
6 hours	5.8	1.7		6.0	1.3		7.0	2.3		
8 hours	5.5	0.9		5.8	1.2		5.9	1.7		
AUC^{+}	62.1	21.2		68.0	18.0		84.3	22.5		*0.0595*
$IAUC_{0\text{-}4hours}$*$^{+}$	8.1	5.3	-10.3	5.4	2.2	-12.5	12.9	8.0	-14.2	*0.1338*
$IAUC_{0\text{-}8hours}$*$^{+}$	-0.2	-2.0	-4.0	-3.4	-9.9	- -30	-5.1	-7.5	-6.1	*0.4281*
Cmin	5.0	1.2		5.4	1.6		5.7	1.8		*0.5681*
Cmax	12.1	4.1		13.6	4.1		16.8	3.8		*0.036*
Tmax*±	2	2	-2	2	2	-2	2	2	-2	*0.3679*
2-0 hours*	-2.3	1.7		-3.3	1.6		-4.8	2.2		*0.0182*
Insulin (pmol/l)										
0 hour	93.0	73.3	-118.1	90.5	42.8	-191.6	110.2	43.7	-277.6	*0.7951*
2 hours	289.1	177.7	-449.9	236.7	124.6	-530.7	281.8	149.6	-477.9	*0.7159*
4 hours	123.6	87.6	-174.4	128.7	62.6	-264.9	240.3	110.2	-523.9	
6 hours	81.8	59.0	-113.3	79.6	39.2	-161.9	150.0	56.6	-397.7	
8 hours	65.1	50.8	-83.4	91.3	53.6	-155.5	126.7	51.8	-310.3	
AUC^{+}	1220.0	953.8	-1560.5	1131.0	643.4	-1988.2	1632.8	785.1	-3395.9	*0.6925*
$IAUC_{0\text{-}4hours}$*$^{+}$	451.8	385.3	-539.5	291.1	193.6	-559.7	418.6	257.0	-778.1	*0.546*
$IAUC_{0\text{-}8hours}$*$^{+}$	361.1	215.5	-632.0	356.7	228.3	-509.4	708.4	372.9	-1090.4	*0.2586*
Cmax	291.3	181.4	-445.1	258.7	150.3	-543.2	294.8	159.9	-477.4	*0.8399*
Tmax*±	2	2	-2	2	2	-2	2	2	-2	*0.4518*
2-0 hours*	233.7	173.5	-252.5	138.2	93.2	-264.4	145.0	106.3	-257.5	*0.6538*
C peptide (nmol/l)										
0 hour	0.73	0.61	-0.88	0.84	0.49	-1.42	1.01	0.56	-1.84	*0.3008*
2 hours	2.11	1.57	-2.84	1.67	1.18	-2.35	1.98	1.38	-2.84	*0.282*
4 hours	1.28	0.90	-1.82	1.10	0.74	-1.61	1.75	1.00	-3.04	
6 hours	0.86	0.63	-1.17	0.72	0.42	-1.25	1.27	0.64	-2.54	
8 hours	0.70	0.50	-0.97	0.64	0.39	-1.05	1.14	0.60	-2.19	
AUC^{+}	10.10	7.97	-12.79	8.58	5.91	-12.46	12.43	7.65	-20.19	*0.1082*
$IAUC_{0\text{-}4hours}$*$^{+}$	3.11	2.71	-4.05	1.74	1.31	-2.25	2.24	2.01	-3.00	*0.0101*
$IAUC_{0\text{-}8hours}$*$^{+}$	4.07	2.79	-5.44	1.73	1.10	-2.01	4.08	2.64	-5.57	*0.0029*
Cmax	2.12	1.59	-2.83	1.68	1.18	-2.39	2.12	1.54	-2.94	*0.1936*
Tmax*±	2	2	-2	2	2	-2	3	3	-3	*0.1174*
2-0 hours*	1.28	1.22	-1.44	0.74	0.55	-0.95	0.73	0.60	-1.24	*0.0222*

Data are geometric mean (1 SD range) except * median (IQR). Units are as tabulated except + units.hour and ± hours

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