Fat metabolism and the metabolic syndrome

Dr Alex S. T. Bickerton

Linacre College

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Oxford Laboratory of Integrative Physiology
Oxford Centre for Diabetes, Endocrinology and Metabolism
Nuffield Department of Clinical Medicine
University of Oxford

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RESPONSIBILITIES

I was responsible for overall study design, collation and analysis of data from the Oxford Biobank, study day design and subject recruitment. On study days I performed all the cannulations and subsequently managed the rest of the day. I also administered the radioactive Xenon, took blood samples, performed plethysmography and indirect calorimetry. Following study days I collated the data, performed the statistical analyses and interpreted the data. In addition, for the first few studies I performed the lipid extractions and mass spectrometry.

The lipid infusions were prepared by Rachel Roberts. Other assistance on study days, including blood sampling and preparation of the test meal was provided variously by Rachel Roberts, Keith Frayn, Fredrik Karpe, Garry Tan, Louise Dennis, Jane Cheeseman, Beryl Barrow, Toralph Ruge and Siobhan McQuaid.

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ABSTRACT

Fat metabolism and the metabolic syndrome

Alex S. T. Bickerton, Linacre College, DPhil thesis, Hilary Term 2008

Background: The metabolic syndrome is associated with an increased risk of diabetes and vascular disease. In order to understand the pathophysiological processes underlying such risk, it is necessary to develop a better understanding of normal fat metabolism and abnormalities associated with the syndrome. The hypothesis tested in this thesis is that specific abnormalities in adipose tissue and muscle fat metabolism characterise the metabolic syndrome.

Methods: Fasting biochemical parameters were measured in a cohort of overweight men with and without the metabolic syndrome. Stable-isotope labeling and arterio-venous difference measurements were conducted in 18 men to elucidate pathways of exogenous and endogenous fat metabolism under fasting and postprandial conditions in adipose tissue and skeletal muscle. In addition, a pilot study of the effects of heat and electrical stimulation on adipose tissue metabolism was undertaken.

Results: **Cohort study** - The prevalence of the metabolic syndrome depended on the definition used. Total cholesterol and apoB were greater in those with the metabolic syndrome than in those without. There was no difference in fasting NEFAs.

Metabolic investigation - There was significant postprandial uptake of NEFA from the circulating NEFA pool by adipose tissue. Chylomicrons were confirmed as the preferred substrate of LPL. There was preferential uptake of FAs derived from chylomicron hydrolysis. There was release of NEFA across muscle. In the metabolic syndrome, adipose tissue NEFA output is lower during fasting and falls less following a meal than in the healthy obese. Clearance of dietary-derived TG is lower across both adipose tissue and muscle in the metabolic syndrome.

Pilot study – Heat increased measures of lipolysis whereas electrical stimulation had no effect.

Conclusions: Fat metabolism in individuals with the metabolic syndrome is characterised by metabolic inflexibility but not insulin resistance.

PUBLICATIONS ARISING FROM THIS THESIS

Publication of material included in this thesis

- Preferential uptake of dietary fatty acids in adipose tissue and muscle in the postprandial period
  Bickerton AST, Roberts R, Fielding BA, Hodson L, Blaak EE, Wagenmakers AJM, Gilbert M, Karpe F, Frayn KN.
  Diabetes 2007; 56:168-76

- Adipose tissue fatty acid metabolism in insulin-resistant men
  Bickerton AST, Roberts R, Fielding BA, Tornqvist H, Blaak EE, Wagenmakers AJM, Gilbert M, Humphreys SM, Frayn KN, Karpe F
  Diabetologia 2008; 51: 1466-74

Publications of material arising from work related to, but not included, in the thesis

- Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding.
  MF-F Chong, L Hodson, **AS Bickerton**, R Roberts, M Neville, F Karpe, KN Frayn, BA Fielding.
  American Journal of Clinical Nutrition 2008; 87: 817-823

- Reduced oxidation of dietary fat after a short term high-carbohydrate diet.
  R Roberts, **AS Bickerton**, BA Fielding, EE Blaak, AJ Wagenmakers, MF-F Chong, M Gilbert, F Karpe, KN Frayn
  American Journal of Clinical Nutrition 2008; 87: 824-831

- Removal of triacylglycerols from chylomicrons and VLDL by capillary beds: the basis of lipoprotein remnant formation.
  F Karpe, **AS Bickerton**, L Hodson, BA Fielding, GD Tan and KN Frayn
  Biochemical society transactions 2007; 35: 472-6.

- The contribution of splanchnic fat to VLDL triglyceride is greater in insulin-resistant than insulin-sensitive men and women: studies in the postprandial state.
  L Hodson, **AST Bickerton**, SE McQuaid, R Roberts, F Karpe, KN Frayn, BA Fielding.
  Diabetes 2007; 56: 2433-41

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  I Dahlman, M Kaaman, T Olsson, GD Tan, **AST Bickerton**, K Wåhlén, J Andersson, E Nordström, L Blomqvist, A Sjögren, M Forsgren, A Attersand, P Arner
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GLOSSARY

AUC, area under the curve

A-V difference, arteriovenous difference

AT, adipose tissue

ATBF, adipose tissue blood flow

apoB, apolipoprotein B

apoE, apolipoprotein E

ATGL, adipose triglyceride lipase

BMI, body mass index

EGIR, European group for the study of insulin resistance

FA, fatty acid

FABP, fatty acid binding protein

FAT/CD36, fatty acid translocase

FATP, fatty acid transport protein

HAS, human albumin solution

HDL, high density lipoprotein

HSL, hormone sensitive lipase

LDF, laser-doppler flowmetry

LDL, low density lipoprotein

LPL, lipoprotein lipase

M, muscle

MRI, magnetic resonance imaging

NEFA, non-esterified fatty acid

NMR, nuclear magnetic resonance

OBB, Oxford Biobank

PPAR, proliferator-activated receptor

SD, standard deviation

SEM, standard error of the mean

TG, triglyceride

TNF-α, tumour necrosis factor-α

TRL, triglyceride rich lipoprotein

V-A difference, venoarterial difference

VLDL, very low density lipoprotein

WHO, World Health Organisation

Chapter 1 : Introduction

The epidemic of obesity is now a global and seemingly unstoppable phenomenon. Data from the health survey for England revealed that in the UK in 2003 43% of men were overweight (BMI 25-30) whilst a further 22% were obese (BMI >30). These numbers are set to rise such that by 2010 around 6.6 million men will be obese as compared to 4.3 million in 2003. The figures for women in the UK are similarly bleak (1). Worldwide, the World Health Organisation (WHO) states that there are now over 1 billion overweight adults, of whom at least 300 million are obese (2).

In the wake of the obesity epidemic follow numerous co-morbidities. Of particular note is the close association between obesity and diabetes (3). Data from the Quality and Outcomes Framework, a primary care based data collection system, shows that the prevalence of diagnosed diabetes is 3.7% in the UK, equating to 1.96 million individuals. The WHO has predicted that the prevalence of diabetes worldwide will rise from 177 million in 2000 to 366 million in 2030, although this may be an underestimate in view of the rate of increase in worldwide obesity (4). Diabetes is not a benign condition. The risk of a first cardiovascular event in a man with diabetes is as high as that of a patient without diabetes who has already had a cardiovascular event (5). Furthermore, the risk of death due to cardiovascular causes is threefold higher in men with diabetes than in those without (6).

On the background of these startling figures, it is salutary to note that the pathophysiological changes underlying the metabolic consequences of weight gain remain poorly understood. The metabolic syndrome, a potent risk factor for both diabetes and vascular disease, may represent a state in which the disturbances of

metabolism that characterize these diseases are already occurring prior to disease manifestation. Debate rages as to whether the metabolic syndrome is, in fact, a useful concept in view of the lack of a unifying pathophysiology and issues of whether the sum of the components of the metabolic syndrome represent any greater risk than the parts (7-10). Identification of specific abnormalities in fat metabolism may provide both a pathophysiological basis for the metabolic syndrome and help to clarify which individuals are actually at risk of the metabolic and vascular complications. Furthermore, such information may assist in the assessment of which, if any, of the various current features used to identify the metabolic syndrome are of most significance. However, in order to understand pathophysiology, it is first necessary to understand normal physiology. In the field of fat metabolism, a number of crucial areas remain poorly understood. Consequently study of the healthy remains as valuable as study of those affected by metabolic disturbance.

1.1 The building blocks (fatty acids, triglycerides and lipoproteins)

Fatty acids (FAs) are long-chain hydrocarbons bound to a carboxyl group. The number of carbon atoms and double bonds within the molecule determine the nomenclature and the physicochemical properties of a FA. FAs circulate either as non-esterified fatty acids (NEFA) bound to albumin or as part of triglyceride (TG) molecules.

A TG molecule is made up of three fatty acids esterified to a glycerol backbone. TGs are highly hydrophobic and are thus carried to and from the tissues within lipoprotein particles.

A lipoprotein particle consists of a core of TG and cholesteryl esters and an outer monolayer of phospholipids, unesterified cholesterol and apolipoproteins. The latter are important proteins that function as structural elements, receptor ligands and regulatory cofactors. Lipoproteins are classified according to their density which reflects the relative proportion of their constituent elements. Thus, chylomicrons and very low density lipoprotein (VLDL) are triglyceride rich in proportion to their protein content and are known collectively as triglyceride rich lipoproteins (TRLs). Low density lipoprotein (LDL), derived from the metabolism of VLDL, is relatively triglyceride poor but cholesteryl ester enriched whilst high density lipoprotein (HDL) has a high protein to lipid ratio.

In addition to structural differences, the various lipoproteins also have different functions; the TRLs deliver TG to the tissues whereas LDL and HDL are responsible for cholesterol transport to and from the periphery respectively.

1.2 Overview of fat metabolism

Fat metabolism can be broadly split into the exogenous and endogenous pathways (Figure 1.1), describing the metabolism of dietary and hepatically derived fat respectively. The two pathways are intimately related. Dietary fat exists largely in the form of TG. TG is hydrolysed in the small intestine to produce FAs and monoglycerides. The FAs and monoglycerides are absorbed across the intestinal epithelium and re-packaged into chylomicrons. These pass directly into the systemic circulation. When chylomicrons reach the peripheral tissues they are partially hydrolysed by the enzyme lipoprotein lipase (LPL). The fate of the resultant FAs and chylomicron remnants varies from tissue to tissue and is discussed in more detail

below. Chylomicrons that pass directly to the liver may be taken up and hydrolysed. The resultant FAs may be stored, oxidised or incorporated into new TRL. The TRLs synthesized in the liver are VLDL. VLDL passes from the liver to the peripheral tissues where it is treated entirely analogously to chylomicrons. VLDL remnants, generated by partial hydrolysis, may either be taken up directly by tissues or undergo more processing within plasma to become LDL.

FAs are a critical metabolic fuel. In health, the concentration of NEFAs in systemic blood varies dependent on the prevailing metabolic conditions. During fasting and exercise NEFA turnover is high since, under these conditions, NEFAs constitute the main source of energy for most metabolic processes. In contrast, NEFA concentrations fall post-prandially, reflecting the predominance of carbohydrate as a fuel source and the need to store dietary derived fat. The systemic concentration of NEFA is dependent on the metabolic processes occurring in many tissues. The metabolism of fat, and hence the flux of NEFA, is highly regulated in each tissue, although the regulatory mechanisms vary and are not yet completely understood.

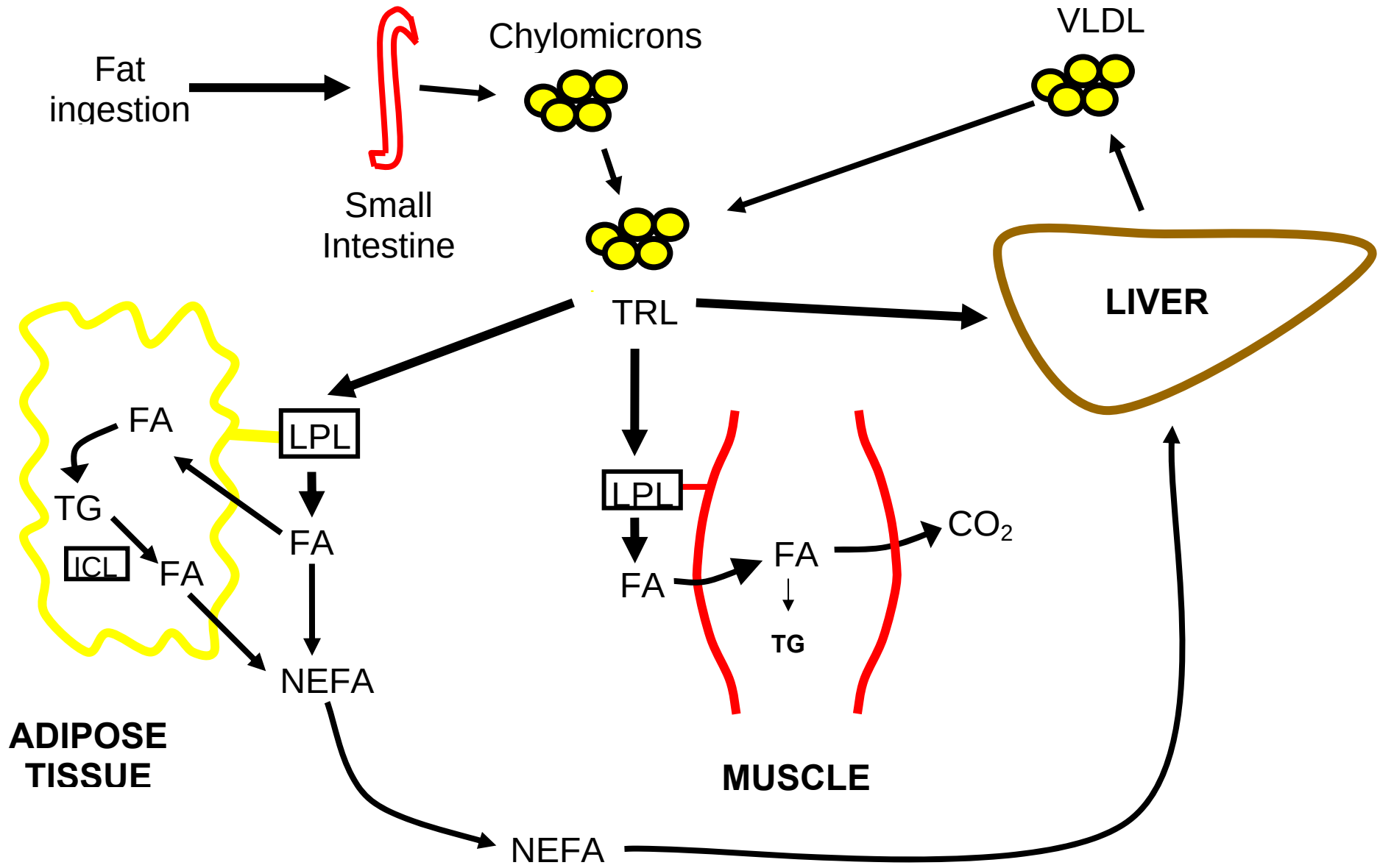

Figure 1.1 Simplified summary of the endogenous and exogenous pathways of fat metabolism (FA, fatty acid: TG, triglyceride: TRL, triglyceride rich lipoprotein: VLDL, very low density lipoprotein: LPL, lipoprotein lipase: ICL, intracellular lipases).

1.2.1 Adipose tissue

Contrary to previous beliefs, adipose tissue is not simply a storage organ for excess calories, but a highly dynamic tissue with a multiplicity of metabolic roles. In addition to being involved in whole body fatty acid balance, adipose tissue also acts as an endocrine organ, secreting a variety of hormones, and an inflammatory mediator, releasing a number of cytokines.

Fatty acid metabolism within adipose tissue is a highly dynamic and carefully regulated process, although, as yet, incompletely understood. In order to illustrate the processes occurring and the shortfall in current knowledge, it is instructive to follow the fate of fatty acids within adipose tissue.

1.2.1.1 TG hydrolysis

As described, NEFAs are largely made available to the adipocyte following hydrolysis of TRL by LPL at the endothelial surface. LPL activity is modulated by insulin in a tissue specific manner. Within adipose tissue, insulin increases LPL activity by mechanisms that are complex and incompletely understood. However, both gene expression and intra-cellular transport are known to be stimulated by insulin (11). As a consequence of the mechanisms by which insulin increases LPL, there is a lag of hours between any rise in insulin concentration and maximal LPL activity. This is entirely appropriate since there is a similar delay between fat ingestion and peak plasma chylomicron concentration. Thus, for several hours following a meal, in response to an acute rise in insulin concentrations, FA availability from hydrolysis of circulating TG is markedly increased. The postprandial

availability of NEFA to adipose tissue is further augmented by the preference of LPL for chylomicron-TG over VLDL-TG (12-14). The fatty acids derived from TG hydrolysis may either be taken up by adipose tissue or, alternatively, pass out of adipose tissue into the systemic circulation (15). The control of this "spill-over" into the systemic circulation is a field of intense research activity. In addition to the NEFAs derived from TG hydrolysis, the adipocyte is also continuously presented with circulating NEFA, largely bound to albumin. Whether these NEFAs contribute in a quantitatively significant way to the NEFA fluxes occurring across adipose tissue under normal physiological conditions is unclear.

1.2.1.2 Fatty acid uptake and re-esterification

On arrival at the adipocyte cell membrane, FAs may be taken into the cell by one of two processes: passive flip-flop/ diffusion (16) or protein-mediated facilitated uptake (17). The relative contribution of each of these mechanisms remains controversial (16, 18). Flip-flop diffusion is dependent on the concentration gradient between the adipocyte cytosol and the capillary lumen. This gradient is largely determined by the balance between intracellular and intravascular TG hydrolysis. In the postprandial state, there is a net fatty acid uptake by adipose tissue as intravascular TG hydrolysis is high whilst intracellular TG hydrolysis is suppressed. During fasting, the situation is reversed, leading to a net fatty acid output.

The proteins involved in the protein-mediated fatty acid uptake process in adipocytes are fatty acid transport protein 1 (FATP1), fatty acid transport protein 4 (FATP4), fatty acid translocase (FAT/CD36) and plasma membrane fatty acid binding protein (FABPpm). The FATPs are a family of closely related membrane bound

proteins expressed in a variety of tissues (muscle, adipose tissue, heart, brain, liver, kidney). Insulin, tumour necrosis factor-α (TNF-α), adiponectin and ligands that activate peroxisome proliferator-activated receptor-γ (PPAR-γ), PPAR-α and PPAR-γ/retinoid X receptor heterodimers have all been demonstrated to have modulatory roles on the expression and actions of the FATP family (19). Regulation is tissue and modulator specific. In adipose tissue, TNF-α downregulates FATP1 expression whilst insulin increases translocation to the cell membrane (20). FATP4 is less insulin modulated and is thus postulated to mediate basal fatty acid uptake (20). FAT/CD36 is another widely expressed membrane bound protein that has been implicated in fatty acid uptake (21). FAT/CD36 expression is upregulated by fatty acids in adipose tissue (22). The precise interactions and cross talk between the various fatty acid transporters remain to be clarified. It is possible that the FATP family represent the proteins that actually carry fatty acids across cell membrane, whilst FAT/CD36 and FABPpm are responsible for accumulating fatty acids at the plasma membrane ready for transportation (17, 23). Once within the adipocyte, FAs are bound to fatty acid binding proteins (FABPs). aP2 is the principal FABP in adipose tissue and is one of a large family of ubiquitously expressed cytosolic proteins that bind fatty acids with high affinity. FABPs have a number of roles within the cell, including shuttling fatty acids between various cellular compartments, regulating intracellular lipid metabolism, expression of inflammatory cytokines and regulation of gene expression (24). On reaching the endoplasmic reticulum (ER) fatty acids are rapidly re-esterified via the phosphatidic acid pathway to form TG which is stored within a lipid droplet.

1.2.1.3 Lipolysis

Once esterified, fatty acids may remain stored within adipose tissue, or, following lipolysis, may be made available for export back out into the circulation.

Hormone sensitive lipase (HSL) has long been seen as the main protein responsible for intracellular lipolysis. However, the observation that HSL null mice exhibited a normal phenotype (25) prompted a search for further intracellular lipases. The result is the recently described adipose triglyceride lipase (ATGL) (26). Although work continues in elucidating the exact function and control of this enzyme, it is known that ATGL hydrolyses triglycerides to diglycerides (26) and is insulin modulated (27).

HSL is believed to perform the next step of the lipolytic process, resulting in the generation of monoglycerides. Monoglyceride lipase, which is not hormonally controlled, then hydrolyses monoacylglycerols to NEFAs and glycerol (28). Modulators of HSL action include insulin (29), nicotinic acid (30), catecholamines (29, 31) and atrial natriuretic peptide (ANP) (32). In order to catalyse lipolysis, HSL requires activation by phosphorylation. There are two pathways of HSL phosphorylation. The first has long been described and depends on cAMP to activate protein kinase A (PKA) which, in turn phosphorylates HSL (31). cAMP concentrations are increased by catecholamines and reduced by nicotinic acid, both of which act via G-protein-coupled receptors. cAMP is also reduced by insulin acting via phosphodiesterase-3B (29, 30). The second, more novel pathway occurs via the cGMP mediated activation of protein kinase G (PKG), with the subsequent phosphorylation of HSL. The stimulus for this latter pathway is ANP, acting via the natriuretic peptide receptor A (32-34).

In order to catalyse lipolysis, HSL must have access to lipid droplets within adipocytes. Perilipins are a family of hydrophobic phosphoproteins that coat lipid droplets and thereby act as a constitutive brake on lipolysis. Phosphorylation of

perilipins by PKA results in conformational change allowing HSL access to the lipid droplet (35). In addition, perilipins may be involved in the translocation of HSL to the lipid doplet (36).

1.2.1.4 Adipose tissue blood flow

As with all aspects of adipose tissue function, adipose tissue blood flow (ATBF) is not a static phenomenon but a dynamic process that varies dependent on a range of physiological stimuli and modulators. In addition, the response of adipose tissue to changing metabolic conditions shows marked inter-individual heterogeneity (37). ATBF is increased by mental stress (38) and exercise (39, 40). However, it is the response to food ingestion that has received most attention. Pure glucose loads (41, 42) and mixed meals (43, 44) both precipitate a rise in ATBF whereas a pure fat load causes no change (45). Thus, there is an intimate relationship between ATBF and nutrient metabolism. This is most pertinently illustrated by the observation that adrenaline infusion increases not only HSL mediated intracellular TG lipolysis, but also ATBF and LPL mediated intravascular TG lipolysis (46). It is likely that the rise in ATBF results in increased substrate availability for LPL. Therefore, in order to maintain physiologically appropriate circulating TG concentrations, ATBF must be highly regulated.

The control of ATBF has received considerable scrutiny. An oral glucose load provokes a far greater response in ATBF than an equivalent glucose and insulin infusion (37). Thus, attention has focused on other stimuli related to food intake. The sympathetic nervous system is intrinsic to adipose tissue function, indeed the effect of catecholamines on HSL has already been alluded to. Studies examining the effects of

β- and α-adrenergic stimulation have shown increased (47-49) and reduced (50) ATBF respectively. These findings, in concert with the observations surrounding the effect of glucose ingestion, provoked the hypothesis that the ATBF response to a meal is a consequence of insulin induced postprandial activation of the sympathetic nervous system. This hypothesis was explored using the combination of a glucose challenge and a "micro-infusion" technique to study the local effect of β- and α-blockade on ATBF (51). In addition, as nitric oxide (NO) is a well recognized global regulator of blood flow, the effect of NO synthase inhibition was simultaneously investigated. This study revealed that NO is the main modulator of resting adipose tissue vascular tone, with some contribution from the α-adrenergic system, whereas the postprandial response is largely under β-adrenergic regulation. β-blockade exerts the same effect on ATBF whether delivered locally, by microinfusion (51), or systemically, by intravenous infusion (52). Thus, the β-adrenergic influence is unlikely to be humoral in origin. This is supported by evidence that patients with high spinal cord injury, a model of sympathetic denervation, show an attenuated lipolytic response under conditions of sympathoexcitation such as mental stress (53). In humans, the route of sympathetic innervation of white adipose tissue remains poorly characterized and thus studies aimed at specifically stimulating the adipose tissue sympathetic supply are not currently feasible. An alternative model that has been developed is intraneural stimulation of a cutaneous nerve. Although mainly sensory in function, there is evidence of sympathetic activity in cutaneous nerves (54). Intraneural electrical stimulation of the lateral femoral cutaneous nerve, which supplies the skin and underlying sub-cutaneous adipose tissue of the anterolateral aspect of the thigh, increased lipolysis (55, 56), however no measurements of ATBF were made. There have been no studies attempting such stimulation of abdominal cutaneous nerves.

1.2.1.5 Hormones and cytokines

Over the last ten years the roles of adipose tissue in inflammation and hormonal signaling have received increasing attention. Adipose tissue is an important source of hormones, termed "adipokines", with leptin and adiponectin being the most well characterized in man, and inflammatory mediators including tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6). As this is not a major focus of the present thesis, I will provide a only brief summary of these factors.

1.2.1.5.1 Leptin

Leptin was the first of the adipokines to be described. Leptin is a 16-kD protein secreted mainly from adipocytes (57). It circulates either free or bound to the extracellular part of the leptin receptor. Leptin is able to cross the blood brain-barrier and act on the arcuate nucleus of the hypothalamus, an area that is key to determining nutrient intake (58). Mutation of the leptin gene was first described in the *ob/ob* mouse (59). Subsequently, administration of the gene product, leptin, to the mice resulted in significant weight reduction (57). These observations initially gave rise to the concept that a similar deficiency in man could precipitate obesity. However, although leptin deficiency *is* associated with an obese phenotype (60), the circulating leptin concentrations in most obese individuals are high rather than low (61). Thus, in the majority, obesity appears to be a state of leptin resistance rather than leptin deficiency. During fasting, leptin concentrations fall rapidly (61). The fall in leptin is associated with the characteristic counterregulatory hormonal response to fasting (62, 63). Therefore, the physiological role of leptin in man is likely to be as a starvation signal rather than an anti-obesity hormone.

1.2.1.5.2 Adiponectin

Adiponectin is an adipokine exclusively expressed in adipocytes (64) and abundantly present in human plasma (65). Adiponectin circulates as a trimer, a hexamer or as a high molecular weight complex of up to six trimers (66, 67). Two adiponectin receptors have been described; adipoR1, widely expressed with high levels in skeletal muscle, and adipoR2, abundantly expressed in liver (68). Both are expressed in adipose tissue (69). Adiponectin has effects on both glucose and fat metabolism. Hepatic glucose production is reduced by adiponectin (70) whilst fatty acid oxidation in both the liver and skeletal muscle is increased (71). Interestingly, although derived from adipocytes, adiponectin concentrations are lower in obesity (65), type 2 diabetes (72) and in some individuals with the metabolic syndrome (73).Conversely, adiponectin concentrations increase following weight loss (72, 74).

1.2.1.5.3 TNFα and IL-6

TNFα and IL-6 are pro-inflammatory cytokines produced in adipose tissue. *In vitro* studies have demonstrated elevated TNFα expression in the adipose tissue of obese individuals (75, 76) with a subsequent fall in expression following weight loss (75). However, this TNFα does not seem to be released into the systemic circulation (77) and thus the pathophysiological significance of this facet of adipose tissue function remains unclear. IL-6 production in adipose tissue is also increased in obesity and falls with weight loss (78). In contrast to TNFα, there is systemic release of IL-6 (77), indeed the plasma concentrations reflect the changes occurring in adipose tissue (78). The molecular metabolic consequences of elevated IL-6 concentrations in man are not fully elucidated. However, *in vitro* study of hepatocyte cell lines reveals

inhibition of insulin signalling by IL-6 (79) and thus IL-6 may play a role in insulin resistance.

1.2.2 Skeletal muscle

1.2.2.1 Intravascular TG lipolysis

Skeletal muscle uses FAs as a metabolic fuel. As for all tissues, FAs are presented either as NEFA bound to albumin or as TG contained within TRL. However, skeletal muscle cannot take up whole TG molecules. Thus, LPL is expressed at the capillary endothelial surface in order to hydrolyse the TG within TRL. In contrast to the situation in adipose tissue, LPL is decreased by insulin in skeletal muscle. Thus LPL activity and mass is high during fasting (80) and low during insulin stimulation (81). As in adipose tissue, the FAs generated by TG hydrolysis may either be taken up into skeletal muscle or spill over into the systemic circulation. Such spillover has been clearly demonstrated in human subjects in vivo (82, 83), however the quantitative and physiological significance of this is unclear.

1.2.2.2 FA uptake and oxidation

As in adipose tissue, there are two main mechanisms of FA uptake in skeletal muscle; passive diffusion down a concentration gradient and protein mediated active transport. FA transporters in skeletal muscle are FAT/CD36 and FATP1 (19, 21). The factors determining the expression and activity of these transport proteins is an area of ongoing research activity. Translocation of both FAT/CD36 and FATP1 to the plasma membrane is increased by insulin (84, 85), whilst a similar effect is seen in response to muscle contraction only in FAT/CD36 (86).

The concentration gradient across the myocyte cell membrane depends in part on the rate of intracellular FA oxidation. The complex, yet elegant, relationship between the uptake and oxidation of both FAs and glucose in skeletal muscle has been an area of research interest for over forty years. Randle and colleagues first described the "glucose-fatty acid cycle" in 1963 (87) and, although there has been much progress in elucidating the precise molecular mechanism, the basic principles of the cycle remain valid. Under fasting conditions, when insulin concentrations are low and FA concentrations high, there is preferential utilization of FA as the major metabolic fuel in muscle. Conversely, following a meal, rising glucose concentrations stimulate insulin secretion which, in turn, suppresses lipolysis and thus circulating FA concentrations, resulting in increased utilization of glucose as a metabolic fuel. A number of studies have shown that elevated FA supply to muscle increases FA oxidation and reduces glucose oxidation and uptake (88-90). Subsequent authors demonstrated that the reverse is true, in that elevated circulating glucose concentrations reduced the FA induced suppression of glucose oxidation (91). Thus, skeletal muscle substrate oxidation is a flexible process dependent on the prevailing metabolic conditions.

The majority of FAs taken up by skeletal muscle are oxidized, however there is also the capability within the myocyte to re-esterify FAs to TG for storage. In healthy individuals, only a very small proportion of FAs are stored as TG, however increased muscle TG has been described in athletes (92, 93), obesity (94) and those with diabetes (92, 95). Strenuous exercise stimulates muscle TG lipolysis (96, 97) and thus it is likely that the elevated muscle TG acts as a fuel depot for the highly trained

athlete. The implications of elevated muscle TG in the context of insulin resistance and diabetes are discussed below

1.2.3 Liver

Lipid metabolism within the liver is extremely complex and a full discussion of all the biochemical pathways involved is beyond the scope of this thesis. Thus, only a brief synopsis pertinent to the studies hereafter described will be presented.

1.2.3.1 Lipid uptake and TG processing

The liver is able to take up both whole TRL particles and NEFA. The apolipoproteins of chylomicron remnants and VLDL act as ligands for the LDL-receptor related protein (LRP) and the LDL receptor respectively in order to facilitate the hepatic uptake of these TRLs. TGs entering the hepatocyte may either be hydrolysed to the constituent FAs or stored. As in other tissues, hepatic NEFA uptake can occur by either simple diffusion or a carrier mediated process via FATP5 (98). The latter appears to be of quantitatively greater significance than the former (99, 100).

1.2.3.2 FA metabolism

FAs within the liver are either oxidized to fuel other metabolic processes or esterified and stored as TG. In addition, the liver is able to synthesise FAs from glucose and amino acids. The mitochondrial β-oxidation pathway is the principal means by which FAs are oxidized, resulting in the production of ketone bodies (acetoacetate and 3-hydroxybutyrate) and CO_2. The principal regulation of FA

oxidation is at the level of the transfer of the FA metabolite fatty acyl-CoA across the mitochondrial membrane. The latter process is facilitated by the enzyme carnitine-palmitoyl transferase-1 (CPT-1). This enzyme is, in turn, inhibited by malonyl-CoA, a product of the metabolism of glucose. Hepatic FA esterification occurs via the phosphatidic acid pathway. It is important to note that insulin stimulates both the production of malonyl-CoA and the esterification of FAs. Therefore, in health, a rise in insulin concentration reduces FA oxidation via a fall in intracellular FA availability and reduced transfer of fatty acyl-CoA to the mitochondria.

1.2.3.3 VLDL production and secretion

VLDL is produced in the hepatocyte by a two stage process involving three different particles (101, 102). The first stage, occurring in the rough endoplasmic reticulum, is the synthesis and subsequent lipidation of apoB100 to form pre-VLDL. This process is catalysed by microsomal triglyceride transfer protein (MTP). The pre-VLDL is then either degraded or passes to a smooth membrane compartment of the ER, where further lipid components are added to produce VLDL2. MTP may also be involved in this further lipidation. VLDL2 is transferred to the Golgi apparatus where further addition of TG results in the production of the mature VLDL1 particle. The main determinants of VLDL production are the supply of FA to the hepatocyte, which may be derived either from the circulation (NEFA or TG) or from intra-hepatic FA synthesis (*de novo* lipogenesis) and the local insulin concentration. The effect of FA supply is reasonably straightforward in that the more FAs available for incorporation into VLDL, the greater the production of VLDL (103). The physiological effect of insulin on hepatic TG production is more complex, however acute hyperinsulinaemia undoubtedly suppresses VLDL production (103-105). This regulation is believed to

occur both via direct effects of insulin on VLDL synthesis (106) and also via the modulation of circulating NEFA (103).

1.3 The metabolic syndrome

1.3.1 Overview

The association of diabetes and hypertension was first noted by Hitzenberger and Richter-Quittner in the early part of the twentieth century (107, 108). This relationship was then further developed by Maraňon and Kylin in the 1920s (109, 110), with the addition of hyperuricaemia by Kylin in 1923 (111). Subsequent to this initial observation, interest in the association of these metabolic abnormalities grew. However, it was not until 1988, when Reaven crystallized these biochemical and biophysical abnormalities into a clinical syndrome (112), that the significance of this association was appreciated. Reaven's original description included insulin resistance, hypertriglyceridaemia (elevated VLDL), hypertension and reduced high density lipoprotein cholesterol (HDL-cholesterol). It is of interest to note that obesity was not a feature. Over the years, the condition has come to be known by a variety of terms including the metabolic syndrome (113-116), plurimetabolic syndrome (117), cardiometabolic syndrome (118), insulin resistance syndrome (119, 120), syndrome "X" (112) and the deadly quartet (121). As interest has grown in the metabolic syndrome, so have the number of associated features. Most definitions now include obesity, however microalbuminuria, inflammation and abnormalities of haemostasis, uric acid metabolism, endothelial, haemodynamic and reproductive function are all recognised associations of insulin resistance and thus have been linked to the metabolic syndrome (122). In an attempt to simplify diagnosis and enable

comparative clinical research, the WHO proposed the first internationally recognized criteria for the identification of the metabolic syndrome in 1999 (116). It is important to remember that the original WHO criteria were put forward as a "working definition to be improved upon in due course". Subsequently the National Cholesterol Education Panel (NCEP-ATP III) (113, 123), the European Group for the Study of Insulin Resistance (EGIR) (114), the American Association of Clinical Endocrinologists (AACE) (122) and, most recently, the International Diabetes Federation (IDF) (115) have all published slightly differing diagnostic criteria (Table 1.1). The WHO, EGIR and AACE definitions all include some form of assessment of insulin resistance. However, the EGIR and AACE use the term "insulin resistance syndrome" rather than "metabolic syndrome" and specifically exclude individuals with type 2 diabetes. The NCEP-ATP III and IDF definitions are designed to be easier to apply in both a clinical setting and for population screening. They do not include any proxy measures of insulin sensitivity, as these are laborious to perform and do not always correlate with the euglycaemic hyperinsulinaemic clamp; often perceived as the gold standard. The fact that there is still no single internationally recognized definition of the metabolic syndrome reflects the diversity of opinion as to the purpose of defining the metabolic syndrome and whether there is a single principal underlying metabolic abnormality. Indeed, the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) have published provocative joint statements questioning the existence, definition and utility of the metabolic syndrome as a diagnostic entity (9, 10). These questions have sparked a lively, but as yet unresolved, debate.

Table 1.1 Definitions of the metabolic syndrome.

Clinical Criterion	WHO (1998)	EGIR (1999)	NCEP-ATP III (2005, modified from 2001)	AACE (2003)	IDF (2005)
Insulin Resistance	IFG, IGT, T2DM, Clamp* **Plus ≥ 2 of the following**	Fasting plasma insulin ≥ 75th percentile **Plus ≥2 of the following**	None **≥3 of the following**	IFG, IGT	None
Adiposity	Men: WHR>0.9 Women : WHR >0.85 AND / OR BMI>30kg/m^2	Men: WC ≥ 94cm Women: WC ≥ 80cm	Men: WC ≥ 102cm Women: WC ≥ 88cm		Population based increased WC **Plus ≥2 of the following**
Triglyceride	≥ 1.7mmol/l	>2.0mmol/l OR on drug treatment	≥ 1.7mmol/l OR on drug treatment	≥ 1.7mmol/l	≥ 1.7mmol/l OR on drug treatment
High Density Lipoprotein	Men < 0.9mmol/l Women < 1.0mmol/l	< 1.0mmol/l OR on drug treatment	Men < 1.03mmol/l Women < 1.3mmol/l OR on drug treatment	Men < 1.03mmol/l Women < 1.3mmol/l	Men < 1.03mmol/l Women < 1.29mmol/l OR on drug treatment
Blood Pressure	≥ 140/90mmHg	≥140/90 OR on drug treatment	Systolic ≥ 130mmHg OR Diastolic ≥ 85mmHg OR On drug treatment	Systolic ≥ 130mmHg OR Diastolic ≥ 85mmHg	Systolic ≥ 130mmHg OR Diastolic ≥ 85mmHg OR On drug treatment
Glucose	IGT, IFG, T2DM	≥ 6.1mmol/l (diabetes excluded)	≥ 5.6mmol/l OR on drug treatment	IFG, IGT	≥ 5.6mmol/l OR T2DM
Other	Microalbuminuria**			Other features of insulin resistance***	

T2DM, type 2 diabetes: BMI, body mass index: WHR, waist-to-hip ratio: WC, waist circumference: IFG, impaired fasting glucose: IGT, impaired glucose tolerance

* hyperinsulinaemic euglycaemic clamp; lowest quartile of glucose uptake for background population

** urinary albumin excretion rate ≥ 20μg/min or albumin creatinine ratio ≥30mg/g

***family history of type 2 diabetes, polycystic ovary syndrome, sedentary lifestyle, advancing age, ethnic groups susceptible to type 2 diabetes

1.3.2 Prevalence of the metabolic syndrome

The prevalence of the metabolic syndrome varies depending on the population studied and the diagnostic criteria used. Data from the Third National Health and Nutrition Examination Survey (NHANES III), gathered between 1988 and 1994, showed approximately 24% of US adults, equating to 47 million US residents, to have the metabolic syndrome (124). However, the prevalence of the metabolic syndrome is markedly dependent on age and ethnicity, with an additional effect of sex in selected groups. The prevalence in the NHANES III dataset increased from 7% in participants aged 20-29 to 44% in those aged 60-69. Mexican Americans demonstrated the highest age-adjusted prevalence at 32%. In this group women had a 26% higher prevalence than men. Data from the UK, gathered over a similar time period, shows the prevalence of the metabolic syndrome in European men, aged 40-69 years, to be 19%, whereas in South Asian and African-Caribbean men of the same age, the prevalence is 29% and 16% respectively (125). Numerous other populations have been studied although between study comparisons can be difficult as investigators frequently modify the recognized definitions dependent on the available information. In comparison to the UK, the prevalence of the metabolic syndrome is similar in Greece and Italy (overall prevalence 23.6% and 17.8% respectively) (126, 127), lower in Finland and China (8.8% and 13.7% respectively) (128, 129) and far higher amongst Japanese Brazilians (47.4%) (130). The effect of age is universal, however the effect of sex is population dependent

Thus far, only the prevalences produced by using the NCEP definition of the metabolic syndrome have been discussed. However, the choice of diagnostic criteria can

also have a marked effect on the observed prevalence. In the aforementioned UK study, striking differences in the prevalence of the metabolic syndrome are seen amongst European women and African-Caribbean men dependent on the definition applied. In European women the prevalence is 14.4% by NCEP criteria compared to the 9.1% using the WHO definition. In contrast, the NCEP criteria find a lower prevalence of metabolic syndrome in the African-Caribbean men than do the WHO criteria (15.5% and 26.5% respectively) (125). Similarly clear differences in prevalence, dependent solely on definition of the syndrome, have been demonstrated in other studies (128, 131-134). It is important to note that the data from the US and UK studies is 10 years old. As already described, the worldwide prevalence of obesity is on the increase and thus the prevalence of metabolic syndrome described above undoubtedly represents an underestimation.

The metabolic syndrome has always been considered a disease of adulthood. However, the rising tide of obesity and consequent metabolic disturbance has not spared the paediatric population. Sixteen percent of US and 22-28% of UK adolescents are overweight or obese. Although the metabolic syndrome is generally uncommon in young people, Weiss *et al* found a prevalence of 50% amongst severely obese adolescents (135). Thus, the metabolic syndrome represents both a current and future threat to health.

1.3.3 Insulin resistance

Since many authorities view insulin resistance as intrinsic to the metabolic syndrome, it is instructive to consider what exactly is meant by the term. The concept of insulin resistance was introduced in 1936 by Himsworth (136) who first described variations in the response of patients with diabetes to a combination of insulin and

carbohydrate. He observed that in some individuals insulin had little or no effect on suppressing hyperglycaemia and put forward the idea of insulin resistance rather than deficiency as the underlying metabolic disturbance. The idea was subsequently developed by Yalow and Berson in 1968 who demonstrated elevated insulin concentrations in those with type 2 diabetes and produced a definition of insulin resistance as "a state (of a cell, tissue, system or body) in which greater than normal amounts of insulin are required to elicit a quantitatively normal response" (137). This definition is still very useful and emphasises that the concept of insulin resistance can potentially apply to any tissue or any substrate on which insulin has an effect.

Numerous methodologies have been described for the assessment of insulin resistance, reflecting the fact that no single technique is ideal under all circumstances. The decision as to which method to employ must take into account the biological question being asked and the population under scrutiny. In addition, when interpreting the results, it is extremely important to consider the circumstances in which the measurement is made. Very different results may be produced by employing the same method to assess insulin resistance during fasting, following a meal or in response to a supraphysiological insulin infusion. Most techniques concentrate on the relationship between insulin and glucose, thereby producing measurements of the effectiveness of insulin action on muscle glucose uptake, hepatic glucose metabolism, whole body glucose metabolism or a combination of all three.

The hyperinsulinaemic euglycaemic clamp is considered by many to be the "gold standard" technique. A variable rate intravenous infusion of glucose is titrated against a fixed rate infusion of insulin to maintain ("clamp") plasma glucose at a pre-determined level. The degree of insulin resistance is calculated from the amount of glucose required to maintain the required plasma glucose concentration once a steady state has been reached (138). The addition of tracers allows statements to be made regarding hepatic glucose metabolism (139). The hyperinsulinaemic euglycaemic clamp is laborious and time consuming. In response, a plethora of simpler techniques such as continuous infusion of glucose with model assessment (140), the frequently sampled intravenous glucose tolerance test with minimal modeling (141) and the insulin sensitivity test (142) have been developed as tools for assessing insulin sensitivity. All of these methods correlate reasonably well with the hyperinsulinaemic euglycaemic clamp (143), but all are still somewhat invasive and time consuming. An alternative approach is the use of mathematical models which require the results of, at most, three blood samples. The most widely adopted is the Homeostasis Model Assessment (HOMA) (144). HOMA can be applied in epidemiological studies using the results of single basal blood samples and correlates well with results from hyperinsulinaemic euglycaemic clamp assessment of insulin resistance (145).

Measurement of the effectiveness of insulin action in relation to other metabolic processes is much less common. Low dose insulin infusions (146) and oral glucose tolerance testing (147-149) have been used to compare the lipolytic response in individuals of varying insulin sensitivity defined in terms of glucose metabolism. The

latter technique is relatively easy to perform and can be applied to relatively large cohorts (148).

1.3.4 Complications of the metabolic syndrome

Clearly, the metabolic syndrome *per se* does not cause any significant mortality or morbidity. However, the pathological significance of the syndrome lies in the attendant risks of vascular disease and type 2 diabetes.

A number of large prospective and retrospective studies have attempted to quantify the excess vascular risk. In the Botnia study, the risk of coronary artery disease and stroke was increased threefold in those with the metabolic syndrome (150). Although this provides some interesting insights into vascular risk, it must be noted that the group defined as having the metabolic syndrome contained a significant number with diabetes. Analysis of the placebo arms of the Scandinavian Simvastatin Survival Study and the Air Force / Texas Coronary Atherosclerosis Prevention Study reveals hazard ratios for the occurrence of coronary disease in subjects with the metabolic syndrome but no diabetes of 1.6 and 1.5 respectively when compared to healthy individuals (151). A very similar risk (hazard ratio 1.8) was seen in post-hoc analysis of the West of Scotland Coronary Prevention Study, a primary prevention statin trial (152).

An increased risk of cardiovascular disease is of particular concern if there is an associated increased risk of cardiovascular mortality. The DECODE study group have looked at 11 prospective European cohort studies involving both men and women. In

non-diabetic subjects with the metabolic syndrome the hazard ratio for cardiovascular mortality, compared with healthy individuals, was 2.26 for men and 2.78 for women. Interestingly, the all cause mortality hazard ratios in the same groups were 1.44 and 1.38 respectively (153). Similarly, analysis of the results of the Kuopio Ischaemic Heart Disease Risk Factor Study revealed risk ratios of 3.4 and 2.08 for coronary heart disease mortality and cardiovascular mortality respectively, in those with the metabolic syndrome compared to those without (128).

Assessment of the risk of vascular disease is dependent on the criteria used to identify the metabolic syndrome in an exactly analogous way to the determination of prevalence. Hunt *et al* compared modified WHO and NCEP criteria in relation to cardiovascular mortality in the San Antonio Heart Study (SAHS). The hazard ratio for cardiovascular mortality, as compared to healthy subjects, in the NCEP identified metabolic syndrome population without diabetes was 2.01. This contrasts starkly with a non-significant ratio of 0.74 in the WHO defined population (133). Similar differences in risk attributable solely to diagnostic criteria were found in the UK population described above. In addition, ethnicity played a clear role in determining coronary risk. The odds ratio for coronary heart disease in South Asian men with the metabolic syndrome was 2.11, as compared to a ratio of 1.6 in European men. When the data were re-analysed excluding those with diabetes from the metabolic syndrome groups, the odds ratio for South Asian men barely changed, whereas the ratio for European men became insignificant (125).

The risk of developing diabetes is even greater than the risk of vascular disease in those with the metabolic syndrome. Factor analysis from a study of Pima Indians identified components of the metabolic syndrome as having a very strong association with the incidence of diabetes (154). In the Kuopio study, the odds ratios for the development of diabetes were 5 – 9, depending on the criteria used to define the metabolic syndrome (131). Similar analysis of the SAHS cohort revealed an odds ratio of 6 (155).

Therefore, overall the metabolic syndrome is associated with a one and a half to threefold increased relative risk of vascular disease, a two to threefold increased relative risk of vascular mortality and a five to ninefold increased likelihood of diabetes. However, it is crucial to remember that the exact risk depends on the definition of the metabolic syndrome used and the population studied.

A large element of the current controversy surrounding the metabolic syndrome concerns whether the increased vascular and metabolic risks of the metabolic syndrome reflect simply an accumulation of risk factors or whether, in fact, the risk conferred by the syndrome as a whole is greater than the cumulative risk of the components. The associated debate questions whether the metabolic syndrome is a reflection of a single pathological process or simply a coincidence of several metabolic disturbances (10). One widely held belief is that insulin resistance represents the unifying underlying pathological process resulting in both vascular and metabolic abnormalities (112). An alternative hypothesis is that the disturbances of lipid metabolism seen in the metabolic

syndrome result in a variety of secondary pathological processes which ultimately lead to vascular and metabolic disease.

1.3.5 The atherogenic dyslipidaemia of the metabolic syndrome

Mention has already been made that the metabolic syndrome is associated with disturbances in lipid parameters. The characteristic pattern is of high TG, low HDL-cholesterol but relatively normal LDL-cholesterol (156, 157). LDL is rich in cholesterol and classically associated with vascular disease. Thus, it is not immediately intuitive to blame the vascular complications of the metabolic syndrome on the associated dyslipidaemia. However, the pattern of high TG and low HDL-cholesterol reflect a pathological change in lipoprotein metabolism resulting in circulating LDL particles that are small, dense and highly atherogenic. In order to understand how these changes occur, it is first necessary to review some aspects of the normal pathways of lipoprotein metabolism that have not yet been covered. HDL is the lipoprotein responsible for the transfer of cholesterol from peripheral tissues to the liver, ultimately for excretion in the bile. This process is termed “reverse cholesterol transport”. HDL is also involved in the regulation of circulating TG. Cholesteryl ester transfer protein (CETP) is a circulating protein that catalyses the exchange of cholesteryl esters and TG between lipoproteins in the blood. The usual physiological role of this protein is to catalyse transfer of cholesteryl esters from HDL to TRLs and the concomitant transfer of TG from TRLs to HDL, particularly in the postprandial period when TRL concentrations are high. The cholesteryl esters then remain within the TRL until taken up by the liver. The HDL-TG is hydrolysed by hepatic lipase in the liver, leaving smaller cholesteryl ester depleted particles which

may then take up further cholesterol from the periphery. Thus, it is clear that in conditions where circulating TRLs are always high, such as the metabolic syndrome, CETP will be active transferring cholesteryl esters out of HDL in exchange for TG from TRLs with the result that circulating HDL cholesterol concentration will be low. In addition, CETP is also able to catalyse the transfer of cholesteryl esters and TG between TRLs and LDL, resulting in LDL particles that are TG enriched and relatively cholesteryl ester poor. The TG in these particles is subsequently hydrolysed, either by hepatic lipase or LPL, resulting in LDL that is small and dense. Compared with larger LDL, small dense LDL is taken up more easily by arterial tissue (158), binds less well to the LDL receptor (159) and is more easily oxidized (160), all of which are features associated with increased atherogenicity. In addition, LDL particle size will determine the number of LDL particles for a given concentration. In other words, if LDL particles are small, a greater number will be present for an equivalent concentration of large LDL particles. Studies evaluating the nature of LDL in the metabolic syndrome have confirmed the association with small dense LDL (161-163). Thus, in the metabolic syndrome, although the total concentration of LDL is not elevated, the LDL particles are not only more atherogenic, but also present in greater numbers.

1.3.6 Triglyceride metabolism and the metabolic syndrome

The aetiology of the hypertriglyceridaemia associated with the metabolic syndrome and other insulin resistant states remains an area of intense research interest. High fasting TG concentrations are intrinsic to the definitions of the metabolic syndrome (113-115, 164). In addition, there is evidence that postprandial TG concentrations are

higher in those with the metabolic syndrome than healthy, non-obese, controls (165). As triglyceride levels are high both fasting and post-prandially, there are likely to be disturbances in both dietary-derived and endogenous TG metabolism in the metabolic syndrome.

1.3.7 Fat metabolism in the metabolic syndrome

1.3.7.1 TG synthesis

Attention has largely focused on the dysregulation of VLDL synthesis. Kinetic studies using stable isotopes to label apolipoproteins have demonstrated increased secretion of VLDL-apoB in abdominally obese men and women under both fasting and post-prandial conditions (166, 167). Indeed, there is a strong positive correlation between visceral fat mass and hepatic secretion of VLDL-apoB, although this is independent of insulin resistance as quantified by HOMA scoring (168). A more recent study has dissected the latter relationship a little further, revealing a strong correlation between HOMA estimated scores of insulin resistance and the hepatic production of VLDL1 but not VLDL2 in healthy subjects (169). This appears to hold true in the context of type 2 diabetes where there is also an overproduction of VLDL1 (170). The increased TG production observed in obesity and diabetes is probably multifactorial in origin. As described earlier, the modulators of VLDL production are insulin concentration in the hepatic portal vein, NEFA supply to the liver and hepatic uptake of circulating TRL and *de novo* lipogenesis within hepatocytes. All of these processes are altered in obesity and insulin resistance. The inhibitory effect of insulin on VLDL production is lost in obesity and type 2 diabetes, suggesting liver specific insulin resistance (105). Elevated

circulating NEFA concentrations have been described extensively in obesity, metabolic syndrome and diabetes and are discussed in more detail below. Hepatic TG uptake is dependent on hepatic TG supply. Thus, the hypertriglyceridaemia associated with obesity, the metabolic syndrome and diabetes leads to a self-perpetuating supply of TRL to the liver. Finally, hepatic *de novo* lipogenesis is increased in subjects with obesity and hyperinsulinaemia (171).

Although high post-prandial TG is well recognized in insulin resistant states (172-174), most human studies have been done in the fasting state and thus there is little information regarding chylomicron synthesis. One study, using a protocol of feeding subjects hourly with a liquid food supplement, demonstrated increased apoB-48 production in hyperinsulinaemic, insulin resistant men. Indeed, there was a direct correlation between fasting plasma insulin and apoB-production rate (175). However, the volume of supplement provided to subjects was dependent on factors such as weight, age and usual physical activity. Thus, oral fat supply was greatest in the individuals with most fat mass and highest insulin levels and, although the fat content of the nutrients ingested did not correlate with apoB-48 production rate, it is difficult to be certain of the precise nature of the relationship between insulin sensitivity and apoB-48 production.

1.3.7.2 TG catabolism

Circulating TG concentrations depend on the balance between TG synthesis and lipolysis. As mentioned, LPL catalyses intravascular TG hdrolysis. LPL activity is reduced in both obesity and type 2 diabetes (176). Indeed, there is a negative correlation

between insulin sensitivity, assessed by hyperinsulinaemic euglycaemic clamp or fasting plasma insulin concentration, and both plasma LPL activity (177) and adipose tissue LPL mRNA content (178). This correlation has been demonstrated under both fasting and post-prandial conditions (179). Thus, it would seem likely that TG hydrolysis is reduced in insulin resistant individuals, however this has proven more difficult to demonstrate. The kinetic studies of isotopically labeled apolipoproteins described above showed no difference in whole body fractional catabolic rate (FCR) in any of the apoB containing lipoproteins between obese and lean men and women (166, 167, 175). This contrasts with the finding of a negative correlation between total adipose tissue mass and FCR of VLDL-apoB (168). In order to clarify such discrepant findings in whole body metabolism, it is useful to examine metabolism within specific tissues. Reduced adipose tissue clearance of TG has been demonstrated in obese individuals compared with lean controls (13, 43). However, the obese groups in those studies were heterogenous, including some individuals with diabetes. In contrast to the findings in adipose tissue, one study of TG clearance across muscle has shown no difference between females with upper body obesity, who are phenotypically analogous to those with the metabolic syndrome, and females with lower body obesity (180). However, this study is difficult to interpret because of a number of confounders including the fact that the upper body obese group contained one subject with diabetes, the study design maintained subjects in a constant fed state, rather than examining the more physiological true post-prandial state and the TG samples from two upper body obese individuals were deemed too lipaemic for reliable analysis. Thus, the handful of studies of TG hydrolysis in insulin resistant states have yielded inconclusive and conflicting results.

In conclusion, obesity is associated with abnormalities of synthesis, and possibly also peripheral clearance, of both chylomicron and VLDL-TG. However, there is only limited information regarding these pathophysiological processes in the metabolic syndrome.

1.3.7.3 NEFA metabolism

A fasting elevation in NEFA has long been recognized in obesity (181, 182) and has also been demonstrated in subjects with diabetes (148, 183). In view of these findings, NEFAs have been put forward as a potential link between insulin resistance and type 2 diabetes (184, 185). Support for this hypothesis comes from the evidence of adverse metabolic sequelae of high NEFA concentrations potentially resulting in abnormal glucose metabolism and atherogenesis.

Elevated NEFAs directly effect pancreatic insulin secretion. This has been shown in humans in vivo by measuring glucose-stimulated insulin secretion during infusions of lipid emulsion over 24– 48 hours. Curiously, the acute and chronic effects of raising NEFA concentrations differ. Acute exposure of subjects to high NEFA concentrations results in increased insulin secretion. However, after 24 hours or more of NEFA exposure insulin secretion is markedly impaired (186, 187). The precise mechanism responsible for the effects of NEFAs on pancreatic function is unknown. The suppressive effect of prolonged exposure has been termed "β-cell lipotoxicity" (188) although whether this is a functional or physical phenomenon remains controversial. Irrespective of the exact

mechanism, the observation of reduced insulin secretion in response to 24-48 hours of NEFA exposure provides compelling evidence for a close link between elevated NEFA concentrations occurring over years, as has been described in the metabolic syndrome, and progressive decline in pancreatic function with subsequent glucose intolerance.

Elevated NEFA concentrations influence the metabolic pathways of both glucose and TG in the liver. The effect of NEFA on hepatic glucose metabolism has yet to be fully clarified. Hepatic glucose production results from a combination of gluconeogensis and glycogenolysis. The balance between these processes is termed hepatic autoregulation. In healthy individuals during fasting, an experimentally produced rise in circulating NEFA concentration results in increased gluconeogenesis but reduced glycogenolysis such that hepatic glucose output is unchanged (189-191). The reverse is also true when NEFA concentrations are suppressed by nicotinic acid (189). The exact mechanism by which elevated NEFA augments gluconeogenesis remains unclear. The suppression of glycogenolysis may be due directly to the elevated NEFA concentrations (192) or, alternatively, may be a consequence of the insulin released in response to the acute rise in circulating NEFA (186, 187). However, studies combining Intralipid and heparin infusions with hyperinsulinaemic clamps, which completely suppress glycogenolysis, have produced mixed results (193, 194). These studies are difficult to interpret since the pharmacological rather than physiological concentrations of insulin used in the clamp studies will have an effect on the balance between gluconeogenesis and glycogenolysis.

In conditions characterized by a chronic elevation of NEFA concentrations hepatic autoregulation may break down. Infusion of Intralipid and heparin precipitated a rise in endogenous glucose production in obese women (195) whilst in patients with type 2 diabetes, nicotinic acid suppression of NEFA resulted in a reduction of both gluconeogenesis and glycogenolysis (191). The molecular explanation for the breakdown in hepatic autoregulation remains unclear, although NEFAs may inhibit insulin mediated pathways of hepatic glucose metabolism and thereby induce hepatic insulin resistance (192).

The pathophysiological effect of high NEFA on muscle metabolism is not exactly as would be predicted from extrapolation of the glucose-fatty acid cycle. As mentioned, in health there is a balance between FA and glucose metabolism in muscle such that increased NEFA supply results in increased FA oxidation with a consequent reduction in glucose oxidation and uptake (88). Thus, it is attractive to hypothesise that in states of increased NEFA supply, such as type 2 diabetes and obesity, muscle glucose uptake will be reduced resulting in elevated circulating glucose. However, the evidence from studies using limb balance techniques to assess muscle specific substrate oxidation has suggested that the situation is somewhat more complex and depends on the prevailing metabolic milieu. In the postabsorptive state fat oxidation is, in fact, lower in subjects with obesity or type 2 diabetes compared with healthy lean controls (196, 197). However, under insulin stimulated conditions, muscle substrate oxidation is not significantly altered in either the obese or the subjects with diabetes. In contrast, lean subjects change from predominantly fat oxidation during fasting to predominantly glucose oxidation during

insulin stimulation. The facility to alter substrate oxidation appropriately to prevailing metabolic conditions has been termed metabolic flexibility (198). Therefore, obesity and type 2 diabetes are characterized by metabolic inflexibility in muscle metabolism. The consequences of this metabolic inflexibility are a failure to take up and oxidize glucose under post-prandial conditions and a failure to oxidize fat when fasting. Thus, glucose is not cleared from the circulation following a meal and fat is stored inappropriately within muscle during fasting, reflected in an increased intramyocellular fat content (92, 94, 199). This is in contrast to the situation in endurance-trained athletes in whom increased intramyocellular fat stores are appropriate sources of energy (92, 97). The molecular mechanism underlying such metabolic flexibility remains to be elucidated. It is possible that the hyperinsulinaemia associated with both obesity and type 2 diabetes results in a maximal suppression of lipolysis under fasting conditions that cannot be augmented by further insulin stimulation. It is important to sound a note of caution when interpreting the studies discussed above since hyperinsulinaemic clamps were used to produce the insulin stimulated conditions. The pharmacological doses of insulin used may have effects that are outside even the pathophysiological hyperinsulinaemia that is seen in insulin resistant states. There have been no studies of muscle substrate oxidation in the metabolic syndrome. It would be particularly pertinent to know whether those with the metabolic syndrome are able to demonstrate metabolic flexibility in the face of changing physiological metabolic conditions.

Therefore, chronically elevated NEFA concentration lead to reduced pancreatic insulin secretion, increased hepatic glucose production and the deposition of fat in tissues

other than adipose tissue. In addition, as NEFA supply is a key determinant of VLDL production, an increased circulating NEFA concentration results in increased hepatic TG synthesis.

The aetiology of high arterial NEFA concentrations in association with insulin resistant states is not fully understood. The classic pathophysiological explanation is a failure of insulin mediated suppression of lipolysis, presumably mainly in adipose tissue. Insulin clamps and oral glucose loads have been used to demonstrate a clear defect in insulin suppression of NEFAs in both obese and lean patients with diabetes (148, 200-203). Similarly, studies using oral glucose tolerance tests, meal tolerance tests and insulin clamps, have demonstrated comparable abnormalities in insulin action amongst first degree relatives of diabetic patients (204, 205). The picture in obesity is slightly less clear. Obesity is, by definition, a state of increased fat mass. Consequently, in obese individuals there is a greater reservoir of fat stores from which NEFAs may arise. There is certainly evidence of higher circulating NEFAs under clamp conditions in obese subjects (43, 206), suggestive of a failure in lipolysis suppression. However, when NEFA turnover and suppression by insulin are expressed per kilogram of fat mass, no abnormality is demonstrated (207).

Although inadequate lipolysis suppression may be important there are other potential mechanisms explaining the high NEFA concentrations associated with the metabolic syndrome. One interesting hypothesis involves the fate of NEFA generated from intravascular TG hydrolysis. As previously described, circulating TG is hydrolysed

by LPL at the capillary endothelium. The resultant NEFAs may then either be taken up by tissues or spill over into the systemic circulation. The control of the fate of these NEFAs is poorly understood, however it is possible insulin mediates the process and that in states of insulin resistance there is increased spillover of NEFA. This has never been previously assessed.

1.3.8 Adipose tissue blood flow and the metabolic syndrome

There are no studies of ATBF in the metabolic syndrome *per se*. However, fasting ATBF is reduced and the postprandial response attenuated in the obese (43, 149, 208-210) and the those with type 2 diabetes (43, 209) when compared with healthy lean controls. In addition, the ATBF response to a meal is related to insulin sensitivity, independently of BMI (149). The mechanism for the impairment in postprandial ATBF response is unlikely to be related purely to hyperinsulinaemia since insulin has little direct effect on ATBF (37). However, as already discussed, ATBF is intimately related to β-adrenergic activity (51, 52). Consequently, it seems more likely that disturbance of sympathetic nervous system activity, as has been described for muscle in association with insulin resistance (211), may underlie the abnormal post-meal ATBF response.

1.4 Summary

FAs are an important source of energy, driving metabolic processes in many tissues. Whole body fat metabolism depends on complex relationships between the tissues. Adipose tissue plays a central role as both a modulator of circulating lipids and an

endocrine organ, whilst skeletal muscle is a major consumer of fatty acids. However, lipid metabolism within adipose tissue and muscle remains incompletely understood. Abnormalities in fat metabolism may represent the pathophysiological link between the metabolic syndrome and the associated vascular and metabolic disease. Thus, it would clearly be of great benefit to improve the understanding of lipid metabolism, on a tissue specific basis, in health and in the metabolic syndrome.

1.5 Aims of this thesis

The aims of this thesis were twofold; firstly, to elucidate normal pathways of FA and TG metabolism in adipose tissue and muscle and secondly to determine how these pathways are altered in those with the metabolic syndrome.

Specific questions I wished to answer included –

- Are dietary-derived and endogenous TG and FAs handled differently in adipose tissue and muscle?
- Is there quantitatively significant uptake of circulating NEFA by adipose tissue?
- Is there spillover into the systemic circulation of FAs derived from intravascular TG hydrolysis in muscle as there is in adipose tissue?
- How does the prevalence of the metabolic syndrome vary dependent on definition in an overweight UK population?
- What are the differences in fasting lipid parameters between equally overweight individuals with and without the metabolic syndrome?

- Does TG handling in peripheral tissues contribute to the hypertriglyceridaemia of the metabolic syndrome?
- Is resistance to insulin mediated suppression of lipolysis in the post-prandial period a feature of the metabolic syndrome?
- Does increased spillover of NEFAs from adipose tissue contribute to the elevated NEFA concentration observed in the metabolic syndrome?

1.6 Summary of chapters

Data are presented in Chapters 3 to 5.

Chapter 2 describes methodology common to all the studies.

Chapter 3 describes a detailed investigation of normal FA and TG metabolism in the subcutaneous abdominal adipose tissue and forearm muscle of overweight subjects.

Chapter 4 describes an analysis of overweight subjects in the Oxford Biobank to elucidate aspects of the metabolic syndrome in that cohort.

Chapter 5 describes a detailed investigation of tissue specific fat metabolism in individuals with and without the metabolic syndrome.

Chapter 2 : Overview of methods and calculations

2.1 Methods

This chapter gives details of methods used to study fat metabolism in humans *in vivo*. These methods and the study protocol were common to all the studies described in this thesis unless otherwise stated.

2.1.1 Arteriovenous differences

Calculation of the difference between arterial and venous concentrations of metabolites across tissues reflects the metabolic fluxes occurring within those tissues (212). Put in another way, if the concentration of a metabolite in arterial blood entering a tissue is greater than the concentration of that metabolite in the venous effluent of that tissue (i.e. a positive A-V difference), then there has been a net uptake of the metabolite within the tissue. Conversely, if the venous effluent contains more of the metabolite than the arterial blood (i.e. a positive V-A difference), then there has been net output. The technique is further refined by including the blood flow through the tissue in the calculation to allow measurement of rate of uptake or release. Thus, the net rate of flux is the product of the A-V or V-A difference and the blood flow. These calculations can be summarized as –

Net Rate of Uptake = A-V Difference x Blood Flow

Net Rate of Release = V-A Difference x Blood Flow

2.1.2 Arterial cannulation

When sampling arterial blood, the assumption is always made that there is no difference between samples taken from different sites. Thus, access to the artery supplying a particular tissue of interest is not essential. Arterial samples can be taken from the radial, brachial or femoral arteries. Cannulation of the femoral artery under aseptic conditions is a straightforward procedure that causes minimal discomfort both during the procedure and subsequently. Therefore, in the studies described in this thesis, femoral arterial cannulation (using an 20 gauge cannula), was used to obtain arterial blood samples. Correct placement of the catheter was confirmed by an oxygen saturation above 95% in arterial blood samples. In addition, on some occasions the cannula was inserted under ultrasound control, in which case correct positioning was directly verified.

2.1.3 Venous cannulation

The tissues of interest in this thesis are muscle and subcutaneous abdominal adipose tissue.

2.1.3.1 Muscle

The venous effluent of forearm muscle is easily sampled by retrograde cannulation (20 gauge cannula) of a deep ante-cubital fossa vein under local anaesthesia (1% lignocaine) (213). A wrist cuff is inflated to above systolic blood pressure (either 30 mmHg above or to 200 mmHg, whichever was higher) for three minutes before taking deep venous samples in order to prevent contamination of the blood from the forearm

vein with blood from the hand. Correct positioning of the cannula was confirmed by the finding of less than 60% oxygen saturation in the drawn blood samples.

2.1.3.2 Subcutaneous abdominal adipose tissue

Sampling the venous effluent of subcutaneous adipose tissue is somewhat more complex. The anterior abdominal depot is separated from the underlying external oblique muscle by a fibrous aponeurosis through which no vessels pass (214). Consequently, cannulation of a vein draining this depot allows sampling of blood draining purely adipose tissue, albeit with a minimal contribution from the overlying skin. Therefore, a modified Seldinger technique, using a 22 gauge × 10cm cannula, has been developed to catheterize a branch of the superficial inferior epigastric vein above the inguinal ligament (214, 215).

2.1.4 Blood flow

A plethora of techniques have been developed for the measurement of local blood flow within tissues. For the studies presented in this thesis, I have used strain gauge plethysmography and the Xenon washout technique to assess forearm muscle and sub-cutaneous abdominal adipose tissue blood flow respectively. However, I will also review some of the other methods available.

2.1.4.1 Forearm muscle

Blood flow through the forearm can be measured invasively by electromagnetic flow meters (216), indicator methods (217), isotopic clearance methods (218), positron

emission tomography (219), laser Doppler flow (220) and nuclear magnetic resonance spectroscopy (221). The non-invasive methods for muscle blood flow assessment include strain gauge plethysmography (213, 222), Doppler ultrasound (223), photoplethysmography (224) and magnetic resonance imaging (MRI) (225).

The insertion of an electromagnetic flow probe is an excellent means of directly measuring blood flow in a vessel. However, this extremely invasive procedure is not appropriate for physiological studies of healthy human subjects. Laser-Doppler flow measurement is a novel method that is analogous to Doppler ultrasound described below but with the use of monochromatic light rather than sound waves (220). This technique requires the insertion of an intramuscular optic fibre and is, thus, also too invasive to form part of very complex metabolic studies. All of the other techniques listed above as "invasive" require an intra-arterial infusion. Muscle blood flow can then be calculated from either dilution of the infusate (thermodilution or dye dilution), detection of a radio-isotope (positron emission tomography) or clearance of an isotope (nuclear magnetic resonance). As discussed previously and below, the protocol for the studies in this thesis already involves infusions of normal saline to maintain patency of the venous catheters as well as an infusion of labeled fatty acid. Thus, both the practicalities of adding an additional infusion and the difficulties that would be presented in interpreting the data resulted in the selection of a less invasive technique for monitoring muscle blood flow.

The principle underlying strain gauge plethysmography is that if the venous outflow of blood from the forearm is occluded without compromising the arterial influx,

the rate of swelling of the forearm will be proportional to the arterial flow. The practical procedure for making such measurements is as follows; blood pressure cuffs are placed on the upper arm and above the wrist whilst a small-bore rubber tube filled with mercury is wrapped around the forearm. The wrist cuff is inflated above systolic blood pressure to prevent blood flow to or from the hand. The upper cuff is then inflated to prevent venous outflow and, as the forearm engorges with blood, the strain gauge is stretched. The arterial flow can be mathematically derived from the rate of stretch, since this is a direct measurement of the rate of forearm distension. Implicit to this technique is the assumption that blood flow to the forearm represents only blood flow to muscle. There are other tissues within the forearm such as adipose tissue and skin. Much of this more superficial flow is eliminated by the wrist cuff (214). However, there is still the potential for variability in forearm skin blood flow to confound the measurement of foream muscle blood flow and consequently make the calculations of forearm substrate flux (described in Section 2.3) difficult to interpret. The most important factor determining skin blood flow is ambient temperature. The studies described in this thesis were carried out in a temperature controlled environment in order to overcome this particular source of error.

Doppler ultrasound assessment of blood flow is based on the observation that erthrocytes will reflect sound waves of a specific frequency, resulting in a change in the frequency of those sound waves. Measurement of the Doppler frequency shift occurring allows calculation of the blood flow. The direction of blood flow and angle of insonation will both affect the Doppler shift measurement and are taken into account in the calculation (223). In the hands of a well trained operator, Doppler ultrasound can be an

extremely sensitive method for the measurement of muscle blood flow. However, the technique is expensive and requires both time and experience to master.

Photoplethysmography is a novel technique that is still being developed (224). The principle underlying this is that variations in the signal detected by a photoreceptor placed adjacent to a light-emitting diode on the skin are related to changes in blood flow in the underlying tissue. Although this method shows promise, more validation and specifically comparison with other techniques, is required before photoplethysmography can be considered a useful methodology.

Magnetic resonance imaging is a well recognized tool for anatomical and pathological imaging. However, by magnetically tagging blood and observing the changes occurring as the blood flows through an imaging slice, it is possible to make calculations of tissue perfusion and blood flow. This technique is termed arterial spin labelling and has been successfully applied to the measurement of muscle blood flow (226). Arterial spin labelling is non-invasive and has advantages over other techniques in that the measurements are not influenced by the blood flow of adjacent tissue. Indeed, it is possible to compare blood flow between muscle groups or even individual muscles (226). However, this technique is severely limited in that the equipment required is cumbersome and exceedingly expensive.

The large number of methods for measuring muscle blood flow reflects the fact no technique is ideal in all circumstances. Strain gauge plethysmography was selected for

the studies in this thesis as it is robust, well validated and can be easily learned and applied, particularly in the context of a complex metabolic study.

2.1.4.2 Adipose tissue

Analagous to the situation in muscle, there are a variety of techniques for measuring adipose tissue blood flow (ATBF) including thermal clearance (227), positron emission tomography (228), laser-doppler flowmetry (LDF) (229), the microdialysis ethanol technique (230) and the ^{133}Xe washout technique (231). Methodological concerns surrounding thermal clearance and the need for access to specialist equipment, that is not generally available, in order to carry out positron emission tomography studies severely limit the application of the first two.

LDF in adipose tissue is based on exactly the same principles as in muscle and, thus, requires insertion of a fibre-optic probe into the subcutaneous adipose tissue. In the single comparative study of the measurement of ATBF that has been performed, LDF and ^{133}Xenon washout correlate reasonably well (229). However, LDF does not yield absolute blood flow values. In addition, subject movement disrupts the measurement of ATBF by LDF and, to some extent, limits the utility of this technique.

Microdialysis is a method for determining interstitial concentrations of a variety of substances. The technique has been applied to numerous tissues, including sub-cutaneous adipose tissue (232). Isotonic fluid is infused through a microdialysis probe placed in the tissue of interest. Hydrophilic substances diffuse across the microdialysis membrane and can then be measured in the probe effluent. ATBF can be assessed by the

addition of a marker of flow (usually ethanol) to the dialysis solvent (230). Although technically simple, the microdialysis ethanol technique is not quantitative and is less responsive to physiological changes than the ^{133}Xe washout technique (42).

The principle underlying the ^{133}Xe washout technique (231) is that the clearance of ^{133}Xe injected into subcutaneous adipose tissue is directly proportional to the blood flow through the tissue. Thus, blood flow is simply calculated as the product of the fractional rate of loss of radioactivity and the partition coefficient of the radioisotope between blood and adipose tissue. ^{133}Xe gas is injected into the anterior subcutaneous abdominal adipose tissue (231) and its disappearance measured using a scintillation detector taped to the anterior abdominal wall (233). The injection is performed 30 minutes prior to any blood sampling. The time lag between injection and sampling allows any hyperaemic response to the injection to resolve and ensures that the ^{133}Xe has equilibrated in the tissue.^{133}Xe is ideal for the measurement of adipose tissue blood flow because it is inert and lipophilic with a high partition coefficient between plasma and adipose tissue. However, this technique is somewhat invasive and requires the use of a radioactive isotope.

2.1.5 Isotopic labelling

Both radioactive and stable isotopes of glycerol and fatty acids have been used to study fat metabolism at both the whole body and tissue-specific level. The rate of appearance of glycerol has been used extensively as a measure of whole body lipolysis (234-236); however the assumptions underlying such calculations have been questioned (237, 238). At the tissue level, radiolabelled glycerol has been used to assess intracellular

and intravascular lipolysis in both adipose tissue (239) and muscle (240). Radioactive tracers are attractive because of the very small quantity of tracer needed and the short duration of study. However, concerns over the standard methodologies used to measure FA specific activity have necessitated the development of very specialized analytical techniques (241). In addition, the radiation exposure and requirement for radioactivity-approved research facilities limits the use of radiotracers.

Such limitations do not apply to the use of stable isotopes of naturally occurring fatty acids. [^{13}C]- and [$^{2}H_2$]-labelled palmitic and oleic acid are the commonly used fatty acids in the majority of isotopic labelling studies (240-242). These fatty acids are present in significant proportion within the circulating NEFA pool and their metabolism has been demonstrated as representative (243). Tracers may be given either orally or by intravenous infusion, depending on the aspect of metabolism under investigation. There are some disadvantages to the use of stable isotopes. The high natural abundance of ^{13}C coupled with the relatively rapid FA turnover means that, if given intravenously, a comparatively high tracer infusion rate, typically 0.04 $\mu mol.kg^{-1}.min^{-1}$ (244), is required. Consequently, a large amount of tracer and albumin are used in the preparation of the infusion. These problems can be ameliorated by the use of uniformly labelled FA and highly sensitive gas chromatography-isotope ratio mass spectrometry. However, mass spectrometry facilities, a pre-requisite for such analysis, are not always easily accessible.

By combining the use of isotopically labelled fatty acids with arteriovenous sampling, the fate of both exogenous and endogenous fatty acids can be traced. For the purposes of the studies in this thesis, [^{13}C]-labelled palmitic acid was given in a test meal

to trace the fate of exogenous fatty acids. [$^{2}H_{2}$]-labelled palmitic acid was complexed to human albumin and infused to trace the fate of circulating NEFA. The assumptions are then made that [^{13}C]-label appearing in TG must be in chylomicrons, at least initially, and [$^{2}H_{2}$]-labelled TG must be VLDL. The methodology for making the [$^{2}H_{2}$]-labelled palmitic acid infusion is described in Section 2.4. The concept of using stable isotopic labelling in the study of fat metabolism is illustrated in Figs 2.1 and 2.2.

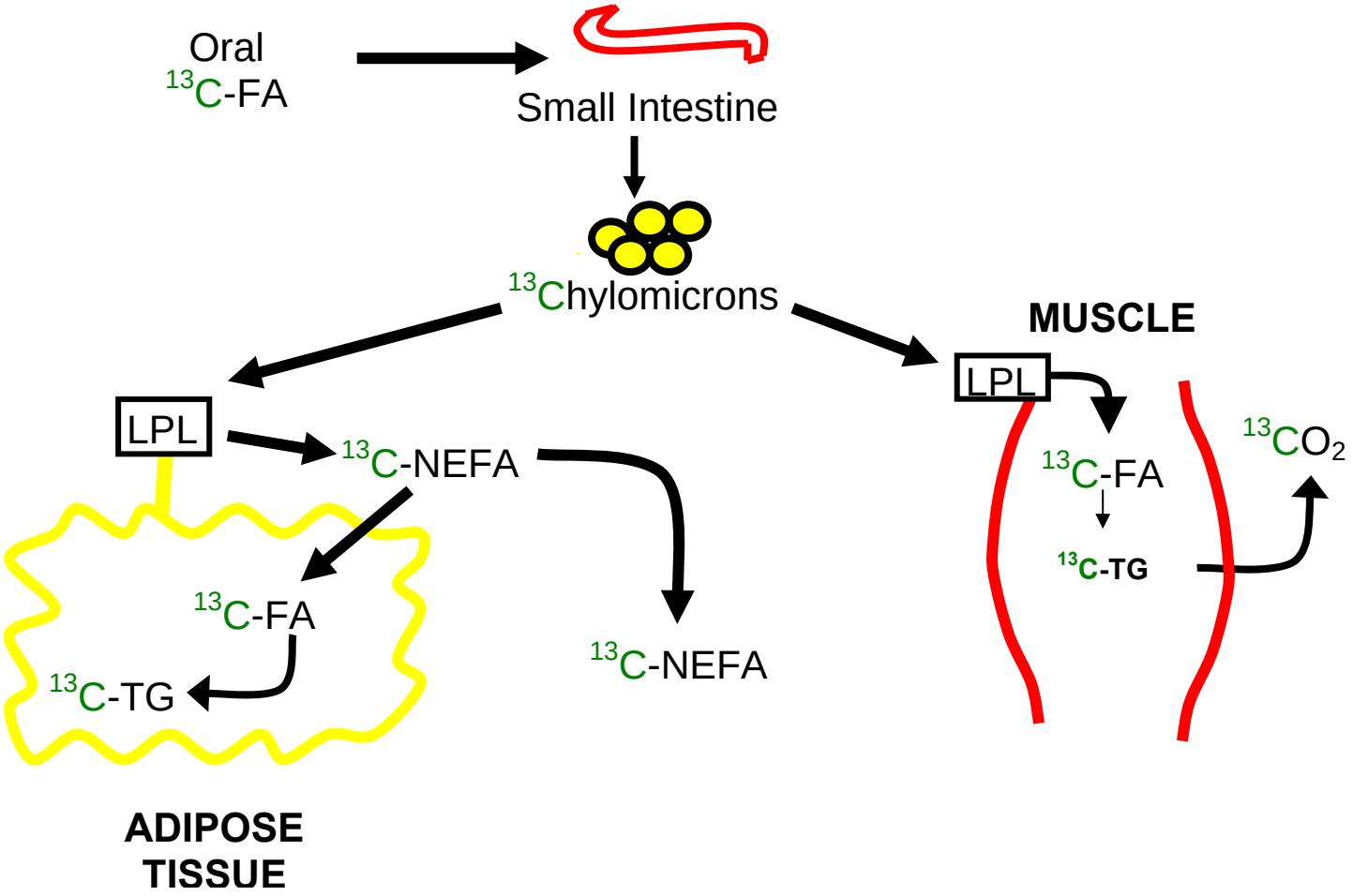

Figure 2.1. Potential metabolic pathways of an orally ingested stable isotopically labelled fatty acid. The oral isotope used in the studies in this thesis was [U-^{13}C]palmitic acid. ^{13}C-NEFA represents ^{13}C-isotopic labelling in the circulating NEFA pool, ^{13}C-FA represents ^{13}C-labelled fatty acids and ^{13}C-TG represents ^{13}C-labelling of TG.

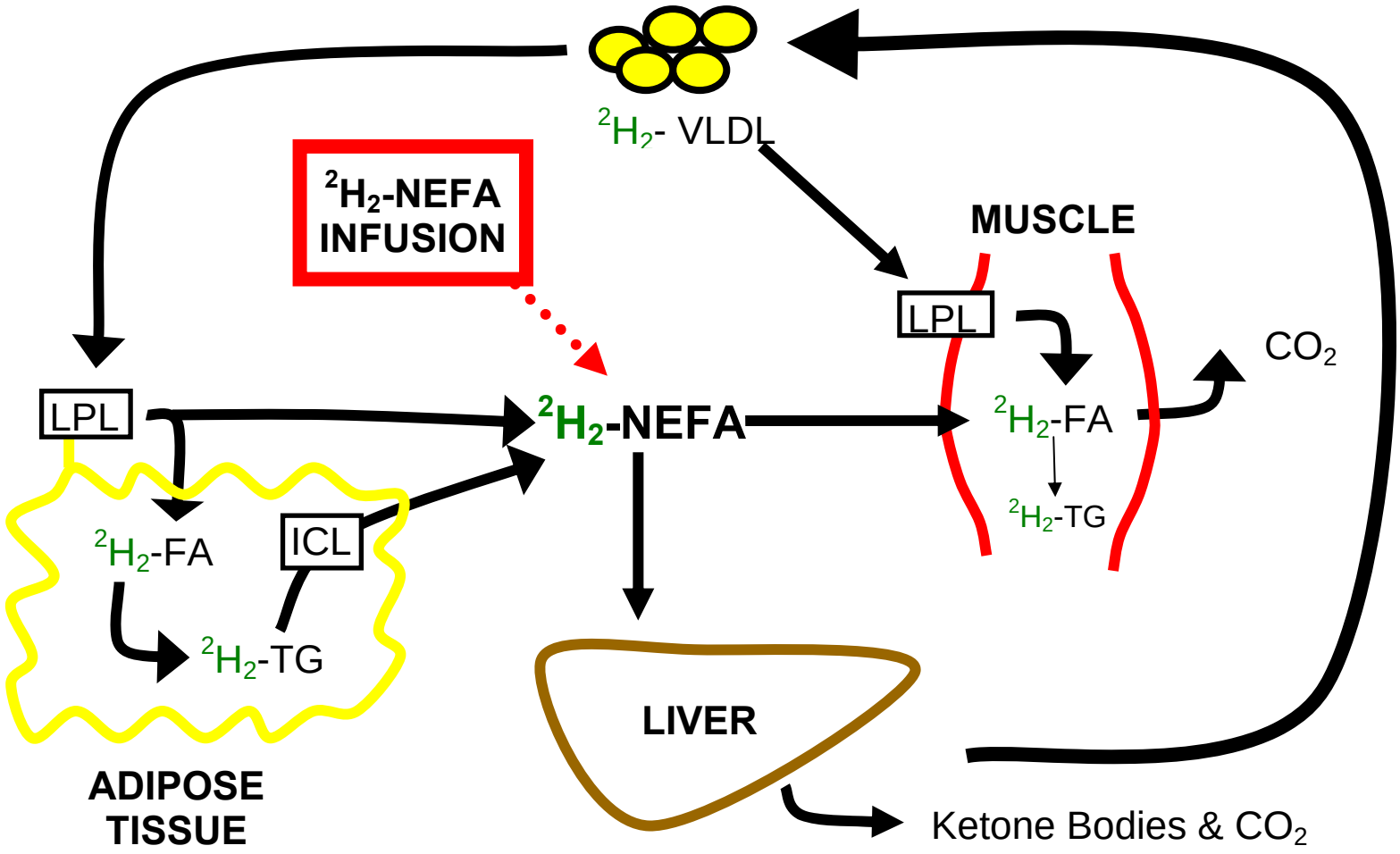

Figure 2.2. Potential metabolic pathways of an infused stable isotopically labeled fatty acid. The infused isotope used in the studies in this thesis was [$^2H_2$]palmitic acid. $^2H_2$-NEFA represents $^2H_2$-isotopic labelling in the circulating NEFA pool, $^2H_2$-FA represents $^2H_2$-labelled fatty acids and $^2H_2$-TG represents $^2H_2$-labelling of TG. ICL are the intracellular lipases, including ATGL, HSL and monoglyceride lipase.

2.1.6 Test meal

This thesis aims to investigate fat metabolism under varying metabolic conditions. Thus, subjects were studied both fasting and following a test meal. A mixed meal (245) (40 g fat, 40 g carbohydrate) was used, aiming to provoke a more physiological metabolic response than would be expected from ingestion of either a pure fat or pure glucose load. In addition, the meal was utilized as a means of administering the oral tracer ([^{13}C]-labelled palmitic acid). The test meal consisted of a chocolate-flavoured liquid fat emulsion (40 g olive oil, 100 mg [U-^{13}C]palmitic acid (isotope purity 98%, CK Gas Products Ltd, Hampshire, UK), 400 mg emulsifier, flavourings), a bowl of cereal (Rice Krispies, Kelloggs UK), 200 ml of skimmed milk and a cup of decaffeinated tea or coffee.

2.1.7 Indirect calorimetry

Indirect calorimetry was developed to measure energy expenditure more easily than the cumbersome direct calorimetry. The latter is a method of measuring heat production by the body requiring specialised equipment (246) that is very restrictive and thereby limited in application. The principle behind indirect calorimetry is that almost all metabolic fuels taken into the body are ultimately completely oxidized to CO_2 and water. Thus, energy expenditure can be calculated from measurements of CO_2 production and O_2 utilisation (247-249). Furthermore, information regarding substrate oxidation can be derived from the same data. Protein oxidation results in the production of nitrogen in addition to CO_2 and water. Thus, the calculations of substrate oxidation also include the

parameter of urinary nitrogen in order to take account of protein metabolism. For the purposes of the studies in this thesis, indirect calorimetry was performed on a whole body basis by the use of a ventilated hood system. A clear plastic hood was placed over the subject's head and air was drawn rapidly through by a pump. Expired air was collected and the CO_2 and O_2 content measured by an on-stream analyzer. The ventilated hood was placed over the subject's head for twenty minutes during which time measurements were made every minute. The first five minutes were considered an equilibration period and the data discarded (250, 251).

2.2 Study protocol

2.2.1 Subject recruitment

2.2.1.1 The Oxford Biobank (OBB)

Subjects were recruited from the Oxford Biobank (OBB); a population-based random collection of healthy men and women aged 30-50 years (252). The Biobank was established to enable the study of genotype-phenotype interactions with special reference to insulin resistance and type 2 diabetes. Volunteers attend for an initial screening visit at which fasting blood samples are taken and a number of biophysical parameters measured. The blood is analysed for insulin, glucose, TG and NEFA concentrations. Subjects have height, weight, waist, hip, blood pressure and skinfold measurements made. In addition, DNA is stored and consent obtained for subsequent genotyping, providing a resource of material for studying both currently recognized as well as potentially important genes involved in the regulation of fat metabolism. Subjects are also asked whether they would

be willing to participate in additional metabolic studies. Using such volunteers allows targeted recruitment of individuals by genotype, phenotype or both as well as the facility to carefully match controls. By the end of the recruitment period there were over 1000 individuals registered with the OBB.

2.2.1.2 Recruitment criteria

The studies presented in this thesis aim to investigate tissue-specific fat metabolism with particular reference to the metabolic syndrome. The definition of the metabolic syndrome used for subject recruitment was that put forward by the EGIR (114): fasting hyperinsulinaemia (the upper quartile of fasting insulin concentration within the OBB population) and two of: hyperglycaemia (fasting plasma glucose $\geq$ 6.1 mmol/l, but non-diabetic), hypertension (systolic/diastolic blood pressures $\geq$ 140/90 mmHg), dyslipidaemia (TG >2.0 mmol/l or HDL-cholesterol < 1.0 mmol/l) or central obesity (waist circumference $\geq$ 94 cm). For the detailed metabolic investigation described below, volunteers were selected to act as controls by matching for age and body mass index (BMI) with the subjects classified as having the metabolic syndrome. Early analysis of the baseline clinical data revealed that the metabolic syndrome and control groups were surprisingly similar in all metabolic variables, differing only in fasting insulin; the single essential criterion necessary for classification. Therefore, when presenting and analyzing the results of the detailed metabolic investigation described below, subjects were also divided according to the National Cholesterol Education Program (NCEP) Expert Panel (Adult Treatment Panel III (ATP III)) (113) definition of the metabolic syndrome; three or more of hyperglycaemia (fasting plasma glucose $\geq$

5.6mmol/l, but non-diabetic), hypertension (systolic ≥ 130 mmHg or diastolic blood pressures ≥ 85 mmHg or both), dyslipidaemia (TG ≥ 1.7 mmol/l or HDL-cholesterol < 1.03 mmol/l) or central obesity (waist circumference ≥ 102 cm). Female subjects were not chosen to avoid the possible confounding effects of menstrual or menopausal status on fat distribution and insulin sensitivity (253-256). Subjects were also excluded if they took any medication known to affect lipid metabolism and on the basis of apoE genotype. As described in Chapter 1, apoE acts as a ligand for the hepatic B/E (LDL) receptor, facilitating the uptake of TRL remnants. There are three isoforms of apoE: apoE2, apoE3 and apoE4, resulting in six phenotypes: E2,2; E3,2; E4,2; E3,3; E4,3; E 4,4. ApoE2 binds poorly to the B/E receptor and consequently individuals who are homozygous for apoE2 have increased circulating TRL remnants (257). Clearly, when recruiting for studies of lipid metabolism it is preferable to exclude individuals who already have a known defect in TG clearance and thus subjects in the OBB homozygous for apoE2 were not recruited. The studies described in this thesis were approved by the Oxfordshire Clinical Research Ethics Committee and all subjects gave written informed consent.

2.2.2 Preparation

Subjects were advised to avoid corn-containing foodstuffs, which are naturally enriched with ^{13}C, for 48 hours prior to a study day. They were also asked to avoid strenuous exercise and alcohol on the day prior to the study day. They attended the department fasting. On arrival, the following anthropometric data were gathered: height, weight, blood pressure, skinfold thicknesses (biceps, triceps, sub-scapular, supra-iliac)

and body composition, estimated by bio-impedance (Bodystat-1500, Bodystat Ltd, Isle of Man, UK). Subjects then rested on a bed whilst the various cannulations were performed using the techniques described above. The superficial epigastric vein was catheterized first, followed by the femoral artery, the deep ante-cubital vein and a superficial vein for administration of the fatty acid infusion. Cannulae were kept open by infusion of normal saline. Once all the catheters were in place, ^{133}Xe gas was injected into the anterior subcutaneous abdominal adipose tissue. A strain gauge and plethysmography cuffs were applied to the arm from which venous blood was sampled.

2.2.3 Sampling protocol

An infusion of [$^{2}H_2$]palmitic acid complexed to human albumin was administered intravenously to the subject at a dose of 0.04 $\mu mol.kg^{-1}.min^{-1}$ (244). The infusion was commenced at least an hour before blood sampling to ensure equilibration of the tracer in plasma. Blood was sampled from the femoral artery, the superficial epigastric vein and the deep ante-cubital vein simultaneously. Three sets of fasting blood samples were taken 30 min apart. Following the meal, blood samples were taken every 30 min for two hours and hourly thereafter, up to six hours. Plethysmography was performed immediately following blood sampling. Breath samples were collected into a breath bag at the same time as the blood samples. Indirect calorimetry was performed using a Deltatrac ventilated-hood indirect calorimeter (Datex, Helsinki, Finland) during the fasting period and on four other occasions over the day. The study day is summarized in Figure 2.3.

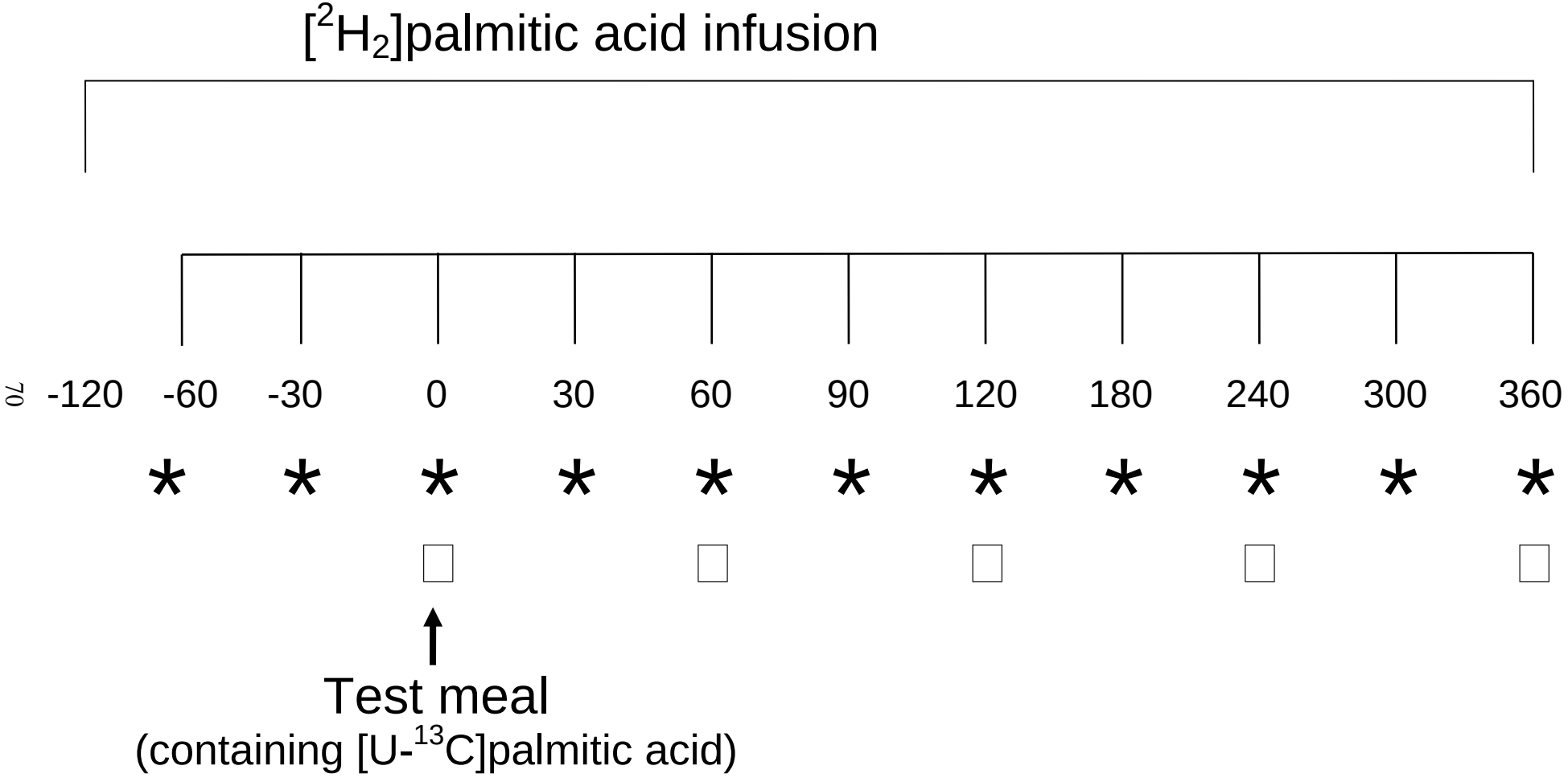

Figure 2.3. Study day sampling protocol. The asterisks (*) represent blood sampling times, the boxes (□) represent indirect calorimetry times.

2.2.4 Sample analysis

2.2.4.1 Biochemical analyses

Blood samples were collected into heparinised syringes (Monovette; Sarstedt, Leicester, UK). A volume of 200 µl of each blood sample was rapidly deproteinized with 7% (wt/vol) perchloric acid. Aliquots of whole blood were taken for measurement of haematocrit, haemoglobin and oxygen saturation. The first-mentioned was necessary for calculations of flux as many of the substrates measured are only present in the plasma fraction. Plasma was separated rapidly from the remaining blood by centrifugation at 4°C. Plasma TG, NEFA, glucose, glycerol and 3-hydroxybutyrate concentrations were measured enzymatically on an ILab 600 Multianalyser (Instrumentation Laboratory, Warrington, UK). The inter-assay coefficients of variation (CV) are shown in Table 2.1. There was insufficient data to calculate intra-assay CVs. The CVs of glycerol and 3-hydroxybutyrate are at the high end of desirability. This reflects both dilution of the original samples with perchloric acid, a necessity in order to deproteinise the samples, and also the fact that some of these results are at the limit of the assay's sensitivity. The Plasma insulin was measured by radio-immunoassay (Linco Human insulin specific RIA kit).

2.2.4.2 Fatty acid analyses

To determine fatty acid composition and isotopic enrichment, total lipids were extracted from plasma, and methyl esters prepared from NEFA and TG fractions (15). Fatty acid compositions (µmol/100µmol total fatty acids) were determined by gas chromatography (GC) (15), and palmitate concentrations calculated by multiplying the proportion of palmitate by the corresponding plasma concentration of NEFA or TG determined enzymatically. [U-^{13}C] and [$^{2}H_2$]palmitate enrichments were

determined simultaneously by gas chromatography-mass spectrometry (GC-MS) using a 5890 GC coupled to a 5973N mass-selective detector (Agilent Technologies UK). The GC was equipped with a DB-Wax 30 m capillary column (from Agilent, i.d. 0.25 mm, film thickness 0.25 μm) and ions with mass-to-charge ratios (m/z) of 270 (M+0), 272 (M+2) and 286 (M+16*)* were determined by selected ion monitoring. Dwell time was 100 ms. Allowance was made for natural enrichment by subtracting a baseline value from each sample enrichment. Tracer-tracee ratios (TTRs) for [U-^{13}C]palmitate, (M+16)/(M+0), and [$^{2}H_2$]palmitate, (M+2)/(M+0), were multiplied by the corresponding palmitate-NEFA or palmitate-TG concentrations to give plasma tracer concentrations. The intra-assay CV for palmitate in the NEFA and TG fractions were 3.6% and 1.7% respectively.

2.2.4.3 Breath gas analyses

Breath CO_2 was resolved from the presence of other gases using a CP-PoraPLOTQ capillary column with dimensions of 27.5 m x 0.32 mm x 10 μm (Varian Limited, Oxford). Splitless injection mode was used, with an injection volume of 40 μl and injector temperature of 110°C. The oven temperature was kept constant at 35°C with a total runtime of 10 min. The column flow was held constant at 1.2 ml/min. Allowance was made for natural enrichment as described above for blood samples.

Table 2.1 Inter-assay CVs for assays performed on the ILab 600 multianalyser

Assay	Mean	CV (%)	Mean	CV (%)
Glucose (mmol/l)	5.0	2.3	13.7	1.6
Triacylglycerol (mmol/l)	1.4	1.9	2.5	1.6
Total Cholesterol (mmol/l)	2.4	2	5.0	1.6
HDL Cholesterol (mmol/l)	0.7	2.3	1.4	1.5
NEFA (µmol/l)	490	2.5	1458	2
Lactate (µmol/l)	193	6.6	1000	6.4
Glycerol (µmol/l)	17	14	57	5.7
3-Hydroxybutyrate (µmol/l)	7.8	20	17	10

2.3 Calculations

2.3.1 Unlabelled metabolites

Concentrations of lipid metabolites in plasma were first converted to whole-blood values by multiplying by (1 – haematocrit). Arterial minus venous differences (A-V differences) and venous minus arterial differences (V-A differences) were calculated. Absolute net flux, as described earlier, is determined as the A-V or V-A difference multiplied by tissue blood flow. In the case of TG, the absolute net flux is also a measure of rate of LPL action, since the removal of TG from plasma within tissues primarily reflects LPL activity. Fractional TG extraction was calculated as the A-V difference divided by the arterial concentration of TG and expressed as a percentage. TG clearance, a measure of the efficiency of substrate removal from the circulation, was calculated as the product of fractional extraction and tissue blood flow (212). Thus, in adipose tissue, these calculations can be summarized as -

Net TG Flux or Rate of LPL action (nmol.100 g tissue^{-1}.min^{-1}) = $\left(TG_a - TG_v\right) \times BF$

Fractional Extraction (%) = $\left(\frac{TG_a - TG_v}{TG_a}\right) \times 100$

TG Clearance (ml.100g tissue^{-1}.min^{-1}) = Fractional Extraction x BF

where TG_a is the arterial concentration of TG (μmol/l), TG_v is the venous concentration of TG (μmol/l) and BF is blood flow (ml.100g tissue^{-1}.min^{-1}). In muscle, blood flow is measured as ml.100ml tissue^{-1}.min^{-1}. Thus, the units for net TG flux (or rate of LPL action) and TG clearance are nmol.100 ml tissue^{-1}.min^{-1} and ml.100ml tissue^{-1}.min^{-1} respectively.

In addition, the net movement of all fatty acids between the plasma and the tissue (transcapillary flux) can be calculated as the difference between NEFA liberated from TG hydrolysis and the NEFA release into or removal from plasma.

$$\text{Transcapillary Flux (μmol/l)} = \left(3 \times \left[TG_a - TG_v\right]\right) - \left(NEFA_v - NEFA_a\right)$$

where, $NEFA_a$ is the arterial concentration of NEFA(μmol/l), $NEFA_v$ is the venous concentration of NEFA(μmol/l). A positive transcapillary flux implies net inward movement of fatty acids across the capillary wall, as would be expected postprandially, whereas a negative transcapillary flux implies a net outward movement of fatty acids, as expected in the fasting state.

Three assumptions are made when applying these various calculations (258). The first is that blood flow is homogenous within the tissue. The second is that there is a steady state in the concentration of metabolites within the arterial and venous blood samples. This is discussed in more detail in Section 3.4. The third is that there is complete hydrolysis of TG molecules into their constituent fatty acids and glycerol. This assumption is supported by the direct measurement of arterio-venous differences

in partial acylglycerols across human adipose tissue, demonstrating that such molecules account for less than two percent of total plasma acylglycerols (259).

2.3.2 Stable isotopes

Fluxes of labelled NEFA and TG were calculated using the same equations as for unlabelled metabolites. In addition, the fate of labelled NEFA and TG can be modelled in order to better understand the differential metabolism of TG dependent on source. Thus, the fractional uptake of [U-^{13}C]palmitate derived from TG hydrolysis by LPL was used to determine the uptake of dietary derived fatty acids, whilst the fractional uptake of non-esterified [$^{2}H_2$]palmitate reflects the uptake of circulating, albumin bound, fatty acids. These calculations are described in detail in Chapter 3. Finally, the rate of appearance of NEFA ($Ra_{(NEFA)}$) gives a whole body estimation of the release of NEFA derived from both intracellular TG stores and following intravascular TG hydrolysis. The details of the calculation of $Ra_{(NEFA)}$ are described in Chapter 5.

2.3.3 Whole body substrate oxidation

Whole body fat and carbohydrate oxidation were calculated from the data obtained by indirect calorimetry. The respiratory exchange ratio (RER) provides an indication of the balance between fat and carbohydrate oxidation and is calculated as CO_2 production divided by O_2 utilisation. The oxidation of 1 mole of glucose produces 6 moles of CO_2 at a cost of 6 moles of O_2 and thus the RER is 1.0. Oxidation of 1 mole of a typical TG molecule utilizes 78 moles of O_2 and produces 55 moles of CO_2 (260) and thus the RER is 0.7. As mentioned, rates of energy expenditure, fat and

carbohydrate oxidation can also be derived from the same data using the following equations (249, 260, 261) -

Fat oxidation (g/min) = $(1.67 \times VO_2) - (1.67 \times VCO_2) - (1.92 \times n)$

Carbohydrate oxidation (g/min) = $(4.55 \times VCO_2) - (3.21 \times VO_2) - (2.87 \times n)$

Energy expenditure (kJ/min) = $(16.32 \times VO_2) + (4.6 \times VCO_2)$

where VCO_2 is the production of CO_2 (l/min), VO_2 is the utilization of O_2(l/min) and n is urinary nitrogen excretion (g/min). For the studies in this thesis, urinary nitrogen was taken as 0.11 mg N.kg body weight^{-1}.hr^{-1} (262).

2.4 Method development

2.4.1 [$^2H_2$]Palmitic acid infusion

In the studies described in this thesis, [$^2H_2$]palmitic acid was used as a tracer of the metabolism of circulating NEFA. The assumption is made that the labeled palmitic acid is treated no differently to endogenous circulating NEFA. In previous studies, several different fatty acids, labeled either with stable or radio-isotopes have been given as infusions to both dogs and humans in order to test the validity of calculations of $Ra_{(NEFA)}$ (241, 263). In all cases, there was no difference in the actual and calculated $Ra_{(NEFA)}$ dependent on either the fatty acid or the type of label used. Furthermore, Evans *et al* used isotopically labeled palmitic and oleic acid to study pathways of NEFA uptake across adipose tissue. Once again, no difference was found between the two tracers (15). It is not possible to directly compare the flux of labeled and unlabeled fatty acids in vivo, however the findings that fluxes are the same irrespective of which fatty acid or what sort of label is used lend support to the assumption that there is likely to be no difference between the metabolic fate of labeled and unlabeled fatty acids.

The simplest and most direct means of labelling the circulating NEFA pool is via an intravenous infusion of labelled fatty acid. However, like all fatty acids, [$^2H_2$]-labelled palmitic acid is highly lipophilic. Fatty acids circulate within blood largely bound to albumin. Thus, in order to make an infusion of labelled fatty acid, it is necessary to complex the [$^2H_2$]-labelled palmitic acid to human albumin (264-266). One of the problems already alluded to in the use of fatty acid infusions is the quantity of labelled fatty acid required to enable the infusion to run at the standard rate of 0.04

$\mu mol.kg^{-1}.min^{-1}$ (244). This was a particular issue for the current studies as many subjects were overweight, necessitating relatively large total masses of isotope. Stable isotopes are very expensive, however the main concern was over the quantity of albumin required. *In vitro*, each molecule of albumin binds approximately six molecules of fatty acid (267-269). If there is too little albumin in the infusion, the fatty acids will precipitate. However, it is not ideal simply to increase the total volume infused as albumin augments the circulating plasma volume (270) and rapid infusion of very high volumes of albumin theoretically results in a risk of hypervolaemic complications. In order to circumvent these problems I developed a technique for ensuring an adequate mass of albumin in a reasonable infusion volume. I calculated the maximum quantity of isotope that could be complexed to the albumin in a standard 500 ml bottle of 4.5% human albumin solution (HAS), using a FA:Albumin molar ratio of 6:1 in my calculations. However, I was aware that fatty acid binding to albumin depends not only on the total quantity, but also on the species of fatty acid (268, 271). Furthermore, HAS already contains five millimoles of octanoic acid which would clearly have an effect on the quantity of palmitic acid that could be added to the albumin. Therefore, I tested the theoretical calculation practically by making up such a solution. I then used this information to determine the maximum weight of subjects permissible in order to run the infusion at the aforementioned rate.

The calculations are summarised as follows: 500 ml of 4.5% HAS contains 22.5 g of albumin (0.331 mmol). Assuming a FA:Albumin ratio of 6:1, the maximum amount of $[^2H_2]$-labelled palmitic acid that could be complexed in one bottle was 1.99 mmol (588 mg). When I made up the infusion with increasing masses of unlabelled palmitic acid, FA precipitation was, in fact, observed when 500 mg (1.69 mmol) of

palmitic acid was added to the 4.5% albumin. The study protocol dictates that the infusion needs to run at 0.04 μmol.kg^{-1}.min^{-1} (244) for at least 7 hours. Thus, the total amount of isotope required is a product of the infusion rate, the weight of the subject and the total time (0.04 x weight x 420). If maximum amount of isotope is 1.69 mmol:

$$\text{Maximum permissible subject weight} = \frac{1.69}{0.04 \times 420} = 100.6 \text{ kg}$$

As central obesity was one of the recruitment criteria, it was likely that there would be subjects weighing in excess of 100.6 kg. HAS is also available as 100 ml bottles of 20%. I therefore withdrew 100 ml of 4.5% HAS from the bottle and replaced it with 100 ml of 20% HAS. The result was a single 500 ml bottle containing 38 g of albumin and the facility to make an infusion for subjects weighing anything up to 166.3 kg. Once again, this was confirmed practically by making up an infusion with unlabelled palmitic acid. As I did not foresee studying subjects weighing over 140 kg, the infusion was made with a maximum of 700 mg of palmitic acid. No fatty acid precipitation was seen. Thus, I was able to maintain a smaller total volume of infusion but make an infusion that allowed studies of very obese subjects.

2.5 Statistical analysis

For all the studies presented in this thesis, data were analysed using SPSS for Windows v11 (SPSS UK, Chertsey, U.K.). Statistical significance was set at $P<0.05$. All data are presented as means±SEM unless otherwise stated. SEM is used in preference to standard deviation for most of the data as the intention is not to describe the variability amongst groups but to describe the precision of the data means obtained. The statistical tests used in each study are described in the relevant chapters.

Chapter 3 : Preferential uptake of dietary fatty acids in adipose tissue and muscle in the postprandial period

3.1 Background

Disturbances in fat metabolism, most notably raised plasma concentrations of NEFA and TG, have been implicated in the pathophysiology of type 2 diabetes and consequent cardiovascular disease (184, 185, 272).

Increased deposition of TG in tissues outside adipose tissue is intimately associated with the development of insulin resistance, the so-called 'fat overflow' hypothesis (273). The pathways by which fatty acids are delivered from, and taken up by, tissues are not well understood even in healthy subjects. In the fasting state lipolysis of stored TG in adipocytes delivers NEFA to the plasma, from where they are taken up by the consuming tissues, mainly skeletal muscle, heart and the liver. However, even in the fasting state, lipoprotein lipase (LPL)-mediated intravascular lipolysis of circulating TG may contribute significantly to NEFA release from adipose tissue (274). In skeletal muscle the relative importance of fatty acid uptake from the action of LPL on circulating TG and of direct NEFA uptake is not well understood. Some studies suggest a major contribution of TG-derived fatty acids (15), whereas others have suggested that this contribution is unmeasurably small (275). In the postprandial state, the situation is further complicated by the delivery of dietary fat as chylomicron-TG, and the suppression of intracellular lipolysis in adipose tissue by insulin. Many studies have suggested that chylomicron-TG is the preferred substrate for LPL and competes with VLDL-TG for lipolysis (12-14). This has not been shown conclusively *in vivo* because of the difficulties of complete separation of chylomicron and VLDL particles. Again, the handling of these sources of TG in muscle is unclear. Direct uptake of NEFA by adipose tissue has been shown during glucose infusion

(240), but it is not known whether this is a physiologically-important pathway for fat deposition in adipose tissue.

I have used a combination of differential stable-isotope labeling of endogenous and meal-derived fatty acids, with arterio-venous tracer and tracee concentration difference measurements across adipose tissue and muscle, to probe the components of fat metabolism in the fasted and fed states. I aimed to quantify the uptake into adipose tissue and skeletal muscle of fatty acids (FA) derived from the following three sources: chylomicron-TG, labelled with [U-^{13}C]palmitic acid, VLDL-TG, labelled endogenously with [$^{2}H_2$]palmitic acid, and circulating NEFA, labelled with [$^{2}H_2$]palmitic acid. In addition, I wished to resolve definitively the issue of lipoprotein species preference as a substrate for LPL hydrolysis.

3.2 Subjects, Materials and Methods

Much of the detail of the methods has already been described in chapter 2. Thus, a brief synopsis only will be presented here with any relevant additional details.

3.2.1 Subjects and Study Protocol

Fourteen healthy male volunteers were studied following an overnight fast. Subjects attended the Clinical Research Unit on one occasion only. Serial blood samples were taken in the fasting state and for 6 hours following consumption of a mixed meal as shown in figure 2.3. Blood was sampled from the femoral artery and veins draining forearm muscle and subcutaneous abdominal adipose tissue in order to measure arterio-venous differences in metabolite concentrations across the tissues.

Subcutaneous abdominal adipose tissue blood flow was measured by ^{133}Xe washout (231). Forearm muscle blood flow was assessed by venous occlusion strain-gauge plethysmography with an inflated wrist cuff (222). Blood flow was measured whenever blood was sampled. The subjects ingested [U-^{13}C]palmitic acid (isotope purity 98%, CK Gas Products Ltd, Hampshire, UK) as part of the meal and were administered an intravenous infusion of [$^{2}H_2$]palmitic acid (isotope purity 97%, CK Gas Products Ltd, Hampshire, UK) complexed to human albumin (infusion rate 0.04 μmol/kg/min). The former acted as a tracer of dietary-derived fatty acids whilst the latter labelled the circulating NEFA pool.

3.2.2 Analyses

Plasma TG, NEFA, glucose, glycerol and 3-hydroxybutyrate concentrations were measured enzymatically. Plasma insulin was measured by radio-immunoassay. Fatty acid composition and isotopic enrichment were measured by GC and GC-MS respectively.

3.2.3 Calculations

Arterio-venous (A-V) and veno-arterial (V-A) differences in all metabolite concentrations (labelled and unlabelled) in whole blood were calculated across adipose tissue and skeletal muscle. Simple and derived flux calculations were made as described in section 2.3.1. In addition, I modelled the fate of FA uptake dependent on source as follows.

3.2.3.1 Fractional uptake of [U-^{13}C]palmitate derived from TG hydrolysis by LPL (Fig. 3.1)

The A-V difference of [U-^{13}C]palmitate in the TG fraction represents the amount of labelled palmitate that is released from lipolysis of [U-^{13}C]-labelled TG by LPL. In adipose tissue, a proportion of the fatty acids (FAs) liberated by LPL will 'spill over' into the systemic circulation (15). This spillover can be measured as the V-A difference of non-esterified [U-^{13}C]palmitate. The difference between the FAs liberated from TG and those passing into the systemic circulation represents chylomicron-FA uptake into adipose tissue. Thus the fractional uptake, or "percentage entrapment" (15), of [U-^{13}C]palmitate into adipose tissue can be calculated as:

Eqn 1. Fractional uptake of [U-^{13}C]palmitate derived from hydrolysis of chylomicron-TG

$$= 100 \times \frac{\text{A - V}[^{13}\text{C}]\text{TG}_{\text{FA}} \; - \; \text{V - A}[\text{U-}^{13}\text{C}]\text{palmitate}}{\text{A - V}[^{13}\text{C}]\text{TG}_{\text{FA}}}$$

where A-V[^{13}C]TG$_{FA}$ is the A-V difference in concentration of labelled palmitate in the TG fraction and V-A[U-^{13}C]palmitate is the V-A difference in concentration of labelled non-esterified palmitic acid. This calculation was also performed for muscle, albeit with the expectation that all FA liberated from TG by LPL would be taken into the tissue (15).

3.2.3.2 Fractional uptake of non-esterified [$^{2}H_2$]palmitate (Fig. 3.2)

[$^{2}H_2$]Palmitate was infused to act as a tracer of both the plasma NEFA pool and of the VLDL-TG (produced in the liver after hepatic uptake of labelled plasma NEFA). In calculating the fractional uptake of [$^{2}H_2$]palmitate from the NEFA pool, the contribution of [$^{2}H_2$]palmitate derived from hydrolysis of [$^{2}H_2$]-labelled TG and escaping tissue uptake ('spillover') must be taken into account. In muscle the

assumption is made that all FAs derived from TG hydrolysis are taken up; however, the same is not true for adipose tissue (5). The fractional uptake of FA derived from VLDL-TG hydrolysis has never been measured in adipose tissue. Thus three models were used to encompass the range of possibilities. The first model, which was also used for the calculation of fractional uptake across muscle, assumes that all of the [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$] labelled TG is taken up into the tissue (0% spillover). Thus, the A-V difference in [$^2H_2$]palmitate represents uptake across the tissue and the fractional uptake can be expressed as:

Eqn 2. Fractional uptake of non-esterified [$^2H_2$]palmitate (**Model 1**)

$$= 100 \times \frac{\text{A - V}[^2\text{H}_2]\text{palmitate}}{\text{Art}[^2\text{H}_2]\text{palmitate}}$$

where Art [$^2H_2$]palmitate is the arterial concentration of [$^2H_2$]palmitate. The second model assumes that none of the [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$]-labelled TG is taken up into the tissue (100% spillover). The A-V difference in [$^2H_2$]TG_{FA} is a measure of the [$^2H_2$]palmitate derived from the hydrolysis of labelled TG. Thus, A-V[$^2H_2$]TG_{FA} is subtracted from the venous [$^2H_2$]palmitate concentration and the fractional uptake can be calculated as:

Eqn 3. Fractional uptake of non-esterified [$^2H_2$]palmitate (**Model 2**)

$$= 100 \times \frac{\text{Art}[^2\text{H}_2]\text{palmitate} - (\text{Ven}[^2\text{H}_2]\text{palmitate} - \text{A - V}[^2\text{H}_2]\text{TG}_{\text{FA}})}{\text{Art}[^2\text{H}_2]\text{palmitate}}$$

where Ven [$^2H_2$]palmitate is the venous concentration of [$^2H_2$]palmitate.

The final model assumes that the uptake of [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$]-labelled TG is identical to the uptake of [^{13}C]palmitate derived from the hydrolysis of chylomicron-TG, calculated above (i.e. VLDL spillover equal to chylomicron spillover). Therefore,

Eqn 4. Uptake of [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$] labelled TG

$$= (1 - \frac{\text{Eqn 1}}{100}) \times \text{A - V}[^2\text{H}_2]\text{TG}_{\text{FA}}$$

Thus, the fractional uptake can be calculated as:

Eqn 5. Fractional uptake of non-esterified [$^2H_2$]palmitate (**Model 3**)

$$= 100 \times \frac{\text{Art}[^2\text{H}_2]\text{palmitate} - (\text{Ven}[^2\text{H}_2]\text{palmitate} - \text{Eqn 4})}{\text{Art}[^2\text{H}_2]\text{palmitate}}$$

3.2.3.3 Total unidirectional fatty acid fluxes

To assess the quantitative significance of unidirectional FA uptake from the circulating NEFA pool across adipose tissue, total NEFA uptake was calculated from the fractional uptake of labelled palmitic acid. Uptake of FAs liberated from TG by LPL could be differentiated from uptake of FAs from the circulating NEFA pool by using the fractional uptake of the appropriate isotope of palmitic acid (i.e. [U-^{13}C]palmitate for FAs from TG hydrolysis and [$^2H_2$]palmitate for circulating FAs).

3.2.3.3.1 TG derived FAs.

The total uptake of palmitate derived from TG hydrolysis was taken as the product of the fractional uptake of LPL-derived [U-^{13}C]palmitate (as in Eqn 1) and the A-V difference in TG-palmitate concentration (i.e. total palmitate made available by LPL). The uptake of total FAs derived from TG hydrolysis was then calculated as the total palmitate uptake divided by the fraction of palmitate in the "pool" of FAs derived from TG hydrolysis, expressed as a percentage. The latter fraction was calculated as follows: The A-V difference in total TG concentration determined the total FAs derived from TG hydrolysis available for uptake. Similarly, the A-V difference in palmitate in the TG fraction was the palmitate derived from TG hydrolysis available for uptake. Dividing the latter by the former determined what

fraction of total FAs derived from TG hydrolysis was made up of palmitate. The calculations can be summarized as:

Eqn 6. Uptake of palmitate derived from TG hydrolysis = $\text{Eqn1} \times \text{A - V TG - palmitate}$

Eqn 7. Palmitate fraction of FAs liberated by TG hydrolysis = $\frac{\text{A - V TG - palmitate}}{\text{A - V TG}}$

Eqn 8. Uptake of total FA from TG hydrolysis = $\frac{\text{Eqn6}}{\text{Eqn7}}$

where A-V TG-palmitate is the A-V difference in TG-palmitate concentration and A-V TG is the A-V difference in TG concentration. This calculation makes two assumptions: Firstly, that, in terms of uptake, adipose tissue does not discriminate between FAs dependent on the TG from which they derive, i.e. FAs liberated from chylomicrons are treated exactly analogously to FAs derived from VLDL (equal spillover). This is the same assumption made in the final model of [$^2H_2$]palmitate uptake above. Secondly, the calculation assumes complete hydrolysis of TG.

3.2.3.3.2 Circulating NEFA.

A similar calculation was made for the uptake of FAs from the circulating NEFA pool. The total palmitate uptake was calculated as a product of the fractional uptake of [$^2H_2$]palmitate, using the third model described above, and the arterial palmitate concentration. The total NEFA uptake was then calculated as the total palmitate uptake divided by the fraction of palmitate in the arterial NEFA pool, expressed as a percentage. The calculations can be summarized as:

Eqn 9. Total non-esterified palmitate uptake = $\text{Eqn5} \times \text{Art palmitate}$

Eqn 10. Fraction of palmitate in NEFA pool = $\frac{\text{Art palmitate}}{\text{Art NEFA}}$

Eqn 11. Total NEFA uptake $= \frac{\text{Eqn9}}{\text{Eqn10}}$

where Art NEFA is the arterial concentration of NEFA.

3.2.4 Statistical methods.

Repeated measures ANOVA with time and, when appropriate, tissue or isotope as within-subject factors was used to identify time effects, differences between tissues, isotope effects, time and tissue interactions and time and isotope interactions. The difference between fasting muscle and adipose tissue TG extraction was assessed by paired t-test.

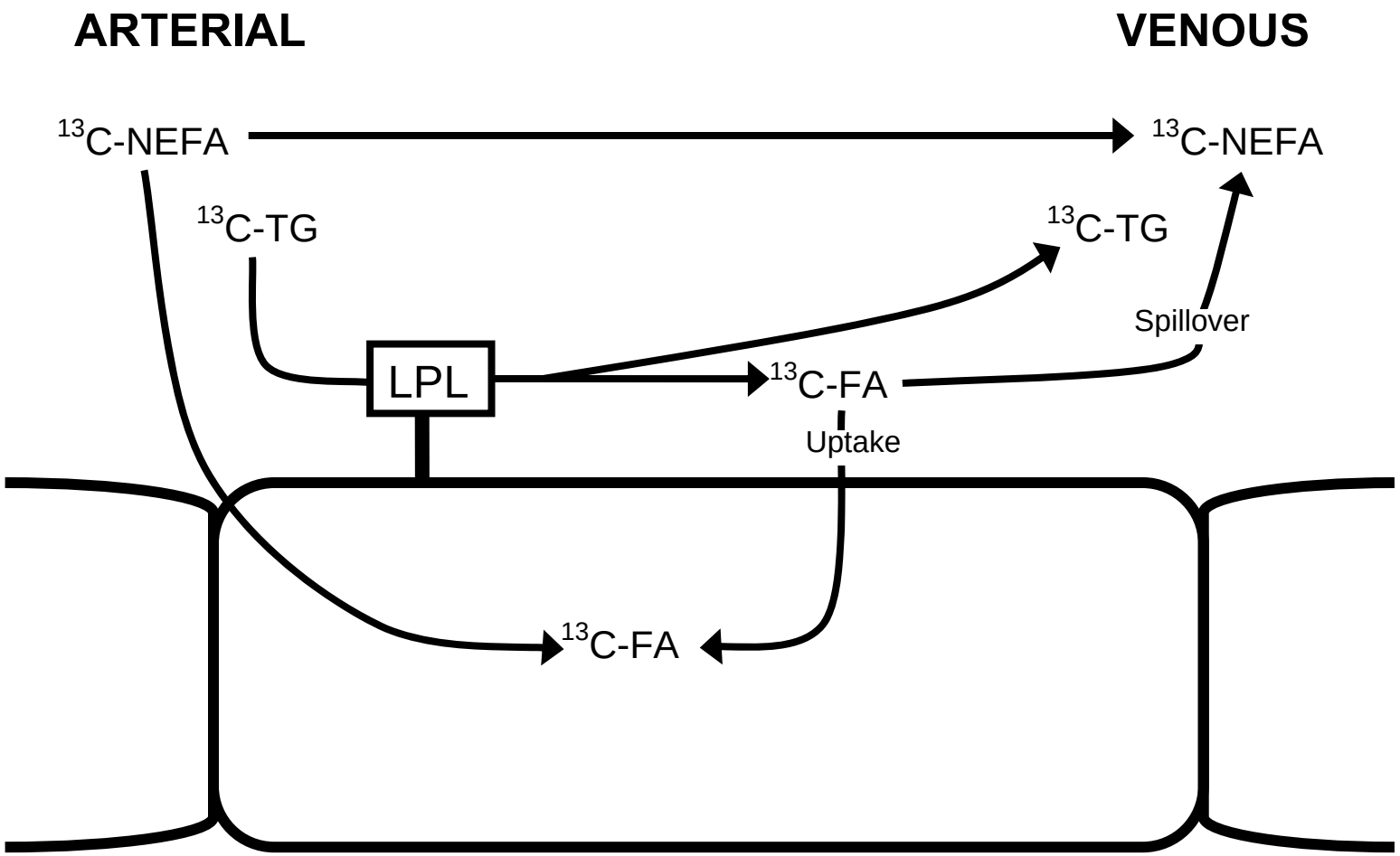

Figure 3.1 Movements of ^{13}C-labelled (dietary-derived) TG and NEFA across tissues. This model provides the basis for the calculation of the fractional uptake of [U-^{13}C]palmitate derived from hydrolysis of ^{13}C-labelled TG by LPL. As discussed in chapter 2, for the early postprandial period, ^{13}C-labelled TG is found entirely within chylomicrons. ^{13}C-NEFA represents ^{13}C-labelled NEFA within the circulating NEFA pool, ^{13}C-FA represents ^{13}C-labelled fatty acid, ^{13}C-TG represents ^{13}C-labelling of fatty acids within TG. In the current study [U-^{13}C]palmitic acid was used as the tracer.

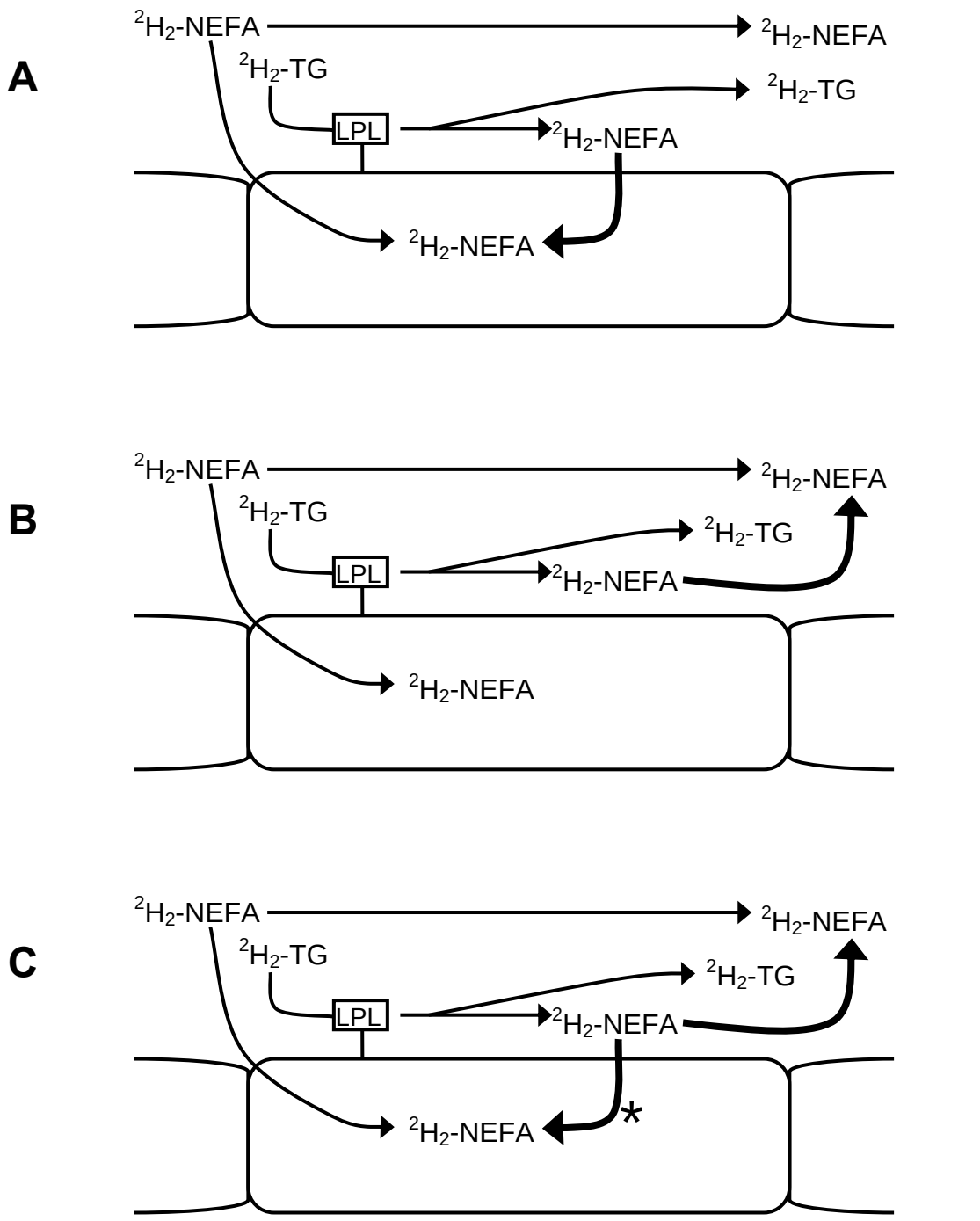

Figure 3.2 Movements of $^2H_2$-labelled TG and NEFA across tissues. These figures illustrate the different models used to calculate the fractional uptake of non-esterified [$^2H_2$]palmitate across adipose tissue and muscle: A: Model 1 assumes that all of the [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$] labelled TG is taken up into the tissue (0% spillover). This was the only model used for muscle. B: Model 2 assumes that none of the [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$]-labelled TG is taken up into the tissue (100% spillover). C Model 3 assumes that the uptake of [$^2H_2$]palmitate derived from hydrolysis of [$^2H_2$]-labelled TG is identical to the uptake of [^{13}C]palmitate derived from the hydrolysis of chylomicron-TG, calculated above (i.e. VLDL spillover equal to chylomicron spillover) and represented as a star on the figure. As discussed in chapter 2, $^2H_2$-labelled TG is found principally within VLDL. Analagously to Fig. 3.1, $^2H_2$-NEFA represents $^2H_2$-labelled NEFA within the circulating NEFA pool, $^2H_2$-FA represents $^2H_2$-labelled fatty acid, $^2H_2$-TG represents $^2H_2$-labelling of fatty acids within TG. In the current study [$^2H_2$]palmitic acid complexed to albumin was infused to act as a tracer within the circulating NEFA pool.

3.3 RESULTS

The clinical characteristics of the subjects are shown in Table 3.1. A summary of the principal study results is presented in Table 3.2. The test meal was given at time 0.

3.3.1 Tissue blood flow and unlabelled metabolites

Adipose tissue blood flow was greater than forearm muscle blood flow, with no statistically significant change in blood flow through either tissue following the test meal (Fig. 3.3). Arterial TG concentrations rose postprandially (Fig. 3.4A). During the fasting period, absolute extraction of TG (A-V difference multiplied by blood flow) was higher across adipose tissue than across muscle (Table 3.2; P=0.011). Following the meal, absolute extraction of TG increased in both adipose tissue and muscle (both P<0.001). Similarly, fractional extraction of TG (A-V difference divided by arterial concentration) was higher during fasting in adipose tissue than in muscle; however, there was no statistical difference between the tissues postprandially (Table 3.2).

Arterial NEFA concentrations fell during the early postprandial period before rising again towards the end of the study (Fig. 3.4B). There was net NEFA uptake across muscle (A-V difference multiplied by blood flow) throughout the study period (Figure 3.5A, Table 3.2), similar to previous studies (15). Net adipose tissue NEFA output (V-A difference multiplied by blood flow) fell following the meal and then increased again later in the postprandial period (Figure 3.5B), similarly to previous studies (15).

3.3.2 Labelled TG and NEFA

3.3.2.1 [^{13}C]-labelled TG and NEFA

[^{13}C]-labelled (dietary) TG appeared in plasma at 60 min and the concentration peaked at 240 min (P<0.001) (Fig. 3.6A). Extraction of [^{13}C]-labelled TG across adipose tissue and muscle was detectable from 60 minutes. Absolute extraction peaked at 120 minutes in adipose tissue and at 180 minutes in muscle. There was greater absolute extraction of [^{13}C]-labelled TG across adipose tissue than across muscle (P=0.017) (Fig. 3.7).

[U-^{13}C]palmitate also appeared in the plasma NEFA pool from 60 minutes but reached a peak concentration later, at 300 minutes (Fig. 3.6B). The arterial plasma [U-^{13}C]palmitate concentration rose between 3h and 5h as the [^{13}C]-labelled TG concentration was falling. In muscle, there was a net uptake of dietary derived FAs throughout the postprandial period. In contrast, there was net efflux of [U-^{13}C]palmitate from adipose tissue, which increased during the study (P<0.001). This must reflect release of dietary FAs, derived from chylomicron hydrolysis, into the systemic circulation (Fig. 3.8A). Increasing release of [U-^{13}C]palmitate from adipose tissue in the later postprandial period would reflect decreasing entrapment of the LPL-derived fatty acids in the tissue, as has been previously described (15).

3.3.2.2 [$^{2}H_2$]-labelled NEFA and TG

The arterial concentration of [$^{2}H_2$]palmitate (the infused tracer) and the tracer-tracee ratio (TTR) did not vary before the meal (Fig. 3.9A), implying that steady state conditions had been reached prior to any blood sampling. Arterial, forearm venous and adipose venous [$^{2}H_2$]palmitate concentrations fell postprandially, but returned to baseline by the end of the study (all P<0.001). I examined the arterio-venous

differences for the [$^2H_2$]palmitate tracer. There was no significant flux of [$^2H_2$]palmitate across adipose tissue during fasting (mean 0.132 nmol.100g^{-1}min^{-1}, 95% CI -0.61 - 0.87) or at 360 minutes (mean 1.67 nmol.100g^{-1}min^{-1}, 95% CI -0.42 - 3.76). However, during the postprandial period there was a net uptake of NEFA by adipose tissue from the circulating NEFA pool from 60 to 300 minutes (Fig. 3.8B). This shows an uptake of plasma NEFA by adipose tissue in the postprandial period, despite an overall net release of NEFA from adipose tissue. There was consistent uptake of circulating NEFA by muscle during the study, which did not vary depending on prandial state (P=0.19) (Fig.3.8B).

[$^2H_2$]-labelled TG was detected in the first blood samples, taken one hour before the meal (mean concentration at T-60 0.19 μmol/l, 95% CI 0.11 - 0.28) and increased consistently throughout the study (P<0.001) (Fig. 3.6A). This reflects incorporation of the infused [$^2H_2$]palmitate tracer into VLDL in the liver. I examined the arterio-venous differences for the [$^2H_2$]-labelled TG as a marker of tissue handling of VLDL-TG. Extraction of [$^2H_2$]-labelled TG was detectable across both adipose tissue and muscle from 60 min (Fig. 3.10A, B). There was no difference in absolute or fractional extraction of [$^2H_2$]-labelled TG between the two tissues (P=0.32, P=0.90 respectively).

3.3.2.3 Comparison of dietary (^{13}C-labelled) and endogenous (^{2}H-labelled) fatty acid handling in adipose tissue and muscle

3.3.2.3.1 Tracer-tracee ratios

As expected, the venous TTR (isotopic enrichment) of [$^{2}H_2$]palmitate was significantly lower than the arterial TTR across adipose tissue (P<0.001) (Fig. 3.9A) because the circulating tracer is diluted by unlabelled fatty acids released from adipose tissue lipolysis. However, an unexpected and striking finding was that the muscle venous TTR of [$^{2}H_2$]palmitate was also significantly lower than the arterial TTR (P<0.001) (Fig. 3.9A). This must imply the release of FAs not labelled with [$^{2}H_2$]palmitate across both tissues. In contrast, when I examined the differences in TTR of [U-^{13}C]palmitate across muscle or adipose tissue, there were no consistent differences in TTR (Fig. 3.9B).

3.3.2.3.2 Labelled TG

Assuming that [^{13}C]-labelled TG is a marker of chylomicron-TG (at least in the early postprandial period) and that [$^{2}H_2$]-labelled TG is a tracer for VLDL-TG, it is possible to compare the handling of these two lipoproteins in the tissues. The fractional extraction of [^{13}C]-labelled TG (marking chylomicrons) was significantly higher across both adipose tissue and muscle than that of [$^{2}H_2$]-labelled TG (marking VLDL) (P<0.001 for adipose tissue and muscle) (Fig. 3.10A, B), implying a preferential hydrolysis of chylomicron-TG over VLDL-TG by LPL in the postprandial period. There was no difference in fractional extraction between the tissues (adipose tissue versus muscle) (Fig. 3.10A, B, data in Table 3.2; P=0.62).

3.3.2.3.3 Labelled FAs

I asked the question: once fatty acids have been liberated from circulating TG by LPL, do they effectively mix with circulating NEFA, or is there preferential uptake by tissues? I calculated the fractional uptake of [^{13}C]palmitate derived from the LPL-mediated hydrolysis of [^{13}C]-labelled TG (Fig. 3.11). This graph may be interpreted as follows: at 60 min after the meal, 87% (SEM 4%) of chylomicron-palmitate liberated via LPL was taken up into adipose tissue, decreasing to 48% (SEM 12%) by 360 min. This fractional uptake of chylomicron-TG palmitate liberated by LPL was consistently greater than that of non-esterified [$^{2}H_2$]palmitate across adipose tissue, irrespective of which model for fractional uptake of [$^{2}H_2$]palmitate originating from hydrolysis of [$^{2}H_2$]-labelled TG across adipose tissue was used (P<0.001 for models 1, 2 and 3) (Fig. 3.11). (These models set outer boundaries for the spillover of VLDL-derived [$^{2}H_2$]-labelled fatty acids.) Thus, in adipose tissue, FAs derived from TG hydrolysis are more likely to be taken up than FAs from the circulating NEFA pool. Given the demonstration of NEFA uptake by adipose tissue in the postprandial period (Fig. 3.8B) I now asked: what is the quantitative importance of this route for adipose tissue fat accumulation in the postprandial period? I therefore calculated the (unidirectional) uptake of total FA derived from TG hydrolysis and compared this with the unidirectional uptake of FAs from the circulating NEFA pool (Fig. 3.12). TG-derived fatty acid uptake was significantly greater than NEFA uptake (P=0.001).

Table 3.1 Subject characteristics

	Mean	Range
Age (yrs)	43	32-54
Weight (kg)	87	73-102
BMI (kg/m^2)	28	23-34
Systolic BP (mmHg)	127	104-143
Diastolic BP (mmHg)	82	73-96
Fasting plasma concentrations		
Glucose (mmol/l)	5.0	4.4-6.0
Insulin (mU/l)	12.2	4.9-22.6
Triglyceride (mmol/l)	1.8	0.7-3.3
HDL-cholesterol(mmol/l)	1.0	0.9-1.7

Table 3.2 Summary of principal study results

	Fasting	Postprandial Period			ANOVA	
		Early	**Mid**	**Late**	**Variable**	**P**
Blood Flow						
Adipose (ml/100g/min)	2.8 ± 0.3	3.0 ± 0.4	2.3 ± 0.2	2.9 ± 0.3	Tissue	0.016
Muscle (ml/100ml/min)	1.8 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	2.2 ± 0.3		
Net NEFA Flux (nmol/100g/min)						
Adipose (Output)	1080 ± 206	423 ± 71	231 ± 33	858 ± 166	Time	<0.001
Muscle (Uptake)	43 ± 31	17 ± 21	69 ± 19	82 ± 32	Time	0.065
NEFA Uptake across Adipose Tissue (nmol/100g/min)						
LPL derived FA	N/A	371 ± 60	380 ± 82	278 ± 52	FA Source	0.001
FA from NEFA pool	N/A	82 ± 23	183 ± 41	-27 ± 54		
Absolute TG Extraction (nmol/100g/min)						
Adipose	120 ± 27	132 ± 20	158 ± 38	146 ± 31	Tissue (fasting)	0.011
Muscle	32 ± 24	113 ± 21	182 ± 41	41 ± 16	Tissue (post-prandial)	0.065
Fractional TG Extraction (%)						
Adipose	5 ± 1	5 ± 1	6 ± 1	6 ± 1	Tissue (fasting)	0.048
Muscle	1 ± 1	6 ± 1	6 ± 1	2 ± 1	Tissue (post-prandial)	0.243
Fractional Extraction Labelled TG across Adipose Tissue (%)						
[^{13}C]	N/A	22 ± 4	11 ± 3	12 ± 3	Isotope	<0.001
[$^{2}H_2$]	N/A	3 ± 2	6 ± 1	4 ± 2		
Fractional Extraction Labelled TG across Forearm (%)						
[^{13}C]	N/A	23 ± 3	10 ± 2	6 ± 2	Isotope	<0.001
[$^{2}H_2$]	N/A	7 ± 1	5 ± 1	2 ± 1		
Fractional Uptake Labelled Palmitate across Adipose Tissue (%)						
[^{13}C]	N/A	83 ± 4	72 ± 5	55 ± 8		
[$^{2}H_2$]						
Model 1		16 ± 6	25 ± 5	-7 ± 9	Isotope	<0.001
Model 2		36 ± 8	63 ± 8	16 ± 15	Isotope	<0.001
Model 3		20 ± 6	35 ± 6	6 ± 12	Isotope	<0.001
Fractional Uptake Labelled Palmitate across Forearm (%)						
[^{13}C]	N/A	103 ± 2	104 ± 3	101 ± 6	Isotope	<0.001
[$^{2}H_2$]	39 ± 4	42 ± 4	46 ± 4	30 ± 4		

The data are expressed as means ± SEM for fasting and postprandial states. The postprandial phase is divided into 2 hour periods; early postprandial (0 - 2 hours), mid postprandial (2 - 4 hours) and late postprandial (4 - 6 hours). Repeated measures analysis of variance (ANOVA) has been used to calculate significant differences between data sets. The parameters under comparison are described in the "variable" column. "tissue" implies a comparison of adipose tissue and muscle, "time" implies a calculation of time effect, "FA source" implies a comparison of LPL-derived and circulating FAs, "isotope" implies a comparison of [^{13}C] and [$^{2}H_2$] labelled TG or FA. ANOVA calculations refer to the whole study period unless otherwise stated.

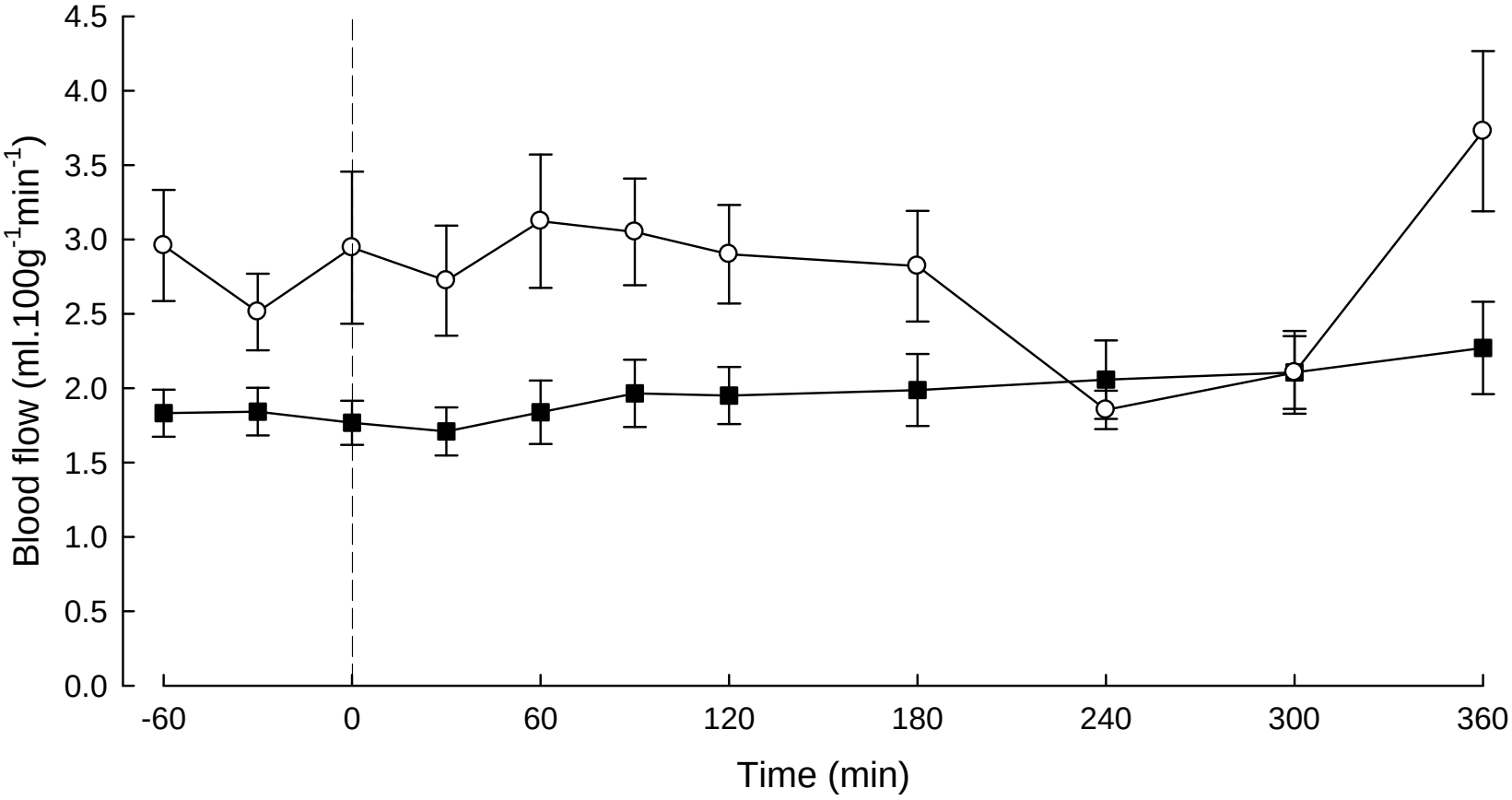

Figure 3.3 Adipose tissue (open circles) and muscle (solid squares) blood flow following a mixed meal. Although there is a small fall in adipose tissue blood flow at 4 and 5 hours, repeated measures analysis of variance revealed no significant time effect in the blood flow in either tissue. Taking the study as a whole, adipose tissue blood flow was greater than muscle blood flow (P=0.016)

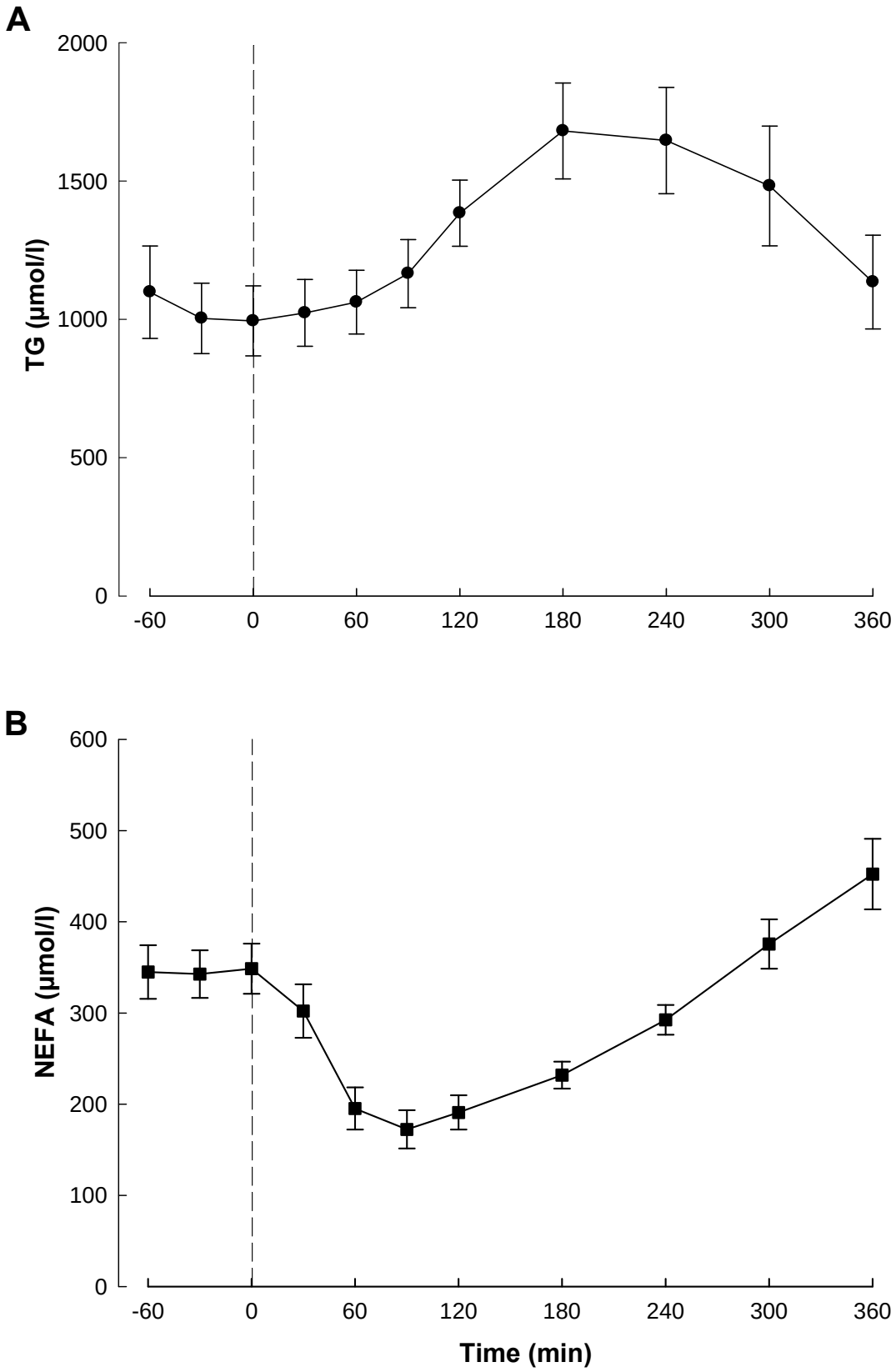

Figure 3.4 Whole plasma arterial concentrations of TG (A) and NEFAs (B) following a mixed meal.

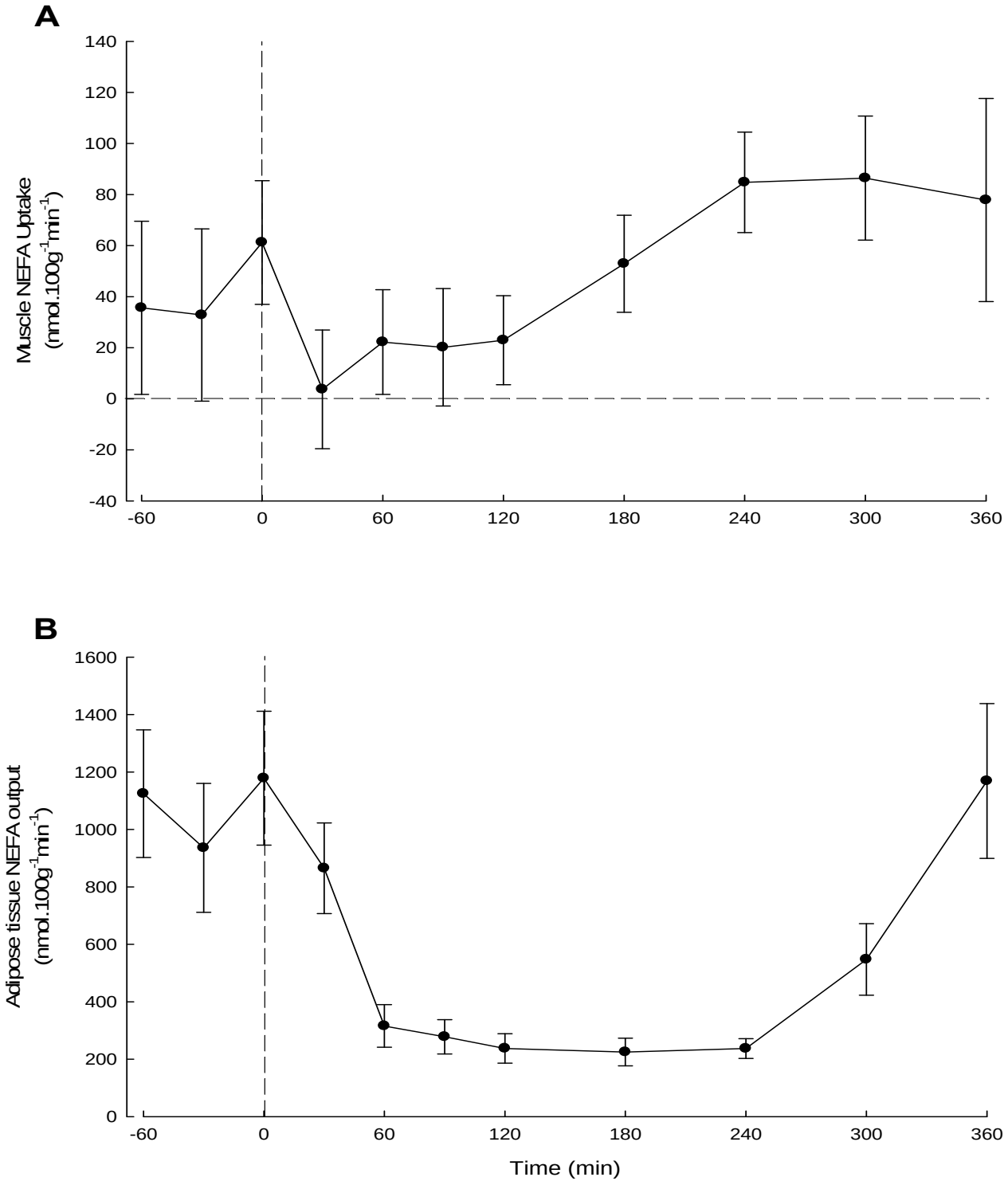

Figure 3.5 Net NEFA fluxes following a mixed meal: A: Uptake across muscle: B: Release across adipose tissue.

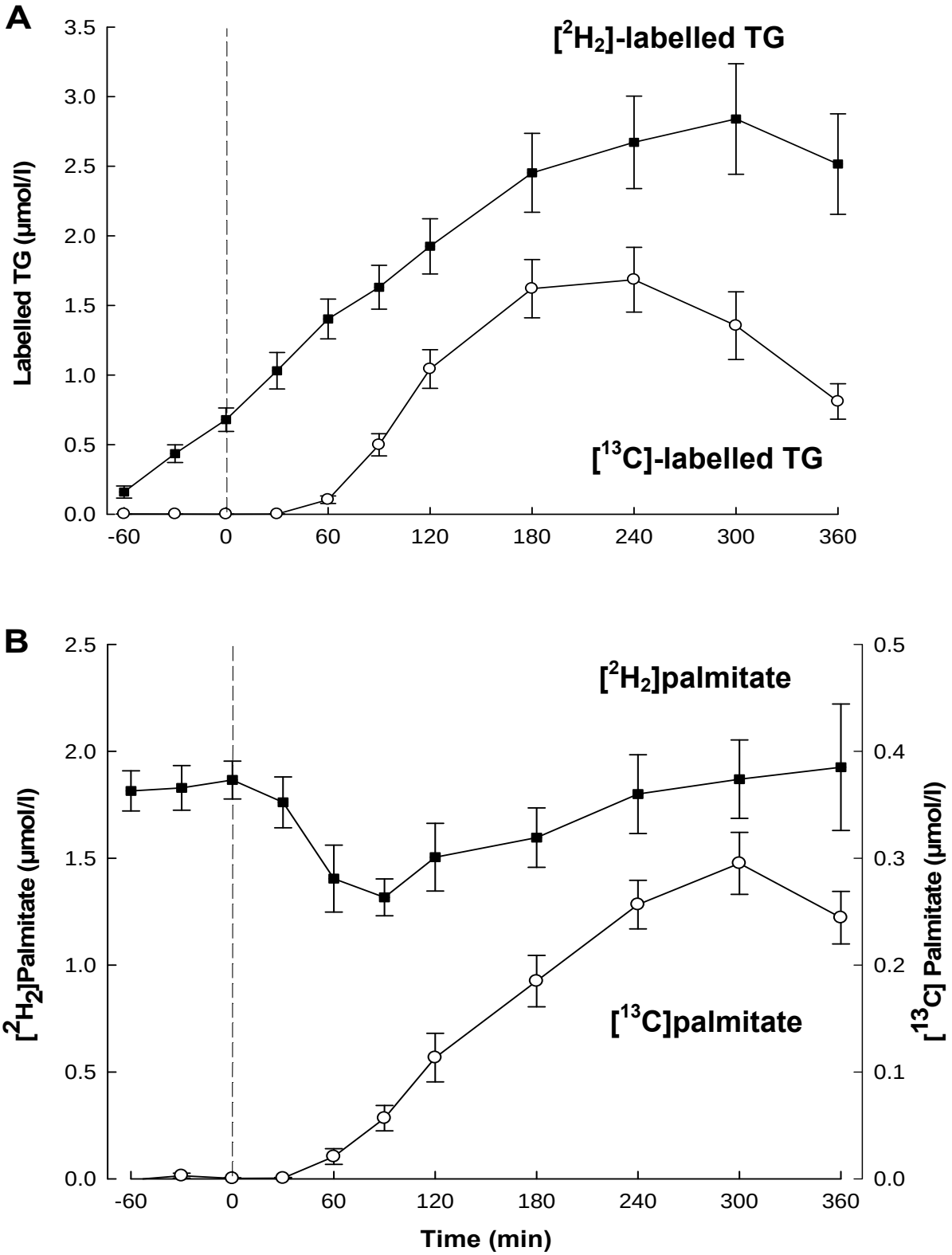

Figure 3.6 Labelled TG and NEFA following a mixed meal. **A**: TG labelled with [$^2H_2$]palmitic acid (solid squares) or [U-^{13}C]palmitic acid (open circles). **B**. Non-esterified [$^2H_2$]palmitic acid (solid squares) and [U-^{13}C]palmitic acid (open circles)

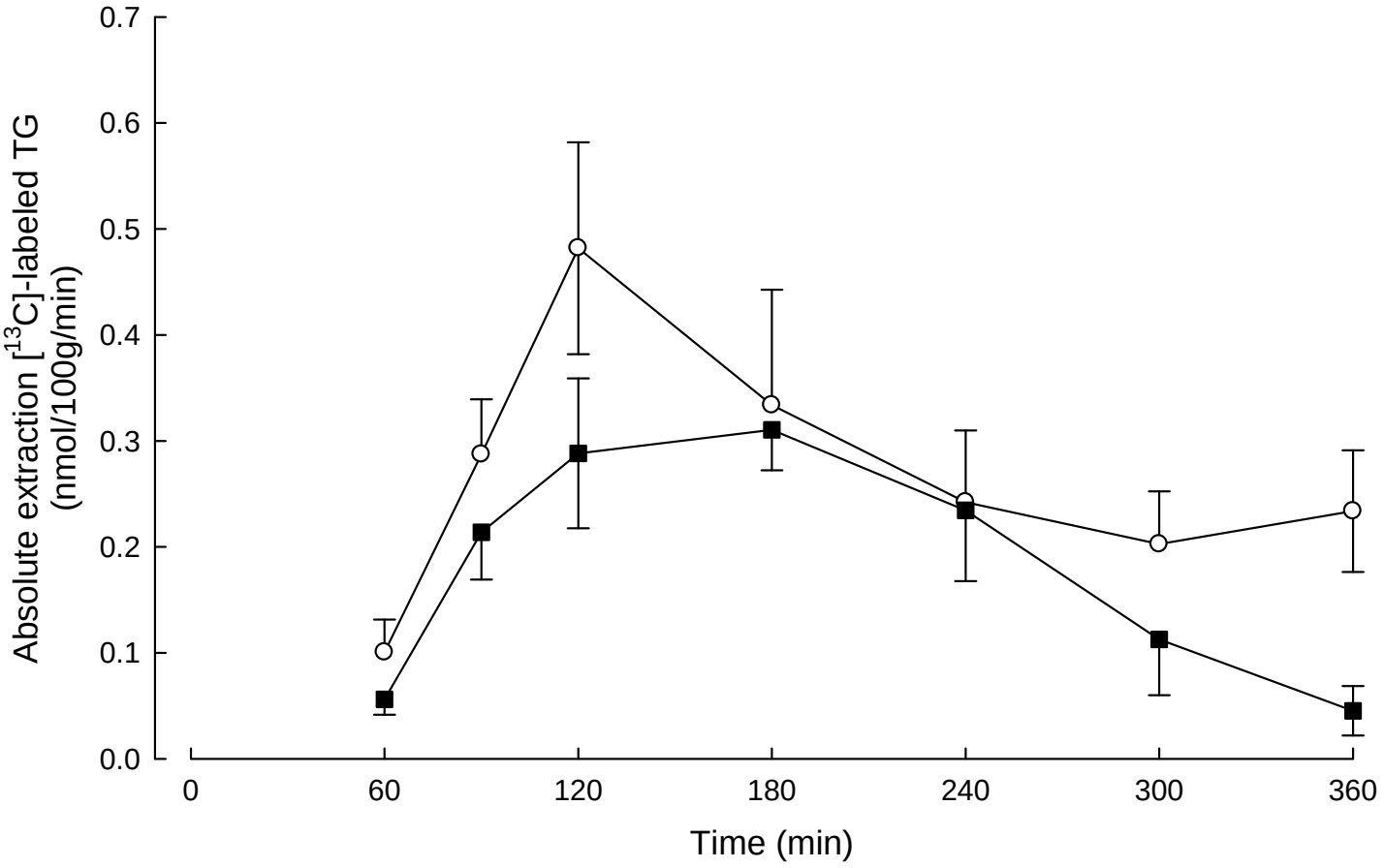

Figure 3.7 Fractional extraction of [^{13}C]-labelled TG across adipose tissue (AT) (open circles) and muscle (M) (solid squares).

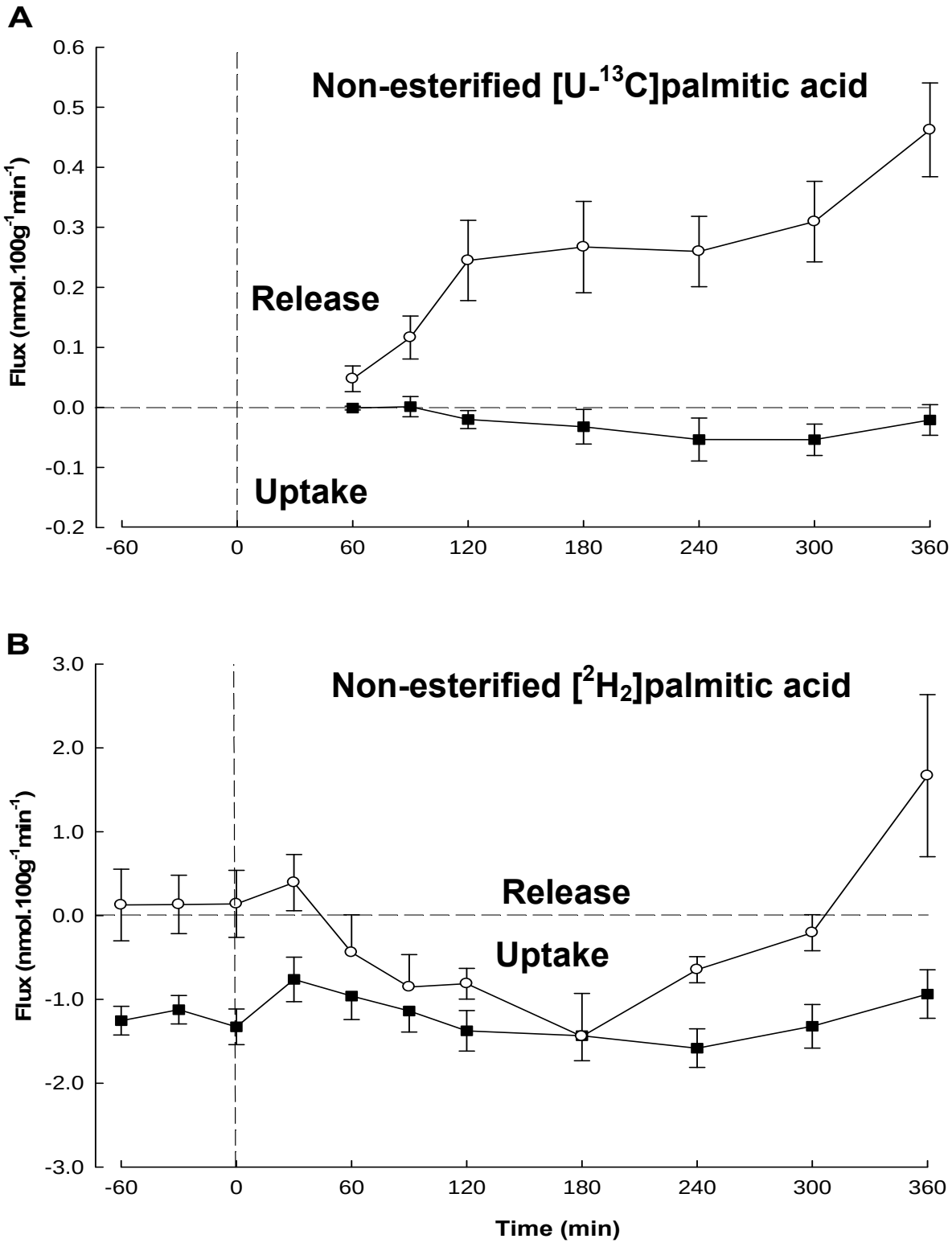

Figure 3.8 Non-esterified [U-^{13}C]palmitic acid (A) and [$^{2}H_2$]palmitic acid (B) flux across adipose tissue (AT) (open circles) and muscle (M) (solid squares) following a mixed meal. Flux is calculated as the V-A difference multiplied by the appropriate tissue blood flow. A positive flux implies output whereas a negative flux implies uptake. [U-^{13}C]palmitic acid was extracted, as expected, across muscle but released across adipose tissue, reflecting the action of LPL on circulating chylomicron-TG. For [$^{2}H_2$]palmitic acid there was consistent extraction across forearm muscle, again as expected, but unexpected uptake during the postprandial period

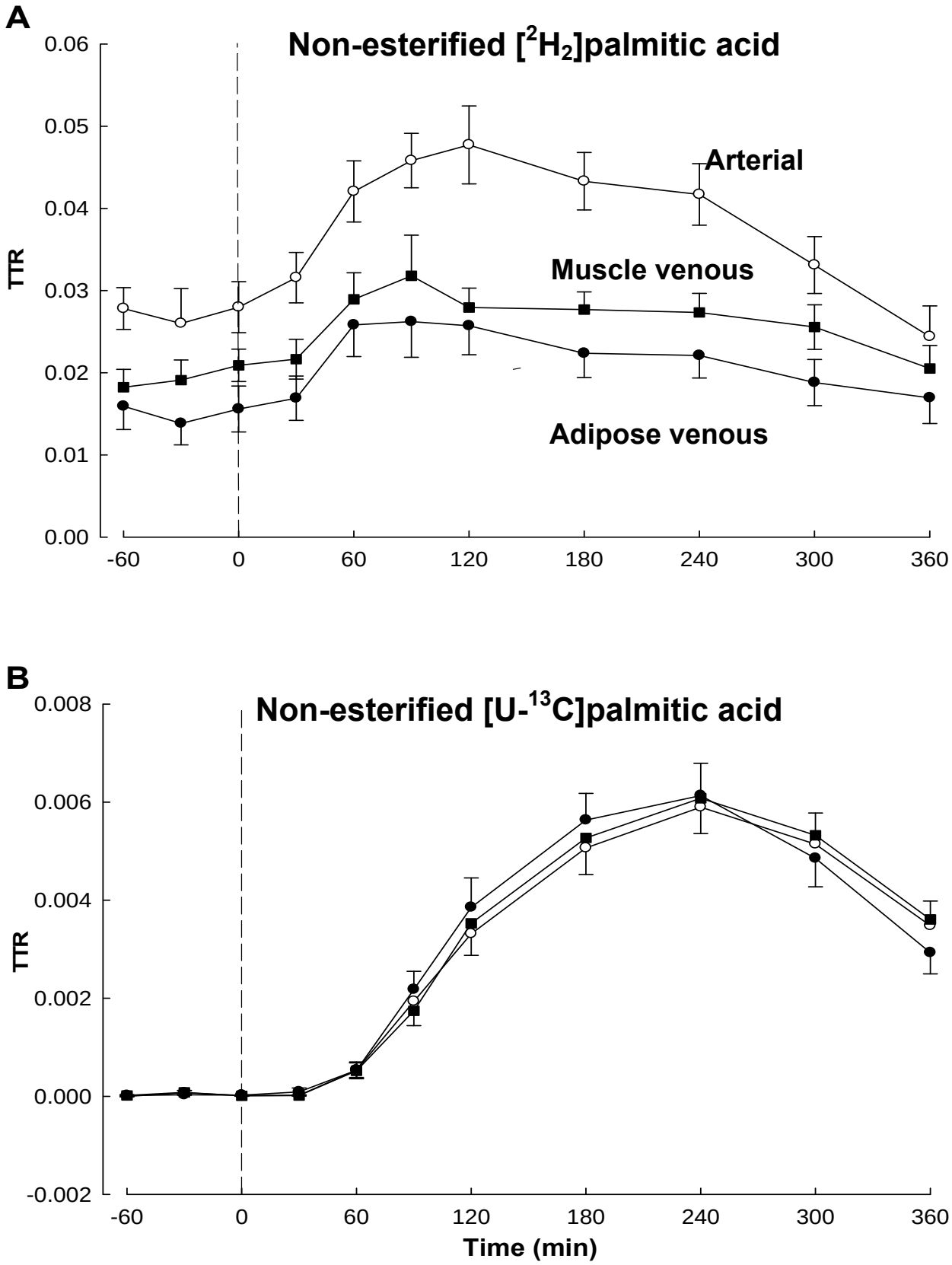

Figure 3.9 Changes in non-esterified [$^2H_2$]palmitic acid (**A**) and [U-^{13}C]palmitic acid (**B**) TTR (isotopic enrichment) following a mixed meal. Arterial (open circles), muscle venous (solid squares) and adipose venous (solid circles) TTRs. Whilst the TTR of [$^2H_2$]palmitic acid was lower in venous samples (from both muscle and adipose tissue) than in arterial plasma, there was no corresponding 'dilution' of the [U-^{13}C]palmitic acid across the tissues.

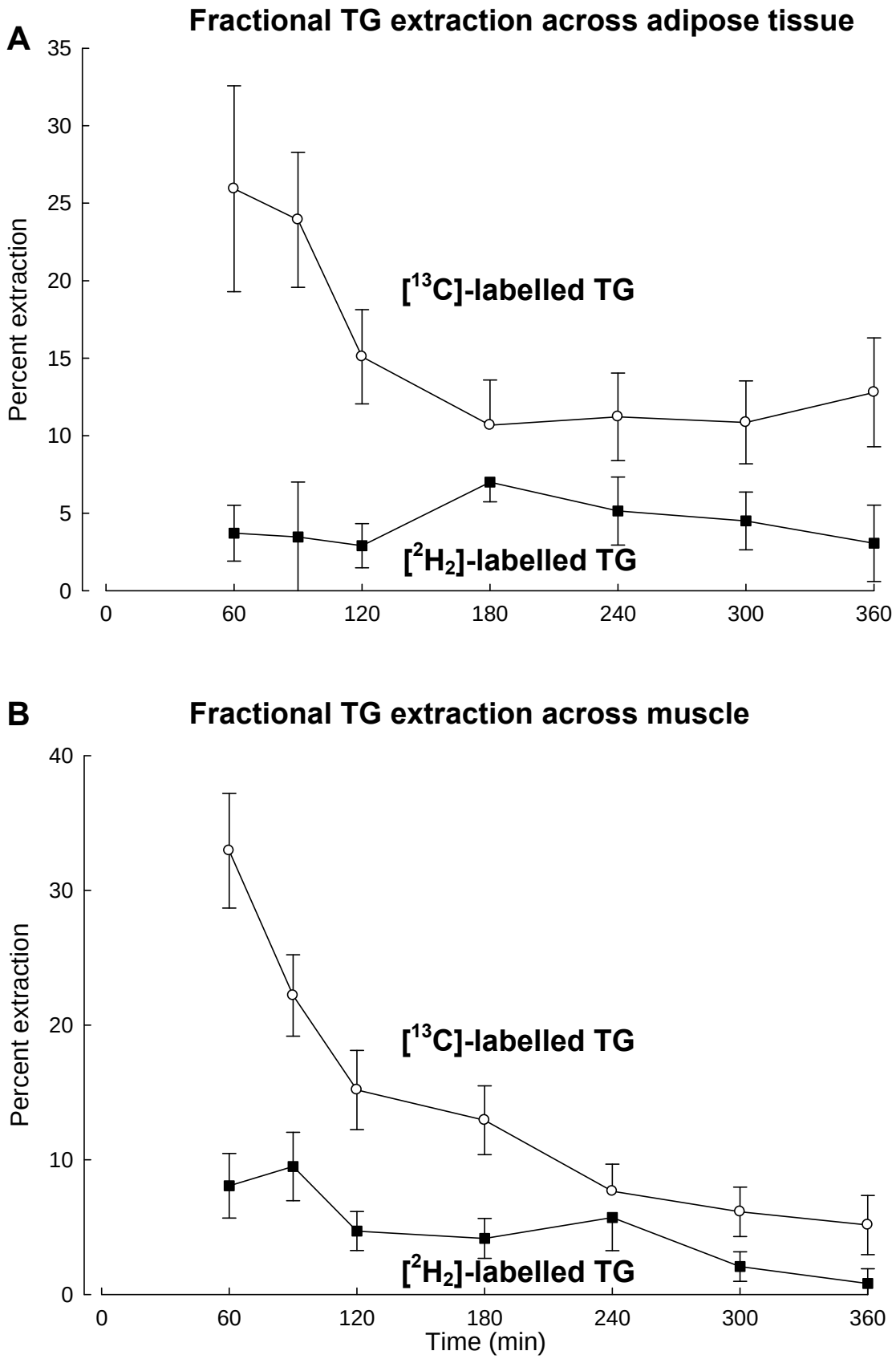

Figure 3.10 Fractional extraction of [^{13}C]-labelled TG (open circles, marking the chylomicron fraction) and [$^{2}H_2$]-labelled TG (solid squares, marking VLDL-TG) across adipose tissue (**A**) and muscle (**B**). Fractional TG extraction was calculated as the A-V difference in labelled TG divided by the arterial concentration of labelled TG and expressed as a percentage. For both tissues, fractional extraction of chylomicron-TG was greater than that of VLDL-TG.

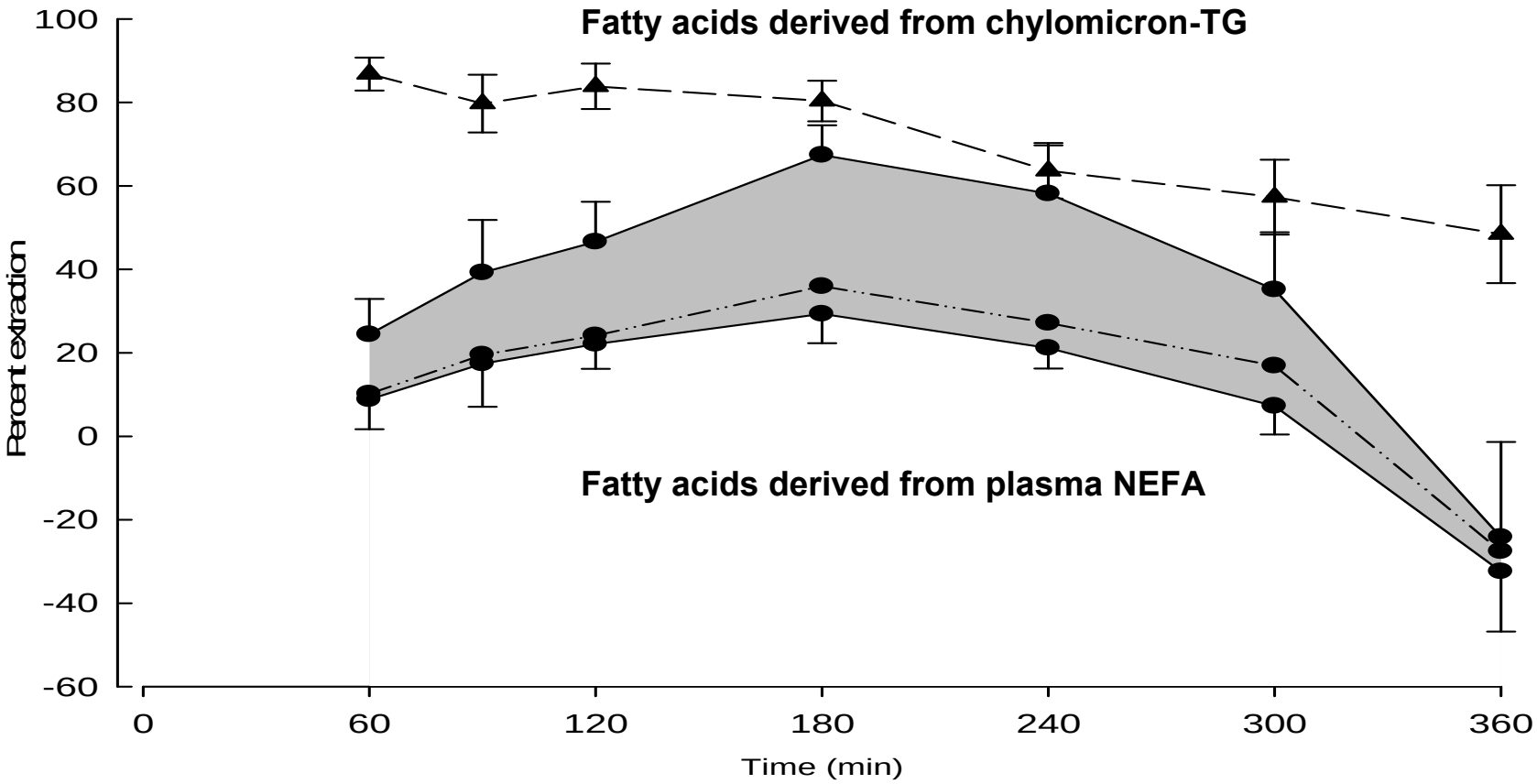

Figure 3.11 Adipose tissue entrapment of LPL-derived fatty acids: comparison of the fractional uptake of [U-^{13}C] palmitic acid derived from chylomicron hydrolysis (black triangles, dashed line) and of [$^{2}H_2$] palmitic acid (black circles, solid line) from the circulating NEFA pool across adipose tissue following a mixed meal. In calculating [$^{2}H_2$] palmitic acid fractional uptake, it is necessary to allow for FAs derived from VLDL hydrolysis. The shaded area represents the range of possible outcomes for [$^{2}H_2$] palmitic acid uptake. The upper border assumes 0% uptake of VLDL-derived FAs (Model 2 in the text) whereas the lower border assumes 100% uptake of VLDL-derived FAs (Model 1 in the text). The dashed line within the shaded area results if the fractional uptake of FAs derived from VLDL hydrolysis is taken to be the same as the fractional uptake of FAs derived from chylomicron hydrolysis (Model 3, Eqns 1-5 in text

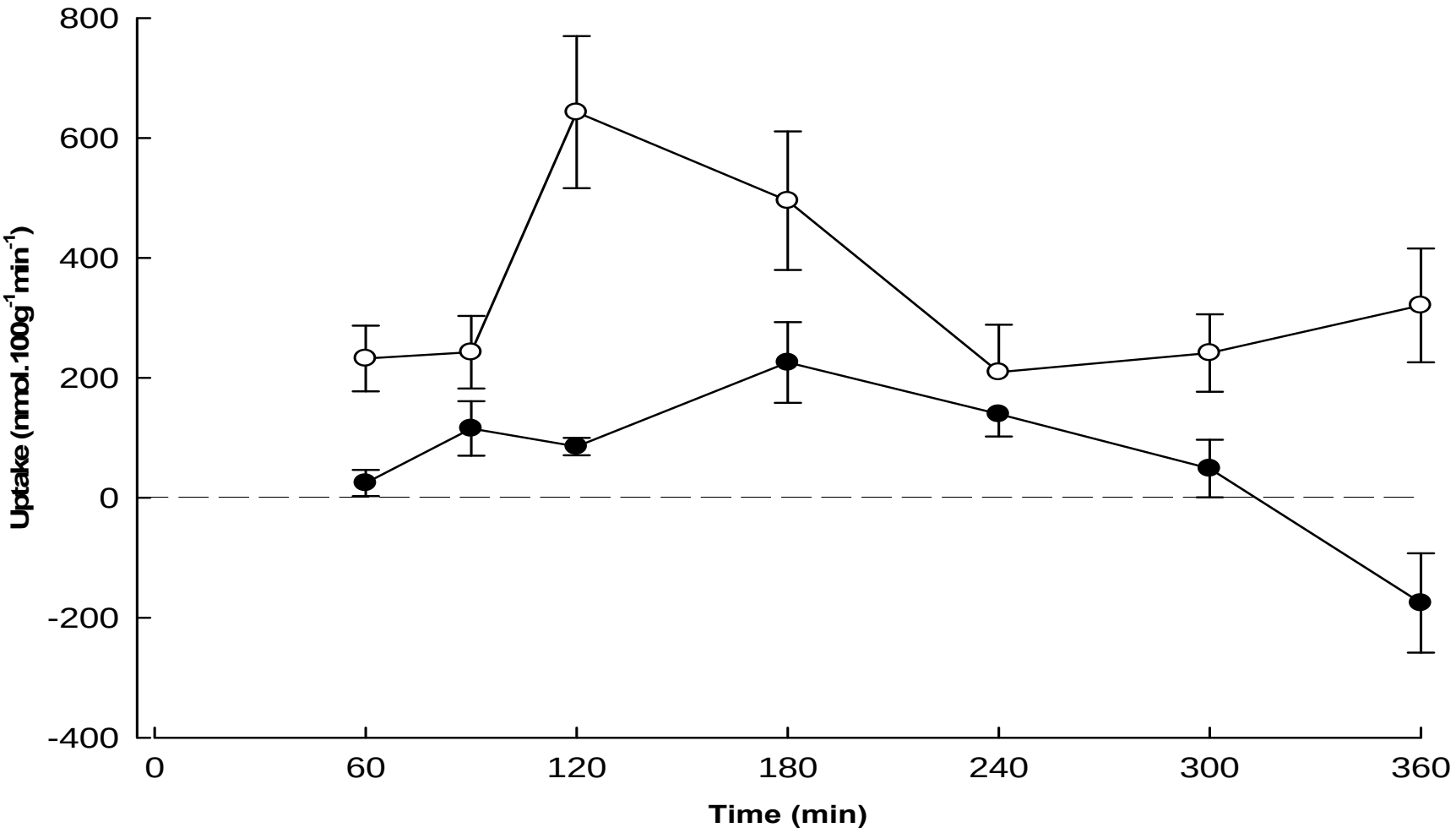

Figure 3.12 Unidirectional adipose tissue uptake of total FAs from the circulating NEFA pool (black circles) and total FAs derived from LPL-mediated TG hydrolysis (white circles) across adipose tissue following a mixed meal (Eqns 6-11 in text).

3.4 DISCUSSION

Disturbances in fat metabolism have been implicated in the pathophysiology of type 2 diabetes and consequent vascular disease (184, 185, 272). However, the pathways of normal fatty acid metabolism are not fully understood. In this study the most striking findings were the postprandial uptake of FAs from the circulating NEFA pool by adipose tissue, the direct confirmation that chylomicrons are the preferred substrate of LPL over VLDL, the preferential channeling of FAs derived from LPL-mediated chylomicron hydrolysis into adipose tissue and the postprandial release of FAs across the forearm.

I have used a combination of stable-isotope tracers to label specifically the chylomicron and VLDL fractions. As the calculations of isotope fractional uptake illustrate, it is essential to measure [$^2H_2$]-labelled TG concentrations when performing isotope infusion studies, since FAs derived from hydrolysis of labelled TG impact on calculations of labelled FA flux. Although in this study I did not examine arterio-venous differences for isolated lipoprotein fractions, this has been done previously without the use of isotopic tracers, and the major result from the current study of preferential extraction of chylomicron- compared with VLDL-TG was consistent with those earlier studies (12-14).

Direct uptake by adipose tissue of circulating NEFA has previously only been demonstrated during glucose infusion (240). The calculation of total NEFA uptake across adipose tissue (Fig. 3.12) illustrates that the postprandial uptake of FAs from the circulating NEFA pool is of quantitative significance. Indeed, during the mid-

postprandial period, over a third of the total FAs taken up by adipose tissue came from the circulating NEFA pool (Table 3.2). The variation in flux depending on the prevailing metabolic conditions suggests that NEFA uptake by adipose tissue is a regulated process.

The source of the NEFAs that are taken up by adipose tissue from the circulating pool is not clear. Unfortunately, the methodology of the current study is not able to answer this question. It is possible that there is simply a continuous, futile cycle of FA release and uptake in all adipose tissue depots, reflecting the dynamic nature of adipose tissue. An alternative view is this is a physiological mechanism to facilitate the redistribution of FAs between adipose tissue depots (276, 277).

The difference between [^{13}C]- and [$^{2}H_2$]-labelled TG extraction across both adipose tissue and muscle implies a preferential hydrolysis of chylomicron-TG by LPL. This has been suggested previously (12-14, 278, 279). However, earlier studies relied on ultracentrifugation for separation of chylomicron-TG and VLDL-TG with the limitation that some small chylomicrons and chylomicron remnants contaminate the VLDL-TG fraction. The use of different stable isotope labels for the chylomicron- and VLDL-TG pools allows a more accurate assessment of LPL preference, especially during the first half of the postprandial period. One possible explanation of the preference for chylomicron-TG is that the larger size of chylomicrons allows them to bind to more LPL molecules per particle than VLDL (280). The difference between the fractional extraction of [^{13}C]- and [$^{2}H_2$]-labelled TG was less marked at the end of the study. Dietary fatty acids may appear in VLDL-TG as early as 3 hours postprandially (281). Thus, by 6 hours

after the meal it is no longer correct to assume that the [^{13}C]palmitate in the TG fraction is restricted to chylomicrons.

The calculation of fractional isotopic uptake across adipose tissue enables differentiation between the uptake of circulating NEFA, represented by the uptake of [$^{2}H_2$]palmitate, and the uptake of FAs derived from chylomicron hydrolysis, represented by the uptake of [U-^{13}C]palmitate. Irrespective of which model of [$^{2}H_2$]palmitate uptake was used, there was a significantly lower uptake of FAs from the circulating NEFA pool as compared to FAs derived from chylomicron hydrolysis. This has been suggested before (240); however, the earlier report did not take into account the contribution of FAs derived from labelled TG hydrolysis in the calculation of NEFA uptake. It is likely that the physical positioning of LPL on the vascular endothelium results in the generation of FAs immediately adjacent to the adipocyte cell membrane. There is evidence of the juxtapositioning of chylomicron particles and the capillary endothelium to support this hypothesis (282, 283). In contrast, circulating NEFA flows more centrally within the lumen of the capillary.

The changes in fatty acid isotope TTR across adipose tissue and muscle are striking. Assuming that there is no discrimination between tracer and tracee for tissue uptake, then a decrease in fatty acid TTR across the tissue must reflect dilution by fatty acids not labelled with the isotope of interest. This is expected in adipose tissue and was seen clearly for [$^{2}H_2$]palmitate. This dilution in [$^{2}H_2$]palmitate enrichment reflects both the release of unlabelled fatty acids from intracellular lipolysis, and (in the postprandial

period) the release of unlabelled- and [U-^{13}C]-palmitate from intravascular chylomicron-TG lipolysis. However, it was unexpected to see almost the same degree of dilution in [$^{2}H_2$]palmitate enrichment across the forearm. That dilution must, again, reflect the release of non-[$^{2}H_2$]-labelled fatty acids from the tissue bed.

Information on the source of these 'diluting' fatty acids comes from the changes in TTR of [U-^{13}C]palmitate across the tissues. In both adipose tissue and muscle, there was no consistent change in [U-^{13}C]palmitate enrichment across the tissue, implying an equal release of labelled and unlabelled fatty acids. In adipose tissue this is not entirely unexpected. Intracellular lipolysis in adipose tissue is suppressed in the fed state. Consequently, there will be no excess release of unlabelled fatty acids and no change in TTR. The data suggest that the major source of dilution of the [$^{2}H_2$]palmitate across adipose tissue in the postprandial state is fatty acid release from intravascular lipolysis of chylomicron-TG. The degree to which this dominates fatty acid release from adipose tissue throughout the postprandial period was unexpected. The dilution of [$^{2}H_2$]palmitate across the forearm in the fasting state (-60 to 0 min on Fig. 3.9A) implies release of unlabelled NEFA from the tissue. This has been observed previously in the leg, where it was attributed to fatty acids released from turnover of intramuscular TG (82). An alternative explanation might be that the forearm also contains some adipose tissue, NEFA release from which dilutes the circulating tracer in the fasting state. After the meal, however, this is likely to be suppressed, and when taken together with the lack of dilution of [U-^{13}C]palmitate, this may imply that in forearm muscle, as in abdominal adipose tissue, there is significant release of fatty acids from intravascular lipolysis of

chylomicron-TG. Despite this, there was no net release of [U-^{13}C]palmitate from intravascular lipolysis of chylomicron-TG in the forearm; rather, there was consistent net uptake (Fig. 3.8) as has been observed before (15). These interpretations apply most strongly to the mid-postprandial period. In the early period there is insufficient labeling of chylomicron-TG, and in the later period [U-^{13}C]palmitate will have recycled as VLDL-TG.

I acknowledge the limitation that my measurements are made in non-steady state conditions following a meal. It has been argued that arterio-venous difference measurements made in non-steady states are difficult to interpret (258). However, more recent measurements of transit times through the forearm suggest that the problem is not as great as once thought (284). I do not know the transit time through subcutaneous adipose tissue, but given the relatively low blood volume and high blood flow compared with resting muscle, it is not likely to be greater. The main results here are based on time-courses that are relatively stable over a period of hours, not on individual time-points, and so I do not feel the problems with non-steady state have affected my conclusions.

In conclusion, the combination of stable isotope methodology and arteriovenous difference measurements has demonstrated new facets of the postprandial deposition of fat in extra-hepatic tissues. This particular combination of techniques is novel in its application to the fasting and postprandial states, although it builds upon earlier seminal studies of fatty acid metabolism in tissues using tracer techniques (83, 240, 285). This study provides novel fundamental knowledge, which can lead to a better understanding of

the abnormalities of fat metabolism in insulin resistance and in type 2 diabetes. These results, and the techniques used, can now be taken forward to examine the effects on adipose tissue and muscle fat metabolism of differing physiological and pathological conditions such as dietary modification and insulin resistance.

Chapter 4 : The metabolic syndrome in the Oxford biobank

4.1 Introduction

The metabolic syndrome describes a collection of clinical features associated with an increased risk of type 2 diabetes and vascular disease (128, 131, 150). In recent years the usefulness of the metabolic syndrome as a clinical entity has come into question, not least because of the failure to identify any single underlying pathophysiological process (9, 286). This is reflected in the variety of definitions of the metabolic syndrome which focus either on adiposity (113, 115), or insulin resistance (114, 116) as intrinsic clinical criteria. Irrespective of the definition used, the metabolic syndrome is associated with a characteristic disturbance in lipid metabolism which may, in fact, underlie both the metabolic and vascular complications. The techniques described in the previous chapters are clearly highly applicable for investigating differences in tissue specific fat metabolism between subjects with the metabolic syndrome and those without. However, before discussing the detailed physiological studies I performed, it is instructive to re-examine the baseline data available for the individuals registered in the Oxford Biobank (OBB).

Application of the various definitions of the metabolic syndrome to the data acquired from large epidemiological studies has shown that the prevalence of the metabolic syndrome depends on the definition used (125). In addition, it is not simply that one definition identifies more individuals than another since not all individuals are similarly classified by the various definitions. The DECODE study group applied the WHO, ATPIII and EGIR definitions of the metabolic syndrome to the DECODE cohorts and demonstrated that only 34% of women and 31% of men were classified as having the

metabolic syndrome by all three definitions (287). The populations in such epidemiological studies are, by definition, heterogeneous, particularly in terms of fat mass. Few studies have assessed whether in cohorts of subjects with similar measures of fat mass, the various definitions of the metabolic syndrome affect the prevalence and the individuals identified as having the syndrome.

The characteristic lipid disturbance of the metabolic syndrome is elevated TG, low HDL-cholesterol and relatively normal LDL-cholesterol (157). These parameters are frequently used to estimate cardiovascular risk (288). However, TG, HDL and LDL concentrations provide only an indirect estimation of the atherogenic potential within the circulation. The argument has therefore been made for the measurement of apolipoprotein B (apo B) to allow a more precise measurement of atherogenic risk (289). The cholesterol and TG content of each of the circulating lipoprotein species varies greatly. However, as discussed in chapter 1, each individual VLDL and LDL particle contains only a single molecule of apo B100, whilst each chylomicron particle and remnant contains only a single molecule of apo B48. Thus measurement of total apo B, including both apo B48 and apo B100, allows determination of the total number of circulating particles with atherogenic potential. There is a direct relationship between apo B concentration and the number of particles taken up and retained by atheromatous plaques (29, 290). Furthermore, there are now a plethora of prospective epidemiological studies showing that the apo B concentration is a better predictor of cardiovascular risk than is that of the more traditional LDL-cholesterol (291, 292). The metabolic syndrome is associated with a significant risk of vascular disease, and thus measurement of apo B concentration may

provide useful information about vascular risk in individuals above and beyond measurement of total cholesterol.

Although not part of any definition of the metabolic syndrome, high NEFA concentrations are linked to abnormalities of glucose homeostasis including reduced insulin secretion (186), increased hepatic glucose output (272) and reduced glucose uptake in muscle (88). Thus, NEFAs have been implicated in the development of insulin resistance and type 2 diabetes (185, 186). Elevated NEFA concentrations have been observed in insulin resistant states such as obesity (181), impaired glucose tolerance and type 2 diabetes (183). Consequently, it has been stated that the metabolic syndrome is also characterized by high circulating NEFA (293). However, there is little information as to whether, in a large cohort, there are differences in circulating NEFA concentrations between those with and without the metabolic syndrome.

Therefore, in order to examine whether two differing definitions of the metabolic syndrome identified the same individuals and what differences exist between those with and without the metabolic syndrome, I aimed to extract the relevant clinical and biochemical data available from the OBB for all moderately overweight males.

4.2 Subjects

Details of the OBB are given in Chapter 2. Potentially the data for all subjects registered with the OBB were available for study. However, as female subjects were not recruited to the detailed physiological study, only the data for males in the OBB (252) were utilised. Subjects with a BMI 25-35 kg/m^2 were included in the present analysis.

This BMI range was selected to encompass the overweight and mildly obese; a representative group of males that included a good sized cohort with the typical features of the metabolic syndrome, but without the additional metabolic complications of gross obesity. Comparisons were made between those with and without the metabolic syndrome defined using the criteria put forward by both the ATP III (113) and the EGIR (114). These definitions were selected because the former is the commonest used in previous studies whilst the latter includes a surrogate measure of insulin resistance, representing a more pathophysiological, as opposed to pure clinical, approach to the metabolic syndrome. In order to ensure clear separation between groups, only individuals with fasting insulin concentrations below the median for the OBB population were included in the control group used in the comparison with the EGIR-defined metabolic syndrome.

4.2.1 Statistical methods

Data are presented as medians±interquartile range and Mann-Whitney U test was used to detect differences between those with and without the metabolic syndrome. Spearman rank correlation coefficients were calculated to examine potential correlations between parameters.

4.3 RESULTS

The biochemical and physical characteristics of the OBB members with the metabolic syndrome and corresponding control subjects are summarized in Table 4.1. There were 372 men with a BMI 25-35 kg/m^2 in the OBB. Of these, 104 (30%) were identified as matching ATP III criteria for the metabolic syndrome and thus the remaining 268 formed the control group. Fifty five (15%) of the 372 men matched the EGIR criteria for the metabolic syndrome, but only 120 (32%) had fasting arterial insulin concentrations below the median for the whole OBB population; the criterion necessary to qualify as a control subject. The two sets of criteria did not identify the same individuals; only 39 subjects were defined as having the metabolic syndrome according to both definitions. The groups were matched for age by either classification. Surprisingly, despite the significant differences in all other parameters measured (See Table 4.1), there was no difference in fasting plasma NEFA concentration between those with the metabolic syndrome and those without, irrespective of which definition of the metabolic syndrome was applied.

There were statistically significant correlations between several measured parameters across the whole study group. These are summarized in table 4.2. Of particular note were the positive correlations between fasting insulin and TG concentrations ($r_s = 0.38$, $P < 0.01$) and between fasting insulin and apo B concentrations ($r_s = 0.14$, $P= 0.01$) (Fig. 4.1). In addition, it is important to note that there was no correlation between fasting insulin and fasting NEFA concentrations.

Table 4.1 Biochemical and physical characteristics of the OBB members studied.

	NCEP-ATP III(113)			EGIR(114)		
	Metabolic Syndrome (n=104)	Controls (n=268)	P	Metabolic Syndrome (n=55)	Controls (n=120)	P
Age (yrs)	43 (40-45)	42 (37-45)	NS	43 (40-45)	42 (35-45)	NS
Weight (kg)	97 (90-103)	86 (81-93)	<0.001	95 (89-102)	85 (81-91)	<0.001
BMI (kg/m^2)	31 (28-32)	27 (26-29)	<0.001	31 (28-32)	27 (26-28)	<0.001
Waist (cm)	104 (100-110)	94 (90-98)	<0.001	102 (98-106)	94 (89-99)	<0.001
Insulin (mU/l)	13.1 (10.0-15.1)	8.5 (6.3-12.3)	<0.001	15.1 (13.5-18.5)	6.0 (5.0-7.2)	N/A
NEFA (µmol/l)	465 (356-594)	417 (306-548)	NS	458 (352-567)	448 (324-561)	NS
TG (mmol/l)	2.1 (1.7-2.7)	1.3 (0.9-1.6)	<0.001	2.6 (2.1-2.9)	1.2 (0.9-1.6)	<0.001
HDL-cholesterol (mmol/l)	1.0 (0.9-1.2)	1.2 (1.1-1.4)	<0.001	1.1 (1.0-1.2)	1.2 (1.1-1.4)	<0.001
Total cholesterol (mmol/l)	5.8 (5.2-6.5)	5.6 (5.0-6.3)	0.06	6.1 (5.5-7.1)	5.6 (5.0-6.4)	0.001
apo B (mg/l)	1015 (826-1215)	937 (797-1107)	0.017	1056 (901-1055)	881 (762-1099)	<0.001
Glucose (mmol/l)	5.7 (5.4-6.0)	5.2 (5.0-5.5)	<0.001	5.7 (5.3-6.0)	5.2 (5.0-5.4)	<0.001
Systolic Blood Pressure (mmHg)	131 (122-139)	123 (118-130)	<0.001	130 (123-143)	125 (118-132)	<0.001
Diastolic Blood Pressure (mmHg)	89 (85-94)	81 (76-86)	<0.001	87 (82-95)	80 (76-85)	<0.001

Data are presented as medians±interquartile range and Mann-Whitney U test was used to detect differences between those with and without the metabolic syndrome.

Table 4.2 Correlations between measured parameters across the whole study group.

		TG	Apo B	HDL-C	NEFA
BMI	r_s	0.27	0.16	-0.27	0.12
	P	<0.01	<0.01	<0.01	0.03
Waist	r_s	0.32	0.18	-0.33	0.13
	P	<0.01	<0.01	<0.01	0.02
Insulin	r_s	0.38	0.14	-0.16	-0.07
	P	<0.01	0.01	<0.01	0.20

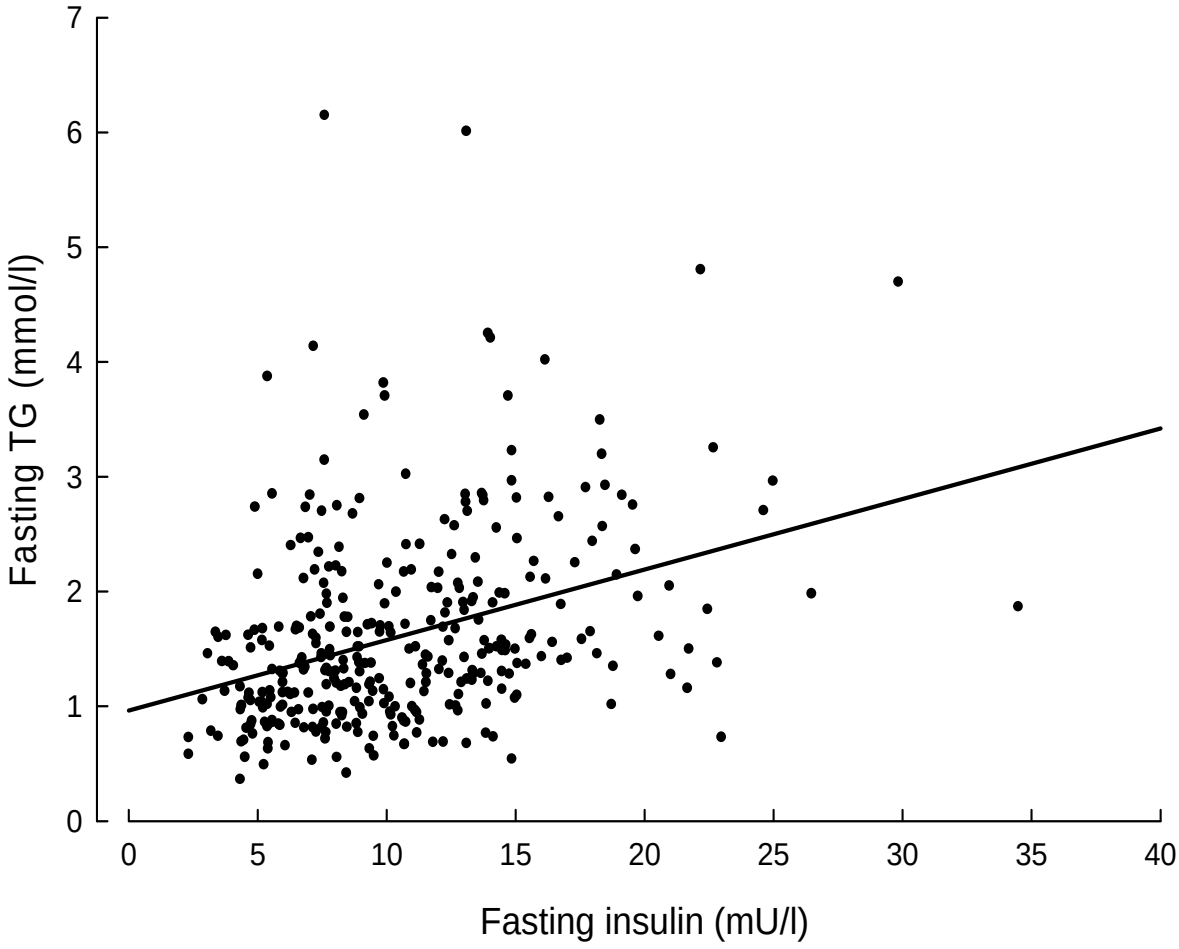

Figure 4.1 Correlation between fasting insulin and fasting TG ($r_s = 0.38$, $P<0.01$)

4.4 DISCUSSION

The metabolic syndrome is common (124), particularly amongst overweight individuals, and is associated with a high risk of vascular (128, 150) and metabolic (131) complications. The key findings in the current analysis of the clinical and biochemical data obtained from overweight males registered with the OBB are as follows: the prevalence of the metabolic syndrome depends on the definition used, and different definitions identify different individuals. Total cholesterol and apo B concentrations were increased in the metabolic syndrome; however, NEFA concentrations were no higher in those with the metabolic syndrome than in those without. Finally, there was an association between the fasting insulin and triglyceride concentrations across the whole study population.

The differences in prevalence of metabolic syndrome attributable to variations in the criteria used for the definition are well recognized (131, 132). Amongst overweight OBB males, the ATPIII definition identified the metabolic syndrome in twice as many individuals as the EGIR definition, broadly in line with a previous epidemiological study (134). The ATPIII definition was designed as a straightforward means of identifying individuals with the metabolic syndrome in day to day clinical practice. No single aetiological process was assumed, although insulin resistance was recognized as an element of the syndrome. This is in contrast to the sentiments of the EGIR, who proposed that insulin resistance is central to the syndrome and thus included relative hyperinsulinaemia as the only mandatory criterion for the definition. In the current analysis, the group with the metabolic syndrome by ATPIII criteria did have significantly

greater fasting insulin concentrations than the healthy controls suggesting that some degree of insulin resistance is indeed characteristic of, if not intrinsic to, the metabolic syndrome.

The pattern of dyslipidaemia observed was characteristic of that observed previously in the metabolic syndrome. The total cholesterol concentration was greater in those with the metabolic syndrome than in the control groups, however, the absolute difference between the groups was small. There are no previous studies simply comparing apo B concentrations in equally obese men with and without the metabolic syndrome. However, population studies have shown a close association between apo B and the metabolic syndrome. Data from the National Health and Nutrition Examination Survey revealed a significantly higher prevalence of the metabolic syndrome, by ATPIII or IDF criteria, amongst participants with a high apo B/apolipoprotein AI (apo AI) ratio (132, 294). These findings confirm those of Wallenfeldt *et al*, who followed a cohort of 61 year old overweight Swedish men. These authors found that two thirds of patients with the metabolic syndrome had high apo B/apo AI ratios. In addition, there were significant correlations between the various elements of the metabolic syndrome, including fasting insulin concentrations, and the apo B/apo AI ratio. Furthermore, as the number of features of the metabolic syndrome rose, so did the apo B/apo AI ratio (295). Data from the Insulin Resistance Atherosclerosis Study have further revealed that apo B is closely correlated to numerous traditional and novel cardiovascular risk factors in patients with the metabolic syndrome (296). Thus, although total cholesterol is a well recognized vascular risk marker, there is increasing argument that the measurement of

apo B provides a more precise measure of vascular risk. This appears particularly pertinent to the metabolic syndrome which is characterized by markedly elevated vascular risk but, at most, a mild elevation in total cholesterol.

The lack of difference in systemic NEFA concentration between the study groups was unexpected. One explanation for this may lie in the utilisation of a control group with similar measures of fat mass to those of the metabolic syndrome group. Previous studies have used lean healthy control groups (148, 293) with very much lower fat mass than the patient groups. Fat mass is one determinant of NEFA concentrations. Although the relationship is not entirely straightforward (238, 293), comparison of groups with very different fat masses is likely to produce differences in systemic NEFA concentrations. This is supported by a closer examination of the literature which reveals that studies comparing groups with similar measures of fat mass have not, in fact, demonstrated differences in fasting systemic NEFA concentrations in first degree relatives of patients with type 2 diabetes (204, 205), in patients with IGT (297) or even in those with frank type 2 diabetes (203, 298, 299). In the present analysis, both groups were overweight and although there was a difference in fat mass, this was clearly not great enough to result in differing circulating NEFA concentrations. Fat mass is only one of a number of moderators of circulating NEFA concentrations. The other, less static influences on NEFA metabolism are considered in detail in Chapter 5.

The association between TG and insulin concentrations is intriguing The modulatory influence of insulin on fat metabolism varies from tissue to tissue however, how these processes are altered in the metabolic syndrome is poorly understood.

In conclusion, examination of the biochemical and clinical data available for overweight men registered with the OBB confirmed that the metabolic syndrome is common, but that the prevalence and individuals identified depend on the definition used. Whilst a modest elevation in cholesterol is seen in the metabolic syndrome, the measurement of apo B adds further information about vascular risk. Finally, in contrast to the prevailing view, elevated NEFA concentrations are not a feature of the syndrome.

The association between TG and insulin concentrations is intriguing and suggests that insulin action or resistance may play some role in the hypertriglyceridaemia of the metabolic syndrome. As described in Chapter 1, the modulation of fat metabolism by insulin is complex and varies from tissue to tissue. Thus, a more detailed examination of fat metabolism in the metabolic syndrome is warranted to help elucidate the observational findings from the OBB. In addition, a firmer understanding of fat metabolism in the metabolic syndrome may enable the development of therapeutic strategies.

Chapter 5 : Fatty acid metabolism and the metabolic syndrome

5.1 Introduction

The classic pattern of lipid parameters observed in the metabolic syndrome is high triglyceride (TG), low HDL-cholesterol, and relatively normal LDL-cholesterol (157); albeit largely composed of small dense LDL particles. As discussed in Chapter 1, the last-named demonstrate marked atherogenic potential (300) and are likely to contribute to vascular disease. This typical dyslipidaemia was confirmed in the Oxford Biobank population in Chapter 4. However, although the data in Chapter 4 suggested a relationship between insulin and TG, the precise pathophysiological basis for the elevation in both fasting and postprandial TG concentrations remains elusive. Production of intestinal and hepatic apolipoproteins is elevated in obesity and correlates with measures of insulin resistance (166, 167, 175). Adipose tissue TG clearance is reduced in obesity (13). In contrast, muscle TG clearance is not different between females with upper body obesity, phenotypically analogous to those with the metabolic syndrome, and females with lower body obesity (180). There is no information on the peripheral clearance of TG in the metabolic syndrome *per se*.

In the analysis described in Chapter 4, I did not find the elevation in systemic NEFA that has previously been observed in other insulin resistant states (181, 183). The proposed mechanism for higher NEFA concentrations in insulin insensitive individuals is resistance to the anti-lipolytic action of insulin in adipose tissue (203). Circulating TG is hydrolysed in the capillary bed of adipose tissue and the fatty acids liberated may either be taken up or may spill over into the systemic circulation. Insulin plays a key role by simultaneously stimulating fatty acid re-esterification and inhibiting intracellular

lipolysis. Both fasting NEFA concentrations and the failure to suppress NEFA under euglycaemic hyperinsulinaemic clamp conditions have been correlated with measures of insulin resistance in healthy individuals (182). Defects in insulin mediated suppression of NEFA concentrations have been shown in obesity (43), in impaired glucose tolerance (148) and in patients with type 2 diabetes (203). In addition, suppression of NEFA following an OGTT is attenuated in individuals with the metabolic syndrome compared to healthy, lean controls (293). My analysis of the data from the OBB provides a snapshot of circulating NEFA concentrations during fasting. Although useful, such data give no information as to the dynamics of fatty acid metabolism occurring in different tissues under varying physiological conditions. There is little such information available regarding adipose tissue fatty acid metabolism and its regulation in the metabolic syndrome. Muscle also makes a significant contribution to whole body TG and NEFA balance, acting mainly as a consumer of NEFA. Abnormalities of muscle fatty acid uptake have been described in other insulin resistant states (301) but the metabolic syndrome *per se* has not been studied.

I hypothesized that abnormal NEFA and TG metabolism in both adipose tissue and muscle may contribute to the dyslipidaemia of the metabolic syndrome and thereby to the metabolic and vascular complications. I set out to use the techniques described in the previous chapters to investigate differences in tissue-specific fat metabolism between subjects with the metabolic syndrome and age- and weight-matched controls. Fatty acid metabolism has been studied in the metabolic syndrome before, but never under fasting and postprandial conditions or in a tissue-specific manner.

5.2 Subjects, materials and methods

Much of the detail regarding subject recruitment, methods and analysis has already been presented in Chapters 2 and 3. Thus, a brief outline only, with any pertinent additional details, will be described here.

5.2.1 Metabolic investigation

5.2.1.1 Subjects

Eighteen overweight male subjects (7 with the metabolic syndrome and 11 controls by ATPIII criteria) were recruited from the OBB for detailed metabolic investigation. No subjects recruited had diabetes. Two subjects, defined as having the metabolic syndrome by ATP III criteria, moved to the control group when re-classified by the EGIR definition, whilst 5 of the control subjects fulfilled the EGIR criteria for the metabolic syndrome. Thus, following re-definition using the EGIR criteria, there were 10 subjects with the metabolic syndrome and 8 controls. The clinical characteristics of the subjects are summarized in Table 5.1. Subjects defined as having the metabolic syndrome by ATP III criteria were matched with controls for age and weight, but not BMI. When the EGIR criteria were applied, the subjects were matched for all of the aforementioned criteria. Data from some subjects have already been described in Chapter 3.

5.2.1.2 Study protocol

Subjects attended the Clinical Research Unit for one full day's detailed metabolic investigation. Serial blood samples were taken in the fasting state and for 6 hours after ingestion of a mixed test meal (40 g fat, 40 g carbohydrate and 100 mg [U-^{13}C]palmitic acid (isotope purity 98%, CK Gas Products Ltd, Hampshire, UK)). Subjects also

received a continuous intravenous infusion of [$^{2}H_2$]palmitic acid (isotope purity 97%, CK Gas Products Ltd, Hampshire, UK) complexed to human albumin (infusion rate 0.04 μmol/kg/min). Subcutaneous abdominal adipose tissue blood flow was measured by ^{133}Xe washout (231). Forearm muscle blood flow was assessed by venous occlusion strain-gauge plethysmography with an inflated wrist cuff (222). Blood flow measurements were made immediately following blood sampling.

5.2.1.3 Analyses

Plasma glucose, 3-hydroxybutyrate, TG and NEFA concentrations were determined enzymatically using an ILab 600 Multianalyser (Instrumentation Laboratory, Warrington, UK). Insulin levels were determined by radioimmunoassay using a commercially available kit (Linco Research, St. Charles, MO). Fatty acid composition and isotopic enrichment were determined by GC and GCMS respectively, as previously described (302).

5.2.1.4 Calculations

Arteriovenous (A-V) and venoarterial (V-A) differences in all metabolite concentrations (labelled and unlabelled) in whole blood were calculated across both adipose tissue and skeletal muscle. Simple and derived flux calculations were made as described in section 2.3. Respiratory exchange ratio (RER) and whole body substrate oxidation rates were calculated from the indirect calorimetry data (260). The production of $^{13}CO_2$ was calculated by multiplying the TTR of $^{13}CO_2/^{12}CO_2$ by the corresponding VCO_2 obtained from indirect calorimetry.

5.2.1.4.1 Rate of appearance of NEFA ($Ra_{(NEFA)}$)

$Ra_{(NEFA)}$ is the rate of appearance of NEFA in the plasma (244). $Ra_{(NEFA)}$ can be calculated on a whole body or tissue-specific basis. However, arteriovenous difference measurements give more information than tissue-specific $Ra_{(NEFA)}$ and consequently only whole body $Ra_{(NEFA)}$ calculations were made in the current study. $Ra_{(NEFA)}$ is calculated from the dilution in plasma of the infused fatty acid tracer. It is assumed that the dilution is predominantly due to the release of unlabelled NEFA following TG hydrolysis. Two other assumptions are also intrinsic to the classic model for calculating Ra. The first is that there is a single circulating NEFA pool into which all NEFAs feed, irrespective of origin, and consequently, $Ra_{(NEFA)}$ represents the sum of tissue NEFA release and intravascular TG hydrolysis (244). The second assumption is that the fatty acid tracer is treated entirely analogously to native circulating NEFA. This has been tested under varying metabolic conditions by comparing the Ra of a number of different tracers with the calculated total NEFA Ra. The data demonstrated that labelled palmitic acid, linoleic acid and oleic acid all provided a reasonable estimate (within 15%) of the total NEFA Ra (303). The calculation of Ra, originally described by Steele and colleagues, was derived from the measurement of glucose turnover under steady state conditions (304). Concerns that Steele's model was inadequate under non-steady state conditions (305) prompted the development of modifications to the equation in order to account for changing metabolic conditions (306). However, a direct comparison of the two equations revealed that the steady state equation is accurate under non-steady state conditions when applied to fatty acid rather than glucose kinetics (241). In the current study, the whole body rate of appearance of NEFA ($Ra_{(NEFA)}$) was derived from arterial tracer-tracee ratios (TTRs) of

[$^2H_2$]palmitate. In order to ensure maximum accuracy and re-validate the earlier methodological comparison (241), Steele's equation for steady-state conditions modified for use with stable isotopes (244) and the equations of Steele modified for non-steady-state conditions (306) were both used to calculate $Ra_{(NEFA)}$. The steady state equation, calculated at each timepoint, is as follows:

$$Ra_{(palmitate)} = \frac{F}{TTR}$$

where F is the rate of the infusion (μmol/kg/min), TTR is the arterial TTR of [$^2H_2$]palmitate.

The non-steady state equation, calculated between consecutive timepoints, was as follows:

$$Ra_{(palmitate)} = \left(\frac{2 \times F}{TTR_a + TTR_b}\right) - \left(\frac{[p \times V] \times [C_a + C_b] \times [TTR_b - TTR_a]}{\left[1 + \left(\frac{TTR_a + TTR_b}{2}\right)\right] \times [TTR_a + TTR_b] \times [b - a]}\right)$$

where a and b are consecutive timepoints, TTR_a is the arterial TTR of [$^2H_2$]palmitate at timepoint a, TTR_b is the arterial TTR of [$^2H_2$]palmitate at timepoint b, C_a is the arterial plasma concentration of [$^2H_2$]palmitate at timepoint a, C_b is the arterial plasma concentration of [$^2H_2$]palmitate at timepoint b, p is the pool fraction (%) and V is the volume of distribution (l/kg). p and V were taken as 1.8% and 0.05 l/kg (242).

For both equations, $Ra_{(palmitate)}$ was converted to $Ra_{(NEFA)}$ as follows:

$$Ra_{(NEFA)} = Ra_{(palmitate)} \times \left(\frac{1}{Fr_{(palmitate)}}\right)$$

where $Fr_{(palmitate)}$ represents the fraction of palmitic acid in the NEFA fraction.

5.2.1.5 Statistical methods

Repeated measures ANOVA was used to determine differences between the groups over the whole study period. Area under the curves (AUCs) and incremental area under the curves (iAUCs) were calculated by the trapezoid method. The unpaired *t*-test was used to compare mean fasting parameters, AUCs and the postprandial fall in $Ra_{(NEFA)}$ between subject groups. Spearman's rank correlation coefficients were used to describe the relationship between fasting net NEFA output and insulin concentration.

5.3 RESULTS

The test meal was given at time 0. All other times on the figures and in the text are expressed relative to time 0. All comparisons between groups refer to the ATP III definition unless otherwise stated. A summary of the principal study results is presented in Table 5.2.

5.3.1 Tissue blood flow and unlabelled metabolites

Adipose tissue blood flow was lower in the subjects with the metabolic syndrome than in the controls over the study period as a whole (P=0.02), although this was most marked during fasting (P=0.04) (Fig. 5.1). Forearm muscle blood flow was similar in the two groups. Fasting and postprandial insulin concentrations were not different between the subjects with the metabolic syndrome and the control group and nor were fasting or postprandial glucose concentrations (Fig. 5.2).

Arterial plasma NEFA concentrations were no higher in subjects with the metabolic syndrome than in the controls (Fig. 5.3A). However, there was a marked difference in net adipose tissue NEFA output per 100 g tissue (V-A difference multiplied by blood flow) (Fig. 5.4A). Under fasting conditions, the subjects with the metabolic syndrome demonstrated a lower net NEFA output (P=0.02) from adipose tissue than the control subjects. Following the meal, net NEFA output was suppressed to similar levels in both study groups. However, as the control subjects demonstrated a higher net NEFA output during fasting, the postprandial fall observed in these subjects was greater than in those with the metabolic syndrome (P=0.02). In addition, there was a significant negative correlation between fasting net NEFA output and insulin concentration in the subjects with the metabolic syndrome, the control group and, therefore, across the whole study population (r_s=-0.53, P=0.03) (Fig. 5.5). In muscle, net NEFA uptake occurred throughout the study and did not differ between the study groups (Fig. 5.6).

Plasma TG concentrations, during both the fasting and post-prandial periods, were higher in the metabolic syndrome than in the control group (P=0.01) (Fig. 5.3B). In addition, the iAUC in TG following the meal was greater in those with the metabolic syndrome (P=0.05). TG extraction by muscle was similar in both groups whilst fasting. However, during the postprandial period, both the fractional extraction (A-V difference divided by arterial concentration) and clearance (fractional extraction multiplied by blood flow) of TG across muscle were lower in the subjects with the metabolic syndrome than in the healthy controls (P=0.01 and 0.03 respectively) (Fig. 5.7A). Fasting TG clearance by adipose tissue was not different between the groups. Postprandially, there was a lower

clearance of TG by adipose tissue (P=0.02) and a trend, albeit not statistically significant, for a lower fractional extraction of TG by adipose tissue in those with the metabolic syndrome compared with controls (Fig. 5.7B).

5.3.2 Labelled TG and NEFA

5.3.2.1 [^{13}C]-labelled TG and NEFA

[^{13}C]-labelled meal-derived TG was detected in plasma from 60 minutes. Subjects with the metabolic syndrome had a higher and later peak arterial concentration of [^{13}C]-labelled meal-derived TG than the healthy controls (P=0.02) (Fig. 5.8A). In addition, over the postprandial period as a whole, the systemic concentration of [^{13}C]-labelled meal-derived TG was greater in the metabolic syndrome group (P=0.05). Adipose tissue clearance of [^{13}C]-labelled meal-derived TG (fractional extraction multiplied by blood flow) was lower in those with the metabolic syndrome (P=0.03) (Fig. 5.8B). Although there was also a trend for muscle clearance of [^{13}C]-labelled dietary TG to be lower in the subjects with the metabolic syndrome, this was not statistically significant. All other measures of [^{13}C]-labelled meal-derived TG removal across both adipose tissue and muscle were similar in the two groups. There was no difference in systemic non-esterified [U-^{13}C]palmitate concentrations between the study groups. Furthermore, all measures of [U-^{13}C]palmitate flux across adipose tissue and muscle were similar in the two groups.

5.3.2.2 [$^{2}H_2$]-labelled NEFA and TG

Arterial [$^{2}H_2$]palmitate concentrations (infused tracer) did not vary before the meal, showing that steady state conditions had been reached prior to any blood sampling.

There was no difference between the study groups in concentrations of [$^2H_2$]palmitate or measurements of flux across either adipose tissue or muscle. Consequently, there was no evidence of reduced NEFA uptake by muscle in those with the metabolic syndrome. Similarly, concentrations of [$^2H_2$]-labelled TG, reflecting incorporation of the tracer into VLDL-TG, were not higher in the subjects with the metabolic syndrome. No difference was demonstrated between the study groups in any of the measurements of [$^2H_2$]-labelled TG extraction across either adipose tissue or muscle.

5.3.2.3 $Ra_{(NEFA)}$

The steady state and non-steady state calculations produced very similar results for $Ra_{(NEFA)}$. When expressed as a function of fat mass, fasting $Ra_{(NEFA)}$ (μmol/min/kg fat mass) was lower in the subjects with the metabolic syndrome (P=0.01) whilst the postprandial fall in $Ra_{(NEFA)}$ was significantly greater in the healthy control group (P=0.04) (Fig. 5.4B). In contrast, when expressed as a function of lean body mass, there was no difference between the groups in any of the aspect of $Ra_{(NEFA)}$.

5.3.3 Measurements of fatty acid oxidation

There was no difference between the study groups in blood 3-hydroxybutyrate concentrations, $^{13}CO_2$ production, RER or whole body substrate oxidation in either the fasting or postprandial periods.

5.3.4 Re-classification of subjects by EGIR criteria

Using the EGIR criteria to define the metabolic syndrome resulted in some interesting changes to the baseline characteristics of the subject groups (Table 5.1) and

the study findings. Following re-definition, there were no statistically significant differences between the study groups in BMI or fat mass, although the difference in waist circumference was maintained. Fasting insulin concentrations are an essential criterion in the EGIR definition and thus were different between those classified as having the metabolic syndrome and those without. The elevated insulin levels were maintained throughout the study in those with the metabolic syndrome (P=0.05) (Fig. 5.9A). In contrast to ATP III-defined subjects with the metabolic syndrome, TG levels were similar in the EGIR-defined metabolic syndrome and healthy control groups (Fig. 5.9B). In addition, the differences in tissue TG clearance were attenuated by re-classification of subjects. The differences in net adipose tissue NEFA output were somewhat more marked, when subjects were re-classified (Fig. 5.10A). The differences in $Ra_{(NEFA)}$ were very similar (Fig. 5.10B).

Table 5.1 Baseline characteristics of subjects dependent on criteria used for the diagnosis of the metabolic syndrome

	NCEP-ATP III(113)			EGIR(114)		
	Metabolic Syndrome (n=7)	Controls (n=11)	P	Metabolic Syndrome (n=8)	Controls (n=10)	P
Age (yrs)	46 (36-52)	42 (32-54)	NS	44 (32-49)	43 (34-54)	NS
Weight (kg)	94 (81-104)	86 (73-99)	NS	93 (73-104)	86 (77-102)	NS
BMI (kg/m^2)	31 (26-35)	27 (25-32)	0.03	28 (25-35)	28 (25-32)	NS
Fat Mass (kg)	21 (17-32)	19 (14-22)	0.01	19 (17-32)	17 (14-28)	NS
Insulin (mU/l)	15.7 (4.9-28.7)	11.9 (6.1-22.6)	NS	15.8 (10.7-28.7)	9.6 (4.9-16.0)	<0.01
Waist (cm)	105 (99-112)	97 (86-108)	<0.01	99 (97-112)	91 (86-110)	0.04
TG (mmol/l)	2.1 (1.1-3.3)	1.1 (0.7-2.4)	0.01	1.3 (0.9-3.3)	1.3 (0.7-2.4)	NS
HDL-cholesterol (mmol/l)	1.0 (0.9-1.2)	1.1 (0.9-1.4)	NS	1.0 (0.9-1.4)	1.1 (1.0-1.3)	NS
Glucose (mmol/l)	5.6 (5.0-6.3)	5.4 (4.4-6.6)	NS	5.8 (5.0-6.6)	5.2 (4.4-6.0)	NS
Systolic Blood Pressure (mmHg)	134 (122-143)	125 (104-142)	NS	130 (104-143)	124 (116-143)	NS
Diastolic Blood Pressure (mmHg)	87 (73-96)	80 (75-98)	NS	86 (75-98)	80 (73-88)	NS

Data are presented as Median (Range). Biochemical data are all fasting plasma concentrations. Statistical differences calculated by unpaired Student's t-test. Comparisons are between subjects with and without the metabolic syndrome defined by NCEP-ATP III criteria on the left and EGIR criteria on the right. The two definitions classified subjects differently (see main text).

Table 5.2 Summary of principal study results

	Fasting			Postprandial		
	Metabolic Syndrome (n=7)	Control (n=11)	P	Metabolic Syndrome (n=7)	Control (n=11)	P
Blood flow						
Adipose (ml/100g/min)	1.8 ± 0.3	2.67 ± 0.2	0.04	2.26 ± 0.19	2.67 ± 0.13	NS
Muscle (ml/100ml/min)	1.9 ± 0.2	1.9 ± 0.2	NS	2.13 ± 0.26	1.92 ± 0.23	NS
Arterial NEFA (μmol/l)	358 ± 21	353 ± 22	NS	284 ± 25	278 ± 11	NS
Arterial TG (mmol/l)	1.3 ± 0.2	0.7 ± 0.1	0.01	1.7 ± 0.2	1.0 ± 0.1	0.01
Net NEFA flux						
Adipose output (nmol/100g/min)	554 ± 244	1260 ± 199	0.02	301 ± 84	593 ± 71	0.02
$Ra_{(NEFA)}$ (μmol/min/kg fat mass)	27.1 ± 2.1	37.8 ± 2.9	0.01	27.0 ± 3.1	30.3 ± 1.8	NS
Muscle uptake (nmol/100g/min)	38 ± 37	52 ± 30	NS	50 ± 23	53 ± 16	NS
Postprandial fall in adipose tissue net NEFA flux (incremental AUC)						
Adipose tissue NEFA output (μmol/100g/min)	N/A	N/A	N/A	-218 ± 157	-728 ± 175	0.02
$Ra_{(NEFA)}$ (μmol/min/kg fat mass)	N/A	N/A	N/A	-1.35 ± 0.34	-3.09 ± 0.65	0.03
TG clearance (ml/100g/min)						
Adipose	0.07 ± 0.04	0.19 ± 0.05	NS	0.05 ± 0.01	0.11 ± 0.02	0.02
Muscle	0.03 ± 0.03	0.04 ± 0.02	NS	0.04 ± 0.01	0.09 ± 0.02	0.03
[^{13}C]-labelled meal-derived TG (from 60 minutes)						
Arterial concentration (μmol/l)	N/A	N/A	N/A	1.39 ± 0.16	0.94 ± 0.14	0.05
Adipose tissue clearance (ml/100g/min)	N/A	N/A	N/A	0.23 ± 0.03	0.45 ± 0.07	0.01
Muscle clearance (ml/100g/min)	N/A	N/A	N/A	0.45 ± 0.14	0.64 ± 0.16	NS
[$^{2}H_2$]-labelled TG						
Arterial concentration (μmol/l)	N/A	N/A	N/A	2.20 ± 0.37	1.76 ± 0.26	NS
Adipose tissue clearance (ml/100g/min)	N/A	N/A	N/A	0.07 ± 0.04	0.15 ± 0.05	NS
Muscle clearance (ml/100g/min)	N/A	N/A	N/A	0.08 ± 0.03	0.08 ± 0.02	NS

The data are expressed as means ± SEM for fasting and postprandial states. The fasting data are means of the -60,-30 and 0 timepoints. The postprandial data are area under the curve (AUC) calculations unless otherwise stated. Although measurable, fasting concentrations of [$^{2}H_2$]-labelled TG were too low to allow meaningful flux calculations. Unpaired Student's *t*-test has been used to calculate differences between subject groups.

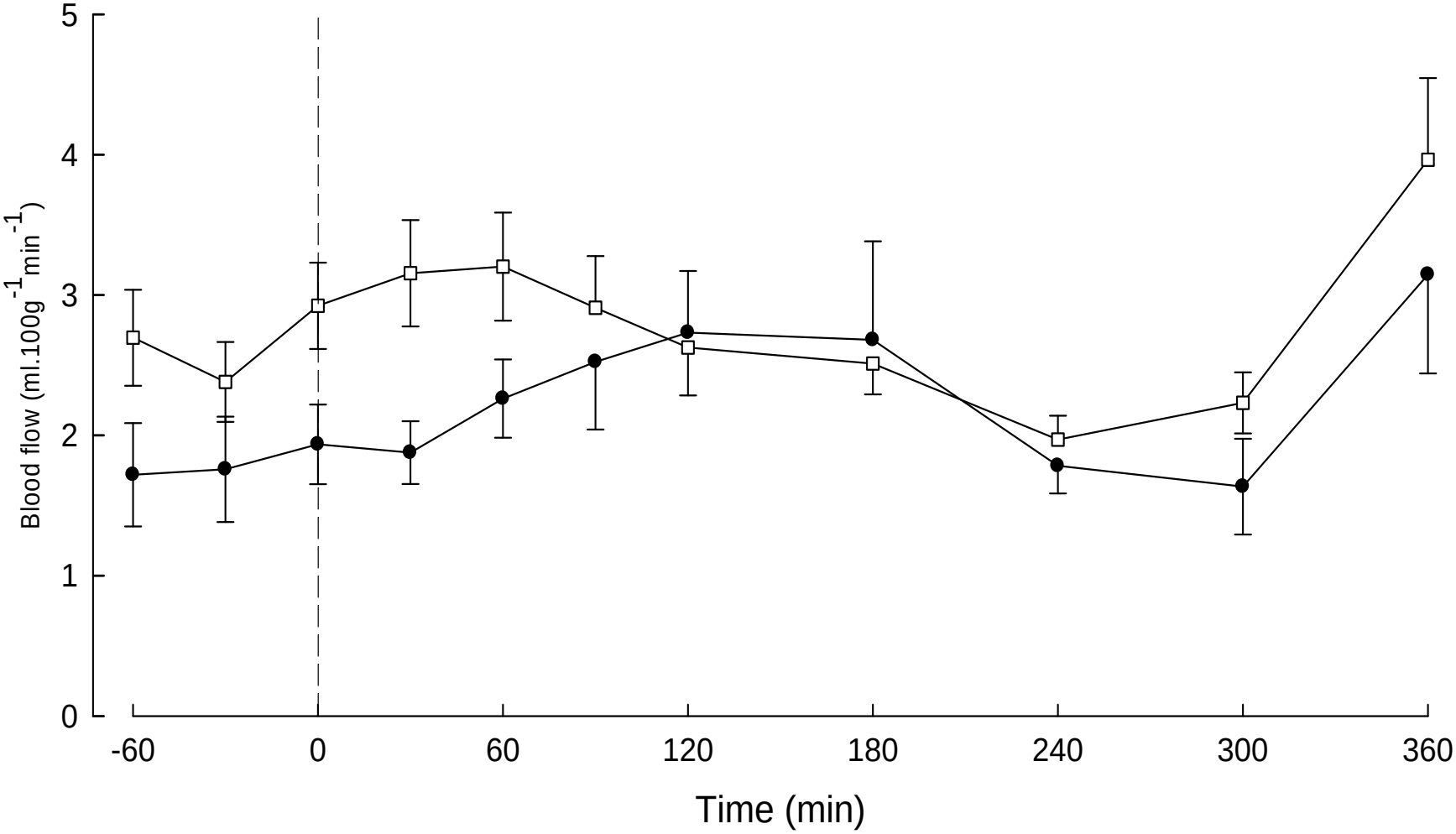

Figure 5.1 Adipose tissue blood flow following a mixed meal in subjects with the ATPIII defined metabolic syndrome (solid circles) and healthy controls (open squares)

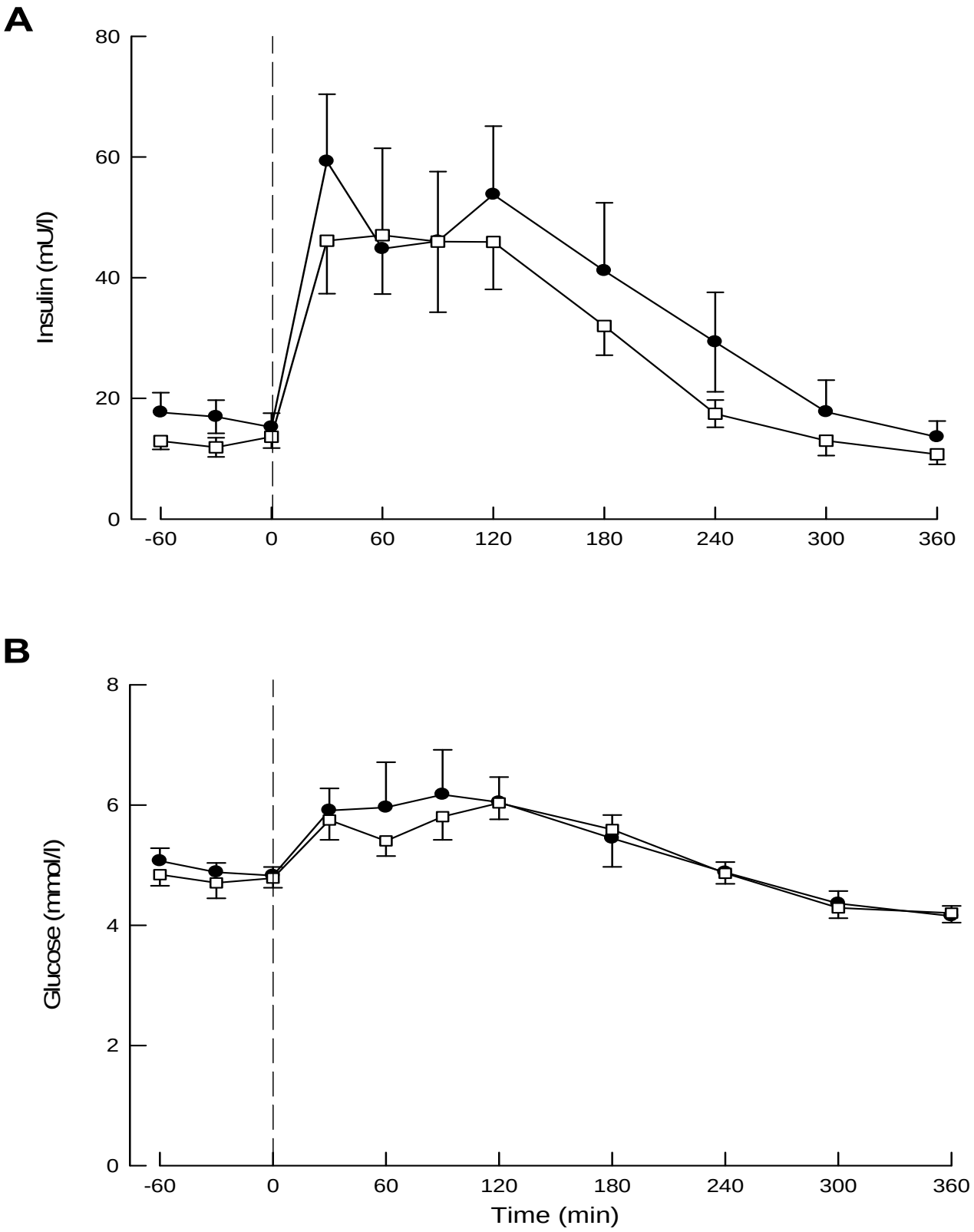

Figure 5.2 Whole plasma arterial concentrations of insulin (A) and glucose (B) following a mixed meal in subjects with the ATPIII defined metabolic syndrome (solid circles) and healthy controls (open squares).

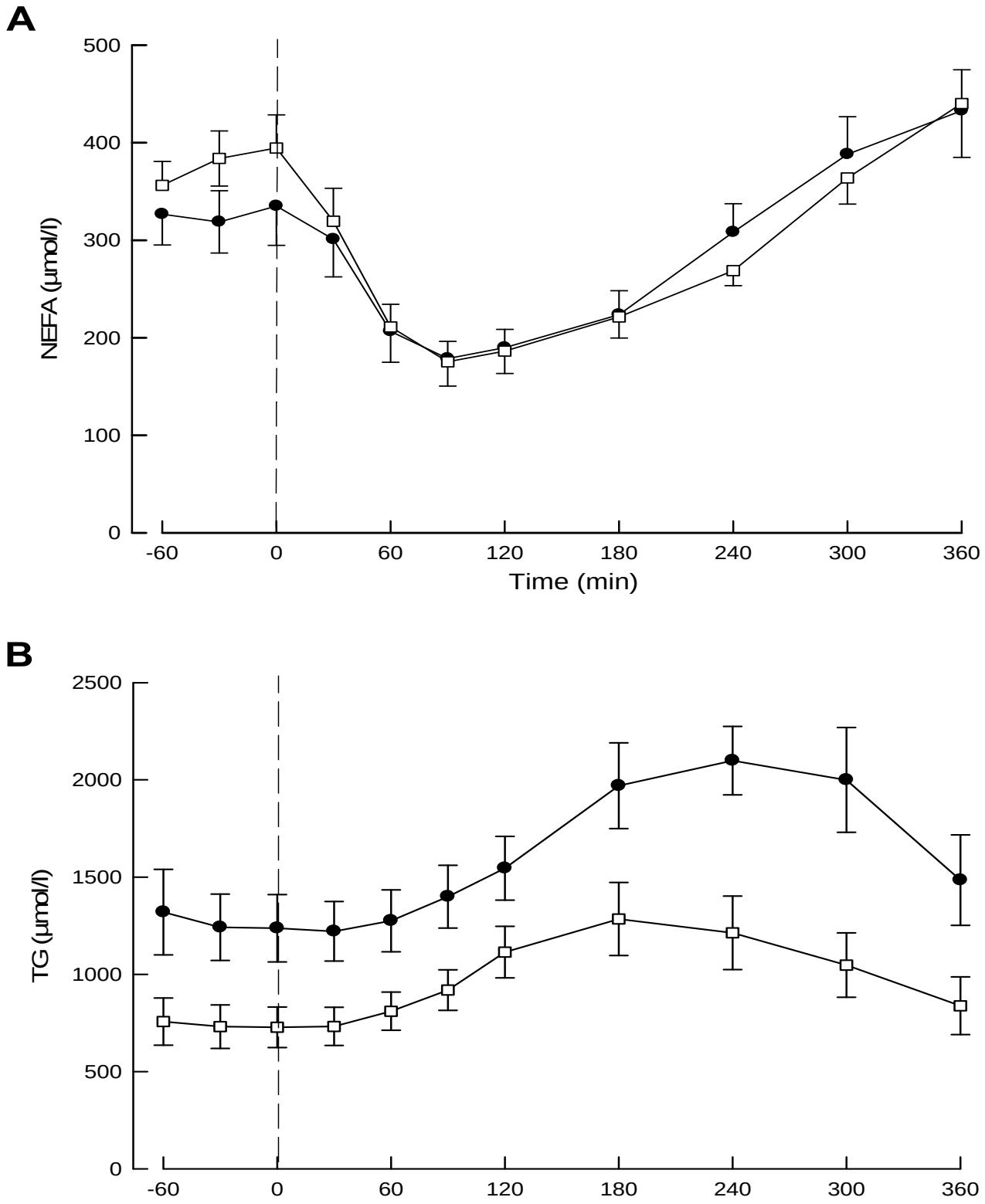

Figure 5.3 Whole plasma arterial concentrations of NEFA (A) and TG (B) following a mixed meal in subjects with the ATPIII defined metabolic syndrome (solid circles) and healthy controls (open squares). The only statistically significant difference between the two groups was in arterial TG (P=0.01).

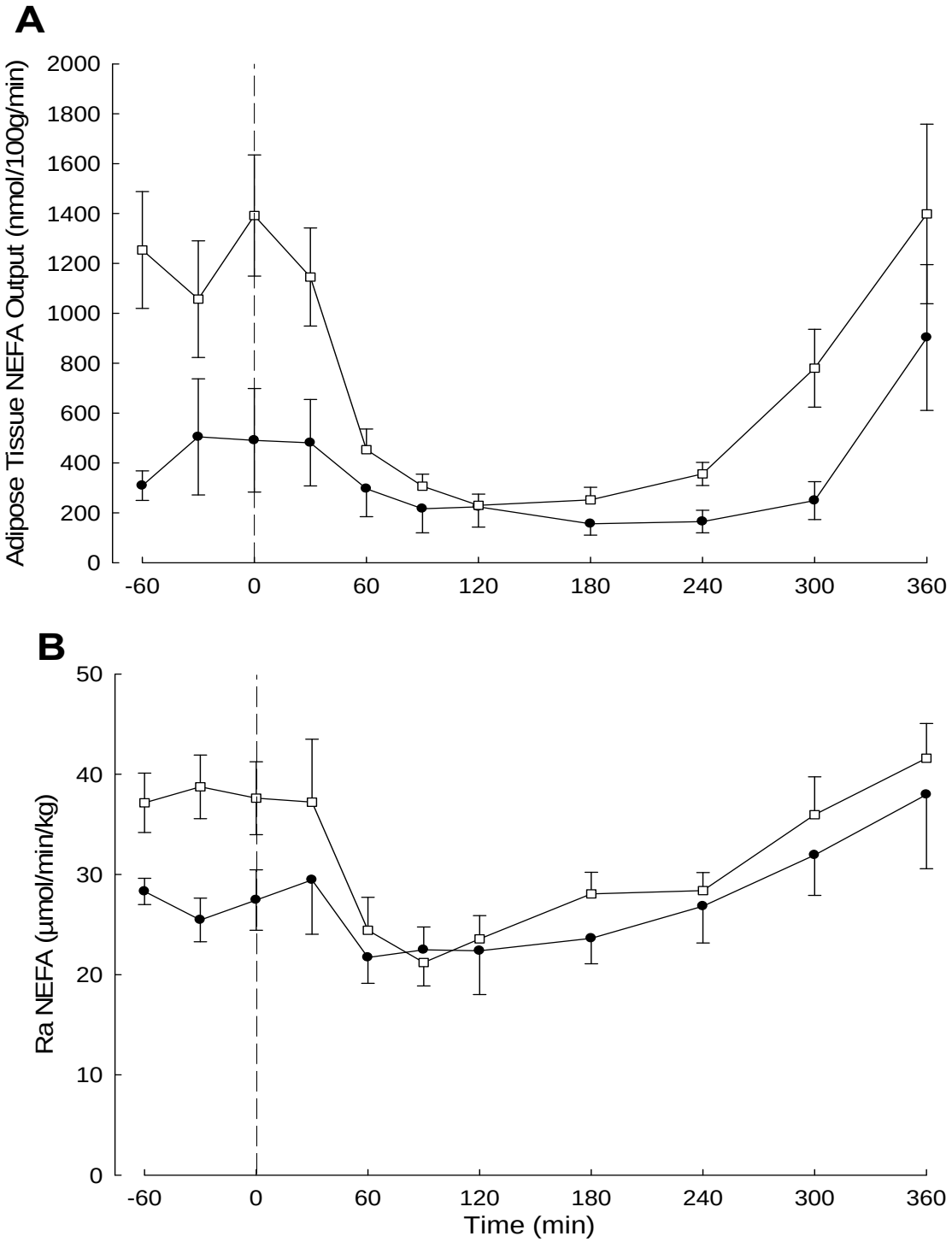

Figure 5.4 Adipose tissue fatty acid flux following a mixed meal in subjects with the ATPIII-defined metabolic syndrome (solid circles) and healthy obese controls (open squares). Net NEFA output from adipose tissue (A) was calculated as the V-A difference in NEFA concentrations multiplied by the blood flow. $Ra_{(NEFA)}$ (B) was calculated using Steele's equation for steady-state conditions modified for use with stable isotopes (244) and is expressed relative to fat mass. Adipose tissue fatty acid output and $Ra_{(NEFA)}$ were lower during fasting (P= 0.02 and 0.01 respectively) and fell less postprandially in the subjects with the metabolic syndrome (P= 0.02 and 0.04 respectively).

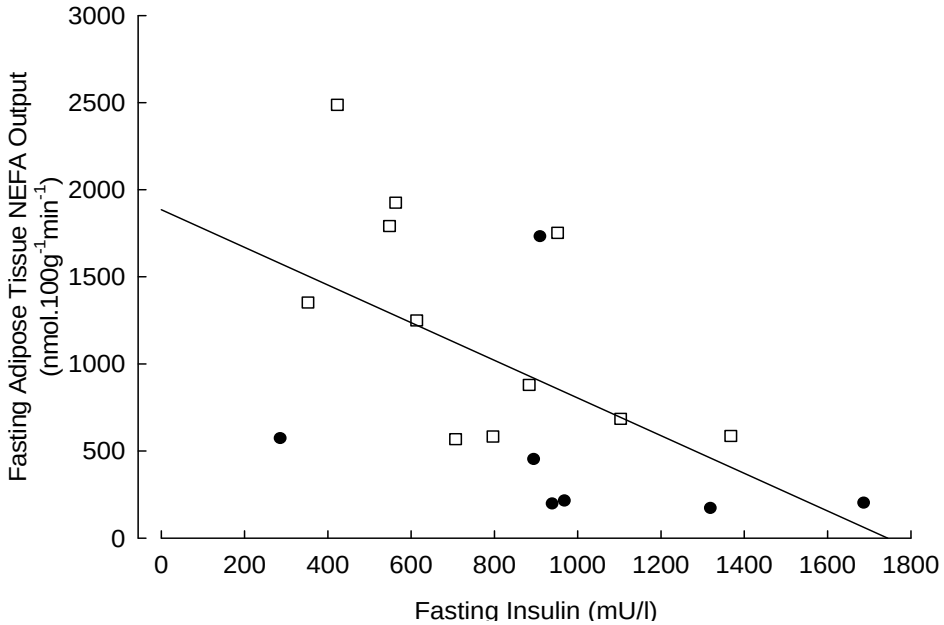

Figure 5.5 Relationship between fasting insulin and fasting net adipose tissue NEFA output, expressed per 100g tissue, in subjects with the metabolic syndrome (solid circles) and healthy obese controls (open squares). There was a negative correlation between fasting net NEFA output and insulin concentration across the whole study population (r_s=-0.53, P=0.02).

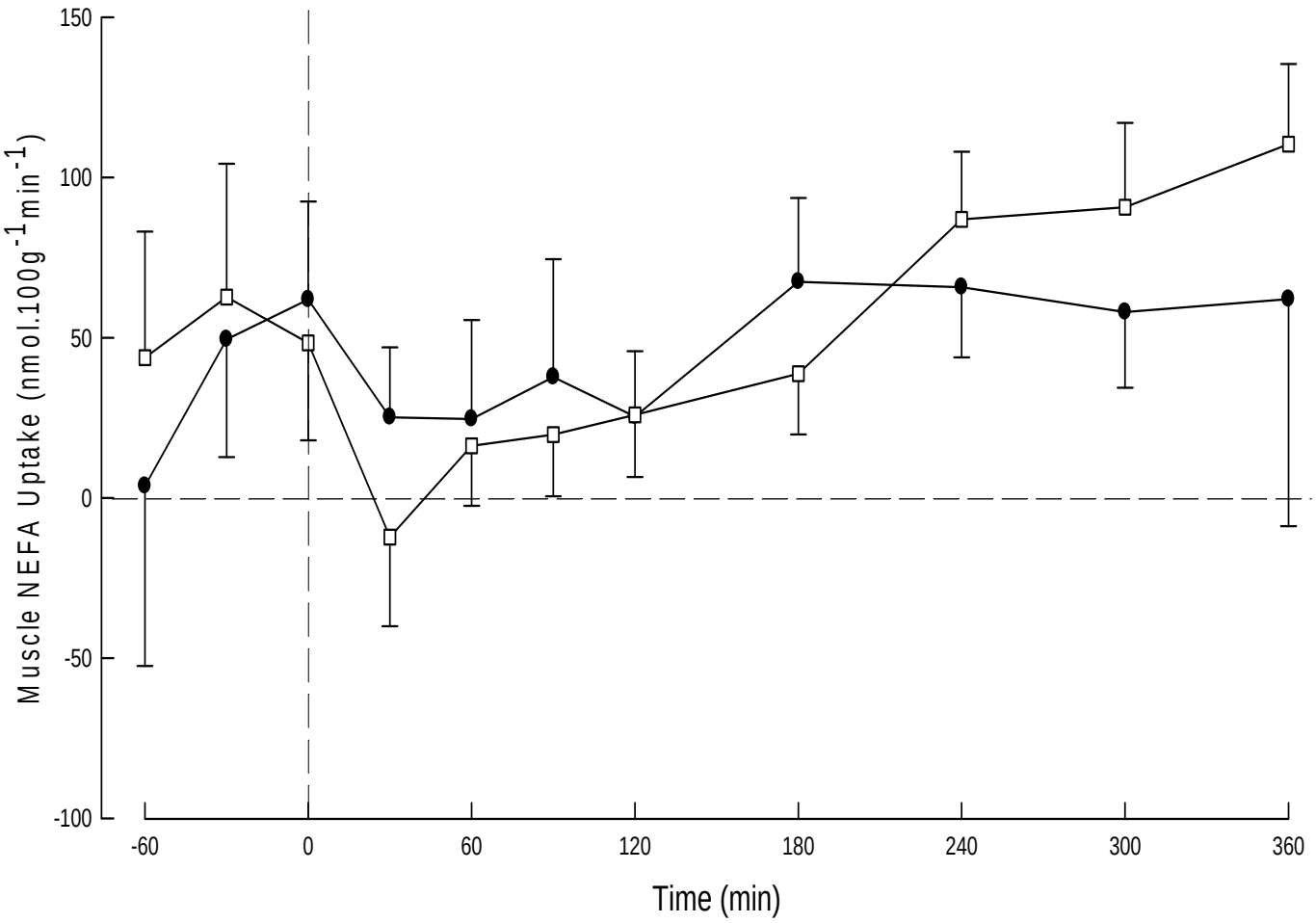

Figure 5.6 Skeletal muscle fatty acid flux following a mixed meal in subjects with the ATPIII-defined metabolic syndrome (solid circles) and healthy obese controls (open squares). Net NEFA uptake by muscle was calculated as the A-V difference in NEFA concentration multiplied by muscle blood flow. There was no difference in uptake of NEFA across muscle between the study groups

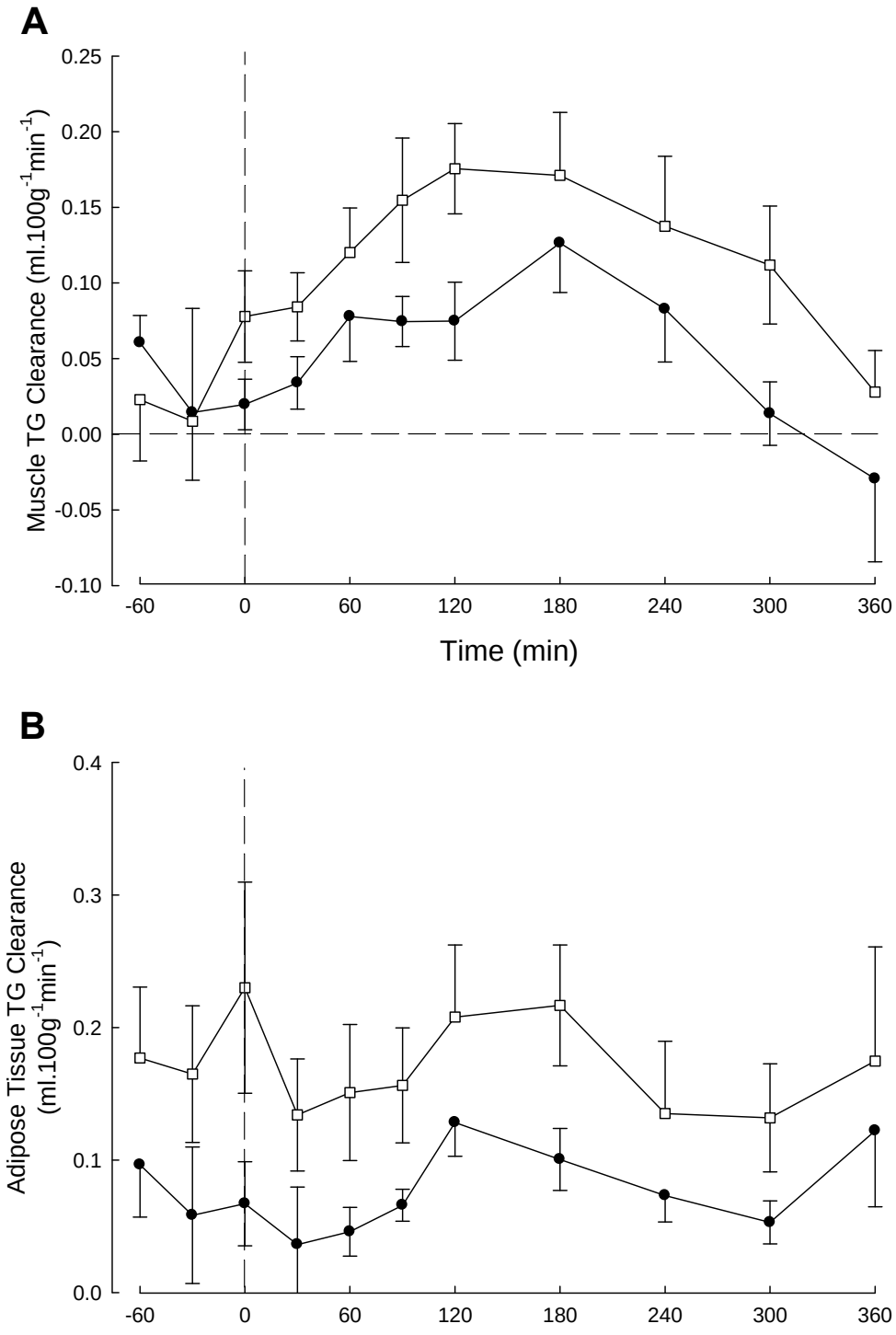

Figure 5.7 Tissue-specific clearance of TG following a mixed meal in subjects with the ATPIII-defined metabolic syndrome (solid circles) and healthy obese controls (open squares). A: Clearance of total TG across muscle. B: Clearance of total TG across adipose tissue. Subjects with the metabolic syndrome cleared less total TG across both muscle (P=0.03) and adipose tissue (P=0.02) following the meal.

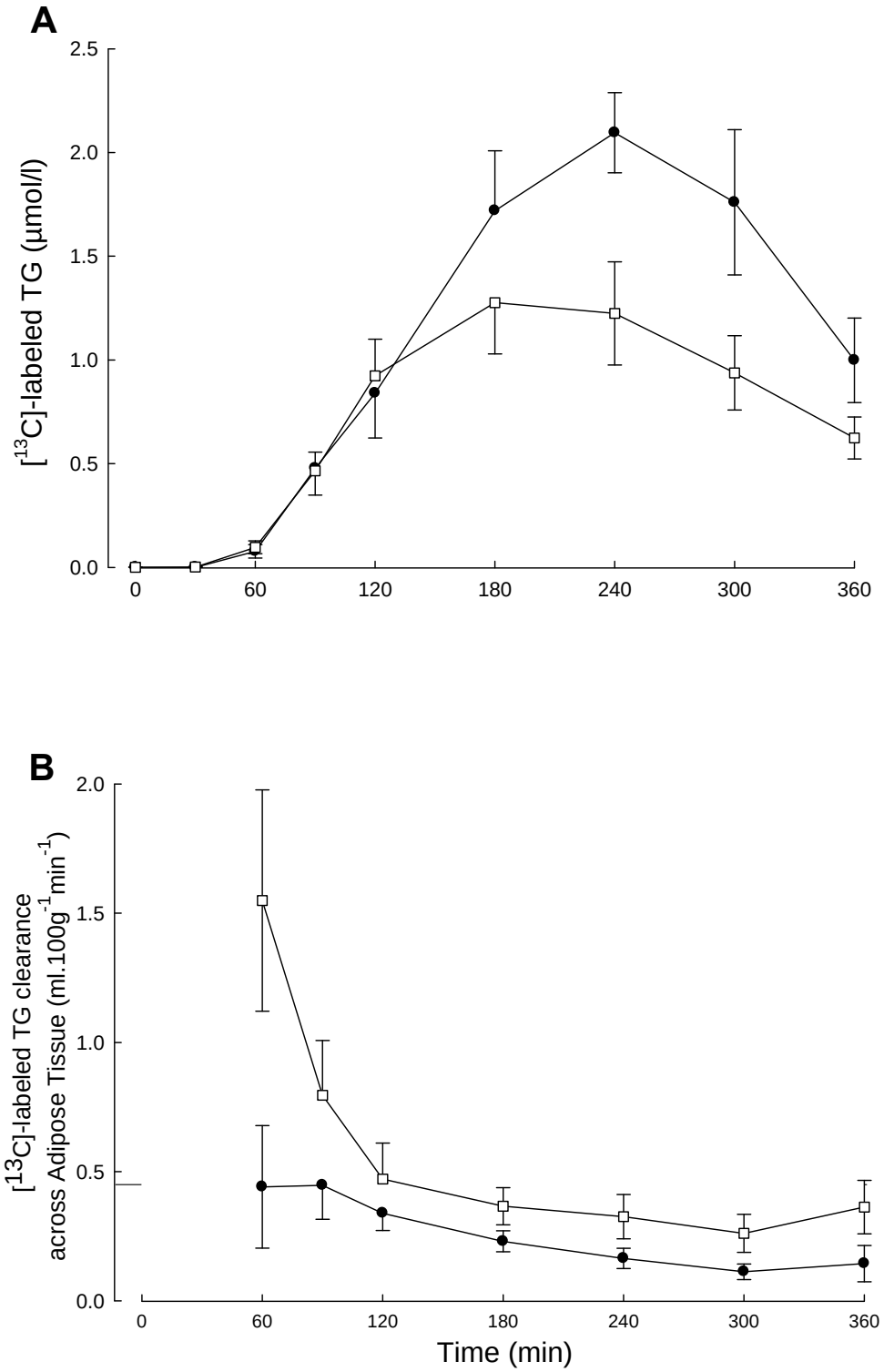

Figure 5.8 Fluxes of [^{13}C]-labelled palmitic acid following ingestion as part of a mixed meal in subjects with the ATPIII-defined metabolic syndrome (solid circles) and healthy controls (open squares). A: Appearance of [^{13}C] labelled TG in the arterial plasma. B: Clearance of [^{13}C] labelled TG across adipose tissue. The clearance was lower in the subjects with the metabolic syndrome than in the healthy controls (P=0.03).

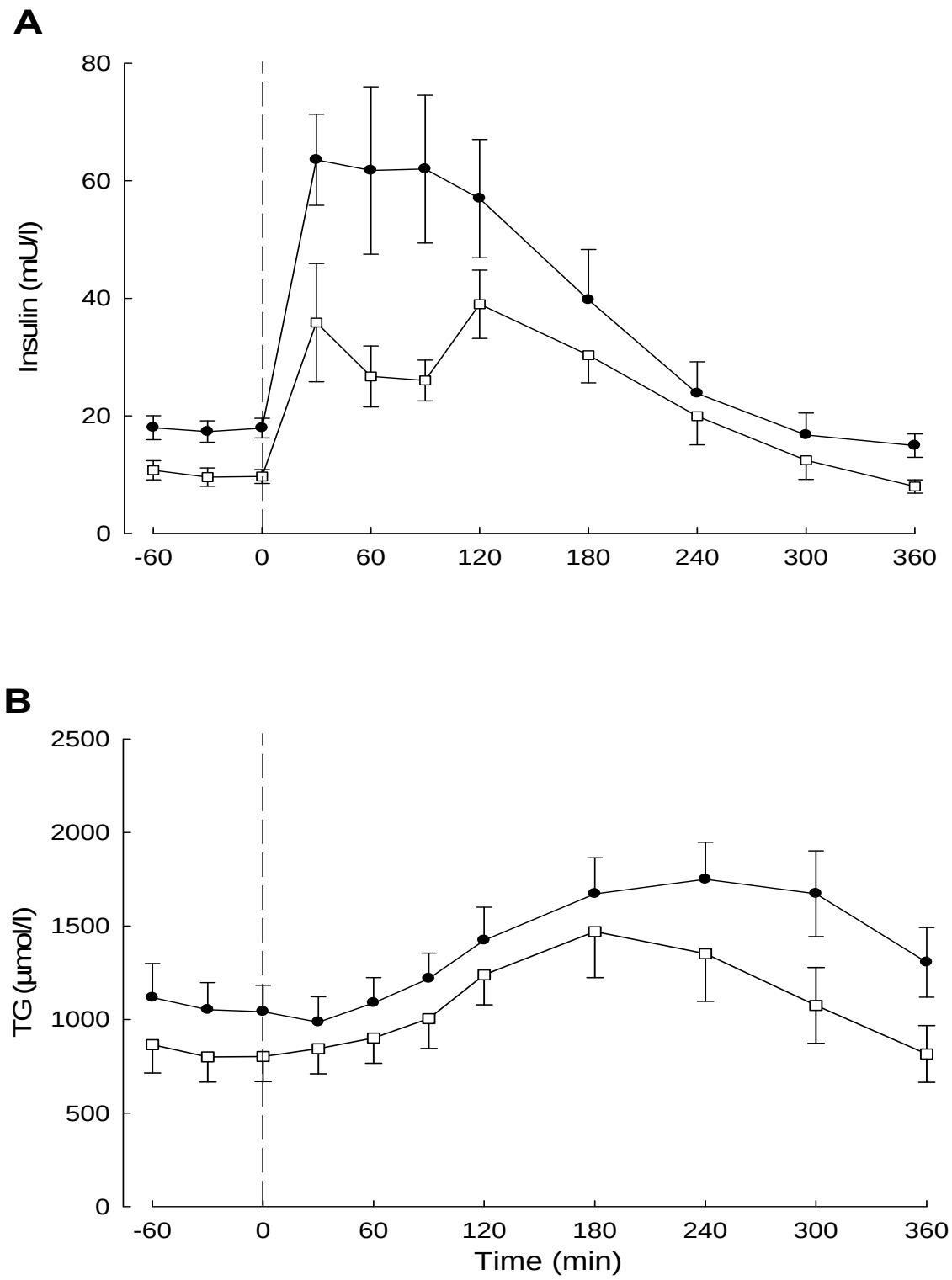

Figure 5.9 Whole plasma arterial concentrations of insulin (A) and TG (B) following re-definition of subjects using the EGIR criteria for the diagnosis of the metabolic syndrome. Both figures represent changes following a mixed meal in subjects with the metabolic syndrome (solid circles) and healthy controls (open squares).

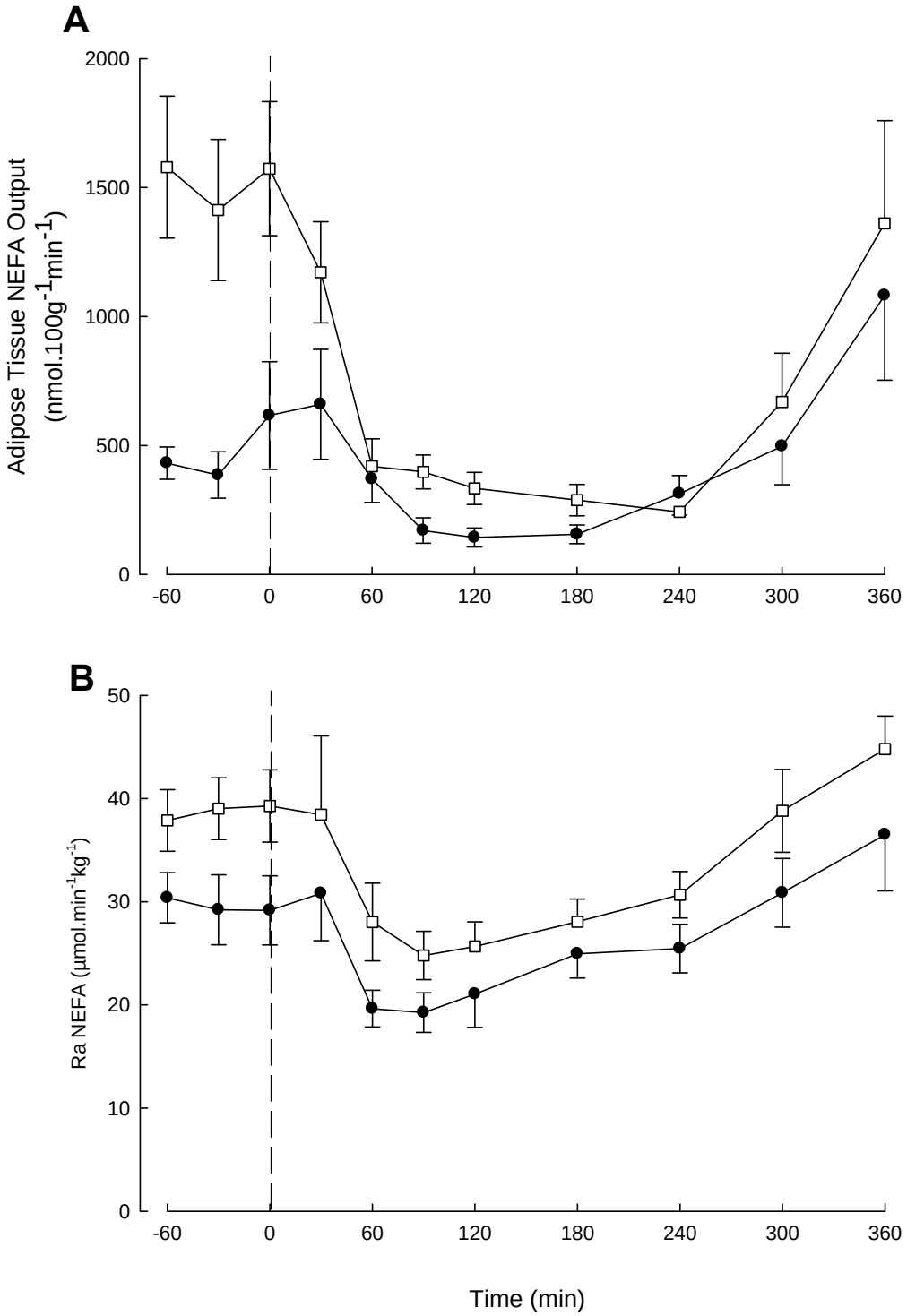

Figure 5.10 Adipose tissue fatty acid flux following re-definition of subjects using the EGIR criteria for the diagnosis of the metabolic syndrome. Both figures represent changes following a mixed meal in subjects with the metabolic syndrome (solid circles) and healthy controls (open squares) A: Net adipose tissue NEFA output. B: $Ra_{(NEFA)}$. The difference in net adipose tissue NEFA output and $Ra_{(NEFA)}$ was maintained following re-classification.

5.4 DISCUSSION

Disturbances in fat metabolism have been implicated in the pathophysiology of type 2 diabetes and consequent vascular disease (184, 185, 272). Two stable isotope tracers were used, in combination with arteriovenous balance techniques, to investigate whether the tissue-specific metabolic fate of both dietary-derived and circulating fatty acids differs between those with the metabolic syndrome and healthy overweight individuals. The most striking findings were that arterial NEFA concentrations did not differ between the two groups, that net adipose tissue NEFA output and $Ra_{(NEFA)}$, each expressed per unit fat mass, were lower during fasting and fell less postprandially in those with the metabolic syndrome and that peripheral TG clearance was lower than in healthy controls. Taken together, these findings demonstrate that the metabolic syndrome is characterized by a lack of dynamism or flexibility (198) in fat metabolism. In the fasting state, when NEFAs should be made available as a metabolic fuel, there is reduced hydrolysis of intracellular TG within adipose tissue and consequently low adipose tissue NEFA output. Similarly, under postprandial conditions, when FAs should be removed from circulating TG for storage, there is reduced intravascular TG hydrolysis, resulting in the persistence of TG within the circulation. The failure to alter fat metabolism in response to changing metabolic conditions would lead to maintained adiposity and an inappropriate supply of fat to non-adipose tissues.

The lack of difference in systemic NEFA concentration between the study groups mirrored the findings of the OBB analysis. The effect of fat mass on circulating NEFA has already been discussed. However, there are number of less

static moderators of NEFA metabolism. The plasma NEFA concentration is determined by the balance between NEFA production from TG lipolysis in adipose tissue and NEFA uptake. The control of these processes is complex and incompletely understood. However, insulin is known to play key roles in the suppression of intracellular TG lipolysis (31), the promotion of intravascular TG hydrolysis (11), and carrier-mediated NEFA uptake (19, 307) in adipose tissue. Thus, it has been suggested that the high NEFA concentrations that have been previously associated with type 2 diabetes and obesity are a reflection of adipose tissue insulin resistance resulting in net increased NEFA output (43). In this study there was a negative correlation across the whole study population between net NEFA output from adipose tissue and insulin concentrations during fasting (Fig. 5.5). In addition, following the meal, both study groups suppressed net adipose tissue NEFA output. An earlier study has shown similar results in patients with type 2 diabetes (245). These findings confirm previous *in vitro* studies demonstrating a maintained anti-lipolytic effect of insulin on isolated adipocytes derived from individuals varying in metabolic health from the healthy lean to patients with diabetes (308, 309). Thus, despite the multiple additional modulators of fatty acid metabolism occurring *in vivo*, in the fasting state adipose tissue retains sensitivity to the systemic insulin concentration across the metabolic spectrum from simple obesity, through the metabolic syndrome to type 2 diabetes.

The maximal suppression of net fatty acid output from adipose tissue was similar to that seen in previous studies of obese individuals (43). However, the difference in decrement in net fatty acid output from adipose tissue suggests that, taken in the round, adipose tissue in subjects with the metabolic syndrome may not be able to respond entirely robustly to prevailing metabolic conditions. The inability of a

tissue to respond appropriately to changes in the metabolic milieu has been termed metabolic inflexibility and has been put forward as a conceptual framework for the pathophysiological events occurring in obesity, insulin resistant states and type 2 diabetes (198). In the present study, the metabolic inflexibility largely reflects the fact that the subjects with the metabolic syndrome have a lower net fatty acid output from adipose tissue in the fasting state.

The lack of difference in muscle NEFA uptake between the two study groups is interesting. The fatty acid concentration gradient across the myocyte cell membrane is a critical determinant of NEFA uptake by muscle. In addition, fatty acid translocase (FAT/CD36) and fatty acid transport protein 1 (FATP1), probably both modulated by insulin, also play important roles in the transport of NEFA into muscle (19, 310). As there was no difference in systemic NEFA or insulin concentrations between the two study groups, the lack of difference in fatty acid uptake is not entirely unexpected. However, this finding contrasts with the impaired uptake of NEFA by muscle that has been demonstrated in obesity (311), in patients with impaired glucose tolerance (312) and in patients with diabetes (313). Thus, individuals with the metabolic syndrome do not appear to demonstrate the same disturbance of fatty acid metabolism in muscle observed in other insulin resistant conditions.

The lack of difference in muscle NEFA uptake undoubtedly contributes also to the finding that $Ra_{(NEFA)}$ expressed as a function of lean body mass did not differ between groups, irrespective of which criteria were used to define the metabolic syndrome. The constituents of lean body mass are the main consumers of NEFA. Thus, the expression of Ra(NEFA) in terms of lean body mass indicates whether, on a

whole body basis, there are differences between groups in terms of NEFA utilisation. There was a difference in fat mass between those with and without the metabolic syndrome by ATPIII criteria and consequently, there was also a difference in lean body mass. However, as there was no difference in $Ra_{(NEFA)}$, it appears unlikely that there are differences in NEFA utilisation on a whole body basis between those with and without the metabolic syndrome, however defined.

This is the first study to show a specific defect of postprandial TG clearance across both muscle and adipose tissue in subjects with the metabolic syndrome. Although earlier work showed similar findings across adipose tissue in obesity (13, 43), the obese groups in those studies were heterogenous, including some individuals with diabetes, and the control groups were lean. Similarly, in the study comparing muscle TG clearance in females with different distributions of body fat (180), the upper body obese group contained one subject with diabetes. Furthermore, the study design maintained subjects in a constant fed state, rather than examining a true post-prandial period. Comparing similarly overweight individuals under physiological conditions and excluding those with diabetes reduced the number of confounding variables in the current study.

The use of stable isotopes allows differentiation between the clearance of dietary–derived ([^{13}C]-labelled) and endogenous ([$^{2}H_2$]-labelled) TG. There was lower clearance of [^{13}C]-labelled dietary TG across adipose tissue in those with the metabolic syndrome. This was especially notable in the early to mid postprandial period (Fig. 5.8b) when nascent chylomicron concentration is greatest. In addition,

there was a trend for a similar difference in clearance across muscle. In contrast, there was no difference between the study groups in the clearance of [$^2H_2$]-labelled TG across either of the tissues studied. These findings suggests that the principal defect is in the clearance of dietary-derived TG. The consequence of reduced postprandial clearance of dietary-derived TG is persistence in the circulation.

There was no difference in any of the measures of oxidation between the study groups. A defect of fasting fatty acid oxidation in muscle has been shown in patients with obesity (196), impaired glucose tolerance (312) and type 2 diabetes (313, 314). The measures of oxidation in the current study examine the oxidation of dietary derived ($^{13}CO_2$) and all fat (RER) on a whole body basis. In addition, the 3-hydroxybutyrate measurements give an assessment of hepatic fatty acid oxidation. Therefore, although it remains possible that subjects with the metabolic syndrome have a deficit in muscle fatty acid oxidation, other tissues appear to compensate for this.

A high fasting insulin is intrinsic to the EGIR definition, providing a proxy measure of insulin resistance. Thus, any difference between the study groups may be due primarily to differences in insulin sensitivity. Net NEFA output from adipose tissue was lower during fasting and suppressed less following the meal in the EGIR-defined metabolic syndrome group. This is likely to be due in large part to the hyperinsulinemia of the EGIR-defined metabolic syndrome subjects, and adds weight to the contention that adipose tissue retains insulin sensitivity in the presence of the metabolic syndrome.

In conclusion, systemic NEFA concentrations are not elevated in subjects with the metabolic syndrome, however defined, when compared with similarly overweight healthy individuals. In addition, the combination of stable isotope methodology and arteriovenous difference measurements has demonstrated that fasting net NEFA output from adipose tissue is lower per unit fat mass with a reduced degree of postprandial suppression. Furthermore, peripheral TG clearance, especially of dietary-derived TG, is impaired in those with the metabolic syndrome. Thus, there is an overall loss of metabolic dynamism in response to changing metabolic conditions. The cellular mechanisms underlying these findings remain to be elucidated, however, adipose tissue resistance to the antilipolytic effect of insulin does not seem to play a significant role. This exemplifies the fact that the resistance to the actions of insulin varies from tissue to tissue and substrate to substrate. Thus, the blanket term "insulin resistance" incompletely describes the complexity of pathophysiological processes occurring in states such as the metabolic syndrome and type 2 diabetes.

Chapter 6 : Summary and Conclusion

6.1 Summary

Fat metabolism is a complex process. Healthy individuals living in industrialized societies consume approximately 80 – 100 g of fat daily (315, 316). This fat is a major source of energy that drives many metabolic processes. Simply oxidizing all fat would be both wasteful and not allow accommodation for times when food is less plentiful. Thus, a significant quantity of fat ingested is stored. A study of US college students demonstrated that amongst the non-obese (BMI<24), the average fat mass was 23.5% (317). Therefore, a 70 kg man carries 16.5 kg of fat. However, even in times of plenty, fat metabolism is dynamic. A bolus of radioactively labelled fatty acid injected intravenously will be cleared from the blood rapidly with a half life of only a few minutes (277). Clearly, with such significant intake of fat, such capacity for storage and such rapid turnover of fatty acids allied to the necessity for the provision of energy, fat metabolism must be tightly regulated and appropriate to the prevailing metabolic conditions. In other words, when energy sources are plentiful fat metabolism must be primarily aimed at storage, whereas at times of need fat must be mobilized to provide the energy necessary for metabolism.

Different organs have different roles in the storage and utilization of fatty acids. Skeletal muscle, the heart, the liver and the kidneys are all able to use fatty acids as oxidisable fuel sources. Fat can be stored in adipose tissue and, to some extent, skeletal muscle and the liver. Fat metabolism in the liver and adipose tissue is particularly complex since both tissues are able to store and oxidise fatty acids as well as synthesizing and hydrolyzing triglycerides. It is especially important to appreciate dynamic nature of adipose tissue which was, for many years, considered simply an

inert storage depot for fatty acids. In addition to local fat metabolism, it is now apparent that adipose tissue is involved in hormonal signaling and inflammation, all of which impact on whole body fat metabolism.

The complex regulatory processes that govern fat metabolism on both a whole body and tissue specific basis are not fully understood. Consequently, I undertook a study to investigate tissue-specific fat metabolism using stable isotopically-labelled fatty acids. In adipose tissue, one of the key findings was characterization of the direct uptake of fatty acids from the circulating NEFA pool. The data presented in Chapter 3 demonstrate that although fatty acids derived from LPL-mediated chylomicron hydrolysis are preferentially chanelled into adipose tissue, uptake from the circulating NEFA pool is of, previously underestimated, quantitative significance. Indeed, over one third of fatty acids taken into adipose tissue during the mid-post prandial period, when chylomicron concentrations peak, are derived from the latter source. In muscle, there was postprandial release across the forearm of fatty acids which are likely to be derived from the hydrolysis of circulating intravascular TG rather than intramuscular TG stores. The findings of these studies are of particular interest as they represent normal physiological responses to a mixed meal. This is in contrast to the use of infusions or ingestion of single nutrients in isolation, which, although producing a more marked response, are less representative of the physiological processes occurring in free living individuals. Taken together the findings in both adipose tissue and muscle illustrate the dynamic nature of fat metabolism and give some indication of the sites of regulation. The precise molecular mechanisms governing these processes remain to be elucidated.

Obesity and type 2 diabetes are conditions in which it is likely that there is an intimate relationship between disease and disturbances in the normal metabolic pathways of fat. This assertion is strengthened by the observation that the burden of morbidity and mortality associated with type 2 diabetes is vascular in origin (5) and thus closely related to abnormalities of circulating lipids (318). The metabolic syndrome describes a cluster of metabolic abnormalities closely associated with type 2 diabetes (131, 154, 155) and vascular disease (128, 151-153). Thus, it is attractive to study fatty acid metabolism in individuals with the metabolic syndrome in order to gain insights into the pathophysiological processes underlying type 2 diabetes and vascular disease. In the large cohort of similarly overweight individuals that I reviewed, there was no difference in arterial NEFA concentrations between those with and without the metabolic syndrome. This was unexpected as the perceived wisdom is that the metabolic syndrome is associated with adipose tissue resistance to the anti-lipolytic effect of insulin (203) and consequently associated with elevated NEFA concentrations (183). Previous studies have compared groups of differing fat masses which may go some way to explaining the observed difference in NEFA concentrations. In addition insulin is only one of a number of modulators of lipolysis and circulating NEFA concentrations. Thus, differences in NEFA between study groups may be due to factors other than adipose tissue insulin resistance. Differences in such factors between those with and without the metabolic syndrome have been little studied and never put forward as an explanation for the perceived differences in circulating NEFAs.

I therefore went on to study how tissue-specific fatty acid metabolism differed between those with and without the metabolic syndrome. The key findings related to

adipose tissue were that despite no difference in arterial NEFA concentrations, net adipose tissue NEFA output and $Ra_{(NEFA)}$, expressed per unit of fat mass, were lower during fasting and fell less postprandially in those with the metabolic syndrome. In addition, during fasting there was a negative correlation between net NEFA output from adipose tissue and insulin concentrations across the whole study population. Furthermore, the postprandial clearance of TG across adipose tissue was lower in those with the metabolic syndrome. Therefore, in the metabolic syndrome, although there is some evidence of sensitivity to the anti-lipolytic effect of insulin, adipose tissue demonstrates a reduced facility to modify fat metabolism dependent on the prevailing metabolic conditions. During fasting, adipose tissue should be mobilizing fat stores, whereas following a meal, adipose tissue should be clearing TG and storing the derived fatty acids. The term "metabolic inflexibility" was coined to describe the failure to appropriately switch between fatty acids and glucose as oxidative fuels for muscle under either fasting or insulin-stimulated conditions (198). However, the same concept can be utilised in the context of the metabolic syndrome to describe the overall loss of metabolic dynamism in adipose tissue metabolism. Thus, in summary, adipose tissue in the metabolic syndrome appears to retain insulin sensitivity, but lose metabolic flexibility.

The mechanistic explanation of such metabolic inflexibility remains elusive. It is possible that there are inherent elements within adipose tissue that adapt to respond inappropriately to those external stimuli which would normally drive fatty acid metabolism in one direction or the other. Potential candidates for such factors include the various transporters and enzyme systems within adipocytes. Alternatively, it may be the external stimuli that determine the variability in adipose tissue metabolism,

whilst adipose tissue itself maintains normal metabolic function. An obvious candidate for such an external modulating factor is insulin. Arterial insulin concentrations are elevated in obesity and the metabolic syndrome (data presented in Chapters 4 and 5), probably reflecting disturbances in glucose rather than fatty acid metabolism. Thus, hyperglycaemia precipitates hyperinsulinaemia, which in turn suppresses the flexibility of adipose tissue to respond appropriately to changes in the metabolic milieu. Finally, it may be a combination of intrinsic and extrinsic factors that together result in reduced metabolic flexibility. Obesity is increasingly recognized as an inflammatory state (319). In addition, obesity is associated with an increased macrophage infiltration of adipose tissue (320, 321) and the release of numerous adipokines, some with local and some with systemic effects (322). Thus, inflammation has the potential to suppress metabolic flexibility through a broad range of mediators. However, whether inflammation is a cause or simply a correlate of adipose tissue dysfunction remains an unanswered question.

The final considerations from a preventative and therapeutic perspective are whether metabolic flexibility, once lost, can be regained and whether a return to metabolic flexibility would reverse the disturbances in metabolism associated with metabolic inflexibility. Lifestyle changes involving weight loss and increased physical activity form the cornerstone of the management of type 2 diabetes and the metabolic syndrome (323). Indeed, intensive lifestyle interventions may prevent the incidence of diabetes (324, 325). As metabolic inflexibility is at the core of adipose tissue dysfunction in the metabolic syndrome, it would seem logical that either weight loss, increased physical activity or both may improve adipose tissue metabolic flexibility. Such studies are yet to be undertaken. However, when assessed in terms of oxidative

substrate selection, metabolic flexibility in obese individuals improves following weight loss (196, 326). Interestingly, in order for all aspects of metabolic flexibility to be improved weight loss needs to be achieved through the combination of reduced caloric intake and increased physical activity. Caloric restriction alone improves insulin stimulated, but not fasting substrate oxidation (196).

6.2 Future directions

The studies described in this thesis pose as well as answer many questions related to fat metabolism. The techniques described can be used to answer some of these questions.

The mechanisms controling the direct uptake by the tissues of circulating NEFA are not understood. The simplest manipulation to study would be artifical elevation or suppression of NEFA by pharmacological means. The combination of the techniques described in this thesis with the measurement of the tissue expression of fatty acid transporters under such conditions might help clarify this issue. Furthermore, there is no description of whether adipose tissue NEFA uptake varies between the lean healthy and the overweight. This is of particular importance bearing in mind the potential deleterious effects of excess circulating NEFA described in Chapter 1.

Further studies would also be of use in dissecting the concept of adipose tissue metabolic inflexibility. Studies carried out prior to and following weight loss, either by caloric restriction alone or in combination with exercise would show whether metabolic flexibility can be reacquired. Furthermore, it would be interesting to

measure inflammatory markers under such conditions to determine whether there is any relationship between inflammation and adipose tissue metabolic flexibility. This, in turn, could be taken forward by pharmacological manipulation of inflammation and subsequent repeat studies.

6.3 Conclusion

In conclusion, the studies in this thesis have shed new light on many aspects of fat metabolism, both healthy and dysfunctional. The key message remains that fat metabolism is complex, dynamic and highly regulated. Within adipose tissue, derangements in the regulation of fat metabolism result in metabolic inflexibility: a reduced capability to respond appropriately to changing metabolic circumstances. Although the mechanism remains unclear, metabolic inflexibility may well represent dysfunction of regulation at many levels, rather than simple resistance to the action of any single modulator of fatty acid metabolism. Further studies are warranted to better understand determinants of normal fat metabolism as well as the aetiology of derangements in adipose tissue function.

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