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Measuring insulin sensitivity and the effect of alternative dietary interventions and exercise on metabolic control

By

Thomas Phillip James Solomon

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Abstract

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The metabolic syndrome is highly prevalent in western society, and the numbers affected by obesity and diabetes continue to rise. This thesis reviews the mechanisms at play and the gaps in the literature that, if filled, may increase knowledge of treatment regimes for affected individuals. Experimentally, it was demonstrated that the oral glucose tolerance test can be a reliable tool to measure insulin sensitivity following adequate dietary and exercise control. Acute and chronic cinnamon ingestion was shown to improve insulin sensitivity. Feeding frequency was found to alter insulin and ghrelin responses and relationships following mixed-meal ingestion. And finally, postprandial lipaemia was found to be attenuated for up to 24 hours following moderate-intensity exercise, illustrating the requirement of daily exercise. In summary, oral glucose tolerance tests are suitable for experimental interventions; and the clinical management of factors associated with the metabolic syndrome should perhaps consider dietary supplements, meal frequency, and exercise timing in addition to the traditional dietary and physical activity guidelines currently in practice.

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Acknowledgments

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Published/Submitted Articles

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The effects of short term cinnamon ingestion on in vivo glucose tolerance
     Abstract published in *Journal of Physiology* (2005) 565(P):C40

Effects of short-term cinnamon ingestion on in vivo glucose tolerance
     In press in *Diabetes Obesity and Metabolism*

The effects of mixed meal feeding frequency on insulin and ghrelin responses in humans
     Under review in *The Journal of Clinical Endocrinology and Metabolism*

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Thesis Contents

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1. General Introduction

1.1 The escalating problem of metabolic diseases

Recent long-term epidemiological evidence shows an increase in the prevalence of obesity [1,2]. Increasing obesity is a growing concern in western developed nations, and currently, according to a Department of Health survey, 65.5% of the population in the UK are overweight (body mass index (BMI) > 25 kg.m^{-2}), and 25.4% are obese (BMI > 30 kg.m^{-2}) (Figure 1.1) [2]. These figures have escalated in the last 10 years with a 20.5% increase in the number of overweight individuals, and a 53.6% rise in obesity (Figure 1.1) [2]. Alarmingly, in the UK 18.3% of children aged under 16 years old are obese, the proportion has risen by 59.8% over a 10 year period [2].

Figure 1.1 – Trends in body mass in the UK
Panel (i) shows body mass in 2004 across an adult age range. Panel (ii) shows changes in body mass from 1993 to 2004. Each bar represents the whole population. Body mass is divided into BMI brackets: obese (> 30 kg.m^{-2}), overweight (25-30 kg.m^{-2}), ideal weight (20-25 kg.m^{-2}), underweight (< 20 kg.m^{-2}). Data adapted from Health Survey for England 2004, Department of Health [2].
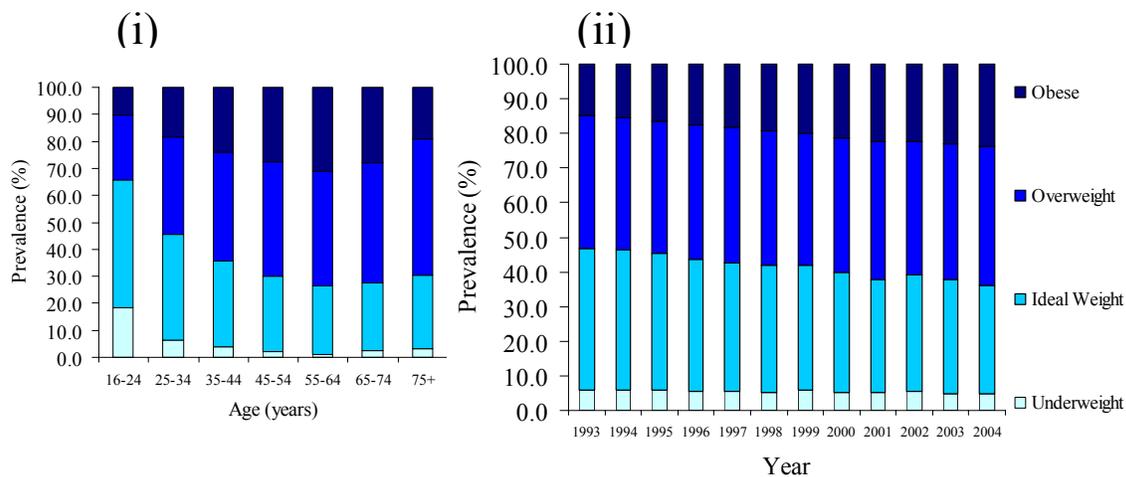
Obesity is an independent risk factor for type 2 diabetes mellitus (T2DM) and symptoms associated with the metabolic syndrome [1,3,4]. Excess body fat, particularly abdominal visceral fat, leads to impaired glucose and lipid metabolism, causing insulin resistance and hyperinsulinaemia [5]. Insulin resistance is a pathological state whereby target tissues are unable to respond to normal circulating concentrations of insulin [5]. This is largely determined by environmental factors, such as dietary intake and physical inactivity [6], yet genetic predisposition is also well documented in

offspring studies [7,8]. In the extreme, these environmental factors, lead to insulin resistance plus pancreatic β-cell dysfunction causing T2DM, a state of metabolic disease characterised by hyperglycaemia and glucosuria [5,9]. Diabetes is diagnosed with the use of fasting plasma glucose (FPG) and postprandial glucose concentrations following an oral glucose tolerance test (OGTT) (see Table 1.1), haemoglobin A1c (HbA1c) levels and clinical judgement.

Table 1.1 – Diabetes diagnostic criteria
Values represent venous plasma glucose concentrations in a non-gestational population. FPG = fasting plasma glucose. 2h-PG = plasma glucose concentration 2 h following a 75 g OGTT. * = ADA recently considered lowering to 5.6 mmol.l^{-1}. Data adapted from Alberti et al (1998) $Diabet$ Med 15: 539-553 [10].

Glycaemic Control	Plasma Glucose Concentration (mmol.l^{-1})	
	FPG	2h-PG
Normal	< 6.1*	< 7.8
Impaired Fasting Glycaemia OR Impaired Glucose Tolerance	6.1 - 7.0	> 7.8
Diabetes mellitus	> 7.0	> 11.0

Detection of diabetes using the OGTT has been shown to improve long-term health outcome and reduce mortality following appropriate treatment [11-13]. If left untreated T2DM patients can develop a number of irreversible problems such as renal dysfunction, retinopathy, nephropathy, neuropathy, pregnancy complications and vascular dysfunction [14]. Ultimately this increases the risk of cardiovascular complications as demonstrated by several longitudinal epidemiological studies, such as the UK Prospective Diabetes Study, the San Antonio Heart Study, and the Framingham Offspring Study [15-17]. McCarty et al (1994) estimates that approximately 80-90% of diabetes is T2DM, and currently in the UK the prevalence of diabetes stands at 4.2% (Figure 1.2), thus there are approximately 2.1 million T2DM patients in Britain [2,18,19]. The number affected has increased by 75% in the past 10 years [2], and it has also been estimated that by the year 2020, approximately 250 million people worldwide shall be affected by T2DM [20]. In addition, approximately 5% of the

National Health Service's budget (c. £3.8 billion per year) is spent on treating and managing diabetes in the UK [21].

Figure 1.2 – Prevalence of diabetes in the UK
Graph shows increasing prevalence of diabetes in adults with time since 1994. Data adapted from Health Survey for England 2004, Department of Health [2].
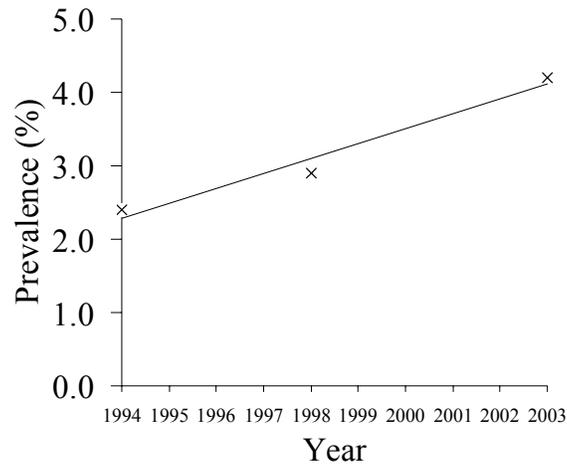
Insulin resistance is also implicated in a multi-factorial disease known as the metabolic syndrome. The metabolic syndrome, originally described by Reaven in 1988, describes a clustering of several risk factors for cardiovascular disease (CVD): abdominal obesity, dyslipidaemia (elevated low-density lipoprotein cholesterol (LDL-c), small dense LDL particles, reduced high-density lipoprotein cholesterol (HDL-c), and elevated triglyceride concentrations), impaired glucose tolerance (IGT), and hypertension [3]. Several diagnostic criteria for the metabolic syndrome are available (Table 1.2), those of the World Health Organisation (WHO), the International Diabetes Federation (IDF) and the National Cholesterol Education Program (NCEP) are the most widespread [10,22,23], and whilst discrepancies in their sensitivity are documented [24], they do provide a useful tool to detect and therefore treat "at risk" individuals. Approximately 25% of the population in the UK are affected with the metabolic syndrome, and those affected have a fivefold greater risk of developing T2DM and are three times more likely to suffer vascular complications [25-27].

Table 1.2 –Diagnostic criteria for the metabolic syndrome [10,22,23]
WHO = World Health Organisation. NCEP ATP III = National Cholesterol Education Program Adult Treatment Panel. IDF = International Diabetes Federation. IGT = impaired glucose tolerance. IFG = impaired fasting glycaemia. † = indicated by T2DM or IFG. * = ADA recently suggested lowering this to 100 mg.dl^{-1} (5.6 mmol.l^{-1}). # = by waist circumference. Adapted from Deen (2004) *Am Fam Physician* 69: 2875-82 [28].

Component	WHO	NCEP ATP III	IDF
	Insulin resistance † plus 2 of following	3 of following	Central obesity # plus 2 of following
Abdominal obesity	Waist to hip > 0.90 MEN (BMI>30 kg.m^{-2}) > 0.85 WOMEN (BMI>30 kg.m^{-2})	Waist circ. > 102 cm MEN > 88 cm WOMEN	Waist circ. > 94 cm MEN > 80 cm WOMEN
Hypertriglyceridaemia	> 150 mg.dl^{-1} (> 1.7 mmol.l^{-1})	> 150 mg.dl^{-1} (> 1.7 mmol.l^{-1})	> 150 mg.dl^{-1} (> 1.7 mmol.l^{-1}) or use of specific treatment
Low HDL-c	< 35 mg.dl^{-1} (< 0.9 mmol.l^{-1}) MEN < 39 mg.dl^{-1} (< 1 mmol.l^{-1}) WOMEN	< 40 mg.dl^{-1} (< 1.04 mmol.l^{-1}) MEN < 50 mg.dl^{-1} (< 1.3 mmol.l^{-1}) WOMEN	< 40 mg.dl^{-1} (< 1.04 mmol.l^{-1}) MEN < 50 mg.dl^{-1} (< 1.3 mmol.l^{-1}) WOMEN
Hypertension	> 140/90 mmHg or use of hypertensive treatment	> 130/85 mmHg or use of hypertensive treatment	> 130/85 mmHg or use of hypertensive treatment
Hyperglycaemia	IGT, IFG, insulin resistance or diabetes	> 110 mg.dl^{-1} (6.1 mmol.l^{-1})*	> 100 mg.dl^{-1} (5.6 mmol.l^{-1}) or previously diagnosed T2DM
Microalbuminuria	Urinary albumin to creatinine ratio > 30 mg.g^{-1} or albumin excretion rate > 20 μg.min^{-1}	- -	- -

As suggested above, diabetes and the metabolic syndrome, conditions underpinned by insulin resistance, both predispose to increased risk of CVD. Epidemiological data illustrates that people with diabetes are 2-3 times more likely to die of CVD [29-31]. The prevalence of CVD increases with age, reflecting age-related changes in body mass and diabetes [2]. Currently in the UK, CVD affects 7.7% of the population, and the number of affected individuals has risen by 25.2% in the last 10 years (Figure 1.3) [2]. CVD is also the leading cause of all-cause mortality and accounts for approximately 36% of deaths annually in the UK [32,33]. In addition, in 2004 CVD cost the NHS £16 billion, further illustrating the large socio-economic impact of metabolic abnormalities in the UK [34].

Figure 1.3 – Prevalence of CVD in UK
Graph shows increased CVD prevalence in adults with time since 1994. Data adapted from Health Survey for England 2004, Department of Health [2].
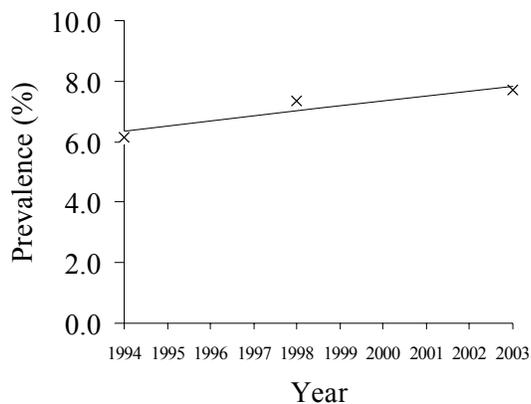
This brief introduction illustrates the escalating problem with metabolic and cardiovascular-related diseases in the UK, and their association with insulin resistance. As will be discussed later in this chapter, lifestyle habits can largely influence such states, and be used to help reduce disease risk. The metabolic syndrome, obesity and T2DM each increase one's risk of developing CVD and dying from its complications. Obesity-induced insulin resistance is the underlying pathology and therefore understanding its onset will aid in the development of interventions to reduce the risk of CVD-related morbidity and mortality.

1.2 Adiposity and insulin resistance

There is much evidence that implicates increased adiposity with diabetes risk and vascular complications (introduced in Section 1.1), showing a strong relationship between excess body-fat and insulin resistance [35-37]. Aging contributes to these metabolic abnormalities, but obesity accelerates their onset [6]. Obesity results from a long-term positive energy balance; energy intake (diet) outweighing energy expenditure (physical activity). Chakravarthy and Booth (2004) stated that the hunter-gatherer lifestyle of our ancestors involved large amounts of physical activity, but such habits are not routine anymore [38]. Recently, technological advances and transportation have led to food abundance and minimal requirement for physical activity. Current statistics show that only 29% of adults in the UK achieve minimum exercise guidelines (30 mins on at least 3 days a week), a statistic that decreases with age and has been in decline over the last 30 years [39,40]. However, whilst high saturated fat and high refined sugar diets are related to the onset of insulin resistance and increased risk of T2DM [41-47], British Heart Foundation data show that fat, sugar and total caloric ingestion has actually reduced in the UK in the last 30 years [40,48]. Therefore, it is probable that sedentary lifestyles are causing energy intake to outweigh energy expenditure, leading to a positive energy balance, explaining the increasing trends in obesity in today's society [49].

1.2.1 The role of adipose tissue

Adipose tissue plays an important role in lipid metabolism by controlling non-esterified fatty acid (NEFA) flux to other peripheral tissues, and by allowing triglyceride hydrolysis and clearance

from the systemic circulation, acting as a buffer to daily lipid flux [50]. In addition, adipocytes also release a number of chemicals and hormones (termed "adipocytokines") that have actions on skeletal muscle, immune cells, neural circuits in the hypothalamus, and other tissues [51,52]. Adipocytokines and FFAs are well documented to have direct effects on insulin signalling and insulin action in muscle, liver and the endothelium [51-53]. Thus, adiposity must be controlled to maintain adequate function and optimum health.

Adipocytes express hormone sensitive lipase (HSL), and lipoprotein lipase (LPL) is found on the endothelial lining of the adipose tissue capillary bed [54,55]. These two enzymes are responsive to hormonal stimulation. Catecholamines upregulate HSL activity, increasing lipolysis, and therefore FFA release into the systemic circulation [54,56], whereas insulin suppresses HSL activity and upregulates LPL activity in adipose tissue in the postprandial period to allow increased triglyceride clearance from the blood [55,56]. Insulin sensitivity of these enzymes is particularly important to maintaining optimal adipose tissue lipid-buffering capacity [50]. With regards to insulin resistance, the site of adiposity is also important. Increased abdominal (android) adiposity appears to be more related to CVD-risk factors than gluteal or femoral (gynoid) adiposity [57-60]. Furthermore, visceral fat has been demonstrated to be more responsive to lipolytic stimuli and less insulin sensitive than subcutaneous fat [61-64], a finding that is particularly important to hepatic insulin resistance as visceral outflow drains directly into the portal vein, thus FFA output from visceral adipose primarily targets the liver [65]. The importance of body fat distribution is exemplified by Klein *et al* (2004), who demonstrated that removing up to 44% of subcutaneous abdominal adipose tissue fails to improve any indices of insulin sensitivity (peripheral or hepatic), markers of CVD-risk, or adipocytokine levels in obese individuals [66].

1.2.2 Insulin action

Insulin exerts numerous actions on a variety of cell types, yet the control of glucose homeostasis is its major function [67]. The most abundant insulin-responsive tissue in mammals is skeletal muscle, which is central to regulation of systemic glucose levels in the postprandial period. Insulin binds to the extracellular domain of the insulin receptor (IR) causing autophosphorylation of specific tyrosine

residues allowing tyrosine kinase activation and phosphorylation of the intracellular insulin receptor substrates (IRS) [67,68]. The IRSs upregulate the phosphoinositol triphosphate pathway by phosphorylating phosphoinositol-3-kinase (PI3K), the key enzyme in the insulin signalling pathway [67,69]. Activation of PI3K propagates a signal to a number of other intracellular kinases (e.g. protein kinase B (PKB/Akt) and protein kinase C (PKC)) that ultimately regulate several cellular functions. These functions include glucose transporter-4 (GLUT-4) translocation from intracellular vesicles to the cell surface membrane, glycogen synthesis, various elongation and initiation factors involved in protein synthesis, various gluconeogenic enzymes involved in hepatic glucose output, enzymes involved in fatty acid synthesis and lipolysis, and other effects on gene expression and transcription [67-69]. In addition, IRS activation has been demonstrated to regulate the mitogen-activated protein kinase (MAPK) pathway involved in cell growth and differentiation and further gene transcription [70,71]. Insulin also has actions in adipose tissue, upregulating LPL activity and suppressing HSL as described above [65]. In the myocardium, insulin regulates development, fuel substrate utilisation, glucose uptake and glycogen synthesis, and cardiac contractility [72-75]. In addition, insulin has an important function in the endothelium upon nitric oxide synthase (eNOS), an enzyme that produces nitric oxide (NO), a freely diffusible compound that plays a key role in smooth muscle relaxation and thus vasodilatation [76,77]. Therefore, postprandially, insulin serves to increase capillary perfusion, allowing increased nutrient and hormonal availability to the tissue bed, it upregulates glucose uptake and glycogen synthesis, promotes protein synthesis and cell growth, suppresses glycogenolysis and glucose output from the liver, and increases adipocyte lipid uptake whilst inhibiting cellular lipolysis. With such diverse actions, anything that alters insulin signalling will have a multitude of outcomes.

1.2.3 Free fatty acids and insulin action

In 1963 Randle proposed that increased FFA availability increased lipid oxidation and was the cause of decreased carbohydrate oxidation in the muscle [78]. The proposed mechanism was that increased levels of acetyl coenzyme A (acetyl CoA) would inhibit pyruvate dehydrogenase (PDH) and increase citrate levels which could inhibit phosphofructokinase (PFK). This would cause reduced glycolytic flux and an accumulation of glucose-6-phosphate (G6P), inhibiting hexokinase (HK) and

thus reducing glucose uptake from the periphery. However this work was demonstrated in rat diaphragm and cardiac muscle, and not human skeletal muscle [78]. More recent work has demonstrated that basal respiratory quotient (RQ) in obese and T2DM is actually elevated thus the glucose: fat oxidation ratio is raised in such patients; and further work investigating lipid infusions during hyperinsulinaemic hyperglycaemic conditions, whilst demonstrating reduced glucose uptake [79-81], show no change in glucose oxidation as a percentage of that taken up into the cell [79,80], plus reductions in glycogen synthesis associated with decreased glycogen synthase activity [82]. Also, hyperglycaemic conditions appear equally able to suppress fat oxidation [83,84]. Additionally, various phosphorus-31 (^{31}P) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy studies have identified reduced glucose uptake and glycogen synthesis, reduced intracellular G6P and free glucose, plus reduced IRS-1-associated PI3K activity in human skeletal muscle during lipid infusion and in insulin resistant groups [85-88]. These findings provide convincing evidence to oppose Randle's original hypothesis.

Recent correlations have been drawn between intramuscular triglyceride (IMTG) accumulation and insulin resistance in skeletal muscle [89-92], and other work also demonstrates increased intrahepatic triglyceride in insulin resistant states [93-95]. However, it is unlikely that this is a direct cause of insulin resistance because skeletal muscle of trained endurance athletes possess high oxidative capacity and high insulin sensitivity, despite elevated IMTG [96]. It is likely, therefore, that in obesity increased intracellular fat derivatives are the cause of reduced insulin action. Recent data indicates that mitochondrial dysfunction in insulin resistance may prevent long-chain fatty acyl coenzyme A (LCFAcylCoA) uptake and oxidation, causing cytosolic accumulation of FFA derivatives [97]. Hyperinsulinaemic hyperglycaemic conditions (similar to those found in T2DM) produce reduced carnitine palmitoyl transferase-1 (CPT-1) activity, the key enzyme in mitochondrial LCFA uptake, via increased levels of its inhibitor malonyl CoA [98,99]. There is also evidence of increased LCFAcylCoA in muscle biopsy samples taken from insulin resistant rats and humans further illustrating their role in the development of insulin resistance [100,101]. Another fat derivative, diacylglycerol (DAG), a hydrolysis product of IMTG, has also been shown to be elevated in models of

lipid-induced insulin resistance [100,102,103]. Direct effects of LCFAcylCoA and DAG upon insulin signalling are documented in muscle and liver, and are known to interfere with protein kinase pathways [53,104-106]. Emerging data shows that DAG activates PKC-θ, which has been implicated in reduced insulin receptor tyrosine kinase activity and IRS-1 tyrosine phosphorylation, and elevated IRS-1 serine phosphorylation [88,100,102,103,107-109]. IRS-1 serine phosphorylation inhibits the insulin signal as demonstrated in insulin resistant models [110-113], whilst PKC-θ knockout mice models demonstrate protection from fat-induced insulin resistance [114]. Therefore it appears that elevated fat derivatives, either from increased adiposity, or induced by diet/infusion, may be a direct cause of insulin resistance.

1.2.3 Adipocytokines and insulin action

As mentioned above, adipose tissue releases several cytokines and hormones. These include leptin, adiponectin, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), resistin, visfatin, plasminogen-activator inhibitor-1 (PAI-1), adipsin, acylation-stimulating protein (ASP), angiotensinogen, insulin-like growth factor-1 (IGF-1), and transcriptional growth factor-β (TGF-β) [52,115]. Amongst these, several have been demonstrated to have direct effects on insulin signalling and inflammatory processes in various tissues.

Leptin has several functions including regulating appetite, energy expenditure, thermogenesis, maturation and reproduction, and the HPA/thyroid/GH axes [115]. Leptin receptors are found in peripheral tissues and the brain [116], and leptin's signalling pathway via PI3K is shared with insulin, providing the potential for interaction [117]. Its serum concentration is directly proportional to fat mass [118,119], and is directly correlated with insulin levels [120]. Leptin can reduce tyrosine phosphorylation of IRS-1 and reduce IRS-1-PI3K binding [121], an interaction central to the insulin signalling cascade. This suggests that hyperleptinaemia may be a direct cause of skeletal muscle insulin resistance. In addition leptin has a direct effect on pancreatic β-cells suppressing glucose-stimulated insulin release [122], further strengthening its role in the pathophysiology of obesity-related disease.

Adiponectin is specific to adipose tissue and is a product of the most abundant gene transcript in adipose tissue [115], yet is downregulated in human obesity and T2DM [123-125]. It augments insulin-induced tyrosine phosphorylation of IRS-1 and PKB/Akt in skeletal muscle [126], thus promoting the insulin signal. Intravenous administration of adiponectin in hypoadiponectinaemic models of obesity results in reduced serum glucose concentrations [126,127] via suppressed gluconeogenesis [127], along with increased skeletal muscle fat oxidation [126]. This has been shown to involve activation of adenosine monophosphate-activated protein kinase (AMPK) [128], the cell's energy sensing enzyme that regulates nucleic transcription of several genes associated with metabolic flux [129]. AMPK activation inhibits acetyl CoA carboxylase (ACC), thus lowering malonyl CoA levels, reducing inhibition of CPT1, indicating a possible mechanism for adiponectin-induced increased fat oxidation [128]. Adiponectin has also been shown to inhibit endothelial nuclear factor-κB (NF-κB), a transcription factor that augments insulin resistance and inflammation [130] (see Section 1.2.4).

Tumour necrosis factor alpha (TNF-α) is an inflammatory cytokine related to the local (adipocyte) and systemic inflammation seen in obesity and T2DM [131]. It is produced mainly by macrophages, but is also expressed by adipose and muscle tissue [115]. Its adipocyte expression is upregulated with increased adiposity and high-fat feeding [132,133], with insulin resistant individuals displaying a negative correlation between serum concentrations of TNF-α and insulin-stimulated glucose transport [134]. Several studies show that TNF-α downregulates peroxisome-proliferator activated receptor γ (PPAR-γ, a nucleic receptor involved in fat oxidation) gene expression, GLUT-4 translocation, glycogen synthase and fatty acid synthase activity, and LPL gene expression [115]. In addition, TNF-α has been demonstrated to have direct effects on insulin receptor autophosphorylation, PKC activity and serine phosphorylation of IRS-1 in muscle tissue and endothelial cells [135-138], thus elevated levels may induce insulin resistance and reduce NO-activated smooth muscle relaxation. Furthermore, as will be discussed below, TNF-α is also involved in the inflammatory responses seen in obese and T2DM states.

IL-6 is another multi-functional cytokine primarily involved in inflammatory responses produced by fibroblasts, the endothelium, monocytes, macrophages, myocytes and adipocytes [115]. Its serum concentration is elevated in obesity [139,140] and T2DM [141], and its expression in adipose tissue, which accounts for around 30% of circulating levels [142], is induced by catecholamines and TNF-α [115,140,143]. IL-6 appears to have functions in immunology, reproduction, bone metabolism, plus roles in glucose and fat metabolism [115]. Recombinant human IL-6 infusion increases whole body glucose disposal, yet also increases hepatic glucose production (HGP) and elevates FPG [144,145]. IL-6 also decreases adipose tissue LPL activity, whilst increasing adipose tissue lipolysis [144,146]. Increased hepatic triglyceride excretion in IL-6 infused rats has been attributed to the increased availability of FFA from adipose tissue as a result of IL-6 induced adipose tissue lipolysis [147]. Further work shows that circulating levels of IL-6 and adipose tissue IL-6 content are inversely correlated with insulin sensitivity [139,148,149], and a gene polymorphism leading to reduced IL-6 levels has been shown to be related to reduced area under the curve following oral glucose tolerance test, reduced insulin concentrations and improved insulin sensitivity [150].

This brief account illustrates that various adipose-derived moieties can have direct effects on insulin signalling, and indirect effects on factors associated with metabolic flux. Many of these are upregulated in obesity (with the exception of adiponectin, which is downregulated) and impede the insulin signal, and therefore, in the event of increased adipose mass, increased adipocytokine levels can induce insulin resistance.

1.2.4 Inflammation and insulin action

In addition to direct effects on muscular and hepatic insulin signalling, fat moieties and adipocytokines are also involved in local and systemic inflammation and vascular dysfunction. This is particularly relevant to the low-grade systemic inflammation seen in obesity and T2DM [151]. Potential links between inflammation and metabolic disease were first demonstrated in 1876 by Ebstein who showed that sodium salicylate (the active component in aspirin) diminished glucosuria in diabetic patients [152]. However, it was not until the 1950s and 60s that relationships between

inflammation and T2DM or obesity were recognised [153-155]. Recent epidemiological studies now confirm such relationships, showing positive correlations between T2DM and markers/mediators of inflammation and acute-phase reactants such as fibrinogen, C-reactive protein (CRP), IL-6, PAI-1 and white cell count [156-161]. In addition, lifestyle intervention in the Diabetes Prevention Programme reduced markers of inflammation and coagulation in IGT individuals, concomitantly with a reduced risk of developing T2DM [162].

The current evidence base illustrates that TNF-α, FFAs and their metabolites, and reactive oxygen species (ROS, systemic markers of oxidative stress), can each activate the c-Jun N-terminal kinase (JNK) pathway, and the IκB kinase-β (IKK-β) pathway (producing NF-κB) [163,164]. The JNK and IKK-β pathways are both involved in inflammation-induced insulin resistance, yet via different mechanisms. JNK promotes serine phosphorylation of IRS-1 [165-167], thus diminishing the intracellular insulin signal, whereas IKK-β activation liberates the transcription factor NF-κB for translocation into the nucleus, where it regulates expression of genes directly involved in insulin resistance and the atherosclerotic process [163,168]. The activity of JNK and IKK-β is elevated with increased adiposity [166,169,170], and in addition to the above, they have also been shown to be activated by advanced glycation end-products [171-173], illustrating the association of hyperglycaemia with inflammatory mechanisms.

Inflammation is an important aspect of insulin resistance. Adipose tissue appears to be a key instigator of inflammatory processes locally and in other tissues such as the liver, the endothelium and muscle. In addition, the liver produces pro-inflammatory cytokines (e.g. TNF-α and IL-6) under elevated FFA conditions and during steatosis [170] (e.g. non-alcoholic fatty liver disease (NAFLD)) via activation of NF-κB. These also propagate local and extra-hepatic (muscle and endothelial) inflammation, leading to insulin resistance [170].

1.2.5 Insulin and capillary perfusion

Recent data have suggested that endothelial dysfunction may impair capillary perfusion of skeletal muscle, reducing its exposure to metabolic substrates, hormones and oxygen [174-176].

Endothelial nitric oxide synthase (eNOS) is necessary for smooth muscle relaxation via the production of NO [76]. Endothelial insulin signalling (via IRS-1 and PKB/Akt) upregulates eNOS activity, and thus insulin is involved in smooth muscle tone and vasodilatation, allowing increased blood flow and perfusion of the tissue [76]. Recent data from Clerk *et al* (2006) shows that in obesity, insulin causes sub-optimal microvascular perfusion in skeletal muscle [177]. Also, research by Laakso *et al* (1990) reports reduced skeletal muscle blood flow in obese and T2DM patients compared with controls [178], whilst further work from Kashyap *et al* (2005) shows that eNOS activity is impaired in T2DM patients [179]. Furthermore, other work demonstrates reduced insulin-mediated capillary perfusion following infusion of lipid [180] or TNF-α [181]. More recent work from Naruse *et al* (2006) demonstrates the involvement of PKC in reduced endothelial NO production in the obese [182], therefore increased FFA availability in obese states might mediate insulin resistance in endothelial cells by similar mechanisms to those discussed above in skeletal muscle. Additionally, the role of inflammation is also important in the capillary perfusion process. TNF-α, oxidised LDL, and lysophosphatidic acid (produced by platelets) all increase nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [183], an enzyme involved in the production of superoxide anions, which react with NO reducing its bioavailability, and thus reducing NO-induced vasodilatation [183,184]. Ultimately, sub-optimal microvascular perfusion shall reduce the availability of insulin and glucose to the skeletal muscle, thus reducing insulin-stimulated glucose uptake.

1.2.6 Overview of insulin resistance

This introduction reflects that insulin resistance is largely determined by the level of adiposity. Whilst the two are closely related, insulin resistance arises in many other conditions besides obesity and T2DM, sometimes independently of body mass (for example, acromegaly, polycystic ovary syndrome (PCOS), Cushing's disease, NAFLD, pregnancy, puberty, lipodystrophy, Prader-Willi syndrome, and others [185]). As described above, adipose tissue may act as a buffer for daily lipid flux. In obesity, FFAs are released into the systemic circulation in great excess of their oxidative needs. In lipodystrophy, at the other extreme of adipose mass, severe insulin resistance arises, and high levels of IMTG and FFA metabolites are also documented [186]. Therefore it is likely that in the

absence of adipose tissue, the circulation, liver and muscle tissue may become the major triglyceride stores, and insulin resistance arises due to the mechanisms discussed above [187]. In animals, fat transplantation into lipodystrophic models, vastly improves insulin sensitivity, and reduces IMTG levels [187,188]. Whilst this illustrates that increased adiposity is not the whole story, this must not lessen the importance of its effects. It is clear that FFAs and adipocytokines are key to the development of skeletal muscle and hepatic insulin resistance, in addition to having direct effects upon endothelial insulin signalling and vascular function via inflammatory responses.

Insulin resistance not only occurs at the level of the tissue (muscle or liver) but also in the vascular endothelium, where an inability of insulin to reduce vascular tone decreases microvascular perfusion and thus the delivery of hormones and nutrients to the muscle tissue. Increased adipose-derived moieties (FFAs, DAG or adipocytokines) may activate a serine/threonine kinase cascade, initiated by PKC, JNK, or IKK-B, leading to IRS serine phosphorylation, which reduces IRS-1-PI3K association and therefore causes reduced activation of insulin-stimulated events downstream of PI3K. Whilst this section has concentrated on the environmental input that causes insulin resistance (i.e. the positive energy balance leading to obesity), several gene defects have been identified that predispose to the development of T2DM [9,189]. However, genetic evolution cannot explain the recent escalating trends in metabolic abnormalities, given the short time course in which this has occurred. Thankfully, epidemiological evidence shows that lifestyle (diet and exercise) intervention can substantially reduce the risk (up to 67%) of T2DM [190-194].

1.3 Treatment interventions for insulin resistant conditions

T2DM and the metabolic syndrome can be treated with pharmaceutical aids [195-199], one of the reasons for the large proportion of the NHS budget spent on diabetes healthcare. Lifestyle also has a large impact on the metabolic impairments of metabolic disease [200]. Exercise and dietary manipulation have been demonstrated to have beneficial effects on the major symptoms of the metabolic syndrome [200] (adiposity, dyslipidaemia, hypertension and hyperglycaemia) and on glycaemic and lipaemic control in diabetic patients [200]. For example, several epidemiological

studies have shown hypocaloric diets and/or increased levels of physical activity to be effective in

reducing symptoms of the metabolic syndrome, improving insulin sensitivity via reduced HGP, and in

improving endothelial function via reduced systemic inflammation [191,201-203]. In addition,

evidence further supports the use of pharmaceuticals and/or lifestyle intervention for affected patients

in order to prevent or delay the onset of cardiovascular disease in at risk individuals [191,204-208].

Furthermore, recent epidemiological evidence from various diabetes prevention studies (e.g. the

American Diabetes Prevention Project (DPP) [191], the Finnish Diabetes Prevention Study (DPS)

[209], the Chinese Da Qing IGT study [193] and a Japanese study [190]) indicate that long-term

dietary and exercise intervention can not only reduce the incidence of diabetes by up to 67%

[190,191,193,209], but also provides a more powerful stimulus than metformin, a routine drug

administered to T2DM patients [194]. The DPP demonstrated that lifestyle (diet and exercise)

alteration reduced the incidence of diabetes more so than treatment with metformin alone in obese IGT

individuals (Figure 1.4).

Figure 1.4 –The Diabetes Prevention Project
    3234 obese IGT individuals were followed for a period averaging 2.8 years with placebo or metformin (2 x 850 mg.day^{-1}) or lifestyle intervention (goal - 7% weight loss plus > 150 min.week^{-1} exercise). Graph (i) shows the change in diabetes incidence over 4 years. Lifestyle modification had a greater effect (58% reduction) than metformin (31% reduction). Graph (ii) demonstrates the marked weight loss following lifestyle alteration compared with metformin administration. Adapted from Knowler *et al* (2003) *New Eng J Med* 346(6):393-403[194].
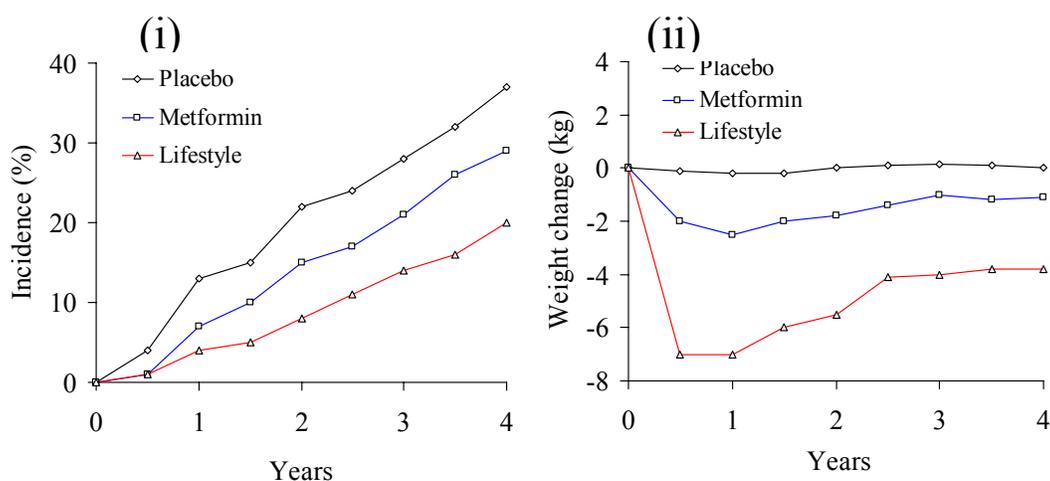
There is clear evidence from epidemiological trials in several countries (UK, US, Finland, Japan, China) that reversal and prevention of metabolic disease and reduction of CVD risk is possible. The data is particularly interesting as besides the direct health benefits it appears that part of the economic burden to healthcare systems may be relieved by patient lifestyle alteration, a relatively low-cost intervention. However, despite this convincing evidence base that diet and exercise are key tools in the prevention and reversal of human metabolic disease, trends in obesity, diabetes and CVD continue to escalate (see section 1.1). Therefore, the currently known effective regimes are either not being prescribed, or are not being adhered to, or there is a lack of public awareness. In addition to improving the communication of the health benefits of lifestyle changes, we could also search for novel therapeutics or interventions that may be more easily carried out by the target group. This thesis investigates some recent alternative approaches to factors associated with insulin sensitivity and the metabolic syndrome. The following two sections (1.4 and 1.5) of this literature review summarise the background knowledge to the key areas investigated in this project.

1.4 Measuring insulin sensitivity

The concept of insulin sensitivity describes the efficiency with which insulin can regulate its functions following a perturbation (e.g. meal ingestion) to the system [185]. Insulin sensitivity follows a continuum where insulin resistant individuals such as T2DM and metabolic syndrome patients have low insulin sensitivity [185]. As described in Section 1.3 various lifestyle or pharmaceutical interventions affect the extent of insulin resistance, therefore it is important that a valid and reliable tool for measuring insulin sensitivity is available for clinicians and academic researchers to further explore the mechanisms that lead to insulin resistance, and to develop appropriate treatment regimes.

The glycaemic clamp protocol, devised in 1966 by Andres *et al* and further developed by Defronzo *et al* (1979), is a technique whereby insulin and glucose are simultaneously infused at known rates [210,211]. Hyperinsulinaemia is maintained so as to maximise peripheral tissue glucose uptake and suppression of HGP, whilst euglycaemia is controlled at the patients individual FPG level [211]. The amount of glucose infused equals the amount of peripheral tissue glucose uptake, thus

providing an insulin sensitivity index (ISI; ISI_{CLAMP}) [211]. Prior to this insulin tolerance tests (ITT) and intravenous glucose tolerance tests (IVGTT) were often used. The ITT was first documented in 1929 by Horgaard *et al* [212]. This involves a single intravenous (IV) insulin injection and the monitoring of blood glucose in the post-injection hypoglycaemic period, and an ISI (ISI_{ITT}) is derived from the disappearance of glucose in the blood [213,214]. The injected insulin bolus suppresses HGP, and thus the ISI_{ITT} gives a peripheral measure of insulin sensitivity [213-215]. The major drawback to the ITT is that the ensuing hypoglycaemia creates undesired symptoms for the patient – cold, nausea, sweating, delirium – and reports of cardiac arrhythmia have been documented [216,217]. The IVGTT is a further technique involving a single IV injection, but of glucose, intended to replicate postprandial conditions. Once again, the subsequent changes in glucose concentrations are monitored and an ISI (ISI_{IVGTT}) is derived from the disappearance of glucose from the systemic circulation [185,218]. As mentioned in Section 1.1, the OGTT is the main technique used in the diagnosis of diabetes. However, several research groups have derived ISIs based on glucose and insulin concentration data from frequently-sampled plasma following a 75 g OGTT [219-223]. These provide a whole body ISI estimate (ISI_{OGTT}) since the postprandial glucose response involves both hepatic and peripheral tissue contributions. In addition to these dynamic tests involving glucose/insulin ingestion/infusion/injections, a number of ISIs have been derived from data in the basal state. The homeostatic model assessment (HOMA) and the quick insulin sensitivity check index (QUICKI) both derive an ISI from FPG and fasting serum insulin (FSI) concentrations [224,225]. These are very simple and rapid tests that are often applied to epidemiological studies. It is documented that because FPG is determined by HGP, ISI_{HOMA} and ISI_{QUICKI} are estimates of hepatic insulin sensitivity [224,225].

The clamp technique allows precise control of insulin and glucose at known low, normal or high levels and can easily be combined with other methodologies (e.g. tracer infusions, magnetic resonance imaging (MRI), positron emission tomography (PET)) to isolate various components of insulin sensitivity, such as peripheral or hepatic sensitivity, glycogen synthesis, or lipolysis [86,226-228]. This makes it the most useful technique for the measurement of insulin sensitivity, and so has become

the "gold standard" tool. However, the clamp technique is not suitable in non-clinical environments where a clinician is not on-hand to provide medical assistance, and a complicated set-up involving infusion pumps, insulin preparation, on-line glucose analysis, and computer algorithms to calculate glucose infusion rates, makes the clamp available only to specialised laboratories [211]. Additionally, the clamp, along with several other techniques, use glucose and insulin injections/infusions that create non-physiological (i.e. prolonged elevation or supraphysiological concentrations) situations that are not representative of free-living situations. A drawback of the techniques involving glucose/insulin infusions/injection (e.g. clamp, ITT and IVGTT) is that they are gut-independent. The literature suggests that several gut peptides (e.g. glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CCK), PYY_{3-36} and ghrelin), released in response to feeding, have effects on energy homeostasis, including pancreatic insulin release, and even direct effects on glucose metabolism [229,230]. Gut-dependent techniques, such as the OGTT may therefore provide a more physiological ISI estimate that incorporates all tissues involved in glucose metabolism into the measure of insulin sensitivity.

Due to the difficulties in acquiring clinical support the clamp is not readily available in our laboratory. The OGTT is a tool of interest because it is gut-dependent, it does not artificially control insulinaemia or glycaemia, and it simulates meal ingestion. ISIs from the OGTT (and ITT, IVGTT, and HOMA [214,218,224,225]) have been validated against the gold standard hyperinsulinaemic euglycaemic clamp protocol [219-223]; however, the reliability of several techniques is unknown. Reliability (i.e. the consistency of repeated measures) of FPG and 2h-PG data from OGTT protocols and ISI_{HOMA} estimates have been assessed and is found to be poor [224,231-234], yet pre-test conditions such as diet and physical activity, two factors that can influence glucose metabolism and insulin sensitivity, were not controlled, thus prompting further work in this area. Another drawback of the OGTT is that it gives a whole body ISI and cannot isolate hepatic from peripheral insulin sensitivity, nor is the rate of appearance of glucose from endogenous and exogenous sources known. For these reasons, we wish to test the reliability of OGTT-derived ISIs following controlled pre-test conditions, in order to establish the best ISI for use in dietary or exercise research interventions; and to

apply isotopic glucose tracers to OGTT protocols to enable quantification of gut- and liver-derived glucose appearance.

1.5 Alternative interventions on the impairments seen in metabolic disease

Either the message regarding health risks of inactivity and obesity is not being effectively communicated to the public, or the public perception is that the effort required to improve their health is too high. Therefore, developing alternative treatment strategies to relieve symptoms of metabolic disease is sensible. Such strategies may include dietary supplementation, meal frequency, and exercise timing.

1.5.1 Cinnamon spice

The first paper to illustrate potential hypoglycaemic effects of cinnamon was from Khan *et al* in 1990, who demonstrated that amongst a number of spices, cinnamon showed the greatest effect in a rat adipocyte glucose utilisation assay [235]. These findings were confirmed more recently by Broadhurst *et al* (2000) and Jarvil-Taylor *et al* (2001) in 3T3-L1 cell lines [236,237]. In 2003 the first human publication became available. Khan *et al* (2003) demonstrated that 1, 3, or 6 g of *Cinnamomum cassia* spice per day for 40 days can reduce FPG, total cholesterol and LDL-c by up to 29% in T2DM patients [238]. Since this publication two further human data sets became available from Mang *et al* and Vanschoonbeek *et al* [239,240]. Mang *et al* (2006) showed similar findings to Khan, but with no effects on haemoglobin A1c (HbA1c) using a 4 month 3 g.day^{-1} intervention in T2DM patients [239]. Vanschoonbeek *et al* (2006) using a 1.5 g.day^{-1} intervention over 6 weeks found no effect of cinnamon in a postmenopausal T2DM group, thus provoking some controversy regarding the effects of cinnamon [240]. The *in vitro* and animal evidence however is more convincing. Cinnamon's phenolic extract may upregulate insulin receptor autophosphorylation, suppress phosphotyrosine phosphatase (PTP-1) inhibition of the insulin receptor, and downregulate glycogen synthase kinase-3β (GSK-3β) activity, thus improving the intracellular insulin signal [241]. Roffey *et al* (2006) also recently showed increased glucose uptake by a phosphoinositol-3-kinase (PI3K) dependent mechanism in 3T3-L1 cell lines, yet downregulated adiponectin expression, an adipocytokine associated with improved insulin

sensitivity, thus complicating the mechanisms [242]. In rats, evidence from Qin *et al* (2003) has shown that long-term cinnamon ingestion can improve fructose-induced insulin resistance via an eNOS dependent mechanism, suggesting improvement in endothelial function, whilst increasing skeletal muscle insulin receptor substrate-1 (IRS-1) phosphorylation and association with PI3K, important interactions in insulin signalling [243,244]. Further work in mice has demonstrated increased insulin receptor and IRS-1 phosphorylation, increased GLUT-4 translocation, plus reduced fasting glucose, total cholesterol and triglycerides, further illustrating the positive effects of cinnamon supplementation [245,246]. Nevertheless, despite the convincing *in vitro* and *in vivo* animal evidence, the picture in humans is less clear, and further work is warranted. The acute effects of cinnamon ingestion, and their persistence, are unknown, and at the time of planning experimental work, only Khan's publication was available, and therefore effects on insulin sensitivity were also unknown.

1.5.2 Ghrelin and feeding frequency

Ghrelin, a 28 amino acid peptide hormone isolated from the oxyntic glands of the stomach, was discovered in 1999 as a ligand for the pituitary growth hormone secretagogue receptor (GHS-R) [247]. Since its discovery over 1200 papers have been published investigating ghrelin's targets and effects, and it is now clear that ghrelin has several endocrine and non-endocrine effects [248]. Perhaps the most well documented function is in appetite regulation in the arcuate nucleus (ARC) and ventromedial nucleus (VMN) in the hypothalamus [248]. Plasma ghrelin concentrations follow a diurnal rhythm, peaking between midnight and dawn, with pulsatile day-long responses [249], peaking prior to meal ingestion and falling in the postprandial period [250-252], suggesting a role for ghrelin in altering hunger before and after ingesting food. Ghrelin responses to meal ingestion are found to reciprocate insulin responses [253], and various data support the theory that insulin is required for postprandial ghrelin suppression [254-258]. This interaction is less pronounced in T2DM patients [259], evidence that insulin resistance may interfere with ghrelin's response and action. Interestingly, Tschop *et al* (2001) demonstrated that ghrelin is downregulated in obese and Pima Indian T2DM individuals [260], with Poykko *et al* (2003) confirming these findings in insulin resistant individuals [261].

Besides its GHS action and orexigenic effects in the hypothalamus, ghrelin has also been implicated in a large number of metabolic and endocrine functions: energy balance, sleep and behaviour, gastric motility and acid secretion, exocrine and endocrine pancreatic function, glucose metabolism, cardiovascular actions and immune function [248]. Ghrelin's effects on metabolism are particularly relevant to metabolic disease especially given its downregulation in insulin resistant states. In 2005 Barazzoni *et al* demonstrated that ghrelin regulates mitochondrial lipid metabolism gene expression and fat distribution in liver and skeletal muscle [262]. Furthermore, Tschop *et al* (2000) and Thompson *et al* (2004) have shown that ghrelin induces adipogenesis [263,264], and Wortley *et al* (2004) have shown that ghrelin deletion increases fat utilisation [265], which provides further evidence for ghrelin's involvement in metabolic flux. This concept is poorly understood in humans and warrants further investigation. In this thesis, pre- and postprandial changes in insulin and ghrelin concentrations are investigated under different feeding regimes to further explore the insulin-ghrelin relationship.

In addition, this thesis also investigates feeding frequency. An abundance of papers can be cited for the effects of meal composition and caloric content on insulin resistance and metabolic abnormalities, yet the concept of meal frequency or snacking is an area that is relatively under represented in the literature [266]. This is surprising given the epidemiological studies of European men and women demonstrating that snacking and light meals contribute to 25-35% of the daily energy intake (EI) and that obesity is related to increased snacking [267,268]. The current studies investigate diets of mixed composition (50-80% carbohydrate, 10-35% fat) fed up to 17 times in a day for 1 to 130 days in various animal and lean, obese and T2DM human cohorts. The evidence base is mixed with regards to metabolism, with the various studies showing positive, negative and null effects of increased meal frequency [269-281]. For example, in humans Bertelsen *et al* (1993) showed that following 6- versus 2-meal isocaloric feeding regimes, increased meal frequency decreased day-long glucose, insulin and free fatty acid concentrations [269]. In contrast, Farshchi *et al* (2005) demonstrated increased insulin, LDL-c, and EI, with reduced thermogenesis following 14 days of increased meal frequency [271]. Finally, Murphy *et al* (1996) compared 12 versus 3 isocaloric meals

per day for 2 weeks and showed no changes in lipaemia (including LPL activity), glycaemic control or insulin [272]. As a result, no clear conclusions can be drawn from the literature as to the optimum feeding frequency regime.

As described above, ghrelin and its involvement in metabolism has become a research focal point. In 2002 Sugino *et al* presented some data investigating the effects of mixed-meal frequency on ghrelin responses in sheep [282]. Following a 12 hour intervention with 2 meals, 4 meals, or *ad libitum* feeding, increased meal frequency inversely correlated with ghrelin area under the curve [282]. In addition, they confirmed the preprandial surges in ghrelin that were documented in previous literature, and they demonstrated reduced peak preprandial ghrelin concentrations with increasing meal frequency [282]. Given ghrelin's involvement with satiety and metabolic flux, these findings may suggest that increased meal frequency suppresses appetite and reduces adipogenesis thus promoting fat oxidation. However EI was not controlled in Sugino's paper and was different between trials, so the different ghrelin response could be due to different calorie intake or different feeding frequency between trials [282]. Similar evidence is not available in humans. Therefore we intend to investigate the ghrelin response to different feeding regimes that are isocaloric.

1.5.3 Exercise and postprandial lipaemia

Following a meal, triglycerides in intestinally-derived chylomicrons and hepatically-derived VLDL compete for the same saturable pathway to be hydrolysed by lipoprotein lipase (LPL), an endothelial lipase found in the capillary bed of peripheral tissues (adipose and skeletal muscle) [283,284]. During this period, elevated circulating triglyceride-rich moieties increase the opportunity for lipid exchange with cholesterol-rich lipoproteins, leading to a depletion of HDL-c and a decrease in the size of LDL, creating a lipaemic profile that is more atherogenic [285]. In addition, during the postprandial period changes in lipid metabolism can increase blood coagulability [286], and fat ingestion *per se* can induce endothelial dysfunction [287,288]. Fortunately, such changes in the healthy human are transient. However, during a normal day when several meals are ingested, postprandial elevation of triglyceride-rich moieties does not completely recover prior to subsequent

meals, and if lipoprotein metabolism is impaired, for example in obesity or insulin resistance, levels of triglyceride-rich lipoproteins may be persistently elevated resulting in increased reverse-cholesterol transport and endothelial dysfunction [289]. Furthermore, a number of studies demonstrate that impaired lipoprotein metabolism is implicated in the development of atherosclerosis [290-299], so reducing postprandial lipaemia may reduce CVD progression.

Physical activity has a large effect on lipid metabolism, and cross-sectional studies indicate that endurance-trained athletes have decreased postprandial lipaemia and increased triglyceride clearance compared with untrained controls [300-305]. This may be partly explained by increased muscle mass and vascularisation, hence increased exposure to the target tissue and capillary bed LPL in athletes. However, when athletes are studied more than 2 days following their last training session, no differences exist compared with controls, thus effects of activity on triglyceride metabolism are only transient and quickly reversed [306,307]. Acute effects of exercise on postprandial lipaemia have been demonstrated by several groups, and they appear to be delayed and transient with no demonstrable effects when exercise is performed less than 1 hour, or as long as 48 hours, before feeding [308-311]. The acute effects of exercise also appear to be related to energy expenditure, as studies comparing various exercise bouts in untrained individuals, e.g. 3 x 30 min versus 1 x 90 min [312], 3 x 10 min versus 1 x 30 min [313], 90 min at 60% VO_2max versus 180 min at 30% VO_2max [309], show similar effects on postprandial lipaemia.

As a therapeutic tool, exercise has a pronounced effect on lipaemia. However, we know little about the timecourse; how long after exercise is the beneficial effect preserved? Moreover, what is the optimum time delay between exercising and feeding to see the biggest effect? Therefore one aspect of this thesis was to investigate the time post-exercise when the beneficial effect on postprandial lipaemia is lost.

1.6 Overview and experimental aims of this thesis

This literature review illustrates the current impact of insulin resistance and its involvement in many diseases. In addition, it has highlighted some possible future research directions. As explained in Section 1.1, trends in obesity and its associated diseases appear to be escalating despite a wealth of knowledge that lifestyle modification can alleviate the onset of such diseases. Therefore, the aim of this PhD project was to provide new information regarding dietary supplementation, feeding frequency, and exercise timing, that may improve existing therapeutic strategies.

As described in Section 1.4, a valid and reliable tool is required to measure insulin sensitivity. Chapter 3 investigates the reliability of validated OGTT-derived ISI estimates due to an absence of such data in the literature. The application of stable isotopic tracers to OGTT protocols was also highlighted as a useful tool for determining glucose appearance. Chapter 4 displays pilot work investigating the quantification of glucose appearance from the gut and the liver during OGTT protocols. Section 1.5.1 illustrated that cinnamon may be a useful therapeutic for impaired glycaemic control, yet evidence in humans is mixed and quite limited. Chapters 5 and 6 make use of one of the OGTT-derived ISI indices to investigate the effects of acute and chronic cinnamon spice supplementation upon insulin sensitivity. In Section 1.5.2, the concept of feeding frequency and the possible involvement of a novel GI peptide, ghrelin, was discussed. Feeding frequency was shown to have mixed results on symptoms of the metabolic syndrome, and recent evidence linked postprandial insulin responses to postprandial ghrelin responses, an association shown to be diminished in T2DM. Chapter 7 investigates the effect of feeding frequency upon insulin and ghrelin responses. Finally, Section 1.5.3 described the literature surrounding the effects of physical activity upon postprandial lipaemia. Exercise was clearly shown to have a delayed and transient effect on triglyceride levels in several studies, however the exact time course of this effect has not been established. Chapter 8 presents data which explores the time delay between exercise and a high fat meal. The aim of this study is to ascertain when the beneficial effect of exercise on lipaemic control is lost.

These topics were chosen after close scrutiny of the literature to identify gaps in the evidence base, or to discover possible novel therapeutics that may be useful in the prevention of metabolic disease. In all studies, a lean but sedentary group was used to study the effects of the intervention. Such subjects are readily-available in our working environment, and were primarily used to assess the potential effects of the intervention prior to further work in patient cohorts.

2. General Methods

The following chapter describes techniques, assays and calculations that are relevant to more than one of the experiments presented in this thesis. This is to avoid unnecessary replication of common methods.

2.1 Ethical approval

All investigations described were granted ethical approval by the Safety and Ethics Subcommittee of the School of Sport and Exercise Sciences. Verbal and written explanations of the experiments were given to all potential subjects. Written consent was obtained from all subjects, who were aware that as volunteers they were free to withdraw from the experiment at any time.

2.2 Blood sample collection

Through the written and verbal explanation of the experiment the subjects were requested to attend on the morning of the investigation having avoided physical activity, because even light physical activity would alter metabolism. Prior to all studies subjects were rested (seated) for 10 minutes, before a resting blood sample was taken from an antecubital vein. Whole blood samples were collected into appropriate vacutainers (BD Vacutainers, Oxford, UK) – non-anticoagulant for serum insulin measurements, sodium fluoride for plasma glucose measurements, and EDTA for plasma ghrelin, triglyceride, and glycerol measures. EDTA vacutainers for ghrelin analysis were pre-treated with 30 µl of the serine protease inhibitor apoprotinin (Sigma, UK) per 300 µl plasma. All samples were kept on ice at 4 °C for no more than 30 min prior to plasma/serum separation. Vacutainers were centrifuged at 3000 g for 10 min at 4 °C, and their plasma/serum constituent separated and stored in 1.5 ml microtubes (Eppendorf UK Ltd, Cambridge, UK) at -70 °C for later analysis.

2.3 Assays

All samples were measured in duplicate and the intra-assay coefficient of variation for each measure is shown in Table 2.1

Table 2.1 – Intra-assay coefficient of variation for each measure in each study

Assay	Chapter	Intra-assay CV (%)
Glucose	3	3.51
	4	3.51
	5	2.40
	6	1.53
[6,6-^{2}H$_2$]-glucose enrichments	4	3.69
[U-^{13}C]-glucose enrichments	4	2.50
Insulin	3	4.12
	4	4.12
	5	5.77
	6	4.38
	7	5.34
Ghrelin	7	3.66
Triglyceride	8	2.04
Glycerol	8	3.64

2.3.1 Glucose

2.3.1.1 Hexokinase method

Plasma glucose concentrations were measured using a COBAS Mira Plus (ABX Diagnostics, Montpellier, France) automated analyser, by an enzymatic assay involving hexokinase and glucose-6-phosphate dehydrogenase and measuring absorbance changes at 340 nm. A 27.8 mmol.l^{-1} calibrator solution and normal (5.15 mmol.l^{-1}) and pathological (14.07 mmol.l^{-1}) human quality control sera (ABX Diagnostics, Montpellier, France) were used. Automated dilutions of the calibrator were made to construct a standard curve.

2.3.1.2 Gas chromatography mass spectrometry (GC-MS)

Prior to GC-MS analysis all samples were deproteinised and derivatised as follows. A 100 μl aliquot of plasma was added to 1 ml of acetone, vortexed and set in ice for 15 min. This mixture was centrifuged at 3000 g at 4 °C for 15 min and the supernatant evaporated under nitrogen gas at 20 °C. The pellet was resuspended in 160 μl of a 1:1 ethylacetate:heptafluorobutyric acid anhydride stock and

incubated at 70 $^{\circ}$C for 60 min. The solution was centrifuged at 3000 g at 25 $^{\circ}$C for 5 min and then evaporated under nitrogen gas again at 20 $^{\circ}$C. The sediment was resuspended in 70 μl of ethylacetate, vortexed, left at room temperature for 15 min and then transferred to a vial ready for gas chromatography analysis. (All chemicals obtained from Sigma, UK.)

The [6,6-^{2}H$_2$]- and [U-^{13}C]glucose enrichments were determined by GC-MS analysis of the heptafluorobutyrate derivatives of glucose on Agilent Technologies GC and mass selective detectors (GC – Agilent Technologies 6890N, UK; Mass selective detector - Agilent Technologies 5973, UK). Ion masses of 519 (molecular ion mass, M), 521 (M+2), and 525 (M+6) were selectively monitored for [6,6-^{2}H$_2$]- and [U-^{13}C]glucose enrichments. Standard enrichment curves ranging 0, 0.5, 1, 1.5, 2, 2.5, and 3 were constructed.

2.3.2 Insulin

Serum insulin concentrations were determined using a commercially available enzyme-linked immunoabsorbant assay (ELISA) kit (DRG Instruments GmbH, Germany), by a solid-phase two-site direct sandwich technique. Absorbance changes were measured at 450 nm. Standards ranging 0, 6.25, 12, 25, 50, and 100 μU.ml^{-1} and human control sera (Bio Rad Lyphochek^{TM} Immunoassay control sera, DRG, Germany) were used. No cross-reactivity with proinsulin is reported by the manufacturer.

2.3.3 Ghrelin

Plasma ghrelin was measured by a competitive ^{125}I-peptide radioimmunoassay (RIA) on unextracted plasma using a commercially available kit (Phoenix Peptides, California, US). A gamma-counter was used to measure counts per million of the samples and standards (1, 2, 4, 8, 16, 32, 64, and 128 pg.tube^{-1}). A standard curve of ghrelin concentration versus bound/free peptide (B/B$_o$ %) was constructed using the following:

$$B\!\!\big/\!\!_{Bo}(\%) = \frac{CPM - NSB}{TB - NSB} \times 100 \qquad [2.1]$$

Where CPM = gamma counts per million of the standard, NSB = CPM of non-specific binding, and TB = CPM of total binding. This assay involves antibodies raised against the C-terminal region, so values stated are for total ghrelin: des-acylated plus octanoylated circulating peptides. Relevant guidelines for the handling of radioactive compounds were followed. No controls were used in this assay. Discrepancy is found between the two commercially available kits from Phoenix Peptides and Linco Research Inc [314]. The two show strong correlation yet the Linco kit measures a factor of 10 higher than the Phoenix kit, and thus there is poor agreement between the two [314]. The majority of previous publications employ the use of Phoenix over Linco, however no information is currently available as to which is the more accurate measure.

2.3.4 Triglyceride

Plasma triglyceride concentrations were measured using a COBAS Mira Plus automated analyser (ABX Diagnostics, Montpellier, France), by an enzymatic assay involving lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase, while measuring absorbance changes at 505 nm. Normal and pathological human quality control sera (ABX Diagnostics, Montpellier, France) and standards ranging 0, 572, 1145, and 2290 $\mu mol.l^{-1}$ were used. Because this assay quantifies triglyceride by measuring glycerol in the sample after lipolysis of the triglyceride with lipase, the triglyceride measures were corrected for free glycerol (see Section 2.3.5).

2.3.5 Glycerol

Prior to analysis samples were deproteinised: 300 µl plasma was added to 30 µl of 1 $mol.l^{-1}$ sulphosalicyclic acid, mixed, and put on ice for 20 min, then centrifuged for 5 min at 3000 g at 4 ^{o}C to separate the supernatant into a fresh tube. Plasma glycerol concentrations were measured using a COBAS Mira Plus automated analyser (ABX Diagnostics, Montpellier, France) by an enzymatic assay involving glycerol kinase, pyruvate kinase and lactate dehydrogenase, while measuring absorbance changes at 340 nm. Glycerol control solution (Sigma, UK) at 573 $\mu mol.l^{-1}$, and standards ranging 0, 115, 229, 458, and 1145 $\mu mol.l^{-1}$ were used.

2.4 Calculations

2.4.1 Insulin sensitivity

Oral glucose tolerance tests (OGTTs) involved consuming 75 g of glucose dissolved in 300 ml of water. Five previously derived insulin sensitivity indices based on OGTT models were used in this thesis: Matsuda (1999), Stumvoll (2000), Soonthornpun (2003), Cederholm (1990), and Belfiore (1998) [219-223]. These were calculated as follows:

$$\text{ISI}_{\text{OGTT}}{}^{\text{Matsuda}} = \frac{10000}{\sqrt{\text{FPG} \times \text{FSI} \times \text{MPG} \times \text{MSI}}} \qquad [2.2]$$

$$\text{ISI}_{\text{OGTT}}{}^{\text{Stumvoll}} = 0.226 - (0.0032 \times \text{BMI}) - (0.000064 \times 2h - \text{SI}) - (0.0037 \times 90\min - \text{PG}) \; [2.3]$$

$$\text{ISI}_{\text{OGTT}}{}^{\text{Soonthornpun}} = \frac{\left(\dfrac{1.9}{6} \times \text{BW} \times \text{FPG}\right) + 520 - \left(\dfrac{1.9}{18} \times \text{BW} \times \text{AUCGlu}\right) - \left(\dfrac{\text{G}_{\text{URINE}}}{1.8} \times 1000\right)}{\text{AUC} - \text{Ins} \times \text{BW}}$$

$$[2.4]$$

$$\text{ISI}_{\text{OGTT}}{}^{\text{Cederholm}} = \frac{75000 + (\text{FPG} - 2h - \text{PG}) \times 0.19 \times 180 \times 1.15 \times \text{BW}}{120 \times \log(\text{MSI}) \times \text{MPG}}$$

$$[2.5]$$

$$\text{ISI}_{\text{OGTT}}{}^{\text{Belfiore}} = \frac{2}{\text{AUC} - \text{Ins} \times \text{AUC} - \text{Glu} + 1}$$

$$[2.6]$$

Where FPG = fasting plasma glucose in mg.dl^{-1}, FSI = fasting serum insulin in μU.ml^{-1}, MPG = mean plasma glucose following OGTT in mg.dl^{-1}, MSI = mean serum insulin following OGTT in μU.ml^{-1}, BMI = body mass index in kg.m^{-2}, 2h-SI = serum insulin concentration 120 minutes following OGTT in μU.ml^{-1}, 90min-PG = plasma glucose concentration 90 minutes following OGTT in mmol.l^{-1}, BW = body weight in kg, AUC-Glu = area under the curve for glucose following OGTT in mmol.l^{-1}.2h, G$_{\text{URINE}}$ = urinary glucose concentration in mmol.l^{-1}, AUC-Ins = area under the curve for insulin following OGTT in μU.ml^{-1}.2h, 2h-PG = plasma glucose 2 h following OGTT in mg.dl^{-1}.

2.4.2 Nonsteady state tracer kinetics

Raw glucose enrichment data were corrected for background enrichment and converted to molecule percent excess (MPE) values, then entered into Steel's model for single-pool nonsteady state tracer kinetics for the calculation of rate of plasma glucose appearance (R_a^{TOTAL}) and rate of plasma glucose disappearance (R_d) [315,316]:

$$R_a^{TOTAL} = \frac{F - \left(pV \times \left(\frac{C_2 + C_1}{2} \right) \times \left(\frac{E_2 - E_1}{t_2 - t_1} \right) \right)}{\left(\frac{E_2 + E_1}{2} \right)} \qquad [2.7]$$

$$R_d = R_a^{TOTAL} - pV \times \frac{C_2 - C_1}{t_2 - t_1} \qquad [2.8]$$

Where F is the tracer infusion rate in $\mu mol.min^{-1}$ corrected for tracer purity (99.0%), pV is the volume of distribution in 160 ml.kg^{-1}, C_1 and C_2 are the plasma glucose concentrations at time 1 and 2 in mmol.l^{-1}, t_1 and t_2 are times at time points 1 and 2 in minutes, and E_1 and E_2 are the plasma [6,6-^{2}H$_2$]glucose tracer enrichments at time 1 and 2. R_a^{TOTAL} and R_d were converted to g.min^{-1} for graphical representation (= $\mu mol.kg^{-1}.min^{-1}$ x kg x 180.2 x 10^{-6}).

Rate of endogenous (R_a^{ENDO}) and exogenous (R_a^{GUT}) glucose appearance into the plasma was calculated according to Jeukendrup *et al* (1999) [317] adapted from Steele *et al* (1968) [318]:

$$R_a^{GUT} = \frac{R_a^{TOTAL} \times \left(\frac{E_2 + E_1}{2} + \left(\frac{C_2 + C_1}{2} \times \frac{E_2 - E_1}{t_2 - t_1} \times pV \right) \right)}{E_{OGTT}} \qquad [2.9]$$

$$R_a^{ENDO} = R_a^{TOTAL} - R_a^{GUT} \qquad [2.10]$$

Where E_1 and E_2 are the plasma [U-^{13}C]glucose tracer enrichment at times 1 and 2, C_1 and C_2 are the plasma glucose concentrations at time 1 and 2 in mmol.l^{-1}, pV is the volume of distribution in 0.160 l.kg^{-1}, and E_{OGTT} is the [U-^{13}C]glucose tracer enrichment of the oral glucose bolus (again this was corrected for tracer purity: 99.0%). R_a^{GUT} and R_a^{ENDO} were converted to g.min^{-1} for graphical representation (= $\mu mol.kg^{-1}.min^{-1}$ x kg x 180.2 x 10^{-6}).

3. Reliability of the oral glucose tolerance test

Co-authors: John Ayuk, Andrew A. Toogood, Andrew K. Blannin, Asker E. Jeukendrup

3.1 Abstract

The gold standard glycaemic clamp methodology is not available to every laboratory group particularly in non-clinical environments. Oral glucose tolerance test-derived estimates of insulin sensitivity have been developed by several research groups and have been validated against the glycaemic clamp method. Yet the reliability of these estimates in repeated measures is not known. This study assessed the reliability of the OGTT in three repeats undertaken by seven male volunteers (age 26 ± 2 years, body mass 74.1 ± 2.4 kg, BMI 23.8 ± 0.6 kg.m^{-2} (mean ± S.E.M.)) following strict pre-test diet and exercise controls. Reliability of fasting glucose and insulin levels, 2 hour glucose and insulin levels, area under the glucose and insulin curves, and various OGTT-derived insulin sensitivity estimates were assessed. The reliability of such measures was quite variable with within-subject coefficients of variation (typical error) ranging from 5.60 to 48.2% for ISI$_{OGTT}$^{Matsuda} and 2h-SI respectively, and intraclass correlation coefficients (ICC) ranging from R = 0.180 to 0.981 for 2h-PG and FSI respectively. However, the reliability of the OGTT-derived insulin sensitivity index from Matsuda *et al* (1999) was high, with a typical error of 5.6% (95% confidence limits, 3.30 to 8.62%), plus an ICC of R = 0.919 (95% confidence limits, 0.545 to 0.988). We conclude that if pre-test diet and physical activity guidelines are adhered to, then some estimates of ISI derived from the OGTT are reliable.

3.2 Introduction

Insulin resistance is the underlying pathology of the metabolic syndrome and diabetes [3,319], increasing the risk of cardiovascular disease [15-17,26,27]. Therefore a reliable, valid and efficient method of measuring insulin sensitivity is crucial to facilitate early diagnosis and treatment of affected or "at risk" individuals. The glycaemic clamp technique is the reference method that gives an estimate of peripheral insulin sensitivity by directly measuring the effects of insulin on glucose utilisation under steady state conditions [211]. However it is not representative of free-living physiology, and not routinely available outside clinical environments. The oral glucose tolerance test (OGTT) is the most commonly performed test to measure glucose tolerance, and, with insulin measurements, gives a cheap and simple estimate of whole body insulin sensitivity in response to a physiological orally-ingested bolus more representative of free-living physiology than the clamp methodology [320]. The OGTT also stimulates gastrointestinal peptide pathways such as ghrelin and PYY_{3-36} which control or influence insulin release and glucose kinetics [229,230,321-323], and therefore indices derived from OGTT data may yield more physiological (meal-related) information. Clinically, fasting plasma glucose (FPG) and two hour post-OGTT plasma glucose (2h-PG) are used as diagnostic tools, yet evidence suggests discrepancies exist between FPG and 2h-PG within varying states of glucose tolerance [324,325], and poor reliability (i.e. poor consistency of repeated measures) of such measures is also reported [224,231-234]. Whilst several groups have presented insulin sensitivity indices (ISI) from OGTT-derived data and validated them against gold standard methodology, the reliability of these values, which is critical to the valid use of the OGTT in clinical or research environments, has not been assessed [219-221]. Our research group wishes to use the OGTT as a tool to assess insulin sensitivity during nutritional interventions due to its simple application, gut-dependent nature, physiological set-up, and due to the lack of regular access to clinical support for the clamp procedure. The aim of this study was to investigate the reliability of several OGTT-derived ISI estimates.

3.3 Methods and materials

Seven lean male volunteers aged 26 ± 2 years, body mass 74.1 ± 2.4 kg, with BMI of 23.8 ± 0.6 kg.m^{-2} (mean ± S.E.M.) were recruited from the local community. All volunteers were deemed healthy

as assessed by a general health questionnaire and their informed written consent was given prior to commencement of the study. Each volunteer underwent OGTTs on three separate occasions over the study period. Each trial was separated by at least five days and dietary record was taken for the two days preceding the first trial to be matched prior to subsequent visits. Subjects were also instructed to refrain from consuming alcohol or caffeine, and from undertaking any non-prescribed exercise for forty-eight hours prior to testing. The protocol was approved by the South Birmingham Health Authority Local Research Ethics Committee.

3.3.1 The Oral Glucose Tolerance Test (OGTT)

Volunteers arrived in the laboratory at 0800 hours following a 12 hour overnight fast, and were studied in a reclined position. An intravenous cannula (BD Venflon, BD, UK) was inserted into an antecubital vein and a fasting blood sample was taken. At t = 0 min participants were instructed to ingest a 300 ml solution containing 75 g of anhydrous D-glucose (Sigma, UK). Blood samples were collected every 15 min until t = 120 min for the determination of glucose and insulin concentrations. Blood samples were collected, processed and stored as described in the General Methods section. Previously derived insulin sensitivity indices based on frequently-sampled OGTT data (Matsuda *et al* (1999), Stumvoll *et al* (2000), Soonthornpun *et al* (2003), Cederholm *et al* (1990), and Belfiore *et al* (1998) [219-223]) were applied to our data and calculated as described in Section 2.4.1 of the General Methods.

3.3.2 Statistical Analysis

Glucose and insulin data were tested for normality and their profiles with time were analysed by two-way (trial*time) ANOVA. Reliability of each OGTT-derived ISI plus fasting and 2 hour glucose and insulin, and area under the curve (AUC) glucose and insulin values, were assessed using retest reliability measures as described by Hopkins *et al* (2000) [326]. Area under the curve was calculated according to the trapezoidal rule. The typical error and the intraclass correlation coefficient (ICC) were calculated for each measure derived from the three OGTT retests in each subject. The typical error is calculated as the within-subject standard deviation for each measure derived from OGTT repeats (i.e.

the random variation in a subject's value between tests), expressed as the percentage coefficient of variation (CV) of the measurement. The ICC is similar to Pearson's correlation coefficient (r) but takes into account the typical error of measurement when more than two repeats have been undertaken. The ICC estimates the level of association between the repeated measures and illustrates the consistency of the rank order of subject's scores on retest [327]. To determine the overall reliability based on these two statistical values, a rank was assigned to each of the OGTT-derived measures, and the measure with the lowest sum of ranks score was chosen as the most reliable estimate of insulin sensitivity. 95% confidence limits (95%-CL) were also calculated for these reliability estimates.

3.4 Results

Figures 3.1 and 3.2 illustrate the changes in glucose and insulin concentrations following OGTT. Each value in each trial (or OGTT repeat) is expressed as mean ± S.E.M. for n = 7. No differences between trials were found (P>0.05).

Figure 3.1 – Plasma glucose concentration profiles following OGTT
The three trials represent the 75 g OGTT retests that were performed following an overnight fast, each separated by at least 5 days. Analysis revealed no significant differences between trials (P>0.05).
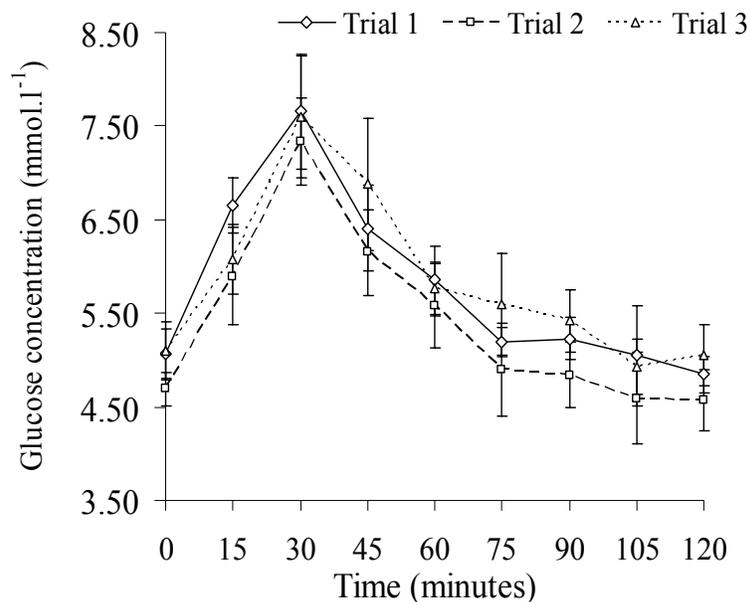
Figure 3.2 – Serum insulin concentration profiles following OGTT
The legend for the trials is the same as Figure 3.1. Analysis revealed no significant differences between trials (P>0.05).
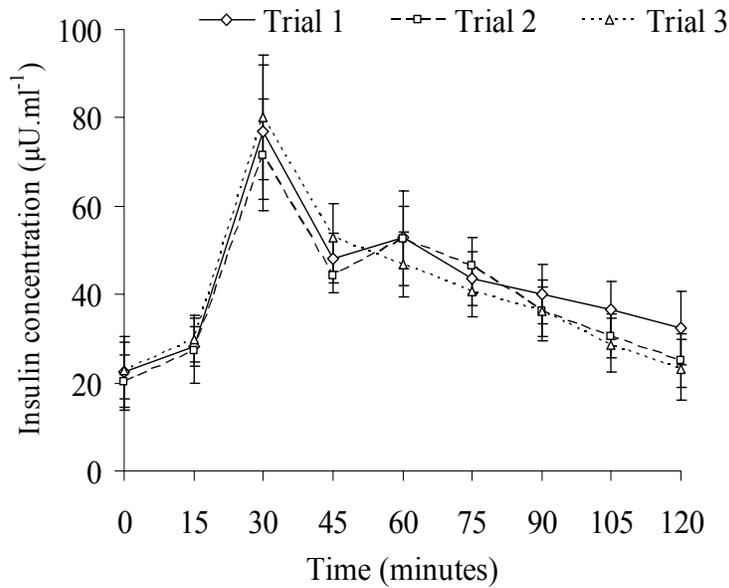
Tables 3.1 and 3.2 illustrate the reliability estimates calculated for each OGTT-derived measure. The typical error (expressed as a percentage CV) is listed in ascending order, whilst the ICCs are listed in descending order. The insulin sensitivity index from Matsuda *et al* (1999) (ISI$_{OGTT}^{Matsuda}$) showed the lowest typical error (Table 3.1) between measurements (CV = 5.60%; 95%-CL 3.30 to 8.62%), whilst 2h-SI showed the largest (CV = 48.2%; 95%-CL 34.6 to 79.0%). Fasting serum insulin (FSI) measures revealed the greatest correlation (Table 3.2) between trials (R = 0.981; 95%-CL 0.876 to 0.997), and 2 hour plasma glucose (2h-PG) had the poorest correlation (R = 0.180; 95%-CL -0.509 to 0.753). ISI$_{OGTT}^{Matsuda}$ had the lowest sum of ranks score (= 4), whilst 2h-PG had the largest (= 20).

Table 3.1 – Typical error of each OGTT-derived measure
   Three 75 g OGTT retests were performed following an overnight fast, each separated by at least 5 days. The within-subject standard deviation (typical error) for each measure was calculated and expressed as a percentage coefficient of variation. These are listed in order of rank. AUC Glu = area under the glucose curve in $mmol.l^{-1}.2h$; AUC Ins = area under the insulin curve in $\mu U.ml^{-1}.2h$; FPG = fasting plasma glucose in $mmol.l^{-1}$; FSI = fasting serum insulin in $\mu U.ml^{-1}$; 2h-PG = 2 hour plasma glucose in $mmol.l^{-1}$; 2h-SI = 2 hour serum insulin in $\mu U.ml^{-1}$; and ISI_{OGTT} = insulin sensitivity index derived from OGTT, the superscript names depict the various ISIs that were calculated.

Measure	Typical Error (CV, %)	95% Confidence Limits		Rank
		Lower	Upper	
$ISI_{OGTT}^{Matsuda}$	5.60	3.30	8.62	1
FSI	8.33	6.23	12.6	2
$ISI_{OGTT}^{Stumvoll}$	8.36	6.25	12.6	3
AUC Ins	8.39	6.27	12.6	4
$ISI_{OGTT}^{Soonthornpun}$	9.14	6.83	13.8	5
FPG	10.1	7.55	15.3	6
$ISI_{OGTT}^{Cederholm}$	10.3	7.68	15.6	7
AUC Glu	12.5	9.31	19.0	8
2h-PG	14.0	10.4	21.4	9
$ISI_{OGTT}^{Belfiore}$	17.6	13.1	27.2	10
2h-SI	48.2	34.6	79.0	11

Table 3.2 – The intraclass correlation coefficient between the three OGTT retrials for each measure
   The measures follow the same legend as Table 3.1, and again are listed in order of rank.

Measure	ICC	95% Confidence Limits		Rank
		Lower	Upper	
FSI	0.981	0.876	0.997	1
$ISI_{OGTT}^{Soonthornpun}$	0.959	0.758	0.994	2
$ISI_{OGTT}^{Matsuda}$	0.919	0.545	0.988	3
$ISI_{OGTT}^{Stumvoll}$	0.887	0.427	0.983	4
AUC Ins	0.868	0.426	0.979	5
$ISI_{OGTT}^{Belfiore}$	0.820	0.187	0.972	6
$ISI_{OGTT}^{Cederholm}$	0.802	0.129	0.969	7
2h-SI	0.692	0.010	0.946	8
FPG	0.278	-0.598	0.852	9
AUC Glu	0.229	-0.618	0.831	10
2h-PG	0.180	-0.509	0.753	11

3.5 Discussion

This study illustrates that the reliability of OGTT-derived measures can be quite variable depending on which one is chosen, with CVs ranging from 5.60 to 48.2% and correlation coefficients ranging from 0.180 to 0.981. However, the reliability of several of these measures, particularly the OGTT-derived insulin sensitivity indices (ISI$_{OGTT}$), is high, with indices calculated according to Matsuda, Stumvoll and Soonthornpun showing typical errors of 5.60, 8.36 and 9.14%, plus correlation coefficients of 0.919, 0.887 and 0.959, illustrating the consistency of the rank order of subject's scores between OGTT retests. It also appears that single glucose and insulin concentrations such as FPG, 2h-PG and 2h-SI are less reliable indices of glucose tolerance or insulin sensitivity.

In a clinical environment the OGTT 2h-PG is used as a second line test to FPG in the diagnosis of impaired glucose tolerance (IGT) and diabetes mellitus [320]. Whilst diagnostic sensitivity (i.e. the percentage of diabetics identified as positive for diabetes using the OGTT) is high, and prognosis is good if disease is detected in its early stages and appropriate treatment is administered, the scientific literature and medical opinion questions the reliability of such OGTT measures (FPG and 2h-PG) [224,231-234,328,329]. However the poor reliability of such measures in these studies is likely attributed to poor pre-test control. Dietary replication and physical activity restriction are imperative for consistency of any meal-ingestion protocol. Studies have shown caloric restriction and physical activity to upregulate glucose uptake mechanisms almost immediately with lasting effects [330,331]. Such controls are not always undertaken in the literature, and particularly in its clinical application the recommendation of an unrestricted diet containing more than 150 g.day^{-1} of carbohydrate for three days prior to the OGTT is perhaps ignored by the patient or even not recommended in the first place [320]. These dietary guidelines seem quite specific, yet with respect to exercise the only recommendation prior to the OGTT protocol is that "the usual physical activity should be undertaken" [320]. These activity guidelines are too vague for a variable that has a large effect on glucose tolerance and insulin sensitivity. In this study, improved reliability was likely due to tight control of diet and exercise for 2 days before the OGTTs; subjects abstained from alcohol, caffeine and exercise, plus replicated their diet during the 2 days before each trial.

Each of the OGTT-derived ISI models have been previously validated against the hyperinsulinaemic euglycaemic clamp [219-223], and in some cases against hyperglycaemic clamps [220], the homeostatic model assessment (HOMA), the quantitative insulin sensitivity check index (QUICKI) and other ISI_{OGTT} models [221]. The models used in this study each showed a strong correlation with ISI_{CLAMP} values in their original papers ($ISI_{OGTT}^{Matsuda}$, r = 0.73, P<0.01 [219]; $ISI_{OGTT}^{Soonthornpun}$, r = 0.87, P<0.01 [221]; $ISI_{OGTT}^{Stumvoll}$, r = 0.79, P<0.01 [220]; $ISI_{OGTT}^{Cederholm}$, r = 0.53, P<0.01 [222]; $ISI_{OGTT}^{Belfiore}$, r = 0.64, P<0.01 [223]), validating their use as tools to measure insulin sensitivity; however no previous reliability experiments on these measures have been undertaken. Our study demonstrates that all of these indices had good reliability, yet by interpreting the retest reliability estimates with their corresponding confidence limits and rank order, it would appear that $ISI_{OGTT}^{Matsuda}$ is the most reliable with a typical error of 5.6%, ranked 1st (Table 3.1, 95%-CL, 3.30 to 8.62%), and an ICC of 0.919, ranked 3rd (Table 3.2, 95%-CL, 0.545 to 0.988), giving it the lowest sum of ranks score of all our measures. These findings along with data from Matsuda *et al* (1999), which showed a correlation coefficient of 0.73 with clamp-derived ISI estimates [219], illustrate the reliability and validity of $ISI_{OGTT}^{Matsuda}$ for use in future research interventions. In addition, this is the sole index to have been validated in a cohort exhibiting a range of insulin sensitive states (normoglycaemic, impaired glycaemic and diabetes) [219]. Therefore, $ISI_{OGTT}^{Matsuda}$ was chosen for future intervention studies in our laboratory.

4. The use of isotopic tracers in oral glucose tolerance tests

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Co-authors: John Ayuk, Andrew K. Blannin, Andrew A. Toogood, Asker E. Jeukendrup

4.1 Abstract

The OGTT is a useful physiological test that determines glucose tolerance. Changes in postprandial glucose and insulin reflect systemic insulin sensitivity. The usefulness of the OGTT in whole-body insulin sensitivity estimation is limited by the fact that the contribution of endogenous glucose production or gastrointestinal glucose absorption to total glucose appearance cannot be determined. In this study four lean male subjects (aged 26 ± 1 years, body mass 73.0 ± 3.9 kg, BMI 23.6 ± 0.9 kg.m^{-2} (mean $\pm$ S.E.M.) underwent a 75 g OGTT combined with a primed intravenous infusion of a $[6,6-^2H_2]$glucose tracer and the ingestion of a $[U-^{13}C]$glucose tracer. Tracer enrichments were determined by GC-MS and applied to Steele's single pool model of nonsteady state tracer kinetics. Results indicate that peak glucose concentrations (8.40 ± 1.08 mmol.l^{-1}) were reached 30 mins after glucose ingestion. In addition, endogenous glucose production was suppressed by $63.8 \pm 3.60\%$ from 0.194 ± 0.012 to 0.070 ± 0.007 g.min^{-1} during the postprandial period. This dual-tracer OGTT tool may be used to assess exogenous glucose appearance and defects in the insulin-responsiveness of endogenous glucose production.

4.2 Introduction

The glycaemic clamp is the gold standard technique for measuring insulin sensitivity [211], yet the oral glucose tolerance test (OGTT) is the preferred method for diabetes diagnostics that gives useful information about glucose tolerance [320]. The OGTT is a physiological test where glucose appearance in the blood is dependent on gastric emptying, intestinal absorption and liver function, and thus it is more representative of free-living situations (meal ingestion) than infusion-based methodology such as the clamp. However, the OGTT, in its simplicity, is also very limited in the information it provides. The changes in glucose concentrations that follow are the result of glucose fluxes into and out of the plasma compartment; a rise in plasma glucose concentration could be the result of reduced tissue glucose uptake, increased hepatic output, or increased intestinal absorption, thus the OGTT provides a whole body measure of glucose tolerance. The application of isotopic tracers to an OGTT allows quantification of flux into and out of such compartments, providing useful information about the existing state of glucose tolerance with regards to the rate of glucose appearance and disappearance [332]. In the 1950s and 1960s Steele *et al* pioneered dual tracer application to metabolism calculating tracer kinetics based on a single-pool model [315,318,333]. Since, several research groups have attempted to further their work, investigating postprandial glucose metabolism during OGTT-type protocols [334,335]. However there is little uniformity in the study designs. This study uses a single-pool dual tracer approach to assess whether it is possible to quantify the rate of glucose appearance from the gut and from endogenous (hepatic) sources, as well as the rate of glucose disappearance from the plasma pool during an OGTT. This is the first study to do so during a standard 2 hour 75 g OGTT.

4.3 Methods and materials

Subjects. Four habitually active male volunteers aged 26 ± 1 years, body mass 73.0 ± 3.9 kg, with BMI of 23.6 ± 0.9 kg.m^{-2} (mean ± S.E.M.) were recruited from the local community. All volunteers were deemed healthy as assessed by a general health questionnaire and provided their informed written consent prior to the study. Volunteers underwent an oral glucose tolerance test

(OGTT) performed with the use of two stable isotopic derivatives of glucose, and were instructed to refrain from consuming alcohol or caffeine and from undertaking any non-prescribed exercise for forty-eight hours prior to testing. The protocol was approved by the South Birmingham Health Authority Local Research Ethics Committee.

Protocol. A $[6,6-^2H_2]$glucose infusate was prepared the night before each study visit. A known trace amount (90 mg.ml^{-1}) of pyrogen-free, 99% purity $[6,6-^2H_2]$glucose (Cambridge Isotope Laboratories, MA) was dissolved in isotonic saline in a sterile environment. The infusate was autoclaved at 120 ^{o}C under high pressure and allowed to cool prior to infusion. A 0.2 μm filter was placed in the infusion line for the intravenous infusions. Volunteers arrived in the laboratory at 0800 hours following a 12 hour overnight fast, and were studied in a reclined position. An intravenous cannula (BD Venflon, BD, UK) was inserted into an antecubital vein on both arms and a 15 μmol.kg^{-1} $[6,6-^2H_2]$glucose prime was administered (t = -60 min) into one of the lines immediately followed by a constant 0.35 μmol.kg^{-1}.min^{-1} infusion of $[6,6-^2H_2]$glucose for 3 hours via a calibrated infusion pump. Infusion rates and primes were based on previous literature and pilot experiments designed to yield an optimum enrichment [317,336]. At t = 0 min participants ingested a 300 ml solution containing 73 g of anhydrous D-glucose (Sigma, UK) and 2 g of $[U-^{13}C]$glucose (Isotec Inc, US). Blood samples were collected at t = -60 min and every 5 mins during the OGTT period for the measurement of glucose concentrations. Glucose tracer enrichments (both $[6,6-^2H_2]$- and $[U-^{13}C]$glucose) were also analysed in plasma samples, infusates, and oral glucose boluses. Tracer kinetics were calculated according to Steele's single-pool, non steady-state model [332,333]. Full details of the assays and calculations of tracer kinetics are specified in the General Methods chapter.

4.4 Results

Figure 4.1 illustrates the plasma glucose kinetics during OGTT showing the mean ± S.E.M. for the four subjects. Peak plasma glucose concentrations (8.40 ± 1.08 mmol.l^{-1}) were reached at t = 30 mins. $[6,6-^2H_2]$glucose enrichment decreased with time whilst $[U-^{13}C]$glucose enrichment increased with time following oral glucose ingestion. Rate of appearance of glucose from the gut (R_a^{GUT})

demonstrated increasing contribution to total rate of glucose appearance (R_a^{TOTAL}) with time, peaking

at t = 80 mins (0.352 ± 0.012 g.min^{-1}), whilst the rate of glucose appearance from endogenous sources

(R_a^{ENDO}) decreased following glucose ingestion from a basal value of 0.194 ± 0.012 g.min^{-1}. Peak

suppression of endogenous glucose production was reached at t = 105 mins, when $R_a^{ENDO} = 0.070 \pm$

0.007 g.min^{-1}. Rate of glucose disappearance increased with time until t = 70 mins (0.456 ± 0.014

g.min^{-1}).

Figure 4.1 – Plasma glucose kinetics during OGTT

Data represents mean $\pm$ S.E.M. A primed 0.35 µmol.kg^{-1}.min^{-1} intravenous infusion of [6,6-^{2}H$_2$]glucose began at t = -60 mins. At t = 0 min a 75 g glucose bolus containing 2 g of [U-^{13}C]glucose was consumed. Panel (i) shows changes in plasma glucose concentrations with time; panel (ii) shows the percentage enrichment of the two tracers in the plasma; panel (iii) shows the rate of glucose appearance into the blood pool from exogenous and endogenous sources; and panel (iv) shows the rate of glucose disappearance from the blood pool.
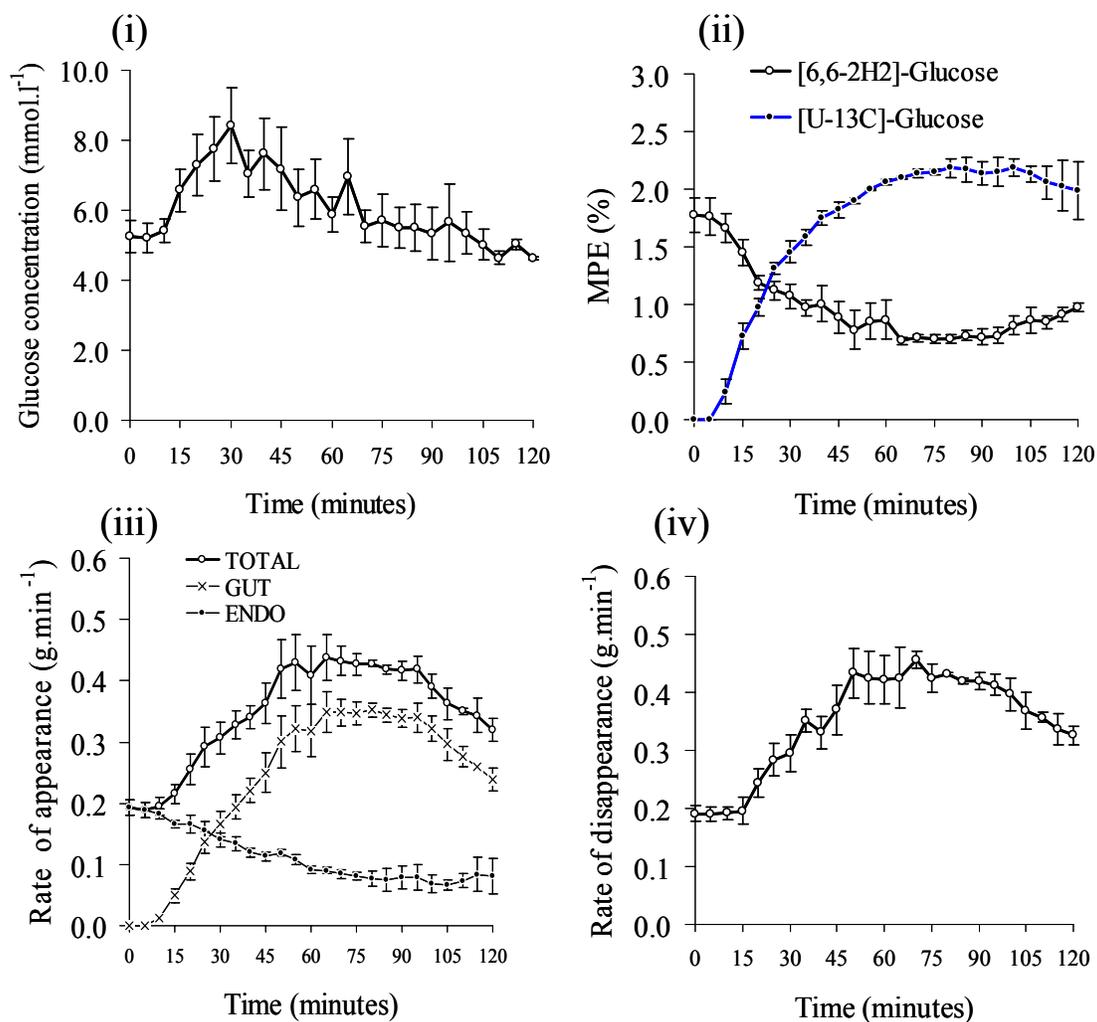
4.5 Discussion

This data demonstrates that the contribution of endogenous glucose production (R_a^{ENDO}) to total rate of appearance is suppressed by $63.8 \pm 3.60\%$ from 0.194 ± 0.012 to 0.070 ± 0.007 g.min^{-1} following oral glucose ingestion, and that the rate of glucose appearance from the gut contributed $63.7 \pm 5.4\%$ of total glucose appearance during a 2 hour OGTT in lean healthy individuals (mean R_a^{TOTAL} for the 2 h period = 0.336 ± 0.018 g.min^{-1}, mean R_a^{GUT} for the 2 h period = 0.214 ± 0.026 g.min^{-1}). In addition, maximal contribution from the gut was 0.352 ± 0.012 g.min^{-1}. This occurred 80 mins following ingestion of the OGTT.

The ability to quantify endogenous glucose production is important for clinical research. Elevated hepatic glucose output in the basal and postprandial state is a key aspect of hyperglycaemia and insulin resistance, and information regarding the liver's response to lifestyle or pharmaceutical interventions is valuable to clinicians and pharmaceutical companies. Tracer-derived estimates from the gold standard clamp technique enables quantification of such variables. However prolonged elevation of insulin during such protocols is not representative of free living situations, and therefore the OGTT is a useful tool to derive such estimates in a physiological postprandial period.

Dual tracer methodology to study glucose kinetics during the OGTT was pioneered by Steele *et al* (1956, 1959, 1968) [315,318,333]. Since, several groups have used dual tracer methods to study glucose metabolism in the postprandial period, these publications are reviewed in Livesey *et al* (1998) and Basu *et al* (2003) [334,335]. Within this literature (references [334,337-349]) there is great variation in the oral glucose loads, sampling frequencies (> every 15 mins) and sampling durations (< 8 hours). In addition, different data sets have been analysed using different compartment models (single versus multiple) and various volumes of plasma glucose distribution. Despite the non-uniformity in methodology, all of these publications have quantified R_a^{TOTAL} and R_a^{ENDO} indicating that basal endogenous glucose production is approximately 0.175 g.min^{-1} (2.5 mg.kg^{-1}.min^{-1}) in normoglycaemic individuals, and that maximal R_a^{GUT} is approximately 0.350 g.min^{-1} (5.0 mg.kg^{-1}.min^{-1}) when a 75 g bolus of glucose is ingested. These values are comparable to those found in the current study: basal $R_a^{ENDO} = 0.194 \pm 0.012$ g.min^{-1} (2.7 mg.kg^{-1}.min^{-1}), maximal $R_a^{GUT} = 0.352 \pm 0.012$ g.min^{-}

[1] (5.0 mg.kg^{-1}.min^{-1}). This suggests that our tool may provide reliable information regarding the rate of glucose appearance following OGTT. However, recent literature from Basu *et al* (2003) and Toffolo *et al* (2006) published after we had completed the current study, suggest that errors in R_a^{ENDO} measurements may arise from rapid changes in postprandial glucose enrichment when using a dual-tracer nonsteady state single-pool methodology [335,350]. These groups have suggested that triple-tracer multiple-pool modelling using variable tracer infusion rates provide better estimates of glucose rates of appearance. However, the complexity of such studies seems to prevent its routine use in clinical studies evaluating the effect of therapeutic interventions.

In addition to Steele's single-pool model used in this and other studies [333], alternative multiple-pool models exist to better estimate glucose kinetics within plasma and interstitial fluid compartments (e.g. Radziuk *et al* 1978 [351], or Mari *et al* 1994 [346]). In addition, several studies have used different values to represent the plasma glucose space. The effectiveness of the Steele equation to accurately calculate R_a may depend on the value of the glucose space [332,352,353]. One of the most recent studies to apply dual stable isotope tracer methodology to the OGTT is that of Livesey *et al* (1998) [334]. This publication performed analysis of dual-tracer OGTT data using a single- (e.g. Steele *et al* 1959 [333]) and a two-pool (Mari *et al* 1994 [346]) model of glucose kinetics with different values for the glucose space. Interestingly, they demonstrated that the model and glucose distribution values have very little effect upon calculated R_a values [334]. The size of the pool (0.160 l.kg^{-1}) used in our analysis was based on recent stable isotope dual-tracer literature (e.g. Jeukendrup *et al* 1999 [317], Delarue *et al* 1996 [349]), however other literature using radioactive dual-tracer methods have used pool sizes ranging from 0.095 to 0.230 l.kg^{-1}. When we apply these ranges to our analysis, it actually makes very little difference to the resultant R_a values. For example, when analysing our data with glucose pool sizes of 0.095 and 0.230 l.kg^{-1}, the resultant basal R_a^{ENDO} values were 0.194 and 0.194 g.min^{-1} respectively, and maximal R_a^{GUT} values were 0.352 and 0.353 g.min^{-1}, respectively. Therefore the size of the glucose space appears to have little effect on the calculated rates of glucose appearance.

This study improves on previous work as stable isotopes, although more expensive, are safer and more ethical than using radioactive moieties which are employed in the majority of tracer studies [332]. Also, by using solely stable isotopes rather than a mixture of radioactive and stable isotopes like several previous publications (e.g. [338,347,354,355]), data analysis will be more rapid. Additionally, our sampling frequency of 5 min is of higher resolution than the 10-30 min frequency used in previous work. This minimises large fluctuations in tracer enrichment between consecutive samples thus minimising variation in R_a during nonsteady-state glucose flux [332]. In addition, unlike the majority of previous publications [339-348], including the dual tracer stable isotope work by Delarue *et al* (1996) [349] and Livesey *et al* (1998) [334], our protocol replicates the standard 2 hour 75 g OGTT used in clinical practice [320], thus illustrating that detection of changes in rate of glucose appearance is possible during a relatively short patient intervention. This current study has however only been undertaken in a relatively small, healthy cohort, and so future work should focus on applying such a tool to individuals with a large range of insulin sensitivity. Additionally, by measuring insulin concentrations, it would be useful to develop an insulin sensitivity index from our OGTT-derived R_a^{ENDO} measure (rate of glucose appearance from the liver per unit of insulin) and validate it against R_a^{ENDO} data from glycaemic clamp methodology.

In conclusion, this study illustrates that estimates of the rate of glucose appearance from endogenous and exogenous sources can be quantified during 2 hour 75 g OGTT protocols using safe stable isotope glucose tracers. These findings indicate that, if applied to an insulin resistance group, valuable information regarding hepatic glucose production may be obtained. Such information would be of use in research to detect specific metabolic abnormalities (e.g. hepatic insulin resistance) and thus find the most effective therapeutic intervention. However, with recent work from Basu *et al* (2003) indicating the uncertainty of single-pool, nonsteady state dual-tracer data, further work is required to determine its reliability and its capacity to measure changes in insulin sensitivity in response to therapeutic interventions in patients with insulin resistance.

5. Effects of acute cinnamon ingestion on insulin sensitivity

Co-authors: Andrew K. Blannin

5.1 Abstract

Cinnamon spice and its phenolic extracts have been demonstrated to exhibit insulin-like actions *in vitro*. In humans the evidence surrounding cinnamon-induced improvements in glycaemia and insulin sensitivity is controversial. In this study, seven sedentary but otherwise healthy male volunteers (aged 26 ± 1 years, body mass 77.0 ± 1.6 kg, and BMI 24.5 ± 0.3 kg.m^{-2}) underwent three oral glucose tolerance tests (OGTT). One trial involved consuming a 5 g placebo with the OGTT (OGTT$_{control}$), another required the ingestion of 5 g of cinnamon with the OGTT (OGTT$_{cin}$), and the other trial involved consuming 5 g of cinnamon 12 hours before the OGTT (OGTT$_{cin12hpre}$). This study demonstrates that cinnamon ingestion reduces the total plasma glucose response to oral glucose ingestion (measured as area under the curve for 2 hours after ingestion) by $-12.9 \pm 4.2\%$ and $-10.0 \pm 3.7\%$ for OGTT$_{cin}$ ($P<0.05$) and OGTT$_{cin12hpre}$ ($P<0.05$) respectively compared with OGTT$_{control}$. Cinnamon also improved insulin sensitivity as assessed by Matsuda *et al* (1999) in both OGTT$_{cin}$ ($P<0.05$) and OGTT$_{cin12hpre}$ ($P<0.05$) trials compared with OGTT$_{control}$. This current study is the first to illustrate that acute cinnamon spice supplementation can improve *in vivo* insulin sensitivity in humans, and that not only are its effects immediate, they also appear to be sustained for 12 hours.

5.2 Introduction

Insulin resistance is the underlying pathology of T2DM and the metabolic syndrome [3]. Insulin sensitivity is easily measured and changes over time can be accurately monitored [185]. Large improvements in insulin sensitivity and other factors associated with the metabolic syndrome are seen following lifestyle or pharmaceutical intervention [191,200] yet prevalence rates continue to escalate [2] and therefore a search for alternative targets or therapeutics is sensible. Cinnamon spice appears to have insulin mimetic properties [235,237]; cinnamon extracts have been shown to increase *in vitro* adipocyte glucose uptake [236,356], and *in vivo* animal studies have demonstrated increased glucose utilisation and upregulation of insulin signalling events, such as increased phosphorylation of the insulin receptor and insulin receptor substrates (IRS-1) [243,244]. However, *in vivo* human evidence is mixed and somewhat limited. Currently there are three published studies in humans. Khan *et al* (2003) demonstrated reduced fasting glucose and lipids (by 18-29%) in T2DM patients following 40 days of 1, 3, or 6 g.day^{-1} *Cinnamomum cassia* feeding [238]; Mang *et al* (2006) showed that 3 g.day^{-1} reduced fasting glucose (by 10%) in T2DM patients after 4 months [239]; yet Vanschoonbeek *et al* (2006) failed to show any effect of a 6 week 1.5 g.day^{-1} feeding regime on fasting measures or insulin sensitivity in postmenopausal T2DM volunteers [240]. At the time of commencing this study, the paper by Khan *et al* (2003) was the only available publication. Their data investigated the effects of cinnamon ingestion on fasting plasma glucose (FPG) but no data were presented on insulinaemia or insulin sensitivity [238]. Also, despite evidence of an effect on FPG it was not known if this was due to the final dose of cinnamon taken 12 hours before the blood sample or an accumulative effect of 40 days cinnamon ingestion [238]. Therefore this study investigated the acute effects of a single cinnamon bolus upon an oral glucose tolerance test (OGTT)-derived insulin sensitivity estimate.

5.3 Methods and materials

Following ethical approval from The School of Sport and Exercise Sciences Safety and Ethics Subcommittee, seven sedentary but otherwise healthy male volunteers, aged 26 ± 1 years, body mass 77.0 ± 1.6 kg, and BMI 24.5 ± 0.3 kg.m^{-2}, were recruited from the local community. All volunteers

were assessed by a general health questionnaire and provided informed written consent prior to commencing the study. Participants made a total of three visits to the laboratory each separated by at least five days. A dietary record was taken for the two days preceding the first test, and subjects were instructed to refrain from consuming alcohol, caffeine, and cinnamon products, and from any exercise for forty-eight hours prior to each trial. The dietary record was for the purposes of diet replication prior to each trial. Trials were assigned in a single-blind randomised cross-over design: a placebo-controlled oral glucose tolerance test ($OGTT_{control}$), an OGTT supplemented with cinnamon ($OGTT_{cin}$), and an OGTT with cinnamon ingested twelve hours prior to the trial ($OGTT_{cin12hpre}$). A 12 h delay between cinnamon ingestion and OGTT was chosen to replicate the delay between final dosing and blood sampling in Khan's publication [238]. Because two trials involved consuming capsules with the OGTT and the other trial involved consuming capsules the night before, the subjects could have been aware of the trial allocation. Therefore, dummy capsules containing placebo were consumed at appropriate times to ensure the same number of capsules were consumed 12 hours before and with each OGTT for each trial to preserve blinding. In addition, to test the effects our chosen placebo may have on glucose clearance, four subjects volunteered to undergo two further OGTTs with and without 5 g of placebo capsules.

5.3.1 $OGTT_{control}$

Subjects arrived in the laboratory at 0800 hours following an overnight fast. An intravenous cannula (BD Venflon, Oxford, UK) was inserted into an antecubital vein and a fasting blood sample was taken. At 0830 hours the OGTT commenced: subjects were instructed to ingest a 75 g bolus of dextrose (Cerestar, Manchester, UK) in 300 ml of water. Blood samples (5 ml) were drawn from the intravenous line at t = 0, 30, 60, 90, and 120 min following ingestion of the drink. During the trial the cannula was kept patent with 5 ml flushes of 0.9% $NaCl_{(aq)}$ saline (Baxter Healthcare, Northampton, UK) following each blood-letting. All volunteers were administered 5 g (= 10 x 500 mg capsules) of vegi-capsulated wheat flour placebo capsules to consume twelve hours prior to (at 2030 hours the previous night following evening meal), and immediately before the OGTT. Wheat flour was chosen in accordance with Khan et al (2003) [238]. All blood sample handling, storage, and analysis

procedures (for glucose and insulin concentrations) are described in the General Methods section (Chapter 2).

5.3.2 OGTT$_{cin}$

Subjects followed the same protocol in this trial as for OGTT$_{control}$, however, 5 g (= 10 x 500 mg capsules) of vegi-capsulated *Cinnamomum cassia* spice (Everythingcinnamon.com, Essex, UK) was ingested with the oral glucose bolus at t = 0 min. Subjects were also given 5 g of vegi-capsulated wheat flour placebo capsules to ingest twelve hours prior to OGTT, i.e. at 2030 hours the previous night following all evening meals. The dietary intake diary was followed as described above, and blood samples were collected and stored in the same manner. A 5 g cinnamon bolus was chosen based on a weighted average of the data from Khan *et al* (2003) that showed that 6 g had a slightly greater effect than 3 g upon FPG [238]; because their data showed doubling the dose did not double the effect this indicated higher doses were unlikely to produce effects on a *pro rata* basis. The data of Khan *et al* (2003) also showed that 1g of cinnamon has little effect on FPG.

5.3.3 OGTT$_{cin12hpre}$

Again the same protocol was followed as for OGTT$_{control}$; however subjects were given 5 g of vegi-capsulated cinnamon to ingest twelve hours prior to the OGTT, i.e. at 2030 hours the previous evening. Also, 5 g of vegi-capsulated wheat flour placebo capsules were ingested with the OGTT at t = 0 min. Again dietary intake diaries were followed as described, and blood samples were collected, stored, and analysed in the same manner.

5.3.4 Insulin sensitivity measurement

Several methods to measure insulin sensitivity are available; these are described in the General Introduction (Section 1.4). In this study the OGTT was used to investigate changes in insulin sensitivity. Estimates derived from OGTT data have been validated elsewhere [219-223] and their reliability is assessed in Chapter 3. As described in Chapter 3 ISI$_{OGTT}$^Matsuda was considered to be the

most reliable insulin sensitivity index for repeated measures interventions and so was selected for this study [219]. Calculations are described in the General Methods section (Equation 2.2).

5.3.5 Statistical Analysis

Data are expressed as mean ± S.E.M. and statistical analysis was carried out with SPSS for Windows 12.0.1 (SPSS Inc, Chicago, US). Raw glucose and insulin data were tested for normality and analysed using two-way (time*trial) repeated measures ANOVA. Main effects were analysed using Bonferroni *post hoc* tests. Raw data was converted to area under the curve (AUC) values by the trapezoidal method and compared using one-way ANOVA. Significant differences were accepted at $P<0.05$. Based on the effect size found in this study, a power calculation, with power set at 0.8 and alpha at 0.05, showed that investigating ten subjects should be sufficient to detect significant differences between trials in a future study.

5.4 Results

Ingestion of wheat flour with an OGTT did not change the glucose response (AUC) to an OGTT compared with an OGTT without flour ingestion (Figure 5.1).

Figure 5.1 – Effect of wheat flour placebo on glucose responses following OGTT
Four subjects volunteered to undergo two further OGTTs with and without 10 x 500 mg (total = 5 g) wheat flour capsules. No differences were found between trials, $P>0.05$. Data represents mean ± S.E.M.
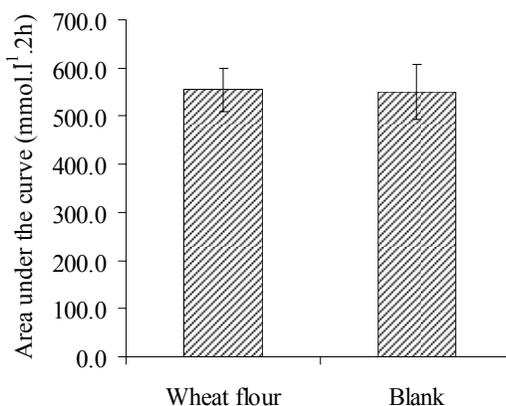
5.4.1 Plasma glucose responses to OGTT in each trial

Analysis of raw data showed a main effect of trial with plasma glucose being lower in OGTT$_{cin}$ and OGTT$_{cin12hpre}$ trials compared with OGTT$_{control}$ (P<0.05, Figure 5.2). Prior to OGTT, fasting plasma glucose measures were taken: 4.89 ± 0.18, 4.75 ± 0.10, and 4.69 ± 0.15 mmol.l^{-1} for OGTT$_{control, cin}$, and $_{cin12hpre}$ respectively. No significant differences were found in these data (P>0.05). Plasma glucose measures at peak (t = 30 mins) and t = 120 mins were also compared, and again no statistical differences between trials were found (P>0.05). The area under the plasma glucose curve was significantly decreased in OGTT$_{cin}$ and OGTT$_{cin12hpre}$ trials by 12.9 ± 4.2% and 10.0 ± 3.7% respectively when compared with OGTT$_{control}$ (P<0.05), but no differences were found between the two cinnamon trials (Figure 5.3).

Figure 5.2 – Plasma glucose responses during OGTT
In each trial a 75 g 300 ml OGTT was ingested. In OGTT$_{control}$, 5 g of placebo capsules were ingested with and 12 h prior to OGTT; In OGTT$_{cin}$, 5 g of cinnamon was ingested with the OGTT and 5 g of placebo was ingested 12 h prior; and in OGTT$_{cin12hpre}$, 5 g of placebo was ingested with the OGTT, whilst 5 g of cinnamon was taken 12 h prior. Glucose concentrations at t = 30 mins were significantly different from baseline in all trials (**, P<0.01). Glucose responses in OGTT$_{cin}$ and OGTT$_{cin12hpre}$ were also significantly different from OGTT$_{control}$ (#, P<0.05) but not from one another (P>0.05). Data represents mean ± S.E.M.

Figure 5.3 – Area under the plasma glucose response curve during OGTT
The three trials follow the same legend as in Figure 5.2. Glucose responses in $OGTT_{cin}$ and $OGTT_{cin12hpre}$ were significantly lower than $OGTT_{control}$ (*, $P<0.05$), though not statistically different from one another ($P>0.05$). Data represents mean ± S.E.M.

5.4.2 Serum insulin responses to OGTT in each trial

Figure 5.4 demonstrates the serum insulin responses to OGTT during the three trials. No differences in insulin responses were found between trials ($P>0.05$). No significant differences were found between trials in fasting serum insulin concentrations ($P>0.05$), nor were any differences found between trials in peak ($t = 30$ min) or $t = 120$ min concentrations ($P>0.05$). Insulin AUC data was not significantly different between trials (Figure 5.5).

Figure 5.4 – Serum insulin responses during OGTT
Again, in $OGTT_{control}$, 5 g of placebo capsules were ingested with and 12 h prior to OGTT; In $OGTT_{cin}$, 5 g of cinnamon was ingested with the OGTT and 5 g of placebo was ingested 12 h prior; and in $OGTT_{cin12hpre}$, 5 g of placebo was ingested with the OGTT, whilst 5 g of cinnamon was taken 12 h prior. Insulin concentrations at $t = 30$, 60, and 90 mins were significantly different from baseline in all trials (**, $P<0.01$). Insulin responses were not different between trials ($P>0.05$). Data represents mean ± S.E.M.
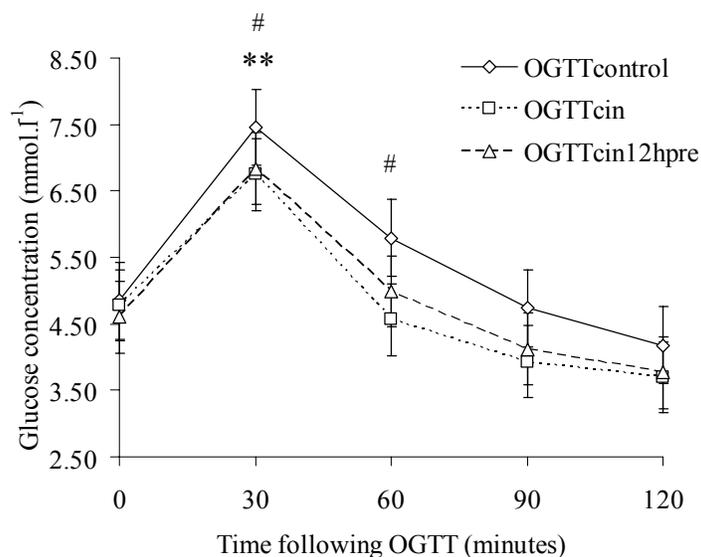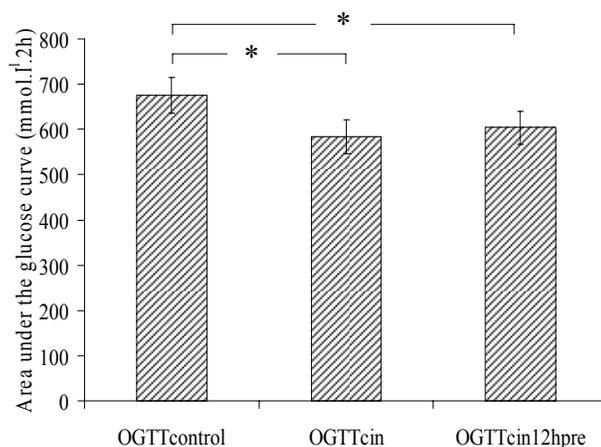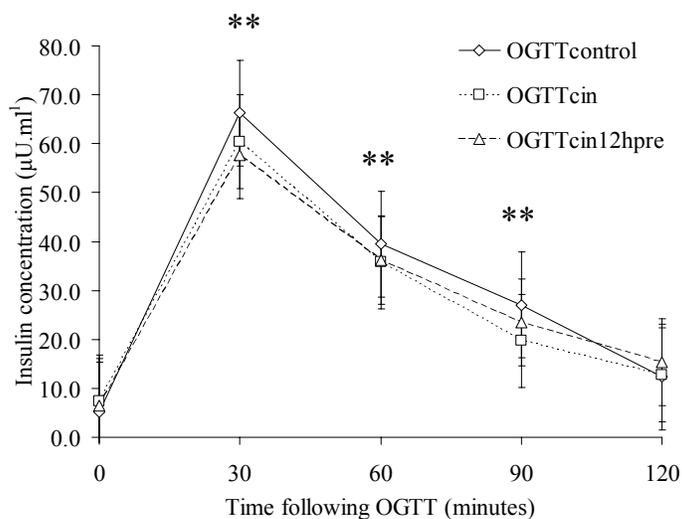
Figure 5.5 – Area under the serum insulin response curve during OGTT
The three trials follow the same legend as in Figure 5.4. Insulin responses were not different between trials (P>0.05). Data represents mean ± S.E.M.
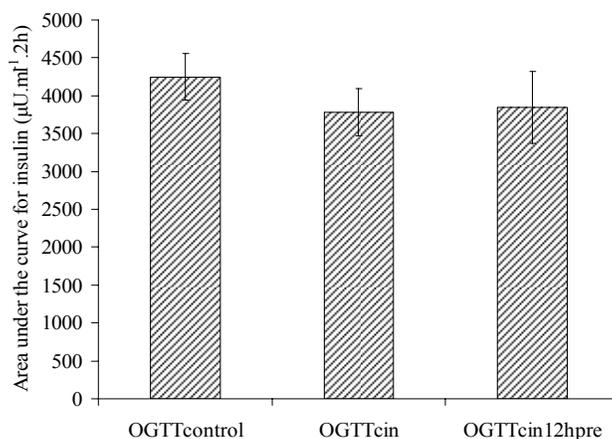
5.4.3 Changes in insulin sensitivity

Figure 5.6 illustrates insulin sensitivity data derived from $ISI_{OGTT}^{Matsuda}$. Insulin sensitivity was

elevated in both the cinnamon trials compared to $OGTT_{control}$ (P<0.05), though they were not different

from one another (P>0.05).

Figure 5.6 – Insulin sensitivity assessed by ISI_{OGTT} (Matsuda *et al*, 1999)
The three trials follow the same legend as in Figure 5.4. The ISI_{OGTT} index derived from Matsuda *et al* (1999) [219] revealed greater insulin sensitivity in the $OGTT_{cin}$ and $_{cin12hpre}$ trials compared to $OGTT_{control}$ (*, P<0.05). Data represents mean ± S.E.M.
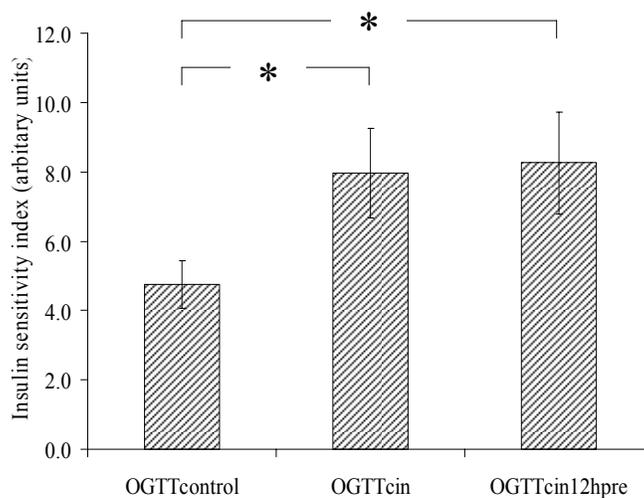
5.5 Discussion

This study demonstrates that a single bolus ingestion of 5 g of cinnamon spice can reduce blood glucose responses to an OGTT and improve insulin sensitivity in inactive but otherwise healthy individuals. These data also show that the effects persist for 12 hours following cinnamon ingestion. The choice of wheat flour as a placebo was shown to have no effect on glucose responses, nor could volunteers distinguish it from the cinnamon capsules.

Elevated fasting and postprandial glycaemia can contribute to the onset of metabolic disease via non-enzymatic protein glycation or toxic effects on the vascular endothelium [357,358]. Improvements in insulin sensitivity can help to maintain blood glucose homeostasis and prevent the detrimental effects of hyperglycaemia [200]. In 1990 it was first described that cinnamon may enhance *in vitro* glucose utilisation in rat adipocytes [235]. Other groups have published data supporting these findings with increased glucose uptake and glycogen synthesis in 3T3-L1 cell lines [236,237]. The cinnamon-induced lowering of *in vivo* glucose responses may indeed be explained by reduced gastric emptying or delayed intestinal absorption [359]; however, our data show no delay in peak glucose concentrations following cinnamon ingestion. In addition, the literature suggests that cinnamon extracts can directly upregulate insulin receptor autophosphorylation and inhibit phosphatase action and glycogen synthase kinase-3β (GSK-3β) activity *in vitro* [241]; and recent *in vivo* evidence in rats shows cinnamon-induced increases in skeletal muscle IRS-1 tyrosine phosphorylation and IRS-1-PI3K association [244], therefore cinnamon's effects on insulin sensitivity may act directly on aspects of the insulin signalling cascade that are directly associated with insulin resistance and T2DM [71,360,361]. Current molecular data however is restricted to animals or *in vitro* work, therefore extrapolation to humans should be treated with caution.

Currently only three publications report cinnamon's effects on glycaemic control and insulin sensitivity in humans [238-240]. Interestingly, Vanschoonbeek *et al* (2006) failed to show any effect of cinnamon ingestion upon OGTT-derived insulin sensitivity estimates [240]. Their study investigated postmenopausal T2DM women and employed 1.5 g.day^{-1} over a 6 week feeding intervention [240]. Khan *et al* (2003) and Mang *et al* (2006) however have reported that cinnamon

ingestion can reduce FPG in T2DM patients following 40 days and 4 months interventions respectively [238,239]. These conflicting findings may be attributed to the doses employed. Khan *et al* (2003) found little effect of 1 g.day^{-1}, yet marked effects at 3 and 6 g.day^{-1}, and Mang *et al* (2006) showed an effect using 3 g.day^{-1}. Vanschoonbeek's study made use of a 1.5 g.day^{-1} cinnamon supplementation, perhaps a level lower than the therapeutic requirement. Khan *et al* (2003) also reported that 20 days following removal of the 40 day cinnamon stimulus, lowered FPG persisted [238]. Whilst our study found no effect on FPG measures it did demonstrate a persistent effect on insulin sensitivity 12 hours after cinnamon ingestion, suggestive that cinnamon may have prolonged effects on events associated with increased insulin sensitivity. It would therefore be sensible to investigate the prolonged effects on insulin sensitivity following removal of the cinnamon stimulus. However, our data are representative of a lean population and so care must be taken if extrapolating these findings to an insulin resistant or obese group.

Differences in findings between groups may also be attributable to the pre-test standardisation. The presently available studies all have discrepancy with respect to dietary and physical activity control, and to the type of drug the patients were taking. For example, the publications by Mang *et al* and Khan *et al* fail to describe dietary or exercise control during their interventions. These are factors that can markedly influence glycaemic control and insulin sensitivity [200], and so care must be taken in comparing such findings. In addition, the data of Mang *et al* (2006), like that of Khan *et al* (2003), described decreased FPG as a result of cinnamon ingestion but also found a correlation between the size of improvement and the pre-intervention FPG [239]. This suggests that improvements in FPG are found with higher pre-trial values, i.e. with poorer glycaemic control. The discrepancies between studies may therefore become clear by looking at the pre-trial FPG values: Khan *et al* (2003) – 11.4-16.7 mmol.l^{-1}; Mang *et al* (2006) – 9.3 mmol.l^{-1}; Vanschoonbeek *et al* (2006) – 8.3 mmol.l^{-1}; present study – 4.9 mmol.l^{-1}. No changes in FPG were found in our data or Vanschoonbeek's data, studies with the lowest pre-intervention values.

Besides FPG, postprandial glucose and insulin levels are also useful markers of insulin sensitivity. The current data adds to the evidence base that cinnamon spice may improve glycaemic

control and insulin sensitivity, and that these effects persist for 12 hours; however, in human patient groups the evidence is varied. The current data in T2DM illustrates improved fasting glycaemia but no effects upon insulin sensitivity, haemoglobin A1c (HbA$_{1c}$), or HDL cholesterol (HDL-c) [238-240]. The literature illustrates that HbA1c and HDL-c are significant markers of glycaemic control and cardiovascular risk prognosis [362-364], and therefore with a lack of evidence with regard to long-term health outcome, further studies are required before cinnamon supplementation can be recommended. Given the *in vivo* animal evidence that cinnamon may upregulate peripheral glucose uptake and intracellular insulin signalling mechanisms; it would be sensible to investigate cinnamon ingestion on such factors in humans, particularly with regard to myocellular glucose utilisation, the major site of insulin-stimulated glucose uptake.

6. Effects of long-term cinnamon ingestion on insulin sensitivity

Co-authors: Lisa Fenton, Amanda Tee, Andrew K. Blannin

6.1 Abstract

Cinnamon spice can improve fasting glycaemia in humans yet data on insulin sensitivity are limited and controversial. Evidence shows prolonged effects on fasting glycaemia following 40 days of cinnamon feeding (Khan *et al* 2003), and on insulin sensitivity 12 hours after a single cinnamon bolus (Chapter 5), but insulin sensitivity data following long term cinnamon feeding are less convincing. In this study, eight sedentary but otherwise healthy male volunteers (aged 25 ± 1 years, body mass 76.5 ± 3.0 kg, BMI 24.0 ± 0.7 kg.m^{-2}; mean $\pm$ S.E.M.) underwent two 14 day interventions involving either cinnamon spice (*Cinnamomum cassia*) or placebo supplementation (3 g.day^{-1}). Placebo supplementation was continued for 5 days following this 14 day period. Oral glucose tolerance tests (OGTT) were performed on days 0, 1, 14, 16, 18, and 20. Cinnamon ingestion reduced area under the glucose curve response to OGTT at day 1 ($-13.1 \pm 6.3\%$ versus day 0; $P<0.05$) but this effect was lost by day 14 of cinnamon supplementation. Cinnamon ingestion reduced the area under the insulin curve response at day 14 ($-27.1 \pm 6.2\%$ versus day 0; $P<0.05$), as well as improving insulin sensitivity as assessed by $\text{ISI}_{\text{OGTT}}^{\text{Matsuda}}$ at day 14 (versus day 0; $P<0.05$). However, these effects were lost during the 5 day period following removal of the cinnamon feeding stimulus at day 14. These data add to the evidence that cinnamon spice can improve *in vivo* glycaemic control and insulin sensitivity in humans, but that effects on insulin sensitivity are not persistent once a prolonged period of cinnamon supplementation is ceased.
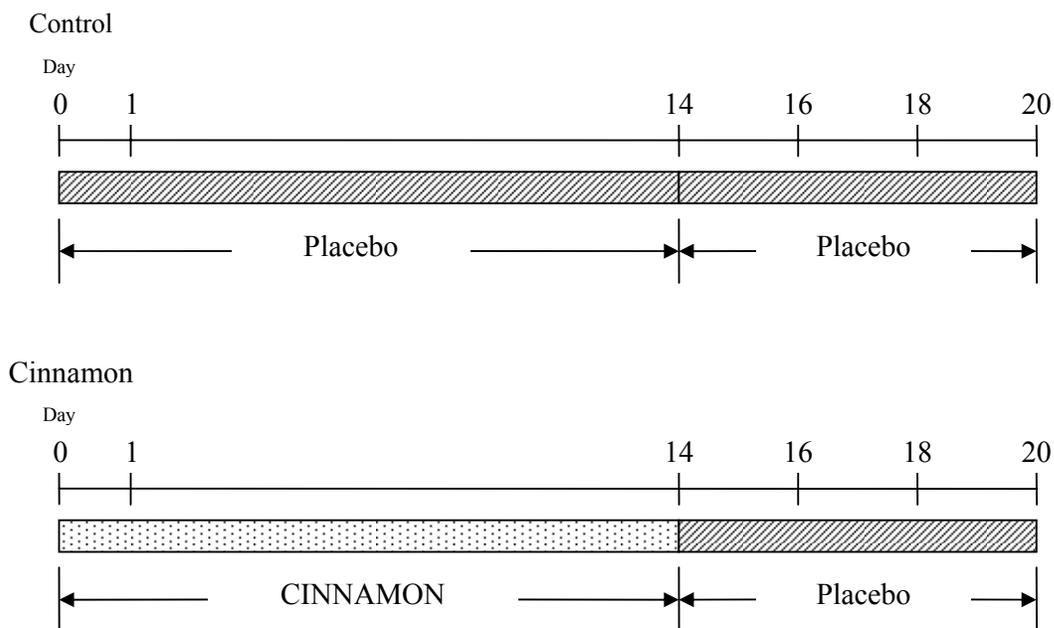
6.2 Introduction

Clear positive effects of cinnamon extracts and cinnamon spice ingestion on glucose utilisation and aspects of insulin signalling have been demonstrated *in vitro* [236,356] and *in vivo* in animals [244,246], yet the *in vivo* human evidence is controversial. Khan *et al* (2003) were the first group to demonstrate that 1, 3, and 6 g.day^{-1} of cinnamon may reduce FPG in T2DM patients [238], a finding confirmed with 3 g.day^{-1} by Mang *et al* (2006) [239], but opposed with 1.5 g.day^{-1} by Vanschoonbeek *et al* (2006) [240]. In addition, Khan *et al* (2003) published evidence that cinnamon ingestion may have lasting effects on FPG (and lipids) up to 20 days post-cessation of supplementation [238]. At the time of commencing this study, Khan *et al* (2003) was the only available study, and no evidence of long term effects of cinnamon ingestion on insulin sensitivity had been presented in humans. The previous chapter presents evidence that an acute 5 g cinnamon bolus can reduce OGTT glucose area under the curve (AUC) and improve insulin sensitivity for up to 12 hours post-ingestion, the next step was to establish if repeated daily ingestion of cinnamon over many days had an accumulative effect on insulin sensitivity. Cinnamon's therapeutic value would, to some extent, be influenced by the preservation of the effect following its withdrawal; therefore we also investigated the time course of insulin sensitivity changes after removal of a 2 week cinnamon feeding intervention.

6.3 Methods and materials

Following ethical approval from The School of Sport and Exercise Sciences Safety and Ethics Subcommittee, eight sedentary but otherwise healthy male volunteers, aged 25 ± 1 years, body mass 76.5 ± 3.0 kg, and BMI 24.0 ± 0.7 kg.m^{-2} (mean ± S.E.M.), were recruited from the local community. All volunteers were assessed by a general health questionnaire and provided informed written consent prior to commencing the study. A dietary record was taken for the two days preceding the first test, and subjects were instructed to refrain from consuming alcohol, caffeine, and cinnamon products, and from any exercise regime during the whole intervention period. The dietary record was for the purposes of diet replication prior to each trial. Each subject completed two 20 day interventions in a single-blind randomised cross-over design: a control intervention (Control trial) and a cinnamon

intervention where cinnamon was consumed during the first 14 days (Cinnamon trial). The interventions were as follows and are summarised in Figure 6.1:

Figure 6.1 – Summary of the intervention periods

Control

Day

| 0 | 1 | | 14 | 16 | 18 | 20 |

Placebo   Placebo

Cinnamon

Day

| 0 | 1 | | 14 | 16 | 18 | 20 |

CINNAMON   Placebo

OGTTs carried out at Day 0, 1, 14, 16, 18, and 20

6.3.1 Control trial

On day 0, volunteers arrived in the laboratory at 0800 hours following an overnight fast. An intravenous cannula (BD Venflon, Oxford, UK) was inserted into an antecubital vein and a fasting blood sample was taken. At 0830 hours subjects were instructed to ingest a 75 g bolus of dextrose (Cerestar, Manchester, UK) in 300 ml of water (OGTT). Blood samples (3 ml) were drawn from the intravenous line at $t = 0, 30, 60, 90,$ and 120 min following ingestion of the drink. During the trial the cannula was kept patent with 3 ml flushes of 0.9% $NaCl_{(aq)}$ saline (Baxter Healthcare, Northampton, UK) following each blood-letting. Blood samples were collected, stored, and analysed as described in the General Methods section. Volunteers returned to the laboratory for further OGTTs at 0800 hours on days 1, 14, 16, 18, and 20, having followed their diet diary. In addition, on days 0-14, subjects were given vegi-capsulated wheat flour placebo pills each containing 500 mg of wheat flour, and instructed

to take 6 pills per day (= 3 g.day^{-1}) at 2030 hours following their evening meal. Further placebo pills were given on days 15-19; again 6 pills were taken at 2030 hours each day.

6.3.2 Cinnamon trial

This period followed exactly the same protocol as the control trial. However, instead of administering placebo capsules on days 0-14, subjects were given 500 mg vegi-capsules containing cinnamon spice (*Cinnamomum cassia*). Volunteers were instructed to consume 6 pills (= 3 g) each day at 2030 hours, following their evening meal. At day 15 subjects consumed 6 x 500 mg wheat flour placebo capsules each evening for the remaining 5 days. OGTTs followed the protocol above and blood collection proceeded in the same fashion. The dose in this study was reduced to 3 g from 5 g in the previous study due to concerns of compliance and potential gastrointestinal problems of ingesting 10 x 500 mg pills per day for 2 weeks. Furthermore, given the evidence that at 3 g.day^{-1} Khan *et al* (2003) demonstrated significant reductions in FPG after 20 days feeding (-13%), 40 days feeding (-18%), plus a persistent reduction 20 days after ceasing cinnamon supplementation (-13%), it was considered likely that 3 g.day^{-1} would provide an effective stimulus. Measures at day 1 were included to confirm the findings from the previous chapter and to enable comparisons to be made between single and accumulative effects of cinnamon ingestion (measures on day 1 and day 14 were compared with values on day 0 to give acute and chronic effects, respectively). Measures were taken at 2 day intervals following removal of the cinnamon stimulus to investigate the time course of post-intervention recovery.

6.3.3 Insulin sensitivity measurement

As described in the previous chapter, ISI$_{OGTT}$^{Matsuda} was considered to be the most reliable index for repeated measures interventions and so was selected for this study [219]. Calculations are described in the General Methods section (Chapter 2).

6.3.4 Statistical Analysis

Data are expressed as mean ± S.E.M. and statistical analysis was carried out with SPSS for Windows 12.0.1 (SPSS Inc, Chicago, US). Raw glucose and insulin data were tested for normality and analysed by three-way (time*day*intervention) repeated measures ANOVA. Main effects were analysed using Bonferroni *post hoc* tests. Raw glucose and insulin data were converted to area under the curve (AUC) values by the trapezoidal method. AUC and insulin sensitivity data were analysed using two-way (day*intervention) repeated measures ANOVA, and again any main effects were analysed using Bonferroni *post hoc* procedures. Significance was achieved when P<0.05.

6.4 Results

6.4.1 Plasma glucose responses to OGTT during each intervention

Figure 6.2 illustrates the glucose responses following OGTT on each day, and Figure 6.3 summarises the AUC data. No significant interactions were present in the OGTT raw glucose profile data (intervention*time – P=0.10, day*time – P=0.11, intervention*day – P=0.09, and intervention*day*time – P=0.13). Statistical analysis of AUC data also failed to show a day*intervention interaction (P=0.13), however a separate analysis comparing day 1 of the cinnamon trial to day 0 of the cinnamon trial revealed decreased AUC glucose (-13.1 ± 6.3%; Figure 6.3, P<0.05). A comparison between day 1 cinnamon and day 1 control revealed a significant difference in glucose responses (-13.9 ± 3.9%; P<0.05). Comparing day 14 of the cinnamon trial to day 0 of the cinnamon trial showed no change (-5.5 ± 8.1%; P>0.05), and a comparison between day 14 cinnamon and day 14 control also showed no changed (-12.3 ± 11.8%; P>0.05). In addition, no significant differences were found between trials for fasting or 2 hour OGTT glucose data (P>0.05).

Figure 6.2 – Glucose concentration profiles following OGTT during each intervention period

OGTTs were undertaken at days 0, 1, 14, 16, 18, and 20 during the intervention period. Bold lines represent the control trial, where 3 g of placebo was administered each day. Dashed lines represent the cinnamon trial, where 3 g of cinnamon spice was administered each day until day 14, from when 3 g of placebo where given until the end of the 20 day intervention. The insert graph on each day represents total area under the glucose curve (AUC) for each OGTT. Glucose AUC was decreased at day 1 in the cinnamon intervention compared to control. * represents significant differences at $P<0.05$. Data represents mean ± S.E.M.

Figure 6.3 – Summary of the area under the OGTT glucose curve on each study day

OGTTs were undertaken at days 0, 1, 14, 16, 18, and 20. CONTROL (white bars) represents 3 g of placebo administered each day. CINNAMON (shaded bars) represents 3 g of cinnamon administered each day until day 14. Analysis of area under the glucose curve following OGTT revealed no intervention*day interaction (P=0.13). However, a separate analysis comparing just Day 1 to Day 0 did demonstrate reduced area under the curve in the cinnamon trial (* indicates P<0.05). No differences within the control trial were found (P>0.05). Data represents mean ± S.E.M.
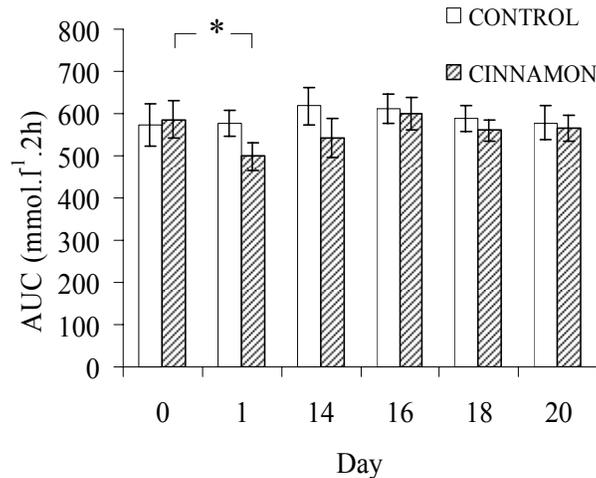
6.4.2 Serum insulin responses to OGTT during each intervention

Figures 6.4 and 6.5 illustrate the insulin responses and AUC during OGTT, respectively. No significant interactions were present in the OGTT raw insulin profile data (intervention*time – P=0.08, day*time – P=0.10, intervention*day – P=0.07, and intervention*day*time – P=0.12). Analysis of AUC data revealed a day*intervention interaction (P<0.05), where AUC at day 14 in the cinnamon trial was significantly reduced compared to day 0 (-27.1 ± 6.2%; Figure 5.5, P<0.05). AUC values at day 14 were also lower in the cinnamon trial than in the control trial (-22.0 ± 10.4%; figure 6.5, P<0.05). No significant differences were found between trials for fasting or 2 hour OGTT insulin data (P>0.05).

Figure 6.4 - Insulin concentration profiles following OGTT during each intervention period.

OGTTs were undertaken at days 0, 1, 14, 16, 18, and 20 during the intervention period. Bold lines represent the control trial, where 3 g of placebo was administered each day. Dashed lines represent the cinnamon trial, where 3 g of cinnamon spice was administered each day until day 14, from when 3 g of placebo where given until the end of the 20 day intervention. The insert graph on each day represents total area under the insulin curve for each OGTT. Insulin AUC was decreased at day 14 in the cinnamon intervention compared to control. * represents significant differences at $P<0.05$. Data represents mean ± S.E.M.
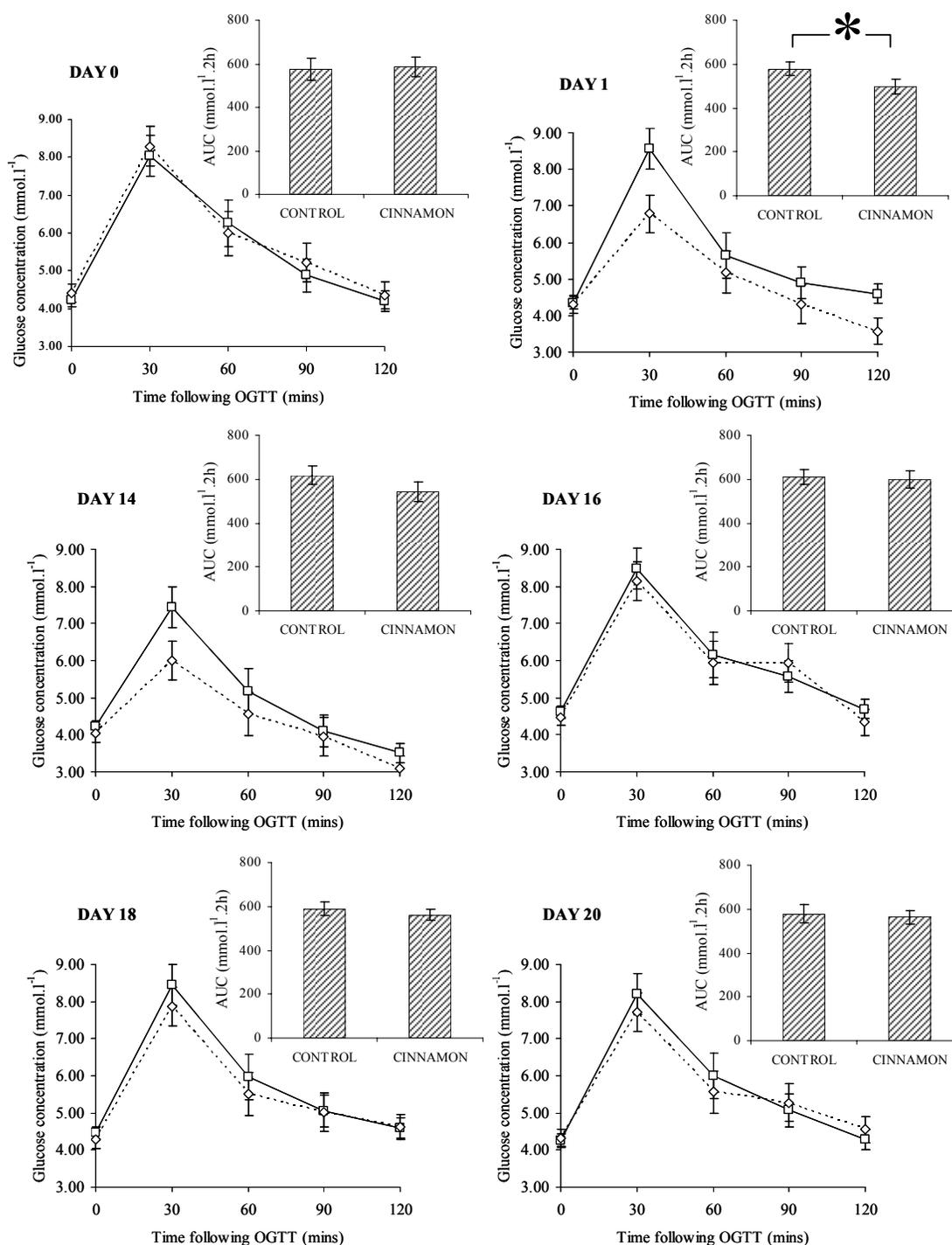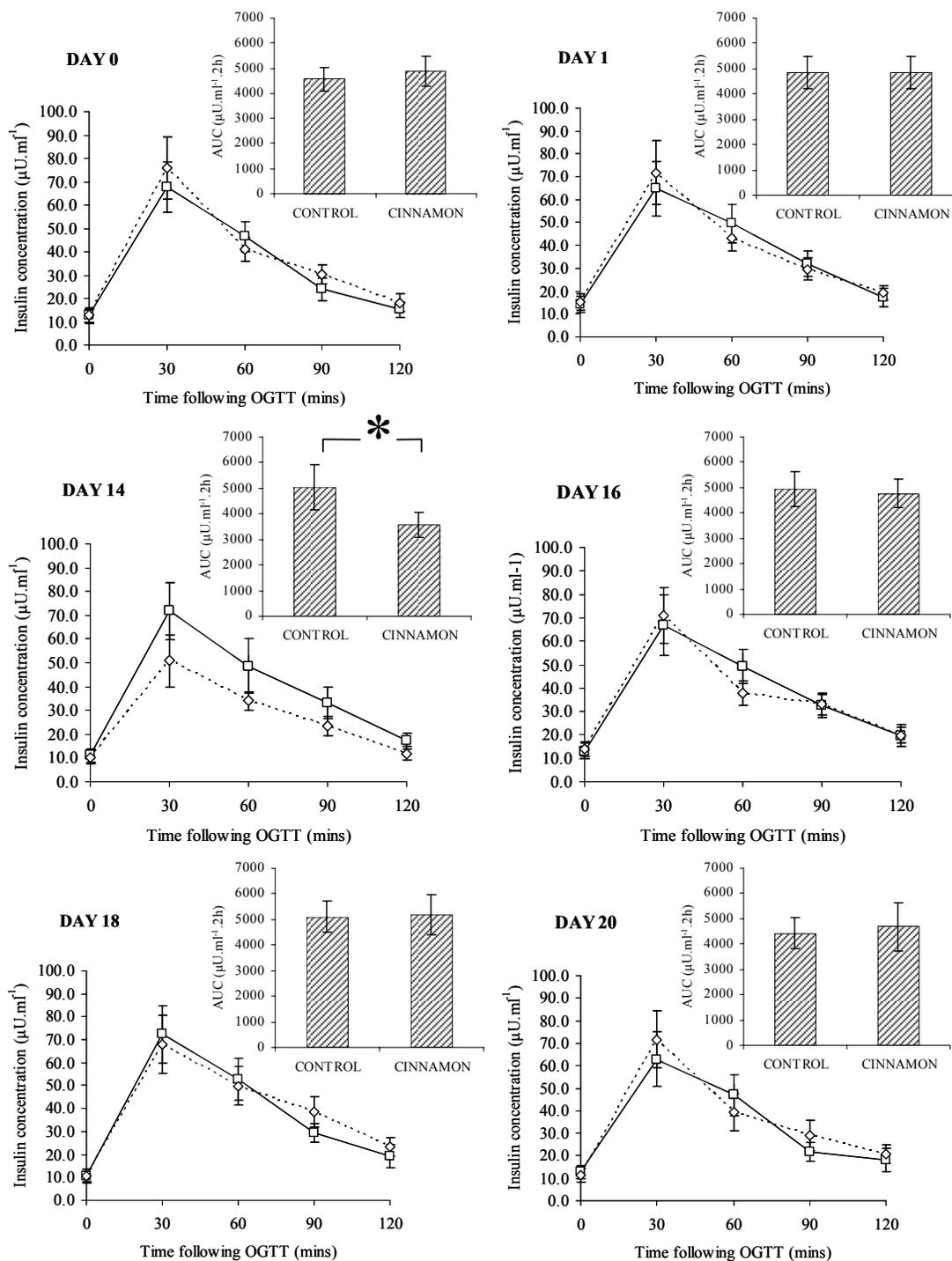
Figure 6.5 – Area under the serum insulin response curve to OGTT on each study day

OGTTs were undertaken at days 0, 1, 14, 16, 18, and 20. CONTROL (white bars) represents 3 g of placebo administered each day. CINNAMON (shaded bars) represents 3 g of cinnamon administered each day until day 14. Analysis of area under the insulin curve following OGTT revealed an intervention*day interaction ($P<0.05$). Further analysis indicated that measures at day 14 in the cinnamon trial were significantly lower than baseline (* indicates $P<0.05$) and all other days ($P<0.05$), and also that the AUC data at day 14 in the cinnamon trial was significantly lower than in the control trial (# indicates $P<0.05$). Data represents mean ± S.E.M.
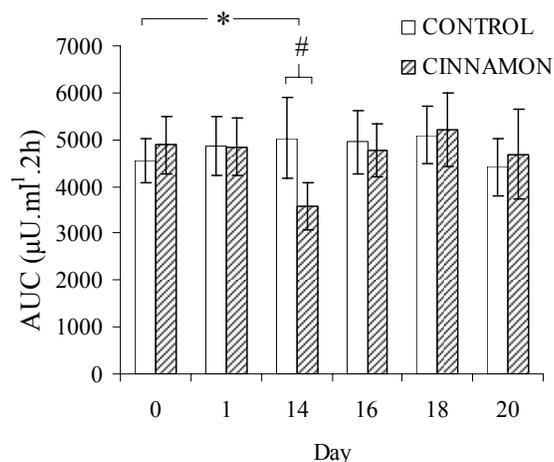
6.4.3 Changes in insulin sensitivity

Figure 6.6 illustrates insulin sensitivity data derived from $ISI_{OGTT}^{Matsuda}$. Analysis showed a day*intervention interaction ($P<0.05$). Further examination revealed that the ISI at day 14 was significantly greater than at day 0 in the cinnamon trial ($P<0.05$), and that measures on day 14 of the cinnamon trial were greater than all days in control trial ($P<0.05$).

Figure 6.6 – Changes in insulin sensitivity during each intervention period.

OGTTs were undertaken at days 0, 1, 14, 16, 18, and 20. The trials follow the same legend as previous figures. The ISI_{OGTT} index derived from Matsuda *et al* (1999) [219] revealed a day*intervention interaction ($P<0.05$) where insulin sensitivity at day 14 was increased compared to day 0 in the cinnamon trial (* indicates $P<0.05$), as well as being significantly greater than all days in the control trial (# indicates $P<0.05$). Data represents mean ± S.E.M.
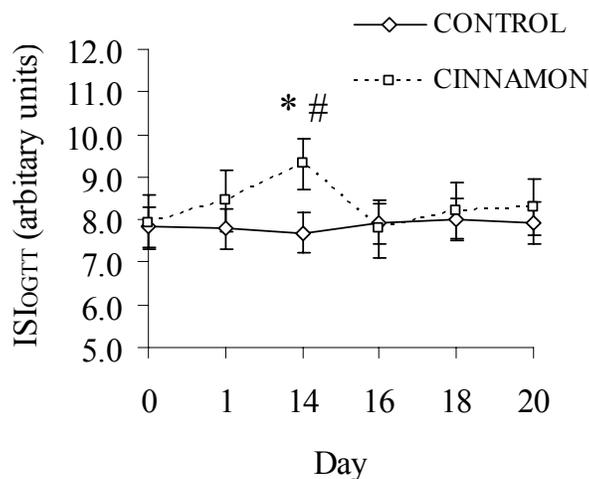
6.5 Discussion

Following a 3 g.day^{-1} supplementation for 14 days, insulin responses to OGTT were reduced (-27%), and insulin sensitivity was improved in inactive but otherwise healthy individuals. However, these data do not show any persistent effects of long term cinnamon supplementation on insulin sensitivity following removal of the cinnamon stimulus. This study also confirms our findings from the previous chapter that an acute bolus of cinnamon spice can reduce glucose responses to OGTT performed 12 hours later (-13%). Although comparisons should be treated with caution, because the two cinnamon chapters used different subjects, the similar drop in the glucose AUC data from ingesting 5 g and 3 g of cinnamon the night before (-10% and -13%, respectively) may suggest that doses greater than 3 g do not provide greater benefit.

This study was perhaps underpowered to detect significant interactions between time, day and trial in raw glucose and insulin concentrations following OGTT. Power calculations, with power set at 0.8 and alpha at 0.05, reveal that based on the effect size in this study, investigating twenty subjects should be sufficient to detect statistically significant changes in a future experiment. This suggests that, with more subjects, significant differences in glucose responses at day 14 may arise, and therefore a more powerful study design may increase the potential value of cinnamon as a therapeutic. Nevertheless significant reductions in insulin AUC data and improvements in insulin sensitivity were detected after 14 days of cinnamon supplementation; illustrating that prolonged cinnamon supplementation may be useful for improving insulin sensitivity. However, these findings should not be directly extrapolated to obese/T2DM populations as the data was obtained using healthy individuals. Despite this our volunteers, although lean (BMI = 24 kg.m^{-2}), did undertake less than the recommended physical activity guidelines [365]. Inactivity is a cardiovascular disease risk factor [200] and a key factor in determining progression along the insulin sensitivity continuum [192,366,367].

The literature investigating long term cinnamon supplementation indicates increased glucose utilisation and increased activity of insulin signalling intermediates in mice [246] and rats [244], reversal of fructose-induced insulin resistance in rats [243], and improved fasting glycaemia and lipids

in humans [238,239]. A recent placebo-controlled study by Vanschoonbeek *et al* (2006) showed no effect of a 1.5 g.day^{-1} cinnamon intervention in postmenopausal T2DM patients [240]. This had a superior study design to the studies of Khan *et al* (2003) and Mang *et al* (2006) with strict pharmaceutical, dietary and exercise restrictions, but used only female subjects, and a relatively low dose, which might possibly account for the null finding [240]. Our data indicates improved insulin sensitivity in a healthy cohort but with no changes in fasting glycaemia. Mang *et al* (2006) demonstrated that large improvements in FPG are only likely in cases of very poor glycaemic control [239]. Our subjects' fasting glycaemia was normal, and therefore improvements would be unlikely. The presence of a persistent effect of cinnamon on glycaemic control seen in Khan's data is useful with regards to cost-benefit analysis, and patient compliance, such that constantly ongoing supplementation is not necessary, and failure to take a dose does not reverse all previous benefits. However, this current study fails to show any persistent effects on insulin sensitivity after stopping cinnamon supplementation. Furthermore, the OGTT glucose AUC data suggests that the effects of cinnamon wear off with continuous supplementation. Insulin AUC data however, was decreased by the accumulative effects of 14 days cinnamon feeding, yet these effects were quickly lost within 2 days of ceasing cinnamon intake. It appears that the effects on postprandial glucose responses are rapid, whereas there is a delayed effect on insulin responses and insulin sensitivity. These mechanisms warrant further attention by investigating glucose uptake at the cellular level, intracellular insulin signalling cascades and transcriptional and translational regulation of genes and proteins involved in glucose metabolism. Imparl-Radosevich *et al* (1998) showed increased insulin receptor autophosphorylation and reduced glycogen synthase kinase-3β (GSK-3β) activity with cinnamon in adipocytes [241], and Qin *et al* (2005) has shown cinnamon-induced increases in skeletal muscle IRS-1 tyrosine phosphorylation and PI3K association in rats [244]. Another study by Qin *et al* (2004) demonstrated that endothelial nitric oxide synthase (eNOS) inhibition prevents the effects of cinnamon in insulin resistant rats [243], suggesting a mechanism involving nitric oxide (NO), a compound implicated in insulin resistance [368]. Cinnamon may activate similar mechanisms in humans, but further work is required.

These findings indicate that in a lean but sedentary male population, long term cinnamon supplementation can improve insulin sensitivity but that the effects are rapidly reversed in the post-intervention period. Despite the strong *in vitro* and *in vivo* animal evidence, the rather conflicting human findings confuse the therapeutic value of cinnamon, particularly when compared to the known impact of pharmaceutical, dietary and exercise interventions on insulin sensitivity and diabetes prognosis [191]. However, the increasing prevalence of obesity and diabetes suggests alternative therapeutic avenues should be explored. Further investigation of the long term effects of cinnamon on insulin sensitivity and intramuscular insulin signalling pathways in humans is required.

7. The effects of feeding frequency on insulin and ghrelin responses

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Co-authors: Edward Chambers, Asker E. Jeukendrup, Andrew A. Toogood, Andrew K. Blannin

7.1 Abstract

Ghrelin, a nutrient-responsive gastrointestinal hormone, has been suggested to be involved in satiety regulation, adipogenesis and fuel oxidation rates. Recent work by Sugino *et al* (2002) demonstrates reduced postprandial ghrelin suppression with increased meal frequency in sheep, but no other variables were measured, nor is similar work available in humans. In this study five healthy male volunteers (aged 24 ± 2 years, body mass 75.7 ± 3.2 kg, and BMI 23.8 ± 0.8 kg.m^{-2}; mean $\pm$ S.E.M.) underwent three 8 hour feeding regimes: fasting (FAST), low-frequency (two meals) ingestion (LOFREQ$_{MEAL}$), and high-frequency (twelve meals) ingestion (HIFREQ$_{MEAL}$). Meals were equicaloric within trials, and total energy intake was equal between feeding trials. Meals consisted of 64% carbohydrate, 23% fat and 13% protein. Total area under the curve for insulin and ghrelin responses did not differ between trials (one-way ANOVA, $P>0.05$). However, the negative relationship between increases in serum insulin and decreases in plasma ghrelin demonstrated during the FAST and LOFREQ$_{MEAL}$ trials ($P<0.05$) was not seen during HIFREQ$_{MEAL}$ (time-series analysis, $P>0.05$). This is the first study to investigate feeding frequency on insulin and ghrelin responses and their relationships in humans.

7.2 Introduction

The recent escalating obesity trend in humans is due to an imbalance between energy intake and energy expenditure [1,2]. Energy intake is influenced by the effect of food's energy density, total caloric content and feeding frequency and the extent to which these alter satiety. Of these factors, feeding frequency has received least attention. Human epidemiological evidence indicates increasing trends in recent years of dietary snacking and increased meal frequency [267,268], and such studies show positive relationships between snacking and increased energy intake and BMI [268], illustrating the potential importance of investigating feeding frequency.

Several gastrointestinal peptides are involved in metabolic processes, and are dysregulated in states of metabolic disease [323,369,370]. One of these peptides, ghrelin, an orexigenic hormone released by the stomach prior to feeding, has also been implicated in the control of fuel metabolism, appetite, and pancreatic insulin release, factors quite central to the onset of metabolic disease [247]. In 2002, Sugino *et al* reported the effects of meal frequency on ghrelin responses in sheep, investigating 2 meals versus 4 meals versus *ad libitum* feeding [282]. It appeared that increased meal frequency resulted in decreased ghrelin responses during the course of the day [282]. However energy intake was not controlled between the different feeding regimes, with 200% of daily energy requirements being ingested in the *ad libitum* trial compared with 120% in the other trials, thus complicating the interpretation of their findings [282].

Various eu- and hyperglycaemic clamp studies in humans demonstrate that postprandial ghrelin suppression appears to be dependent on insulin release [256,258,371], and in insulin-withdrawn type 1 diabetics, postprandial ghrelin suppression is not apparent [256]. In insulin resistant states, such as obesity and T2DM where fasting ghrelin is downregulated [260], the magnitude of the postprandial ghrelin suppression is also smaller [372]. This is surprising given the hyperinsulinaemia that prevails in such states, and suggests that insulin sensitivity may be important to ghrelin responses. Sugino *et al* (2002) did not report insulin concentrations, and the effects of feeding frequency on concomitant insulin and ghrelin responses are not currently known. Therefore, this study investigated the effects of meal frequency during equicaloric feeding regimes upon the responses of insulin and ghrelin.

7.3 Method and materials

Following ethical approval from The School of Sport and Exercise Sciences Safety and Ethics Subcommittee, five active but otherwise untrained male volunteers, aged 24 ± 2 years, body mass 75.7 ± 3.2 kg, with BMI 23.8 ± 0.8 kg.m^{-2} (mean ± S.E.M.), were recruited from the local community. All volunteers were assessed by a general health questionnaire and provided informed written consent prior to commencing the study. Participants had to make a total of three visits to the laboratory each separated by at least five days. A dietary record was taken for the day preceding the first test, and volunteers were instructed to refrain from consuming alcohol, caffeine, and from any exercise for twenty-four hours prior to each trial. The dietary record was for the purposes of diet replication before each trial. Participants were studied in a reclined position for the duration of all trials (though habitual activity for toilet visits was permitted), and each completed three 8 hour dietary interventions in a randomised cross-over design: a fasting control trial (FAST), a low-frequency meal trial (LOFREQ$_{MEAL}$), and a high-frequency meal trial (HIFREQ$_{MEAL}$). The meals provided in the trials had a macronutrient composition intended to replicate typical foods eaten during the day: 64% carbohydrate (1.93 g.kg^{-1}.LOFREQ$_{MEAL}$$^{-1}$, 0.32 g.kg^{-1}.HIFREQ$_{MEAL}$$^{-1}$), 23% fat (0.30 g.kg^{-1}.LOFREQ$_{MEAL}$$^{-1}$, 0.05 g.kg^{-1}.HIFREQ$_{MEAL}$$^{-1}$), and 13% protein (0.42 g.kg^{-1}.LOFREQ$_{MEAL}$$^{-1}$, 0.07 g.kg^{-1}.HIFREQ$_{MEAL}$$^{-1}$), and provided approximately 66% of the daily recommended intake (1667 kcal of recommended 2500 kcal for a 70 kg man) [48], the final 33% being an evening meal of the volunteers choice after completion of each trial. Trial meals consisted of white bread, Nutrigrain bars, apples, and cheddar cheese. The total energy intake was identical in each trial; however the number of meals was varied as explained below.

7.3.1 FAST

Volunteers arrived in the laboratory at 0800 hours following a 12 hour overnight fast, an intravenous cannula (BD Venflon, Oxford, UK) was inserted into an antecubital vein and a fasting blood sample was taken. Venous blood samples (3 ml) were drawn from the intravenous line every 10 min until t = 8 hours to be later analysed for insulin and ghrelin concentrations. (See General Methods section for details of blood sample collection, storage and analysis). During the trial the

cannula was kept patent with 3 ml flushes of 0.9% $NaCl_{(aq)}$ isotonic saline (Baxter Healthcare, Northampton, UK) following each blood-letting. Each participant was given 14.3 ml.kg^{-1} body mass of water to consume *ad libitum* throughout the trial. This volume of water corresponded to 1 litre per 70 kg of body mass, which was considered appropriate for the intervention. At the end of the trial the cannula was removed and the individual was sent home and instructed to return to the laboratory in 5-7 days, fasted, having followed their dietary intake record.

7.3.2 LOFREQ$_{MEAL}$

The experimental protocol for this trial was identical to the FAST trial, except at t = 0 and 4 hours, participants consumed a 4.95 g.kg^{-1} body mass mixed meal (composition described above). Subjects were given 15 min to ingest each meal. As well as the controlled intake of food, subjects were again asked to consume 14.3 ml.kg^{-1} body mass of water *ad libitum* throughout the trial.

7.3.3 HIFREQ$_{MEAL}$

This trial was identical to the FAST and to LOFREQ$_{MEAL}$ trials except that 0.825 g.kg^{-1} body mass mixed meals were administered every 40 mins throughout the trial commencing at t = 0 mins, making a total of twelve meals. Participants were given 5 min to finish their meals. Again, the same volume of water was provided for ingestion *ad libitum*.

In the feeding frequency literature there is no consistency in the number or composition of meals administered. The only available one-day intervention by Bertelsen *et al* (1993) used an 8 hour period, so that was replicated here [269]. The choice of 2 and 12 meals was made in relation to the range of meal frequencies in the available publications [269-275]: 1 to 3 (low-frequency) versus 6 to 17 (high-frequency).

7.3.4 Statistical Analysis

Data are expressed as mean ± S.E.M. and significant differences were accepted at P<0.05. Raw insulin and ghrelin data were tested for normality and analysed by two-way (trial*time) repeated measures ANOVA. Main effects were analysed using Bonferroni *post hoc* tests. Raw insulin and

ghrelin data were converted to area under the curve (AUC) values by the trapezoidal method and trials were compared using one-way ANOVA. These analyses were carried out with SPSS for Windows 12.0.1 (SPSS Inc, Chicago, US). Due to the influence of feeding on preprandial ghrelin concentrations documented in previous publications, i.e. preprandial rises and postprandial falls in plasma ghrelin concentration in proportion to caloric intake [250,251,373], in this study fasting ghrelin concentrations in each trial were also compared using one-way ANOVA to assess the effect the presentation of different sized meals in LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ may be having.

In order to analyse the ghrelin response patterns in each of the three trials, and highlight the differences otherwise not detected by ANOVA, a pulse analysis was performed using Cluster 8, a sub-program of Pulse_XP software (Pulse_XP, Virginia, US), to identify peaks and nadirs in the ghrelin data, as described by Veldhuis *et al* (1986) [374]. Cluster 8 is a statistically-based peak detection algorithm which locates significant increases and decreases (clusters) of hormone concentrations within a data series and has been used in several hundred endocrine publications. Cluster 8 does not assess hormone-hormone interaction but determines whether the data series of a single hormone represents pulsatile secretion rather than assay noise. This analysis then provides information about the pulse characteristics: number of peaks, number of nadirs, peak duration, area under the peak, etc, so that one can study changes in pulse characteristics under different conditions [374]. Such analysis is carried out using pooled t-testing on the experimental replicates. A peak is defined as a significant increase in concentration followed by a significant decrease ($P<0.05$). A nadir is defined as a significant decrease followed by a significant increase ($P<0.05$). Pulse analysis and peak detection is an important phenomenon in endocrine systems as it is thought that endocrine glands signal to their target tissues via episodic hormonal secretion [374-376]. Pulse_XP has been used in several hundred publications to investigate endocrine pulsatility. Pulse analysis of the insulin data was not possible due to the irregular sampling frequency (see Figure 7.1), upon which the software cannot work.

In order to examine the relationships between insulin and ghrelin responses, a time-series analysis was applied to the data similar to that described by Box *et al* (1970) and Carroll (1977) [377,378]. For each subject, Pearson correlation coefficients (r) were calculated between insulin and

ghrelin concentrations synchronised in time. Each subject's r value was converted to a *z*-score (a statistical normality score), the mean *z*-score for all subjects was calculated, this mean *z*-score was converted back to an r value (the mean correlation coefficient for all subjects), and the statistical significance (P) for the correlation was read from critical values tables. These calculations were performed in each of the three trials. Such analysis was carried out because previous publications have shown that changes in insulin concentrations appear to regulate changes in ghrelin concentrations [254-258,371]. In addition, to investigate any possible time delay between such relationships, the same calculations were made for correlations between insulin values and the ghrelin values measured 10, 20, 30, 40, 50, 60, and 70 mins later. This was carried out because Cummings *et al* (2001) showed that the postprandial fall in ghrelin appears to be delayed after the rise in insulin [250]. Furthermore, in other examples of endocrine system synergy (e.g. ghrelin and growth hormone [379]) there is often a time-delay between such responses. The 70 min period was chosen in retrospect as, by examining the ghrelin/insulin profiles, a 70 min delay captured the largest postprandial changes in insulin and ghrelin. Note that due to the irregular sampling of insulin (see Figure 7.1), the correlations between insulin and ghrelin were performed between 15 data points only, at t = 0, 20, 40, 60, 80, 120, 180, 240, 260, 280, 300, 320, 360, 420, and 480 min. This reduces the power of this analysis compared to a more frequent insulin sampling rate where more data points would be available to investigate these correlations.

7.4 Results

7.4.1 Serum insulin responses

Figure 7.1 shows serum insulin responses during the three trials. Two-way ANOVA revealed a main effect of time (P<0.01), trial (P<0.01) and a time*trial interaction (P<0.01). Peak insulin concentrations of the two meals in LOFREQ$_{MEAL}$ were different from one another (83.0 ± 7.6 versus 57.1 ± 7.3 $\mu U.ml^{-1}$; P<0.05). During the HIFREQ$_{MEAL}$ trial serum insulin concentrations reached a plateau (mean concentration, 33.9 ± 7.7 $\mu U.ml^{-1}$) during the 8 hour intervention period. During the FAST trial insulin steadily decreased over time from 15.6 ± 6.5 to 12.7 ± 6.9 $\mu U.ml^{-1}$ (P>0.05). Responses were similar in all subjects.

Figure 7.1 – Serum insulin responses to mixed meal ingestion.

The three trials, FAST, HIFREQ$_{MEAL}$, and LOFREQ$_{MEAL}$, represent 8 hour intervention periods where no meals, 12 meals, or 2 meals were ingested, respectively. Meals within trials were equicaloric, and total caloric consumption between HI- and LOFREQ$_{MEAL}$ trials was identical. Data differed from baseline (t = 0 mins) at many time points: all except t = 20 mins in HIFREQ$_{MEAL}$; and all except t = 180, 240, 260, and 480 mins in LOFREQ$_{MEAL}$ (# or * indicates P<0.05; ## indicates P<0.01). No differences were found during FAST (P>0.05). A main effect of trial and further post hoc analysis indicated that LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ were significantly different to FAST (P<0.05) but not to one another (P=0.13). Data represents mean ± S.E.M.
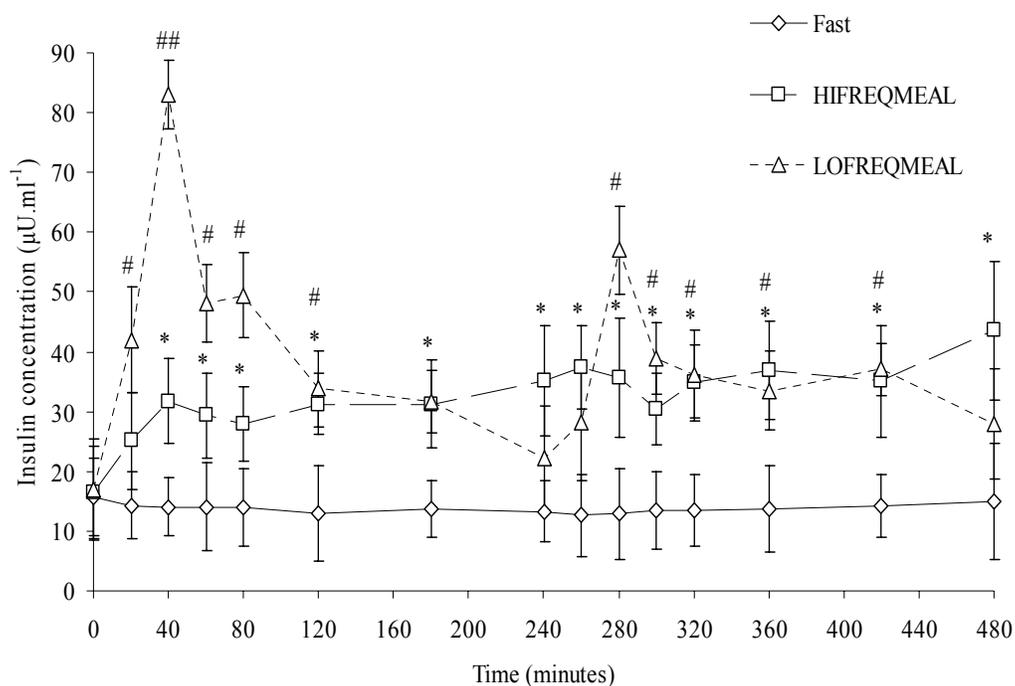
Total area under the insulin response curves for the 8 hour periods were increased in LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ trials by 172 ± 37% and 142 ± 18% respectively when compared with FAST (P<0.05), but no differences were found between the two meal trials (P=0.18, Figure 7.2).

Figure 7.2 – Area under the serum insulin response curves following meal ingestion.
The three trials depicted on the x-axis are described in brief in Figure 7.1. Total insulin responses (AUC) for the 8 hour period were greater in HIFREQ$_{MEAL}$ and LOFREQ$_{MEAL}$ than in FAST (* indicates $P<0.05$) but not different from one another ($P=0.18$). Data represents mean ± S.E.M.

7.4.2 Plasma ghrelin responses

Figure 7.3 illustrates the plasma ghrelin concentrations during each intervention. Analysis revealed a main effect of time ($P<0.01$), trial ($P<0.01$) and a time*trial interaction ($P<0.01$). Postprandial ghrelin nadirs between the two meals in LOFREQ$_{MEAL}$ were not different (205 ± 10 versus 200 ± 9 pmol.l^{-1}; $P>0.05$). During FAST ghrelin steadily increased with time from 253 ± 9 to 315 ± 9 pmol.l^{-1}, reaching significance at several time points ($P<0.05$) (see Figure 7.3). No differences were found between fasting preprandial ghrelin concentrations ($P>0.05$), although the comparison between fasting ghrelin in LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ trials approached statistical significance ($P=0.08$). Responses were similar in all subjects. The three trials are represented as separate graphs in Figure 7.3 to aid clarity.

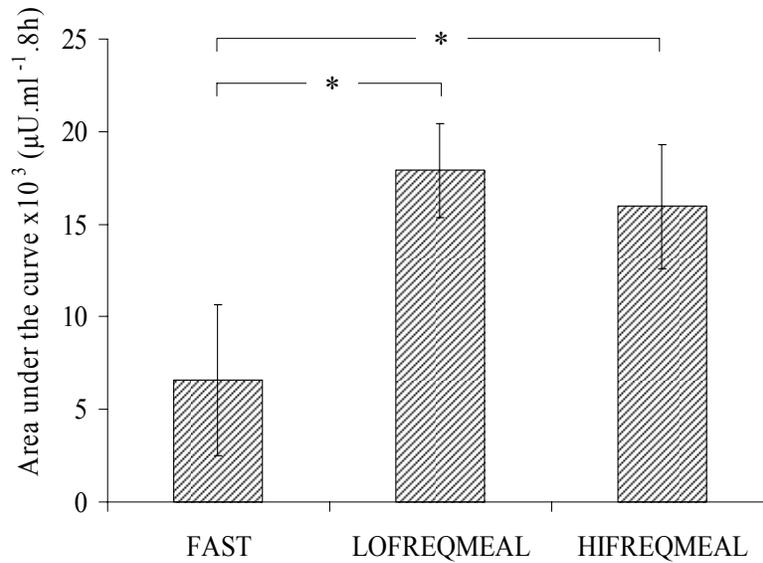Figure 7.3 – Plasma ghrelin responses to mixed-meal ingestion

The three trials, FAST, HIFREQ$_{MEAL}$, and LOFREQ$_{MEAL}$, represent 8 hour intervention periods where no meals, 12 meals, or 2 meals were ingested, respectively, as indicated by bold arrows. Data differed from baseline (t = 0 mins) at many time points in FAST and LOFREQ$_{MEAL}$ (* indicates $P<0.05$; ** indicates $P<0.01$), no change from baseline was found in HIFREQ$_{MEAL}$ ($P>0.05$). A main effect of trial and further post hoc analysis revealed that LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ were different from FAST ($P<0.01$) but not different from one another ($P>0.05$). No differences were found between fasting (preprandial) ghrelin concentrations in each trial ($P>0.05$), however LOFREQ$_{MEAL}$ versus HIFREQ$_{MEAL}$ approached significance ($P=0.08$). Pulse analysis revealed different numbers of significant peaks and nadirs between trials, illustrating the difference in ghrelin secretion patterns during the different feeding interventions. The flat-line inserts are arbitrary representations of the significant peaks and nadirs during the trial. Data are expressed as mean ± S.E.M.
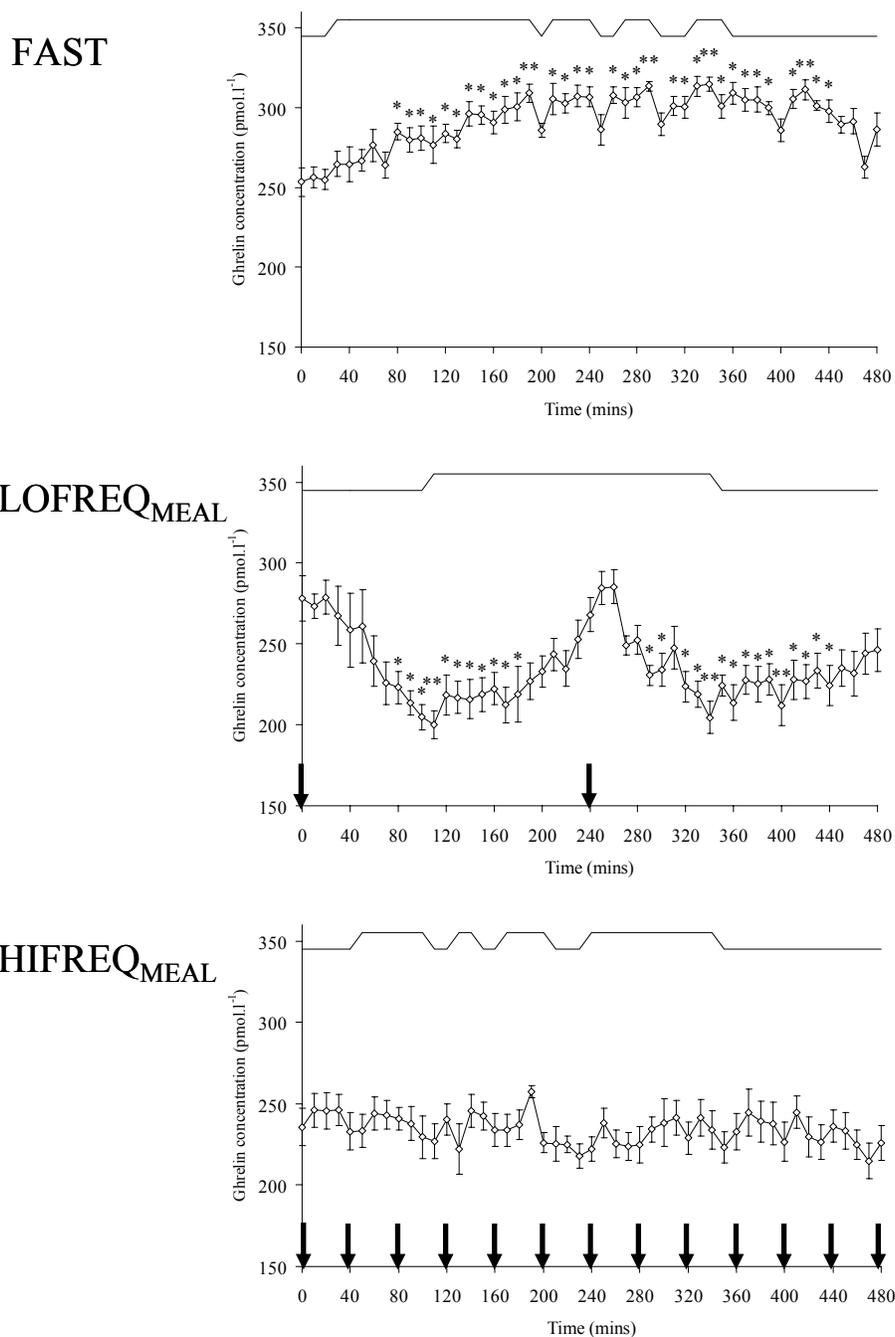
Compared with FAST, total area under the ghrelin response curves for the 8 hour intervention periods were decreased by $19.4 \pm 6.4\%$ and $20.2 \pm 4.5\%$ during $LOFREQ_{MEAL}$ and $HIFREQ_{MEAL}$ trials respectively (P<0.05), but no differences were found between the two meal trials (P>0.05, Figure 7.4).

Figure 7.4 – Area under the plasma ghrelin response curves following meal ingestion.
The three trials depicted on the x-axis are described in brief in Figure 7.3. Total ghrelin responses for the 8 hour period were lower in $HIFREQ_{MEAL}$ and $LOFREQ_{MEAL}$ than FAST (* indicates P<0.05) but not different from one another (P>0.05). Data represents mean $\pm$ S.E.M.
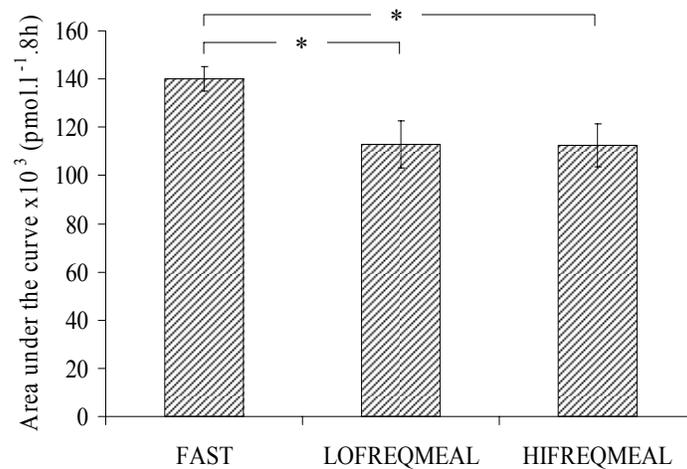
7.4.3 Ghrelin pulse analysis

Figure 7.3 illustrates the significant peaks and nadirs from the pulse analysis (illustrated by the flat line inserts), showing that ghrelin exhibited four peaks and four nadirs during FAST, one peak and two nadirs during $LOFREQ_{MEAL}$ and four peaks and five nadirs during $HIFREQ_{MEAL}$. Table 7.1 shows further information generated by the pulse analysis.

Table 7.1 – Ghrelin pulse analysis

FAST, LOFREQ$_{MEAL}$, and HIFREQ$_{MEAL}$ represent the same trials as described in Figure 7.3. Ghrelin responses between LOFREQ$_{MEAL}$ and HIFREQ$_{MEAL}$ were distinct as depicted by the different number of significant peaks and nadirs, and different peak and nadir characteristics. Matching superscript letters in each row represent statistically significant differences between trials ([a], [d] and [f] represents P<0.001; [b] and [e] represents P<0.01; [c] and [g] represents P<0.05. These comparisons demonstrate significant differences in ghrelin pulsatility between the different feeding trials. Data represents mean ± S.E.M.

Ghrelin pulse characteristics	FAST	LOFREQ$_{MEAL}$	HIFREQ$_{MEAL}$
Number of peaks	4	1	4
Peak width (min)	70.0 ± 66.8 [e]	230 [a e]	55.0 ± 38.7 [a]
Peak height (pmol/l)	311 ± 3.51 [d f]	285 [a d]	247 ± 7.13 [a f]
Peak area (pmol/l.min)	1455 ± 2169 [e]	8331 [a e]	496 ± 420 [a]
Number of nadirs	4	2	5
Nadir width (min)	5.00 ± 17.3 [e g]	45.0 ± 35.3 [c e]	20.0 ± 7.0 [c g]
Nadir level (pmol/l)	287 ± 1.86 [d f]	199 ± 18.5 [b d]	226 ± 7.0 [b f]

7.4.4 Insulin-ghrelin interactions

Figure 7.5 indicates the temporal relationship between the two hormones during the three trials as depicted by a time-series analysis. In the FAST trial there was a negative correlation between insulin and ghrelin concentrations (P<0.05). During LOFREQ$_{MEAL}$, there was no direct correlation (simultaneous insulin and ghrelin values, see insulin leads ghrelin by 0 mins on Figure 7.5) between insulin and ghrelin, however there was a negative correlation when insulin led ghrelin responses by 20, 40 and 50 mins (P<0.05). During HIFREQ$_{MEAL}$ no significant relationship existed between insulin and ghrelin responses (all time delays P>0.05).

Figure 7.5 – Relationships between insulin and ghrelin responses.

The three charts represent the time-series analysis in each trial. Correlation coefficients were calculated for relationships between insulin and ghrelin responses over the 8 hour period. These were calculated when the two variables were synchronised in time (insulin correlated with simultaneous ghrelin value, see "insulin leads ghrelin by 0 mins"), and relationships were also assessed between the insulin concentrations and the ghrelin concentrations observed 10, 20, 30, 40, 50, 60, and 70 mins later. The insert graph on each chart illustrates the trends in ghrelin (bold line, $pmol.l^{-1}$) and insulin (dotted line, $\mu U.ml^{-1}$) concentrations in the corresponding trial. During FAST there was a negative relationship between insulin and ghrelin (* represents $P<0.05$). During LOFREQ$_{MEAL}$ there was a negative correlation between insulin and ghrelin responses, reaching significance when insulin led ghrelin by 20, 40 and 50 mins (* indicates $P<0.05$). During HIFREQ$_{MEAL}$ there were no significant associations between insulin and ghrelin ($P>0.05$).
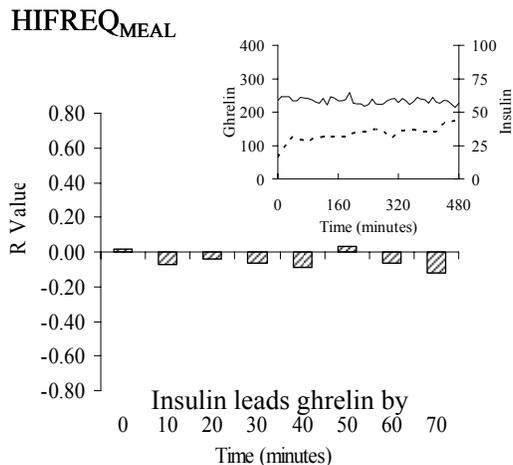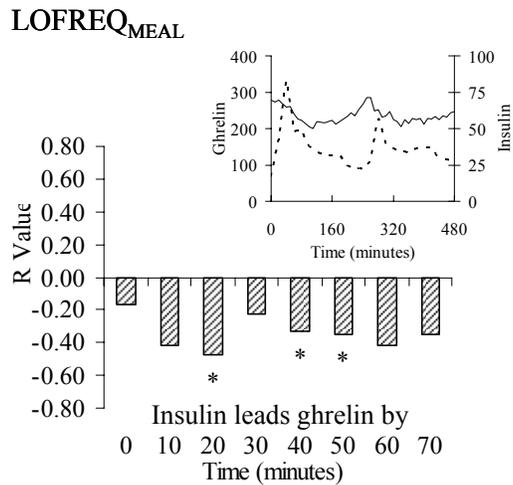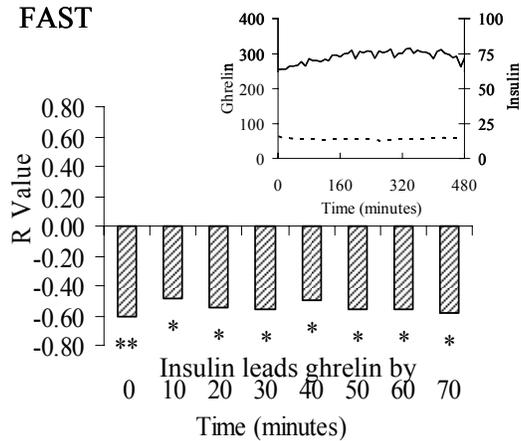
7.5 Discussion

This data demonstrates that whilst the insulin and ghrelin responses to different feeding frequency regimes are quite different (Figures 7.1, 7.3, Table 7.1), such feeding patterns have no effect on the total (AUC) day long responses when caloric load is controlled (Figures 7.2 and 7.4). During a period of prolonged fasting, it was clear that insulin and ghrelin concentrations have an inverse relationship (Figure 7.5 FAST). This study also illustrates that during a period of low-frequency meal ingestion serum insulin concentrations are inversely related to plasma ghrelin concentrations (Figure 7.5 LOFREQ$_{MEAL}$), but that when the same total caloric load is consumed in smaller individual meals across the day, this insulin-ghrelin relationship is not observed (Figure 7.5 HIFREQ$_{MEAL}$).

The time-series analysis of the two hormones was carried out to investigate the effects of meal frequency on the insulin-ghrelin association reported in the literature; nutrient-induced rises in insulin may be required for postprandial ghrelin suppression [256,258,371]. Besides direct correlations, correlations with time delays of up to 70 mins between insulin and ghrelin values were also analysed to determine whether changes in insulin induce a delayed response in ghrelin. During a period of fasting, our data shows that insulin falls slightly and ghrelin rises slightly with time, causing an inverse correlation between the two variables (Figure 7.5, FAST). During a period of low-frequency feeding (2 meals in 8 hours), the results illustrate that although there is an insulin-ghrelin relationship, there is a delay (of approximately 20 minutes) between responses of the two hormones (Figure 7.5, LOFREQ$_{MEAL}$). No significant correlation exists when no time-delay between the hormone concentrations is applied (i.e. correlation of insulin with simultaneous ghrelin values, see insulin leads ghrelin by 0 mins, LOFREQ$_{MEAL}$ on Figure 7.5). This illustrates that nutrient-induced rises in insulin may cause postprandial ghrelin suppression with a 20 min delay. Finally, during a period of high-frequency meal ingestion (12 meals in 8 hours), either the hormone-hormone interaction is lost or insulin does not fluctuate sufficiently to influence ghrelin. However, during FAST where changes in insulin concentrations are small (Figure 7.1) there is a significant relationship between the two hormones (Figure 7.5), illustrating that only small changes in insulin are required for effects on

ghrelin, thus it is more likely that high-frequency meal ingestion actually reduces the insulin-ghrelin interaction. Whilst these are novel findings, these analyses are only correlations from which exact causality cannot be determined, and, due to the irregular insulin sampling frequency, the loss of power associated with fewer time points being analysed means that such interpretations must be made with caution and may indeed explain the loss of significant hormonal relationships in HIFREQ$_{MEAL}$. An improved study design would be to sample insulin and ghrelin regularly and frequently at identical intervals. Despite this, several studies have documented that insulin is required for postprandial ghrelin suppression [254-259,371]. Additionally, Anderwald *et al* (2003) showed reduced insulin-stimulated ghrelin suppression in T2DM patients, illustrating the importance of insulin in regulating ghrelin [259]. The exact importance of an insulin-ghrelin relationship is unclear, but with insulin's known involvement in metabolic flux and ghrelin's implications with orexigenic neuropeptide networks [380], mitochondrial lipid metabolism gene expression [262] and fuel substrate selection [265], such a relationship may provide a useful avenue for metabolic research. Our findings indicate that high-frequency feeding may be detrimental to insulin's control of ghrelin responses, a problem seen in T2DM patients [259]. However with only correlation analyses to make this interpretation, suggesting that increased feeding frequency contributes to metabolic disease would be purely speculative and further work would be required to explore this concept.

The expense of ghrelin measurement limited this study to n = 5, however Sugino *et al* (2002) used the same subject number to show that increased feeding frequency decreased total ghrelin (AUC) responses [282]. The findings here show no differences in day-long AUC insulin or ghrelin responses, yet with only five subjects, the analysis was perhaps underpowered. Based on the effect size calculated in this study, a power calculation, with power set at 0.8 and alpha at 0.05, indicated that eight subjects should be sufficient to achieve statistically significant differences between trials in a future study. Several comparisons between the HIFREQ$_{MEAL}$ and LOFREQ$_{MEAL}$ trials approached significance and therefore a further study with a larger cohort may detect an effect. This would be interesting in that increased meal frequency may indeed reduce day-long insulin responses when energy intake is controlled. By examining the baseline data on Figure 7.3, it is also evident that some difference may

exist between fasting ghrelin concentrations prior to meal ingestion. The comparison between HIFREQ$_{MEAL}$ and LOFREQ$_{MEAL}$ again approached significance (P=0.08) and therefore with a larger sample size, it may be that a larger caloric load presented to an individual may cause a larger preprandial ghrelin surge. Preprandial ghrelin surges, triggered by visual or olfactory stimuli, are likely to provide a meal initiation signal that activate orexigenic neuropeptide pathways in the hypothalamus [52,381]. There is evidence illustrating calorie-dependent postprandial ghrelin suppression [251], but a calorie-dependent effect on the preprandial ghrelin surge would be a novel finding. In retrospect, more preprandial measures in the hour before feeding would have allowed greater insight into preprandial ghrelin changes.

The exact importance of the postprandial ghrelin decline is unknown, but again it may be involved in satiety regulation. Recent work implicating ghrelin with adipogenesis and metabolic flux [262,264,265], suggests that this peptide, like insulin, may also be involved with postprandial nutrient storage and oxidation. Changes in typical ghrelin responses in the postprandial period may therefore disrupt such systems. Although this study shows that total ghrelin responses (AUC) are not altered by feeding frequency, the pulse analysis (Figure 7.3, flat-line, and Table 7.1) reveals clear differences in the ghrelin response pattern between trials, and so further research in this area is required to establish the importance of such changes in ghrelin secretion. A loss of insulin-regulated ghrelin fluctuations due to high-frequency feeding may affect ghrelin's control of satiety and metabolic flux, yet this is speculative, and further work is required. Additionally, the data in Table 7.1 confirms findings from Koutkia *et al* (2004) [382] that showed pulsatile ghrelin responses during a fasting period with similar peak characteristics to those found here, yet pulsatility during feeding periods has not been reported in humans before and so our data adds new information to this limited evidence base.

The meals used in this study were of mixed composition (64% carbohydrate, 23% fat and 13% protein) within the WHO macronutrient intake ranges [383], and were provided in equicaloric amounts during the two feeding interventions, controlling total energy intake. In a free living environment, data suggests that snacking is correlated to increased energy intake, and that snacks are generally high sugar or high fat foods [277,384,385]. Our meals derived 13% of total calories from free sugars, and

23% from fats, perhaps not representative of a usual snack. However, the definition of "snack" also causes problems for such investigations. Is a snack a smaller portion of a typical meal taken more frequently throughout the day, or does a snack represent a high-sugar/high-fat food taken between meals in addition to typical meals [386,387]? The two definitions could change both the research design and the subsequent results. Here a snack was chosen to represent a smaller-sized portion of a meal that was eaten more frequently throughout the day. In this approach we were able to isolate the effect of feeding frequency from the combination of factors involved in a free living situation such as feeding frequency and energy density of the snacks consumed. If high-sugar or high-fat snacks were eaten in addition to usual meals resulting in a hypercaloric energy intake it is probable that increased insulin and decreased ghrelin trends would be seen. This is however, speculative, and further work is needed to answer that question.

These data were recorded from lean healthy volunteers, so care must be taken in predicting the outcome in a patient group. The current literature is mixed with regards to the efficacy of increased feeding frequency (or snacking) regimes in causing or treating metabolic anomalies. A number of studies report a positive impact of increased meal frequency on factors such as lipaemia, thermogenesis, and fasting glycaemia [269,275,276], whilst other studies show the opposite [271,277-279], and further data show that no differences exist [272,280,281]. The observation in this study that increased feeding frequency may disrupt the insulin-ghrelin relationship may be relevant to diminished regulation of ghrelin seen in insulin resistance (discussed earlier). This is relevant to the increased snacking habits seen in our society [267,268], however these suggestions require further investigation. This study provides the only such data in humans, and therefore further work is prudent, particularly regarding the long-term effect of meal frequency. Also, given the inconclusive evidence in the literature regarding feeding frequency and its metabolic implications, large randomised controlled trials are required to resolve speculation that the current increases in snacking habits contribute to the epidemic increases in the frequency of obesity and T2DM.

8. The effect of time-delay between exercise and high-fat meal ingestion on postprandial lipaemia

Co-authors: Andrew K. Blannin

8.1 Abstract

Postprandial lipaemia in humans is a useful marker of cardiovascular disease risk. It is well documented that low- to moderate-intensity exercise reduces postprandial lipaemia; however it is not fully understood how long this effect may persist. In this study eight healthy volunteers, aged 21 ± 1 years, body mass 72.3 ± 2.3 kg, and BMI 23.0 ± 0.7 kg.m^{-2} (mean $\pm$ S.E.M.) undertook three 90 minute bouts of moderate intensity exercise (walking at 55% heart rate reserve) either 12, 24, or 48 hours prior to a 6 hour oral fat tolerance test (OFTT; 71 kJ.kg^{-1}; macronutrient composition 1.22 g.kg^{-1} fat, 1.21 g.kg^{-1} carbohydrate, and 0.20 g.kg^{-1} protein). Total triglyceride responses (AUC) to OFTT were attenuated by $24.6 \pm 5.6\%$ in the 12 hour trial ($P<0.01$) and $19.9 \pm 7.7\%$ in the 24 hour trial ($P<0.05$) compared with the 48 hour trial. Peak triglyceride concentrations were also reduced following the 12 hour trial compared with the 48 trial (1113 ± 97.5 vs. 1526 ± 126 µmol.l^{-1} $P<0.01$), yet no differences were seen in fasting triglyceride measures ($P>0.05$). These findings suggest that a moderate-intensity exercise stimulus carried out 12 or 24 hours ahead of a high fat meal exerts a larger effect on postprandial lipaemia than when carried out 48 hours before. These findings may be attributed to the delayed and transient exercise-induced increase in lipoprotein lipase activity or exposure (due to increased capillary perfusion), or due to suppression of hepatic very low density lipoprotein production.

8.2 Introduction

Postprandial lipaemia is a useful marker of cardiovascular disease (CVD) risk [388]. Prolonged presence of triglyceride-rich lipoproteins in the circulation increases the opportunity for lipid exchange with cholesterol-rich lipoproteins, leading to events that promote atherogenesis such as a depletion of high density lipoprotein cholesterol and a decrease in the size of low density lipoproteins [285,389]. Besides this, the presence of triglycerides in the circulation activates inflammatory mechanisms in the endothelium, causing low-grade chronic inflammation, as indicated by increased levels of acute-phase proteins (e.g. CRP, fibrinogen, and PAI-1) which are also associated with elevated atherosclerotic risk [160]. Thus, circulating triglycerides may contribute to the vascular atherosclerotic process if levels are persistently elevated. In addition, as described in Chapter 1, elevated intracellular fatty acid derivatives, which ultimately are derived from circulating triglycerides, can contribute to inflammation and impede the insulin signal causing insulin resistance [100-103,163,164].

A single aerobic exercise bout can attenuate postprandial lipaemia in sedentary [308] and physically active [390] populations. It is also well documented that endurance-trained individuals exhibit decreased plasma triglyceride concentrations in both fasted and postprandial states [391], partly as a result of increased skeletal muscle triglyceride uptake via enhanced activity of heparin-releasable lipoprotein lipase (LPL), the rate limiting enzyme in triglyceride clearance [392,393]. Endurance athletes also possess a large, well vascularised muscle mass and this may increase the availability of endothelial binding sites, in addition to the amount of LPL [391]. Thus, exercise can be a useful tool to reduce postprandial lipaemia.

The effects of continuous versus intermittent exercise, exercise intensity and long-term training regimes upon triglyceride responses to high-fat meal ingestion are well documented [312,394-396]. The time course of lipaemic benefits following an exercise stimulus has also been investigated. Zhang *et al* (1998) demonstrated a marked improvement with exercise carried out 12 h prior to a fat meal compared with 1 h before [308], and Hardman *et al* (1998) have shown that the benefit on postprandial lipaemia remains until 60 h post-exercise [397]. The data of Zhang *et al* (1998) and Hardman *et al*

(1998) suggest the optimal time delay is longer than 1 h, but shorter than 60 h. Furthermore, one of the key mechanisms, increased LPL activity, begins to increase 4 h post-exercise [398,399] and is still elevated 24 h following the stimulus [392]. Hence, it is clear that exercise-induced improvements in postprandial lipaemia are delayed and transient. In addition, a period of de-training in athletes reduces LPL activity [400], and physical inactivity has been shown to suppress LPL activity compared to active controls [401]. The exact time course of the transient lipaemia-suppressing effects is unknown. This information would be useful when writing physical activity guidelines, particularly regarding the aspect of frequency. Therefore, the aim of this study was to investigate the time course of the beneficial effect by comparing the effect of a prolonged exercise bout carried out 12-, 24- or 48 h prior to a high fat meal. Although not available at the time of commencing this study, a publication by Zhang *et al* (2004) recently demonstrated reduced lipaemia following a 12 h but not a 24 h delay between prolonged moderate-intensity exercise and a high fat meal in obese hypertriglyceridaemic patients, suggesting that the beneficial effects are lost in the 12-24 h post-exercise period [402]. The current study presents similar data but in a lean, healthy cohort.

8.3 Method and materials

Following ethical approval from The School of Sport and Exercise Sciences Safety and Ethics Subcommittee, eight lean healthy volunteers, aged 21 ± 1 years, body mass 72.3 ± 2.3 kg, and BMI 23.0 ± 0.7 kg.m^{-2} (mean $\pm$ S.E.M.), were recruited from the local community. All volunteers were assessed by a general health questionnaire and provided informed written consent prior to commencing the study. Each subject completed three interventions in a randomised cross-over design, each involving ingestion of a high fat meal (OFTT) that followed a moderate-intensity exercise bout carried out either 12, 24, or 48 h previously. Each exercise bout consisted of a 90 min treadmill walk at 55% of heart rate reserve (55% age-predicted HRR), recorded using a Polar A3 Heart Rate Monitor (Polar Electro, Kempele, Finland). Treadmill speed was maintained at 4 mph whilst gradient was set to attain the desired HRR. Exercise duration and intensity was chosen to replicate protocols from previous publications [403,404]. A dietary record was taken for two days preceding the first test for the purpose of diet replication prior to each subsequent trial, and subjects were instructed to refrain

from consuming alcohol, caffeine, and from any exercise regime for forty-eight hours prior to each trial. The test meals matched those of previous literature assessing exercise-induced postprandial lipaemia to allow comparison with such data [312,403]: 71 kJ.kg^{-1} body mass with a macronutrient composition of 1.22 g.kg^{-1} fat (66% of calories), 1.21 g.kg^{-1} carbohydrate (29% of calories) and 0.20 g.kg^{-1} protein (5% of calories), consisting of whipping cream, ice cream, raisins, walnuts, and digestive biscuits.

8.3.1 Exercise 48 hours before oral fat tolerance test (48 h trial)

Subjects arrived in the laboratory at 0800 hours and completed a 90 min treadmill walk as described above. Following the exercise bout subjects were instructed to return to the laboratory 48 hours later, again at 0800 hours, following an overnight fast. An intravenous cannula (BD Venflon, Oxford, UK) was inserted into an antecubital vein and a fasting blood sample was taken. The high-fat test meal (OFTT) was then administered at t = 0 h and further 5 ml venous blood samples were drawn from the intravenous line at t = ½, 1, 2, 4 and 6 h. During the trial the cannula was kept patent with 5 ml flushes of 0.9% NaCl $_{(aq)}$ isotonic saline (Baxter Healthcare, Northampton, UK) following each blood sample. Plasma was stored at -70 ºC and triglyceride and glycerol concentrations were measured as described in the General Methods. Water was consumed *ad libitum* throughout. At the end of the trial the cannula was removed and the subject was sent home and instructed to return to the laboratory in 5-7 days, having followed their dietary intake record. A 48 h delay between exercise and OFTT was used as a control because in previous studies OFTTs in control (no exercise) trials were administered following 48 h abstinence from exercise, thus assuming the loss of exercise effects at this time point [307,312,405]. In addition, Aldred *et al* (1995) demonstrated no improvement in postprandial lipaemia following a 48 h delay between a 12 week exercise training regime and OFTT [307]. In this study an exercise trial 48 h prior to OFTT was used as a control so that factors such as time invested, contact with investigators and calories expended were equal between trials.

8.3.2 Exercise 24 hours before oral fat tolerance test (24 h trial)

The experimental set-up for this trial was identical to the 48 h trial. However, instead of a 48 h preprandial exercise bout, the 90 min walk was undertaken 24 h prior to the OFTT at 0800 hours.

Again, water was permitted *ad libitum*, and blood collection, storage and analysis followed the same practise.

8.3.3 Exercise 12 hours before oral fat tolerance test (12 h trial)

The experimental set-up was again identical to other trials, except the 90 min walk was undertaken 12 h prior to the OFTT, at 2000 hours, the night prior to the test meal. Water was again permitted *ad libitum*, and blood collection, storage and analysis followed the same practise.

The time delays between exercise and OFTT (24 and 12 h) were chosen because the available literature at the time of commencing this study indicated that exercise-induced improvements in postprandial lipaemia or LPL activity are evident from 8- to 24 h post-exercise [392,406,407], but are lost beyond delays of 2 days or more [308,397].

8.3.4 Statistical Analysis

Data are expressed as mean ± S.E.M. and statistical analysis was carried out using SPSS for Windows 12.0.1 (SPSS Inc, Chicago, US). Prior to statistical analysis triglyceride data was corrected for free glycerol (see General Methods for details). Triglyceride data was tested for normality and analysed using two-way (time*trial) repeated measures ANOVA. Main effects were analysed using Bonferroni *post hoc* tests. Raw triglyceride data was converted to area under the curve values by the trapezoidal method and trials were compared using one-way ANOVA followed by Bonferroni *post hoc* tests. Fasting (t = 0 min), peak (t = 120 min), and final (t = 360 min) triglyceride measures were also compared by one-way ANOVA, again followed by Bonferroni *post hoc* tests. Significant differences were accepted at P<0.05.

8.4 Results

8.4.1 Heart rate data during exercise trials

Average heart rates during the final hour (steady state) of each trial were 136 ± 2, 136 ± 2, and 138 ± 3 beats per minute (~55% HRR) for the 12, 24 and 48 h trials, respectively. These values were not significantly different between trials (P>0.05).

8.4.2 Plasma triglyceride responses to high-fat meal ingestion

Figures 8.1 and 8.2 show plasma triglyceride responses to OFTT during the three trials. Plasma triglyceride responses were significantly decreased in the 12 h and 24 h trials by $24.6 \pm 5.6\%$ ($P<0.01$) and $19.9 \pm 7.7\%$ ($P<0.05$) respectively when compared with the 48 h trial, but no differences were found between the 12 h and 24 h trials ($P>0.05$). No significant differences were found in fasting plasma triglyceride measures taken before the OFTTs: 605 ± 35, 595 ± 47, and 669 ± 119 μmol.l^{-1} for the 12, 24 and 48 hour trials, respectively ($P>0.05$). Peak (t = 120 mins) triglyceride concentration was significantly lower in the 12 h trial compared with the 48 h trial ($P<0.01$), but no differences were found at t = 360 mins ($P>0.05$).

Figure 8.1 – Plasma triglyceride responses following meal ingestion.
The three trials, 12 h trial, 24 h trial, and 48 h trial, represent a 90 min moderate-intensity exercise bout carried out 12, 24, or 48 h prior to a high fat meal. Triglyceride concentrations at t = 60, 120, 240, and 360 mins were significantly different from baseline in all trials (** indicates $P<0.01$). Triglyceride responses in the 12 h trial were lower compared with the 48 h trial ($P<0.05$) but not the 24 h trial ($P=0.08$). The 48 h trial was not different to the 24 h trial ($P>0.05$). No differences were found in fasting or t = 360 min triglyceride measures between trials ($P>0.05$), however peak triglyceride concentrations (t = 120 mins) were lower in the 24 h trial compared to the 48 h trial ($P<0.01$). Data represents mean $\pm$ S.E.M.
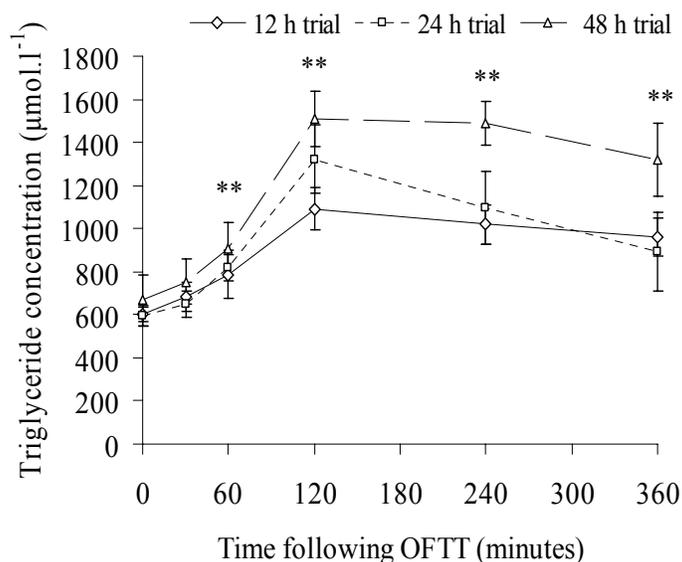
Figure 8.2 – Area under the plasma triglyceride response curve during meal ingestion
The 12 h trial, 24 h trial, and 48 h trial, once again represent a 90 min moderate-intensity exercise bout carried out 12, 24, or 48 h prior to a high fat meal. Triglyceride responses in the 12 h trial and the 24 h trial were significantly lower than in the 48 h trial (** indicates $P<0.01$; * indicates $P<0.05$) though not different from one another ($P>0.05$). Data represents mean $\pm$ S.E.M.
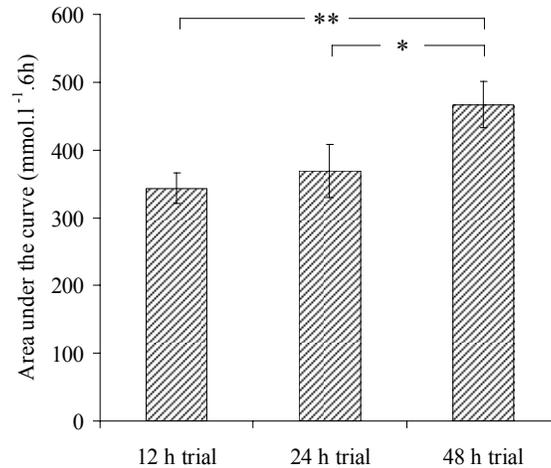
8.5 Discussion

This study demonstrates that in lean healthy individuals the effects of a prolonged moderate-intensity exercise bout upon postprandial lipaemia are altered by the time course between the exercise stimulus and meal ingestion. A time delay of 12 and 24 h between a 90 min moderate-intensity walk and OFTT had a beneficial effect (25% and 20%, respectively) on circulating postprandial triglycerides when compared to a delay of 48 h, yet no effects on fasting plasma triglyceride concentrations were seen. In contrast to the findings of Zhang *et al* (2004), who employed a similar study design in obese hypertriglyceridaemic patients [402], this study demonstrates that the lipaemic benefits are still present 24 hours after a single exercise bout in a lean healthy population. In concordance with previous studies, these beneficial effects are gradually lost as the time delay between exercise and meal is increased.

Triglyceride concentrations in the postprandial period are determined by chylomicron appearance from the gut, hepatic VLDL-TG output, and peripheral tissue triglyceride hydrolysis by LPL [389]. Although these factors were not measured in this study it is worth speculating about the mechanisms in an attempt to interpret the current findings. Meal ingestion stimulates pancreatic

insulin release which downregulates muscle LPL activity and upregulates adipose LPL activity [398,408-411]. Endothelial LPL provides the rate limiting step for triglyceride hydrolysis and clearance from the systemic circulation, and an exercise stimulus has been shown to increase skeletal muscle LPL activity in a delayed and transient manner [399,412]. Evidence suggests that skeletal muscle LPL activity is elevated at up to 24 h post-exercise [304], possibly explaining the effects of exercise carried out 12- and 24- compared with 48 h prior to OFTT in this study. During exercise, Kiens *et al* (1998) showed that, intramuscular triglyceride (IMTG) stores are reduced, and in the post-exercise recovery period, increased skeletal muscle LPL activity may serve to replenish these stores [413]. In addition, both meal ingestion and exercise can increase muscle tissue capillary perfusion thus allowing increased delivery of substrates to the LPL on the capillary endothelium [414]. However, no data is available to show the effect of exercise on insulin-enhanced capillary perfusion when there is a delay of many hours between the exercise and the meal. Further studies are required to establish if prior exercise causes a greater insulin action on capillary perfusion. Finally, exercise has been shown to reduce serum VLDL-TG concentrations [415] and hepatic output [416], and increase ketone levels [417], indicative of increased hepatic fat oxidation. However, there is no direct evidence of exercise-induced suppression of hepatic VLDL-TG output in humans. Furthermore, no studies have investigated the time course of this exercise-induced change in hepatic contribution.

Zhang *et al* (2004) studied the effects of a similar protocol in obese middle-aged hypertriglyceridaemic and impaired glucose tolerant male subjects, and found attenuated postprandial lipaemia 12 h following a 60 min moderate-intensity exercise bout when compared with a 24 h delay [402]. In contrast, our data indicates no significant difference between 12- and 24 h delays between exercise and OFTT. This may be explained by the increased duration of exercise used during our trials, and thus increased energy expenditure, possibly prolonging the effects of our stimulus upon postprandial lipaemia.

The use of a 48 h delay was used as the control trial in this study for reasons explained in the Methods and Materials above (section 8.3.1). It is possible that in our study a 48 h delay between exercise and meal may still be having an effect on postprandial lipaemia, however given the current

evidence that exercise-induced increased LPL activity is apparent 24 h after but not 48 h after exercise [304,406], and that the literature shows no effects of exercise on triglyceride levels 2 days post-stimulus [307,397,405], it is unlikely that a "no exercise" trial would differ from our $OFTT_{48hpre}$ trial. However, the lack of a "no exercise" control trial is a design weakness of this study, as previous literature used different subjects and therefore extrapolations cannot be accurately drawn. A further limitation of this study may be the lack of oxygen consumption (VO_2) data. Whilst stating VO_2 is typical of exercise studies, it has been clearly demonstrated that exercise duration and intensity is not an important determinant of postprandial lipaemia if energy expenditure is consistent [289]. Our subjects exercised at 55% HRR, levels comparable with heart rates observed in previous studies. These levels were maintained in each trial, thus energy expenditure would be equal between trials.

Assuming that 48 h after exercise there is a minimal effect on postprandial lipaemia, this study demonstrates that a 90 min moderate-intensity walk carried out 12- or 24 h prior to a high fat meal improves postprandial lipaemia, a marker of CVD risk, in lean healthy individuals. Given the significant loss of the beneficial effect between 24 and 48 hours after exercise this work suggests physical activity should be performed daily in order to maintain optimal lipaemic control.

9. General Discussion

9.1 General Findings

The following five paragraphs summarise the novel findings that this thesis adds to the current literature.

9.1.1 Reliability of the OGTT

Chapter 3 demonstrated that some OGTT-derived measures of insulin sensitivity (ISI_{OGTT}) are reliable when pre-test diet and exercise is standardised. It was concluded that $ISI_{OGTT}^{Matsuda}$ was the most reliable measure, and together with previous observations that this variable is a valid estimate of insulin sensitivity measured against the clamp, $ISI_{OGTT}^{Matsuda}$ was considered the most appropriate measure to investigate insulin sensitivity during repeated measures experiments.

9.1.2 The use of stable isotope tracers in OGTT protocols

Dual tracer methodology was applied to the OGTT technique in Chapter 4, allowing the determination of glucose rate of appearance into the systemic circulation from exogenous (meal) and endogenous (hepatic) sources during the standard 2 hour 75 g OGTT. Use of this technique in a patient group may allow monitoring of treatment (pharmaceutical or lifestyle) interventions upon hepatic insulin sensitivity.

9.1.3 Cinnamon ingestion and insulin sensitivity

The work in Chapter 5 showed that acute cinnamon ingestion (a single 5 g bolus) improved insulin sensitivity by up to 13%, with a persistent effect lasting 12 hours. Chronic cinnamon feeding (3 g.day^{-1} for 14 days) was also shown to improve insulin sensitivity in Chapter 6, though these effects were not accumulative or persistent in the 6 day period following removal of the cinnamon feeding stimulus. Given the relatively small improvements seen in these studies, and the mixed evidence in the literature as to the effect of cinnamon in humans in vivo [238-240], further work is required before cinnamon may be considered a therapeutic supplement for states of insulin resistance.

9.1.4 Feeding frequency, serum insulin and plasma ghrelin responses

The findings in Chapter 7 supported evidence that insulin may regulate postprandial ghrelin responses. Also, this hormone-hormone interaction appeared to have a time delay of approximately 20 minutes. Feeding frequency had no effect on total day-long (AUC) insulin or ghrelin responses, yet it was demonstrated that the insulin-ghrelin relationship seen during low-frequency (2 meals.day^{-1}) feeding was less apparent when the same total caloric load was ingested in twelve equicaloric meals (high frequency feeding). Eating frequency may, therefore, affect insulin's control of ghrelin release.

9.1.5 Exercise and postprandial lipaemia

When compared to a 48-hour delay between moderate-intensity exercise and a high-fat meal, postprandial lipaemia was suppressed by up to 25% when the exercise bout was carried out 12 or 24 hours prior to the meal. These data from Chapter 8 suggest that the beneficial effect of exercise on circulating triglycerides is lost beyond 24 hours post-stimulus in lean healthy subjects. This illustrates that daily exercise should be recommended in order to maintain optimal lipaemic control.

9.2 Further investigation

The recent rise in obesity and diabetes prevalence (General Introduction, Section 1.1) demonstrates the urgent need for effective therapies. The large diabetes intervention studies in the US, Finland, China and Japan [190,191,193,209] have generated convincing evidence that lifestyle alteration via diet and increased physical activity is a powerful therapeutic tool. However, this knowledge has not stopped the rise in metabolic disease (General Introduction, Section 1.1). Therefore, the communication of this knowledge to the public is either failing or compliance by the public to these known effective therapies is poor. The information from such intervention studies is available in both scientific and lay publications, however, the health benefits of lifestyle alteration are not directly taught in the UK school education system, and therefore such information has to be actively sought. In addition, the Department of Health's statistics show that the recommended levels of physical activity are only achieved by 29% of the population in the UK [39,40], demonstrating that, in addition to poor education, compliance to the known effective therapeutic methods is also poor. In

order to tackle this problem, current education must be improved, or researchers must search for alternative therapeutic methods to which compliance may be easier. This PhD project added new information to recent alternative research directions investigating symptoms of the metabolic syndrome. Whilst patient or "at risk" cohorts were not used, statistical differences were achieved in lean, healthy, yet minimally active volunteers, and therefore it would be prudent to continue such investigations in patient groups.

In Chapter 3, ISI_{OGTT} measures were demonstrated to be reliable. Reliability of these measures has not been investigated in insulin resistant individuals, nor have several of these ISI indices been validated against the gold standard glycaemic clamp methodology across a range of insulin sensitive states and therefore this remains an avenue for future work. Chapter 4 described the use of dual tracer methodology during the standard 2 h 75 g OGTT in lean healthy individuals. Adaptation of this tool into a patient population would also be sensible. If it were demonstrated that the hepatic contribution to the rate of glucose appearance was increased in obese individuals, then this would provide a good basis for the use of this new tool in monitoring treatment regimes targeted at improving hepatic insulin sensitivity. Chapters 5 and 6 provide evidence for the positive effects of cinnamon ingestion on glycaemic control and insulin sensitivity. In animal and cell lines, several research groups have elucidated some of the mechanisms at play [235-237,241-246], however, very little is known regarding the effects of cinnamon ingestion in humans at the molecular and cellular level. With the use of mass spectrometry it may be possible to measure the active component of cinnamon (e.g. MHCP or cinnamaldehyde [236,356]) in the blood, to calculate its rate of appearance and half life. However, whilst some data concerning cinnamon's active component is available, describing a phenolic lipid-soluble compound [236,356], its cellular targets or kinetics are not fully understood. Further work may also focus on cinnamon's regulation of the insulin signalling cascade in human skeletal muscle, as has been demonstrated in rats and mice by Kim *et al* (2006) and Qin *et al* (2003, 2004) [243-246]. Chapter 7 further investigated recent findings that insulin may be responsible for postprandial ghrelin suppression. The correlation analyses performed here cannot determine causality, but do concur with existing evidence that changes in insulin are likely to affect ghrelin responses [254-259]. The exact

physiological significance of an insulin-ghrelin association is not yet known, however the two hormones are nutrient-responsive and involved in regulating energy balance, and therefore further work studying such factors would be wise. Feeding frequency is a factor that receives little attention in comparison to meal composition and caloric intake [266]. The available literature shows mixed evidence regarding its effects on factors surrounding metabolic disease [269-281], thus large-scale randomised controlled studies are required [266]. Some *in vitro* evidence suggests that prolonged hyperinsulinaemia may have detrimental effects on the liver, increasing VLDL-TG output, a factor associated with the metabolic syndrome [418,419]. Prolonged periods of high-frequency feeding may result in a state of hyperinsulinaemia during the day, and therefore it would be interesting to investigate such effects *in vivo* in future work. Finally, Chapter 8 concluded that moderate-intensity exercise should be performed daily in order to maximise lipaemic benefits. With findings from Zhang *et al* (2004) [402] in obese subjects, the time course of exercise effects are now quite well established, however the mechanisms of exercise-induced triglyceride suppression are not fully understood. In humans, it is well documented that increased activity of LPL accounts for increased triglyceride clearance from the systemic circulation [304,398,399,412,420,421]. However, the evidence also suggests that LPL is not the sole factor involved in lowered triglyceride levels following exercise because basal triglyceride uptake is not altered by exercise despite reduced triglyceride concentrations [417], and LPL activity 18 hours post-exercise has been shown to be unaltered despite lowered postprandial lipaemia [422]. Whilst work in animal models has confirmed that exercise may reduce hepatic VLDL-TG production [416,423], only measures of plasma VLDL and 3-hydroxybutyrate concentrations have been made in such studies in humans [415,417]. It would therefore be useful to investigate the time course effects of exercise on the lipid partitioning between adipose tissue, liver and muscle to further understand the mechanisms involved.

9.3 General conclusion

Chapter 1 demonstrates the great impact that metabolic abnormalities associated with insulin resistance have on mortality, morbidity and global health care costs and economy. Despite a clear understanding of the lifestyle factors that lead to such conditions, and the interventions that can be

applied to relieve symptoms and reduce morbidity, Department of Health statistics clearly show that trends in obesity, diabetes and CVD continue to rise (Figures 1.1, 1.2, and 1.3; [2]). This PhD project identified and extended the investigation of some recent viewpoints surrounding metabolic abnormalities that may prompt alternative treatment strategies. Besides further validating the OGTT as a useful research tool, this thesis demonstrates that dietary supplementation with cinnamon, meal frequency, and exercise time course may be important factors to consider when prescribing nutritional and physical activity lifestyle alterations.

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