ANABOLIC AND INSULIN RESISTANCE IN HUMANS IN HEALTH AND DISEASE

by

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ABSTRACT

Anabolic resistance is the reduced muscle protein synthetic response to stimuli, such as feeding and exercise, and is implicated in muscle loss in a variety of conditions. It often coexists with insulin resistance, which describes the attenuated response of the body to the effects of insulin on, for example, glucose disposal. Therefore, in order to help develop ways to combat muscle loss, it is crucial to understand more about how these two types of resistance interact.

The overall aim of the studies described in this thesis was to determine whether reduced anabolic response to feeding is associated with insulin resistance in (a) middle-aged males with obesity and in (b) adult and paediatric patients with chronic inflammatory bowel disease (Crohn's disease, CD). The arterio-venous net balance technique, across the forearm, was used to test the anabolic and insulin responses to oral nutrition.

In the paediatric CD study (Chapter 4) patients in remission were found to have a neutral net amino acid (AA) balance in response to a mixed meal, while controls were in positive balance. This was associated with reduced fat free mass z-scores but not insulin resistance. In male CD net AA balance was negative and associated with lower appendicular lean mass, higher levels of muscle fatigue and reduced habitual dietary protein intake, relative to controls, but not insulin resistance.

Neither anabolic nor insulin resistance was found in middle-aged men with obesity (Chapter 3) in response to milk protein and dextrose ingestion, with

prior oral fat ingestion, or in a group of adult CD patients in active disease in response to a mixed meal (Chapter 5). Moreover these groups did not as a whole have reduced muscle mass.

In summary, the findings of this thesis suggest that anabolic resistance does not always associate with insulin resistance, but does appear to associate with reduced muscle mass, in the populations studied. This implies that treatments to restore and/or maintain muscle mass should focus on improving anabolic response to food, using high protein diets and exercise. The testing and development of such interventions, tailored to different populations, are therefore warranted.

PUBLICATIONS

Abstracts

Moran G, Walker A, Nixon A, Devadason D, Muhammed R, Tsintzas K, Kirkham S, Stephens F (2018). OTH-003 Paediatric Crohn's disease patients in remission have a reduced skeletal muscle protein balance after feeding. *Gut*. BMJ Group **67** (S1): A59-A60.
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Papers

Davies A, Nixon A, Muhammed R, Tsintzas K, Kirkham S, Stephens FB, Moran GW (2020). 'Reduced skeletal muscle protein balance in paediatric Crohn's disease.' Clin Nutr. 39 (4): 1250-1257.
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Tsintzas K, Jones R, Pabla P, Mallinson J, Dong-hyun Kim, Barrett D, Cooper S, Davies A, Taylor T, Gaffney C, Chee C, van Loon L, Stephens FB (2020). 'Effect of acute and chronic dietary fat ingestion on postprandial skeletal muscle protein synthesis rates in middle-aged, overweight and obese men.' Am J Physiol Endocrinol Metab. 318 (3): E417-E429.
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DECLARATION

All of the work presented in this thesis was the result of procedures carried out by myself, including all *in vivo* human trials, blood and urine sample collection, preparation and biochemical analysis, expired gas, ultrasound, handgrip and questionnaire analysis, with the following exceptions:

Deep venous blood sampling, ultrasound measurements of the brachial artery and DEXA scans were carried out by Aline Nixon (Chapters 4 and 5) and Joanne Mallinson (Chapter 3). Cannulations were performed by Aline Nixon, Tariq Taylor, Natalie Shur and members of the paediatric and adult gastroenterology teams at QMC. Consultants Sian Kirkham and Rafeeq Muhammed led paediatric patient recruitment and Gordon Moran adult recruitment. Analysis of plasma FFA and serum insulin, TAG, and glycerol was performed by Sally Cordon, cytokines by Lissa Sutherland and testosterone by Robert Jones. Melanie Marshall and Maab Ali analysed diet diary and participant questionnaires. Metabolomic sample preparation, ultra high performance liquid chromatography mass spectrometry, multivariate and univariate analysis was carried out by Professor Warwick Dunn and his team at the Phenome Centre Birmingham.

I hereby declare that the present thesis has been composed by myself and that it is a record of the work performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted in any other application for a higher degree and all sources of information have been appropriately referenced.

Amanda Davies

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ABBREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
a-v	arterio-venous
AA	amino acids
ASMI	appendicular skeletal muscle mass index
AUC	area under the curve
BCAA	branched chain amino acids
BMI	body mass index
CCK	cholecystokinin
CD	Crohn's disease
CHO	carbohydrate
Con	control
COX	carbohydrate oxidation
CRP	c-reactive protein
DEXA	dual-energy X-ray absorptiometry
DHA	docosahexaenoic acid
EAA	essential amino acids
EGTA	ethyleneglycol-Bis-β-aminoethylether tetraacetate
eIF	elongation factor
EPA	eicosapentaenoic acid
FCP	faecal calprotectin
FFA	free fatty acids
FFM	fat free mass
FM	fat mass
FO	fish oil

FOX	fat oxidation
FOXO	forkhead box transcription factor
FSR	fractional synthetic rate
GAP	GTPase-activating proteins
GH	growth hormone
GI	gasto-intestinal
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HbA1c	glycated haemoglobin
HBI	Harvey Bradshaw index
HGP	hepatic glucose production
HOMA-IR	homeostatic model assessment of insulin resistance
IBD	inflammatory bowel disease
IDC	indirect calorimetry
IGF-1	insulin-like growth factor 1
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IPAQ	international physical activity questionnaire
IR	insulin receptor
IRS	insulin receptor substrate
LBM	lean body mass
LM	lean mass
LPL	lipoprotein lipase
MAFbx	muscle atrophy F-box

mitoFSR	mitochondrial protein fractional synthetic rate
mitoPS	mitochondrial protein synthesis
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
mTORC	mammalian target of rapamycin complex
MUFA	monounsaturated fatty acids
MuRF1	muscle RING-finger protein-1
myoFSR	myofibrillar fractional synthetic rate
myoPS	myofibrillar protein synthesis
n3PUFA	omega−3 polyunsaturated fatty acids
NAMPT	nicotinamide phosphoribosyltransferase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OGTT	oral glucose tolerance test
P38 MAPK	p38 mitogen-activated protein kinase
P70S6K	p70 S6 kinase
PB	protein breakdown
PDC	pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PI3K	phosphoinositide 3-kinase
PKCθ	protein kinase C theta
PS	protein synthesis
PUFA	polyunsaturated fatty acids
Rab	ras-like small GTP-ase

REE	resting energy expenditure
RER	respiratory exchange ratio
Rheb	GTP-ase Ras homolog enriched in brain
ROI	region of interest
sarcoFSR	sarcoplasmic protein fractional synthetic rate
sarcoPS	sarcoplasmic protein synthesis
SD	standard deviation
SDS	standard deviation score
SEM	standard error of the mean
SFA	saturated fat
T2DM	type 2 diabetes mellitus
TAG	triacylglycerides
TBM	total body mass
TCA	tricarboxylic acid cycle
THL	tetrahydrolipstatin
TNFα	tumour necrosis factor alpha
TSC1 & 2	tuberous sclerosis proteins 1 and 2
UPS	ubiquitin proteasome system

1 GENERAL INTRODUCTION

1.1 INTRODUCTION

Anabolic resistance describes the reduced protein synthetic response of skeletal muscle to anabolic stimuli such as feeding and exercise (Rennie and Wilkes 2005; Breen et al. 2013). It is implicated in muscle loss in a variety of conditions including critical illness (Rennie 2009), inflammation (Lang, Frost, and Vary 2007), obesity (Chevalier et al. 2005a), ageing (Wall et al. 2015) and disuse (Glover et al. 2008; Wall et al. 2016). A common factor in all of these situations is insulin resistance, which describes the attenuated response of target tissues, primarily skeletal muscle, adipose tissue and liver to the actions of insulin (McGarry 2002; DeFronzo and Tripathy 2009). Not only do these two types of resistance frequently coexist but insulin cell signaling pathways link to pathways involved in protein metabolism (Brunet et al. 1999; Kamei et al. 2004).

In order to design effective treatments to reduce muscle loss it is important to understand the relationship between insulin and anabolic resistance. For example, if insulin resistance were to lead to anabolic resistance then effectively the therapeutic focus could be on improving insulin sensitivity. The relationship between anabolic and insulin resistance will be explored in two groups at risk from both types of resistance: individuals with obesity / who are overweight, and patients with chronic inflammatory disease. The studies in this thesis are designed to further our understanding of the relationship between anabolic and insulin resistance in order to help develop ways to combat muscle loss in a variety of conditions.

1.2 ANABOLIC RESISTANCE

The idea of anabolic resistance and its link to muscle loss came to the fore in the mid 2000s. It was well understood that muscle loss was a result of negative protein balance i.e. where the rate of muscle protein breakdown (MPB) exceeds that of muscle protein synthesis (MPS). Indeed, it is the balance between these two processes which determine whether muscle mass is reduced, maintained or increased.

Anabolic resistance has been shown by several studies in the elderly (Guillet et al. 2004; Cuthbertson et al. 2005; Wall et al. 2015). Guillet et al. (2004) show reduced MPS in elderly vs. young controls under hyperinsulinaemic and hyperaminoacidaemic conditions. Cuthbertson et al. (2005) demonstrate reduced MPS in the elderly vs. young controls at various doses of EAA (0, 2.5, 5, 10, 20, 40g) under hyperinsulinaemic conditions. Wall et al. (2015) also show that response to 20g casein in the elderly is reduced vs. young controls. However an increase in MPB has not been consistently demonstrated (Volpi et al. 2000; Wilkes et al. 2009). Volpi et al. (2000) found, in response to the oral administration of amino acids and glucose, that there was no difference between the elderly and young controls in MPB although MPS was reduced in the elderly. Whereas Wilkes et al.'s (2009) data suggest that a moderate rise in insulin (from 5 to 15 μIU/mL) under euglycaemic isoaminoacidaemic conditions resulted in reduced suppression of MPB in older vs. younger subjects.

A raft of studies involving reduced physical activity including immobilisation (Glover et al. 2008; Wall et al. 2016), bed rest (Biolo et al. 2004; Tanner et al. 2015) and a reduction in daily activity (Breen et al. 2013) have demonstrated anabolic resistance and suggest this is of greater importance in muscle loss in disuse than an increase in MPB (Glover et al. 2008; Phillips 2009; Marimuthu, Murton, and Greenhaff 2011). In critical illness, including sepsis, although increased MPB appears to be a major contributor to atrophy there is also evidence that anabolic resistance is present (Crossland et al. 2008; Rennie 2009; Puthucheary et al. 2013) although this is disputed by others (Klaude et al. 2012). The presence of anabolic resistance has also been established in people with obesity (Chevalier et al. 2005a; Pereira et al. 2008; Murton et al. 2015; Beals et al. 2016). Furthermore, anabolic resistance can be induced acutely in young healthy volunteers by intravenous lipid administration (Stephens et al. 2015).

Although clearly defined, anabolic resistance is not yet fully characterized, with the relative contributions of physical activity and nutrition, elevated intramuscular lipid species, insulin resistance and inflammation to its development remaining unclear. Similarly, it remains equivocal whether increasing anabolic stimuli and/or improving insulin sensitivity and/or reducing inflammation can restore anabolic sensitivity to 'normal' levels or simply improve it.

Studies using high quantities of protein/amino acids (AA) and/or exercise have been shown to abolish anabolic resistance in the elderly (Moore et al. 2015), people with obesity (Chevalier et al. 2015) and type 2 diabetes mellitus

(T2DM) (Bassil et al. 2011). For example, Moore et al. (2015) show, with regression and breakpoint analyses on a series of 6 studies providing oral doses of EAA from 0-40g, that the same FSR can be reached with both young and elderly participants although the required dose is higher in older subjects. Optimum protein intake per meal for young subjects to achieve a myofibrillar FSR of 0.06%/hr is 0.25g/kg LBM whereas to achieve the same FSR elderly participants needed 0.61 g/kg LBM. This contrasts with Cuthbertson et al.'s (2005) findings where increasing protein doses over a similar range did not result in parity of FSR between young and older subjects. Other studies in the elderly (Houston et al. 2008) and using immobilisation (Glover et al. 2008) have only found an improvement and not normalisation.

Reducing hyperglycaemia in post-operative cancer patients (Biolo et al. 2008) increased MPS and produced a neutral protein balance while better glycemic control in those with T2DM (Gougeon et al. 2000) normalised protein metabolism to match that of controls with obesity. Biolo et al. (2008) tested the effects on protein metabolism of conventional versus intensive insulin treatment i.e. euglycaemia versus moderate hyperglycaemia in post-operative cancer patients. Euglycaemia resulted in increased protein synthesis and neutral net protein balance as assessed by leg arterio-venous balance of stable isotope tracers. They also observed reduced skeletal muscle leucine oxidation, increased skeletal muscle glutamine production (positive PDH response to insulin facilitating conversion of glucose to glutamine), reduced whole body phenylalanine turnover and improved insulin sensitivity. They suggest that during insulin induced euglycaemia muscle protein synthesis is stimulated by the increased leucine availability and possibly insulin itself and

whole body proteolysis is reduced by the increase in systemic amino acid availability.

Gougeon et al. (2000) used 15N glycine and methyl histidine (3MH) excretion to investigate whole body protein metabolism and skeletal muscle catabolism respectively, in patients with diabetes, with and without oral hypoglycaemic agents, versus controls with obesity, during an isocaloric diet. They found a reduction in glycaemia, an improvement in insulin sensitivity and in nitrogen balance, to the level of controls, in patients with diabetes taking oral hypoglycaemic agents. The main factor in improving protein balance was a reduction in protein catabolism, which was suggested to be due to increased insulin sensitivity, which in turn suppressed protein breakdown.

Induction of remission in inflammatory bowel disease (IBD) has been shown to increase muscle mass (Subramaniam et al. 2015) but not necessarily normalize it (Werkstetter et al. 2013). Neither have the specific site(s) of anabolic resistance been elucidated. Although cell-signaling pathways involved in MPS and MPB and linked to insulin have all been implicated (Stephens et al. 2015; Murton et al. 2015; Beals et al. 2016) and will be explored further in a subsequent section.

While a reduction in the availability of plasma AA could also affect muscle protein balance, anabolic resistance has been demonstrated in the absence of any reduction in availability of AA, for example in people with obesity (Beals et al. 2016) and in the elderly (Durham et al. 2010), although not consistently so (Rasmussen et al. 2006). Moreover a recent paper has shown that

supplementation of essential amino acids (EAA) with a nitric oxide precursor (l-arginine) does not improve anabolic response in the elderly despite increased tissue perfusion (Mitchell et al. 2017) suggesting defects may reside at the level of the muscle.

Further the presence of ‘anabolic inflexibility’ has now been shown in the elderly i.e. a reduced ability to increase MPS in response to feeding which was negatively correlated with basal MPS (Wall et al. 2015). Intriguingly this idea is similar to that of metabolic inflexibility where in cases of insulin resistance individuals have a high fasting respiratory quotient (RQ) and show a reduced ability to respond to feeding by increasing carbohydrate oxidation (COX) any further (Kelley et al. 1999).

1.3 INSULIN RESISTANCE

While much is known from the considerable amount of research conducted into insulin and insulin resistance over the past 100 years much is still to be characterised. Broadly insulin acts to increase skeletal muscle glucose disposal and reduce hepatic output of glucose, as well as suppress the output of free fatty acids (FFA) from adipose tissue (Björnholm and Zierath 2005). Insulin also increases blood flow via nitric oxide dependent mechanisms (Steinberg et al. 1994) and improves skeletal muscle protein balance by reducing MPB and acting as a permissive agent in increasing MPS (Greenhaff et al. 2008).

In skeletal muscle the activation of the insulin-signaling cascade stimulates glucose uptake via translocation of GLUT4 transporters to the cell membrane (Furtado et al. 2002). Insulin binding causes the auto-phosphorylation of tyrosine residues on the insulin receptor (IR), which in turn results in tyrosine phosphorylation of insulin receptor substrate (IRS) (Wilden et al. 1992). IRS then binds to phosphoinositide 3-kinase (PI3K) which in turn activates the protein kinase Akt (protein kinase B) (Backer et al. 1992; Alessi et al. 1996). Akt activates AS160 which causes the translocation of GLUT4 to the cell membrane and so inward transport of glucose (Sano et al. 2003). Insulin also stimulates phosphorylation of intracellular glucose (Kruszynska et al. 1998) as well as its subsequent synthesis to glycogen (Cohen, Nimmo, and Proud 1978) or oxidative disposal (Huang et al. 2003). The activity of several enzymes involved in the oxidation of glucose are positively affected, with perhaps the most pivotal being the rate limiting step in COX where pyruvate is

converted to acetyl CoA, by the pyruvate dehydrogenase complex (PDC) (Connaughton et al. 2010). PDC is a multi-enzyme complex with pyruvate dehydrogenase phosphatase (PDP) as the stimulatory component, and pyruvate dehydrogenase kinase (PDK) inhibiting the reaction.

Lipid infusion studies in healthy volunteers have given insight into the development of insulin resistance in skeletal muscle. They have shown that excess lipid can cause an increase in fat oxidation (FOX) and reduction in COX via attenuated suppression of PDK4 while glucose is diverted to be stored as glycogen (Chokkalingam et al. 2007; Tsintzas et al. 2007). As lipid derivatives accumulate in the muscle they have a negative impact on the insulin signaling cascade (Hotamisligil et al. 1996; Jiang et al. 1999) and so result in a reduction in glucose transport (Boden et al. 1991). Indeed impaired glucose transport (Cline et al. 1999), phosphorylation (Kelley et al. 1996; Kruszynska et al. 1998), storage (Damsbo et al. 1991) and oxidation (Kim et al. 2006) are all found in type 2 diabetes mellitus (T2DM).

Lack of physical activity is also thought to induce skeletal muscle insulin resistance by decreasing the activity of signaling proteins involved in glucose uptake, phosphorylation and conversion to glycogen (Biensø et al. 2012). Additionally, inflammatory cytokines e.g. tumor necrosis factor alpha (TNFα) interfere with the insulin-signaling cascade in a similar manner to lipid derivatives. They activate kinases e.g. c-Jun N-Terminal Protein Kinase 1 (JNK1) & protein kinase C theta (PKCθ), which phosphorylate IRS1 on serine residues thus inhibiting its ability to phosphorylate PI3K (Hotamisligil et al. 1996; Hirosumi et al. 2002). They have also been found to reduce the

expression of other insulin signaling proteins (Stephens and Pekala 1991) as well as inhibit Akt activity via the stimulation of ceramide synthesis (Holland et al. 2011). The ability of insulin to suppress hepatic glucose production (HGP) (Brøns et al. 2009) and FFA release from adipose tissue are also reduced by excess lipid availability (Jensen et al. 1989).

In insulin resistant individuals, a result of reduced glucose uptake by muscle and increased HGP, glycaemia is increased and lipaemia also rises as fatty acid release from adipose tissue is insufficiently suppressed. In response hyperinsulinaemia ensues which initially keeps at least fasting glycaemia under control. However, a vicious cycle of deteriorating insulin sensitivity can then result. Increased plasma (Ellis et al. 2000) and intramyocellular lipid (Dresner et al. 1999) cause further reductions in skeletal muscle insulin sensitivity, hyperinsulinaemia reduces insulin receptors and decreases glucose uptake even more (Del Prato et al. 1994), the switch between FOX and COX with feeding becomes impaired (metabolic inflexibility) (Kelley et al. 1999) and hyperglycaemia additionally down-regulates glucose transport (Zierath et al. 1996; Creager et al. 2003). Hyperglycaemia and hyperlipaemia also affect vascular reactivity (Creager et al. 2003) and can therefore reduce nutrient delivery to the muscle.

Yet as long as insulin producing pancreatic beta cells can compensate by producing more insulin, insulin resistance, rather than diabetes is present. But once beta cell function becomes impaired, due to over-stimulation by hyperglycemia and hyperlipidaemia, then insulinopenia and fasting

hyperglycaemia result and as such frank diabetes is apparent (DeFronzo, Bonadonna, and Ferrannini 1992).

Both the improvements seen in glycaemic control with bariatric surgery (Guidone et al. 2006) and the work of Roy Taylor has shown that it may be possible for people with diabetes to go into remission, even those who have had diabetes for up to 6 years (Taylor 2016; Taylor et al. 2018; Lean et al. 2018). The 'twin cycle' hypothesis suggests that the accumulation of hepatic and pancreatic fat, cause diabetes (Taylor 2008). Roy Taylor's group has shown that if this excess fat can be reduced, with a very low calorie diet, then glycaemic control can be recovered (Lim et al. 2011). This is thought to be possible as a reduction in liver fat restores hepatic glucose output to normal levels and a reduction in pancreatic fat can improve beta cell function and therefore insulin production. This does not however preclude the role of skeletal muscle insulin resistance in the development of diabetes. Any factor which contributes to hyperinsulinaemia will tend to increase fat deposition in the liver (Taylor 2008).

In order to facilitate the consideration of the links between anabolic and insulin resistance the regulation of skeletal muscle protein synthesis and breakdown will be explored in the next section.

1.4 SKELETAL MUSCLE PROTEIN METABOLISM

1.4.1 Muscle protein synthesis

1.4.1.1 Cell signaling and its regulation

In vitro studies have demonstrated that the major site of regulation of protein synthesis is at mRNA translation (Holz et al. 2005; Hershey, Sonenberg, and Mathews 2012). They have done this by investigating whether quantity of RNA or efficiency of translation are linked to acute increases in MPS. For example (Chesley et al. 1992) showed that an increase in MPS in humans after a bout of resistance exercise was not associated with increases in RNA, rather an increase in RNA activity (amount of protein synthesised per unit time per unit of RNA), suggesting acute regulation of MPS is translational rather than transcriptional.

mRNA translation is switched on by the phosphorylation of a cascade of signaling proteins. Central to this cascade is the protein kinase mTORC1, the mammalian target of rapamycin complex 1 (Schmelzle and Hall 2000; Gingras, Raught, and Sonenberg 2001). Downstream of mTORC1 is p70S6 kinase (p70S6k), which phosphorylates ribosomal protein S6 and stimulates protein translation (Anthony et al. 2000). Eukaryotic initiation factor 4E-binding protein 1 (4EBP1) is also phosphorylated by mTORC1 causing the dissociation of elongation factor 4E (eIF4E), which is then free to associate with eIF4G forming the pre-initiation complex eIF4F and initiating protein translation (Holz et al. 2005).

The insulin-signaling cascade is linked to mTORC1 via a series of signaling proteins downstream of Akt. Akt inhibits the GTPase activator protein complex (GAP) of tuberous sclerosis proteins 1 and 2 (TSC 1 & 2) (Manning et al. 2002), which in turn inhibit the GTP-ase Ras homolog enriched in brain (Rheb) (Inoki et al. 2003). Rheb activates mTORC1 when GTP is bound to it (Long et al. 2005). Thus the inhibition of TSC 1 and 2 by Akt results in the formation of Rheb + GTP and so activation of mTORC1. Independently of mTORC1, Akt also stimulates mRNA translation via glycogen synthase kinase 3 (GSK-3) and elongation factor 2 (eLF2) (Welsh and Proud 1993). *In vitro* studies therefore suggest that insulin stimulates at least 2 pathways linked to protein synthesis.

mTORC1 is also controlled by amino acids and this has been shown in humans (Dickinson et al. 2011). *In vitro* studies suggest this is via the Ras-like small GTP-ase (Rab) pathway (Sancak et al. 2008) and not via Rheb (Dennis et al. 2011). Rab are thought to facilitate the localization of mTORC1 to the lysosomal membrane where it associates with Rheb GTP (Sancak et al. 2008). *In vitro*, Rheb or Rag can activate mTORC1 but it can only be maximally stimulated by both (Dennis et al. 2011). This is in agreement with *in vitro* studies and those in rodents showing that both insulin and amino acids can stimulate mTORC1 activity independently but that their effect is additive (Patti et al. 1998; Anthony et al. 2002).

In addition to amino acids and insulin, insulin like growth factor (IGF)-1 and growth hormone (GH) both increase MPS *in vitro* likely through similar signaling cascades to insulin (Rommel et al. 2001) as both can bind to IRS-1

(Myers et al. 1993; Argetsinger et al. 1995) and GH stimulates production of IGF-1 (D'Ercole, Stiles, and Underwood 1984). mTORC1 is also stimulated by exercise (Drummond et al. 2009) and gonadal hormones such as testosterone (Basualto-Alarcón et al. 2013).

Negative regulators of PS include cytokines, glucocorticoids, lipid and inactivity. Work in rodent muscle suggests the cytokine TNFα attenuates phosphorylation of mTOR, 4EBP1 and S6 (Lang and Frost 2007) and it has been shown to decrease MPS in cultured human myoblasts (Frost, Lang, and Gelato 1997). Furthermore, increased mRNA of cytokines TNFα and interleukin (IL)-6 in muscle of critically ill patients has been associated with reduced phosphorylation of not only mTOR and downstream effectors p70S6K and 4E-BP1 but also Akt and GSK3 (Constantin et al. 2011). Glucocorticoids have been shown to inhibit mTOR signaling *in vitro* and in rodents (Wang et al. 2006). Lipid infusion in healthy volunteers has been associated with reduced phosphorylation of 4EBP1 and reduced MPS (Stephens et al. 2015). Some lipid infusion studies in rats (Yu et al. 2002) and in humans (Dresner et al. 1999; Belfort et al. 2005) suggest lipid metabolites inhibit proximal insulin signaling, which could also contribute to reduced MPS. However, other human lipid infusion studies suggest negative effects of lipid on glucose transport and glucose oxidation are independent of insulin signaling pathways (Storgaard et al. 2004; Tsintzas et al. 2007; Høeg et al. 2011). Immobilisation reduces MPS but several human *in vivo* studies (de Boer et al. 2007; Glover et al. 2008; Kilroe et al. 2020) have not shown this to be associated with a reduction in mTORC1 signaling or gene expression, suggesting other mechanisms may also be involved.

Increases in signaling proteins *in vitro* do not always directly translate into increases in MPS in humans *in vivo.* Although EAA have been shown to increase both mTORC1 signaling and MPS in humans (Atherton et al. 2010), the activation of mTORC1 endured long after MPS had been reduced. MPS with pulse feeding has been shown to be equivalent to bolus feeding but in the absence of the detection of mTORC1 activity (Mitchell et al. 2015). (Greenhaff et al. 2008) found although Akt and p70S6K phosphorylation increased in line with insulin concentrations (under 4 different levels of insulin clamp) there was no corresponding change in MPS. Indeed (Murton et al. 2015) suggested that Akt activity in fact mirrors insulin concentration, while mTOR activity represents extracellular amino acid levels, rather than either indicating the level of protein synthesis. Therefore while *in vitro* data linking key signaling cascades to MPS is relatively clear, this has not been replicated in humans. Human *in vivo* studies, which elucidate changes in signaling proteins that correlate with changes in protein metabolism, are therefore required. Consequently, it is important to emphasize what actually happens with regard to protein balance in humans *in vivo*.

1.4.1.2 MPS in humans *in vivo*

In humans *in vivo*, infusion of amino acids in the fasted state has been found to increase protein synthesis producing a positive protein balance (Liu et al. 2002; 2006). However, even at high concentrations insulin only increases MPS in the presence of amino acids (Gelfand and Barrett 1987). In healthy volunteers (Fujita et al. 2006) showed that an intermediate (0.2 mU/kg/min) rather than high (0.4 mU/kg/min) dose of insulin administered to the leg in fact

stimulated leg PS the most, as the high dose decreased blood amino acid concentrations and blood flow was not sufficiently increased to compensate. They concluded that hyperinsulinaemia can increase MPS if it increases blood flow and amino acid delivery to the muscle. This is backed up by a recent systematic review and meta-analysis (25 studies) of the actions of insulin in relation to MPS, which concluded that there was no effect of insulin on MPS except when AA delivery was increased (Abdulla et al. 2016). Interestingly it would appear that this applies to an increase in AA generally and not to BCAA alone (Everman et al. 2016). Hence the general view in humans is that insulin has a permissive effect on MPS.

AA on the other hand produce a dose response increase in MPS (Bohé et al. 2003) up to a maximum dubbed 'muscle full' (Atherton et al. 2010). Maximum stimulation of MPS by AA is thought to be achieved by 3g leucine (Wilkinson et al. 2013), 10g of EAA (Cuthbertson et al. 2005) or 30g of high quality protein e.g. lean beef (Symons et al. 2009). However a recent study by (van Vliet et al. 2018) suggests that leucine, in the absence of the availability of other essential amino acids, does not stimulate MPS.

Necessarily crucial to the stimulation of MPS is the delivery of these AA to the muscle. Insulin's positive effects on blood flow are well known (Vollenweider et al. 1993; Fujita et al. 2006) and mixed meals (Vincent et al. 2006) and EAA (Mitchell et al. 2013) are also known to increase blood flow via insulin. Furthermore, AA have been shown to increase blood flow at fasting levels of insulin (Greenhaff et al. 2008).

Additionally, AA and insulin modulate each other's activity in humans. Insulin increases disposal of plasma AA (Fukagawa et al. 1986) by stimulating AA transport (Biolo, Declan Fleming, and Wolfe 1995; Fryburg et al. 1995). Effects on specific AA transporters have been demonstrated e.g. increased transport of AA by the 'A' system in humans during hyperinsulinaemia (Bonadonna et al. 1993). Inhibition of MPB by insulin also reduces availability of AA for MPS (Castellino et al. 1987). The net effect of insulin is therefore to reduce plasma amino acid concentrations. Whereas amino acids have been shown to inhibit glucose disposal (Krebs et al. 2002). The mechanism for this inhibition is thought to be via S6 kinase which phosphorylates serine residues on IRS-1 and therefore attenuates P13K activity (Tremblay et al. 2005).

1.4.1.3 Other regulators of MPS in humans

Human studies have shown IGF-1 (Russell-Jones et al. 1994; Liu et al. 2006) and GH (Fryburg, Gelfand, and Barrett 1991) stimulate protein synthesis. Further important anabolic agents include resistance exercise (Wolfe 2006) and testosterone (Forbes 1985).

Conversely MPS is down-regulated when these anabolic stimuli are scarce, indeed protein synthesis is reduced in starvation (Essén et al. 1992). Reduced MPS is also associated with inflammatory conditions (Rennie 2009). Indeed (Puthucheary et al. 2013) demonstrated reduced MPS in critically ill patients on day 1 of their admission to intensive care, but this had returned to normal by day 7. Network analysis of muscle metabolomics in the same subjects demonstrated a close relationship between diminished anabolic

signaling and inflammatory pathways (Puthucheary et al. 2018). Note that in some conditions however, notably burns (Biolo et al. 2002), protein turnover overall can be significantly increased.

Attenuated MPS has also been found in conditions associated with chronic inflammation such as cancer cachexia (Emery et al. 1984; Williams et al. 2012). Direct effects of cytokines on MPS in humans have however been difficult to establish. Infusion of TNFα into healthy humans, that increased plasma concentrations to levels equivalent to those seen in chronic conditions, was not found to reduce either whole body or MPS (Petersen et al. 2009). Whereas acute administration of interleukin (IL)-6 has been shown to reduce protein turnover in humans (van Hall et al. 2008) but it was suggested that this was due to an indirect effect of the concomitant reduction in circulating amino acids. Indeed cytokines may impact on muscle protein balance in several indirect ways such as by suppressing GH/IGF (Elijah et al. 2011), modulating blood flow (Groeneveld et al. 1999), increasing insulin resistance (Marette 2002) and increasing cortisol (van Hall 2012).

1.4.2 Muscle protein breakdown

Originally the definition of anabolic resistance was based on reduced MPS (Rennie and Wilkes 2005). However this definition has been expanded by one of the original authors (Rennie 2009) to include possible effects on MPB. This is because it is the balance between MPS and MPB that will determine whether muscle mass is lost, maintained or gained. Furthermore it was net BCAA balance, as a proxy for protein balance, that was measured in the

studies in this thesis. Therefore a consideration of MPB was thought to be useful context.

1.4.2.1 Cell signaling and its regulation

There are thought to be 3 main systems involved in protein degradation: calpains; lysosomal; and ubiquitin-proteasome (UPS). Calpains are proteases which breakdown the cytoskeleton of myofilaments and are activated by calcium and inhibited by calpastatin (Parr et al. 1992; Costelli et al. 2001). Within lysosomes, proteases breakdown extracellular and membrane proteins after their uptake by endocytosis, and cell proteins by autophagy (Scott and Klionsky 1998). However it is the UPS that is thought to break down the majority of cell proteins (Rock et al. 1994) including myofibrillar proteins (Solomon and Goldberg 1996). In rodents UPS has been shown to be activated by ubiquitin ligases muscle atrophy F–box (MAFbx) and muscle specific RING finger-1 (MuRF1) (Bodine et al. 2001; Lecker et al. 2004).

In vitro amino acids and insulin have been shown to attenuate MPB (Sadiq, Hazlerigg, and Lomax 2007) likely via forkhead box transcription factors (FOXO1 and 3) (Sandri et al. 2004). Upstream of FOXO is Akt, and the phosphorylation of Akt reduces the activity of FOXO (Brunet et al. 1999) and therefore proteolysis (Kamei et al. 2004). *In vitro* studies by the Goldberg group have also shown that lysosomal activity is influenced by transcription factors such as FOXO3, which are inhibited by insulin signaling via Akt (Zhao et al. 2007). It is important to highlight that the Akt/FOXO axis is one possible

link between synthetic and proteolytic pathways and further that this axis links insulin to both sides of the protein balance equation. Moreover FOXO binds to PDK suggesting a link to the down-regulation of COX in both muscle atrophic and insulin resistant conditions (Furuyama et al. 2003).

Pro-inflammatory cytokines are associated with the stimulation of MPB (Costelli et al. 1993). In muscle of critically ill patients increased mRNA of cytokines TNFα and IL-6 has been associated with increased mRNA and protein of MAFbx and MuRF1 (Constantin et al. 2011). Indeed TNFα, has been shown to induce MPB in rats (Goodman 1991; Zamir et al. 1992; Llovera et al. 1997). *In vitro* experiments suggest more than one mechanism for TNFα-induced stimulation of the UPS: via p38 MAPK (Li et al. 2005) and another transcription factor often associated with inflammation: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Li and Reid 2000). IL-1 and IL-6 have also been shown to increase protein breakdown in rats (Zamir et al. 1991; Goodman 1994; Haddad et al. 2005). Whereas IGF-1 has been shown to reduce MPB *in vitro* (Latres et al. 2005).

Ubiquitin ligases are regulated by insulin and amino acids in humans (Gelfand and Barrett 1987). Indeed studies have shown a reduction in MPB concurrent with a reduction in MAFbx protein levels (Greenhaff et al. 2008) or vice versa (Jones et al. 2004). However some human studies in atrophic situations have not shown consistent increases in atrogin activity (de Boer et al. 2007) or increases in atrogin mRNA and FOXO phosphorylation with concurrent decreases in Akt phosphorylation (Doucet et al. 2007). It seems likely that in muscle loss driven by inflammation the Akt/FOXO pathway is involved

(Crossland et al. 2008; Constantin et al. 2011) whereas in immobilisation this appears not to be the case (de Boer et al. 2007).

1.4.2.2 MPB in humans *in vivo*

MPB is decreased by insulin and this effect is enhanced when AA concentrations are low (Gelfand and Barrett 1987). Although maximal suppression of MPB by insulin occurs at relatively low concentrations of the hormone c.15 mIU/L (Wilkes et al. 2009), insulin's main contribution to positive protein balance is a reduction in MPB rather than an increase in MPS (Russell-Jones et al. 1994; Liu et al. 2006; Greenhaff et al. 2008). This was elegantly demonstrated by (Liu et al. 2006) in healthy volunteers whose protein kinetics were measured using stable isotope tracers in response to varying levels of insulin (up to 110 mU/L above fasting levels). Although the full range of insulin concentrations shifted negative fasting protein balance to positive this was achieved only through significant reductions in PB rather than increases in PS. AA also attenuate MPB in humans (Svanberg et al. 1996) and several studies have demonstrated the ability of BCAA specifically to reduce MPB (Louard, Barrett, and Gelfand 1990; MacLean, Graham, and Saltin 1994; Shimomura et al. 2006). Studies in post-operative patients have suggested that this effect may even be specific to the ketoacid product of leucine metabolism, alpha-ketoisocaproic acid (KIC) (Sapir et al. 1983; Francois et al. 1984).

1.4.2.3 Other regulators of MPB in humans

MPB has been shown to be significantly increased in critical illness in humans (Klaude et al. 2012; Puthucheary et al. 2013). However, evidence for the direct elevation of MPB in chronic inflammatory conditions in humans is lacking. Yet mRNA of elements of the UPS have been shown to be 2-4x higher in cancer cachexia vs. those with benign disease (Williams et al. 1999). More direct measures of MPB e.g. using stable isotope tracers in combination with a-v difference or excretion of 3-methylhistidine would be preferable. However, in the absence of these measurements mRNA can give an indication, with the caveat that changes in mRNA may not reflect changes in MPB.

Studies where TNFα has been infused directly into healthy humans are equivocal with both systemic and local infusions having been reported to have no effect on muscle protein turnover (De Blaauw et al. 1997; Petersen et al. 2009) or result in an increase in muscle protein breakdown during local infusion (Bach et al. 2013). Despite this TNFα has been associated with muscle loss in several conditions including reduced activity (Breen et al. 2013), cancer (Utech et al. 2012), COPD (Remels et al. 2010) and Crohn's disease (Subramaniam et al. 2015). Although this may well be due to a reduction in MPS as opposed to an elevation of MPB.

1.5 LINKS BETWEEN ANABOLIC AND INSULIN RESISTANCE

In cell signaling terms the Akt/mTORC1 and FOXO pathways have been established as a key link between insulin signaling and both MPS and MPB pathways. It is also known that insulin and AA modulate each other's activity. Moreover, anabolic and insulin resistance coexist in many conditions where muscle atrophy is present. To understand this relationship better, a series of human *in vivo* studies will be discussed. In order to provide context and further evidence for a relationship between the two a brief discussion of studies in a related group, people with T2DM, will precede a focus on the two main models employed in this thesis: people with obesity/overweight and the chronic inflammatory bowel disease, Crohn's disease.

1.5.1 Type 2 diabetes mellitus

An obvious group to consider when investigating links between insulin and anabolic resistance are people with T2DM. (Pereira et al. 2008) compared whole body protein metabolism in people with T2DM and obesity vs. controls with obesity under baseline and hyperinsulinaemic-euglycaemic-isoaminoacidaemic clamp conditions. Medication related to diabetes and lipid metabolism was suspended one week prior to the experimental trials. They found an overall negative correlation between fasting insulin levels and whole body protein net balance across all subjects. Under insulin clamp conditions whole body protein balance was blunted in males due to the failure of PS to increase in response. Change in protein synthesis during the clamp was correlated both with an index of fasting insulin sensitivity, the homeostatic

model assessment of insulin resistance (HOMA-IR), and glucose disposal during the clamp. In women PS did increase in response to the clamp and therefore in females there was no further effect of T2DM in addition to obesity (and its associated level of insulin resistance), when compared with lean females from a previous study (Chevalier et al. 2004). Inhibition of PB was not different between groups. This suggests that although insulin resistance is related to anabolic resistance in both sexes, T2DM confers greater anabolic resistance in men but not women.

Attenuated whole body protein synthetic response to an hyperinsulinaemic-euglycaemic-isoaminoacidaemic clamp resulting in a neutral/negative protein balance together with reduced glucose disposal has also been found in young, male and female glucose tolerant offspring of people with T2DM with normal BMI (Burgos et al. 2016). Indeed, positive correlations between rate of glucose disappearance and whole body PS as well as change in whole body protein net balance with the clamp were found. Once again there were no differences in the suppression of whole body PB. This suggests a link between insulin and anabolic resistance prior to the development of T2DM and independent of obesity.

Conversely, (Cuthbertson et al. 2017) assessed muscle fractional synthetic rate (FSR), of 2 different muscle fractions, in response to an oral bolus of both 10 and 20g EAA in elderly vs. aged matched subjects with T2DM while insulin was maintained at post-absorptive levels. They found no difference in myofibrillar (myo)FSR between groups although sarcoplasmic (sarco)FSR was reduced in the group with T2DM at the 20g dose. Any differential

response of muscle sub-fractions (e.g. contractile vs. oxidative) to anabolic stimuli may lead to a better understanding the specific effects and consequences of anabolic resistance at a cellular level. For example effects on muscle could predominantly affect quantity (contractile) or quality (oxidative). A clearer understanding would better facilitate the development of solutions.

Signaling proteins also differed between groups in the Cuthbertson et al. (2017) study with the increase in mTOR and 4E-BEP1 activation in the elderly not present in T2DM. The authors concluded that anabolic resistance was not involved in sarcopenia in T2DM. However there did not appear to be vast differences between the groups for example fat mass was the only anthropometric variable to be significantly different. Furthermore, subjects with T2DM had well-controlled glycaemia with removal of oral hypoglycaemic agents only on the morning of the study. Little information was supplied on the insulin sensitivity of controls except that post-absorptive levels of insulin and glucose were lower than in subjects with T2DM. Furthermore, supplementary material stated that the number of subjects for each treatment was only four, except in the assessment of basal synthesis for the subjects with T2DM where n=8. It is also possible that the EAA stimulus was too great to show up any differences as the same author showed previously that 10g of EAA was sufficient to stimulate maximal protein synthesis in young healthy subjects, although it did elicit an attenuated response in the elderly. No comparison was made to young controls in this study and it may be that T2DM does not confer any greater level of anabolic resistance than is already present with ageing. Although there was no clear measure of physical

activity, all subjects were described as physically active. Taken together it is possible that all of these factors contrived to produce no difference in myoFSR between subjects with T2DM and age-matched controls in this case.

Yet (Manders et al. 2012) also found no difference in whole body protein synthetic response or muscle FSR or whole body PB between subjects with T2DM and non-diabetic controls. However very large amounts of protein and carbohydrate (134g and 268g respectively) were used in the protocol and the authors themselves suggested more 'meal-like' quantities should also be tested. Indeed in a study comparing the effect of hyperaminoacidaemia vs. isoaminoacidaemia on whole body protein kinetics in sedentary males with T2DM and obesity (Bassil et al. 2011) found hyperaminoacidaemia restored whole body protein balance to normal levels in subjects with T2DM (vs. young lean controls). Thus, the discrepancies between these study findings may largely relate to the amount of protein given and suggest that while anabolic resistance may be present in people with T2DM, it can be overcome with increased protein/AA intake.

However more recently, 20g protein has been used to test postprandial skeletal muscle anabolic response in older subjects with T2DM vs. age-matched controls (Kouw et al. 2015). No differences in basal or postprandial mixed muscle FSR were found between groups once again suggesting that T2DM may not confer any further anabolic resistance than premature ageing. Yet both basal and postprandial concentrations of insulin and leucine were increased in subjects with T2DM vs. control, which the authors speculated may have been part of a compensatory mechanism protecting MPS.

Taken together the evidence seems to suggest that the level of insulin resistance of people with T2DM may not confer any greater level of anabolic resistance than that associated with obesity and / or ageing. If indeed anabolic resistance is similar between these populations then atrophy rates may also be expected to be comparable. However, if muscle protein breakdown is elevated further in T2DM (unknown) than the other conditions this may not be the case.

As such, while the weight of evidence suggests a relationship between the two types of resistance in humans, it is not certainly not clear-cut. It is possible that anabolic resistance does not link to rates of atrophy or that there are so many potentially contributing factors it has been difficult to remove all of these from experimental comparisons. In particular the consideration of both sides of the protein balance equation is thought to be important in order to establish any potential links.

Two populations at risk from developing both insulin and anabolic resistance will be examined in this thesis: people with obesity; and those with chronic inflammatory disease. Previous studies are discussed in the following section.

1.5.2 Obesity

The proportion of adults in the UK who are overweight (BMI >24.9-<30.0) and who have obesity (BMI >29.9) has reached epidemic proportions at 35.6% and a further 28.7% respectively (Baker 2019). Excess body fat mass is associated with a wide range of metabolic complications (Kelley et al. 1999;

Sheehan and Jensen 2000; Mittendorfer 2011) and reduced quality of life (Søltoft, Hammer, and Kragh 2009; Kearns et al. 2013). Fat mass (Abate et al. 1995; Goodpaster et al. 1997) and intramyocellular lipid (IMCL) accumulation (Pan et al. 1997; Krssak et al. 1999) is correlated with insulin resistance and some studies show lipid metabolites negatively impact on insulin signaling (Itani et al. 2002; Holland et al. 2007; Corcoran, Lamon-Fava, and Fielding 2007).

There has been controversy in recent years as to whether 'metabolically benign' obesity, as characterised by (Stefan et al. 2008) or a 'fat but fit' (Fogelholm 2010) phenotype exists. The latter systematic review suggested individuals with obesity who are fit had a lower risk of all cause mortality than those who were normal weight but unfit. Yet the fit individuals with obesity still had a greater risk of T2DM and greater prevalence of cardiovascular risk factors than those with normal weight and low fitness. The 'fat but fit' idea has also been countered by a more recent systematic review of the effect of metabolic health on all cause mortality in groups split by BMI (Kramer, Zinman, and Retnakaran 2013). Moreover, a very large cohort study of >1.3m Swedish men demonstrated the risk of all cause mortality was 30% lower in normal weight than in fit men with obesity (Högström, Nordström, and Nordström 2016).

Many studies have shown a link between obesity and anabolic resistance (Guillet et al. 2009; Murton et al. 2015; Beals et al. 2016; Smeuninx et al. 2017), but this has not been consistently demonstrated (Beals et al. 2017; Tran et al. 2018; Kouw et al. 2019). Indeed the term sarcopenic obesity has

been coined to describe the coexistence of the two conditions (i.e. age-related loss of muscle mass and quality, plus BMI 30+).

Studies investigating insulin and anabolic resistance in populations who are overweight can broadly be divided into 2 groups. Firstly, amino acid infusion studies under various clamp conditions and secondly, those using oral ingestion of dietary protein as a stimulus.

1.5.2.1 Amino acid infusion studies

The majority of the amino acid infusion studies reviewed in this section have demonstrated the presence of insulin resistance in response to various clamp conditions in overweight subjects vs. controls. While all studies have also found some evidence of modified protein metabolism in individuals with obesity/overweight, not all have demonstrated anabolic resistance. Some also reported a negative correlation between fat mass and rates of protein synthesis in the fasted (Guillet et al. 2009; Tran et al. 2018) and in the fed state (Murton et al. 2015).

Although the main focus of (Pereira et al. 2008) was subjects with T2DM vs. controls with obesity, the latter were also compared with a previous study from the same lab in lean subjects (Chevalier et al. 2004). Middle-aged men with obesity had reduced whole body PS and protein balance vs. young male lean volunteers in response to a hyperinsulinaemic-euglycaemic-isoaminoacidaemic clamp, although whole body protein balance was still positive in men with obesity. Across males with obesity (and T2DM) protein balance was negatively correlated with fasting insulin concentrations. Change

in PS (fasted to fed) was also correlated with a range of measures of insulin resistance including whole body fasted indices (HOMA-IR) and glucose disposal during clamp. No differences in whole body PB were observed between groups but reduced suppression of PB was correlated with fasting insulin.

In 2015 the same group (Chevalier et al. 2015) used a hyperinsulinaemic-hyperglycaemic-hyperaminoacidaemic clamp protocol with middle-aged men with obesity who were also shown to have reduced whole body glucose disposal and protein balance vs. younger lean controls. In contrast to the hyperinsulinaemic-euglycaemic-isoacidaemic clamp studies lower whole body protein balance in the subjects with obesity vs. controls was mainly driven by the attenuated suppression of whole body PB ($p=0.06$) rather than reduced whole body PS. Not only were subjects with obesity insulin resistant in comparison to controls but whole body PB may have also been negatively affected by the clamp's hyperglycaemia as this is known to increase PB in healthy subjects (Flakoll, Hill, and Abumrad 1993). Whole body protein balance in subjects with obesity had been found to be -65% in comparison to lean subjects in isoaminoacidaemic conditions (Pereira et al. 2008) but with hyperaminoacidaemia this gap was reduced to -32%. The fact that the subjects with obesity in were significantly older than the lean controls may well have contributed to this gap.

Despite the age mismatch total, myo- and sarco-FSR were not different between groups and neither was 4EBP1 phosphorylation. S6K1 phosphorylation was reduced in the subjects with obesity vs. controls and was

linked to reduced IRS1 activation perhaps reflecting the previously mentioned link between these two signaling proteins as part of a feedback loop between AA and insulin signaling rather than a deficit in MPS. This study suggests increased AA availability can improve protein balance in people with obesity via an increase in PS but not entirely rescue it as PB remains increased.

An earlier study from a different group (Guillet et al. 2009) deployed an hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp to study the anabolic response to AA in young male adults with obesity. These obese subjects were also found to have reduced whole body PS and protein balance as well as reduced glucose disposal vs. young lean controls. The suppression of whole body PB was not attenuated in the subjects with obesity unless their higher insulin levels were taken into account. Mixed muscle FSR in the subjects with obesity did respond to clamp stimulation but did so from a lower base than controls such that with clamp it was lower in the obese vs. controls. Interestingly mitochondrial (mito)FSR was reduced in the subjects with obesity in the fasted state and with clamp, and did not respond to the clamp stimulus.

(Murton et al. 2015) also used a hyperinsulinaemic-euglycaemic-hyperaminoacidaemic clamp and, in accordance with Guillet et al. (2009), found reduced glucose disposal and attenuated MPS but this time in older men with obesity vs. controls and measuring myofibrillar rather then mixed muscle FSR. This was despite a similar increase in mTOR phosphorylation with AA infusion in both groups. However, Murton et al. (2015) found no difference in muscle strength between subjects with obesity and controls as

well as reduced expression of mRNA of proteins involved in mitochondrial biogenesis and oxidative phosphorylation. This links to the reduced in mitoFSR found in Guillet et al. (2009) and suggests that muscle quality in people with obesity may be diminished.

Furthermore, the hyperaminoacidaemia in Guillet et al. (2009) and Murton et al. (2015) still resulted in reduced MPS in subjects with obesity vs. controls which contrasts with Chevalier et al.'s (2015) findings that MPS could be normalised by hyperaminoacidemia. While total AA provision in Chevalier et al. (2015) was less than in Murton et al. (2015) (22g vs. 28g respectively) it contained >50% more BCAA incl. c. 3.1g leucine vs. 1.6g in Murton et al. (2015). It is therefore possible that the greater provision of BCAA/leucine in Chevalier et al. (2015) stimulated MPS in the subjects with obesity to a greater extent than the smaller quantity used by Murton et al. (2015). This could support Chevalier et al.'s assertion that anabolic resistance in people with obesity can, at least in part, be ameliorated by increased AA with the caveat that a certain threshold of BCAA intake may need to be reached. Age of participants is unlikely to have been a factor as although subjects in Chevalier et al. (2015) were younger than in Murton et al. (2015) (44.3 yrs. vs. 66.4 yrs.) those in Guillet et al. (2009) were even younger (24.4 yrs.). Neither are insulin levels implicated as they were similar during clamp conditions at c. 80mIU/L in both Murton et al. (2015) and Chevalier et al. (2015) and were similar between subject groups.

Murton et al. (2015) also measured leg protein metabolism using the arterio-venous balance technique in combination with a phenylalanine tracer and

somewhat surprisingly found leg PB to be lower in older subjects with obesity vs. age-matched controls. This contrasts with whole body findings of attenuated suppression of PB in the previously described studies and the expectation that in the presence of insulin resistance suppression of PB may be attenuated. These findings suggest that the situation in muscle, which is estimated to account for only 25% of whole body PB (Heslin et al. 1992), is different from the whole body.

A recent study by (Tran et al. 2018) found lower fasting MPS in male and female subjects in their 30s with obesity in response to AA infusion, in accordance with Guillet et al. (2009) but in contrast to the other studies (Chevalier et al. 2015; Murton et al. 2015). Tran et al. (2018) suggested subjects with obesity in their study had reduced fasting MPS because they were more insulin resistant (higher fasting glucose and HBa1C vs. controls) than those in Chevalier et al. (2015) and Murton et al. (2015). However, Guillet et al.'s (2009) subjects were relatively insulin sensitive, having a HOMA-IR no different from control at 1.89 (calculated from the average fasting glucose and insulin values available) suggesting Tran et al.'s (2018) assertion may not be correct. IGF-1 levels were reduced in the subjects with obesity in the study by Tran et al. (2018), which could also have contributed to reduced fasting MPS, but were not measured by Guillet et al. (2009). Furthermore, in contrast to Guillet et al. (2009) MPS response to AA infusion was greater in Tran et al.'s (2018) subjects with obesity vs. controls such that fed MPS was no different to control. It is unlikely that this compensatory response was driven by the higher insulin in individuals with obesity vs.

controls, as subjects with obesity also had higher insulin than controls in the study by Guillet et al. (2009).

MitoPS was also lower in the post-absorptive state in the study by Tran et al. (2018) and was similar to controls with AA infusion, although the fasted to fed change was not significant in subjects with obesity. Actual quantity of BCAAs infused was similar to the study by Guillet et al. (2009) (11.5g vs. 11.7g respectively) and muscle biopsy timing (4hrs) was aligned. BMI of participants was similar (Tran et al. 2018 34.4 vs. Guillet et al. 2009 32.5), although % body fat mass was at least numerically greater in Tran et al. (2018) (33.1% vs. 29.3%). Further Tran et al.'s (2018) volunteers were over a decade older than Guillet et al.'s (2009). It is possible therefore that the subjects with obesity in these two studies were at a different stage in terms of development of obesity and/or insulin resistance leading to some sort of compensatory responses to protect MPS in the Tran et al. (2018) group that were not yet present in the Guillet et al. (2009) group.

1.5.2.2 Protein ingestion studies

Excepting Tran et al. (2018), the most recent studies investigating anabolic response in people with obesity have been designed to be more physiological i.e. use oral doses of whole protein but like the infusion studies have found mixed results.

(Beals et al. 2016) fed 3 groups of young male and female subjects (healthy weight, overweight and those with obesity) lean pork equivalent to 36g protein. In accordance with Murton et al. (2015), but in contrast to Chevalier

et al. (2015), they found myoPS reduced in overweight and obese vs. healthy weight. However, mTOR phosphorylation did not increase from high basal levels in response to protein ingestion in those who were overweight or with obesity, implicating some sort of signaling impairment. This contrasts with Murton et al. (2015) where signaling responses were no different to controls.

Reduced myoPS in Beals et al. (2016) was also associated with fat mass, yet overweight participants did not show any clear signs of developing insulin resistance as assessed by HOMA-IR and oral glucose tolerance test (OGTT). This led Beals et al. (2016) to conclude that anabolic resistance may develop prior to insulin resistance. However, this claim would need to be supported by measurement of muscle insulin sensitivity.

The lean pork ingested by subjects in the study by Beals et al. (2016) contained c. 3.3g leucine (BCAA content unavailable) i.e. similar to the quantity of leucine given to subjects in the Chevalier et al. (2015) study. It is estimated that on average 76% of ingested leucine in the fed state reaches the circulation (Hoerr et al. 1991). Therefore, the equivalent plasma level in Beal et al.'s (2016) subjects may rather have been closer to 2.5g making it intermediate between the amounts supplied by the Murton et al. (2015) (1.6g) and Chevalier et al. (2015) studies (3.1g) and as such it may not have been sufficient to overcome anabolic resistance in the obese. Although Mike Rennie's work did suggest a 'ceiling' in terms of the stimulation of FSR by ingesting EAA's in the elderly (Cuthbertson et al. 2005) this may not be apparent in other populations, feeding whole protein may give different results and importantly only one half of the protein balance equation was considered.

Indeed increasing anabolic stimuli may affect breakdown rates as well as synthetic rates, both of which contribute to net protein balance. Furthermore in terms of potentially overcoming anabolic resistance different stimuli may have different effects and therefore, for example, exercise could augment the effect of extra protein.

A subsequent paper, presenting different data from the same study (Beals et al. 2017), reported that mitoPS was increased with protein ingestion in both the healthy weight group and those with obesity. This is hard to reconcile with the lack of response of mitoPS to AA infusion found in Guillet et al. (2009) especially as both sets of subjects with obesity were young and sedentary. Guillet et al. (2009) did study only men and Beals et al. (2017) a mixed sex group and differences between the sexes in at least the whole body PS response to AA have been reported (Pereira et al. 2008). However, the level of insulin resistance of subjects with obesity also differed between studies. As previously mentioned fasting insulin resistance of subjects with obesity in Guillet et al. (2009) was no different from controls whereas in Beals et al. (2017) it was significantly greater both vs. their own controls and vs. Guillet et al.'s (2009) subjects with obesity (Beals et al. 2017 subjects with obesity HOMA-IR: 5.82 vs., Guillet et al. 2009 HOMA-IR: 1.89). It is possible therefore that the different findings could be because the subjects in these two studies were at a different stage in the development of insulin resistance. However, it should be noted that insulin assays were not harmonised prior to 2010 and therefore the figures from the Guillet et al. (2009) study may not be comparable with the later studies.

Furthermore, it is conceivable that some sort of compensatory mechanism may have protected the mitoPS response to protein ingestion as insulin resistance progressed in the study by Beals et al. (2017), echoing the findings of Tran et al. (2018). It is possible that insulin was involved in this. Although absolute insulin concentrations were greater in the study by Guillet et al. (2009) compared to Beals et al. (2017), the difference between subjects with obesity and controls in the former study was less pronounced (insulin levels of subjects with obesity were 2-fold higher than in controls) than in the latter study (where insulin concentrations in the obese were 3-fold higher than in controls). This data also suggests that fasting insulin resistance is not associated with a reduction in mitoPS.

(Smeuninx et al. 2017) investigated MyoPS responses to 15g milk protein in young lean, old lean and old subjects with obesity. The increase in MyoPS from fasted to fed state was greater in young lean than old lean and there was no significant increase in MyoPS seen from fasted to fed in old subjects with obesity, which suggested that both age and obesity confer some level of anabolic resistance. Post-prandial MyoPS rates were not only negatively associated with fat mass but also physical activity levels in older subjects, as assessed by step count.

(Kouw et al. 2019) investigated anabolic resistance in response to 25g of whey protein in relatively active middle-aged males (step count >7000/day) with obesity. Despite finding reduced postprandial PS and PB and therefore protein balance at a whole body level in subjects with obesity vs. lean there was no difference in postprandial myoPS. Using standard values for

composition of whey protein from cow's milk (Rafiq et al. 2016) the dose provided by Kouw et al. would have contained approx. 4g BCAA and 2g of leucine. Thus, after a first splanchnic pass the dose of leucine would likely have been similar to Murton et al. (2015) (i.e. c.1.5g) where a difference in myoPS between lean and obese was found. As such this study suggests that physical activity may reduce the attenuation of MPS in people with obesity.

Indeed (Hulston et al. 2018) showed no difference in muscle FSR in response to resistance exercise in young, recreationally active lean males and females vs. those with obesity. This raises the possibility that not only increased protein but also exercise could be used to combat anabolic resistance in people with obesity. Further (Smiles et al. 2019) used lipid infusion to induce anabolic resistance in middle-aged overweight males and to test if the attenuated mixed muscle PS response could be recovered by a bout of exercise. They showed that mixed muscle PS in response to ingestion of 30g protein in the presence of elevated FFA could be restored to control levels with exercise. This suggests physical activity can acutely as well as chronically enhance MPS in people with obesity. In fact physical activity status could provide an explanation for the discrepancies found between studies. However, it is very difficult to accurately compare physical activity status, particularly across studies with the information available. Further as physical activity is also linked to insulin resistance it is very hard to disentangle what is causal.

Taken together all of this evidence suggests that anabolic resistance is present in people with obesity but may vary according to level of stimulation

by AA, muscle fraction measured (mixed, sarco, myo, mito), level and/or stage of metabolic health and/or obesity, age and physical activity level. Additionally, the evidence suggests that it may be possible to ameliorate anabolic resistance in people with obesity using a combination of increased protein and physical activity. Furthermore, it appears that body fat mass and insulin resistance may be associated with anabolic resistance under certain conditions. Although one of the studies described (Beals et al. 2016) suggested anabolic resistance may precede insulin resistance the authors did not measure muscle insulin resistance and were extrapolating from whole body measures. Moreover evidence from two studies (Tran et al. 2018; Beals et al. 2017) suggests that as yet undefined compensatory mechanisms to protect postprandial synthetic responses may occur once fasting insulin resistance is apparent. The exact sequence of development and the coexistence of insulin and anabolic resistance in overweight individuals and people with obesity are therefore yet to be resolved.

1.5.3 Crohn's Disease

Inflammatory states are associated with both insulin resistance (Gump et al. 1974) and anabolic resistance (Rennie 2009). However, while anabolic resistance has been found in critically ill patients, little work has been done in those with chronic inflammatory disease. Crohn's disease (CD) is an autoimmune disease of the gastrointestinal (GI) tract with approximately one quarter of cases occurring in childhood (Sawczenko et al. 2001). Up to 60% of patients (Schneider et al. 2008) are thought to have reduced muscle mass and this is not always associated with reduced BMI (Adams et al. 2017).

Reduced muscle mass in CD has been linked to decreased muscle function (Werkstetter et al. 2012; Lu et al. 2016) reduced bone mass and density (Dubner et al. 2009; Bryant et al. 2015), reduced physical activity (van Langenberg, Papandony, and Gibson 2015), fatigue and diminished quality of life (Irvine, Zhou, and Thompson 1996). Not only will investigating anabolic and insulin resistance in CD contribute to our understanding of their relationship but it will also help in designing treatments to combat low muscle mass and improve quality of life in these patients.

1.5.3.1 Pathophysiology

CD is a chronic inflammatory bowel disease with a hallmark of discontinuous inflammation. Disease can occur at any site within the GI tract, although the disease location is commonly in the distal ileum and it may include inflammatory, stricturing and penetrating disease behaviours. In addition to clinical presentation and endoscopic and imaging investigations, markers of inflammation such as c-reactive protein (CRP) and faecal calprotectin (FCP) are routinely used to assess disease activity and active inflammation respectively (Gomollón et al. 2017). CD is characterized by periods of active disease and remission, with deep remission generally defined as complete and/or sustained mucosal healing with symptom resolution (Ruemmele et al. 2014).

1.5.3.2 Medical management

Current medical management guidelines for paediatric patients (Ruemmele et al. 2014) and adults (Gomollón et al. 2017; Gionchetti et al. 2017) include a

variety of therapies for induction and maintenance of remission which will be briefly discussed here in order to illustrate their possible effects on anabolic and insulin resistance and muscle mass. Key to induction of remission is treatment with corticosteroids and particularly in paediatric patients nutritional therapy (exclusive enteral nutrition). Corticosteroids are used because of their anti-inflammatory properties (Brattsand and Linden 1996) and they have been shown to blunt TNFα synthesis in monocytes (Steer et al. 2000). However they also stimulate MPB and attenuate MPS leading to an overall reduction in muscle mass (Hasselgren 1999). Indeed glucocorticoids have been shown to attenuate phosphorylation of 4E-BP1 in the presence of amino acids in healthy humans (Liu et al. 2004). Thus, as corticosteroids are known to retard growth as well as cause osteoporosis (McDonough, Curtis, and Saag 2008) they are used sparingly in paediatric as well as adult patients. Further, they stimulate gluconeogenesis and reduce skeletal muscle glucose uptake and disposal (Kuo et al. 2015) and as such are also associated with insulin resistance (Capristo et al. 1999).

Immunomodulators, such as thiopurines and methotrexate, are used to maintain remission. There are no known effects of these immunosuppressant drugs on skeletal muscle protein metabolism. Neither are widely known to affect insulin sensitivity although an *in vitro* study showed 6-mercaptopurine to have insulin sensitizing effects (Liu et al. 2013), and a study using methotrexate in rodents (Russo et al. 2012) came to the same conclusions.

Anti-TNFα (biological) therapy is used to both induce and maintain remission, particularly in patients refractory to other treatments and/or with high disease

burden. As previously discussed not only is TNFα implicated in the attenuation of MPS and promotion of MPB (Constantin et al. 2011) but it has also been shown to induce muscle insulin resistance (Plomgaard et al. 2005). Thus, the inhibition of TNFα should have positive effects on both anabolic response and insulin sensitivity. Infliximab (anti-TNFα) has been shown to increase muscle volume and strength in adult CD (Subramaniam et al. 2015) and reduce whole body protein turnover in paediatric patients (Steiner et al. 2007). However its effects on insulin sensitivity are equivocal with some studies showing no effect (Gentile et al. 2002; Koutroubakis et al. 2009) while a more recent study found positive effects (Paschou et al. 2018).

Aminosalicylates, which have both anti-inflammatory and immunomodulatory properties, may also be used in those with mild disease (Lim and Hanauer 2004; Ruemmele et al. 2014) but have no known adverse effects on anabolic or insulin sensitivity. The use of corticosteroids and anti-TNFα medication in particular should therefore be taken into account when investigating anabolic and insulin resistance in CD.

1.5.3.3 Nutritional status

Although the prevalence of obesity in patients with Crohn's disease is estimated to be close to that of the general population (Steed, Walsh, and Reynolds 2009; Nic Suibhne et al. 2013), undernutrition is still often seen in both children and adults (Marcil et al. 2019) and is prevalent during acute exacerbations and hospitalization (Nguyen, Munsell, and Harris 2008). The presence of malnutrition is likely due to a reduction in food intake, food

avoidance (Bergeron et al. 2018) and/or cytokine-induced anorexia (Plata-Salamán 1998; Bannerman et al. 2001), and malabsorption (Vaisman et al. 2006). Energy intake has been negatively associated with disease activity in some studies (Hodges et al. 1984; Rigaud et al. 1994) whereas another study found it to be comparable in active disease and controls (Wardle et al. 2018). Similar energy intake between patients in remission and controls has also been found (Filippi et al. 2006; Taylor et al. 2018). CD is also associated with anaemia as well as reduced levels of many vitamins and minerals, notably vitamin D and those with antioxidant properties (Gerasimidis, McGrogan, and Edwards 2011).

Growth retardation (Griffiths et al. 1993; Sawczenko and Sandhu 2003) and pubertal delay (Ballinger, Savage, and Sanderson 2003) are commonly found in paediatric disease and are associated with malnutrition and inflammation (Tigas and Tsatsoulis 2012).

1.5.3.4 Energy expenditure and fat oxidation

Higher fasted (Varille et al. 1996; Al-Jaouni et al. 2000; Zhao et al. 2015; Sammarco et al. 2017) and fed (Mingrone et al. 1999) resting energy expenditure (REE) have been reported in CD and linked to reduced body weight (Barot et al. 1982). Whereas other studies have found REE to be similar to controls (Schneeweiss et al. 1999) and no correlation between disease severity and REE (Wiskin et al. 2009; Takaoka et al. 2015). Several studies including those finding no difference in REE have however reported increased FOX in the fasted (Al-Jaouni et al. 2000; Schneeweiss et al. 1999;

Nishida et al. 2013) and fed states (Mingrone et al. 1999; Al-Jaouni et al. 2000). These reports are largely confined to subjects in active disease/those who are underweight, although this was not the case with the subjects in the study by Mingrone et al. (1999). Collectively, these findings imply changes in REE are largely linked to nutritional status while fuel oxidation may be influenced by both nutritional and disease status.

1.5.3.5 Low muscle mass and strength

Reduced muscle mass is prevalent in adult (Bryant et al. 2013; Zhang et al. 2017) and paediatric (Thayu et al. 2007; Bechtold et al. 2010; Ward et al. 2017) CD. Importantly studies have shown reduced fat free mass (FFM) to be more prevalent than reduced BMI (Bryant et al. 2013; Molnár et al. 2017; Adams et al. 2017). A systematic review of body composition in IBD by Bryant et al. (2013) found 28% of CD patients had reduced FFM although only 5% were underweight. Molnár et al. (2017) found reduced FFM in 30% and low BMI in 21% of 136 CD outpatients. Furthermore, in a retrospective study of 90 IBD patients (85% CD) starting anti-TNFα medication 45% of patients had reduced muscle mass (as measured by abdominal CT scan) (Adams et al. 2017). Interestingly of those who had reduced muscle mass 39% were underweight (BMI < 18.5), 41.5% had a normal BMI but 19.5% were overweight or obese, although the median BMI for those with reduced muscle mass was 19 vs. 24 for those with normal muscle mass. These studies demonstrate that reduced muscle mass is not confined to those who are underweight and is therefore not only driven by undernutrition. Reduced muscle mass is negatively correlated with disease duration (Schneider et al.

2008) and is not only present in active disease (Burnham et al. 2005) but persists in remission (Sylvester et al. 2009). Taken together these findings suggest that reduced muscle mass in CD is a result of both malnutrition and inflammation, which are both linked to anabolic and insulin resistance.

Several studies in adults have found reduced muscle strength in CD vs. controls (Wiroth et al. 2005; Valentini et al. 2008; Lu et al. 2016). In a patient group containing individuals in both active disease and remission (63% active and 37% remission) Lu et al. (2016) found reduced handgrip strength in CD, although once normalized for skeletal muscle mass the differences disappeared. Reduced handgrip strength associated with reduced muscle mass has also been found in paediatric patients with mild/inactive disease (Werkstetter et al. 2012). However, in 2 studies of adult CD patients in remission, a reduction in strength was found in CD vs. controls despite no differences in lean mass between groups (Wiroth et al. 2005; Valentini et al. 2008). This implies potential deficits in quality as well as quantity of muscle mass negatively impact on function in CD.

1.5.3.6 Physical activity levels

The question of the contribution of physical activity to low muscle mass in CD is an interesting one, as it is unclear whether it is a cause or consequence. Certainly, lower levels of total activity (van Langenberg, Papandony, and Gibson 2015) and lower levels of intense activity (Vogelaar et al. 2015) have been found in CD. Physical activity levels are inversely correlated with self-reported fatigue and CD patients report that their activity levels are limited by

their disease (DeFilippis et al. 2016; Tew, Jones, and Mikocka-Walus 2016). Exercise has been shown to be safe and effective in improving fatigue (Engels, Cross, and Long 2018) and quality of life (Loudon et al. 1999; Ng et al. 2007), as well as being a positive driver in the maintenance of remission (Jones et al. 2015). Furthermore, it has been shown to increase lean mass in CD in remission (Cronin et al. 2019). This further highlights the importance of the level of physical activity in terms of both insulin and anabolic sensitivity.

1.5.3.7 Insulin resistance

The presence of fasting hyperinsulinaemia has lead to the finding of whole body insulin resistance (HOMA-IR) in active disease (Bregenzer et al. 2006; Valentini et al. 2009). Furthermore TNFα infusion in healthy humans has been reported to decrease insulin sensitivity by inhibiting phosphorylation of Akt (Plomgaard et al. 2005). However patients in remission have been found to be insulin sensitive (Capristo et al. 1999).

1.5.3.8 Protein metabolism

Only one study has investigated protein metabolism in adult patients with active CD (Powell-Tuck et al. 1984), while 3 studies have been conducted in paediatric patients (Motil et al. 1982; Thomas et al. 1992; Steiner et al. 2007). In 1984 Powell-Tuck et al. measured 24hr whole body protein turnover in undernourished adult IBD patients (84% CD) during parenteral or enteral feeding. Measurement of whole body PS was via the stable isotope tracer ^{15}N glycine, given intravenously and its recovery in urinary ammonia. Whole body PB was assessed via methyl histidine excretion. Powell-Tuck et al.

(1984) demonstrated that whole body PS and PB were positively correlated with severity of disease.

An increase in whole body protein turnover with active disease has also been found in paediatric patients (Thomas et al. 1992). This study measured fasting whole body protein turnover with ^{13}C leucine infusion and showed that treatment inducing remission, either steroids or an elemental diet, reduced protein turnover. Steiner et al. (2007) had similar findings when investigating protein turnover with leucine, phenylalanine and tyrosine stable isotope tracers in paediatric CD pre and post infliximab infusion. However, although both whole-body fasting and fed protein turnover were decreased post infusion and clinical status improved, whole body protein balance was no different after infusion in either state. This suggests that high protein turnover in active disease can be reduced with standard treatments but protein balance remains unaltered.

Motil et al. (1982) investigated whole body protein turnover in the fed state (assessed with ^{13}C leucine infusion) in paediatric patients, pre- and post-nutritional supplementation. Nutritional supplementation was delivered overnight via nasogastric tube and equivalent to a 40% increase in protein and energy requirements. In contrast to the previous studies the authors found whole body protein turnover increased with nutritional supplementation. This may have been because these patients had growth failure and the response seen was indicative of an improvement in nutritional status (Golden, Waterlow, and Picou 1977) rather than an improvement in disease activity. Their post-supplementation protein turnover was not compared with a control

group and the level of disease activity post supplementation is not known. Although an overall increase in net protein balance with supplementation did not reach significance, whole body weight was increased and FFM increased, as assessed by whole body potassium. However, lean mass was not measured directly and whether any increase in FFM would have continued and become normalised is not known.

A 2-year longitudinal study of lean mass in paediatric patients (Sylvester et al. 2009) recruited at diagnosis, showed a significant improvement in lean mass but this was not normalised by the end of the study despite patients being in clinical remission. Furthermore, a study investigating the association of reduced lean mass with signaling proteins involved in protein metabolism in adult CD (active disease and remission) found Akt and 4EBP1 phosphorylation in skeletal muscle to be reduced in CD regardless of disease activity vs. controls (van Langenberg et al. 2014). There were however no differences in GSK-3β phosphorylation while mTORC1 was not measured. Interestingly a lower ratio of phosphorylated/total Akt was associated with reduced habitual physical activity and vitamin D3 in CD. The involvement of Akt implicates the insulin/IGF-1 signaling axis and there was a trend towards an association of lower ratios of phosphorylated/total Akt with higher serum IGF-1 (whereas CD were found to have lower IGF-1 than controls overall). Protein and mRNA levels of muscle atrophy markers (Mafbx and MuRF1) were no different to controls. This suggests a reduction in MPS but not MPB signaling thus raising the possibility that anabolic resistance may be present in CD and implicated in persistent muscle mass deficits.

1.5.4 Summary

In summary, anabolic and insulin resistance are often found to coexist but knowledge of any co-development and relationship in humans *in vivo* remains incomplete. While several studies have investigated these factors in obesity we are no closer to understanding their relative temporal development. Furthermore, very little work has been carried out in chronic inflammatory disease examining anabolic and insulin resistance despite the high prevalence of reduced muscle mass in this population.

1.6 AIMS

The main aim of this thesis was to test the hypothesis that reduced anabolic response to feeding (anabolic resistance) is associated with skeletal muscle insulin resistance in (a) overweight/obesity in adults, and (b) chronic inflammatory bowel disease (CD) in adults and paediatric patients.

Firstly, in a crossover study design, anabolic and insulin responses were assessed in an overweight/obese population using carbohydrate and protein meals taken orally, with or without prior ingestion of a high fat meal. Fat pre-ingestion (pre-loading the muscle with lipid) has been used as a method to acutely induce insulin resistance (Boden et al. 1991; Tsintzas et al. 2007). Hence if it also induces anabolic resistance this would suggest that they are linked. The acute induction and measurement of both insulin and anabolic sensitivity in response to oral protein and carbohydrate, subsequent to an oral fat load, has not previously been attempted in people with obesity.

Secondly, in paediatric CD in remission skeletal muscle anabolic and insulin responses to an oral mixed meal were compared to age- and BMI-matched controls. Patients in remission have previously been shown to have persistently reduced muscle mass but the relationship between anabolic and insulin resistance has not been investigated in this group.

Finally, anabolic and insulin resistance was examined in adult CD in active disease vs. healthy controls, matched for age and BMI, to elucidate to what extent they are associated with chronic systemic inflammation and reduced muscle mass.

2 GENERAL MATERIALS AND METHODS

2.1 STUDY SUBJECTS

2.1.1 Healthy volunteers

Healthy volunteers were recruited for all the studies in this thesis (Chapters 3,4 and 5) by advertisements placed across Nottingham University and Nottingham University Hospitals campuses. Direct mail to local male inhabitants aged 40-60 (Chapter 3) and Facebook advertisements to parents within the local area were also used to recruit healthy children to match the paediatric patients (Chapter 5). All participants were given a detailed written and verbal briefing of the study protocol and ample opportunity to ask questions before agreeing to participate. It was made clear that they were free to withdraw at any time. None of the subjects had given blood or taken part in another study within the preceding 3 months. All studies were approved by the University of Nottingham Medical School Ethics Committee. After consent subjects completed a general health questionnaire and underwent a short medical screening. This involved measurements of heart rate, blood pressure (lying and standing), height and weight, a 12-lead ECG and a small blood sample. The blood samples were analysed by the Department of Clinical Chemistry, QMC, Nottingham, for full blood count and hepatic, renal and clotting functions.

2.1.2 Crohn's Disease outpatients

Adult and paediatric Crohn's Disease (CD) outpatients were screened for appropriate medical inclusion criteria and classified as being in remission or active disease (see Chapters 4 and 5 for details) and referred to the study by their consultant physician. Study briefings and informed consent (parental consent and participant assent ≤15 years old) were performed by a member of the research team on the day of a standard clinical visit where possible or on a separate occasion by prior arrangement with the subject. Studies reported in Chapters 4 and 5 were approved by the NHS Health Research Authority, West Midlands – Coventry and Warwickshire Research Ethics Committee. Medical cover was available for the duration of all study visits and full experimental records were kept.

2.2 MAIN TECHNIQUES

2.2.1 The arterio-venous balance technique

The principle technique used in the experimental visits across all studies in this thesis (Chapters 3, 4 and 5) was the arterio-venous (a-v) balance technique (Andres, Cader, and Zierler 1956), which allows the estimation of nutrient net balance across a tissue bed. Overall forearm net balance provides a good estimate of forearm muscle net balance for several reasons. Firstly 80-85% of forearm blood flow is estimated to be through muscle (Andres et al. 1954), and 85% of glucose disposal during an hyperinsulinaemic-euglycaemic clamp is taken up by muscle (DeFronzo et al. 1981). Furthermore, the potential contamination of venous drainage by adipose tissue is thought to be reduced in the forearm vs. leg, largely due to the relative size of the depots (Guo and Jensen 2013). Although the absolute volume of muscle in the leg is much larger and would therefore potentially be more representative of skeletal muscle as a whole. Cannula placement, as discussed in a forthcoming section, is also important. The use of a deep perforating vein, draining largely muscle tissue is preferred to a superficial vein, which potentially includes more skin drainage.

Forearm a-v balance was therefore used to derive forearm muscle net balance of the following nutrients: branched chain amino acids (BCAA); glucose; and free fatty acids (FFA) under fasted and fed conditions. Therefore estimations of forearm muscle protein net balance, insulin sensitivity and fatty acid metabolism respectively, were produced. The

forearm was used in preference to the leg for the reason stated above, ease of cannulation and because one site in the paediatric CD patients was to be used for intravenous drug administration later in the day.

Differences between arterialized-venous and deep venous concentrations of each nutrient were multiplied by brachial artery blood flow and corrected for forearm lean mass (see blood sampling section for details of cannulae placement). Uptake or release of nutrients by skeletal muscle varies greatly as a result of blood flow, especially during postprandial periods, so raw a-v difference is insufficient to quantify net balance when steady state conditions are not present (Coppack et al. 1990). Correction for lean forearm muscle mass was made to normalise data for the amount of tissue present and facilitate inter-subject comparison.

The following equation was used for a given nutrient (N):

Net balance of N (μmol/min/100g of forearm lean mass)

= (Blood flow (ml/min) x ([N]arterialised – [N]venous (mmol/L or μmol/L)}/1000 (not for glucose)/ {lean mass (g) x 100}.

Blood flow was derived from ultrasound measurements (Toshiba Aplio 300, Cannon medical systems Ltd, Crawley, UK) of brachial artery diameter and blood velocity. Brachial artery diameter was measured 3 times, per sampling point, at both systole and diastole using images where the intima was clearly visible. The mean of these measurements in a ratio of 2/3:1/3, diastolic: systolic was used to derive the area of the blood vessel. Blood velocity was

measured once per sample time point. The equation for the calculation for blood flow is:

Brachial artery blood flow (ml/min)

= Cross sectional area of blood vessel (cm^2) x blood velocity (cm/s)

Where cross sectional area of blood vessel (cm^2)

= [π x (((mean diameter at systole (mm) / 10) x 1/3) x ((mean diameter at diastole (mm) / 10) x 2/3))]

All blood flow measurements were taken in the 2 minutes preceding blood sampling for consistency and to ensure the a-v balance results were as accurate as possible. Note that blood rather than plasma flow was used as haematocrit was not measured in these studies.

Forearm lean mass was measured by Dual-energy X-ray absorptiometry (DEXA) (Luna Prodigy, GE Healthcare, Little Chalfont, UK). Images from the whole body DEXA scan were subsequently analysed using the custom analysis function within the Lunar Corporation Analysis Software (Version 9.30) to derive forearm lean mass. The forearm was defined as extending from the proximal edge of the radial head where it joins the lateral epicondyle of the humerus to the tip of the distal phalanx. A rectangular region of interest (ROI) was created initially with the upper horizontal edge of the rectangle aligned with the proximal edge of the radial head. The lower vertices of the ROI were then adjusted to incorporate the distal phalanx whilst excluding the tissue mass of the leg and trunk. A note was taken of which arm had the

venous cannula at each visit and it was that arm's lean mass that was used to standardise nutrient uptake.

The key benefits of the a-v balance technique are: the range of nutrients and indices derived; the high degree of temporal resolution; and that it is minimally invasive. These advantages are highlighted in making a comparison with the use of stable isotope essential amino acid tracers in combination with muscle biopsies. The latter technique is commonly deployed to derive muscle protein synthesis (MPS) (Nair, Halliday, and Griggs 1988) and can be used in combination with the a-v balance technique to calculate muscle protein breakdown (MPB) (Barrett et al. 1987). This would have provided a level of specificity (MPS & MPB) not afforded by the a-v balance technique alone, which instead estimates overall AA balance. However, not only would muscle biopsies not have been a suitable technique with the paediatric cohort in Chapter 4, but with one, maximum two, fed sampling points temporal resolution would have been low. In contrast, with a-v balance, measurements were taken every 15 (Chapter 3) or 20 minutes (Chapters 4 and 5). Furthermore, the use of one stable isotope tracer and muscle biopsy would mean only protein metabolism could be investigated (although multiple tracers could be deployed), whereas a-v balance allows measurement of muscle metabolism of all key nutrient groups simultaneously. The use of one stable isotope tracer also assumes the selected amino acid mirrors the action of other amino acids. In the studies in this thesis all three BCAA's were measured providing a larger sample of essential amino acid (EAA) activity. Although phenylalanine is now widely used, due to its lack of metabolism by skeletal muscle. To further minimize the use of invasive techniques it would

have been possible to calculate whole body nitrogen balance by collecting urine and analyzing urea content. However this would have provided limited information at the whole-body level and not necessarily have mirrored skeletal muscle metabolism (Liu and Barrett 2002). As such a-v balance was chosen to measure muscle net balance of a range of nutrients.

2.2.2 Dual-energy X-ray absorptiometry (DEXA)

Baseline DEXA scans (Luna Prodigy, GE Healthcare, Chicago, USA) were taken to provide measurements of total and regional body composition in all studies (Chapters 3, 4 and 5). DEXA works on the principle that the attenuation of X-rays by bone, fat and skeletal muscle are different and so measurements of the mass of each tissue can be derived (Ellis 2000). Appendicular lean mass (LM) (sum of lean mass in the limbs measured by DEXA) and ASMI (appendicular lean mass (kg) / height $(m)^2$) were calculated to give more precise indices of skeletal muscle mass than total lean mass alone.

Total body lean mass was used to standardise substrate oxidation calculations in order to facilitate comparison between subjects. Measurements of forearm lean mass were also used to standardise forearm nutrient uptake (BCAA, glucose and FFA) in all studies and strength measurements in the Crohn's disease studies (Chapters 4 and 5).

2.2.3 Indirect calorimetry (IDC)

IDC was used in all studies to measure inspired volumes of oxygen and expired volumes of carbon dioxide in order to calculate whole body substrate oxidation of subjects in the fasted and fed states (Frayn 1983). Cosmed (Cosmed srl, Rome, Italy) was used for the Crohn's studies (Chapters 4 and 5) and GEM (GEM nutrition Ltd, Daresbury, UK) for the study in Chapter 3. Both systems used a ventilated hood. At the start of each visit turbine and gas calibrations were performed for Cosmed and 2 overall calibrations for GEM. In between sessions a further gas calibration or overall calibration, respectively, was carried out. The first 5 minutes of data was discarded to allow for a steady state to be achieved and the mean of the following 15 minutes of data was used to provide the basis for the calculations of: substrate oxidation; respiratory exchange ratio (RER); and resting energy expenditure (REE).

Substrate oxidation values can be calculated because the volumes of oxygen needed and carbon dioxide produced from standard fats and carbohydrates are known. (Péronnet and Massicotte 1991) equations were used in the Crohn's disease studies (Chapters 4 and 5):

$$\text{Rate of fat oxidation (FOX) (g/min)} = 1.695\ VO_2 - 1.701\ VCO_2$$

$$\text{Rate of carbohydrate oxidation (COX) (g/min)} = 4.585\ VCO_2 - 3.226\ VO_2$$

Where volume of carbon dioxide (VCO_2) & volume of oxygen (VO_2) inputs are in litres per minute (l/min).

Note: Péronnet and Massicotte use glucose as a standard molecule for carbohydrate COX and the average composition of human adipose tissue triacylglycerol (Berry et al. 1986) for FOX.

In the study in Chapter 3 nitrogen excretion was derived from the analysis of urea concentrations in both urine and plasma. Therefore Frayn's (1983) equations (Frayn 1983), which take into account protein oxidation, were used:

$$FOX\ (g/min) = 1.67VO_2 – 1.67VCO_2 – 1.92n$$

$$COX\ (g/min) = 4.55VCO_2 - 3.21VO_2 – 2.87n$$

Where VCO_2 & VO_2 inputs are in litres per minute (l/min) and n = nitrogen excretion rate (g/min) (see urea analysis for the calculation of n).

Note: Frayn uses glucose as the standard molecule for COX and palmitoyl-stearoyl-oleoyl-glycerol for FOX and takes the values for protein from Magnus Levy 1907.

Where protein oxidation had been calculated VO_2 and VCO_2 were adjusted to produce a standard non-protein RER:

$$Adjusted\ VO_2 = (0.746 \times COX\ (g/min) + (2.03 \times FOX\ (g/min)) + (0.966 \times n\ (g/min)$$

$$Adjusted\ VCO_2 = (0.746 \times COX\ (g/min) + (1.43 \times FOX\ (g/min)) + (0.782 \times n\ (g/min)$$

$$Adjusted\ RER = volume\ of\ CO_2\ (ml/min) / volume\ of\ O_2\ (ml/min)$$

Substrate oxidation rates were converted from g/min to kJ/ min using standardised values for the number of kJ in a gram of a. fat: 39.4; and b.

carbohydrate: 15.6kJ. They were further standardised by total body lean mass as described above (DEXA).

2.2.4 Handgrip dynamometer

An assessment of forearm muscle function was undertaken at the end of the meal tolerance test in both of the Crohn's disease studies (Chapters 4 and 5). Participants performed 12 maximal static voluntary contractions, with the same time interval between contractions, using an handgrip dynamometer (MIE medical research Ltd. Leeds, UK), with both dominant and non-dominant arms. The peak contraction was taken as maximal handgrip isometric strength (kg), usually achieved within the first 2-3 contractions. Level of fatigue was derived from the difference in peak strength and strength measured at the end of 12 maximal contractions (mean of the last 3) (White et al. 2013). This method of endurance testing has been validated by White et al. (2013) and is argued to be superior to testing by sustained grip strength as the latter relies too much on the motivation of participants.

Participants were familiarised with the protocol during screening and the same trained operator both gave instructions and took measurements from all participants (Bandinelli et al. 1999). A trained operator and a clear reproducible protocol were the key reasons (over and above a reliable instrument) given by Bandinelli et al. (1999) when demonstrating high reproducibility in taking strength measurements using a handgrip dynamometer. The measurements were not repeated at different time points during the visit in case fatigue became a factor and it caused disruption to the

physiological measurements being taken. Muscle strength measurements were standardized for forearm muscle size (see a-v balance section), and in the paediatric study (Chapter 4) for height and age, using the method developed by (Rauch et al. 2002), to facilitate comparison.

2.2.5 Metabolomics

Plasma samples were extracted and analysed by ultra high performance liquid chromatography mass spectrometry (UHPLC-MS) at the Phenome Centre Birmingham. For details of extractions, raw data processing, QC filtering and multivariate data analysis see Appendix 1. For details of univariate analysis see Chapter 3. This was an untargeted analysis, hence although QC samples were used no metabolite standards were deployed. As such results were normalised to total sample peak area (normalised %), and are therefore semi-quantitative and not entirely analogous to concentrations. After assigning each metabolite to a metabolite class and univariate analysis data were supplied by the Phenome Centre Birmingham. For details of subsequent data analysis see Chapter 3.

2.2.6 Blood sampling

Participants were asked to rest in a semi-supine position on a bed and after an injection of 1% lidocaine to anaesthetise the area, a cannula was inserted in a retrograde fashion into a superficial vein on the dorsal surface of the dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand (Gallen and Macdonald 1990). To ensure arterialisation initial blood samples were taken after a

minimum of 20 minutes in the hand-warming unit. A second cannula was placed in a deep antecubital vein in the non-dominant forearm, also in a retrograde fashion (Greenfield 1961). Both of these cannulae were kept patent with a saline drip and used for blood sampling. In terms of the a-v balance method therefore the arterialised-venous sample from the hand was the 'arterial' sample and the sample from the contralateral forearm the 'venous' sample. Samples were drawn concurrently, by two different individuals. Although both shunting and volume of distribution of a particular metabolite affect transit time (Macdonald 1999) we concluded that staggering sampling timings to fit three different metabolite transit times was impractical. Particularly as this would have involved making assumptions about the volume of distribution in patients with chronic inflammatory disease, which may well have differed from controls.

Ideally the venous sample is taken from a deep perforating vein in order to minimize the contribution of skin and adipose tissue to the sample. Ultrasound guided insertion was used to place the cannula into a deep perforating vein via a superficial antecubital vein. The operator guided the cannula past the bifurcation to ensure the tip was in the descending branch. This technique was used to locate and confirm deep vein cannulation in all adult volunteers (Chapters 3 and 5). In the paediatric study (Chapter 4) however a more superficial vein was used in all participants as the majority of patients were due to have an infusion of anti-tumor necrosis factor alpha (TNFα) therapy using the same cannula that afternoon. This may mean that less of a difference would be detected between the arterial and venous samples in that study, as the more superficial vein is likely to be contaminated

with skin and adipose tissue drainage. Amount of blood drawn and frequency of sampling varied according to study and is detailed within the methods sections of the experimental chapters.

Whole blood samples for glucose estimation were immediately decanted into micro-tubes, containing sodium fluoride and heparin and kept on a roller for 3 minutes before determination of glucose concentration. Further portions of whole blood were immediately decanted into 2 lithium heparin tubes, (i) with added Ethyleneglycol-Bis-β-Aminoethylether Tetraacetate (EGTA) (15 µl / ml whole blood) and (ii) with EGTA and tetrahydrolipostatin (THL) (at 10 µl / ml whole blood). THL was used in order to maintain representative lipid fractions in samples to be analysed for free fatty acids (FFA). Samples were then centrifuged at 4°C at 4400rpm for 10 minutes and the separated plasma stored at -80°C until further analysis. Plasma was subsequently analysed for (i) BCAA concentrations and (ii) FFA concentrations in all studies included within this thesis. Plasma was also analysed for CRP and cytokines in both Crohn's disease studies (Chapters 4 and 5), for testosterone in the paediatric Crohn's study (Chapter 4) and urea concentrations in the study in Chapter 3. A final portion of blood was left to clot for at least 20 min at room temperature before centrifugation at 4°C at 4400rpm for 10 minutes. The serum was decanted and stored at -80°C until analysed for insulin and triacylglycerol (TAG) concentrations in all studies and additionally for glycerol in the study in Chapter 3.

2.2.7 Urine collection

Urine was collected directly into plastic bottles at baseline, 3.5 hours and 7 hours in the study in Chapter 3. Total volume was measured at each time point and a 1.75ml sample was taken and frozen at -80°C until analysis of urea content (see indirect calorimetry above and urine metabolite analysis below).

2.2.8 Blood metabolite analysis

2.2.8.1 Blood glucose

Whole blood samples (c. 0.5ml) were analysed for glucose (mmol/L) by a glucose analyser (YSI 2300 STATplus, Yellow Springs Instruments, Ohio, USA) 3 minutes after being drawn. A study in our lab (unpublished data) has shown that the concentration of glucose stabilized after this period. This method was used to ensure that the readings of arterialised and venous samples were comparable and therefore facilitate the validity of the a-v balance method. Arterialised samples were always measured prior to venous samples.

2.2.8.2 Plasma branched chain amino acids (BCAA)

In measuring muscle protein balance it is expedient to measure a representative sample of essential amino acids (EAA) knowing that their appearance can result only from dietary intake and / or protein breakdown and disappearance from protein synthesis and / or oxidation. The BCAA, leucine, isoleucine and valine, are a relatively homogenous group of EAAs

that make up approximately 17% of circulating amino acids in healthy adults aged 21-38 (Hammarqvist et al. 2010) and comprise 14-18% of muscle protein (Layman and Baum 2004). As they account for 35–40% of dietary EAAs in body protein (Riazi et al. 2003) they are therefore quantitatively the most important group of EAAs thus providing a best estimate of total muscle protein balance.

Plasma (i) was analysed for BCAA concentrations by spectrophometric assay based on the method proposed by (Beckett et al. 1996). Leucine dehydrogenase (Sigma-Aldrich Company Ltd, Dorset, UK) was diluted in 25 mM sodium phosphate buffer (pH 7.2). A 0.2 M glycine-KCL-KOH buffer adjusted to pH10.5 with 2 M KOH was used. Leucine dehydrogenase deaminates all 3 BCAA's reducing NAD to NADH:

$$\text{Leucine} + NAD^+ \Leftrightarrow \alpha\text{-ketoisocaproate} + NADH + H^+ \quad [1]$$

$$\text{Isoleucine} + NAD^+ \Leftrightarrow \alpha\text{-ketomethylvalerate} + NADH + H^+ \quad [2]$$

$$\text{Valine} + NAD^+ \Leftrightarrow \alpha\text{-ketovalerate} + NADH + H^+ \quad [3]$$

The rate of appearance of NADH can be derived from the difference in absorbance (at 340nm) measured by spectrophotometer (Spectra max 190, Molecular Devices, San Jose, USA) prior to enzyme addition and once the reaction is complete at 60 minutes. The concentration of BCAAs (μmol/L) is calculated with reference to a known set of leucine standards. The intra-assay coefficient of variation was 4.4%.

2.2.8.3 Plasma free fatty acids (FFA)

Plasma total FFA concentrations were analysed by enzymatic-colorimetric assay (Wako NEFA C test kit, FUJIFILM Wako Diagnostics U.S.A. Corporation) according to the manufacturers instructions. Non-esterified fatty acids (NEFAs) are first converted to Acyl CoA by Acyl-CoA-Synthetase (ACS) (reaction 4) then oxidized by Aycl-CoA-Oxidase (ACOD) to hydrogen peroxide (H_2O_2) (reaction 5). Peroxidase (POD) finally facilitates the donation of oxygen from H_2O_2 to condense 3-Methyl-N-Ethyl-N-aliline (MEHA) and 4-aminophenazone to produce a red pigment (reaction 6).

$$\text{NEFA} + \text{ATP} + \text{CoASH} \rightarrow \text{Acyl-CoA} + \text{AMP} + \text{PPi} \quad [4]$$

$$\text{Acyl-CoA} + O_2 \rightarrow \text{2,3-trans-Enoyl-CoA} + H_2O_2 \quad [5]$$

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{MEHA} \rightarrow \text{Quinoneimine-color} + 4H_2O \quad [6]$$

The intensity of the red pigment produced is proportionate to the concentration of FFA and is measured by spectrophotometer at 540nm (Pentra 400 automated analyser, Horiba Medical Instruments, Montpellier, France). The concentration of FFAs (mmol/L) is then calculated with reference to a set of standards by regression analysis. The intra-assay coefficient of variation was 1.8%.

2.2.8.4 Serum insulin

Serum insulin concentrations were analysed by radioimmunoassay (Millipore Human insulin specific kit) according to the manufacturers instructions. The

kit works on the double antibody technique (Morgan and Lazarow 1962). Briefly insulin competes with a labelled tracer antigen (^{125}I-labelled human insulin) for binding with a human insulin antiserum. Binding sites are initially saturated with tracer but the addition of non-labelled insulin displaces the tracer in a manner proportionate to the amount of non-tracer present. Bound and non-bound tracer fractions are separated and measured using a gamma counter (Cobra II auto gamma, model no. 5005, Canberra Packard). Insulin concentrations (mIU/L) are then determined using a programme, which matches counts to the results from a series of known standards (algorithm: 4 parameter logistic plot using % isotope bound). R^2 were >0.9995. Note that any values below the lowest standard (3.125mIU/L) were calculated by extrapolating the curve. Intra-assay variation was 5.2%.

The forearm muscle glucose net balance was the primary measure of muscle insulin sensitivity used in all of the studies in this thesis. The Matsuda index was also used as an index of postprandial whole-body insulin sensitivity (Matsuda and DeFronzo 1999; DeFronzo and Matsuda 2010). The lower the index the higher the level of insulin resistance.

$$\text{Matsuda Index} = 10000 / (\sqrt{} G_0 * I_0 * G_{mean} * I_{mean})$$

Where G_0 = fasting plasma glucose (mg/dl), I_0 = fasting serum insulin mIU/L,

G_{mean} = mean plasma glucose concentration during MTT (mg/dl) 0-120mins and

I_{mean} = serum insulin concentration during MTT (mIU/L) 0-120mins.

2.2.8.5 Serum triacylglycerols (TAG)

Serum TAG concentrations were analysed by colormetric enzymatic assay (ABX Pentra Triglycerides CP, Horiba, Kyoto, Japan). Triglycerides are hydrolysed to glycerol and FFAs by lipoprotein lipase (LPL) (reaction 7). Glycerol is phosphorylated by glycerokinase (GYK) (reaction 8). The oxidation of Glycerol-3-phosphate by glycerol 3 phosphate oxidase (GPO) produces H_2O_2 (reaction 9). 4-aminoantipyrine and p-chlorophenol are then condensed by peroxidase (POD) using oxygen from H_2O_2 to form quinoneime dye (reaction 10).

$$\text{Triglycerides} + H_2O \rightarrow \text{glycerol} + \text{fatty acids} \quad [7]$$

$$\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol-3-phosphate} + \text{ADP} \quad [8]$$

$$\text{Glycerol-3-phosphate} + O_2 \rightarrow H_2O_2 + \text{DHAP} \quad [9]$$

$$2H_2O_2 + \text{4-AAP} + \text{p-Chlorophenol} \rightarrow \text{Quinoneimine} + 4H_20 \quad [10]$$

The intensity of the dye, measured at 540nm by spectrophotometer, is proportionate to the concentration of glycerol and therefore TAG (mmol/L). The intra-assay coefficient of variation was 2.2%.

2.2.8.6 Serum free glycerol

This assay was only performed for samples from the study reported in Chapter 3. FFA concentrations were unexpectedly similar between visits during the first 3 hours of the study and so serum glycerol was measured to

provide further insight into FFA metabolism. Serum free glycerol concentrations were analysed by colorimetric enzymatic assay (Sigma F6428). The set of enzymatic reactions used are similar in principle to those described for the estimation of TAG above, minus the initial hydrolysis stage. Although the dye produced is the same, the intermediary enzymes and products differ, largely due to the use of a different manufacturer's reagent.

The phosphorylation of glycerol by ATP is catalyzed by glycerol kinase (GK) and produces glycerol-1-phosphate (G-1-P) and ADP (reaction 11). Glycerol phosphate oxidase (GPO) then catalyzes the oxidation of G-1-P to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) (reaction 12). H_2O_2 is coupled with 4-aminoantipyrine (4-AAP) and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine (ESPA) by POD producing quinoneimine dye (reaction 12).

$$\text{Glycerol} + \text{ATP} \rightarrow \text{G-1-P} + \text{ADP} \quad [11]$$

$$\text{G-1-P} + O_2 \rightarrow \text{DAP} + H_2O_2 \quad [12]$$

$$H_2O_2 + \text{4-AAP} + \text{ESPA} \rightarrow \text{Quinoneimine dye} + H_2O \quad [13]$$

Spectrophometric analysis and derivation of glycerol concentration (mmol/L) was as described for TAG above. The intra-assay coefficient of variation was 1.76%.

2.2.8.7 Plasma cytokines

Plasma concentrations of inflammatory cytokines are often used as markers of systemic inflammation and were therefore measured in the subjects in the two Crohn's disease studies (Chapters 4 and 5). Concentrations of inflammatory cytokines: tumor necrosis factor (TNF)α; interleukin (IL)-6 and IL-1β were measured in baseline (over-night fasted) arterialized plasma samples by colorimetric sandwich ELISA (Duoset Elisa, R&D systems, Minneapolis, US) (TNFα - DY210-05, IL-6 - DY206-05 & IL-1β -DY201-05).

All assays were performed according to manufacturer's instructions with the following exception: carbonate-bicarbonate buffer (Sigma-Aldrich) was used to dilute the capture antibody as optimisation revealed higher signals were produced than with the recommended phosphate buffered saline (PBS).

All three ELISA's work on the same principle, where the cytokine of interest is bound by both a capture and a detection antibody. The detection antibody is biotinylated so when streptavidin-horseradish peroxidase (HRP) is added, the biotin element of the detection antibody reacts with the streptavidin. With the addition of a substrate solution containing H_2O_2 and tetramethylbenzidine (TMB), HRP catalyses the reduction of H_2O_2 to water while TMB acts as a hydrogen donor causing the production of a blue dye. Finally a stop solution containing sulphuric acid (2N H_2SO_4) is added and the dye changes to yellow. The amount of yellow dye present is then measured by spectrophotometer at 450nm and is proportional to the concentration of target cytokine in the sample. Concentrations (pg/ml) are then computed using a known set of

standards. Spline analysis was used to interpolate values from the standard curves (Prism V7.0).

2.2.8.8 Plasma bioavailable testosterone

Testosterone is well-known to have anabolic effects on muscle mass (Brodsky, Balagopal, and Nair 1996) and androgens have been found to be reduced in paediatric CD (Ballinger, Savage, and Sanderson 2003). It was therefore decided to measure testosterone concentrations in plasma of subjects in the paediatric study (Chapter 4) to see if there was any association with muscle mass. Bioavailable testosterone was measured in baseline (over-night fasted) arterialized plasma samples, diluted 10x, by colorimetric ELISA (Testosterone parameter assay kit KGE010, R&D systems, Minneapolis, US), according to manufacturer's instructions. The principle of the assay is one of competitive binding. Testosterone in the sample competes with horseradish peroxidase (HRP)-labeled testosterone for sites on a monoclonal antibody already bound to a goat anti-mouse antibody on the plate. As per the cytokine assays HRP reacts with H_2O_2 and TMB in a substrate solution producing a blue dye. A stop solution containing sulphuric acid (2N H_2SO_4) is then added turning the dye yellow. The amount of dye present is then measured by spectrophotometer at 450nm and is inversely proportional to the concentration of testosterone (ng/ml) in the sample. A 4-parameter logistic regression (elisaanalysis.com) was used for the analysis. The intra-assay coefficient of variation was 4%.

2.2.8.9 Plasma urea

In order to estimate whole body nitrogen excretion, measurement of the concentration of urea in both plasma and urine is necessary. It is however only an estimation as urea doesn't account for all the nitrogen excreted in urine (c.90%) (Salazar 2014). Nitrogen excretion can then be used to calculate whole body net protein oxidation and non-protein RER providing more accurate measures of FOX and COX as described in the IDC section above. Rather than estimating nitrogen excretion a general estimate of protein oxidation of 15% can be assumed (Tentolouris et al. 2008). However it was considered important to derive an estimate of protein oxidation per subject in the study in Chapter 3 as obese subjects have been shown to oxidise higher levels of protein than healthy subjects (Bruce et al. 1990). See the following urine metabolite analysis section for details of the assay and calculations used to derive nitrogen excretion rate.

2.2.9 Urine metabolite analysis

2.2.9.1 Urea

Urinary urea and plasma urea concentrations were analysed by spectrophometric assay (Randox laboratories Ltd. UK, UR221). Urine samples were first diluted to 1 in 20 in ddH_2O to ensure they were within the range detectable for range of the assay. Plasma samples were analysed undiluted. Urease hydrolyses urea producing ammonia and CO_2 (reaction 14). Glutamate dehydrogenase (GLDH) then combines the ammonia with α-oxoglutarate and NADH producing glutamate and NAD^+ (reaction 15).

$$\text{Urea} + H_2O \rightarrow 2NH_3 + CO_2 \quad [14]$$

$$2\ \alpha\text{-oxoglutarate} + 2NH_4^+ + 2NADH_2 \rightarrow \text{L-glutamate} + 2NAD^+ + 2H_2O \quad [15]$$

The rate of disappearance of NADH was therefore derived from the difference in absorbance measured by spectrophotometer (Spectra max 190) at 340nm and 37°C at t = 0 and once the reaction was complete at t = 30. The concentration of urea (g/ml) was calculated with reference to a known set of urea standards.

Calculation of nitrogen excretion is as follows:

Urinary N (g/min) = ((Urinary urea (g/ml) * 0.47) * Urine volume (ml)) / recovery time (mins)

Where N = nitrogen and 0.47 takes account of the fact that only 47% of urea is nitrogen.

Urinary nitrogen excretion is then corrected for any changes in the urea pool during recovery as measured in plasma:

Correction factor (g/min) = (((pre-recovery plasma urea concentrations (g/L) - post-recovery plasma urea concentrations (g/L)) * 0.47) * (0.57 * body mass (kg)))) / 1000 * time difference between sampling points (mins)

Where total body water is estimated as approx. 57% of body mass.

Corrected N excretion rate = Urinary N excretion (g/min) +/- correction factor (g/min)

2.3 STATISTICAL ANALYSIS

Two of the three studies in this thesis (Chapters 4 and 5) compared Crohn's disease patients' response to a meal stimulus with control subjects. As such a two-way analysis of variance (ANOVA) with repeated measures for one factor (time) was deployed to analyse plasma metabolites and blood flow. If significant differences were found Sidak's multiple comparisons were used to investigate them. Where time was not a variable i.e. subject characteristics, baseline metabolites and hormones, body composition, muscle function, patient reported outcome measures (PROMS), energy intake and energy expenditure unpaired student's t-tests were used. If data were not normally distributed, as determined by a Shapiro-Wilk test (cytokines and hormones in Chapter 4) a Mann-Whitney test was deployed. Incremental area under the curve was also calculated to demonstrate glucose net uptake in response to feeding.

Similar analyses were used for the study reported in Chapter 3. However, as it was designed as a randomized cross-over study, data were examined by repeated measures one-way ANOVA or where time was also a variable repeated measures 2-way ANOVA. Post hoc analysis to locate which differences between the 3 visits were significant was conducted using Tukey's multiple comparisons. Incremental area under the curve was used to analyse glucose and insulin responses to milk protein and dextrose ingestion. Total area under the curve was used for BCAA and FFA responses, in order to provide a clear picture of overall postprandial net balance, thus indicating net uptake or efflux.

All data were presented as mean +/- SEM except for non-parametric tests where median and interquartile range were used. P value of <0.05 was considered significant. Data analysis was undertaken with Prism software V.7.0 (La Jolla, San Diego, US) and Microsoft Excel for Mac 2011 V14.7.7 (Microsoft, USA).

3 THE EFFECT OF PRIOR ORAL FAT INGESTION ON SKELETAL MUSCLE INSULIN AND ANABOLIC RESPONSES TO MILK PROTEIN AND DEXTROSE INGESTION ON MEN WITH OBESITY

3.1 INTRODUCTION

Middle-aged men with obesity are a population at risk from type 2 diabetes mellitus (T2DM) (Kahn and Flier 2000) and the age-related loss of muscle mass and function (Rosenberg 1997), sarcopenia (Baumgartner 2000). Further T2DM is thought to exacerbate sarcopenia (Park et al. 2009). These conditions place a huge burden on public health and social care provision (Allender and Rayner 2007; Scarborough et al. 2011; Margaris 2017) and are associated with a significant reduction in the quality of life of the individual (Søltoft, Hammer, and Kragh 2009; Kearns et al. 2013). It is therefore important to understand the effects of dietary fat ingestion on insulin and anabolic resistance (hallmarks of T2DM and sarcopenia, respectively) in this population in order to design preventative measures.

Men with obesity have been shown to be less insulin sensitive and have a reduced anabolic response compared to healthy controls (Pereira et al. 2008; Guillet et al. 2009; Chevalier et al. 2015; Beals et al. 2016). Insulin resistance has been induced acutely by high fat meals in men with obesity whereas anabolic resistance was not measured (Xiao et al. 2006; Jans et al. 2012; Wuesten et al. 2005). Anabolic and insulin resistance have been induced acutely in healthy volunteers by 7hr lipid infusion, with insulin sensitivity measured during a 3-h hyperinsulaemic-euglucaemic clamp and anabolic resistance by muscle synthetic response (fractional synthetic rate, FSR) to a 21g bolus of amino acids (Stephens et al. 2015). Higher levels of intramyocellular long chain acyl-CoA and acetylcarnitines were found in the lipid visit, thus associating lipid accumulation with insulin and anabolic

resistance. In another study in healthy volunteers whole body insulin resistance but not skeletal muscle anabolic resistance (FSR) was shown in response to lipid infusion beginning 10hrs before an EAA bolus of 7g (Katsanos et al. 2009). It was proposed by Stephens et al. that the contrasting results were due to the lack of hyperinsulinaemia in the Katsanos et al. study. Lower levels of insulin may have resulted in attenuated suppression of fat oxidation (FOX) thus preventing sufficient intramyocellular accumulation of lipid to induce skeletal muscle anabolic resistance and possibly muscle insulin resistance although the latter was not measured. However, it is not known whether insulin resistance and anabolic resistance will both be induced by oral lipid load in middle-aged men with obesity.

Oral fat loads have been shown to induce muscle insulin resistance equivalent to lipid infusion in healthy young subjects (Nowotny et al. 2013). It has also been shown that the composition of the lipid load can result in differential effects, at least on insulin sensitivity (Jans et al. 2012; Xiao et al. 2006). In both insulin resistant men (Jans et al. 2012) and overweight non-diabetic subjects (Xiao et al. 2006) polyunsaturated fatty acids (PUFA) had insulin sensitising effects as compared to saturated fatty acids (SFA). Indeed a recent systematic review and meta-analysis of the effects of PUFA, monounsaturated fatty acids (MUFA), SFA and carbohydrate concluded that the substitution of PUFA for SFA and carbohydrate had beneficial effects on glycaemia and insulin sensitivity in the long term (Imamura et al. 2016).

Some studies investigating n3PUFA (eicosapentaenoic acid (EPA, 20:5) and docosaoentaenoic acid (DHA, 22:6)) have reported beneficial effects on

insulin sensitivity and muscle protein balance in humans. Improved insulin sensitivity vs. n6PUFA has been shown acutely in healthy volunteers (Stephens et al. 2014) and after chronic supplementation (Delarue et al. 2006). Positive effects of DHA and EPA have been found on insulin signaling *in vitro* and in rodents, providing a possible mechanism for acute improvements in insulin sensitivity (Taouis et al. 2002; Le Foll et al. 2007; Lanza et al. 2013; Capel et al. 2015). Although not all studies have shown chronic benefits (Glauber et al. 1988; Fasching et al. 1991; Lalia et al. 2015). Those studies that have suggest advantages may instead be linked to alterations in cellular membrane composition (Aas et al. 2006) for which at least 2 weeks of supplementation is thought to be needed (McGlory et al. 2014). A significant increase in FSR in both young and elderly individuals (+33% & +50% respectively), possibly due to the stimulation of mTOR, has been reported after 8 weeks of 4g fish oil supplementation (1.86g EPA & 1.50g DHA) (Smith et al. 2011b; 2011a), although no improvement in insulin sensitivity was seen in either study. n3PUFA may also reduce muscle protein breakdown by suppressing the ubiquitin proteasome system via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as indicated by murine (Whitehouse and Tisdale 2001) and *in vitro* studies (Wang et al. 2013). This may be mediated by activation of nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ) which inhibits NF-κB (Magee et al. 2012). n3PUFA's positive effect on protein balance may translate into a real change in muscle mass in humans *in vivo* with studies investigating n3PUFA supplementation (4g/day FO) reporting significant increases in lean mass in 6 weeks in healthy subjects (Noreen et al. 2010)

through to 6 months in healthy older adults (Smith et al. 2015). It is not known however whether positive effects on anabolic response would be seen acutely in subjects with obesity.

The aim of this study was to test the anabolic and insulin sensitivity responses to an oral protein and carbohydrate load, subsequent to the ingestion of 3 different test meals containing: 1. saturated fat (SFA); 2. fish oil (FO) (36% replacement of SFA by fish oil); and 3. no fat (Control), in a randomized crossover study in middle-aged men with obesity. The testing of different fatty acids has been included to understand whether lipid per say or specific types of lipid are involved in acute induction of insulin and or anabolic resistance. Postprandial anabolic and insulin sensitivity responses by skeletal muscle would be assessed by forearm skeletal muscle arterio-venous (a-v) balance of BCAA and glucose respectively.

The specific objectives of this study were to examine: 1. the effects of an oral SFA load on skeletal muscle anabolic and insulin resistance in middle-aged men with obesity and; 2. whether the replacement of some of the SFA by fish oil derived n3PUFA would alter these effects; 3. identification of any specific lipids, via metabolomics, that may be associated with any differences between test meals in terms of insulin and / or anabolic sensitivity. It is hypothesized that SFA will cause both a reduced anabolic response and insulin resistance as compared to control and that these effects will be attenuated when a proportion of SFA is substituted with fish oil.

3.2 METHODS

3.2.1 Subjects

Male subjects, aged 40-60, were recruited through advertisements on the Nottingham University and Nottingham University Hospitals Trust campuses and via direct mail to local inhabitants of appropriate age. All participants were overweight or classified as obese with a BMI of ≥27 but otherwise reported being in good health.

3.2.2 Outcome measures

The primary outcomes were forearm skeletal muscle protein net balance AUC (an index of anabolic sensitivity to dietary protein ingestion) and forearm skeletal muscle glucose net balance (an index of skeletal muscle insulin sensitivity to dietary CHO ingestion). Secondary outcomes were: circulating glucose and insulin area under the curve (AUC) and Matsuda index (Matsuda and DeFronzo 1999; DeFronzo and Matsuda 2010) (indices of whole body insulin sensitivity); forearm skeletal muscle free fatty acids (FFA) net balance; identification of plasma lipid species via metabolomics; whole body metabolic rate and fuel oxidation; and body composition.

3.2.3 Experimental protocol

Participants attended the laboratory on 3 separate occasions, at least 1 week apart, and underwent 3 different treatments in a randomized crossover study (Figure 3.1). On each visit they ingested an oral test meal at time zero, then 4

hours later a bolus of milk protein (0.3g/kg) and dextrose (0.8g/kg) in 4ml/kg of water. The oral test meal was a hot chocolate-flavoured drink containing either of 2 fat emulsions, saturated fat (SFA) or fish oil (FO) (lipid trials), or water (control trial). The SFA trial included 0.7g/kg of palm stearin (AKOSTEAR/SG) (Newens et al. 2011). Composition of palm stearin per 100g: 99.9g fat of which 63g saturates, 30g monounsaturates and 7g polyunsaturates and energy content was 3696kJ. For the second lipid trial (FO) part of the palm stearin was replaced by fish oil (Croda, UK) such that it contained 0.45g/kg of palm stearin and 0.25g/kg fish oil. Fish oil contained: 810mg/g of Omega-3 (expressed as FFA), split as follows between DHA 690mg/g and EPA 80mg/g. The amount of fish oil in the FO meal was designed to be similar to the amount of fish oil previously added to an intravenous lipid emulsion that was shown to reduce lipid induced insulin resistance in healthy young humans (Stephens et al. 2014).

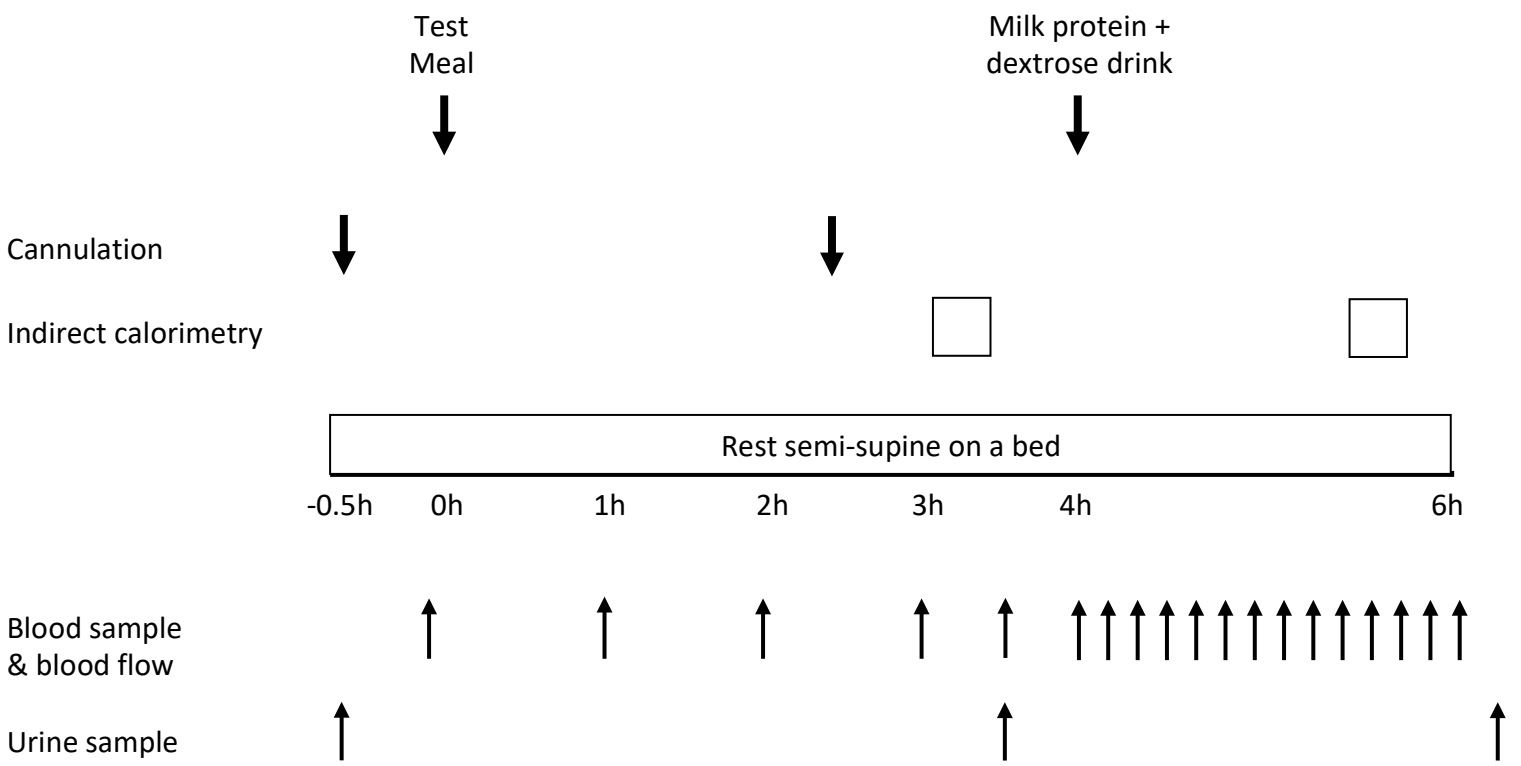


Figure 3.1 Schematic diagram of the study design

Participants reported to the laboratory at 0800, following an overnight fast, having abstained from strenuous exercise and alcohol for the previous 48 hours. On the first visit, their body composition was assessed by DEXA. Subsequently participants were asked to void their bladder then rest in a semi-supine position on a bed while a cannula was inserted in a retrograde fashion into a superficial vein on the dorsal surface of the dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand (Gallen and Macdonald 1990) (see General Methods). After baseline blood samples participants ingested one of the three test meals (in a maximum of 10 min), after which the experimental clock was started. Arterialised-venous blood samples (6ml) were taken every hour for the first 4 hours for the determination of arterialised-venous glucose, insulin, branched chain amino acids (BCAA), free fatty acids (FFA), TAG and glycerol. After the blood sample at 3h indirect calorimetry (GEM Nutrition Ltd, Daresbury, UK) was performed for 20 min to assess metabolic rate and fuel oxidation. At 3.5 hours a further blood sample was taken and bladder voided. Additionally, during the first 4 hours a second cannula was placed into a deep perforating vein, via a superficial antecubital vein, in the forearm contralateral to the first cannula for deep venous blood sampling. This was inserted into the descending vein using ultrasound guidance (see General Methods). Brachial artery blood flow was measured in the same arm using Doppler ultrasound (Toshiba Aplio 300). Immediately post the 4hr (t=240 min) blood sample the milk protein and dextrose drink was ingested. From 4h, every 15 min, blood flow was measured immediately prior to blood samples being taken concurrently from both cannulas. Samples

taken on the hour and half hour were 7ml arterialised-venous and 5ml deep venous and at quarter past and quarter to the hour were 5ml and 3ml respectively. Sampling enabled the calculation of forearm net balance of glucose every 15 min and BCAA and FFA every 30 min prior to and in response to the ingestion of the milk protein and dextrose drink. Indirect calorimetry was repeated at t=380 min in order that whole body metabolic rate and fuel oxidation could be compared pre and post milk protein and dextrose drink. A final urine sample was taken after the final blood samples at t=420 min. Urine samples were used to calculate nitrogen excretion and derive non-protein RER.

3.2.4 Blood & urine metabolite analysis

Blood glucose levels were measured using Yellow Springs Instrument Analyzer, YSI, 2300 STAT PLUS. Plasma separated from Ethyleneglycol-Bis-(β-Aminoethylether) Tetraacetate (EGTA) treated blood was analysed for BCAA concentrations by spectrophometric assay (Beckett et al. 1996). Plasma samples further treated with tetrahydrolypostatin (THL) were analysed for FFA by colorimetric kit (NEFA C, Wako). Serum separated from arterialised-venous blood was analysed for insulin concentration by radioimmunoassay (Milipore, Human insulin specific kit) and triacylglycerol (TAG) and glycerol by colorimetric kits (ABX Pentra Triglycerides CP and Sigma F6428 respectively). Urine and plasma (separated from EGTA treated blood) were analysed for urea by spectrophometric assay (Randox laboratories Ltd. UK, UR221) (See General Methods for details).

3.2.5 Metabolomics

Metabolomic data (non-polar) from arterialised plasma samples t=0, t=240, t=330 and t=420 min were obtained in the form of normalised sample total peak area (%) (see Appendix 1 for full methods). Two-way ANOVA (visit x time) had been performed on data from: (a) t=0 vs. 240 min to locate any changes associated with fat ingestion and from (b) t=240 vs. 330 vs. 420 min to locate changes in response to milk protein and dextrose, with or without prior fat ingestion, using the software MetaboAnalyst (Xia et al. 2015). p-values were reported after correction for multiple testing using the Benjamin-Hochberg method.

All non-polar metabolites that had been found to have significant main effects are reported. Fatty acid content of lipid metabolites was researched using lipid maps (https://www.lipidmaps.org).

To facilitate interpretation and to be able to visualize changes between time-points data was converted to fold changes for t=0-240 and t=240-420 min as follows:

Fold change = mean of normalised total peak area for metabolite feature (1) at t=240 min / mean of normalised total peak area for metabolite feature (1) at t = 0 min

As such positive numbers indicate that a particular metabolite was increasing in plasma between those time points and a negative number suggest that it was decreasing.

3.2.6 Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) and have been analysed using repeated measures one-way analysis of variance (ANOVA) or where time is also a variable, two-way ANOVA (visit x time). Where significant differences were found post-hoc analysis (Tukey's multiple comparisons) were used. Area under the curve (AUC) has been calculated to determine forearm skeletal muscle glucose net uptake and BCAA net balance in response to milk protein and dextrose ingestion. AUC has also been calculated for arterialised-venous glucose and insulin responses. Fractional extraction of both glucose and BCAA across the forearm was calculated as follows: ((glucose or BCAA a-v difference (mmol/L or μmol/L)) / arterialised glucose or BCAA (mmol/L or μmol/L)) * 100. As fractional extraction is independent of blood flow it can help demonstrate the contribution of blood flow to net nutrient balance. AUC of fractional extraction of glucose and BCAA was calculated. P value of <0.05 was considered significant. Data analysis was undertaken with Prism software V.7.0 (La Jolla, San Diego, US) and Microsoft Excel for Mac 2011 V14.7.7 (Microsoft, USA).

3.3 RESULTS

3.3.1 Subject characteristics & body composition

Eight subjects aged 52.8 ± 2.0 yrs. were recruited (Table 3.1). On average the group suffered from obesity: BMI 31.8 ± 1.4 kg/m^2 (range 26.8-40.1) with a fat mass (FM) of 34.2 ± 2.7 kg, equating to 35 ± 2% of total body mass (TBM). Lean body mass (LBM) was 61 ± 2% of TBM (59.0 ± 2.3 kg), appendicular LM 26.8 ± 1.0 and ASMI (appendicular skeletal muscle mass index) 8.8 ± 1.0 kg/m^2. In comparison to reference values for males aged 50-59 (Imboden et al. 2017) all lean mass metrics for this group of subjects were c.50th or 60th centile suggesting they did not have reduced lean mass in comparison to an age and sex matched reference population.

Table 3.1: Subject characteristics & body composition

	n=8
Age (yrs)	52.8 ± 2.0
Height (m)	1.75 ± 0.03
Weight (kg)	96.5 ± 4.2
BMI (kg/m^2)	31.8 ± 1.4
FM (kg)	34.2 ± 2.7
BMC (kg)	3.4 ± 0.2
LBM (kg)	59.0 ± 2.3
FM/TBM (%)	35 ± 2
LBM/TBM (%)	61 ± 2
Appendicular LM (kg)	26.8 ± 1.0
ASMI (kg/m^2)	8.8 ± 1.0
Right forearm LM (kg)	1.52 ± 0.06
Left forearm LM (kg)	1.47 ± 0.07

All values are means ± SEM. FM = fat mass, BMC = bone mineral content, LBM = lean body mass, TBM = total body mass, LM = lean mass, ASMI = appendicular skeletal muscle mass index. Note that two participants had a DEXA hemiscan (right side) and so left forearm values include values for right arms from 2 subjects.

3.3.2 Whole body physiology

3.3.2.1 Insulin sensitivity

Arterialised plasma glucose declined slightly between t=0 to t=240 min but then increased sharply in all visits post ingestion of the protein and dextrose drink, peaking 45-60 min post drink ingestion (SFA: 6.6 ± 0.43 mmol/L and FO: 7.5 ± 0.58 mmol/L at t=285 min and Con: 8.2 ± 0.35 mmol/L at 300 min) (time effect: $p<0.0001$) (Figure 3.1A). Plasma glucose then reduced in all visits but only returned to fasting levels in Con by t=420 min (4.7 ± 0.25 mmol/L) whereas SFA and FO remained elevated at 5.7 ± 0.29 mmol/L and 5.7 ± 0.39 respectively. Glucose excursion was therefore significantly different between visits (interaction effect $p<0.0001$). Glucose AUC in response to protein and dextrose (t=240-420 min) however was not different between visits (main effect $p=0.085$) (Figure 3.1B). Although a difference between responses was detected in glucose AUC calculated over the first 2 h post protein and dextrose ingestion (main effect $p=0.015$) (data not shown), post hoc testing showed that this was driven by the greater glucose AUC in Con vs. SFA ($p=0.026$), whereas the difference between Con and FO was not significant. The overall shape of the serum insulin curves showed a similar pattern to glucose (time effect $p<0.0001$) except that there was no statistical difference between visits either when expressed over time (Figure 3.1C) or as AUC (Figure 3.1D). The Matsuda index did not differ between visits either and on average was 4.7 ± 0.89 in Con, 5.11 ± 0.93 in SFA and 4.06 ± 0.59 in FO, with <4.3 considered to be insulin resistant (Gutch et al. 2015) (Figure 3.1E).

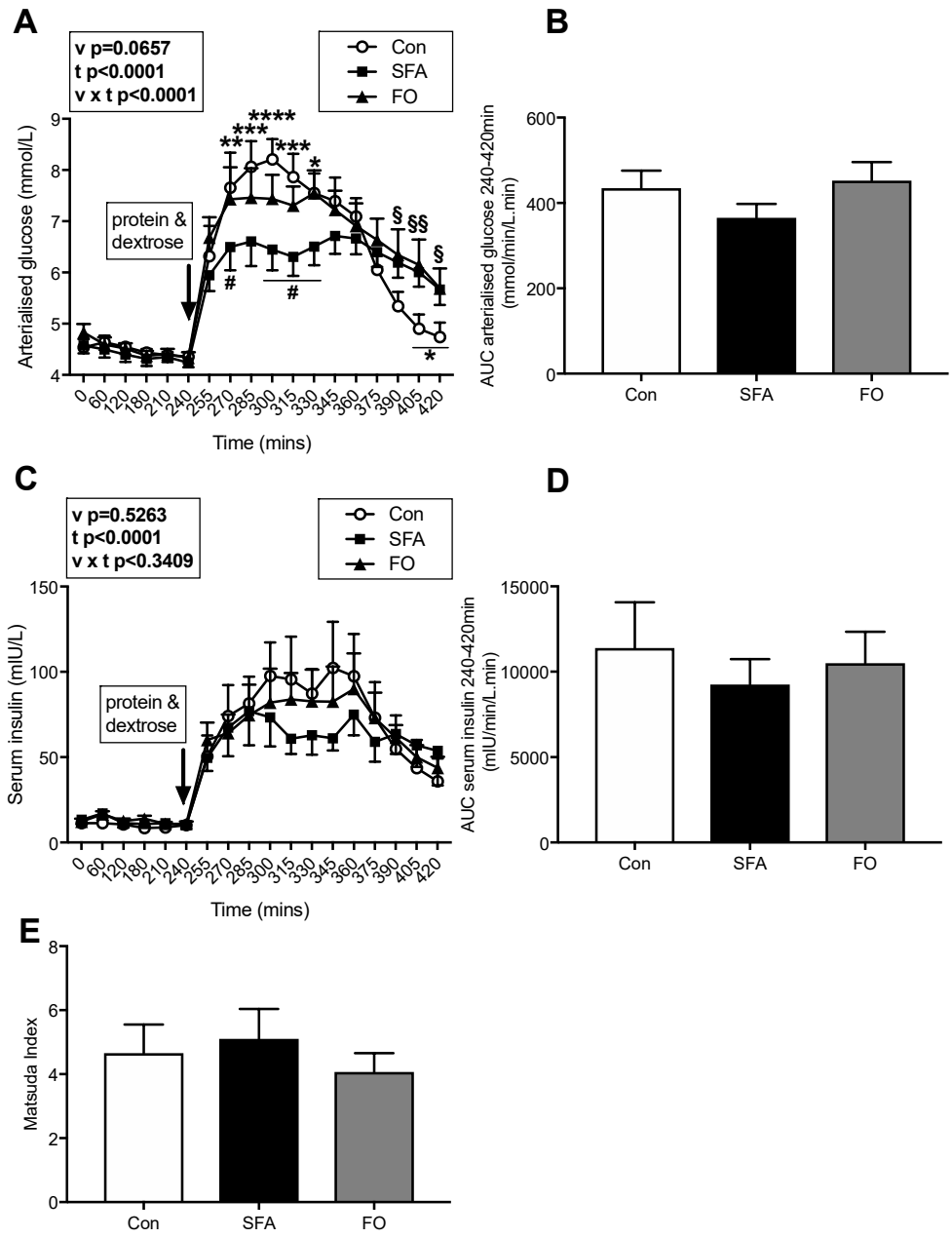


Figure 3.1 Indicators of whole body insulin sensitivity: arterialised plasma glucose concentrations over time (A) and AUC (B); serum insulin over time (C) and AUC (D); and Matsuda index (E).

Values are means ± SEM. Con = control, SFA = saturated fat & FO = fish oil visits. Results of 2-way or 1-way ANOVA are shown if any main effects were significant: v = visit effect, t = time effect and v x t = interaction effect. Any significant differences from post-hoc analysis are marked: * = Con vs. SFA, § = Con vs. FO and # = SFA vs. FO (* = p<0.05, ** = p<0.01, *** = p <0.001, **** = p<0.0001). Matsuda index is an index of postprandial insulin sensitivity where the lower the index the higher the level of insulin resistance. Note that n=7 for insulin (C&D) and Matsuda index (E) as one subject was a significant outlier (i.e. >2 standard deviations beyond the mean).

3.3.2.2 Energy expenditure

REE was greater 140 min after the protein and dextrose drink in all visits (time effect p=0.001) than prior to it and there was a strong trend towards a difference between the visits (visit effect p=0.052) (Figure 3.2A). Post hoc analysis showed this was driven, at least in part, by the difference in post drink REE between Con: 5.2 ± 0.19 kJ/hr/kgLBM and FO: 6.3 ± 0.35 kJ/hr/kgLBM (p=0.025). There were no significant differences between visits in pre drink REE. The increase in postprandial REE was 4.0 ± 2.1% in Con, 4.3 ± 2.9% in SFA and 7.6 ± 2.2% in FO, although these differences were not statistically significant. Non-protein RER prior to the drink was 0.93 ± 0.04 in Con, 0.82 ± 0.02 in SFA and 0.88 ±0.02 in FO and post was 0.96 ± 0.02, 0.86 ± 0.01 and 0.88 ± 0.01 respectively (visit effect p=0.002) (Figure 3.2B). An overall time difference did not reach significance largely because there was no change in pre and post RER in the FO visit. Post hoc testing revealed that pre drink RER was significantly lower in SFA than Con (p=0.0002), while FO was significantly higher than SFA (p=0.023) but no different to Con. Post drink RER in both SFA and FO was significantly lower than Con (p=0.001 and p=0.009 respectively). There was a time difference pre and post drink ingestion in COX (p=0.003), with COX being higher post drink in all visits, but there were no differences between visits (Figure 3.2C). In terms of FOX there were no time effects but there was an overall difference between visits (visit effect p=0.024) (Figure 3.2D). Post hoc testing revealed no further significant differences.

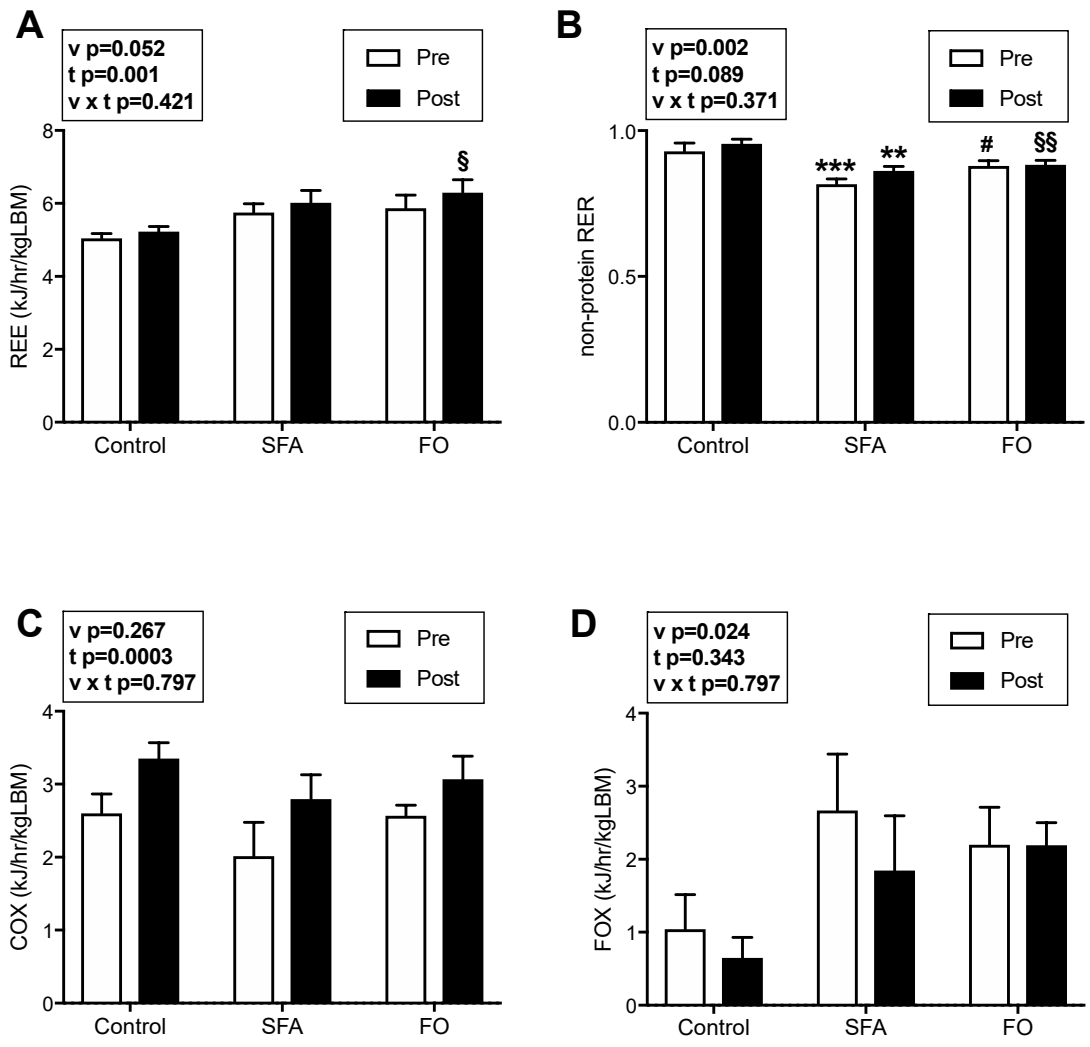


Figure 3.2 Whole body energy metabolism: resting energy expenditure (REE) (A); respiratory exchange ratio (RER) (B); carbohydrate oxidation (COX) (C); and fat oxidation (FOX) (D).

Values are means ± SEM. SFA = saturated fat and FO = fish oil visits. Results of 2-way ANOVA are given: v = visit effect, t = time effect and v x t = interaction effect. Any significant differences found in post-hoc analysis are marked: * = Con vs SFA, § = Con vs. FO and # = SFA vs. FO (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). Note that n=6 in the control visits as there were technical problems with indirect calorimetry during two visits.

3.3.2.3 Lipid metabolism

Arterialised plasma FFA increased from t=0 through to t=240 min in all visits, but after the ingestion of the protein and dextrose drink (t=240) reduced significantly to >50% below baseline by t=420 min (time effect $p<0.0001$) (Figure 3.3A). The pattern of decline during the final 3h of the study differed between visits as demonstrated by the detection of both interaction ($p<0.0001$) and visit effects ($p=0.0015$). Post hoc testing comparing mean differences between visits showed there were significant differences between Con and both lipid visits (SFA $p=0.001$, FO $p=0.016$). FFA concentrations reduced faster in Con vs. lipid such that they were lower in Con than SFA at t=270-420 min ($p<0.05$) and FO at t=270-360 min ($p<0.05$). Between t=330-390 min FFA levels in FO were also lower than SFA ($p<0.05$). Both interaction ($p<0.0001$) and time effects $p<0.0001$ were found for serum TAG (Figure 3.3B). TAG remained constant throughout during the Con visit at baseline levels (c. 1.2 mmol/L). In lipid visits TAG rose steadily after ingestion of both lipid drinks (t=0-240min) then steeply between t=240-270 min, coinciding with the ingestion of the protein and dextrose drink. From a peak of 2.5 ± 0.5 mmol/L in SFA (t=300 min) and 2.6 ± 0.6 mmol/L in FO, levels fell in both lipid visits to concentrations comparable with Con in FO by t=420 min but were still slightly elevated in SFA at that time ($p<0.05$). Levels of serum TAG in SFA and FO were therefore significantly different from Con between t=180-420 and t=180-390 respectively, but not different from each other.

Serum free glycerol concentrations were close to double in Con vs. lipid between t=120-240 min but dropped to less than half of lipid in Con between

t=330-390 min (interaction effect $p<0.0001$, time effect $p<0.0001$) (Figure 3.3C). Levels in all 3 visits were similar by t=420 min.

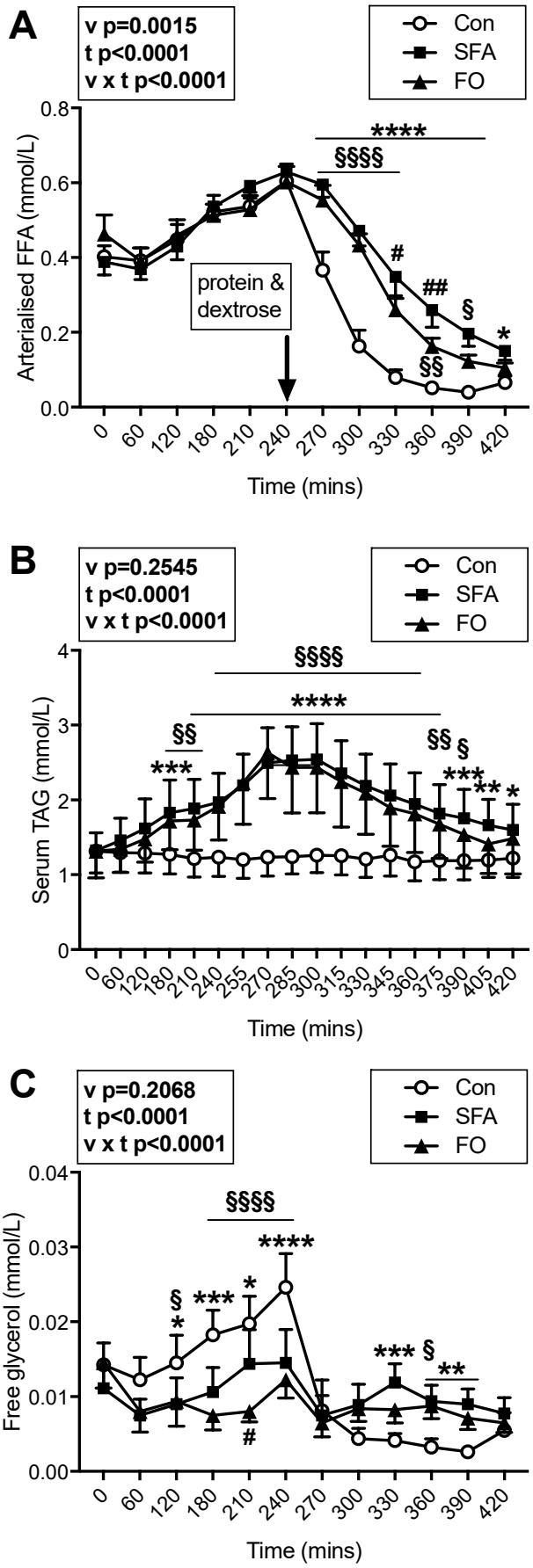


Figure 3.3 Whole body lipid metabolism: arterialised plasma FFA concentrations (A); arterialised serum TAG concentrations (B); and serum free glycerol (C).

Values are means ± SEM. Con = control, SFA = saturated fat and FO = fish oil visits. Results of 2-way ANOVA are given: v = visit effect, t = time effect and v x t = interaction effect. Any significant differences found in post-hoc analysis are marked: * = Con vs. SFA, § = Con vs. FO and # = SFA vs. FO (* = p<0.05, ** = p<0.01, *** = p <0.001, **** = p<0.0001).

3.3.3 Metabolomics

Over 12,000 lipid metabolites were detected in the arterialised plasma samples. 25 metabolites had significant time effects t = 0-240 min, but no visit or interaction effects were found (Table 3.2). Post milk protein and dextrose challenge (t = 240-360) 96 metabolites had significant time effects and 69 were different between visits, although there were no interaction effects (Table 3.3). Just over half of those that were different between visits after the protein and dextrose drink were TAG (35/69), with the next largest category DAG (7/69). More of the TAG that were different between visits were increasing in FO (28/35) than in SFA (15/35) (Figure 3.4). Figure 3.4 highlights the differences in TAG profiles between the visits. The TAG species going up in FO are skewed to the right i.e. contain relatively longer chain FA vs. those in SFA. However all of the TAG detected to have changed significantly by visit contained long or very long chain FA, ranging from C50 to C66. EPA and DHA could be seen in all of the top 5 most changed TAG for FO, however 2 of these TAG (62:15 & 60:12) were also in the top 5 most changed for SFA. There was a clearer split in DAG species between visits with 6/7 increasing in FO while none of these increased in either Con or SFA (Figure 3.5). The 3 DAG which were most increased in FO were all variants of C40:9, the vast majority of which (39/40 as per lipid maps) contain either DHA or EPA.

Table 3.2 Summary of metabolites with significant main effects by class (t=0-240 min)

Metabolite class	Time effect	Visit effect	Interaction effect
Total number of significant metabolites (p<0.05)	25	0	0
Acyl carnitine	4	0	0
Ceramides and sphingolipids	1	0	0
Diacylglyceride	2	0	0
Fatty acids and fatty esters	4	0	0
Glycerophospholipids	4	0	0
Lysine metabolism	2	0	0
Lysoglycerophospholipids	2	0	0
Nucleosides and nucleotides	1	0	0
Other class	2	0	0
Oxidised fatty acids	2	0	0
Triacylglyceride	1	0	0

Significant results from 2-way ANOVA (visit x time) t=0 vs. t=240 min of non-polar metabolomic data, grouped into classes. 25 metabolites were found to have significant time effects, with no other significant effects.

Table 3.3 Summary of metabolites with significant main effects by class (t=240-420 min)

Metabolite class	Time effect	Visit effect	Interaction effect
Total number of significant metabolites (p<0.05)	**96**	**69**	**0**
Acyl amino acids	1	2	0
Acyl carnitine	16	3	0
Ceramides and sphingolipids	1	1	0
Diacylglyceride	11	7	0
Fatty acids and fatty esters	14	1	0
Glycerophospholipids	8	4	0
Lysine metabolism	1	0	0
Lysoglycerophospholipids	3	6	0
Mixed class	6	0	0
Monoacylglycerides	3	1	0
Nucleosides and nucleotides	0	3	0
Other class	4	1	0
Oxidised fatty acids	9	2	0
Purine metabolism	1	0	0
Short chain organic acids	2	0	0
Sterol and steroid metabolism (incl. Vit D)	9	2	0
Triacylglyceride	2	35	0
Vitaimin A metabolism	1	0	0
Vitamin E metabolism	3	1	0

Significant results from 2-way ANOVA (visit x time) t=0 vs. t=240 min of non-polar metabolomic data, grouped into classes. 96 metabolites were found to have significant time effects and 69 had significant visit effects, but there were no interaction effects.

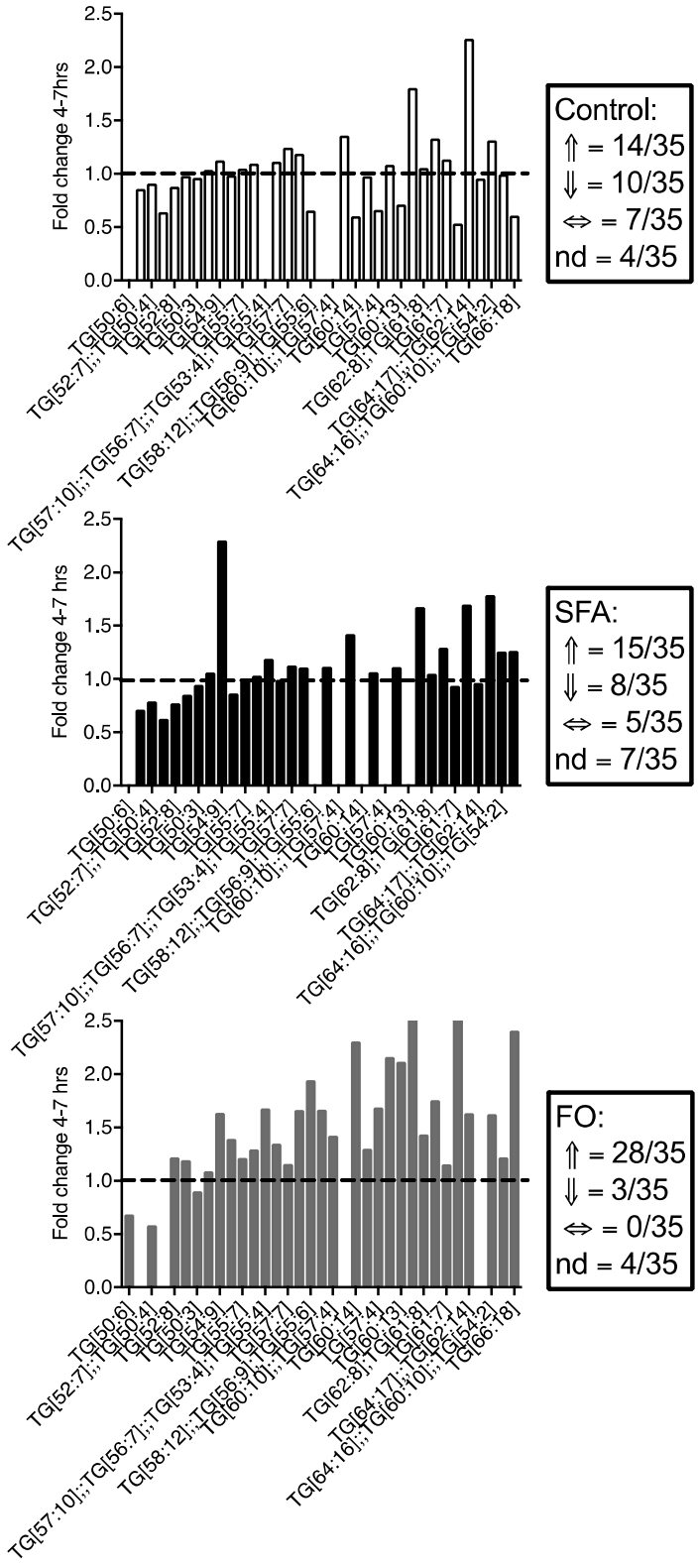


Figure 3.4 Summary of TAG fold changes by visit t-240-420 min

All TAG shown are significant for visit effects (2-way ANOVA) during this time period. They are ranked left to right in terms of chain length. The number increasing (🡅), decreasing (🡇), no change (🡄🡆) or not detected (nd) are shown by visit

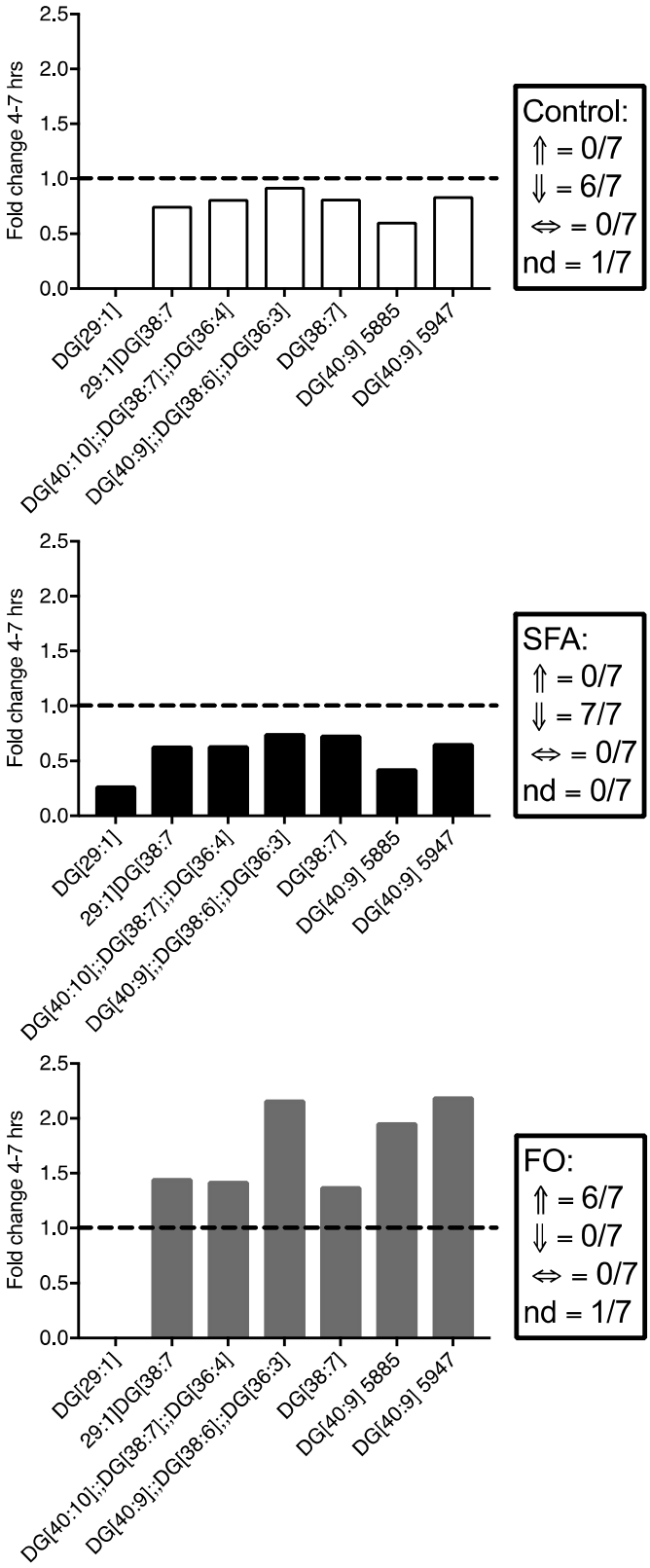


Figure 3.5 Summary of DAG fold changes by visit t-240-420 min

All DAG shown are significant for visit effects (2-way ANOVA) during this time period. They are ranked left to right in terms of chain length. The number increasing (🡅), decreasing (🡇), no change (🡄🡆) or not detected (nd) are shown by visit.

3.3.4 Muscle physiology

3.3.4.1 Skeletal muscle insulin sensitivity

Glucose a-v differences increased post protein and dextrose drink ingestion (t=240 min) and remained elevated between t=270-375 min before dropping back (Figure 3.6A) (time effect $p<0.0001$). There was a trend ($p=0.088$) towards an interaction effect with Con appearing to have a greater (0.83 ± 0.24 mmol/L t=270 min) and more sustained peak a-v difference than SFA which peaked at 0.64 ± 0.22 mmol/L t=315 min, while FO was intermediate (peak t=330 min 0.78 ± 0.13 mmol/L). Blood flow was highly variable between subjects (range at baseline: 10-168 ml/min and at peak: 15-254 ml/min) and so although numerically it appeared to be higher in FO and lower in Con no statistical difference was found between visits (Figure 3.6B). Neither were there any time effects. Mean postprandial blood flow for the entire postprandial period (t=255-420 min) was 97 ± 18 ml/min in FO, 88 ± 17 ml/min in SFA and 82 ± 15 ml/min in Con, with none of these differences statistically significant. Any emerging difference between visits in glucose a-v difference was therefore nullified once blood flow was taken into account hence there was no difference in glucose net uptake between visits (Figure 3.6C). Glucose net uptake increased in all groups post drink at t=270 min, remaining higher than baseline throughout (time effect $p<0.0001$). Overall glucose uptake, when expressed as AUC (t=240-420 min) was 440 ± 87 μmol.min/100gforearmLM in Con, 372 ± 101 μmol.min/100gforearmLM in SFA and 336 ± 82 μmol.min/100gforearmLM in FO, with no statistical differences between them (Figure 3.6D). However, there was a trend towards an

interaction in fractional glucose extraction (p=0.070) (Figure 3.6E). Indeed a main effect was found in AUC % glucose extraction p=0.025 (t=240-420 min) with Con having the highest fractional extraction. Post hoc testing showed % glucose extraction was lower in FO than Con (p=0.041) (Figure 3.6F).

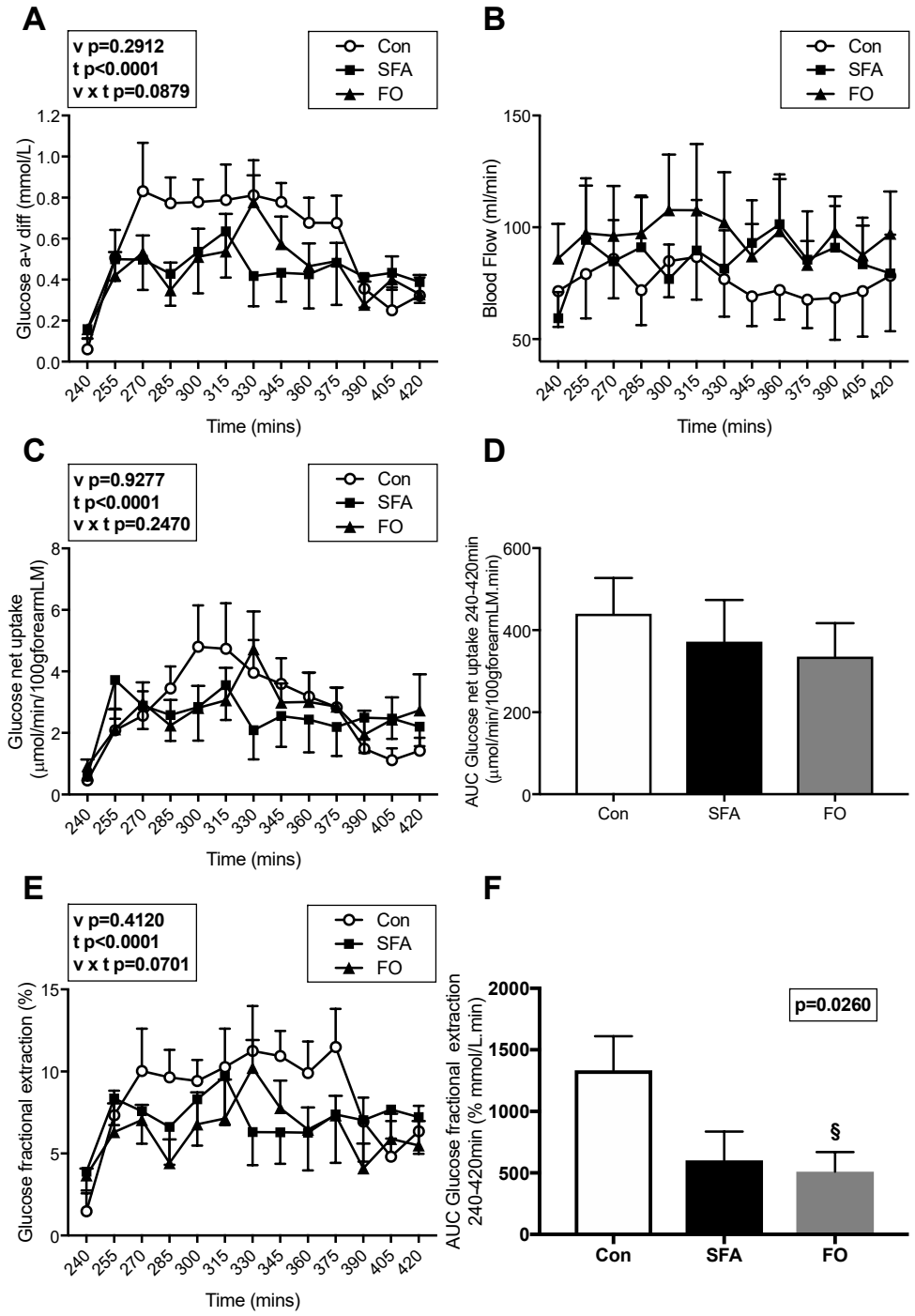


Figure 3.6 Skeletal muscle insulin sensitivity: glucose arterio-venous (a-v) difference across the forearm (A); brachial artery blood flow (B), glucose net uptake across the forearm over time (C) and AUC (D); and fractional glucose extraction over time (E) and AUC (F).

Values are means ± SEM. Con = control, SFA = saturated fat and FO = fish oil visits. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect. Any significant differences found in post-hoc analysis are marked: § = Con vs. FO, $p<0.05$.

3.3.4.2 Skeletal muscle anabolic response

Arterialised plasma BCAA concentration increased in response to protein and dextrose in all visits, peaking at t=270 min in Con and SFA at 429 ± 84 μmol/L and 329 ± 51 μmol/L respectively, while FO peaked at t=300 min at 367 ± 39 μmol/L (time effect p=0.002) (Figure 3.7A). BCAA concentrations then fell back in all visits and were no different to baseline from t=330 min. There were no significant differences between visits. Neither did BCAA a-v difference differ between visits and a time effect failed to reach significance (p=0.092) (Figure 3.7B). There was however an interaction effect detected in BCAA net balance (p=0.040) (Figure 3.7C). Con responded positively to the protein and dextrose drink peaking with a net positive balance of 0.37 ± 0.32 μmol/min/100gforearmLM at t=270 min. Post hoc testing showed that this was significantly different from FO (p=0.0003), which was still in negative balance at this time (-0.25 ± 0.20 μmol/min/100gforearmLM). There was only a trend towards a difference at t=270 min between Con and SFA however (0.08 ± 0.11 μmol/min/100gforearmLM) (p=0.087). Con remained in positive BCAA net balance until t=420 min while FO achieved a positive balance by t=300 min which was then maintained. SFA dipped into negative balance t=300-330 min but then regained positive balance. However, all visits achieved positive net BCAA balance across the entire 3h postprandial period when expressed as AUC and there were no differences between visits (Figure 3.7D). There was an interaction effect (p=0.044) in fractional BCAA extraction across the forearm tissue (Figure 3.7E). Post hoc analysis showed extraction in SFA was significantly reduced vs. Con at t=240 min (p=0.044) and FO extraction was significantly lower than both Con (p=0.010) and SFA (p=0.010)

at t=270 min. However there were no significant differences between visits overall in AUC BCAA fractional extraction t=240-420 min (Figure 3.7F).

3.3.4.3 Skeletal muscle lipid metabolism

There was a time effect ($p<0.0001$) found in FFA a-v difference, reflecting the reduction seen in the response to the protein and dextrose drink followed by stabilisation (Figure 3.8A). There were no differences between visits. FFA net balance, whether considered across the time course (Figure 3.8B), or expressed as AUC (Figure 3.8C) did not differ either. In all visits participants were in overall positive net FFA balance postprandially (t=240-420 min): Con 18.7 ± 11.3 μmol.min/100gforearmLM, SFA 35.9 ± 11.8 μmol.min/100g forearmLM and FO 27.4 ± 9.9 μmol.min/100gforearmLM.

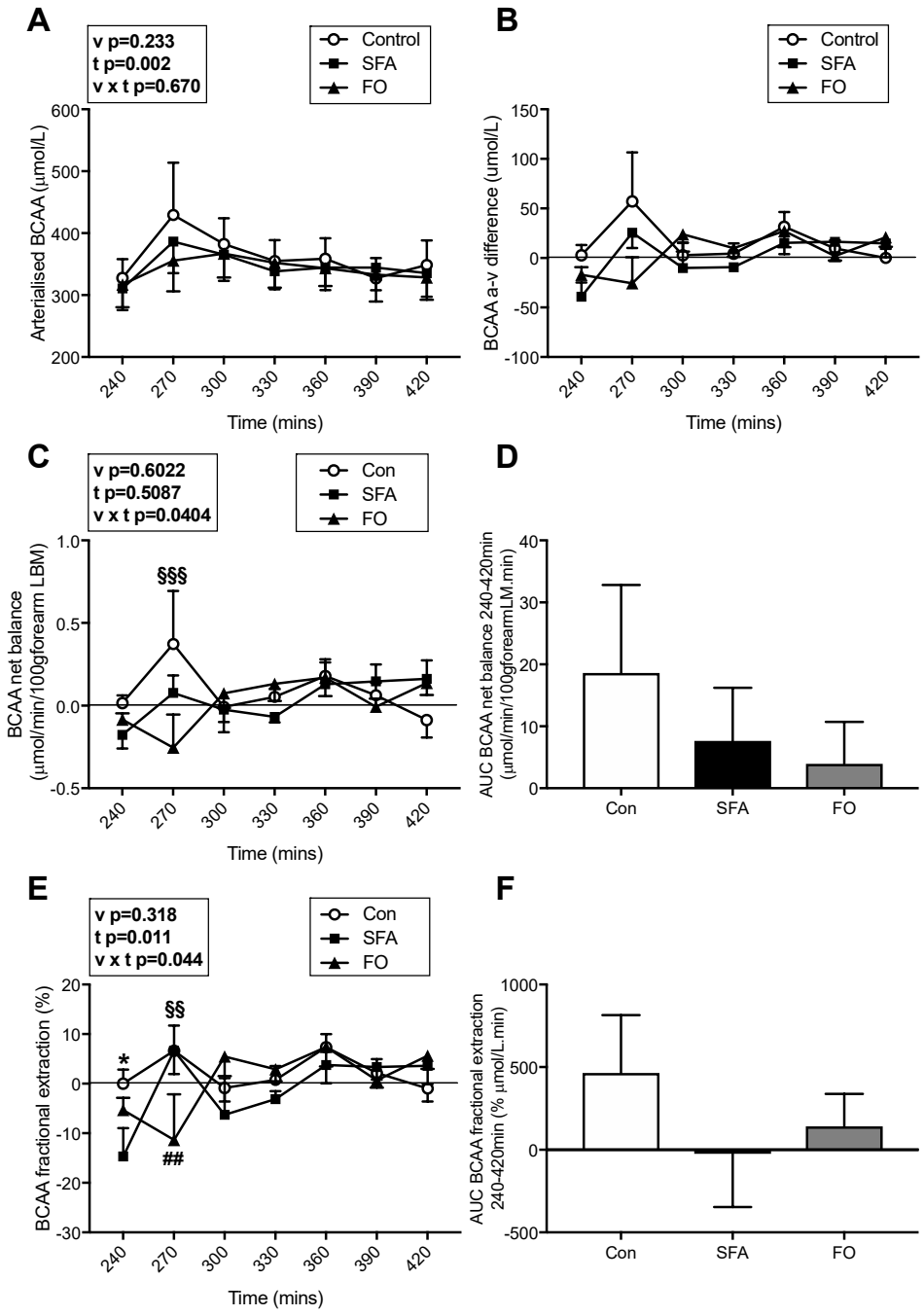


Figure 3.7 Indicators of skeletal muscle anabolic response: arterialised plasma BCAA concentrations (A); BCAA arterio-venous (a-v) difference across the forearm (B); BCAA net balance across the forearm over time (C) and AUC (D); fractional BCAA extraction over time (E) and AUC (F).

Values are means ± SEM. Con = control, SFA = saturated fat and FO = fish oil visits. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect. Any significant differences found in post-hoc analysis are marked: * = Con vs SFA, § = Con vs. FO and # = SFA vs. FO (* = p<0.05, ** = p<0.01, *** = p <0.001). .

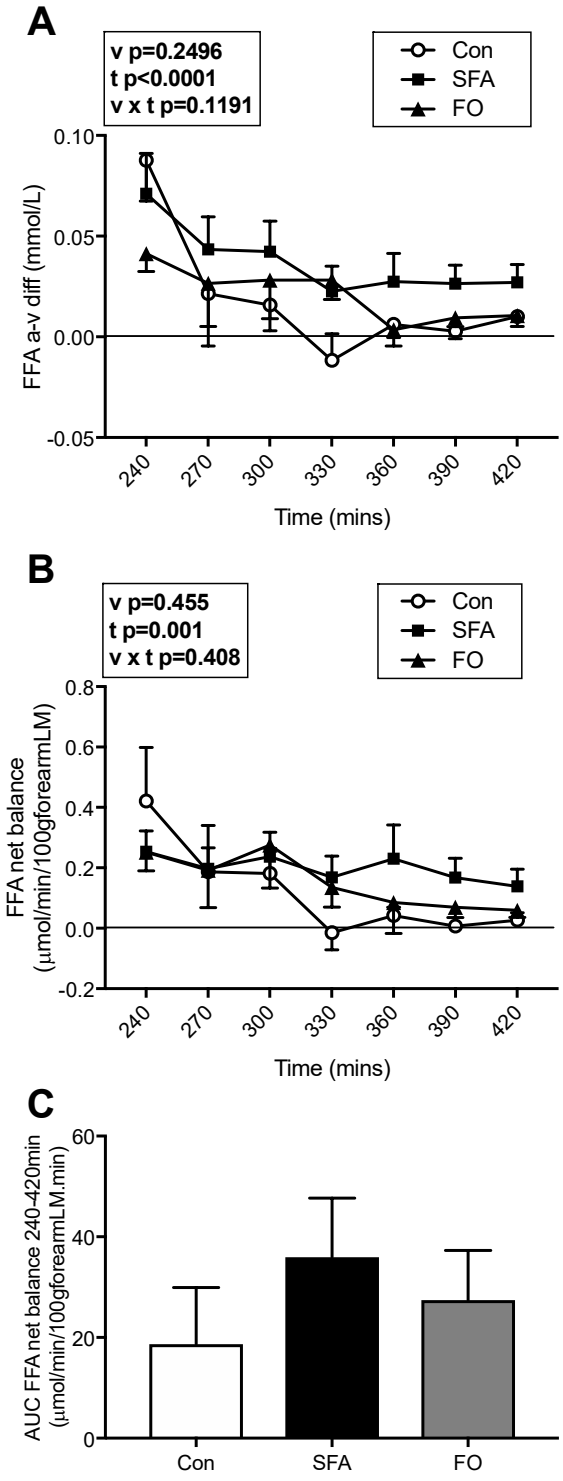


Figure 3.8 Skeletal muscle FFA metabolism: FFA arterio-venous (a-v) difference across the forearm (A), FFA net balance across the forearm over time (B) and AUC (C).

Values are means ± SEM. Con = control, SFA = saturated fat and FO = fish oil visits. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect.

3.4 DISCUSSION

3.4.1 Introduction

The primary aim of the present study was to test if an oral saturated fat load would induce both skeletal muscle anabolic and insulin resistance in middle-aged men with obesity. Secondarily, whether the replacement of some of the saturated fats by fish oil would attenuate these effects. In contrast to the hypothesis, SFA didn't induce muscle anabolic or insulin resistance, as demonstrated by no significant differences between visits in forearm BCAA net balance or glucose net uptake when expressed as AUC. Although there was an interaction effect in BCAA net balance overtime, suggesting a different pattern of response between visits. Further, Con glucose excursion was greater than SFA, but not FO, although there was no difference in overall insulin response between visits. Glucose fractional extraction across the forearm was higher in Con vs. lipid suggesting that increased blood flow prevented attenuated glucose uptake in lipid, despite not being statistically higher vs. Con. The main novel finding relating to FO was that it produced the highest REE post protein and dextrose drink, as even though there was a postprandial increase in COX no reduction was seen in FOX. This is the first study to investigate anabolic and insulin responses to acute oral administration of SFA and part replacement of SFA by FO in middle-aged men with obesity.

3.4.2 Whole body glycaemic & insulinaemic responses

In the current study neither whole body nor skeletal muscle insulin resistance was detected in response to an oral lipid load of 0.7g per kg (SFA or SFA and FO) in middle-aged men with obesity. However, several studies investigating the effects of oral fat loads in healthy males (López et al. 2008), those with hyperlipoproteinaemia but normal glucose tolerance (Lopez et al. 2011) and overweight subjects / those with obesity (Wuesten et al. 2005; Xiao et al. 2006; Jans et al. 2012) have found both insulin resistance and contrasting patterns of glucose and insulin excursion to the current study. Lopez et al. found a similar glucose response across a range of meals from those high in SFA to MUFA but higher insulin responses in SFA in both healthy males and those with hyperlipoproteinaemia but normal glucose tolerance (Lopez et al. 2011). Thus as more insulin was needed to maintain similar blood glucose levels whole body insulin resistance was detected in SFA and to a lesser extent in MUFA vs. Con. In contrast to the present study there was no separate glucose (and protein) stimulus i.e. test meals were mixed. They were not liquidised, had a higher overall energy content (3.35MJ) and were tested against a low fat, carbohydrate rich, but hypoenergetic control. It follows that insulin excursion would be higher in the hyperenergetic lipid visits.

The effect on skeletal muscle insulin sensitivity of meals with different fatty acid compositions (SFA, MUFA and PUFA - rich in n3PUFA)) has also been tested in insulin resistant (HOMA-IR > 2.5) overweight men and those with obesity (Jans et al. 2012). The main difference with the current study was that these meals were mixed (61% fat, 32% CHO and 7% protein).

Postprandial insulin sensitivity was measured by PGI (AUC glucose x AUC insulin x 10^{-6}) with higher values indicating greater insulin resistance. Insulin sensitivity was shown to be reduced in SFA vs. PUFA and both insulin and glucose excursions were highest in SFA.

A study in individuals who were glucose tolerant (as assessed by OGTT) but overweight or suffering from obesity tested the effects of different fat emulsions (SFA, MUFA, PUFA – rich in n6 PUFA) ingested regularly over a 24h period vs. control (water) on insulin sensitivity (Xiao et al. 2006). Plasma glucose was similar amongst treatments and no different to baseline whereas insulin decreased in Con but not in the lipid groups. After 24h of fat ingestion subjects underwent a hyperinsulinaemic-euglycaemic clamp and insulin sensitivity was found to be similar between MUFA, PUFA and Con but attenuated in SFA. Although insulin sensitivity was tested after pre-loading with fat the regular ingestion over 24h plus use of clamp are key differences with the present study.

In Wuesten et al. a large oral fat load of 1.5g/kgLBM high in n6PUFA was followed by an i.v. glucose challenge at t = 4h. Significantly increased insulin levels were found in healthy and in subjects with obesity vs. no-fat control and as such insulin resistance was detected in lipid. Although the timing of the delivery of glucose (minus protein) 4h subsequent to the fat load was similar to the present study it was delivered i.v. This would perhaps have been expected to produce a lower insulin response vs. oral delivery due to the absence of stimulation by incretins (Lindgren et al. 2011). However, in contrast to the present study, insulin was greater in the lipid trial.

Thus in all of these studies SFA (or n6PUFA) ingestion insulin resistance was induced by the stimulation of hyperinsulinaemia. Increased insulin excursion with lipid has been proposed to be as a result of hyper-stimulation of beta cells by lipid (Holness et al. 2004), stimulation of increased release of the incretin, glucagon like peptide 1 (GLP-1) (Beysen et al. 2002) and reduced clearance of blood insulin in the face of high plasma FFAs (Balent et al. 2002; Wuesten et al. 2005). Some evidence of reduced insulin clearance may be seen in the shape of the insulin curves for lipid visits in the present study or this may simply reflect the lower glucose excursion. Insulin secretion is also thought to be influenced by the degree of saturation and chain length of fatty acids (Stein et al. 1997) with n3PUFA proposed to reduce insulin secretion in comparison to SFA (Holness et al. 2004). Together these data may help explain why the insulin response was highest in SFA followed by PUFA and then Con in these studies. However it was not possible to measure c-peptide in the current study to confirm possible effects on secretion/clearance of insulin.

Conversely, and in accordance with the current study, many studies investigating the effect of fat on glycaemic responses in humans (Estrich et al. 1967; Collier, McLean, and O'Dea 1984; Owen and Wolever 2003; Moghaddam, Vogt, and Wolever 2006; Sun et al. 2014) have found fat to reduce glycaemia. All of these studies compared glucose and insulin responses to CHO ingested alone, with CHO in combination with fat and/or protein. Collier et al., Owen and Wolever and Sun et al. all used everyday foods in their experiments, while Moghaddam et al. used glucose and corn oil, but all consistently found the addition of fat decreased glycaemic response vs.

CHO alone. Further in comparison to carbohydrate, fat has been shown to initially decrease postprandial glycaemic excursion (1-3 hrs.) but then extend it thereafter (Freckmann et al. 2007), which also fits with current data.

Although there are multiple differences in protocol between all of these experiments it is possible that one key factor influenced whether lipid induced hyperinsulinaemia, as in the first raft of studies or whether glycaemia was attenuated, as in the second group. It has been noted since the start of the 20th century that the presence of fat in the duodenum slows gastric emptying (Thomas 1957; Welch et al. 1987). The mechanism for delayed gastric emptying is thought to be via the release of Cholecystokinin (CCK) and GLP-1 which have both been shown to reduce gastric emptying in a dose-dependent manner in rats (Young, Gedulin, and Rink 1996), although neither were measured in the current study.

It is likely, but unconfirmed, that the lower glucose excursion in SFA vs. Con in the present study can be explained by the slower gastric emptying effect of lipid (Collier, McLean, and O'Dea 1984), even though fat ingestion was 4 h prior to the protein and dextrose challenge. Ingestion of fat 30 min prior to carbohydrate has previously been shown to slow gastric emptying and therefore reduce circulating glucose, insulin and gastric inhibitory polypeptide's (GIP's) responses to feeding in patients with T2DM but stimulate GLP-1 levels (Gentilcore et al. 2006). A study into gastric emptying rates showed 50% of a high fat drink (400g, 58% fat) was still present in the stomach at 160 min post ingestion (Castiglione, Read, and French 2002). This suggests that a significant proportion of the lipid meal in the current study

would still have been present in the stomach and duodenum when the participants drank the protein and dextrose drink. As the delivery of glucose by-passed the gastrointestinal tract in Wuesten et al. (2005) this may also explain why insulin was significantly increased in response to glucose despite prior fat ingestion.

Indeed it appears that choice of control and experimental conditions (i.e. using clamp or not and method of delivery of nutrients) determine whether lipid delays gastric emptying or not. If a high calorie CHO (and protein) control is used in the absence of clamp, whether delivery of nutrients is as a mixed meal or staggered, it is likely that high SFA ingestion will lead to a comparative delay in glucose absorption and therefore reduced glycaemia. If however a high SFA meal was compared only to high fat meals of different FFA composition (and) a hypoenergetic control, a comparative delay in glucose absorption is unlikely and hyperinsulinaemia may ensue.

Arterialized glucose in FO was however similar to Con in the present study. The addition of n3 PUFA to a SFA rich meal has been shown to increase gastric emptying (Robertson et al. 2002). The increased gastric emptying with n-3PUFA was reported to be as a result of slower CCK release and an attenuated GLP-1 response. Plasma glucose was not however measured in that study. However it is likely that this study explains why glucose excursion in FO was similar to Con in the current study.

It is interesting that BCAA appearance in the blood in SFA was not similarly delayed. It is difficult to explain this discrepancy but it could be because the

SFA test meal had already stimulated a gastric response, which can last for several hours (Merki et al. 1991) and therefore the stomach was already primed with pepsin to breakdown the milk protein. Speculatively, as this would not have been the case in the control visit, the faster gastric emptying in Con but slower gastric response in SFA could have cancelled each other out leading to the finding of no difference in arterialized BCAA concentration between the visits.

3.4.3 Skeletal muscle insulin and anabolic resistance

While reduced gastric emptying of CHO ingested 4h after lipid may explain the pattern of insulin and glucose excursions and the absence of whole body insulin resistance, it is still surprising that insulin resistance was not found at the level of skeletal muscle. Indeed the higher arterialized-venous blood glucose concentrations in Con would have been expected to drive an increase in glucose uptake in Con and FFAs are known to suppress glucose transport (Dresner et al. 1999; Kashyap et al. 2004; Belfort et al. 2005). Higher fractional glucose extraction and a trend towards greater a-v difference were present in Con, but did not translate into higher glucose uptake. This was because blood flow in lipid visits was numerically, if not statistically higher, than Con. Lipid has been claimed to reduce blood flow in the periphery (Sidery et al. 1991; Liu et al. 2011), have no effect (Szendroedi et al. 2012) or increase it (Raitakari et al. 2000). The differences in findings are likely due to the method of delivery of fat (oral vs. iv.), the composition of the meals, type and site of measurement and level of hyperinsulinaemia induced. Indeed in Raitakari et al. (2000) it is likely an increase in blood flow was driven by

hyperinsulinaemia. However, there was a lack of hyperinsulinaemia in the lipid visits in the current study.

Although there was no difference in insulin AUC between visits there was actually a trend towards a greater peak insulin concentration (t=285-360) in Con rather than SFA (98 mIU/L vs. 73 mIU/L respectively) (p=0.066). Yet although the differences were not significant mean, FO blood flow was 1.3x that of Con and SFA was intermediate between the two. In which case a mechanism independent of hyperinsulinaemia may be implicated. An increase in blood flow, mediated by endothelial-derived vasodilators, has been found in non-human primates as insulin sensitivity reduced with a high fat diet (Chadderdon et al. 2016). Thus, as the effectiveness of insulin to stimulate increased blood flow via NO-dependent mechanisms and so glucose uptake into the muscle declines, blood flow may be increased through other pathways to compensate. It is possible that such a mechanism was present in the subjects in the current study. Furthermore, beneficial effects of FO on vascular function have been found previously, with significant increases in plasma n3PUFA able to reverse impairment by SFA of flow mediated dilatation (FMD) (Newens et al. 2011).

An alternative explanation for the findings of no skeletal muscle insulin resistance is that the protocol failed to induce significant intramyocellular lipid accumulation in lipid visits. The test drinks were provided 4 hours before the protein and glucose bolus in order to pre-load the muscle with lipid. 3-4hrs has previously been shown to be necessary to show an impact on muscle insulin sensitivity (Boden et al. 1991; Tsintzas et al. 2007). FFA uptake was

not measured until t=240 min, immediately prior to the ingestion of dextrose and milk protein, at which point there were no significant differences between trials. Neither were muscle biopsies taken for analysis of lipid metabolites nor any differences detected by metabolomics, so it is not possible to confirm whether significant lipid accumulation was achieved.

It is interesting that plasma FFA were not elevated in the lipid trials in comparison to Con prior to the ingestion of the protein and dextrose drink. This may be due to a combination of factors. Firstly, prolonged fasting is known to increase FFA concentrations (Browning et al. 2012), which could explain why FFA concentrations also increased in Con. Indeed higher levels of serum glycerol in Con prior to the ingestion of dextrose and milk protein (t=120-240), suggest higher levels of endogenous lipolysis and post drink ingestion lower levels (t=330-390) imply the suppression of this process. Secondly, when lipid is administered intravenously heparin is routinely used to activate lipoprotein lipase (LPL) and therefore release FFA from TAG (Schalch and Kipnis 1965), but no such stimulus was used in the present study. Thirdly, whether the preservative THL is used in plasma samples to be analysed for FFA may also produce significant differences. Observations by our group suggest that despite the use of heparin with lipid infusion there is little increase in FFA observed when THL is used. Furthermore that FFA diffuse quickly down a concentration gradient into tissue and therefore do not remain elevated in plasma for long.

Finally, not all studies attempting to induce insulin resistance via oral lipid load have seen an increase in FFAs (Nowotny et al. 2013; Pedrini et al. 2006),

over and above the expected increase seen over time with fasting (Browning et al. 2012). It is interesting to note that Nowotny et al. did use THL, although it appears that Pedrini et al. did not. However, Nowotny et al. (2013) suggested adipose tissue was taking up FFAs derived from chylomicron TAG due to the stimulation of LPL by incretins (Eckel, Fujimoto, and Brunzell 1979; Kim et al. 2000) released in response to lipid ingestion, which acted to reduce FFA 'spillover' into the blood. This implies tissue uptake of FFAs in the lipid visits could still have been greater than Con, either because FFA's diffused rapidly into muscle and/or they were derived from the chylomicron TAG.

TAG levels were higher in lipid visits than Con by 180 min and remained so until 390 min in FO and 420 min in SFA. (Coppack et al. 1990) found positive a-v differences of FFA and TAG across the forearm demonstrating that TAG is taken up into the forearm muscle. Furthermore, high levels of TAG have also been shown to induce insulin resistance in the absence of elevated FFAs (Pedrini et al. 2006). An *in vitro* study in cultured rat muscle cells, by the same group suggested this was via inhibition of insulin signaling (Pedrini et al. 2005). They found attenuated glucose uptake and glycogen synthesis accompanied by reduced activity of Akt and glycogen synthase but not attenuated phosphorylation of IRS or PI3K in the presence of TAG-rich lipoproteins.

Arterialized FFA concentrations were significantly higher in both lipid visits vs. Con after the milk protein and dextrose drink. This was presumably as a result of the significant rise in insulin further stimulating LPL to the extent that FFA's spilled over into the circulation in the lipid visits (Pruneta-Deloche et al.

2004) in tandem with the suppression of endogenous lipolysis by insulin (Elia et al. 1988). There was no difference in FFA muscle uptake between visits post dextrose and protein ingestion however.

On balance it seems unlikely that high levels of lipid did not enter the muscle. Instead it may be that fat oxidation was able to remain sufficiently high in order to handle the excess lipid, possibly as a result of attenuated inhibition by relatively low levels of insulin or even some level of insulin resistance. Certainly after the protein and dextrose load fat oxidation was 2.9x the rate of Con in SFA and 3.4x in FO. Therefore attenuated inhibition of FOX, rather than reduced of uptake of FFA, may have contributed to the findings of similar muscle insulin sensitivity and anabolic response between visits.

Another potential explanation is that if insulin resistance was already developing in the current subjects it may have meant that the crossover study design failed to show up any differences. In support (Kashyap et al. 2004) demonstrated the negative impact of increased plasma FFA on insulin signaling and glucose uptake in healthy volunteers but failed to find any impact on those with a family history of T2DM. There is some evidence that our subjects were at least borderline insulin resistant at the whole body level e.g. HOMA-IR of 2.9 (<2.5 considered to be insulin sensitive (Gutch et al. 2015)) and fasting insulin of 12.29 mIU/L (>9microIU/ml identified 80% of patients with T2DM (Johnson et al. 2010)). Although conversely, some metabolic flexibility was demonstrated in SFA with FOX reducing and carbohydrate oxidation (COX) increasing in response to the protein and dextrose drink as RER rose from 0.82 to 0.86. However, although several

studies showing insulin and / or anabolic resistance have been conducted either in healthy subjects (Stephens et al. 2015; Katsanos et al. 2009) or have made comparisons with healthy controls (Pereira et al. 2008; Guillet et al. 2009; Chevalier et al. 2015), others have studied subjects with obesity using a crossover design (Jans et al. 2012). As previously described, in Jans et al. (2012) men c.60 yrs. with obesity drank liquid test meals high in SFA, MUFA or PUFA. Differences were detected with greater postprandial insulin sensitivity, attenuated uptake of fatty acids from TAG and increased intramyocellular lipid turnover in PUFA vs. SFA. It is unlikely therefore that the crossover design contributed to our findings.

The heterogeneity of the subject group may have meant that we were unable to detect any differences between visits. There were considerable variations, for example in serum insulin concentrations, blood flow and BCAA a-v differences in this group as demonstrated by the magnitude of the SEMs. Furthermore the range in Matsuda index (calculated from data from the Con trial) was 1.9-8.8, despite the removal of an outlier (due to insulin concentrations >2 SD from the mean).

The absence of muscle insulin resistance, whether due to compensatory mechanisms increasing blood flow, insufficient intramyocellular accumulation of lipid due to sufficient rates of FOX, heterogeneity of subjects or a combination of all of these factors may also explain the absence of anabolic resistance. In support 0.9g per kg intravenous (i.v.) lipid induced both insulin and anabolic resistance in healthy young men in response to a 21g bolus of AA during a hyperinsulaemic-euglycaemic clamp (Stephens et al. 2015).

While there were several differences in protocol between studies it is likely that particularly the clamp-induced hyperinsulinaemia, was instrumental in producing the contrasting results. The insulin peak in SFA in the current study was 50% lower in than the peak in Stephens et al.. It is possible that this lower level of insulin did not significantly attenuate fat oxidation in lipid visits and so prevented sufficient accumulation of intramuscular lipid metabolites to cause not only insulin but also anabolic resistance via common insulin signaling and protein synthetic pathways (Lang 2006; Masgrau et al. 2012; Stephens et al. 2015). Indeed this is the argument advanced by Stephens et al. when explaining the differences in findings between their study and (Katsanos et al. 2009). In the latter study insulin resistance was found in the absence of anabolic resistance in healthy young men, the fat load was >3x (10.17Mj) greater than in Stephens et al. and a smaller quantity of EAA (7g) was used. Lipid drove a significantly greater insulin excursion than control in Katsanos et al. but the absolute insulin peak was 70% lower than in Stephens et al. presumably due to the absence of carbohydrate co-administration. In the context of a small EAA stimulus and potential suppression of insulin signaling by lipid metabolites, it is also possible that the relatively greater insulin response in the lipid trial, may have contributed to an increase in muscle protein synthesis, therefore producing no difference vs. control in Katsanos et al.. The greater insulin response but similar glucose excursion however meant that whole body insulin resistance was found in lipid vs. Con. The absence of glucose co-ingestion in that study could also have resulted in high levels of FOX via the Randle cycle (Randle et al. 1963) and so attenuated accumulation of intramyocellular lipid, which in turn did not

impede insulin signaling and muscle protein synthesis. In contrast to Katsanos et al., in the present study insulin excursion was not greater in the lipid trials, while glucose excursion was greater in Con than SFA. This supports the interpretation that lower insulin concentrations may have failed to sufficiently suppress FOX and so prevent intramyocellular lipid accumulation and their detrimental effects on muscle insulin sensitivity and anabolic response in lipid visits.

It should be noted that although no overall difference in level of anabolic response, as assessed by AUC, was found between trials, an interaction effect was found in terms of the pattern of anabolic response over time. This could demonstrate the potential of the pre-ingestion of fat to at least decrease the initial response to protein (and dextrose) intake. It would be interesting to investigate whether this finding is reproducible and what the potential mechanisms might be. However while lipid may impact on immediate anabolic response, overall anabolic resistance was not found postprandially.

3.4.4 The effects of n3PUFA

The most interesting finding relating to FO was that postprandial REE was significantly greater than Con ($p=0.025$). This is in contrast to the findings of Jans et al. who observed no difference in energy expenditure between trials with acute oral administration of SFA, MUFA and PUFA (PUFA contained 20g of fish oil: 18% EPA and 12% DHA). Results from studies investigating medium term effects (15 days - 12 wks.) of fish oil supplementation on RMR are equivocal, finding both an increase (Couet et al. 1997; Gerling et al. 2014)

and no change (Bortolotti, Tappy, and Schneiter 2007; Noreen et al. 2010; Jannas-Vela et al. 2017). These contrasting findings are likely due to differences in study design, with low subject numbers, lack of control of background diet and differing doses of n3PUFA and periods of supplementation all potentially impacting on the results. Those studies observing an increase in RMR (Couet et al. 1997; Gerling et al. 2014) found the difference disappeared when adjusted for LM suggesting n3PUFA may have increased RMR via an increase in LM.

The higher energy expenditure in FO vs. Con in the present study appeared to be due to a postprandial increase in COX with no concomitant decrease in FOX. Therefore it is possible that in FO, fat was preferentially partitioned towards oxidation vs. storage in contrast to SFA (Couet et al. 1997; DeLany et al. 2000; Beermann et al. 2003). Additionally carbon chain length and degree of unsaturation of fatty acyl-CoA's may have meant that more energy was expended in oxidising FO (EPA C20:5(n-3) & DHA C22:6(n-3)) vs. SFA (C16:0) (Yu et al. 2005). Further a reduction in body fat has been reported in short term fish oil supplementation trials (3-6wks) in humans (Couet et al. 1997; Noreen et al. 2010). Conversely, FOX was reduced in an acute study during an hyperinsulinaemic euglycaemic clamp, when an increase in glucose disposal was achieved via the substitution of 1/3 of n6PUFA lipid infusion by n3PUFA in healthy volunteers and (Stephens et al. 2014). The greater skeletal muscle PDC activity found in n3PUFA vs. n6PUFA was suggested to have resulted from reduced allosteric inhibition by muscle acetyl CoA, levels of which were found to be reduced in n3PUFA. Although not measured directly this implies muscle FOX was reduced in n3PUFA vs. n6 PUFA in that

study. Yet the expression of the gene encoding NAMPT (nicotinamide phosphoribosyl transferase) in skeletal muscle was 50% greater in n3PUFA than in n6PUFA in Stephens et al.. NAMPT increases NAD availability, which is a limiting factor on maximal oxidative capacity (Frederick et al. 2015). Speculatively in the present study therefore the rate of the tricarboxylic acid (TCA) cycle could have been increased by n3PUFA via greater availability of NAD. Indeed n3PUFA have also been shown to increase expression of several enzymes involved in the TCA in murine studies (Ahmed et al. 2014). Consequently reduced inhibition of PDC via lack of acetyl CoA accumulation may have allowed an increase in COX despite the maintenance of FOX in FO. The mechanism for maintaining FOX in the face of increased COX in FO requires further investigation. Nevertheless the high postprandial REE found in this study is a novel finding providing greater insight into how n3PUFA may exert beneficial metabolic effects. Particularly in the context of the findings of some chronic studies where n3PUFA supplementation has reduced FM (Couet et al. 1997; Noreen et al. 2010) and increased LM (Couet et al. 1997; Gerling et al. 2014), further studies designed to elucidate the potential benefits of n3PUFA and practical ways of incorporating it into the diet of populations at risk from muscle loss are warranted.

3.4.5 Metabolomics

Metabolomic data suggests more long chain TAG, many of which likely contained EPA and DHA, were increasing in arterialised plasma in FO than SFA and Con t=240-420 min. There could be several explanations for this. Firstly, that these TAG species were still appearing from the gut in the FO

visit. If this were indeed the case then it would perhaps suggest that absorption rate varies between fatty acids. However, a study measuring the fractional absorption of different fatty acids suggests that absorption of palmitate, EPA and DHA are relatively similar (McKimmie, Easter, and Weinberg 2013). This was because although chain length was found to be inversely proportional to fractional absorption, a greater degree of unsaturation had the opposite effect, thus counterbalancing differences in chain length. Furthermore, the proposed relatively quicker gastric emptying of FO in comparison to SFA (Robertson et al. 2002) implies that appearance in blood should have been quicker not slower than SFA. Moreover there was no difference in total serum TAG t=240-420 between FO and SFA suggesting that there was no difference in TAG appearance between the visits.

An alternative explanation for more longer chain TAG increasing in FO than SFA t=240-420 min is that adipose tissue and/or skeletal muscle were taking up fewer longer chain fatty acids in the FO visit. Once again the absence of a difference in total TAG t=240-420 between FO and SFA and the fact that there was no compensatory increase in other TAG in SFA plasma makes this unlikely. Taken together this implies that the differing TAG profiles in blood may simply reflect the profile of the ingested fatty acids (Heath et al. 2007; Hodson, Skeaff, and Fielding 2008). This is also a probable explanation for the findings in terms of DAG. In FO long chain DAG species containing DHA and EPA, were increasing t=240-420 min but this was not the case for SFA or Con.

3.4.6 Limitations & future studies

The main limitation of this study was the heterogeneity of the subject population, particularly in terms of insulin sensitivity. An initial glucose tolerance test was not performed and therefore the insulin sensitivity of the subjects was not known at recruitment. Neither were the details of habitual diet or physical activity collected, both of which may have affected responses to the experiment. On the one hand the oral delivery of nutrients was a strength as it is much more physiological than i.v. delivery and clamp. However, it seems oral delivery with the current protocol may have caused a difference in gastric emptying between visits, which had a significant effect on the results. With only one measurement of FFA a-v difference prior to protein and dextrose ingestion and the absence of a-v data on TAG throughout, it was not possible to confirm that significantly greater uptake of fat by forearm muscle occurred in the lipid visits. Furthermore, the metabolomics techniques deployed appeared not to be sensitive enough to detect differences in a-v balance of lipid metabolites between visits. A 2-step process, where key metabolites were identified using an untargeted approach, which was then followed by a targeted analysis, using standards to derive concentrations of key metabolites, may have been more successful. In line with similar studies it is also important to highlight that the FO visit was still majority SFA with only 36% substitution with n3PUFA.

3.4.7 Conclusions

In summary, in contrast to the original hypothesis, pre ingestion of oral SFA did not induce insulin and anabolic resistance in response to dextrose and protein in men with obesity. Despite the absence of these deleterious effects the part substitution of SFA with n3PUFA still produced positive effects in terms of greater postprandial REE. This could be a key benefit of n3PUFA which may partially explain reduced fat mass found in chronic supplementation studies (Couet et al. 1997; Noreen et al. 2010; Gerling et al. 2014). While further studies are required, this suggests that part replacement of SFA by n3PUFA in the diet of men with obesity may have beneficial effects on metabolic health.

4 REDUCED SKELETAL MUSCLE PROTEIN BALANCE IN PAEDIATRIC CROHN'S DISEASE

Please note that this chapter has been previously published under the same title (Davies et al. 2020).

4.1 INTRODUCTION

In the era of biological therapy and rising obesity in the general population (NCD Risk Factor Collaboration (NCD-RisC) 2017) malnutrition is less prevalent in paediatric Crohn's disease (CD), though still an apparent complication in a substantial minority (Gerasimidis, McGrogan, and Edwards 2011; Vasseur et al. 2010; Marcil et al. 2019). Many of these patients also have disproportionately lower skeletal muscle mass (Thayu et al. 2007; Bechtold et al. 2010). Reduced muscle mass is seen in active disease (Burnham et al. 2005), persists in remission (Sylvester et al. 2009) and is negatively correlated with disease duration (Schneider et al. 2008). Low muscle mass has been linked to decreased muscle function (Wiroth et al. 2005; Werkstetter et al. 2012; Lu et al. 2016), low bone mass and density (Dubner et al. 2009; Werkstetter et al. 2013), reduced physical activity (Werkstetter et al. 2012), fatigue and impaired quality of life (Irvine, Zhou, and Thompson 1996).

Skeletal muscle mass is determined by the balance between muscle protein synthesis and breakdown, rates of which determine protein turnover. Children in active disease a have high protein turnover (Motil et al. 1982). However, whereas conventional medical treatments such as corticosteroids (Thomas et al. 1992), elemental diets (Thomas et al. 1992) and biological therapies (Steiner et al. 2007) reduce fasting protein breakdown, they also reduce fasting protein synthesis, resulting in no change in net fasting protein balance when patients are in remission.

Protein synthesis is stimulated by protein nutrition, particularly the branched chain amino acids (BCAA). Twelve weeks of elemental diet induce partial normalisation of muscle cross-sectional area with no further change at 52 weeks even when patients are in remission (Werkstetter et al. 2013). Once the inflammatory burden is reduced, patients in remission may continue to have reduced muscle mass versus controls (Sylvester et al. 2009), despite receiving apparently adequate protein nutrition (Motil et al. 1982; Diamanti et al. 2009).

This suggests that reduced muscle mass in active disease is driven by inflammation and a sub-optimal muscle protein synthetic response to protein feeding, termed anabolic resistance (Breen et al. 2013) that may persist in remission. Anabolic resistance is thought to be the main driver of muscle loss in critical illness, ageing and disuse (Rennie 2009). Low levels of physical activity may be implicated in the persistence of anabolic resistance in patients in remission.

We hypothesise that reduced muscle mass and function in paediatric CD patients could be associated with anabolic resistance. We aimed to measure skeletal muscle protein balance in a cohort of paediatric CD patients and age- and BMI-matched controls in both the overnight-fasted and fed states.

4.2 MATERIALS AND METHODS

4.2.1 Study population

We aimed to recruit male and female, stable, asymptomatic CD outpatients (age 11-18 years). Relevant CD symptoms were measured through a Harvey Bradshaw index (Harvey and Bradshaw 1980). Patients were approached from Nottingham University Hospitals Trust and Birmingham Women's and Children's NHS Foundation Trust existing patient population. Disease activity was quantified through C-reactive protein (CRP), faecal calprotectin (FCP) and ileocolonoscopy or magnetic resonance imaging (MRI). Active disease was defined as CRP >5mg/dl or FCP >250μg/g or the presence of ulceration at ileocolonoscopy or MRI scan. In the absence of these findings patients were classed as being in deep remission. Any CD-related medication apart from corticosteroids within 3 months prior to recruitment was permitted. Age-, gender- and BMI-matched healthy volunteers were recruited through advertisements at both institutions, through local press, and social media posts to parents. All potential participants were given comprehensive written and verbal explanations of the study before giving written informed consent (parental consent at ≤15yrs) and were free to withdraw at any time. Participants completed a general health questionnaire and underwent a short medical screening prior to participation. The study was approved by the Health Research Authority (15/WM/0285) on the 26th July 2016 with the study conforming to the recognised standards of the Declaration of Helsinki.

4.2.2 Outcome measures

The primary outcome was forearm skeletal muscle protein net balance under fasted and fed conditions. Secondary outcomes were; forearm skeletal muscle glucose net balance; forearm and whole body insulin sensitivity; resting and fed metabolic rate, daily physical activity; forearm muscle isometric strength and fatigability; appendicular lean mass (LM) and appendicular skeletal muscle index (ASMI); daily energy intake and dietary macronutrient composition; and markers of active disease (interleukin (IL)-1β, IL-6, TNFα, CRP and FCP). We investigated the effect of gender and disease activity on the primary outcome in an exploratory sub-analysis.

Differences between arterialised venous and venous concentrations (a-v difference) of BCAA, and glucose, multiplied by brachial artery blood flow and corrected for forearm lean mass were used to determine the net balance of these nutrients across the forearm under fasted and fed conditions. Positive values indicated net tissue uptake, whereas negative values indicated net release of nutrients. The following equation was used for a given nutrient (N):

Net balance of N (μmol/min/100g of forearm lean mass) = {Blood flow (ml/min) x ([N]arterialised – [N]venous (mmol/L or μmol/L)}/1000 (not for glucose)/ {lean mass (g) x 100}.

Lean mass (LM) was measured by Dual-energy X-ray absorptiometry (DEXA) (Luna Prodigy, GE Healthcare). The Matsuda index was used as an index of whole body insulin sensitivity (Matsuda and DeFronzo 1999; DeFronzo and Matsuda 2010), with a lower index indicating a higher level of insulin resistance. Muscle strength measurements were standardized for muscle size, as well as for height and age,

using the method developed by (Rauch et al. 2002), to facilitate comparison. Appendicular LM (sum of lean mass in the limbs measured by DEXA) and ASMI (appendicular lean mass (kg) / height $(m)^2$) were calculated to give more precise indices of skeletal muscle mass than total lean mass alone.

4.2.3 Experimental protocol

Participants reported to the laboratory at 0800, following an overnight fast, having abstained from strenuous exercise for the previous 48 hours. On arrival, their body composition was assessed by DEXA. Body mass, whole body, and regional body composition and body mass index were calculated. Subsequently, participants were asked to rest in a semi-supine position on a bed while a cannula was inserted in a retrograde fashion into a superficial vein on the dorsal surface of the dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand (Gallen and Macdonald 1990). A second cannula was placed in an antecubital vein in the non-dominant forearm. Both these cannulas were used for blood sampling. After baseline blood samples, and measurements of brachial artery blood flow (in the non-dominant arm), as measured by Doppler ultrasound (Toshiba Aplio 300), and resting energy expenditure by indirect calorimetry (Cosmed, Italy), all participants ingested a 220ml bottle of Ensure plus nutrition shake (t = 0). This meal provided 330kcal, consisting of 30% of energy as fat (11g), 53% of energy as carbohydrates (44g), and 17% of energy as protein (14g). This meal composition mirrors dietary recommendations and specifically the protein content equates to 32% of the daily protein requirements of the CD subjects (0.75g/kg bw).

Arterialized-venous (2 ml) and venous (2 ml) blood were obtained concurrently from the heated hand vein and antecubital vein along with brachial artery blood flow measurements at t = 0 and every 20 minutes thereafter for 2 hours, so that forearm muscle net balance of amino acids and glucose could be calculated in the fasted and fed states. At t = 100 a final indirect calorimetry was performed providing resting energy expenditure (REE) and respiratory exchange ratio (RER) to compare with fasting levels.

At the end of the 2h postprandial period, an assessment of forearm muscle function was undertaken. Participants performed 12 maximal static voluntary contractions using a dynamometer (MIE medical research Ltd. UK), with both dominant and non-dominant arms. The peak contraction was taken as maximal handgrip isometric strength (kg), usually achieved within the first 2-3 contractions. Level of fatigue was derived from the difference in peak strength and strength measured at the end of 12 maximal contractions (mean of the last 3) (White et al. 2013). Participants were familiarised with the protocol during screening and the same trained operator both gave instructions and took measurements from all participants (Bandinelli et al. 1999). SDS (standard deviation scores) were calculated for height dependent and weight dependent forearm strength as previously shown (Rauch et al. 2002).

4.2.4 Blood metabolite, cytokine & hormone analysis

Blood glucose levels were measured using Yellow Springs Instrument Analyzer, YSI, 2300 STAT PLUS. Plasma separated from Ethyleneglycol-Bis-(β-Aminoethylether) Tetraacetate (EGTA) treated blood was analysed for BCAA concentrations by spectrophometric assay (Beckett et al. 1996). Serum separated from arterialised-

venous blood was analysed for insulin concentration by radioimmunoassay (Milipore, Human insulin specific kit), CRP was measured by ELISA at the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham, as part of the initial screening process or in baseline blood samples. Inflammatory cytokines, tumour necrosis factor (TNF)α; interleukin (IL)-6; IL-1β, and bioavailable testosterone, were measured in baseline (over-night fasted) arterialized plasma samples by colorimetric ELISA (R&D systems, Minneapolis, US) according to manufacturer's instructions.

4.2.5 Assessment of physical activity, habitual dietary intake & anthropometrics

Step counts measured using a pedometer (Omron, Kyoto, Japan) for 3-days in advance of the study visit and self-reported levels of physical activity, using short form International Physical Activity Questionnaire (IPAQ) (Craig et al. 2003) were used to assess habitual physical activity levels. Routine energy intake was measured using a 3-day paper-based food diary completed by participants in the days preceding their study visit (1 weekend day and 2 week days). Great care was taken in briefing participants, checking diaries during the study visit and the same operator analysed all the diaries using Nutritics software (Dublin, Ireland) in an effort to minimise inaccuracy. Anthropometrics were collected by a single investigator using a standard protocol and z-scores were calculated for height and BMI (WHO Growth Reference data for 5-19 year olds) and for fat mass (FM) and fat free mass (FFM) (McCarthy et al. 2014).

4.2.6 Statistical analyses

The parametric or non-parametric nature of the data was determined with a Shapiro-Wilk test. Parametric data are presented as mean ± standard error of the mean (SEM) and non-parametric as median plus interquartile range (IQR). Parametric data has been examined using t-tests or where time is also a variable, two-way analysis of variance (experimental group x time). Where data were not normally distributed, these were analysed using a Mann-Whitney test. Area under the curve (AUC) has also been calculated to illustrate glucose net uptake in response to feeding. P value of <0.05 was considered significant. Data analysis was undertaken with Prism software V.7.0 (La Jolla, San Diego, US)

4.3 RESULTS

4.3.1 Subject characteristics

Twenty CD participants (15.6 ± 0.5 yrs; BMI 20.6 ± 0.9 kg/m^2) were recruited (Table 4.1). Mean number of years since diagnosis was 4.3 ± 0.6. All CD participants were asymptomatic with an HBI of < 4. Nine CD participants had evidence of disease activity with a mean FCP of 808 ± 225ug/g, mean CRP of 2.2 ± 1.2 mg/dl or evidence of ulceration at ileocolonoscopy or magnetic resonance enterography (MRE). The other eleven CD participants were in deep remission with mean FCP of 61 ± 12ug/g and CRP of 0.3 ± 0.2 mg/dl and absence of disease activity at ileocolonoscopy or MRE. Eleven participants were being treated with anti-TNF therapy (Adalimumab or Infliximab) while the rest were naïve to biological therapies. Five CD participants had a history of intestinal resection for stricturing (n=3) or penetrating (n=2) disease behaviour. See supplementary table for individual CD participant details (Appendix 2.1). Nine healthy control (Con) participants (16.0 ± 0.6 years; BMI 20.7 ± 0.6 kg/m^2) were recruited and matched to CD participants. Although there were no significant differences in anthropometrics between groups 10% of CD patients had a BMI z-score of <-2. It was not possible to cannulate two of the female CD participants, so for mechanistic studies in CD n=18. All endoscopic or imaging investigations were undertaken as part of the patients' standard care.

Table 4.1 Subject characteristics of control and Crohn's disease patients

	Con n=9	CD n=20[w] / n=18[v]	p value
Age (yrs)[w]	16.0 ± 0.6	15.6 ± 0.5	n.s.
Height (m) [w]	1.71 ± 0.05	1.66 ± 0.02	n.s.
Height-for-age (z-scores) [w]	0.5 ± 0.4	0.1 ± 0.2	n.s.
Weight (kg) [w]	60.7 ± 3.7	56.8 ± 2.7	n.s.
BMI (kg/m^2) [w]	20.7 ± 0.6	20.6 ± 0.9	n.s.
BMI-for-age (z-scores) [w]	0.03 ± 0.27	-0.2 ± 0.3	n.s.
Years since diagnosis [w]	n/a	4.2 ± 0.6	n/a
HBI [w]	n/a	1 ± 0	n/a
FCP (µg/g) [w]	n/a	132 ± 41	n/a
CRP (mg/dl) [w]	<5 ± 0	2.6 ± 0.6	n.s.
IL-1β (pg/ml)[v]	169 (37.5-517)	454 (30.5-1835)	n.s.
IL-6 (pg/ml) [v]	101 (50-335)	83 (28-287)	n.s.
TNFα (pg/ml) [v]	37 (0-111)	212 (0-661)	p=0.078
Testosterone (ng/ml) [v]	1.8 (1.4-8.2)	9.0 (3.1-16.6)	p=0.031

All values are means ± SEM except for non-parametric test results (cytokines & testosterone) where values are median and interquartile range and z-scores, which were calculated for height-for-age and BMI-for-age using WHO standards. Statistically significant differences ($p<0.05$) and trends ($p<0.1$) are listed and n.s. = no significant differences between groups. CD=Crohn's disease, Con=Control, HBI=Harvey Bradshaw Index, FCP=faecal calprotectin, CRP=C-reactive protein, IL-1β=Interleukin 1 beta**,** IL-6=Interleukin 6, TNFα=tumour necrosis factor alpha, w = n=20 and v = n=18.

4.3.2 Muscle physiology: protein and glucose metabolism

Arterialized plasma BCAA (BCAA) peaked at t = 40 in response to feeding (time $p<0.0001$) (Figure 4.1A). Con but not CD were in positive forearm muscle BCAA balance (Figure 4.1B), although both groups mounted an initial response to feeding (t = 0 to t = 20) (Figure 4.1C).

Glucose a-v difference across the forearm increased in response to feeding, with this and glucose net uptake not different between CD and Con suggesting no skeletal muscle insulin resistance (Figures 4.2A & C).

4.3.3 Body composition and muscle function

There was a significant difference between Con and CD FFM z-scores (0.10 and -0.84 respectively, $p=0.015$) but only trends towards a difference in LBM, appendicular LM and ASMI (Table 4.2). All other body composition measures were comparable between Con and CD. No differences were found in muscle strength or fatigue although there was a trend for CD to fatigue more than Con in the dominant arm.

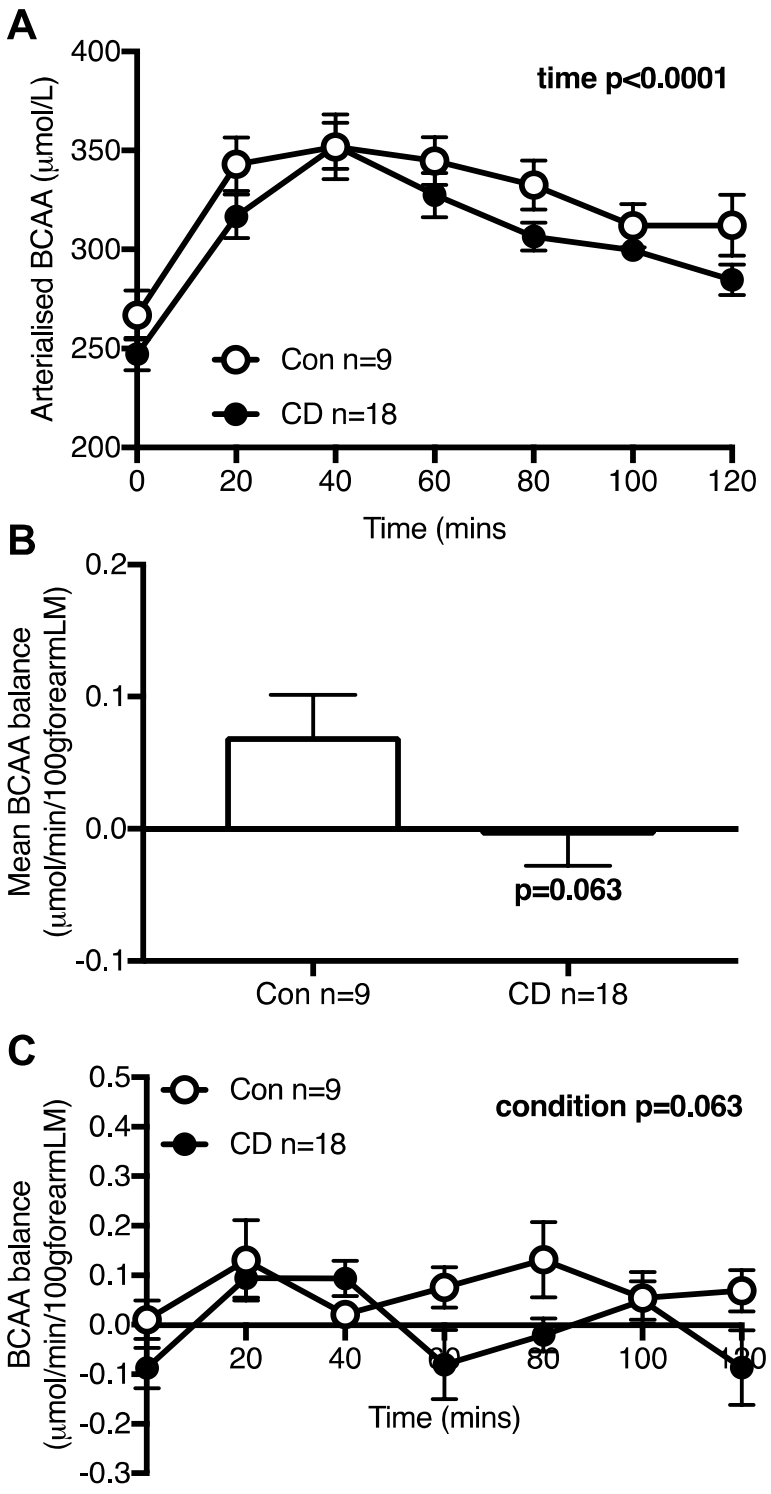


Figure 4.1 Indicators of protein metabolism in CD vs. Con: arterialized plasma BCAA concentrations (A), mean BCAA net balance across the forearm standardised for forearm lean mass (B) and BCAA net balance across the forearm over time, standardised for forearm lean mass (C).

Values are means ± SEM. Significant differences ($p<0.05$) & trends ($p<0.1$) are marked. BCAA=branched chain amino acids, CD=Crohn's disease, Con=Control.

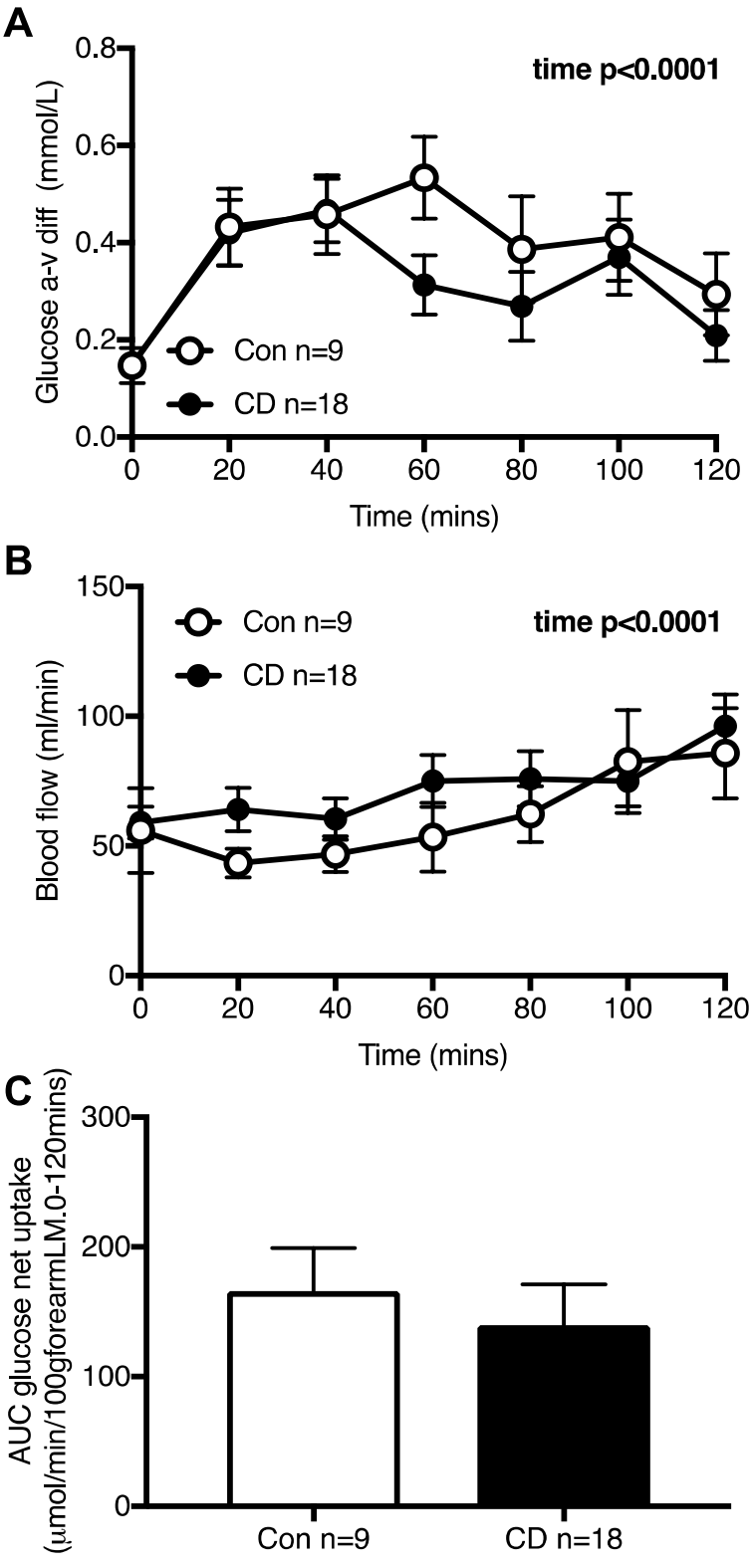


Figure 4.2 Skeletal muscle insulin sensitivity in CD vs. Con: glucose arterio-venous difference across the forearm (A), brachial artery blood flow (B) and AUC glucose net uptake across the forearm standardized for forearm lean mass (C).

Values are means ± SEM. Significant differences ($p<0.05$) & trends ($p<0.1$) are marked. CD=Crohn's disease, Con=Control.

Table 4.2 Body composition and muscle function

	Con n=9	**CD** n=20[w] / n=18[v]	**p value**
Body composition[w]			
FM (z-scores)	0.08 ± 0.37	0.90 ± 0.40	n.s.
FFM (z-scores)	0.10 ± 0.44	-0.84 ± 0.15	p=0.015
LBM (kg)	46.0 ± 4.5	39.3 ± 1.5	p=0.084
Appendicular LM (kg)	22.9 ± 2.6	18.7 ± 0.8	p=0.057
ASMI (kg/m^2)	7.6 ± 0.4	6.8 ± 0.2	p=0.052
Muscle function[v]			
Fatigue (%) dom	18 ± 3	26 ± 2	p=0.061
Fatigue (%) non-dom	21 ± 4	23 ± 2	n.s.
Strength dom (kg/kg forearm LM)	23.8 ± 1.3	25.6 ± 1.5	n.s.
Strength non-dom (kg/kg forearm LM)	23.9 ± 1.1	24.3 ± 1.4	n.s.
Age dependent FS SDS dom	-1.41 ± 0.35	-1.50 ± 0.22	n.s.
Age dependent FS SDS non-dom	-1.62 ± 0.31	-1.90 ± 0.21	n.s.
Height dependent FS SDS dom	-1.13 ± 0.35	-0.93 ± 0.34	n.s.
Height dependent FS SDS non-dom	-1.36 ± 0.33	-1.37 ± 0.36	n.s.

All values are means ± SEM, except standard deviation scores (SDS) calculated for height and age dependent strength (see methods) and z-scores calculated for FM & FMM using age and sex appropriate reference data (see methods). Statistically significant differences ($p<0.05$) and trends ($p<0.1$) are listed and n.s. = no significant differences between groups. CD=Crohn's disease, Con=Control, FM=fat mass, FFM=fat free mass, LBM=lean body mass, LM=lean mass, ASMI= appendicular skeletal muscle index, FS=forearm strength, dom=dominant arm, non-dom=non dominant arm, w = n=20 and v = n=18.

4.3.4 Whole body physiology: insulin sensitivity & energy expenditure

Arterialized blood glucose and serum insulin levels increased in both groups post-feeding (t = 20) and peaked at t = 40, (time $p<0.0001$), (Figures 4.3A & B). Glucose concentrations then dropped back, stabilizing above fasting levels by t = 60, whereas insulin continued to decline until t = 120. CD response to feeding in terms of these parameters and Matsuda index was not different to Con and therefore no whole-body insulin resistance was detected (Figures 4.3A - C). REE and RER increased post-feeding (t = 0 vs. t = 20-120) in CD and Con (all $p<0.05$) with no differences between groups (Table 4.3).

4.3.5 Cytokine analyses

No differences in TNFα, IL-1β and IL-6 were observed between CD and Con groups although there was a trend for higher TNFα in CD (Table 4.1).

4.3.6 Testosterone levels

Testosterone levels were significantly higher in CD (median 9.0 ng/ml, IQR: 3.1-16.6) when compared to Con (1.8ng/ml, 1.4-8.2, $p=0.031$) (Table 4.1).

4.3.7 Physical activity and diet

Neither activity levels nor total energy intake differed between groups (Table 4.3).

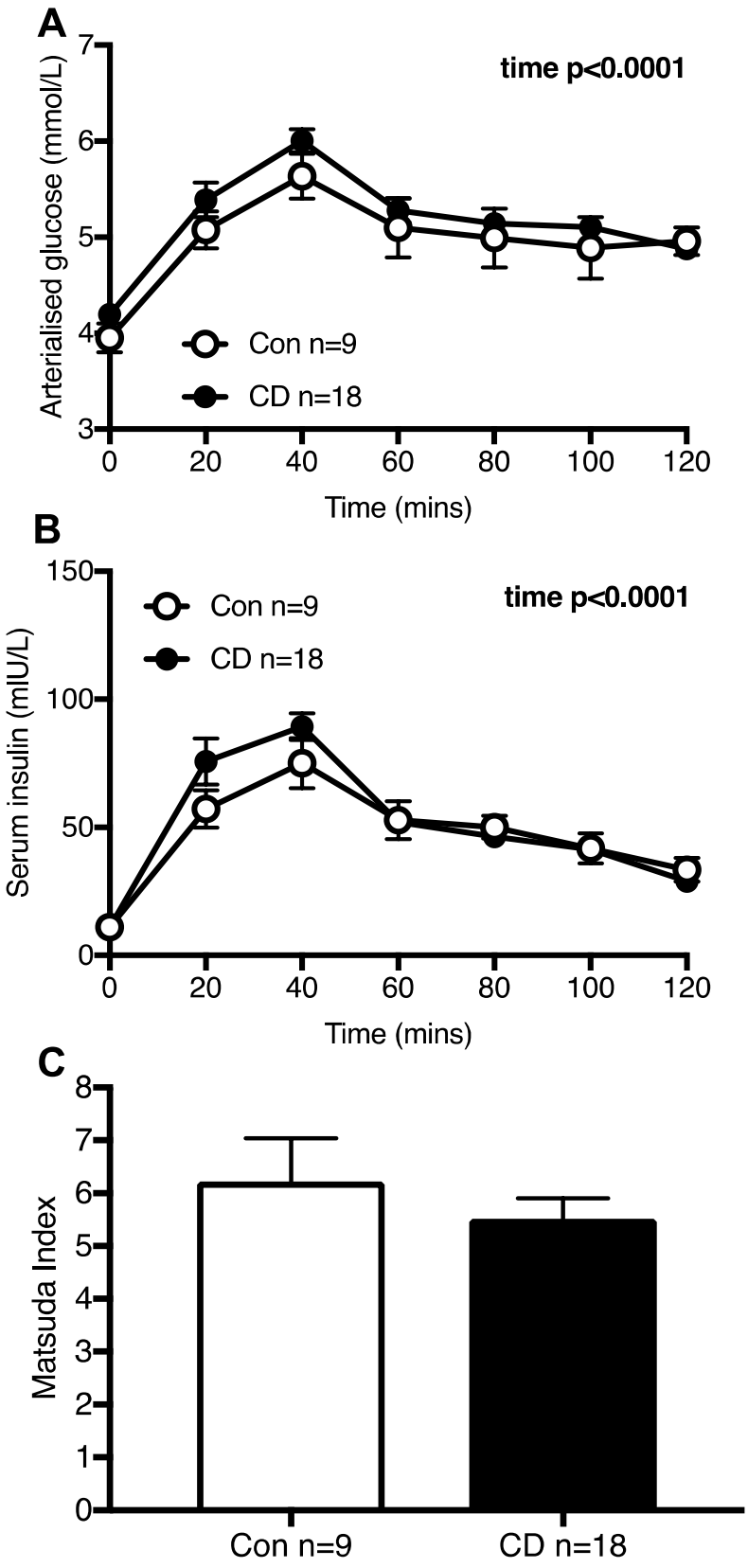


Figure 4.3 Indicators of whole body insulin sensitivity in CD vs. Con: arterialized blood glucose concentrations (A), serum insulin (B) and Matsuda index (C). Values are means ± SEM. Significant time effects are marked. CD=Crohn's disease, Con=Control.

Table 4.3 Energy intake & energy expenditure

	Con n=9	**CD** n=20[w] / n=18[v]	**p value**
Energy intake[w]			
Energy intake (kJ/day)	9124 ± 1081	8333 ± 512	n.s.
Protein intake (kJ/day)	1531 ± 173	1350 ± 93	n.s.
Protein intake (g/day)	90 ± 10	79 ± 5	n.s.
Recommended protein intake (g/day)	46 ± 3	43 ± 2	n.s.
Indirect calorimetry[v]			
REE - fasted (kJ/hr/kgLBM)	6.1 ± 0.3	5.9 ± 0.4	n.s.
REE - post-feeding (kJ/hr/kgLBM)	6.5 ± 0.3[*]	6.0 ± 0.4[*]	n.s.
RER – fasted	0.81 ± 0.02	0.76 ± 0.05	n.s.
RER - post-feeding	0.87 ± 0.02[*]	0.80 ± 0.05[*]	n.s.
Physical Activity[w]			
Pedometer (no of steps/day)	8056 ± 849	7831 ± 725	n.s.
IPAQ (total MET-mins/week)	6196 ± 2158	4443 ± 994	n.s.

All values are means ± SEM. Statistically significant differences between fed and fasted states in both groups are marked *=p<0.05. n.s. = no significant differences between groups. CD=Crohn's disease, Con=Control, IPAQ: international physical activity questionnaire, w = n=20 and v = n=18.

4.3.8 Sub-analyses by sex & disease activity

Results from the sex and disease activity sub-analyses are shown in Appendix 2. Male CD participants (CDM) n=11, were in overall negative BCAA balance, in contrast to the positive balance of male control participants (ConM) n=5, (p=0.049) (Appendix 2.2F). CDM also had lower levels of arterialised BCAA than ConM at baseline and post feeding (condition p=0.027) (Appendix 2.2B). CDM had a lower height for age when compared to ConM (z-scores -0.2 and 0.5 respectively, p=0.021) (Appendix 2.3) and appendicular LM was 24% lower than in ConM (p=0.034) (Appendix 2.4). CDM fatigued significantly more than ConM in the dominant arm (p=0.014) (Appendix 2.5). Protein intake was lower in CDM (p=0.026) with 75 ± 5g/kg body mass/day reported in CDM and 105 ± 15g/kg/day reported in ConM (Appendix 2.7).

There was a difference in skeletal muscle insulin sensitivity in active (n=9) versus deep remission (n=11) (Appendix 2.8D and L). In active disease forearm glucose net uptake was more than 2-fold greater than deep remission (p=0.036) implying skeletal muscle insulin resistance in CD in deep remission. Serum insulin also peaked sooner in active disease, at 20 min, versus 40 min in deep remission (interaction p=0.0014) (Appendix 2.9H). Active disease had higher serum IL-6 levels than deep remission (median: 206 pg/ml, IQR: 83-390 vs. 41 pg/ml, 0-135, respectively; p=0.025). There was however no difference in anabolic response between those in active disease and deep remission (Appendix 2.2H&L).

4.4 DISCUSSION

We hypothesised that the reduced muscle mass in CD could be driven through anabolic resistance of skeletal muscle to a mixed oral meal that may persist even when in clinical remission. Con but not CD were in a positive net AA balance and this was associated with reduced FFM z-scores. However, CD participants were able to mount an initial positive BCAA balance response to feeding and so may not be completely anabolically resistant as hypothesised. Protein balance, and the ability to increase muscle protein mass, is also determined by muscle protein breakdown. The overall neutral net balance observed in the current study may suggest that muscle protein synthesis and/or breakdown are affected. Male CD participants had an overall negative AA balance, with associated lower appendicular LM, higher levels of muscle fatigue and reduced habitual dietary protein intake. Moreover, CD patients with active disease had similar net muscle AA balance, but a higher skeletal muscle glucose uptake, compared to those in deep remission. These exploratory findings may imply that anabolic resistance plays a role in the aetiology of reduced LM in males irrespective of disease status. These findings will need to be validated in a larger cohort.

Adolescent CD patients may therefore require more protein per meal compared to age-matched controls in order to maintain a positive AA balance and muscle mass. This is the first study to have investigated skeletal muscle AA balance in paediatric CD.

Findings of reduced muscle mass in paediatric CD in remission are congruent with the majority of the literature (Burnham et al. 2005; Thayu et al. 2007; Sylvester et al.

2009; Bechtold et al. 2010). Reduced muscle function has been previously found in paediatric CD patients with mild disease and in clinical remission (Werkstetter et al. 2012).

CD participants in the current study still consumed more protein than recommended and their consumption was above National Diet and Nutrition Survey (NDNS) 2014-16 ('NDNS: Results from Years 7 and 8 (Combined) - GOV.UK' n.d.) average values. CD: 79g and NDNS: 67g (NDNS aged 11-18 and weighted to match our gender split). However, a higher protein recommendation may be appropriate as a linear relationship between amino acid availability and protein balance holds true above current recommended intake levels because amino acids suppress muscle protein breakdown as well as stimulate protein synthesis (Deutz and Wolfe 2013). An overall increase in protein intake, rather than specific amino acid supplements, has recently been suggested to be most effective in both stimulating muscle protein synthesis and suppressing muscle protein breakdown (Cholewa et al. 2017). Greater anabolic response to higher doses of protein in healthy adults has been shown to be largely due to the suppression of protein breakdown with only a small increase in protein synthesis (Kim et al. 2016). Despite experimental studies indicating leucine is key in stimulating the mTOR pathway (Kimball et al. 1999) chronic supplementation has not consistently produced positive results (Cholewa et al. 2017). It has now been demonstrated that stimulation of muscle protein synthesis by protein ingestion is not solely due to leucine (van Vliet et al. 2018). A study in CD adults showed that protein supplementation equivalent to a 25% increase in protein intake for 16 weeks improved LM; though no detail of protein turn-over was available (Machado et al. 2015). Our observation of a lower net AA balance in the face of adequate amino acid availability would further support the notion of an impaired anabolic response in CD.

Testosterone levels were raised in CD. This contrasts with previous studies that have reported both reduced androgens and delayed maturation in CD (Ballinger, Savage, and Sanderson 2003; DeBoer and Denson 2013), though it is likely that these studies reported on total rather than bioavailable testosterone but did not specify.

Insulin inhibits protein breakdown and has a permissive effect in stimulating protein synthesis via the Akt/mTOR signaling pathway (Proud 2006; Lai et al. 2012). Whole body insulin resistance has been found in CD patients with active disease (Bregenzer et al. 2006) with TNFα being shown to reduce insulin sensitivity in humans (Plomgaard et al. 2005). In the present study skeletal muscle insulin sensitivity is similar between CD and Con as has been previously shown (Capristo et al. 1999). However skeletal muscle glucose uptake was higher in active disease vs. deep remission. Although plasma TNFα levels were similar between these groups, plasma IL-6 was significantly higher in patients with active disease. Chronically elevated levels of IL-6 have been implicated in insulin resistance and reduced skeletal muscle mass (Haddad et al. 2005). Conversely acute administration of IL-6 has been found to increase glucose disposal (Carey et al. 2006). It remains unclear why skeletal muscle insulin sensitivity may be higher in active disease vs. deep remission.

Higher basal metabolic rate per FFM ratio and diet-induced thermogenesis have regularly been reported in CD, which could explain lower body mass (Varille et al. 1996). However, in accordance with the current study, several other studies in both paediatric CD (Wiskin et al. 2009) and adults (Schneeweiss et al. 1999; Takaoka et al. 2015) have found no relationship between the two.

The association of reduced AA balance with reduced FFM z-scores in CD is a possible explanation as to why patients fail to gain muscle mass even when in remission. CD may not be able to re-build muscle mass because they cannot maintain a positive AA balance in response to feeding. In a study of young adult males with inflammatory bowel disease, a fifth of participants had significantly reduced muscle mass raising the possibility that these LM deficits persist into adulthood (Bryant et al. 2015). A longitudinal study would be needed to investigate this.

The main limitation of this study is the relatively small sample size that will not allow detailed sub-analyses by disease activity and sex. Moreover, it is cross-sectional and acts as a precursor to a larger prospective study. Furthermore, although reduced serum Vitamin D has been implicated in reduced LM (Mager et al. 2018) values were unavailable in this cohort.

A key strength was our use of the a-v balance technique giving a holistic view of the anabolic response and high temporal resolution. The discovery that CD can respond to anabolic stimuli, albeit to a lesser extent than healthy controls, has important implications for treatment.

In conclusion, we have shown that reduced FFM z-scores in CD patients are associated with a reduced AA balance in response to feeding, thus providing a possible explanation for the persistence of low muscle mass in children regardless of disease activity. Moreover, male CD participants were in overall negative AA balance with associated lower appendicular LM, height for age, fatigue in the dominant arm and protein intake. This is an interesting preliminary observation that

requires further elucidation. A large prospective interventional study is warranted to confirm the effects of disease activity and gender, protein intake and exercise, with the aim of restoring muscle mass to healthy levels in children and young adult CD patients.

5 INVESTIGATING THE RELATIONSHIP BETWEEN ANABOLIC AND INSULIN RESISTANCE AND REDUCED MUSCLE MASS IN ADULTS WITH ACTIVE CROHN'S DISEASE

5.1 INTRODUCTION

In Chapter 4 paediatric CD patients in clinical remission had reduced protein balance in response to a mixed meal and this was associated with reduced FFM z-scores but not insulin resistance. The prevalence of reduced muscle mass in adult CD patients has been estimated to be between 21% (Bryant et al. 2015) and 61% (Zhang et al. 2017). As in adolescent patients, it has been linked with reduced muscle function (Valentini et al. 2008; Lu et al. 2016), osteopenia (Bryant et al. 2015), fatigue and lower quality of life (Wiroth et al. 2005).

Reduced muscle mass in adult CD has also been associated with reduced phosphorylation of Akt and 4EBP-1 suggesting a diminution of muscle protein synthetic signalling (van Langenberg, Della Gatta, Hill, et al. 2014). Muscle protein breakdown signalling targets E3 ubiquitin ligases (MuRF-1 & atrogin-1) were also investigated but were found to be no different from control. As discussed in Chapter 1 only one study of protein balance in adult CD has been found, which measured whole body protein balance in the fed state (Powell-Tuck et al. 1984). Findings in that study are in accordance with those in paediatric patients (Motil et al. 1982; Thomas et al. 1992; Steiner et al. 2007) showing increased protein turnover in active disease, which is positively correlated with disease severity. Insulin resistance has been shown in active disease (Bregenzer et al. 2006) and TNFα has been shown to reduce insulin sensitivity by inhibiting phosphorylation of Akt (Plomgaard et al. 2005). Yet those in remission appear to be insulin sensitive (Capristo et al. 1999).

It is not known therefore whether anabolic resistance is also implicated in reduced muscle mass in adult CD patients and whether it is associated with insulin resistance in active disease. It was therefore planned to replicate the paediatric protocol in adult CD in active disease to answer these questions.

It is hypothesized that anabolic resistance will be associated with reduced lean mass in adult CD in active disease and that these patients will also be insulin resistant.

5.2 METHODS

5.2.1 Subjects

Male and female CD outpatients with active disease (aged 18-75) were recruited from Nottingham University Hospitals Trust. Disease activity was defined through objective markers of inflammation: faecal calprotectin (FCP) of >250 µg/g, C-reactive protein (CRP) of >5 g/dL, recent ileocolonoscopy, computed tomography [CT], magnetic resonance [MR] enterography showing active disease. CD symptoms were measured with a Harvey-Bradshaw Index [HBI] score (>5) recorded at inclusion. Any CD-related medication apart from corticosteroids within 3 months prior to recruitment was permitted.

Age-, sex- and BMI-matched healthy volunteers were recruited through advertisements on the Nottingham University and Nottingham University Hospitals Trust campuses.

5.2.2 Outcome measures

The primary outcome was forearm skeletal muscle BCAA net balance under fasted and fed conditions. Secondary outcomes were; forearm skeletal muscle glucose net balance; forearm and whole body insulin sensitivity; forearm skeletal muscle FFA net balance; resting and fed metabolic rate, daily physical activity; forearm muscle isometric strength and fatigability; appendicular lean mass (LM) and appendicular skeletal muscle index (ASMI); daily energy intake and dietary macronutrient composition; and markers of

active disease (IL-1β, IL-6, TNFα, CRP and FCP). The effect of sex on the primary outcome was investigated in an exploratory sub-analysis.

5.2.3 Experimental protocol

Participants reported to the laboratory at 0800, following an overnight fast, having abstained from strenuous exercise and alcohol for the previous 48 hours. On arrival, their body composition was assessed by DEXA. Subsequently, participants were asked to rest in a semi-supine position on a bed while a cannula was inserted in a retrograde fashion into a superficial vein on the dorsal surface of the dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand (Gallen and Macdonald 1990). A second cannula was placed in an antecubital vein in the non-dominant forearm. Both these cannulas were used for blood sampling (see General Methods for details). After baseline blood samples, and measurements of brachial artery blood flow (in the non-dominant arm), as measured by Doppler ultrasound (Toshiba Aplio 300), and resting energy expenditure by indirect calorimetry (Cosmed, Italy), all participants ingested a 220ml bottle of Ensure plus nutrition shake (t = 0). This meal provided 330kcal, consisting of 30% of energy as fat (11g), 53% of energy as carbohydrates (44g), and 17% of energy as protein (14g). This meal composition mirrors dietary recommendations and specifically the protein content equates to 24% of the daily protein requirements of the CD subjects (0.75g/kg bw).

Arterialized-venous (2 ml) and venous (2 ml) blood were obtained concurrently from the heated hand vein and antecubital vein along with brachial artery blood flow measurements at t = 0 and every 20 minutes thereafter for 2 hours, so that forearm muscle net balance of amino acids, glucose and FFA could be calculated in the fasted and fed states. At t = 100 min a final indirect calorimetry was performed providing resting energy expenditure (REE) and respiratory exchange ratio (RER) to compare with fasting levels.

At the end of the 2h postprandial period, an assessment of forearm muscle function was undertaken (See General Methods).

5.2.4 Blood metabolite, cytokine & hormone analysis

Blood glucose levels were measured using Yellow Springs Instrument Analyzer, YSI, 2300 STAT PLUS. Plasma separated from Ethyleneglycol-Bis-(β-Aminoethylether) Tetraacetate (EGTA) treated blood was analysed for BCAA concentrations by spectrophometric assay (Beckett et al. 1996). Serum separated from arterialised-venous blood was analysed for insulin concentration by radioimmunoassay (Milipore, Human insulin specific kit). CRP was measured by ELISA at the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham, as part of the initial screening process or in baseline blood samples. Inflammatory cytokines: TNFα; IL-6 and IL-1β were measured in baseline (over-night fasted) arterialized plasma samples by colorimetric ELISA (R&D systems, Minneapolis, US) according to manufacturer's instructions. (See General Methods for details for all assays).

5.2.5 Assessment of physical activity & habitual dietary intake

Step counts measured using a pedometer (Omron, Kyoto, Japan) for 3-days in advance of the study visit and self-reported levels of physical activity, using short form International Physical Activity Questionnaire (IPAQ) (Craig et al. 2003) were used to assess habitual physical activity levels. Routine energy intake was measured using a 3-day paper-based food diary completed by participants in the days preceding their study visit (1 weekend day and 2 week days). (See General Methods for further details.)

5.2.6 Patient-related outcome measures (PROMS)

The Inflammatory bowel disease questionnaire (IBDQ) (Irvine, Zhou, and Thompson 1996), Short Form 36 Health Survey (SF-36) (Ware and Sherbourne 1992), the Inflammatory Bowel Disease fatigue (IBD-F) patient self-assessment scale (Czuber-Dochan et al. 2014), European quality of life 3 dimensions (EQ-3D) (EuroQol Group 1990) and the Hospital anxiety and depression scale (HADS) (Zigmond and Snaith 1983) were also completed by all patients.

5.2.7 Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) and any potential differences between CD and Con have been examined using t-tests or where time is also a variable, two-way analysis of variance (condition x time). Where significant differences were found post-hoc analysis (Sidak's multiple comparisons) were used. Area under the curve (AUC) has been

calculated to illustrate glucose net uptake in response to feeding. P value of <0.05 was considered significant. Data analysis was undertaken with Prism software V.7.0 (La Jolla, San Diego, US)

5.3 RESULTS

5.3.1 Subject characteristics

Eight CD participants (41.3 ± 4.5 yrs; BMI 26.9 ± 1.5 kg/m^2) were recruited (Table 5.1). Mean number of years since diagnosis was 14.5 ± 4.6 and mean age at diagnosis was 27 ± 4 yrs. Only one participant was diagnosed prior to the age of 18. All CD participants had active disease with a mean FCP of 749 ± 275 µg/g, mean CRP of 7 ± 3 mg/dl. Three participants were being treated with anti-TNFα therapy (Adalimumab or Infliximab), three with Thiopurines (Mercaptopurine) and one with both (Supplementary table in Appendix 3). One patient was not undergoing any pharmacological therapy neither did they have any history of pharmacological use. 5/8 CD participants had a history of intestinal resection for stricturing or penetrating disease behaviour. See supplementary table in Appendix 3 for individual CD participant details. All endoscopic or imaging investigations were undertaken as part of the patients' standard care.

Eight healthy control (Con) participants (41.2 ± 4.3 years; BMI 25.1 ± 1.1 kg/m^2) were recruited and matched to CD participants (Table 5.1). There were no significant differences in anthropometrics between groups. Results of the cytokine assays (TNFα, IL-1β and IL-6) are not presented as the number of detectable results was so low. However, it should be noted that the samples were analysed in the same batch as the paediatric samples (from Chapter 4) where cytokines were detected. This suggests that TNFα, IL-1β and IL-6 levels were too low to be detected in samples from adult Con and CD.

Table 5.1 Subject characteristics of Con and CD patients

	Con	CD	p	ConM	CDM	p	ConF	CDF	p
	n=8	n=8 n=5[b]		n=4	n=4		n=4	n=4 n=1[b]	
Age (yrs)	41.2 ± 4.3	41.3 ± 4.5	n.s.	38.9 ± 4.6	39.4 ± 5.4	n.s	43.6 ± 7.7	43.1 ± 8.0	n.s
Height (m)	1.8 ± 0.03	1.7 ± 0.03	n.s	1.8 ± 0.04	1.8 ± 0.04	n.s.	1.7 ± 0.02	1.6 ± 0.03	n.s.
Weight (kg)	78.3 ± 3.3	78.5 ± 6.0	n.s	83.0 ± 4.7	87.0 ± 3.7	n.s.	73.7 ± 3.9	69.9 ± 10.2	n.s.
BMI (kg/m^2)	25.1 ± 1.1	26.9 ± 1.5	n.s	24.8 ± 1.3	28.0 ± 1.2	n.s.	25.4 ± 1.9	25.7 ± 2.8	n.s.
Diagnosis (yrs)	n/a	14.5 ± 4.6	-	n/a	12.8 ± 1.6	-	n/a	16.3 ± 9.8	-
HBI	n/a	4 ± 2	-	n/a	2 ± 1	-	n/a	6 ± 3	-
FCP (µg/g)[b]	n/a	749 ± 275	-	n/a	751 ± 355	-	n/a	742	-
CRP (mg/dl)	5 ± 3	7 ± 3	n.s	6 ± 5	11 ± 5	n.s.	4 ± 2	4 ± 4	n.s.

All values are means ± SEM. There were no significant differences between groups. CD=Crohn's disease, Con=control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD, HBI=Harvey Bradshaw Index, FCP=faecal calprotectin, CRP=C-reactive protein, b= reduced n number (CD n=5 and CDF n=1).

5.3.2 Body composition and muscle function

There were no significant differences in body composition between groups (Table 5.2). Appendicular LM was 24.5 ± 2.3 kg in Con 23.7 ± 2.4 kg in CD. Neither were there differences between Con and CD in muscle function (Table 5.3). On average subjects fatigued to a similar level in both arms (c.17%) and also had similar levels of strength. Average strength in the dominant arm was 29.2 ± 2.5 kg/kgforearmLM in Con and 24.0 ± 2.4 kg/kgforearmLM in CD.

5.3.3 Energy expenditure

REE increased post-feeding (t=0 vs. t=105 min) as expected in Con from 5.8 ± 0.2 to 6.4 ± 0.3 kJ/hr/kgLBM and in CD from 5.9 ± 0.3 to 6.5 ± 0.4 kJ/hr/kgLBM (time effect p=0.0003) with no differences between groups (Table 5.4). However there were no time differences in RER, COX or FOX. There were no differences between groups in any of these metrics.

Table 5.2 Body composition

	Con	CD	P	ConM	CDM	P	ConF	CDF	P
	n=8	n=7		n=4	n=4		n=4	n=3	
FM (kg)	24.5 ± 3.3	28.6 ± 3.5	n.s.	21.3 ± 4.2	28.1 ± 3.4	n.s.	27.7 ± 5.2	29.2 ± 8.0	n.s.
BMC (kg)	3.2 ± 0.2	2.9 ± 0.2	n.s.	3.5 ± 0.3	3.3 ± 0.2	n.s.	2.8 ± 0.2	2.3 ± 0.3	n.s.
BMD (g/cm^3)	1.3 ± 0.05	1.2 ± 0.04	n.s.	1.3 ± 0.1	1.3 ± 0.03	n.s.	1.2 ± 0.1	1.1 ± 0.1	n.s.
LBM (kg)	50.6 ± 4.0	49.5 ± 4.1	n.s.	58.1 ± 5.6	55.7 ± 3.8	n.s.	43.2 ± 2.1	41.4 ± 5.5	n.s.
LBM/TM (%)	65 ± 4	61 ± 3	n.s.	70 ± 5	64 ± 3	n.s.	59 ± 5	58 ± 3	n.s.
Appendicular LM (kg)	24.5 ± 2.3	23.7 ± 2.4	n.s.	29.2 ± 2.8	28.0 ± 2.1	n.s.	19.8 ± 1.1	18.0 ± 1.9	n.s.
ASMI (kg/m^2)	7.7 ± 0.5	8.0 ± 0.7	n.s.	8.7 ± 0.7	9.1 ± 0.8	n.s.	6.8 ± 0.2	6.5 ± 0.5	n.s.

All values are means ± SEM. There were no significant differences between groups. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con, CDF=female CD, FM=fat mass, BMC=bone mineral content, BMD=bone mineral density, LBM=lean body mass, TM = total body mass, LM=lean mass, ASMI= appendicular skeletal muscle index.

Table 5.3 Muscle function

	Con	CD	P	ConM	CDM	P	ConF	CDF	P
	n=8	n=8[v] / n=7[w]		n=4	n=4		n=4	n=4[v] / n=3[w]	
Fatigue (%) D[v]	17 ± 4	15 ± 3	n.s.	19 ± 7	16 ± 5	n.s.	15 ± 3	14 ± 6	n.s.
Fatigue (%) ND[v]	22 ± 5	13 ± 2	n.s.	25 ± 11	13 ± 4	n.s.	19 ± 4	13 ± 1	n.s.
Strength D (kg/kgforearmLM)[w]	29.2 ± 2.5	24.0 ± 2.4	n.s.	24.9 ± 3.9	25.5 ± 3.9	n.s.	33.5 ± 1.2	22.1 ± 3.0	0.01
Strength ND (kg/ kgforearmLM)[w]	27.5 ± 2.3	24.6 ± 2.1	n.s.	24.0 ± 2.9	25.0 ± 3.1	n.s.	31.1 ± 2.6	24.0 ± 3.3	n.s.

All values are means ± SEM. Statistically significant differences ($p<0.05$) are listed and n.s.=no significant differences between groups. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con, CDF=female CD, D=dominant arm, ND=non dominant arm . v = n=8 and w = n=7.

Table 5.4 Energy expenditure

	Con	CD	p	ConM	CDM	p	ConF	CDF	P
	n=8	n=7		n=4	n=4		n=4	n=3	
REE – fasted (kJ/hr/kgLBM)	5.8 ± 0.2	5.9 ± 0.3	n.s.	5.7 ± 0.3	5.5 ± 0.3	n.s.	5.9 ± 0.2	6.4 ± 0.4	n.s.
REE – fed (kJ/hr/kgLBM)	6.4 ± 0.3***	6.5 ± 0.4***	n.s.	5.9 ± 0.3*	6.0 ± 0.5*	n.s.	6.8 ± 0.3**	7.1 ± 0.1**	n.s.
RER – fasted	0.77 ± 0.01	0.81 ± 0.03	n.s.	0.78 ± 0.02	0.82 ± 0.04	n.s.	0.76 ± 0.02	0.79 ± 0.04	n.s.
RER – fed	0.80 ± 0.02	0.80 ± 0.02	n.s.	0.81 ± 0.03	0.82 ± 0.02	n.s.	0.80 ± 0.02	0.79 ± 0.02	n.s.
COX – fasted (mg/min/kgLBM)	1.5 ± 0.3	1.9 ± 0.5	n.s.	1.7 ± 0.3	2.5 ± 0.8	n.s.	1.4 ± 0.5	1.2 ± 0.2	n.s.
COX – fed (mg/min/kgLBM)	2.4 ± 0.4	2.3 ± 0.4	n.s.	2.2 ± 0.7	2.7 ± 0.6	n.s.	2.5 ± 0.6	1.8 ± 0.7	n.s.
FOX – fasted (mg/min/kgLBM)	1.9 ± 0.1	1.7 ± 0.3	n.s.	1.7 ± 0.2	1.3 ± 0.3	n.s.	2.0 ± 0.2	2.2 ± 0.2	n.s.
FOX – fed (mg/min/kgLBM)	1.8 ± 0.2	1.8 ± 0.2	n.s.	1.6 ± 0.4	1.5 ± 0.2	n.s.	1.9 ± 0.3	2.3 ± 0.2	n.s.

All values are means ± SEM. There were no significant differences between groups (n.s.) but significant differences between fed and fasted states are marked *=p<0.05, **=p<0.01 & ***=p<0.001. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con, CDF=female CD, REE=resting energy expenditure, RER=respiratory exchange ratio, COX=carbohydrate oxidation, FOX=fat oxidation.

5.3.4 Whole body physiology

5.3.4.1 Insulin sensitivity

Arterialized blood glucose levels rose post-feeding (t=0-20 min), peaking on average at t=60 min (at 5.9 ± 0.5 mmol/L in Con and at 6.1 ± 0.4 mmol/L in CD) and remained significantly above baseline in both groups at the end of the study (t=120 min) (time effect $p<0.0001$) (Figure 5.1A). There was no difference between Con and CD.

Serum insulin levels also increased in all groups post-feeding and peaked at t=40 min (time $p<0.0001$) (Figure 5.1D). There was a high degree of variation in this peak for CD (75 mIU/L ± 22.3), which meant no difference was detected with Con (46 mIU/L ± 3.9). No differences were detected between groups in serum insulin response overall or in Matsuda Index (Figure 5.1G). Matsuda index was in Con 6.5 ± 0.8 and 8.1 ± 2.0 in CD (with <4.3 indicative of insulin resistance (Gutch et al. 2015)).

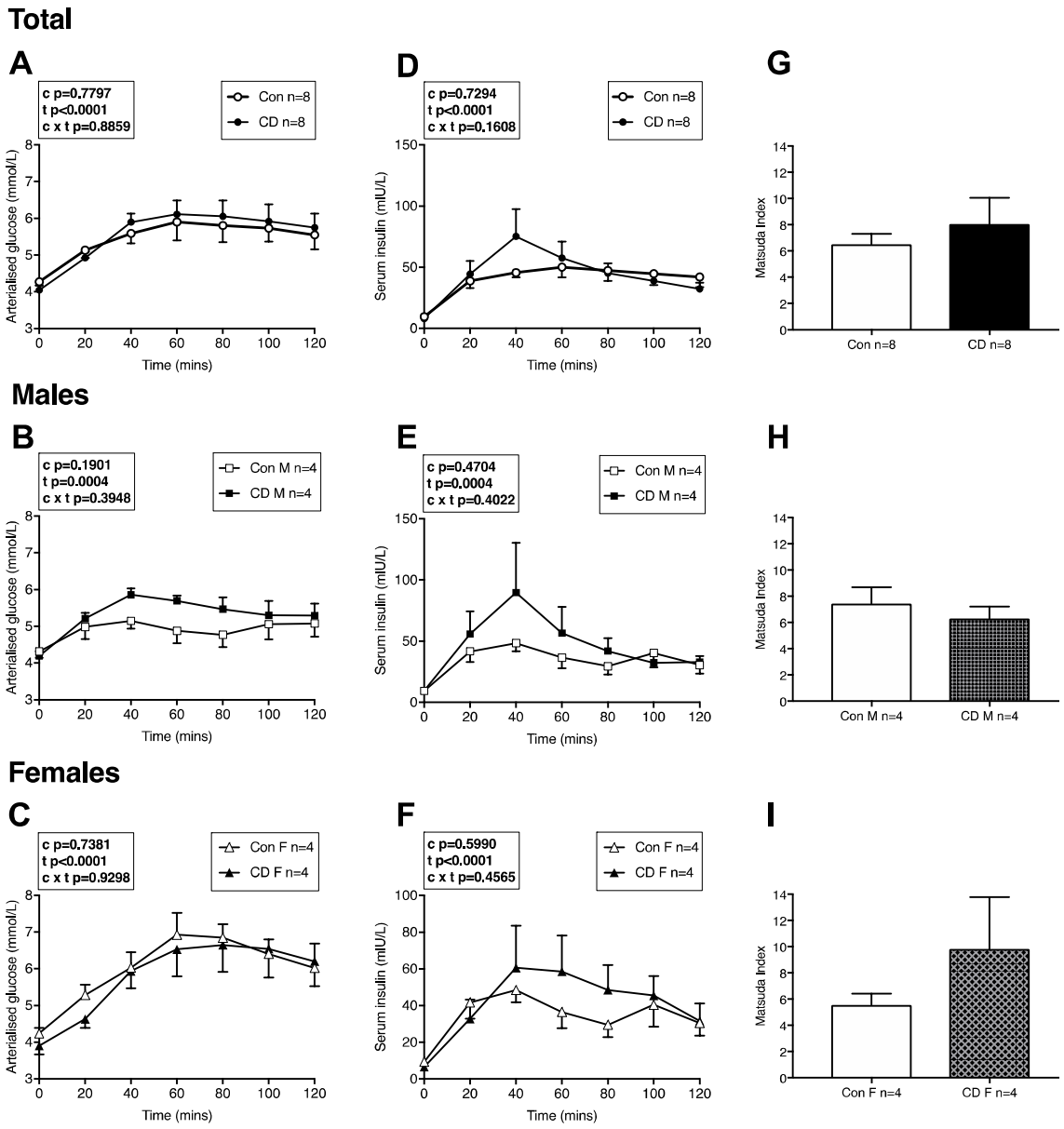


Figure 5.1 Indicators of whole body insulin sensitivity: arterialized blood glucose concentrations (A-C), serum insulin (D-F) and Matsuda index (G-I).

Values are means ± SEM. CD=Crohn's disease, Con=control, M=male and F=female. A,D,G = Con vs. CD, B,E,H = male subjects only, C,F,I = female subjects only. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect. Matsuda index is an index of postprandial insulin sensitivity where the lower the index the higher the level of insulin resistance.

5.3.5 Muscle physiology

5.3.5.1 Skeletal muscle insulin sensitivity

Glucose a-v difference across the forearm increased in response to feeding from 0.13 ± 0.04 mmol/L to a peak of 0.84 ± 0.12 mmol/L in Con at t=60 min and from 0.11 ± 0.02 mmol/L to a peak of 0.54 ± 0.13 mmol/L in CD at t=40 min (time effect $p<0.0001$) (Figure 5.2A). CD a-v difference was already in decline by t=60 min and at only just over half the level of Con at 0.45 ± 0.13 mmol/L, but there was only a trend towards an interaction effect ($p=0.068$). There was a time effect ($p=0.007$) detected in blood flow, which was higher in the second hour of the study than the first, but there were no differences between Con and CD (Figure 5.2D). Glucose net uptake was not different between CD and Con either, suggesting no difference in skeletal muscle insulin sensitivity (Figure 5.2G).

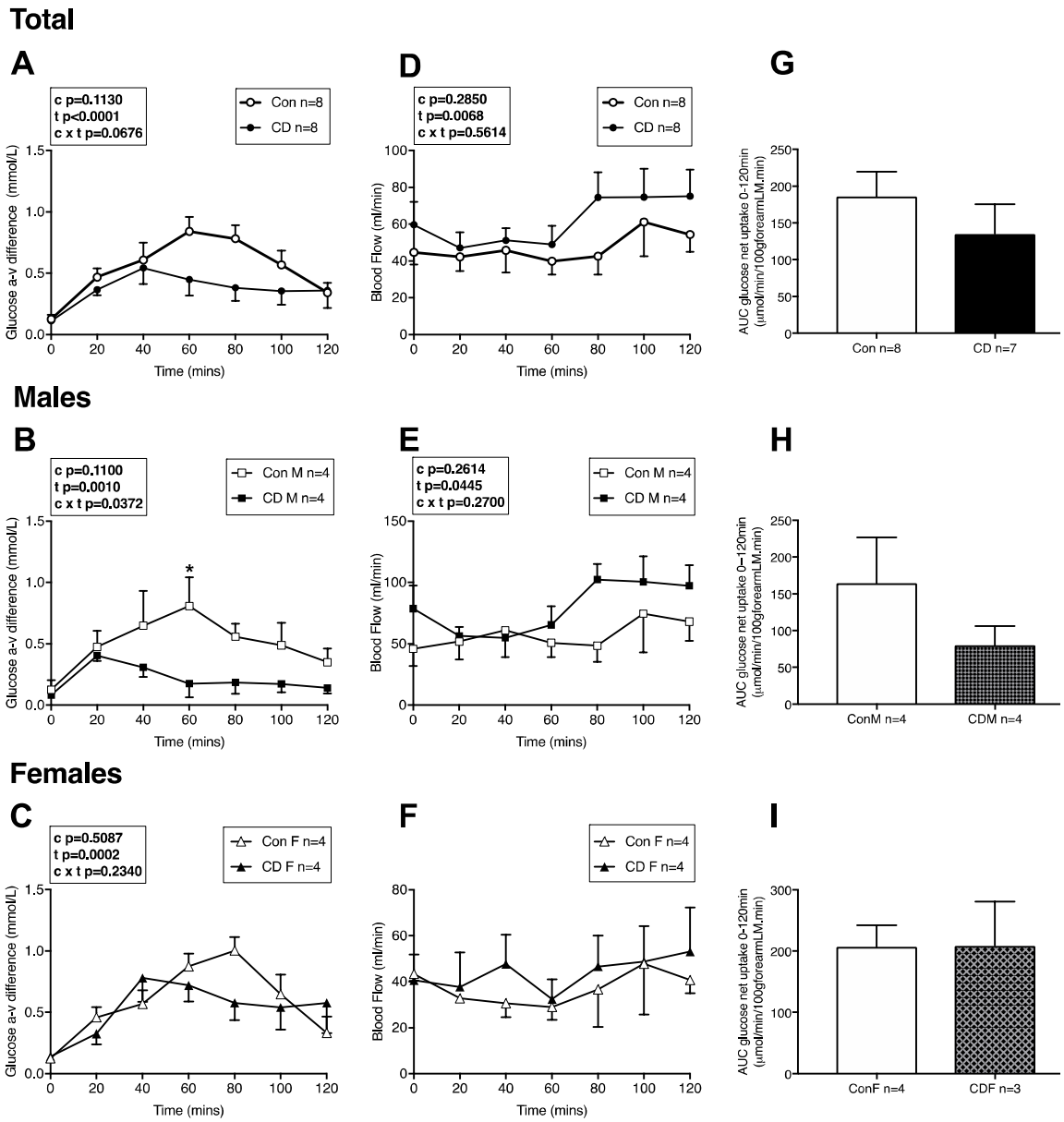


Figure 5.2 Skeletal muscle insulin sensitivity: glucose arterio-venous difference across the forearm (A-C), brachial artery blood flow (D-F) and AUC glucose net uptake across the forearm standardized for forearm lean mass (G-I).

Values are means ± SEM. CD=Crohn's disease, Con=control, M=male and F=female, BCAA=branched chain amino acids. A,D,G = Con vs. CD, B,E,H = male subjects only, C,F,I = female subjects only. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect.

5.3.5.2 Skeletal muscle anabolic response

From similar fasting concentrations of on average 247 ± 11 μmol/L in Con and 254 ± 11 μmol/L in CD, arterialized plasma BCAA peaked in response to feeding at t = 40 min at 300 ± 14 μmol/L and 348 ± 18 μmol/L respectively (time effect $p<0.0001$) (Figure 5.3A). There was no overall difference between Con and CD. Neither BCAA net balance over time (Figure 5.3D) or mean BCAA net balance (Figure 5.3G) were different between Con and CD. Mean BCAA net balance was on average neutral.

5.3.5.3 Skeletal muscle lipid metabolism

Arterialized plasma FFA concentrations were suppressed following feeding in all groups and reached a steady state, significantly lower than fasting levels, by t=80 min (time effect $p<0.0001$ (Figure 5.4A). FFA concentrations, a-v difference and net balance were similar between CD and Con (Figures 5.4 A,D,G). There were no differences between groups in TAG, which remained relatively constant t=0-100 min at c. 1mmol/L (Figure 5.4J).

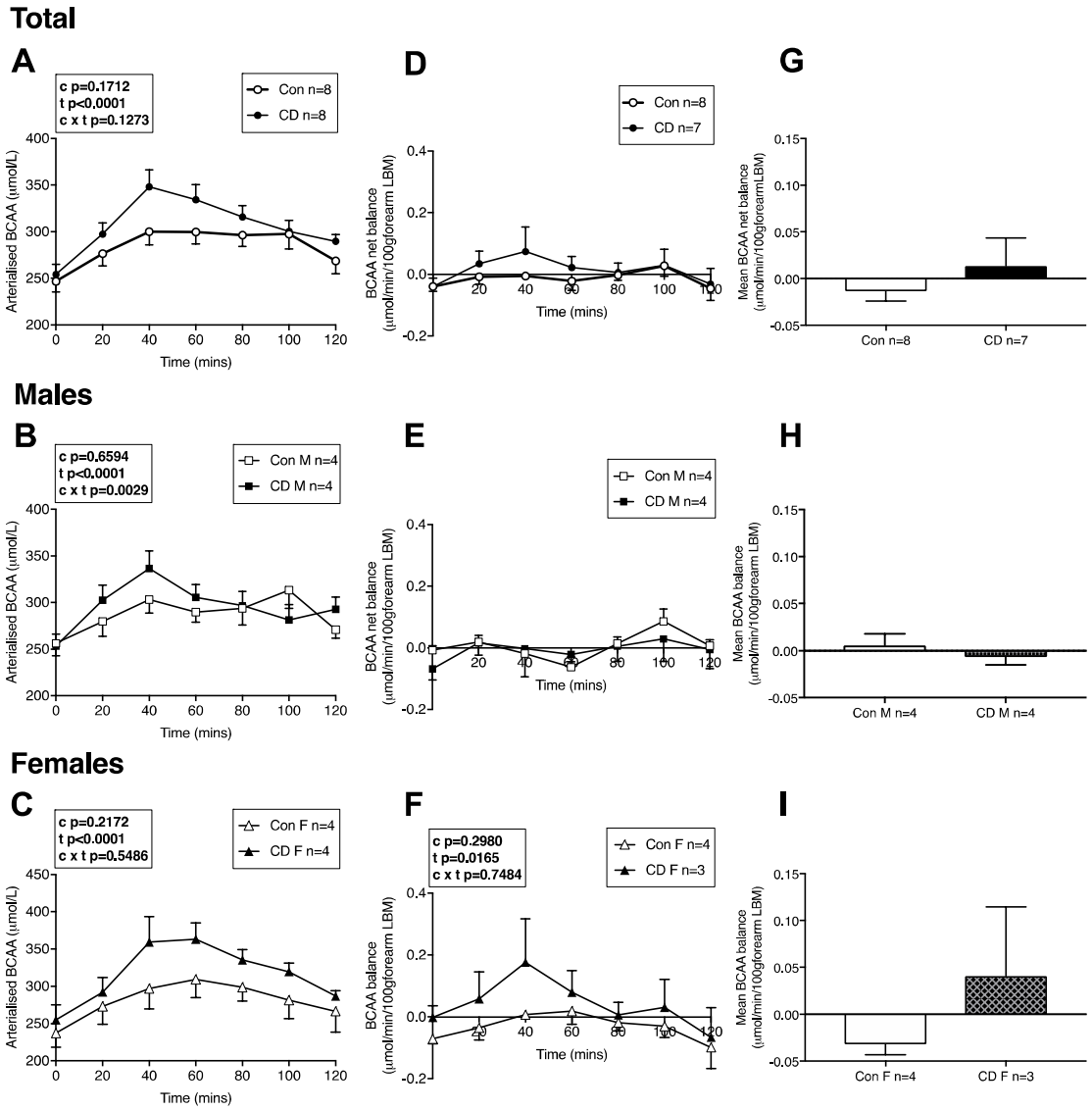


Figure 5.3 Indicators of protein metabolism: arterialized plasma BCAA concentrations (A-C), mean BCAA net balance across the forearm standardized for forearm lean mass (D-F) and BCAA net balance across the forearm, over time, standardized for forearm lean mass (G-I).

Values are means ± SEM. CD=Crohn's disease, Con=control, M=male and F=female, A,D,G = Con vs. CD, B,E,H = male subjects only, C,F,I = female subjects only. Results of 2-way or 1-way ANOVA are shown if any of the main effects were significant: v = visit effect, t = time effect and v x t = interaction effect.

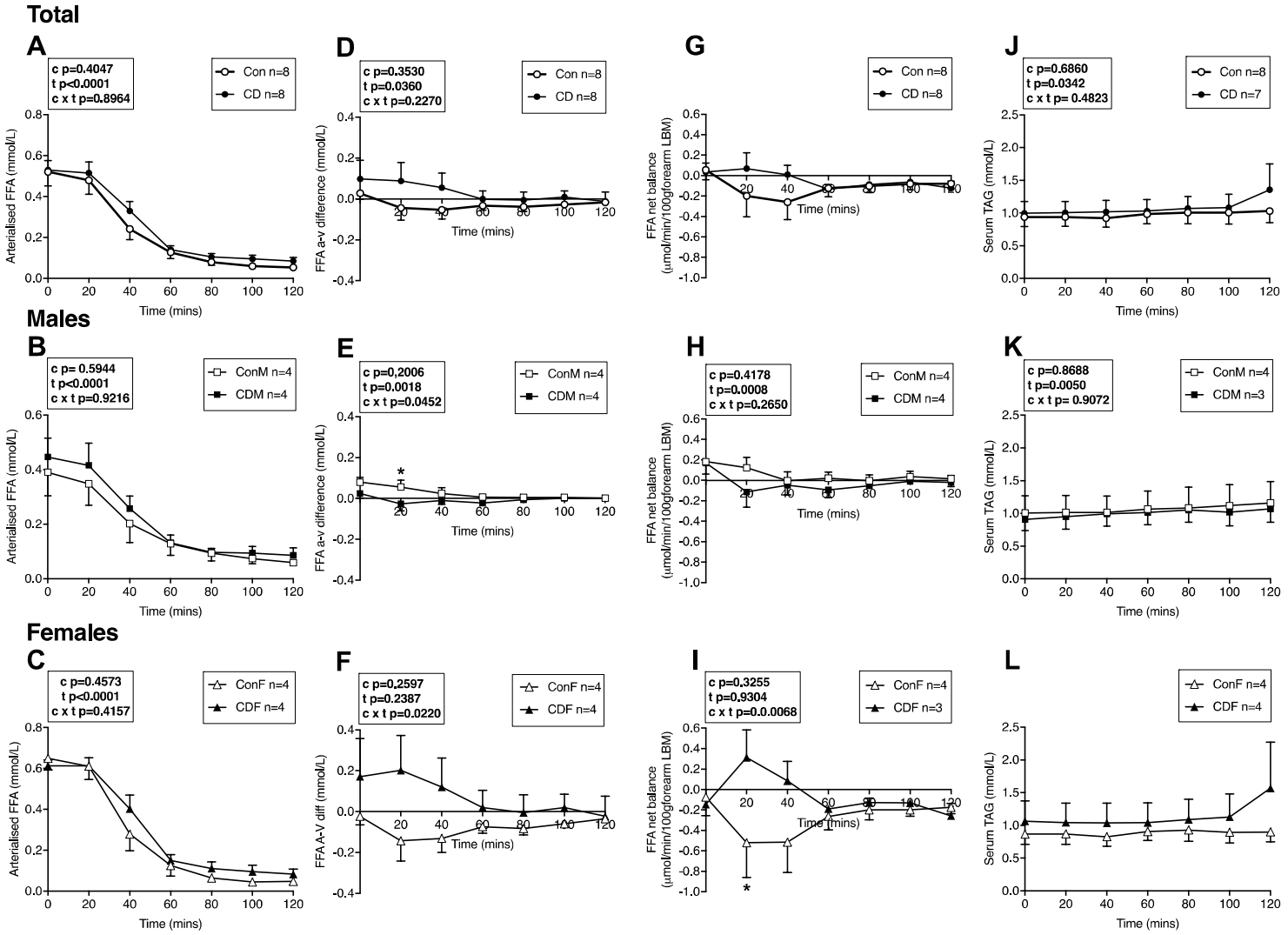


Figure 5.4 Skeletal muscle lipid metabolism: arterialized plasma FFA concentrations (A-C), FFA arterio-venous difference across the forearm (D-F), FFA net balance across the forearm (G-I) & arterialized serum TAG concentrations (J-L).

Values are means ± SEM. CD=Crohn's disease, Con=control, M=male & F=female. A,D,G,J = Con vs. CD, B,E,H,K = male only, C,F,I,L = female only. Results of 2-way or 1-way ANOVA are shown if any main effects were significant: v= visit, t= time & v x t= interaction effect.

5.3.6 Physical activity and diet

Activity levels did not significantly differ between Con and CD whether assessed by pedometer or IPAQ (Table 5.5). There were no differences in energy intake between groups or in any of the 3 main nutrients (Table 5.6). Protein intake exceeded current dietary guidelines of 0.75g / kg bw / day, by 24% in Con and by 41% in CD.

5.3.7 Patient reported outcome measures (PROMS)

Key issues highlighted in SF-36 were general health (33 ± 5) and energy/fatigue (43 ± 6) with the lower the score the more severe the problem (Table 5.7). Pain scored 78 ± 6, indicating it was less of an issue, but it is clearly still a problem for CD as it received the highest score in EQ-3D (1.6 ± 0.2) (1= no problem and 3 = severe). Anxiety and depression were moderate to low issues at 0.9 ± 0.2 and 0.5 ± 0.1 respectively, where 0 is low and 3 is high. IBDQ scores were on average 5.0 ± 0.4 (1=poor & 7=optimum) suggesting quality of life is somewhat affected by CD. IBD-fatigue mean scores were: 10.1 ± 1.3 for section 1, indicating subjects had moderate fatigue (although a score of 11-20 indicates severe fatigue) and 27.7 ± 6.0 for section 2, showing fatigue had a moderate effect on daily activities (moderate effect is defined as a score of 1-60). There were no significant differences between genders in any of the PROMS.

Table 5.5: Physical activity

	Con	CD	p	ConM	CDM	p	ConF	CDF	P
	n=8	n=7		n=4	n=4		n=4	n=3	
Pedometer (000's steps/day)	10.0 ± 1.6	7.5 ± 0.9	n.s.	13.5 ± 2.0	8.1 ± 1.4	0.072	6.5 ± 0.2	6.9 ± 1.1	n.s.
IPAQ (total 000's MET-mins/wk)	7.0 ± 3.2	7.8 ± 3.5	n.s.	8.7 ± 4.8	10.1 ± 5.0	n.s.	3.8 ± 2.6	6.1 ± 5.2	n.s.

All values are means ± SEM. n.s. = no significant differences between groups and a trend ($p<0.1$) is marked. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con, CDF=female CD, IPAQ=international physical activity questionnaire. N numbers are as specified in column headers except for IPAQ where CDM n=3 & ConF n=2 meaning Con n=6 and CD n=7.

Table 5.6: Energy intake

	Con	CD	p	ConM	CDM	p	ConF	CDF	P
	n=8	n=7		n=4	n=4		n=4	n=3	
Energy intake (MJ/day)	7.5 ± 1.2	7.3 ± 0.7	n.s.	8.7 ± 2.3	8.2 ± 1.2	n.s.	6.3 ± 0.7	6.5 ± 0.8	n.s.
Carbohydrate intake (MJ/day)	3.6 ± 0.6	3.3 ± 0.4	n.s.	4.5 ± 1.1	3.7 ± 0.7	n.s.	2.8 ± 0.3	2.9 ± 0.5	n.s.
Fat intake (MJ/day)	2.6 ± 0.5	2.6 ± 0.3	n.s.	2.9 ± 1.0	3.0 ± 0.6	n.s.	2.3 ± 0.4	2.2 ± 0.3	n.s.
Protein intake (MJ/day)	1.2 ± 0.2	1.4 ± 0.1	n.s.	1.3 ± 0.3	1.5 ± 0.3	n.s.	1.2 ± 0.2	1.3 ± 0.2	n.s.
Protein intake (g/day)	73 ± 9	83 ± 9	n.s.	77 ± 16	88 ± 16	n.s.	70 ± 12	77 ± 9	n.s.
Recommended protein intake (g/day)	59 ± 3	59 ± 4	n.s.	62 ± 4	65 ± 3	n.s.	55 ± 3	52 ± 8	n.s.

All values are means ± SEM. There were no significant differences between groups (n.s.). CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con, CDF=female CD, N numbers are as specified in column headers.

Table 5.7 Patient-related outcome measures (PROMS)

		CD	CDM	CDF
		n=8	n=4	n=4
SF-36	Physical functioning	85 ± 8	95 ± 2	75 ± 16
	Physical health limitations	66 ± 12	63 ± 13	69 ± 24
	Emotional problem limitations	88 ± 13	75 ± 25	100 ± 0
	Energy/fatigue	43 ± 6	38 ± 6	48 ± 10
	Emotional well being	78 ± 7	71 ± 12	85 ± 7
	Social functioning	73 ± 10	72 ± 16	75 ± 14
	Pain	78 ± 6	84 ± 7	73 ± 10
	General Health	33 ± 5	33 ± 3	34 ± 10
EQ-3D	Mobility	1.1 ± 0.1	1.0 ± 0.0	1.3 ± 0.3
	Self-Care	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
	Usual activities	1.1 ± 0.1	1.0 ± 0.0	1.3 ± 0.3
	Pain	1.6 ± 0.2	1.5 ± 0.3	1.8 ± 0.3
	Anxiety	1.1 ± 0.1	1.3 ± 0.3	1.0 ± 0.0
	VAS	72.1 ± 4.1	78.3 ± 1.7	67.5 ± 6.3
	TTO	0.85 ± 0.05	0.88 ± 0.1	0.82 ± 0.1
HADS	Anxiety (mean)	0.9 ± 0.2	0.7 ± 1.3	1.0 ± 0.3
	Depression (mean)	0.5 ± 0.1	0.5 ± 1	0.5 ± 0.3
IBD- QOL	IBD - QOL (mean)	5.0 ± 0.4	5.2 ± 0.3	4.8 ± 0.7
IBD – Fatigue	Section 1	10.1 ± 1.3	9.0 ± 1.4	11.3 ± 2.2
	Section 2	27.7 ± 6.0	29.3 ± 7.5	26.1 ± 10.5

All values are means ± SEM. There were no significant differences between groups. CD=Crohn's disease, CDM=male CD, CDF=female CD, SF-36=Short Form 36 Health Survey, EQ-3D=European quality of life 3 dimensions, VAS=visual analog scale, TTO=time trade-off values, HADS=Hospital anxiety and depression scale, IBD-QOL=The Inflammatory bowel disease quality of life questionnaire, IBD-F=the Inflammatory Bowel Disease fatigue patient self-assessment scale where section 1 identifies the level and duration of fatigue and section 2 assesses the impact of fatigue on daily activities.

5.3.8 Sub-analysis by sex

While n numbers are very small for any sub-group analysis (n=4) this section is included to illustrate potential differences between the sexes in response to a mixed meal. Arterialised glucose, regardless of condition, peaked later in females than males (t = 60 min vs. t = 40 min) (Figures 5.1C and B). CDM glucose peak was higher than ConM at 5.9 ± 0.2 mmol/L and 5.2 ± 0.2 mmol/L respectively (t test p=0.041) (Figure 5.1B). CDM also had significantly attenuated glucose a-v difference vs. ConM (interaction effect p=0.037) (Figure 5.2B). CDM a-v difference had already returned close to baseline levels after 60 min to 0.18 ± 0.11 mmol/L while at the same time point ConM a-v difference peaked 4.5x higher at 0.81 ± 0.24 mmol/L. The glucose a-v difference in males did not however translate into a difference in net uptake (Figure 5.2H). This was largely because blood flow was more than double ConM at t=80 min (102 ± 13 ml/min vs. 49 ± 13 ml/min) (t test p=0.025) and remained high until the end of the trial (Figure 5.2E).

An interaction effect was found in arterialised BCAA in males (p=0.003) (Figure 5.3B). BCAA concentrations in male CD were initially higher in response to the meal than in ConM but were lower at t=100 min. AUC of arterialized BCAA showed CDM to have raised levels in comparison to ConM (t test p=0.047) (data not shown). However, any differences did not translate into positive forearm muscle BCAA balance and there was no difference in forearm muscle balance between ConM and CDM (Figures 5.3E and H).

There were interaction effects in FFA a-v difference in both male (p=0.045) and female (p=0.022) sub-analyses (Figures 5.4E and F). In CDM this was largely driven by a reduced a-v difference in comparison to Con at t=20 min (-0.03 ± 0.03 mmol/L vs. +0.06 ± 0.03 mmol/L respectively) (post hoc p=0.037). Yet higher blood flow in CDM meant that this did not in fact result in reduced FFA uptake versus ConM and both groups reached neutral balance from t=40 min (Figure 5.4H). Conversely, in CDF, FFA a-v difference was positive at baseline and t=20-40 min, whereas in ConF it was negative (Figure 5.4F). The interaction effect remained in terms of FFA net balance in females (p=0.007) implying net uptake of FFA in CDF immediately after feeding, in contrast to the efflux seen in ConF (Figure 5.4I). In fact post hoc testing showed that at t=20 min CDF uptake was significantly different from ConF (p=0.020).

Although pedometer readings in CDM were numerically lower than Con M, 8.1k steps ± 1.4 and 13.5k ± 2.0 steps respectively, the difference was not significant (p=0.072) (Table 5.5). Nor were there any differences in the IPAQ totals. ConF were significantly stronger in the dominant arm than CDF, 33.5 ± 1.2 kg/kgforearmLM vs. 22.1 ± 3.0 kg/kgforearmLM respectively (p=0.010) (Table 5.3).

5.4 DISCUSSION

The primary aim of the current study was to ascertain if anabolic resistance to an oral mixed meal was associated with reduced skeletal muscle mass in adults with active CD. Secondarily, to understand if anabolic resistance was associated with insulin resistance. No differences in anabolic response or muscle insulin sensitivity were found between CD and Con. Furthermore, there were no differences between groups in body composition or muscle function. This is the first study to investigate the anabolic response to a meal in adult CD.

The variability in reported prevalence of low muscle mass in the adult CD population is high, ranging from 21% (Bryant et al. 2015) to c.60% (Schneider et al. 2008; Zhang et al. 2017). This is likely a result of the heterogeneity of the study populations (disease duration & severity and pharmacological treatment) and the inconsistent definition of reduced muscle mass and / or sarcopenia used. The definition of sarcopenia has been expanded beyond the elderly in the clinical literature to mean the loss of muscle mass and function more generally (Santilli et al. 2014; Cruz-Jentoft et al. 2019). In terms of its measurement there are a plethora of ways of defining it from simply reduced muscle mass vs. Con, or one or two standard deviations below a population mean (Baumgartner et al. 1998; Schneider et al. 2008; Zhang et al. 2017), to those incorporating various measures of function including handgrip strength (Cawthon 2015; Lu et al. 2016). Measurement of reduced muscle mass itself may also be made by a range of techniques and

assessed at a whole body or more specific level e.g. appendicular LM or ASMI.

Pharmacological history is also likely to impact on the prevalence of reduced muscle mass and seven of eight CD patients in the current study were being treated or had been treated with anti-TNFα medication. Infliximab has been shown to improve volume of the quadriceps femoris muscle in adults after 3 infusions (16 weeks), although there was no further improvement after 4 infusions (25 weeks) (Subramaniam et al. 2015).

Taking all of this into account it would have been expected to find a prevalence of low muscle mass of between 20 and 40% in CD in the current study. In fact, based on a definition of reduced appendicular lean mass of >1 SD below the sex-specific control mean, 38% of CD subjects (2/4 female and 1/4 male) did have reduced muscle mass. Interestingly, all of these individuals had a negative mean BCAA balance: -0.053, -0.011 and -0.011 μmol/min/100g forearm LM (vs. CD average of 0.014 μmol/min/100g forearm LM). However, the low prevalence of reduced muscle mass in CD in the current study, in conjunction with the large variation in male muscle mass and small sample size meant that no difference was found between Con and CD.

No difference in strength was found between CD and Con in the present study. Reduced muscle strength in CD has been found in groups with reduced LM (Lu et al. 2016) and without (Wiroth et al. 2005). Yet the reduced strength found by Wiroth et al. was only in the lower limbs, which were not tested in the current study and not in the upper limbs, which were. Therefore

the current results, showing no difference in strength between CD and Con in the upper limbs, have precedent. The high prevalence of anti-TNFα medication may also have contributed to these findings. In the study investigating the effect of infliximab on muscle mass and function muscle strength was also increased after 16 weeks with some further gains at 25 weeks (Subramanian et al. 2015). But it is not known whether these improvements normalised these indices as no comparison with controls or reference data was made. The mechanism of action was proposed to be the reduction of TNFα leading to prevention of NF-κB activation, which in turn would both reduce proteolysis (via Akt activation and inhibition of FOXO4 and E3 ubiquitin ligases (Cai et al. 2004), and increase muscle synthesis (via MyoD transcription (Guttridge et al. 2000) however none of these were measured.

Fatigue was a key symptom reported by CD in the current study in accordance with previous findings where over 50% of CD report fatigue as a key symptom (Singh et al. 2011; van Langenberg and Gibson 2014). A correlation between objectively measured and self-reported fatigue has also been demonstrated (van Langenberg, Della Gatta, Warmington, et al. 2014). This was not the case in the present study where, although moderate levels of fatigue were reported in PROMS, handgrip fatigue was not found to be greater in CD vs. Con. However it is now well accepted that fatigue is multifactorial (van Langenberg and Gibson 2014) and it is possible that the fatigue reported in the present study was cognitive rather than physical.

Postprandial insulin resistance was not detected in CD in this study either at the whole body level (Matsuda Index) or in terms of skeletal muscle (glucose uptake across the forearm). No other studies found have measured glucose net uptake across the forearm in order to assess insulin sensitivity in CD. Higher fasting concentrations of insulin have previously been found in active CD vs. Con leading to reduced insulin sensitivity as measured by HOMA-IR (Bregenzer et al. 2006; Valentini et al. 2009). However, although significantly different from Con, HOMA-IR was still within the normal range in CD in both of these studies (i.e. <2.5 (Gutch et al. 2015)). Furthermore HOMA-IR is an index of fasting insulin sensitivity and largely reflects the insulin sensitivity of the liver.

Hyperinsulinaemia and reduced insulin sensitivity has also been found in response to OGTT in CD (Bregenzer et al. 2006). Although 8/17 patients in Bregenzer et al. were being treated with steroids there was no difference found in insulin sensitivity between these patients and those who were not. However, none of the subjects in that study were being treated with anti-TNFα medication whereas the majority in the present study were or had been. Even though previous studies have shown no effect of infliximab on insulin sensitivity (Gentile et al. 2002; Koutroubakis et al. 2009) a more recent study demonstrated a reduction in fasting insulin concentration and improvement in HOMA-IR after 6 months (Paschou et al. 2018). It is likely that there was no effect in Gentile et al. because the subjects were already insulin sensitive. Moreover level of insulin sensitivity is likely to be not just related to disease activity and pharmacological treatment but to a multitude of other factors including physical fitness and diet. Thus not only the different measures of

insulin sensitivity and high prevalence of anti-TNFα medication but similar diet and activity levels in CD subjects vs. Con may explain why this cohort was not found to be insulin resistant.

The paediatric study of patients in remission, described in Chapter 4, did suggest a link between an attenuated anabolic response and reduced muscle mass. It might therefore have been expected to find such an association in adults who were in active disease. Indeed the adults had active symptoms and all had objective markers of inflammation. Whereas the paediatric cohort were in remission, with half in deep remission (i.e. the bowel was objectively healed), and while the other half had some disease they were asymptomatic. There are many possible reasons why no difference in anabolic response was found between Con and CD in the current study. But it may simply be that muscle mass and function were not reduced. We hypothesized that a reduced anabolic response would be associated with reduced muscle mass and without the presence of the latter it is perhaps unsurprising the former was not observed. Further, only one of the adult patients had been diagnosed with CD as a child, thus disease burden in the current cohort may be lower in comparison to a cohort who had earlier disease onset. Speculatively, perhaps persistent sarcopenia in adult CD is more likely if a deficit in muscle mass is produced while suffering from the disease during puberty and/or with overall greater disease burden over time. Indeed the subject diagnosed as a teenager did have reduced appendicular lean mass versus control (> 1SD below the control mean). A cross sectional study comparing those diagnosed in childhood with those who were not or a longitudinal study would be need to

investigate this. Patients should also be assessed for disease burden to understand if near constant active disease is implicated.

Adult CD subjects were not found to be insulin resistant nor were elevated cytokines detected. Indeed a negative correlation between levels of IL-6 and TNFα with muscle mass and function has been established in the elderly (Visser et al. 2002; Schaap et al. 2006). Although one study (van Langenberg, Della Gatta, Hill, et al. 2014) found reduced muscle mass and reduced phosphorylation of key signalling proteins in the muscle synthetic pathway in CD with lower TNFα levels than Con. Only one of the patients in the current study was anti-TNFα naïve and all except one were receiving current pharmacological therapy, likely explaining low levels of cytokines. Taken together all of these factors may mean that body composition differences were unlikely to be present in the current group.

It is also possible that the nutritional stimulus provided was not sufficient to elicit a response. Indeed mean BCAA net balance was effectively neutral in both groups suggesting a limited anabolic response to the meal. Nevertheless the 14g protein provided is equivalent to 24% of CD daily requirement based on 0.75g / kgbw (59g) and 17% of actual CD intake (83g). This compares well with the 12g protein eaten at breakfast by adults aged 19-64 (in a sample taken from NDNS, 2008–2014 n=3619) and equivalent to 17% of daily intake (72g +/- 23) (Gaal et al. 2018). Furthermore the main protein source in Ensure plus is milk protein, which is estimated to contain 39% EAA (Gorissen et al. 2018). In which case the dose of EAA given was c.5.5g and as little as 3g EAA has been shown to increase FSR (Bukhari et al.

2015). Moreover, using a large amount of protein as an experimental stimulus has the potential to mask any potential differences (Paddon-Jones et al. 2004; Katsanos et al. 2009; Wall, Cermak, and van Loon 2014). It is therefore considered important that a relatively small dose of protein, which is comparable to real life, is used in order to detect any anabolic resistance. Importantly, there is a statistical difference between fasted and fed BCAA balance for all participants (-0.040 vs. 0.006 μmol/min/100gforearm lean body mass, p=0.023) suggesting 14g was sufficient stimulus to elicit a response. Finally, in contrast to the paediatric study, there were no differences in protein intake between Con and CD.

Overall no difference was detected in physical activity between CD and Con in this study. This could also have contributed to absence of anabolic resistance and reduced muscle mass (Breen et al. 2013). But is in contrast with several studies where lower levels of activity (van Langenberg, Della Gatta, Hill, et al. 2014), lower intensity of activity (Vogelaar et al. 2015) and a reduction in both have been shown in CD (van Langenberg, Papandony, and Gibson 2015). In the current study male CD had lower pedometer readings than ConM and as such it maybe that the study was not sufficiently powered to detect a difference using the techniques we deployed. Studies using accelerometry (van Langenberg, Papandony, and Gibson 2015) and those with large sample sizes (Tew, Jones, and Mikocka-Walus 2016) have detected differences. Further, CD patients generally report that their activity levels are limited by disease and physical activity levels are inversely correlated with self-reported fatigue (DeFilippis et al. 2016; Tew, Jones, and Mikocka-Walus 2016).

There were two interesting findings in relation to the indirect calorimetry results. Firstly, the absence of time effects across all groups between the fasted and fed states in RER, COX and FOX. Although an expected postprandial increase in REE was observed, any change in the aforementioned metrics was not. This could indicate some metabolic inflexibility in the study population or suggest that the sample size was not sufficient to demonstrate the expected differences in combination with a relatively small meal stimulus. Secondly, was the lack of any differences between Con & CD. Increased fasting (Al-Jaouni et al. 2000; Zhao et al. 2015; Sammarco et al. 2017) and fed REE (Mingrone et al. 1999) have both been reported in CD, whereas other studies have shown there to be no difference in either (Schneeweiss et al. 1999). It is likely that there are competing forces affecting REE in CD (Schneeweiss et al. 1999; Nishida et al. 2013) with malnutrition tending to suppress REE and active disease to increase it. In support, subjects in a study reporting similar fasted REE between control and CD were in clinical remission (Mingrone et al. 1999), whereas subjects on pharmacological treatment had reduced REE in comparison to those without treatment / pre treatment (Sammarco et al. 2017; Nishida et al. 2013). Therefore, depending on the balance between disease activity and malnutrition it would be quite possible to find lowered, similar or raised REE vs. controls. It follows therefore that CD in the current study, who were in active disease, on average slightly overweight and on pharmacological treatment, had a similar REE to controls. Increased fasted (Capristo et al. 1999; Schneeweiss et al. 1999; Nishida et al. 2013) and fed FOX in CD has also been a common finding (Mingrone et al. 1999; Al-Jaouni

et al. 2000). Neither an increase in fasted or fed FOX was found in the present study. However both under-nutrition (Mingrone et al. 1999) and disease activity (Al-Jaouni et al. 2000) could drive increased FOX. In support the majority of these studies featured underweight subjects and/or those in active disease. The features of the present study group may not therefore be conducive to elevated FOX.

Studies suggest that energy intake is reduced when disease activity is high (Hodges et al. 1984; Rigaud et al. 1994) and comparable to controls when disease activity is low / patients are in remission (Filippi et al. 2006; Taylor et al. 2018). Even when energy intake is reduced protein may not be below recommended levels but evidence of protein malnutrition may remain (Hodges et al. 1984; Sousa Guerreiro et al. 2007). The findings of no difference in energy intake between CD and Con and protein consumption exceeding RDA in CD in the present study are therefore consistent with the literature.

Active disease status did not appear to confer higher levels of the following cytokines: TNFα, IL-6 & IL-1β in CD vs. Con in this study. However, all but one patient were being treated pharmacologically and only 1 was anti-TNFα naïve. In a review of serum cytokine levels in IBD (Korolkova et al. 2015) showed equal numbers of studies had found elevated and equivalent levels of TNFα and IL-1β in active CD vs. Con. They also found 14 studies showing increased IL-6 in active disease and 9 with similar levels to controls. This suggests that the serum levels of cytokines may not be a good biomarker of

disease activity and/or cytokine expression. It may therefore be necessary to measure muscle expression.

The current study also suggested several sex differences. CDM had higher blood flow than ConM at 80mins whereas no differences were detected between CDF and ConF. This resulted in the obviation of potential reductions in glucose uptake in CDM vs. ConM, that were apparent as significantly reduced a-v differences. This finding is redolent of those seen in Chapter 3 where overweight / obese men had reduced a-v difference but not reduced glucose uptake due to increased blood flow in response to acute lipid challenge and Chapter 4 where the same observation was made in paediatric male CD in response to meal challenge. It is possible therefore that a similar compensatory mechanism aimed at maintaining glucose uptake in the face of attenuated NO-stimulated vasodilation is in play, which requires further investigation.

Arterialised plasma BCAA AUC in response to feeding was higher in CDM than ConM but not in CDF vs. ConF. This contrasts with the findings of lower levels of plasma BCAA found in a majority male adult CD cohort (Hisamatsu et al. 2012) where they were linked to reduced dietary protein. CDM in the current study however ate 35% more protein than recommended levels and their protein consumption did not differ from ConM. A further study showed a negative correlation between BCAA concentrations and disease activity (Chiba, Suzuki, and Matsumoto 2018). However patients in this study had active disease and so rather than being linked to disease activity it is possible that increased BCAA in this group is part of a ‘metabolic signature’ linked to

insulin resistance (Newgard et al. 2009). Increased plasma BCAA have been proposed as both a cause (Tremblay et al. 2005; Newgard et al. 2009) and a consequence (Boulet et al. 2015; Gannon, Schnuck, and Vaughan 2018) of insulin resistance. A possible mechanism for increased BCAA in insulin resistance is a reduction in BCAA catabolism via attenuation of the inhibition of branched chain α-keto acid dehydrogenase (BCKDH) complex by insulin (Nellis et al. 2002). Although insulin sensitivity was not different to Con in the current study there were clear indications from CDM in terms of attenuated glucose a-v difference vs. Con and non-significant differences in peak insulin that there was some perturbation in insulin sensitivity. Therefore it is possible that plasma BCAA is increased in CDM due to a spillover from decreased BCAA catabolism. This speculative hypothesis requires further investigation.

Net uptake of FFA in CDF, driven by positive FFA a-v difference, was seen postprandially, particularly at t = 20 min. This appeared to be at the level of the muscle, as arterial levels of FFA were not raised. Interestingly, increased uptake does not appear to be reflected in increased FOX, at least at a whole body level. Neither is there evidence of insulin resistance in CDF, which could be expected if there is accumulation of intra-myocellular fats. It is possible therefore that the results reflect the contribution of adipose tissue to FFA forearm balance. Post-prandial FFA flow in adipose tissue will be much greater than in muscle (Coppack et al. 1990) and than in other nutrients (average contribution of muscle to forearm balance 80-85% (Andres et al. 1954; DeFronzo et al. 1981). Greater a-v difference may therefore reflect either greater FFA extraction by adipose tissue or reduced release in the fed

state. This is perhaps a more plausible explanation than significantly greater up take of FFA by CDF into the muscle after a meal.

Both Con & CD females exhibited a later postprandial plasma glucose peak than males (i.e. 60 min vs. 40 min). In studies comparing male and female glucose metabolism several gender differences have been found (Ahmed, Gannon, and Nuttall 1976; Basu et al. 2006; Keramida and Peters 2017; Mauvais-Jarvis 2018). However in terms of the time course of postprandial plasma glucose responses studies have not reported a difference (Ahmed, Gannon, and Nuttall 1976; Basu et al. 2006). Yet in studying graphs presented by Basu et al. there could be a small time lag in female vs. male glucose response. If so this could have been exaggerated in the current study due to the lower frequency of sampling (20 vs. 10mins).

In the present study ConF were stronger than CDF. Some studies have found effects to be more pronounced in males (Geerling et al. 1999; Valentini et al. 2008) while Wiroth et al. (2005) found that CD females had lower muscle function than CD males even once adjusted for FFM. However CDF mean strength in the dominant arm of 22.1 +/- 3.0 kg / kg forearm LM was similar to ConM at 24.9 +/- 3.9 kg / kg forearm LM suggesting that it may be ConF that is unusually strong (33.5 +/- 1.2 kg / kg forearm LM) in this case. Therefore it is unlikely that CDF were actually weaker than average.

5.4.1 Limitations and future studies

The key limitation of this pilot study was small sample size, in particular making any sub-analysis by sex tentative at best. Further the low prevalence

of reduced muscle mass in this group is likely to have contributed to the finding of no difference between CD and Con in anabolic response. A larger follow up study powered for sex and where subjects are screened for low muscle mass is therefore warranted. Such a study could investigate the association between age at disease onset and disease burden with muscle mass and function. Novel insights from sub-analysis by sex included reduced glucose a-v differences, increased plasma BCAA, and increased blood flow in CDM. Speculatively hints of insulin resistance in CDM may be linked to the increased plasma BCAA concentrations and increased blood flow may be a compensatory mechanism. Not only do these elements require further study but the need to take into account the rise of obesity, as well as the traditional danger of under-nutrition, in the study and treatment of CD is highlighted.

5.4.2 Conclusions

In summary, no difference in anabolic response, insulin sensitivity, body composition or muscle function was found between CD and Con in the present study. Yet the absence of differences does not preclude a link between these factors and further investigation is required. Particularly as those subjects that did have reduced appendicular LM did have negative mean BCAA balance across the forearm in response to a mixed meal. As a consequence of this study we now have much more information on the metabolic responses to a mixed meal in active CD.

6 GENERAL DISCUSSION

The principle aim of this thesis was to test the hypothesis that attenuated anabolic response to feeding is associated with skeletal muscle insulin resistance in two distinct populations a) middle-aged males with obesity and b) paediatric and adult patients with chronic inflammatory bowel disease (CD). This hypothesis was tested using the a-v balance technique across the forearm to assess the anabolic and insulin responses to oral loads of carbohydrate and protein, subsequent to the ingestion of oral fat loads of different fatty acid composition in the group with obesity (Chapter 3) or to a mixed meal in the CD studies (Chapters 4 and 5).

The primary finding of this thesis does not fully support the original hypothesis that anabolic and insulin resistance are necessarily associated. In the paediatric study (Chapter 4) CD patients were found to have neutral net AA balance in response to a mixed meal while controls were in positive balance. This neutral balance was not associated with skeletal muscle insulin resistance but was associated with reduced FFM z-scores. In male CD net AA balance was negative and was associated with lower appendicular lean mass, higher levels of muscle fatigue and reduced habitual dietary protein intake, when compared with controls, but not insulin resistance. In the remaining studies in this thesis, describing the responses of the adults with obesity and CD patients in active disease, to oral nutrition (Chapters 3 and 5, respectively), neither anabolic resistance nor insulin resistance were found. Furthermore, in neither case did participants have reduced muscle mass. Therefore while anabolic resistance may not always be associated with insulin

resistance it has been shown that it is associated with reduced muscle mass in paediatric patients with CD.

One study, Beals et al. (2016), has previously found anabolic resistance in the absence of whole-body insulin resistance in subjects with obesity in response to the ingestion of 36g of protein (lean pork), although skeletal muscle insulin resistance was not measured. This contrasts with the majority of the literature on subjects with obesity, which has shown that the two types of resistance coexist. Together the paediatric CD study (Chapter 4) in this thesis and Beals et al. (2016) may demonstrate that in some cases anabolic resistance can exist independently of insulin resistance. In which case treating insulin resistance in cases of muscle loss would not be sufficient. Treatment options would instead likely include a combination of the main two anabolic stimuli: increased protein intake and exercise. Indeed it has been shown in subjects with obesity, T2DM and in the elderly that increased protein intake/ hyperaminoacidaemia (Chevalier et al. 2015; Manders et al. 2008; Moore et al. 2015) and exercise-stimulated muscular contraction (Wall et al. 2012; Kouw et al. 2019; Smiles et al. 2019) can reduce anabolic resistance. Although the paediatric patients in Chapter 4 already had protein intakes well above recommended levels (+84%), male CD protein intake was significantly lower than male controls. Moreover it has been previously shown in adult CD that 25% extra protein on top of habitual intake (which was at recommended levels) does increase lean mass (Machado et al. 2015). A combination of resistance and aerobic exercise in CD has also been shown to increase muscle mass (Cronin et al. 2019). Therefore, a follow up intervention study,

in a paediatric cohort, testing various levels of supplemental protein intake and exercise programs, is required.

Such interventions could also be tested in an adult cohort in active disease who had been screened for reduced appendicular LM versus a healthy reference population (>1 SD below the mean). No differences in anabolic or insulin resistance in adult CD were found in the study described in Chapter 5. The most likely explanation for this is because as a group they did not have reduced muscle mass. Interestingly, the three subjects who did have reduced appendicular LM, also had negative net AA balance across the forearm in response to a mixed meal. Therefore, screening for reduced appendicular LM in a similar cohort would be a sensible strategy before testing the aforementioned interventions. It may also be the case that persistently reduced muscle mass in adulthood is linked to disease activity/onset during adolescence. Indeed only one of the subjects in the adult CD study in Chapter 5 had been diagnosed as a teenager and he did have reduced appendicular LM versus control. The two other subjects who had reduced appendicular LM were not diagnosed as children but as very young adults (19 and 23 yrs). Moreover early disease onset could be a marker for overall disease burden and as such this should also be investigated. Therefore a future adult study could recruit subjects with both childhood and adult disease onset and assess disease burden over time to test these hypotheses.

Alternatively, the Beals et al. (2016) subjects, although insulin sensitive at the whole-body level, may have been insulin resistant at the level of the muscle. Furthermore, it is also possible that the CD subjects in Chapter 4, who were in

remission at the time of the investigation could have previously been insulin resistant. Indeed postprandial muscle insulin sensitivity (as indicated by glucose a-v differences) in male CD did show a trend towards being reduced when compared with male control subjects (p=0.067). It is tempting to speculate that insulin resistance may have been necessary to originally induce anabolic resistance, which may persist once insulin resistance has recovered.

This idea could be analogous to the reduction in FOX that may be apparent in people post-obesity. Lower postprandial FOX in people post-obesity versus those who had never had obesity has been demonstrated in several studies despite weight loss and improved insulin sensitivity (Astrup et al. 1994; Raben et al. 1994; Larson et al. 1995). The implication of this reduced ability to burn fat is that those who have previously had obesity are likely to need to habitually consume a low fat diet in order to avoid putting weight back on (Astrup et al. 1994). Speculatively, in the same way, once someone has been anabolically resistant, while anabolic response could be somewhat improved with increased insulin sensitivity, as has been demonstrated by (Gougeon et al. 2000) in T2DM, it may be that some level of anabolic resistance and/or inflexibility persists. Changes in muscle cell signaling could be part of the potential mechanism for this.

However, mTOR signaling has been found to be attenuated in elderly T2DM compared with elderly Con (Cuthbertson et al. 2017), normal in older subjects with obesity (Murton et al. 2015) and hyperactive in the fasted state with no increase in feeding in young subjects with obesity (Beals et al. 2016).

Theoretically, this could represent a cycle of initial hyperactivity followed by normalisation and finally attenuation with advanced age and deterioration in metabolic health but further studies are required to confirm this notion. Nevertheless, if some sort of anabolic inflexibility persists despite recovery of insulin sensitivity, a high protein intake and/or increased physical activity may still be needed in order to avoid a reduced anabolic response and resultant reduction in muscle mass/quality. To test this idea it would be necessary to perform a series of cross sectional studies, possibly in people with obesity, at various stages of the development of weight gain and insulin resistance.

An additional aim of the study in participants with obesity in Chapter 3 was to investigate whether varying the composition of the dietary fat preload would produce different metabolic responses to subsequent co-ingestion of CHO and protein. While there were no differences between visits in terms of anabolic and insulin resistance, resting energy expenditure after the ingestion of the dextrose and milk protein drink was greater with n3PUFA (but not SFA) than Con. This was associated with maintenance of the pre-drink rate of FOX while postprandial COX was appropriately increased. Although previous studies have found that n3PUFA may exert beneficial effects by channeling excess fat into storage (Aas et al. 2006), the current study findings imply that, at least acutely, n3PUFA may be able to reduce fat accumulation by maintaining its oxidation in the presence of a large carbohydrate (and protein) challenge. These may not be incompatible findings as a recent study in human myotubes has shown EPA increases fatty acid cycling (storage, lipolysis and oxidation) vs. palmitate (Løvsletten et al. 2018). Therefore it may in fact be that n3PUFA exert their beneficial effects through increased ‘futile’

cycling. This would need to be tested in different human populations *in vivo* both acutely and chronically in order to be confirmed.

Other novel findings include the negation of potential differences in glucose uptake via increased blood flow. In all 3 studies in this thesis there was either a trend towards an interaction indicating a reduced glucose a-v difference in the treatment groups (SFA in Chapter 3, CDM in Chapter 4 and CD adults in Chapter 5) or in male adult CD (Chapter 5) a significant difference. However, in none of these cases did this manifest itself as attenuated glucose uptake, due to the positive effects of treatment on blood flow. It is intriguing that this finding was present in all of the studies in this thesis, suggesting a common compensatory mechanism may be in play, which is currently unknown. Yet, as discussed in Chapter 5, (Chadderdon et al. 2016) found non-NO dependent increases in blood flow in the face of developing insulin resistance in non-human primates, suggesting a possible line of enquiry. A first step would be to aim to reproduce the findings of increased blood flow in response to lipid in a group at risk from insulin resistance e.g. overweight/ individuals with obesity. Such a study would involve measuring plasma concentrations of eicosanoid metabolites and NO availability to ascertain which mechanisms were implicated. Indeed it would be fascinating to pursue a series of studies with the aim of mapping out the body's compensatory responses to various nutritional challenges. This could contribute to the development of treatments to focus on complementing or harnessing such mechanisms to improve anabolic and insulin sensitivity in at risk populations.

Finally, the studies in this thesis also suggest there may be sex differences in protein metabolism and insulin sensitivity in patients with CD. In the paediatric CD study in Chapter 4, the neutral protein balance in response to a mixed meal was driven by the negative net balance in boys. Furthermore, some studies, but not all, have found reduced muscle mass to be more severe in boys (Bechtold et al. 2010; Werkstetter et al. 2011) and more prevalent in men (Bryant et al. 2015). Differences in protein metabolism between the sexes in obesity have already been highlighted previously (Chevalier et al. 2005b; Pereira et al. 2008). Although both men and women with obesity were found to be anabolically resistant in comparison to lean controls an additional deteriorating effect of T2DM was found only in men. Taken together this may suggest that males have the potential to be affected more severely in terms of anabolic resistance than females. It is not known why this may be the case but it could be related to their greater muscle mass or even that anabolic resistance could be associated with testosterone signaling. Studies comparing anabolic response between the sexes in various conditions as well as the development of targeted remedies are therefore warranted.

The key limitations of the studies in this thesis were small sample size and that they were cross sectional and acute rather than longitudinal in nature. Furthermore, it should be noted that MPS and MPB were not measured directly. The a-v balance technique used in all studies provides a measure of net protein balance and as such the contribution of synthesis and breakdown cannot be separated. Moreover muscle protein oxidation also contributes to net balance but was not measured. Whole-body protein oxidation was

estimated by calculating nitrogen excretion rate from urinary and plasma concentrations of urea in subjects with obesity in Chapter 3 and no significant differences were found between trials. Whole-body protein oxidation was not assessed in the CD trials and so it is not possible to suggest whether differences in oxidation contributed to differences in protein net balance across the forearm. Nevertheless, this could be investigated in a follow up study and does not negate the finding of altered anabolic response to a mixed meal in paediatric CD, when compared with control subjects. Conversely, a strength of the a-v balance technique is that it does provide an overall picture of protein net balance which ultimately determines whether muscle mass is gained or lost over a period of time. Moreover this technique affords the measurement of the net balance of multiple nutrients allowing the co-assessment of tissue insulin sensitivity to glucose along with indices of fat metabolism. Although accuracy of results could have been improved by measuring haematocrit in order to convert blood flow to plasma flow.

Additionally, the oral administration of nutrients in these studies instead of using clamps is a key element, which helps to more closely replicate 'real-life' circumstances. While infusion and clamp studies are invaluable in being able to isolate and test key potential variables, it is also crucial to understand metabolic responses in more 'real-life' situations. This may have unintended consequences, like the possible differences in gastric emptying suggested to have caused the reduced glycaemic excursion in the SFA trial versus control in the study in Chapter 3. However this approach has also demonstrated the potential presence of compensatory measures taken by the body to preserve muscle nutrient uptake e.g. the increased blood flow found in all studies.

In summary, the key finding of this thesis was that anabolic resistance does not always coexist with insulin resistance. This implies that treatments to restore and/or maintain muscle mass in at risk populations should not only focus on improving insulin resistance but interventions to increase anabolic response to food, including high protein diets and exercise. Future studies testing such interventions in a variety of populations are therefore warranted.

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APPENDIX 1 – METABOLOMICS METHODS

Please note that these methods were supplied by Professor Warwick Dunn (Phenome Centre Birmingham).

Sample preparation

Plasma samples were extracted to separate low molecular weight metabolites from other biochemicals including proteins, RNA and DNA. Samples were thawed and extracted on ice. For the analysis of water-soluble metabolites applying a HILIC assay, 180 µL of acetonitrile (LC-MS grade, LiChrosolv, Merck) was added to 60 µL of plasma followed by vortex mixing (15 seconds), centrifugation (13,000×*g*, 15 min) and transfer of the clear supernatant to a glass LC autosampler vial (VI-04-12-02RVG 300µL Plastic, Chromatography Direct, UK). For the analysis of lipid metabolites, 180 µL of isopropyl alcohol, IPA (LC-MS grade, LiChrosolv, Merck) was added to 60 µL of plasma followed by vortex mixing (15 seconds), centrifugation (13,000×*g*, 15 minutes) and transfer of the clear supernatant to a glass LC autosampler vial (VI-04-12-02RVG 300µL Plastic, Chromatography Direct, UK). A single pooled QC sample was prepared by combining 25 µL aliquots of all biological samples where adequate sample volume remained and vortex mixing (2 minutes). Aliquots (60 µL) of the pooled QC sample were extracted as defined above.

Ultra High Performance Liquid Chromatography-Mass Spectrometry

The samples were analysed applying two Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) methods using a Dionex UltiMate 3000 Rapid Separation LC system (Thermo Fisher Scientific, MA,

USA) coupled with a heated electrospray Q Exactive Focus mass spectrometer (Thermo Fisher Scientific, MA, USA). Polar extracts were analysed on a Accucore-150-Amide-HILIC column (100 x 2.1 mm, 2.6 µm, Thermo Fisher Scientific, MA, USA). Mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in 95% acetonitrile/water and mobile phase B consisted of 10 mM ammonium formate and 0.1% formic acid in 50% acetonitrile/water. Flow rate was set for 0.50 $mL.min^{-1}$ with the following gradient: t=0.0, 1% B; t=1.0, 1% B; t=3.0, 15% B; t=6.0, 50% B; t=9.0, 95% B; t=10.0, 95% B; t=10.5, 1% B; t=14.0, 1% B, all changes were linear with curve = 5. The column temperature was set to 35 °C and the injection volume was 2 µL. Data were acquired in positive and negative ionisation modes separately within the mass range of 70 – 1050 *m/z* at resolution 70,000 (FWHM at m/z 200). Ion source parameters were set as follows: Sheath gas = 53 arbitrary units, Aux gas = 14 arbitrary units, sweep gas = 3 arbitrary units, Spray Voltage = 3.5kV, Capillary temp. = 269 °C, Aux gas heater temp. = 438°C. Data dependent MS2 in 'Discovery mode' was used for the MS/MS spectra acquisition using following settings: resolution = 17,500 (FWHM at m/z 200); Isolation width = 3.0 m/z; stepped normalised collision energies (stepped NCE) = 25, 60, 100%. Spectra were acquired in three different mass ranges: 70 – 200 m/z; 200 – 400 m/z; 400 – 1000 m/z.

Non-polar extracts were analysed on a Hypersil GOLD column (100 x 2.1mm, 1.9 µm; Thermo Fisher Scientific, MA, USA). Mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in 60% acetonitrile/water and mobile phase B consisted of 10 mM ammonium formate and 0.1% formic acid in 90% propan-2-ol/water. Flow rate was set for 0.40 $mL.min^{-1}$ with the

following gradient: t=0.0, 20% B; t=0.5, 20% B, t=8.5, 100% B; t=9.5, 100% B; t=11.5, 20% B; t=14.0, 20% B, all changes were linear with curve = 5. The column temperature was set to 55 °C and the injection volume was 2µL. Data were acquired in positive and negative ionisation mode separately within the mass range of 150 – 2000 *m/z* at resolution 70,000 (FWHM at m/z 200). Ion source parameters were set as follows: Sheath gas = 50 arbitrary units, Aux gas = 13 arbitrary units, sweep gas = 3 arbitrary units, Spray Voltage = 3.5kV, Capillary temp. = 263 °C, Aux gas heater temp. = 425 °C. Data dependent MS2 in 'Discovery mode' was used for the MS/MS spectra acquisition using following settings: resolution = 17,500 (FWHM at m/z 200); Isolation width = 3.0 m/z; stepped normalised collision energies (stepped NCE) = 20, 50, 80%. Spectra were acquired in three different mass ranges: 200 – 400 m/z; 400 – 700 m/z; 700 – 1500 m/z.

A Thermo ExactiveTune 2.8 SP1 build 2806 was used as instrument control software in both cases and data were acquired in profile mode. Quality control (QC) samples were analysed as the first ten injections and then every seventh injection with two QC samples at the end of the analytical batch. Two blank samples were analysed, the first as the 6th injection and then the second at the end of each batch.

Raw data processing

Raw data acquired in each analytical batch were converted from the instrument-specific format to the mzML file format applying the open access ProteoWizard software (Kessner et al. 2008). Deconvolution was performed

with XCMS software according to the following settings of Min peak width (4 for HILIC and 6 for lipids); max peak width (30); ppm (12 for HILIC and 14 for lipids); mzdiff (0.001); gapInit (0.5 for HILIC and 0.4 for lipids); gapExtend (2.4); bw (0.25); mzwid (0.01) (C. A. Smith et al. 2006). A data matrix of metabolite features (*m/z*-retention time pairs) vs. samples was constructed with peak areas provided where the metabolite feature was detected for each sample. Putative annotation of metabolites or metabolite groups was performed by applying the PUTMEDID-LCMS workflows operating in the Taverna workflow environment (Brown et al. 2011). We applied 5 ppm mass error and a retention time range of 2 s in feature grouping and molecular formula and metabolite matching. As different metabolites can be detected with the same accurate *m/z* (for example, isomers with the same molecular formula), multiple annotations could be observed for a single detected metabolite feature. Also, a single metabolite could be detected as multiple molecules, particularly as a different type of ion (e.g., protonated and sodiated ions). Throughout, the term “metabolite” refers to either single metabolites or groups of molecules with the same retention time and the same accurate *m/z*. All molecules were annotated according to guidelines for reporting of chemical analysis results, specifically to Metabolomics Standards Initiative level 2 (Sumner et al. 2007).

QC filtering and multivariate data analysis

The data for pooled QC samples were applied to perform QC filtering. For each metabolite feature detected QC samples 1-8 were removed and the relative standard deviation and percentage detection rate were calculated.

Metabolite features with a RSD > 30% and a percentage detection rate < 70% were deleted from the dataset. Missing values in the data were replaced by values applying k nearest neighbour (kNN) missing value imputation followed by normalisation to sample total peak area (as a percentage), glog transformation and Pareto scaling prior to data analysis. Principal Components Analysis (PCA) was then performed to assess the technical variability (measured by the replicate analysis of a pooled QC sample) and biological variability as part of the quality control process using the software MetaboAnalyst (Xia et al. 2015).

Univariate data analysis

Data were normalised to sample total peak area (as a percentage) and were defined as normalized concentration (%), no missing value imputation or scaling was performed and glog transformation was performed prior to data analysis. 2-way ANOVA was applied in the software MetaboAnalyst (Xia et al. 2015) with two factors (time and diet) with all p-values reported after correction for multiple testing using the Benjamin-Hochberg method.

APPENDIX 2 – PAEDIATRIC CROHN'S DISEASE STUDY SUPPLEMENTARY DATA

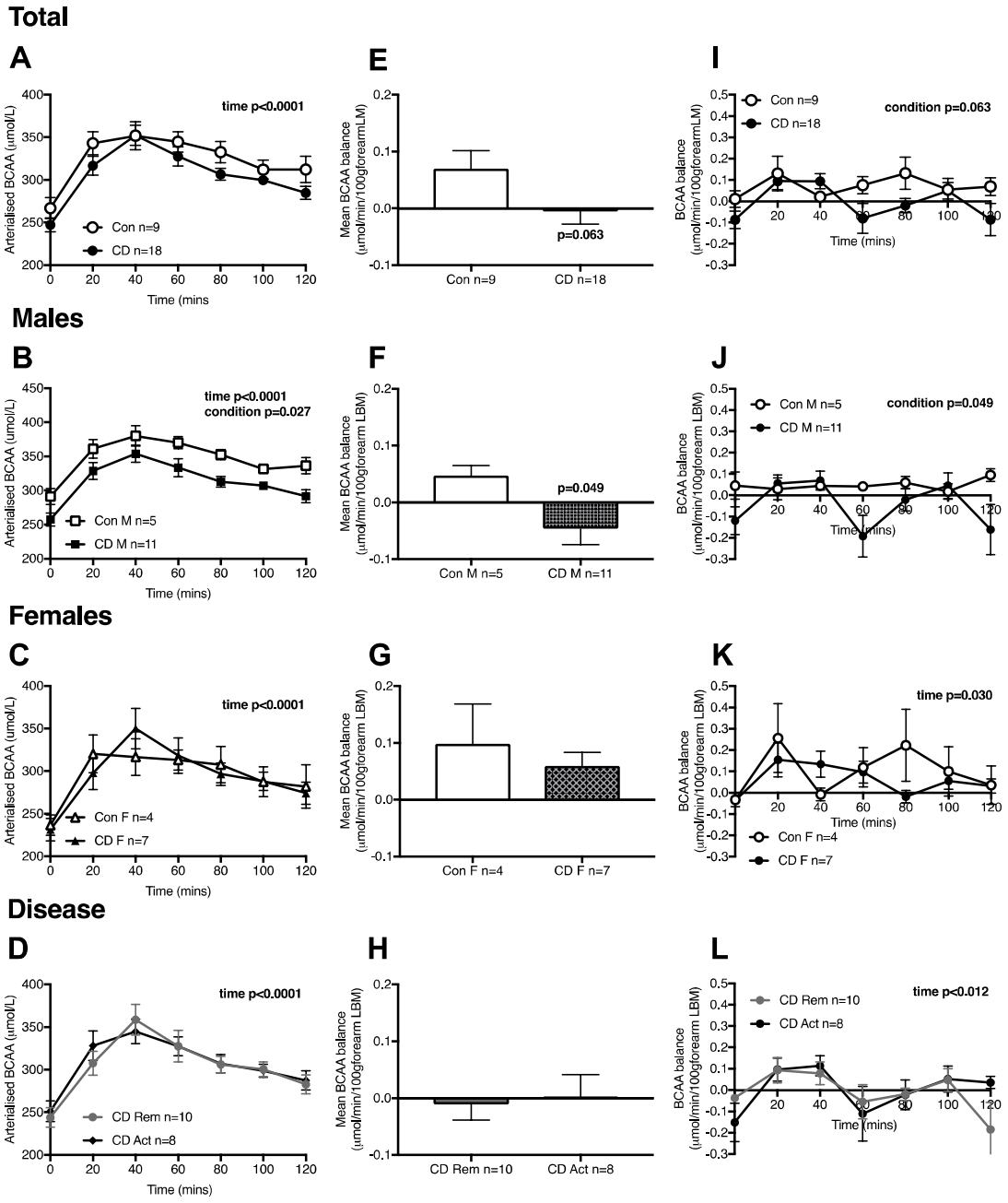
Appendix 2.1 Supplementary data: individual patient demographics

CD participant	Gender	Disease Duration (years)	Surgical history	HBI	Treatment at inclusion	Disease Phenotype	CRP	FCP	Endoscopy or MRE
1	F	9	R hemicolectomy	1	Azathioprine, sulfasalazine	A1aL3B2	0	n/a	Ulcerated and strictured ileocolic anastomosis
2	F	7	n/a	8	Azathioprine, mesalazine	A1aL2B1	9	n/a	Patchy diffuse ileocolic ulcerations
3	M	1	n/a	0	Azathioprine, mesalazine	A1L2L4B1	6	1800	
4	M	1	n/a	0	Azathioprine, mesalazine	A1L2B1	0	1468	
5	M	3	n/a	1	Azathioprine, mesalazine	A1L3L4B1	0	459	
6	M	9	n/a	0	Infliximab, methotrexate	A1L2B2	0	405	Ulcerated colonic stricture
7	M	7	n/a	0	Infliximab, mercaptopurine	A1L3B1	0	419	

8	F	2	n/a	1	Adalimumab, methotrexate, mesalazine, module	A1aL1B1	0	291	
9	F	7	n/a	1	Infliximab, azathioprine	A1aL3B1	0	813	
10	F	2	n/a	0	Azathioprine, mesalazine	A1L2B1	0	51	
11	F	2	n/a	0	Azathioprine, mesalazine	A1L3B1	0	124	
12	M	6	R hemicolectomy	2	Mesalazine	A1L1B3	0	30	
13	M	3	n/a	0	Azathioprine, mesalazine	A1L3B1p	0	n/a	Normal MRE
14	M	3	n/a	0	Infliximab, azathioprine, mesalazine	A1aL3B1	0	76	
15	M	6	n/a	0	Infliximab, azathioprine	A1L1B1	0	72	
16	M	3	R hemicolectomy	0	Infliximab, azathioprine	A1L1B2	0	30	
17	F	5	R hemicolectomy	2	Infliximab,	A1L1B3	0	74	

					azathioprine				
18	F	2	n/a	1	Infliximab, azathioprine	A1L3L4B1	0	30	
19	M	5	R hemicolectomy	0	Adalimumab, azathioprine	A1aL3B2p	1	n/a	normal colonoscopy
20	F	5	n/a	0	Adalimumab, azathioprine	A1aL3L4aB1p	2	n/a	normal colonoscopy

Detailed individual demographics including data relating to gender, disease duration (years), surgical history, clinical symptoms as measured through Harvey Bradshaw Index (HBI), concomitant therapy at inclusion, disease location as per the Montreal Classification, C-reactive protein (CRP) in mg/dl and faecal calprotectin (FCP) in ug/g at inclusion, and recent ileocolonoscopy or magnetic resonance enterography (MRE) detailing disease activity.



Appendix 2.2 Indicators of protein metabolism: arterialized plasma BCAA concentrations (A-D), mean BCAA net balance across the forearrm (E-H) and BCAA net balance across the forearm over time (I-L).

Values are means ± SEM. CD=Crohn's disease, Con=Control, F=female, M=male, CD Rem=CD in remission and CD Act=CD in active disease. A,E,I = Con vs. CD, B,F,J = male subjects only, C,G,K = female subjects only and D,H,L = CD subjects only, split between those with active disease (Act) and those in remission (Rem). Significant differences ($p<0.05$) & trends ($p<0.1$) are marked. BCAA=branched chain amino acids.

Appendix 2.3 Subject characteristics of paediatric Con and CD patients by gender

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=20	n=5	n=11	n=4	n=9
Age (yrs)	16.0 ± 0.6	15.6 ± 0.5	16.1 ± 1.0	15.9 ± 0.7	15.9 ± 0.9	15.2 ± 0.7
Height (m)	1.71 ± 0.05	1.66 ± 0.02	1.79 ± 0.1	1.68 ± 0.03	1.60 ± 0.03	1.63 ± 0.03
Height for age (centile)	63.6 ± 11.0	50.7 ± 6.6	81.0 ± 8.6	40.7 ± 8.8*	41.8 ± 17.8	62.8 ± 8.7
Weight (kg)	60.7 ± 3.7	56.8 ± 2.7	65.9 ± 5.5	57.9 ± 3.7	54.3 ± 2.5	55.4 ± 4.1
BMI (kg/m^2)	20.7 ± 0.6	20.6 ± 0.9	20.3 ± 0.9	20.3 ± 1	21.2 ± 1.0	20.8 ± 1.5
Years since diagnosis	n/a	4.3 ± 0.6	n/a	4.2 ± 0.8	n/a	4.3 ± 0.9
HBI	n/a	1.5 ± 0.4	n/a	0.8 ± 0.2	n/a	2.2 ± 0.9
FCP (µg/g)	n/a	132 ± 41	n/a	118 ± 64	n/a	146 ± 55
CRP (mg/dl)	<5 ± 0	<5 ± 0.4	<5 ± 0	<5 ± 0.6	<5 ± 0	<5 ± 0.6
IL-1β (pg/ml)	303 ± 92	1397 ± 540	396 ± 135	1420 ± 715	187 ± 110	1355 ± 878
IL-6 (pg/ml)	166 ± 52	236 ± 113	244 ± 78	316 ± 170	70 ± 13	92 ± 62
TNFα (pg/ml)	72 ± 36	484 ± 145	122 ± 63	519 ± 200	22 ± 22	420 ± 208
Testosterone (ng/ml)	4.6 ± 1.3	15.8 ± 4.4*	7.0 ± 1.6	15.0 ± 4.1	1.6 ± 0.2	17.1 ± 10.3

All values are means ± SEM except for non-parametric test results (cytokines & testosterone) where values are median and interquartile range and z-scores, which were calculated for height-for-age and BMI-for-age using WHO standards. Statistically significant differences ($p<0.05$) = * are marked. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD, HBI=Harvey Bradshaw Index, FCP=faecal calprotectin, CRP=C-reactive protein, IL-1β=Interleukin 1 beta, IL-6=Interleukin 6, TNFα=tumour necrosis factor alpha.

Appendix 2.4 Body composition of paediatric Con and CD patients by gender

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=20	n=5	n=11	n=4	n=9
FM (kg)	12.3 ± 1.7	16.1 ± 2.0	9.7 ± 1.4	12.9 ± 2.3	15.5 ± 2.9	20.1 ± 3.1
BMC (kg)	2.5 ± 0.2	2.2 ± 0.1	2.8 ± 0.3	2.2 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
BMD (g/cm^2)	1.14 ± 0.02	1.07 ± 0.02	1.16 ±0.04	1.06 ± 0.03	1.12 ± 0.01	1.08 ± 0.03
LBM (kg)	46.0 ± 4.5	39.3 ± 1.5	53.4 ± 6.4	42.8 ± 1.9	36.6 ± 1.2	35.0 ± 1.5
LBM/TBM (%)	75 ± 3	70 ± 2	80 ± 4	75 ± 3	68 ± 4	65 ± 3
Appendicular LM (kg)	22.9 ± 2.6	18.7 ± 0.8	27.2 ± 3.6	20.6 ± 1.0*	17.5 ± 0.8	16.4 ± 0.8
ASMI (kg/m^2)	7.6 ± 0.4	6.8 ± 0.2	8.2 ± 0.7	7.3 ± 0.2	6.8 ± 0.3	6.1 ± 0.2

All values are means ± SEM except z-scores calculated for FM & FMM using age and sex appropriate reference data (see methods). Statistically significant differences ($p<0.05$) = * are marked. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD.

Appendix 2.5 Muscle function of paediatric Con and CD patients by gender

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=18	n=5	n=11	n=4	n=7
Fatigue (%) dom	18 ± 3	26 ± 2	14 ± 2	25 ± 2*	24 ± 4	28 ± 5
Fatigue (%) non-dom	21 ± 4	23 ± 2	22 ± 6	26 ± 3	20 ± 5	17 ± 4
Strength dom (kg/kg forearm LM)	23.8 ± 1.3	25.6 ± 1.5	24.5 ± 1.6	25.4 ± 1.9	23.0 ± 2.2	25.8 ± 2.8
Strength non-dom (kg/kg forearm LM)	23.9 ± 1.1	24.3 ± 1.4	24.8 ± 1.6	24.4 ± 1.8	22.8 ± 1.4	24.2 ± 2.5
Age dependent SDS dom	-1.41 ± 0.35	-1.50 ± 0.22	-0.92 ± 0.33	-1.60 ± 0.27	-2.03 ± 0.55	-1.36 ± 0.40
Age dependent SDS non-dom	-1.62 ± 0.31	-1.90 ± 0.21	-1.04 ± 0.33	-1.88 ± 0.26§	-2.34 ± 0.27	-1.94 ± 0.37
Height dependent SDS dom	-1.13 ± 0.35	-0.93 ± 0.34	-0.98 ± 0.47	-0.73 ± 0.41	-1.31 ± 0.59	-1.23 ± 0.62
Height dependent SDS non-dom	-1.36 ± 0.33	-1.37 ± 0.36	-1.11 ± 0.46	-1.04 ± 0.45	-1.68 ± 0.51	-1.90 ± 0.60

All values are means ± SEM except standard deviation scores (SDS) calculated for height and age dependent strength (see methods). Statistically significant differences between groups ($p<0.05$) = * and trends ($p<0.1$) = § are marked. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD, dom=dominant arm, non-dom=non dominant arm.

Appendix 2.6 Energy expenditure for paediatric Con and CD patients by gender

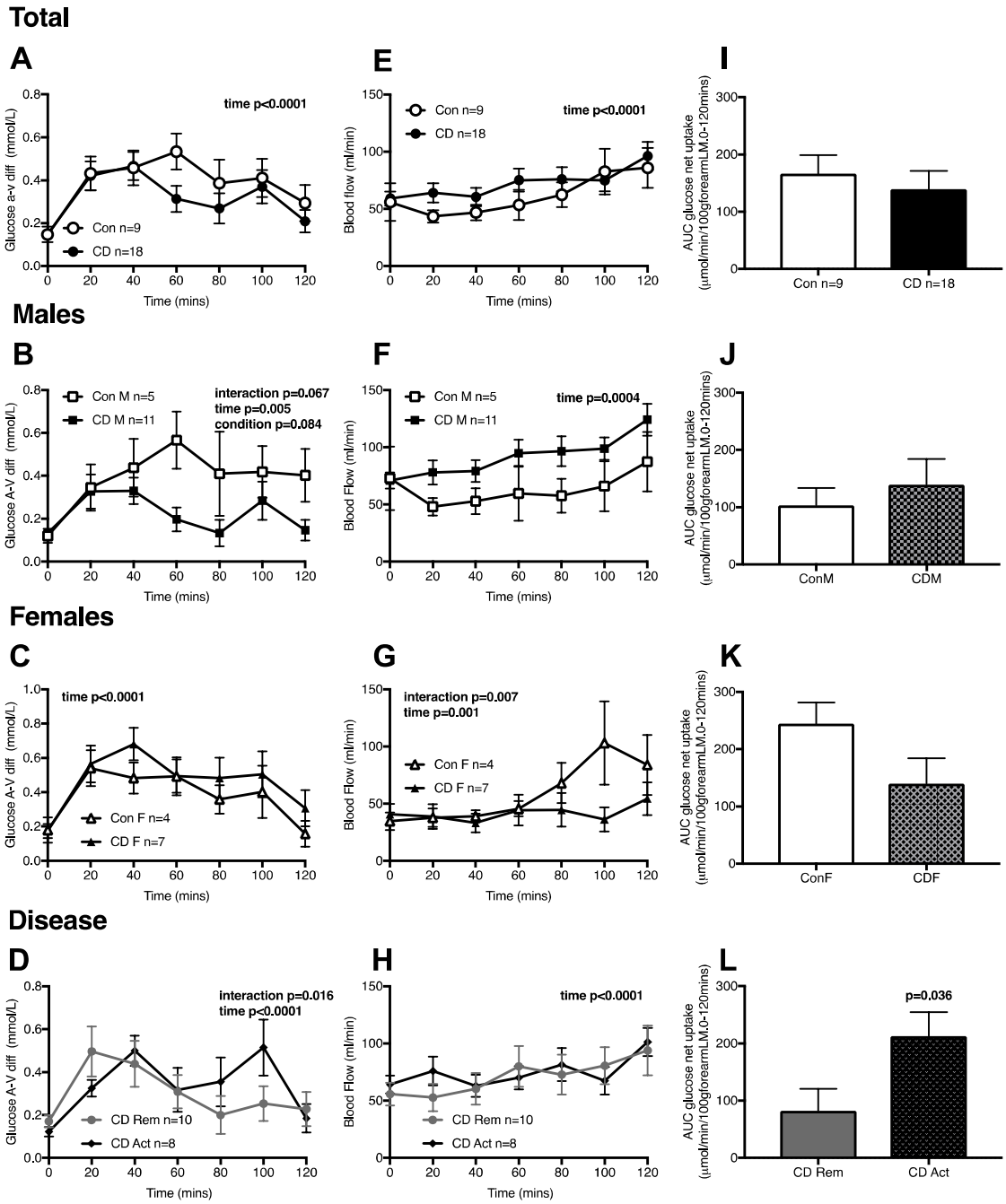
	Control	CD	ConM	CDM	ConF	CDF
Indirect calorimetry	n=9	n=18	n=5	n=11	n=4	n=7
REE - fasted (kJ/hr/kgLBM)	6.1 ± 0.3	5.9 ± 0.4	5.7 ± 0.4	6.3 ± 0.3	6.6 ± 0.4	5.2 ± 0.9
REE - post-feeding (kJ/hr/kgLBM)	6.5 ± 0.3^{T}	6.0 ± 0.4^{T}	6.1 ± 0.5	6.3 ± 0.2	7.0 ± 0.3^{T}	5.7 ± 1.0^{T}
RER – fasted	0.81 ± 0.02	0.76 ± 0.05	0.81 ± 0.03	0.78 ± 0.01	0.81 ± 0.05	0.73 ± 0.13
RER - post-feeding	0.87 ± 0.02^{T}	0.80 ± 0.05^{T}	0.85 ± 0.03^{T}	0.86 ± 0.02^{T}	0.88 ± 0.02	0.70 ± 0.12
COX - fasted (mg/min/kgLBM)	2.4 ± 0.6	2.2 ± 0.4	2.2 ± 0.5	1.9 ± 0.3	2.7 ± 1.2	2.6 ± 0.9
COX - post-feeding (mg/min/kgLBM)	3.9 ± 0.4^{T}	3.1 ± 0.4^{T}	3.3 ± 0.5^{TT}	3.7 ± 0.5^{TT}	4.7 ± 0.5	2.2 ± 0.6
FOX - fasted (mg/min/kgLBM)	1.6 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	1.7 ± 0.4	1.2 ± 0.4
FOX - post-feeding (mg/min/kgLBM)	1.2 ± 0.2$^{\S T}$	1.3 ± 0.2$^{\S T}$	1.3 ± 0.3^{T}	1.2 ± 0.2^{T}	1.1 ± 0.2	1.6 ± 0.4
Physical Activity	n=9	n=20	n=5	n=11	n=4	n=9
Pedometer (no of steps/day)	8056 ± 849	7831 ± 725	8286 ± 1488	6979 ± 870	7767 ± 766	9002 ± 1176
IPAQ (total MET-mins/week)	6196 ± 2158	4443 ± 994	5654 ± 2984	4022 ± 1557	7099 ± 3622	4910 ± 1270

All values are means ± SEM. Statistically significant differences between fed and fasted states in both groups are marked ^{T}=p<0.05 and trends (p<0.1) $^{\S T}$. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD, REE=resting energy expenditure, RER=respiratory exchange ratio, COX=carbohydrate oxidation, FOX=fat oxidation, IPAQ=international physical activity questionnaire.

Appendix 2.7 Energy intake for paediatric Con and CD patients by gender

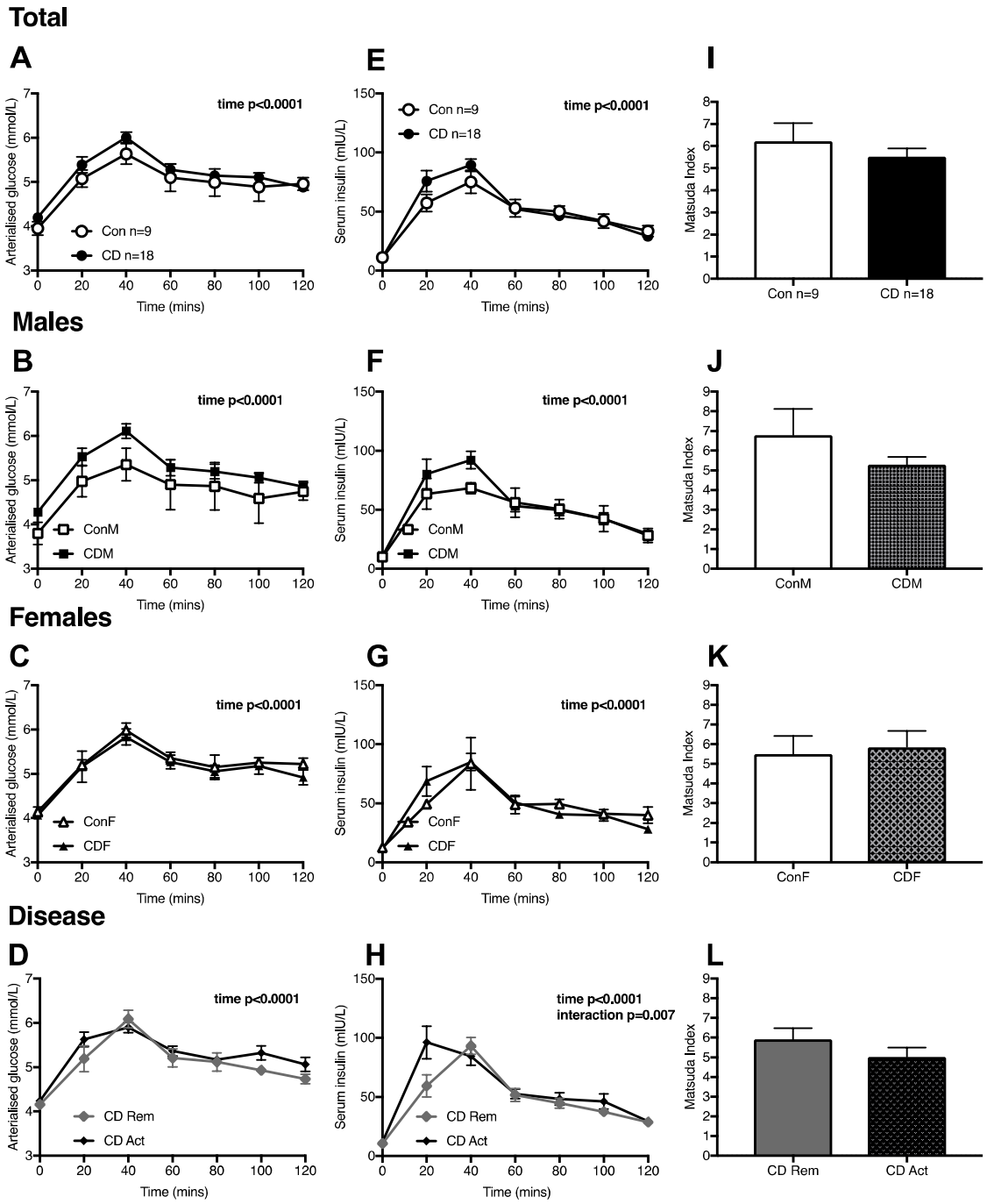
	Control	CD	ConM	CDM	ConF	CDF
Food diaries	n=9	n=18	n=5	n=11	n=4	n=7
Energy intake (kJ/day)	9124 ± 1081	8333 ± 512	10547 ± 1669	8381 ± 624	7345 ± 687	8258 ± 944
Carbohydrate intake (kJ/day)	4299 ± 595	4099 ± 355	4901 ± 935	4162 ± 544	3547 ± 570	4001 ± 372
Fat intake (kJ/day)	3294 ± 490	2884 ± 182	3863 ± 803	2946 ± 161	2584 ± 255	2787 ± 415
Protein intake (kJ/day)	1531 ± 173	1350 ± 93	1784 ± 255	1273 ± 81*	1214 ± 108	1469 ± 204
Protein intake (g/day)	90 ± 10	79 ± 5	105 ± 15	75 ± 5*	71 ± 6	86 ± 12
Recommended protein intake (g/day)	46 ± 3	43 ± 2	49 ± 4	43 ± 3	41 ± 2	44 ± 3

All values are means ± SEM. Statistically significant differences between groups ($p<0.05$) = * are marked. CD=Crohn's disease, Con=Control, ConM=male con, CDM=male CD, ConF=female con & CDF=female CD.



Appendix 2.8 Skeletal muscle insulin sensitivity in CD vs. Con: glucose arterio-venous difference across the forearm (A-D), brachial artery blood flow (E-H) and AUC glucose net uptake across the forearm (I-L).

Values are means ± SEM. CD=Crohn's disease, Con=Control, F=female, M=male, CD Rem=CD in remission and CD Act=CD in active disease. A,E,I = Con vs. CD, B,F,J = male subjects only, C,G,K = female subjects only and D,H,L = CD subjects only, split between those with active disease (Act) and those in remission (Rem). Significant differences ($p<0.05$) & trends ($p<0.1$) are marked.



Appendix 2.9 Indicators of whole body insulin sensitivity:arterialized blood glucose concentrations (A-D), serum insulin (E-H) and Matsuda index (I-L).

Values are means ± SEM. CD=Crohn's disease, Con=Control, F=female, M=male, CD Rem=CD in remission and CD Act=CD in active disease. A,E,I = Con vs. CD, B,F,J = male subjects only, C,G,K = female subjects only and D,H,L = CD subjects only, split between those with active disease (Act) and those in remission (Rem). Significant time effects are marked.

APPENDIX 3 – ADULT CROHN'S DISEASE PATIENTS SUPPLEMENTARY DATA

Appendix 3 Disease history by adult CD subject

CD subject	Gender	Disease Duration (years)	Surgical history	HBI	Treatment at inclusion	Disease Phenotype	CRP	FCP	Endoscopy or MRE
1	F	10	None	13	Nil	A2L3B1	<5	n/a	Active TI disease
2	F	45	R hemicolectomy, transverse colectomy, defunctioning loop ileostomy	4	Infliximab	A2L3B3	<5	329	SES-CD = 10
3	F	1	None	1	Adalimumab	A3L2B2	14	1659	SES-CD = 10
4	M	16	R hemicolectomy, ileocolic resection	0	Nil	A1L3B3p	20	340	SES-CD = 5
5	M	10	Small bowel resection	0	Infliximab, mercaptopurine	A2L3B2	<5	>1800	Multi-focal stricturing small bowel CD
6	F	9	R hemicolectomy	5	Mercaptopurine	A2L3B1	<5	742	n/a
7	M	15	small bowel resections x 2	1	Nil	A2L3B2	5	566	Multifocal stricturing small bowel CD
8	M	10	None	5	Mercaptopurine	A2L3B3	20	298	n/a

Detailed individual demographics including data relating to gender, disease duration (years), surgical history, clinical symptoms as measured through Harvey Bradshaw Index (HBI), concomitant therapy at inclusion, disease location as per the Montreal Classification, C-reactive protein (CRP) in mg/dl and faecal calprotectin (FCP) in ug/g at inclusion, and recent ileocolonoscopy or magnetic resonance enterography (MRE) detailing disease activity.