# THE IMPACT OF N-3 PUFA INGESTION ON METABOLIC, MOLECULAR AND EPIGENETIC RESPONSES TO A SHORT-TERM HIGH-FAT DIET

By

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## ABSTRACT

Obesity is widely considered a primary risk factor for type 2 diabetes (T2D). However, less is known about the early adaptive responses to short-term periods of high-fat energy excess (HFEE). Previous reports detailing whole-body adaptation to fat and energy oversupply are equivocal, perhaps, in part, owing to use of different experimental protocols, varying durations of dietary manipulation and participant cohorts with individuals of varying characteristics.

In addition to use of different dietary protocols between studies, alterations in functional end-point measures due to the type of dietary fat consumed warrants consideration. Daily n-3 PUFA intake, commonly obtained from pelagic fish oil (FO) consumption, has been shown to positively associate with insulin sensitivity in epidemiological studies and thus may be a useful dietary strategy for slowing insulin resistance development.

**Chapter 2** of this thesis extends previous literature by demonstrating that 6 d HFEE (150 % habitual energy intake; 60 % of energy from fat) does not clearly alter whole-body insulin sensitivity, irrespective of FO consumption. However, investigation of metabolism at the tissue level, as presented in **Chapter 3** of this thesis, offers insight into a potential tissue-specific level of regulation that precedes whole-body regulation. Skeletal muscle insulin signalling protein (*e.g.* protein kinase B (PKB)) activity, levels of certain ceramide species, and AMPK α2 activity were altered following HFEE and may explain the early maladaptive responses to short-term HFEE. Moreover, FO intake as 10 % of total fats mediated some of these molecular

responses, including PKB and AMPK α2 activity, reflecting possible functional effects of FO at the subcellular level.

Regulation of these metabolic / molecular responses at both the tissue and whole-body level can be explained, in part, by genetic predisposition, environmental influence and more recently epigenetics, including microRNAs (miRNAs). In **Chapter 4**, we characterised the plasma and skeletal muscle miRNA responses to HFEE and oral glucose ingestion. We demonstrate transient changes in levels of certain miRNAs following oral glucose ingestion in both tissue types and in response to HFEE in skeletal muscle. However, no significant correlations between basal plasma and skeletal muscle miRNA levels were observed, suggesting that our candidate plasma miRNAs may be co-ordinating functional changes in other tissue types. Plasma miR-145-5p and skeletal muscle miR-204-5p predicted a significant proportion of the variance in mean whole-body insulin sensitivity change in response to HFEE. These data indicate that these miRNAs may be useful biomarkers of insulin resistance development following HFEE.

A constraint of this thesis is that all conclusions are made within the context of statistically unaltered insulin sensitivity. Therefore, future investigations of diet-induced maladaptation should consider establishing a time course of insulin resistance development in response to HFEE, or use different study populations. Populations that are more susceptible to T2D development, *e.g.*, overweight, sedentary individuals would be of particular interest. These data would aid development of a working model of diet-induced insulin resistance that has more direct application to T2D progression and extends the data presented herein.

**THANK YOU**

Throughout my life I have been immensely proud to be part of a variety of teams, socially, professionally and in sporting contexts. I believe in the collective power of teams to excel beyond individual abilities and I can say with absolutely certainty that I would not be submitting this thesis if it wasn't for a whole host of remarkable people who have made a contribution to this body of work; some large, some small, but none insignificant.

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## ABBREVIATIONS

**AMPK** 5' adenosine monophosphate-activated protein kinase
**ATP** Adenosine triphosphate
**CPT-1** Carnitine palmitoyltransferase 1
**DAG** Diacylglycerol
**DTNB** Beta dystrobrevin
**DTT** Dithiothreitol
**EDTA** Ethylenediaminetetraacetic acid
**EGTA** Ethylene glycol tetraacetic acid
**FA(s)** Fatty acid(s)
**FO** Fish oil
**FOXO1** Forkhead box protein O1
**GSK(-3β)** Glycogen synthase kinase (-3β)
**GLUT** Glucose transporter
**HFEE** High-fat energy excess
**IRS** Insulin receptor substrate
**LCFA-CoA** Long-chain fatty acid-coenzyme A
**$MgCl_2$** Magnesium chloride
**miRNA** microRNA
**NaCl** Sodium chloride
**NaF** Sodium fluoride
**$Na_3(Ov)_4$** Sodium orthovanadate
**OGTT** Oral glucose tolerance test
**PDK** Phosphoinositide-dependent kinase
**PI3K** Phosphoinositide 3-kinase
**PIP2** Phosphatidylinositol 4,5-biphosphate
**PIP3** Phosphatidylinositol (3,4,5)-triphosphate
**PKB** Protein kinase B
**PKC** Protein kinase C
**PMSF** Phenylmethanesulfonylfluoride
**PP2A** Protein phosphatase 2A
**PUFA** Polyunsaturated fatty acid(s)
**RISC** RNA-induced silencing complex
**SBTI** Soybean trypsin inhibitor
**T2D** Type 2 Diabetes
**TBC1D4** TBC1 domain family member 4
**TG(s)** Triglyceride(s)
**TRIS** 2-Amino-2-hydroxymethyl-propane-1,3-diol

## LIST OF PUBLICATIONS

### Articles published:

Macnaughton LS & **Wardle SL** (2015) Caught in the CrossFire? J Physiol 000.0: 1-10 (**see, Appendices**).

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Macnaughton LS, **Wardle SL**, Tipton KD (2015) Comment on viewpoint: What is the relationship between acute measures of MPS and changes in muscle mass? J Appl Physiol 118: 498-503 (**see, Appendices**).

McGlory C, **Wardle SL**, Macnaughton LS (2013) Pattern of protein ingestion to maximise muscle protein synthesis after resistance exercise. J Physiol 591: 2969-2970 (**see, Appendices**).

### Articles in preparation:

**Wardle SL**, Macnaughton LS, McGlory C, Witard OC, Ferrando AA, Galloway SR, Moran CN, Tipton KD. Whole body insulin sensitivity is not clearly altered following consumption of a six day, high-fat energy excess diet, with or without dietary fish oils, in healthy, young males.

McGlory C, **Wardle SL**, Scott F, Macnaughton LS, Hamilton DL, Galloway SDR, Dick JR, Bell JG, Phillips SM, Tipton KD. Influence of 8 wk of n-3 PUFA supplementation on muscle protein synthesis in response to protein feeding and resistance exercise.

### Accepted abstracts for conference presentation:

**Wardle SL**. MicroRNA expression in the development of insulin resistance (*Invited oral presentation at Physiology 2015; 7th July 2015, Cardiff, UK*).

**Wardle SL**, McGlory C, Macnaughton LS, Witard OCW, Whitfield PD, Galloway SR, Moran CN, Tipton KD. Six days high fat overfeeding alters skeletal muscle ceramide expression prior to changes in whole-body insulin sensitivity in young healthy males *(Oral presentation at Physiology 2014; 30th June 2014, London UK).*

**Wardle SL**, Macnaughton LS, McGlory C, Witard OCW, Ferrando AA, Galloway SR, Moran CN, Tipton KD. Six Days High Fat Overfeeding Does Not Alter Whole-body Insulin Sensitivity in Young Healthy Males *(Poster presentation at the American Diabetes Association (ADA) 74th Scientific Sessions; 15th June 2014, San Francisco, USA).*

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# **CHAPTER 1** General introduction.

## *1.0. Introduction*

Macronutrient and energy excess positively associate with obesity, insulin resistance and type 2 diabetes (T2D; (1,2)). The proportion of 16-64 year olds classified as overweight or obese in Scotland increased from 52.4 to 61.9 % from 1995 to 2012 (3). Moreover, of all T2D individuals with their BMI on record, 87.1 % were either overweight or obese (4). In a recent meta-analysis, being overweight or obese was associated with a 3 or 7 times greater risk of developing T2D, respectively, than normal body weight (5). These data highlight the significant implications of positive energy balance for T2D risk.

Current estimated worldwide prevalence rates of diabetes stand at 347 million people (6), with T2D accounting for 90 % of this figure (7). T2D is characterised by insulin resistance, *i.e.*, the failure of key organs to respond effectively to insulin secretion from the pancreas (8,9). T2D-associated complications include cardiovascular disease, renal failure, retinopathy (possibly leading to blindness), and neuropathy (possibly leading to limb amputation) (2,10). The annual cost to the NHS from T2D-related illness is around £9 billion (11), with 10 % of the healthcare budget in Scotland given to diabetes care (12). With increasing T2D prevalence rates, a substantial burden is being placed on the national healthcare budget and thus health and economic policy.

The sharp rise in T2D prevalence rates over the preceding decades raises a 'gluttony *vs.* sloth' debate regarding the primary cause of energy imbalance (13). Both increased energy intake and decreased physical activity undoubtedly contribute to a more positive energy balance. However, over the past 40 years and for the first time in history, food availability has exceeded human requirements, particularly in the Western world (14,15). Increased food availability has contributed to energy overconsumption, particularly in Western, well-developed countries (16), and has coincided with increasing T2D prevalence rates. Investigation of energy excess and its role in T2D development is central to understanding T2D pathology and associated metabolic perturbations.

The study of insulin resistance and T2D spans several experimental models that include human, rodent and cell-based systems. In addition to chronic models of insulin resistance (*e.g.* overweight / obese / T2D individuals), acute models can be used to investigate early responses leading to T2D onset. Lipid and heparin infusions (*e.g.* (17)), feeding of a high-fat meal or diet (*e.g.* (18,19)), and exercise restriction (*e.g.* (20)) all are commonly used models of acute insulin resistance. However, arguably the most valid model to study energy excess is feeding of a hyperenergetic meal or diet. Investigations of this nature have been less frequent than investigations using other models, and with largely varied findings. Establishing a working model of short-term energy excess leading to insulin resistance would benefit future investigations into the early adaptive responses to overfeeding and T2D development.

Pharmacological (ant)agonists / agents, exercise prescription and dietary manipulation all have been investigated as strategies to prevent or slow T2D onset (2). Increased dietary or supplemental intake of n-3 polyunsaturated fatty acids (n-3 PUFA(s)), commonly obtained from pelagic fish oil (FO), is one candidate dietary strategy. Epidemiological studies demonstrate a positive relationship between habitual dietary intake of n-3 PUFAs and metabolic health (21,22). The mechanisms governing this association may relate to a more permeable cell membrane resulting from n-3 PUFA incorporation that alters signalling and nutrient transport between cells (23–25). Enhancing the delivery of nutrients, particularly glucose, into tissue will increase insulin sensitivity, perhaps slowing T2D onset. Therefore, consumption of n-3 PUFAs during a period of energy excess may be a viable strategy for reducing insulin resistance development.

Environmental influences including dietary manipulation, combined with genetic predisposition underpin the development of T2D. However, environmental exposure also is likely coded into the biological system by epigenetic mechanisms (26,27). Epigenetic mechanisms are involved in gene regulation and include the non-coding RNA molecules, microRNAs (miRNAs) (28). Heritable changes in phenotype or gene expression that occur in the absence of changes in the DNA sequence are considered epigenetic in nature (29). The main modes of miRNA action on gene expression are twofold; *i*) to inhibit translation initiation of nascent mRNA transcripts and / or *ii*) degradation of mRNA transcripts (30,31). These processes result in a reduced expression of genes encoded by the targeted mRNA transcripts. The role of the mRNA target as a stimulator or inhibitor of a given pathway will determine the direction of change in flux through the pathway as a result of this reduced mRNA expression.

MiRNAs are tightly regulated and integral for 'normal' whole-body metabolic regulation (32). However, altered miRNA levels have been shown in a variety of disease states including T2D and atherosclerosis (33,34), suggesting miRNAs may be useful biomarkers of disease and are likely important in health maintenance and adaptations to diet.

A significant body of research exists characterising the influence of nutritional interventions on insulin sensitivity, yet the precise molecular mechanisms underpinning these adaptive responses are incompletely understood. What is known, is that a series of phosphorylation cascades involving insulin signalling through protein kinase B (PKB) play a crucial, defining role in glucose uptake in insulin sensitive tissues (*e.g.* (8,35–37)). Therefore, in this chapter, an overview of insulin signalling first will be presented, followed by a critical review of existing studies that characterise adaptive responses to short-term lipid and energy oversupply. The focus for discussion of these studies will be on glucose metabolism, lipid metabolism and epigenetic regulation. Cumulatively, the aim of this chapter is to provide the reader with an appreciation for the philosophy of this thesis, including the existing evidence base and principal rationale for the experimental data presented herein.

### *1.1. Insulin signalling*

Carbohydrate feeding is the primary stimulus for insulin secretion (38,39). Glucose, from carbohydrate digestion, is *sensed* by the pancreatic β-cells which are clustered in islets with a high degree of vascularisation (38). This highly vascularised network augments the transport of blood to, and from, the pancreas and enables greater

nutrient exchange across the tissue bed (38). The glucose transporter (GLUT) isoform, GLUT2, expressed in pancreatic β-cells, is deployed to the plasma membrane in an insulin-independent manner and is the first β-cell micro-domain to be exposed to, and thus *sense*, circulating blood glucose (38). Following entry of glucose into the cell via GLUT2 transporters, glucose is phosphorylated by glucokinase to form glucose-6-phosphate (38). Glucose influx enhances glucose metabolism and flux through the tricarboxylic acid (TCA) cycle; leading to a raised adenosine triphosphate (ATP): adenosine diphosphate (ADP) ratio from both glycolytic and oxidative metabolism (38,39). As a result, plasma membrane depolarisation (via closure of the ATP-sensitive potassium channels) mediates subsequent calcium influx by stimulating the opening of voltage-dependent calcium channels (39,40). The fusion and exocytosis of insulin-containing granules is stimulated from this sudden calcium influx - leading to insulin secretion into the circulation for action on tissue (38,39).

Insulin stimulates a series of post-translational modifications during the period from insulin binding to its receptor, to insulin-mediated glucose uptake. This series of post-translational modifications forms a signalling cascade and is considered an amplification process (41). Amplification through the signalling cascade means that even a small activation of proximal genes can cause significant activation of downstream targets and thus glucose uptake (41). The insulin signalling cascade is best characterised in skeletal muscle, but liver and adipose tissue also are primary insulin-responsive tissues with important integrated functions in glucose homeostasis (**Figure 1.1**; (42,43)).

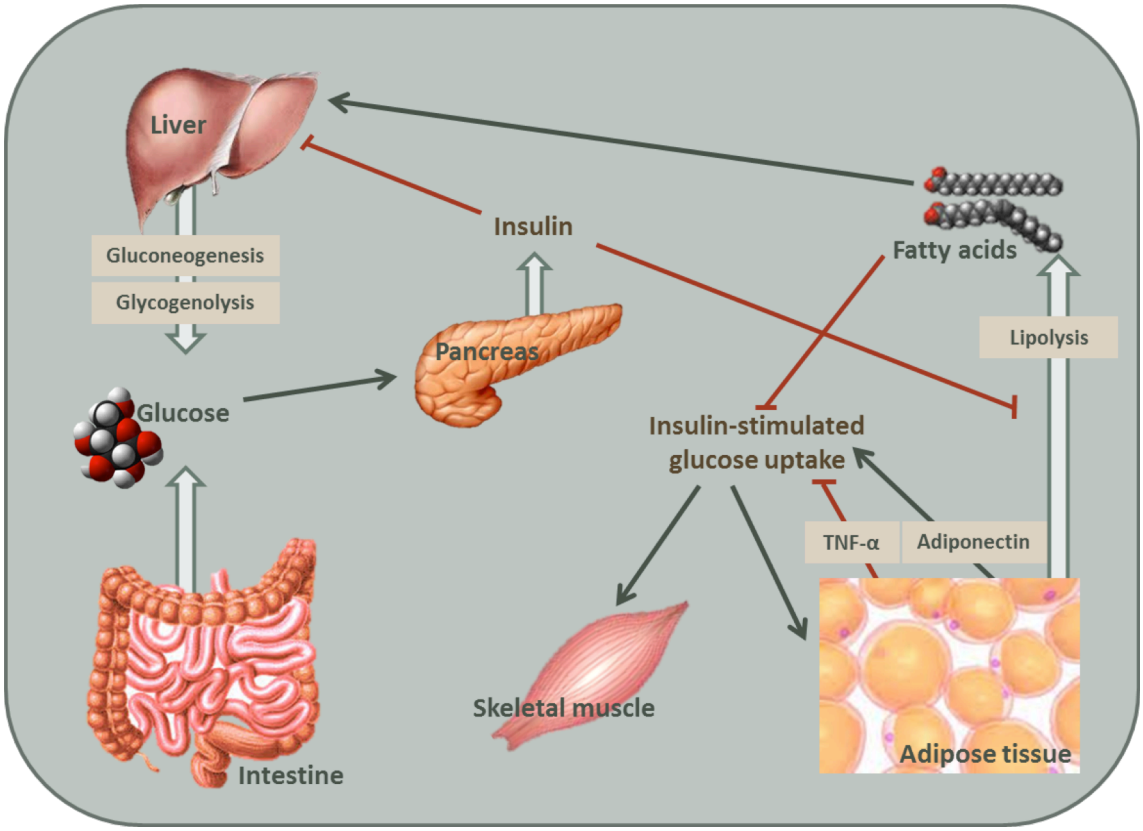



**Figure 1.1** Integrated tissue cross-talk for insulin-stimulated glucose and lipid homeostasis. Insulin has inhibitory actions on glucose production and FA mobilisation, promoting glucose uptake into skeletal muscle and adipose tissue. Green arrows denote stimulation / increase, flat red lines denote inhibition. Adapted from (287).

### *1.1.1. Skeletal muscle*

Skeletal muscle is an important site of blood glucose regulation, accounting for approximately 75 % of whole-body non-oxidative glucose disposal (9,44,45). Several reports have reviewed the skeletal muscle insulin signalling process in detail (9,35,37,46,47), and a schematic is depicted in **Figure 1.2A**.

Binding of insulin leads to autophosphorylation of the insulin receptors on the muscle membrane via activation of the tyrosine residues within the receptors (46). These autophosphorylated, active receptors promote recruitment of a variety of docking proteins with key substrates being the family of insulin receptor substrates (IRS) and

in particular, IRS-1 (46). Human IRS-1 contains several tyrosine and serine / threonine phosphorylation sites that can interact with downstream signalling proteins. In an insulin sensitive state, IRS proteins are phosphorylated on their tyrosine residue by the lipid kinase, phosphoinositide 3-kinase (PI3K) (47). PI3K consists of both a regulatory and catalytic subunit, with different, but complementary, features. It is the regulatory subunit that interacts with IRS proteins, whereas the catalytic subunit promotes the phosphorylation of phosphoinositols located within the plasma membrane (9,47). One particular catalytic action of PI3K is the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) at the plasma membrane (9). It is the generation of PIP3 in particular that drives subsequent downstream actions (35).

One of the key downstream effectors of glucose uptake is PKB. Three ubiquitously expressed isoforms of PKB exist in skeletal muscle, with PKB1 and PKB2, rather than PKB3, involved in insulin signal transduction (9,35). Phosphorylation of PKB at two key sites, $Thr^{308}$ and $Ser^{473}$, is necessary for full protein activation (9,48) and the phosphoinositide-dependent kinase (PDK) proteins are important in this process (9). Specifically, PDK1 and PDK2 are thought to act on PKB1 and PKB2, respectively (9). The PDK proteins also stimulate phosphorylation of atypical protein kinase C (aPKC), which amplifies the signal for glucose uptake. PKB acts as a branching point for glucose uptake, glycogen synthesis and anabolic signalling (49). Glycogen synthase kinase (GSK)-3 is inhibited by the phosphorylation of PKB, which results in the removal of glycogen synthesis suppression. The subsequent increase in glycogen synthesis further augments the signal for glucose uptake by reducing intracellular glucose concentrations (46). The other mechanism of PKB is phosphorylation of its

substrate, TBC domain family member 4 (TBC1D4) (35). When TBC1D4 is phosphorylated, the Rab-GTPase proteins are activated. Upon activation of the Rab-GTPase proteins, suppression of the facilitated glucose transporter, GLUT4, is released, enabling GLUT4 translocation to the plasma membrane (9,35). GLUT4 is the predominant skeletal muscle isoform of the glucose transporter family members. Once at the plasma membrane, GLUT4 facilitates the transport of glucose from the bloodstream into tissue (9,35). The net effect of insulin-mediated glucose uptake is decreased blood glucose concentrations and increased skeletal muscle glycogen synthesis and / or glucose oxidation.

### *1.1.2. Liver*

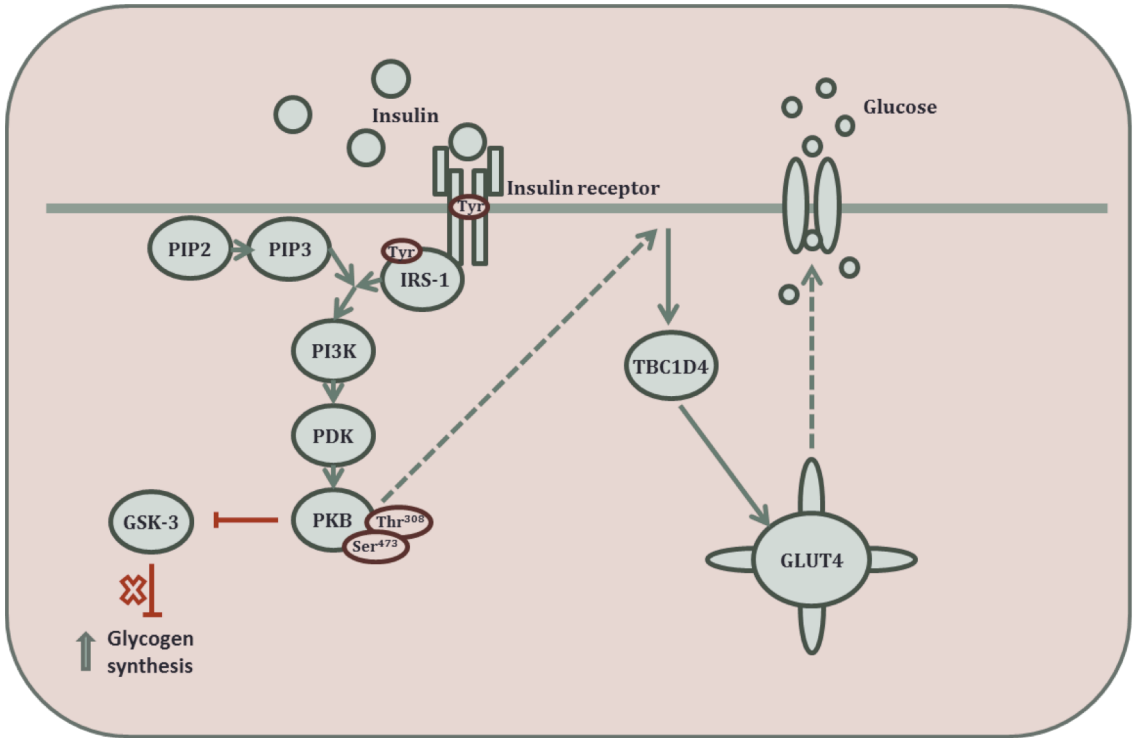
Glucose homeostasis is regulated by insulin action on liver tissue. Insulin promotes glucose uptake into liver via many of the same mechanisms as skeletal muscle, albeit culminating with GLUT2, rather than GLUT4, translocation (50,51). However, arguably the most significant role of insulin within the liver is suppression of hepatic glucose output (50,52). Suppression of glucose output from liver will reduce the contribution of endogenous glucose to the plasma glucose pool in order to offset the increased glucose availability from exogenous sources. Another insulin-mediated mechanism within the liver is the stimulation of glycogen synthesis which occurs via two primary mechanisms. First, glycogen synthase is activated following PKB-mediated phosphorylation and thus inactivation of GSK-3β (50). Second, gluconeogenic enzymes are suppressed by forkhead box protein O1 (FOXO1) (50). The combination of reduced glucose output from the liver and increased glucose

uptake into both skeletal muscle and liver facilitates the restoration of blood glucose concentrations to their normal level.

### *1.1.3. Adipose tissue*

Adipose tissue is a storage depot for triglyceride (TG) but also has functional relevance within the context of energy, and glucose, homeostasis (53). Adipose tissue is regarded as an efficient buffer for substrate (and in particular fatty acid (FA)) flux during postprandial periods (53). Insulin contributes to this buffering action via suppression of lipolysis and reduced mobilisation of FAs from adipose tissue (53). A reduction in circulating FA levels will cause a switch towards carbohydrate metabolism based on fuel availability. This switch in fuel utilisation creates a concentration gradient for glucose uptake into skeletal muscle and liver as more glucose is being oxidised within the cell. Thus, insulin action on adipose tissue will promote glucose uptake into tissue.

**A. Insulin sensitive state**




**B. Insulin resistant state**

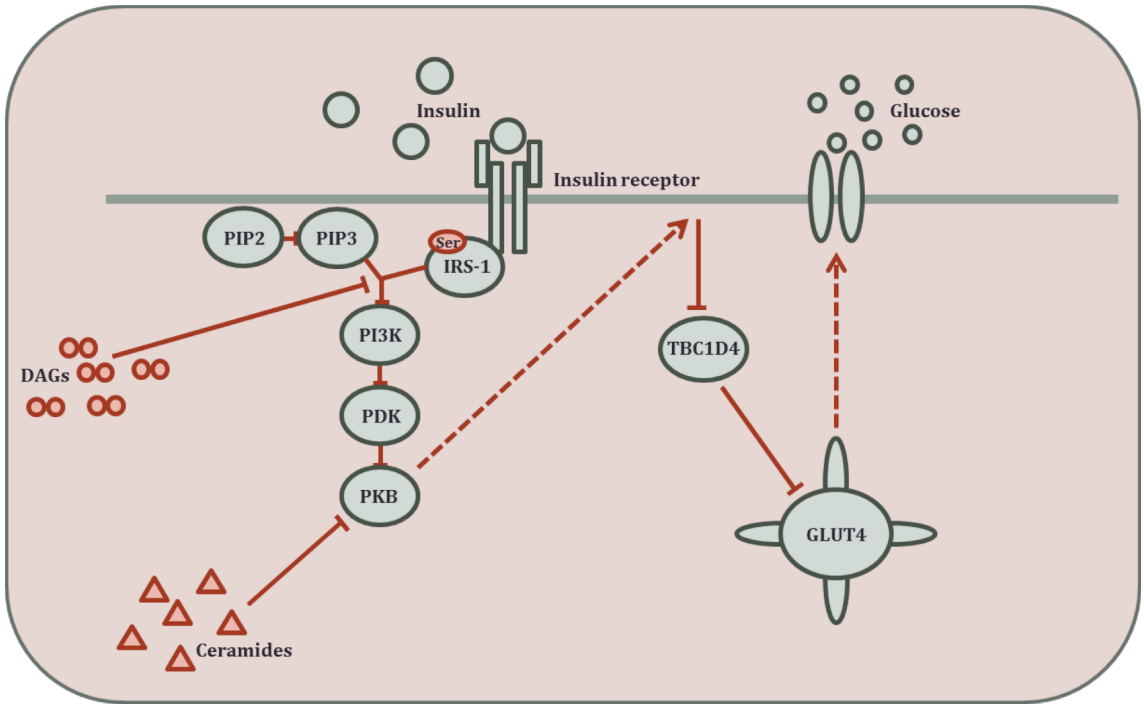



**Figure 1.2** Insulin signalling pathway in an insulin-sensitive state (A) and insulin-resistant state (B). Broken lines indicate membrane translocation, arrows denote phosphorylation and flat-ended lines denote inhibition of phosphorylation. With an increase in cellular DAGs and ceramides, functional phosphorylation of key signalling intermediates including IRS-1 and PKB is reduced. Reduced phosphorylation of these signalling proteins results in impaired glucose uptake.

*1.2. Insulin resistance*

Defects in insulin action are different between type 1 diabetes (T1D) and T2D. The inability to produce enough insulin is a key feature of T1D (54), whereas T2D sufferers are able to produce sufficient insulin but are unable to effectively respond to this insulin stimulus (35). The failure of key organs to respond effectively to normal circulating levels of insulin is termed insulin resistance (8,9). An effort to maintain euglycaemia in the early stages of insulin resistance development is made by increasing insulin secretion from the pancreas (8). However, following this period of compensated insulin resistance, glucose uptake is impaired despite elevated insulin secretions (8). For these reasons, hyperinsulinemia and / or hyperglycaemia reflect an insulin-resistant state.

The mechanism by which glucose uptake is impaired in insulin-resistant states was widely investigated in the 1990s. Glycogen synthesis plays a major role in glucose metabolism in the postprandial state, and is the primary driver of glucose uptake (55–57). However, glycogen synthesis is impaired in T2D (58,59) and is largely responsible for the associated insulin resistance-mediated hyperglycaemia (55). The rate of muscle glycogen synthesis is regulated by glycogen synthase activity (60,61), hexokinase II activity (62–64) and glucose transport (65). Glucose transport is considered the primary rate limiting step for glycogen synthesis (55,66–68). Glucose transport is limited by insulin signal transduction and thus the responsiveness of peripheral tissue to elevated insulin levels (9,35,44,65,69).

Impaired insulin signal transduction in insulin-resistant states is largely characterised by a blunted phosphorylation of key kinases within the pathway (see,

**Figure 1.2B**). Reduced tyrosine phosphorylation of IRS-1 (and increased dysfunctional serine / threonine phosphorylation) (70), reduced PI3K activation (71) and reduced PKB phosphorylation (72,73) are all consistent features of insulin resistance / T2D. Reductions in phosphorylation of these kinases, indicative of protein activation in this instance, result in impaired insulin signal transduction, reduced GLUT4 translocation to the plasma membrane and reduced glucose uptake into the cell.

The mechanisms regulating defective phosphorylation of kinases within the insulin signalling cascade are multifactorial. However, lipid metabolism is considered a major player in the (dys)regulation of these proteins (37,50,74–76). Excess energy and lipid intake is associated with an accumulation of lipid derivative intermediates that downregulate insulin signalling protein activation (77). These lipid derivatives include diacylglycerol (DAG), long-chain fatty acyl-CoA (LCFA-CoA) and the sphingolipid, ceramide (78). DAGs and ceramides are probably the most widely investigated lipid derivatives within the context of insulin resistance, and herein, will form the basis for discussion of lipid-mediated insulin resistance.

Insulin resistance is associated with elevated skeletal muscle TG levels (79,80). However, high TG levels also are regularly observed in highly trained athletes (79), creating what is termed the athlete's paradox. This paradoxical finding was a key factor leading to early investigations into the role of DAGs in insulin resistance as a way to explain (dysfunctional) high TG levels in the insulin-resistant state (81). DAG synthesis occurs synonymously with TG synthesis, thus increases in both DAGs and TGs are common traits of insulin resistance (80,81). Moreover, DAGs are known to

allosterically activate protein kinase C (PKC) isoforms involved in serine phosphorylation of IRS-1 and thus impaired insulin signal transduction (81). Using lipid / heparin infusion methodology in healthy humans, Itani and colleagues (81) demonstrated an increase in skeletal muscle DAG content and PKC activity, concomitant with reduced insulin sensitivity (81). Moreover, *in vitro* data demonstrated a SFA-mediated increase in DAG content in human (82) and mouse myotubes (83). These increases in DAG content occurred in combination with impaired insulin-stimulated glucose uptake (82) and inhibition of insulin-stimulated GSK-3β and PKB phosphorylation (83). Taken together, skeletal muscle DAG content inversely associates with insulin sensitivity, perhaps at the level of IRS-1 and / or PKB.

Ceramides are another lipid derivative involved in insulin resistance. Ceramides are synthesised via two primary pathways including *de novo* synthesis from palmitate and recycling of sphingosine via the salvage pathway, catalysed by ceramide synthase (84). Ceramide synthesis is regulated by adipokine concentrations, SFA availability, inflammation and endoplasmic reticulum stress (84,85). Ceramide action is primarily through the secondary intermediates PKCζ and protein phosphatase 2A (PP2A) which dephosphorylate PKB (84–87), resulting in decreased distal insulin action and reduced glucose uptake into the cell (84,85). Considering impaired glucose uptake into tissue is a hallmark of T2D, recent attention has been afforded to ceramides in an endeavour to better understand the development of insulin resistance.

Intramuscular ceramide levels have been implicated in T2D pathology with increased skeletal muscle ceramide levels observed in obese, insulin-resistant individuals

compared to controls (72,88). Moreover, data from *in vitro* work highlight a causal relationship between increased ceramide levels and insulin resistance. Palmitate-induced ceramide production causes inhibition of insulin signalling at the level of PKB (reduced phosphorylation) (89) in a dose-dependent manner (87). Ceramides now are commonly associated with human insulin resistance (77,90,91). Whereas the action of ceramides is on proteins more distal to those inhibited by DAGs, the resultant outcome of decreased glucose uptake is the same with increases in either lipid derivative. Thus, lipid metabolism is a key area of investigation for studies designed to unravel mechanisms of insulin resistance development.

*1.3. High fat feeding - Impact of energy excess on insulin signalling*

Given that chronic overfeeding is a key stimulus for the development of obesity and insulin resistance, investigation of the early adaptive responses to short-term, high-fat energy excess (HFEE) may help inform the basis for T2D progression. Chronic overfeeding is associated with elevated circulating FFA and TG levels (92,93). Historically, elevated lipid levels were thought to impair glycolytic carbohydrate metabolism via inhibition of phosphofructokinase (94,95). Reduced glycolytic flux inhibits glucose metabolism and results in a concentration gradient that is not in favour of glucose uptake. This concept was coined the Randle Cycle after work by Sir Phillip Randle and colleagues in the 1960s (94,95). Specifically, their research, conducted in heart and skeletal muscle preparations, demonstrated that an increase in circulating FAs (*e.g.* from fat consumption or increased adipose tissue lipolysis) leads to an accumulation of acetyl-CoA and citrate (from elevated rates of fat oxidation via Krebs Cycle metabolism). Raised levels of acetyl-CoA and citrate cause a

concentration gradient to develop whereby an excess of these products inhibits the catalysing activities of pyruvate dehydrogenase and phosphofructokinase, respectively. Inhibition of these enzymes reduces glycolytic flux resulting in a build-up of glucose-6-phosphate inside the cell. Another high product concentration gradient is created from increased glucose-6-phosphate levels, resulting in reduced activity of hexokinase and limiting glucose uptake into the cell. Limited glucose uptake into the cell results in a state of hyperglycaemia; one of the defining characteristics of insulin resistance and T2D (8). Thus, based on this mechanism, carbohydrate metabolism can be regulated by fat metabolism.

These studies performed by Randle *et al.* (94,95) were devised on the concept that metabolically active tissues, such as cardiac and skeletal muscle, have the capacity to readily switch between carbohydrate and fat metabolism depending on the energy status of the cell (96). Whereas Randle and colleagues focussed much of their work on the metabolic switch to fat oxidation, the opposite switch (*i.e.* from fat metabolism to carbohydrate metabolism) also occurs. Mechanisms associated with increased carbohydrate metabolism can lead to an inhibition of fat metabolism. For example, increased glucose metabolism has been linked to elevated levels of malonyl-CoA within the cell (96). Malonyl-CoA is carboxylated from acetyl-CoA by acetyl-CoA carboxylase (ACC) (96). Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase (CPT)-1 activity; a major control point in the transport of fats into the mitochondria for oxidation (96). LCFA-CoAs will accumulate intramuscularly in the absence of β-oxidation (medium chain lengths can enter the mitochondria for oxidation without the need for transporter proteins (97)) and FAs will be diverted towards esterification (96). This increased FA environment and drive towards FA

esterification also can lead to the accumulation of lipid derivatives. As discussed previously, a build-up of these lipid derivatives have been implicated in insulin resistance due to their impact on the activity of proteins within the insulin signalling pathway. The concept of carbohydrate metabolism regulating fat metabolism is particularly pertinent within the study of short-term overfeeding as reduced, or unchanged, circulating FA and TG levels are regularly observed (19,98,99). These observations are in contrast to the increased concentrations reported in obesity (92,93,100,101), and which are integral to the mechanistic action of the Randle cycle. Therefore, the relationship between carbohydrate and fat metabolism is likely dependent upon the metabolic status of the cell.

The effects of high-fat energy excess (HFEE) are multi-faceted and are not completely understood in human skeletal muscle. Existing studies designed to investigate the metabolic responses to HFEE are detailed in **Table 1.1**. The lack of consistency in experimental design, including participant cohorts, duration and degree of overfeeding, and selected end-point measures, make it difficult to directly compare study results. However, the general consensus appears to be of a detrimental effect of overfeeding, particularly with regards to perturbation at the tissue level. Thus, short-term HFEE protocols may be a valid and useful model for investigation of metabolic disease development.

**Table 1.1** Studies detailing a high-fat overfeeding dietary intervention in human participants.

| REFERENCE | PARTICIPANTS | FEEDING INTERVENTION | KEY FINDINGS |
|---|---|---|---|
| **Adochio** *et al.* (99) | 21 healthy, lean males and females (20-45 y). | Crossover design. 5 d energy surplus (40 %) with increased energy from FAT or CHO. HF diet: 50 % FAT, 30 % CHO, 20 % PRO. | No change in whole-body insulin sensitivity (glucose disposal rate) following HF feeding. Significant increase in insulin-stimulated serine phosphorylation of IRS-1 in skeletal muscle at 5 d. |
| **Alligier** *et al.* (102) | 44 healthy males (18-55 y). | 56 d energy surplus (760 kcal): 70 g of lipids (mostly saturated and monounsaturated) added to habitual diet. | Significant increase in body mass, body fat percentage and HOMA index at 14 and 56 d. Upregulation of genes involved in lipid metabolism at 14 d, with enhanced expression of genes involved in remodelling pathways at 56 d. |
| **Bachmann** *et al.* (103) | 12 young, healthy males (29.9 ± 1.5 y). | 3 d HF diet (2,667-2,943 kcal·d$^{-1}$; 30-35 % CHO, 55-60 % FAT, 11-16 % PRO) Mostly SFA. | Significant decrease in GIR of clamp (indicative of decreased insulin sensitivity) following HF feeding. Significant increase in IMCL levels of glycolytic muscle. |
| **Bakker** *et al.* (104) | 12 Dutch South Asians and 12 Dutch Caucasians (19-25 y); all healthy non-exercisers with a family history of T2D. | 5 d HF energy excess – habitual dietary consumption with the addition of 375 mL cream per day (1,275 kcal·d$^{-1}$; 94% fat). | Increased basal plasma glucose and insulin levels and decreased insulin-stimulated glucose disposal (likely due to decreased non-oxidative glucose disposal) post-HF feeding in SA only. |
| **Brons** *et al.* (19) | 26 healthy, young males (29-30 y). | 5 d energy surplus (50 %) with 60 % FAT, 32.5 % CHO, 7.5 % PRO). Equal contribution of SFA, MUFA and PUFA. | Whole-body (peripheral) insulin sensitivity was unaltered by high-fat overfeeding; however, hepatic glucose production and fasting glucose levels were significantly increased following the diet. On the muscle level, mitochondrial function was not significantly altered. |

| | | | |
|---|---|---|---|
| **Cornier** *et al.*(98) | 13 lean and 9 obese subjects post-weight loss (10 % of total body mass; termed reduced obese) healthy males and females (25-45 y). | 3 d energy surplus (50 %) with 50 % CHO, 30 % FAT and 20 % PRO. Balanced PUFA and SFA contributions. | Reduced GIR (indicative of decreased insulin sensitivity) in lean females, with no change in lean males or reduced obese individuals post-feeding. Lean females also had impaired insulin-mediated suppression of hepatic glucose production. Glucose disposal was unaltered irrespective of group. |
| **Cornford** *et al.* (105) | 9 healthy, non-obese males and females (24 ± 1 y). | 14 d energy surplus (~ 4000 kcal·d$^{-1}$); 50 % CHO, 35 % FAT, 15 % PRO. | Significant reduction in whole-body insulin sensitivity (mixed meal tolerance test) at 24 d. Non-significant increase in intramyocellular lipids and no change in skeletal muscle markers of insulin resistance or inflammation. |
| **Horton** *et al.*(106) | 9 lean (28.6 ± 5.4 y) and 7 obese (37.6 ± 5.3 y) males. | Crossover design. 14 d energy surplus (50 %) from CHO or FAT. | Greatest body mass gains in the first 7 d of overfeeding. The majority of excess energy was stored as fat with no increases in fat oxidation on the HF diet. |
| **Hagobian & Braun** (107) | 6 males (30 ± 8 y) and 3 females (23 ± 2 y). All healthy and regular aerobic exercisers. | 3 d energy surplus (25%): 57 % CHO, 28 % FAT, 15 % PRO. | Significant decrease in insulin sensitivity, inferred by Matsuda, non-significant increase in HOMA-IR. |
| **Heilbronn** *et al.*(108) | 40 healthy, sedentary males and females (21-65 y). | 28 d energy surplus (1250 kcal·d$^{-1}$) with 45 % FAT, 40% CHO, 15 % PRO. | Significantly increased fasting glucose, fasting insulin and insulin resistance (HOMA-IR) following overfeeding. Altered serum lipid profiles and increased circulating ceramide levels with increased fat storage in liver and adipose tissue. |
| **Hulston** *et al.* (109) | 17 healthy, active individuals randomised to one of two groups; HF-control (n=9 (7M, 2F); 24 ± 2 y) or HF-probiotic (n=8 (7M, 1F); 25 ± 2 y). | 7 d energy surplus (50 %) with 65 % FAT, ~23 % CHO, ~12 % PRO. Probiotic supplements consumed twice daily by probiotic group during the 7 d feeding intervention and also the preceding 3 wk. | Matsuda insulin sensitivity was significantly reduced following control HF overfeeding. However, probiotic supplementation prevented the decrements in insulin sensitivity observed with the control group. |

| | | | |
|---|---|---|---|
| **Jordy** *et al.* (110) | 11 young, healthy, moderately trained males (23.8 ± 0.9 y). | 3 d HF (77 % total energy (mostly MUFA)) energy excess (75%) with 13 % PRO, 10 % CHO. | Significant increase in HOMA-IR (decreased insulin sensitivity) following HF feeding, with increased IMTG and decreased skeletal muscle glycogen over the same time-period. No change in protein content or location of FAT/CD36 or FABPpm (FA transporters). |
| **Meugnier** *et al.* (111) | 8 lean young men (23 ± 1 y). | 28 d energy surplus (760 kcal): 70 g of lipids (mostly SFA and MUFA) added to habitual diet. | Significantly increased body mass and waist circumference after 14 and 28 d overfeeding. Altered expression of 55 genes in skeletal muscle, relating to TG synthesis and lipolysis inhibition. SREBP1 considered a key target in short-term overfeeding. |
| **Samocha-Bonet** *et al.* (112) | 40 healthy, sedentary males and females (37 ± 2 y). | 28 d energy surplus (1250 kcal·d$^{-1}$) with 45 % FAT, 40% CHO, 15 % PRO. | Significantly increased fasting glucose, fasting insulin, insulin resistance (HOMA-IR), and oxidative stress following overfeeding. Some increases in mitochondrial electron transport proteins and PGC-1α at Day 3, but no changes in lipid oxidation or mitochondrial enzyme activity at Day 28. |
| **Shea** *et al.*(113) | 8 lean and 8 obese males (healthy, weight stable, 19-29 y). | 7 d energy surplus (40 %): 50 % CHO, 35 % FAT, 15 % PRO. | HOMA-IR was unaltered by overfeeding. Differential expression of 6 genes involved in lipid metabolism, energy production and immune function between obese and lean groups. |
| **Tam** *et al.*(114) | 36 males and females (21-59 y), some postmenopausal and / or family history of T2D. | 28 d energy surplus (1250 kcal·d$^{-1}$) with 45 % FAT, 40% CHO, 15 % PRO). | Significant declines in insulin sensitivity post-HF feeding, concomitant with total body mass and fat mass gains. However, subcutaneous adipose tissue inflammation was unaltered at the same time-point. |
| **Walhin** *et al.*(115) | 26 healthy active males (25 ± 7 y), 14 of whom were on energy surplus only diet. | 50 % energy surplus for 7 d, with simultaneous step reduction to < 4000 steps·d$^{-1}$. | Decreased insulin sensitivity after 7 d energy surplus (via diet and activity manipulation), with altered expression of several genes involved in lipid and glucose homeostasis. |

| | | | |
|---|---|---|---|
| **Wulan** *et al.* (116) | 20 healthy males (10 Whites, 10 South Asian). | 4 d hypercaloric, HF diet (50 % energy excess, 25 % CHO, 60 % FAT, 15 % PRO). | Significant decrease in insulin sensitivity, inferred by the oral glucose insulin sensitivity index, irrespective of group but no change in HOMA-IR. |

In studies of crossover design where participants have engaged in more than one overfeeding protocol, only the fat overfeeding protocol has been included for evaluation.

*1.4. Impact of n-3 PUFAs on insulin sensitivity*

Given the current global T2D prevalence rates, strategies that prevent or slow associated declines in insulin sensitivity are clearly attractive for the mass population. One such dietary strategy is consumption of n-3 PUFAs. Data are starting to accumulate regarding the efficacy of n-3 PUFA intake for reducing metabolic disease risk.

n-3 PUFAs are a class of polyunsaturated fatty acids that have a double bond located on the third carbon from the methyl end of the fatty acyl chain (23). The n-3 PUFA, α-linolenic acid (ALA; C18:3n-3) is an essential FA (23) and is used as substrate for the synthesis of other n-3 PUFAs, including the highly metabolically active eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) (23). Despite these synthetic pathways, dietary ingestion remains the primary source of EPA and DHA. Oily, pelagic fish, including salmon, mackerel and trout are key dietary sources of EPA and DHA (117). Thus, fish oil (FO) is often used to increase consumption of n-3 PUFAs.

EPA and DHA synthesis from ALA occurs primarily in the liver and involves a series of desaturation and elongation reactions (23). The rate of EPA and DHA synthesis from ALA is limited by the availability of Δ6-desaturase; an enzyme that is competitively used in the synthesis of longer chain n-6 PUFAs (*e.g.* arachidonic acid (AA; C20:4n-6)) from linoleic acid (23). Due to this enzyme competition, the n-3 class of PUFAs are often considered in combination with n-6 PUFAs. A high ratio of consumed n-6 PUFAs to n-3 PUFAs (n-6/n-3) is commonly observed in Western societies and is associated with disease risk (118).

Epidemiological data provide rationale for the potential protective effects of high n-3 PUFA consumption on CV disease. Historically, high intake of n-3 PUFAs, for example, from whale and seal blubber routinely consumed by Inuit populations, was associated with lower CV disease prevalence within this population (24,119–121). However, it must be acknowledged that traditional Inuit populations also had highly physically active lifestyles (122); the benefits of which cannot easily be discerned from their dietary habits. Nonetheless, data are beginning to accumulate from other populations in support of the inverse relationship between n-3 PUFA intake and CV mortality (24,123–125). Moreover, pathologies including cancer, neurodegeneration, and autoimmune diseases have been associated with a high n-6/n-3 ratio (23,118,126), suggesting increased n-3 PUFA intake may be protective against the development of these disorders. Whereas associative epidemiological data are largely in support of the benefits of n-3 PUFA for a host of pathologies (23), randomised controlled trials are less clear (reviewed in (127)) and the mechanisms behind any n-3 PUFA-mediated effects still require a degree of clarity.

Dietary or supplemental n-3 PUFA intake is reflected by increased n-3 PUFA composition of the erythrocyte (128,129), adipocyte (130), and myocyte (128,131); although the temporal incorporation of n-3 PUFA differs between tissues (130). The intracellular lipid environment, which is largely reflective of dietary lipid intake, is closely matched to the composition of cell membranes (132). Thus, increasing the amount of n-3 PUFAs in the diet increases their proportion within the cell membrane (130). This alteration is often at the expense of n-6 PUFAs, particularly the n-6 PUFA, AA. The incorporation of n-3 PUFAs into cell membrane phospholipids is purported

to alter membrane function and signalling (133–135). The kinked nature of the FA side-chain due to multiple unsaturated bonds renders the phospholipid membrane more permeable due to gaps between adjoining lipids (136). In contrast, a highly saturated cell membrane is tightly packed with limited space in between lipids for cell endocytosis and exocytosis (136). Thus, n-3 PUFA intake may result in preferable membrane composition for cell signalling.

Increased consumption of n-3 PUFAs results in several other pleiotropic adaptations, additional to increased membrane permeability. These adaptations include a decreased production of prostaglandins, reduced inflammation, and reduced platelet formation (25). Obesity and T2D are characterised by low-grade inflammation (137), which is associated with accumulation of intramyocellular lipids (138). Acute inflammation is a well-adapted, protective response. However, the chronic inflammation that is characteristic of many pathologies including T2D is dysfunctional (139,140). Several studies, therefore, have investigated the anti-inflammatory effects of n-3 PUFAs for effectively counteracting the inflammation-associated pathology of insulin resistance.

The anti-inflammatory effects of n-3 PUFAs are manifested in eicosanoid metabolism. Eicosanoids are generated from both n-3 and n-6 PUFAs and their metabolism impacts inflammatory / immune responses (137). Substrate competition exists between the n-3 PUFAs, EPA and DHA, and the n-6 PUFA, AA (137). Generally, eicosanoids produced from EPA and DHA have anti-inflammatory actions, compared to the pro-inflammatory tendencies of eicosanoids generated from AA (137). Since dietary intake of n-3 PUFAs results largely in a displacement of AA within the plasma

membrane, n-3 PUFA intake may be a viable strategy for increasing the production of anti-inflammatory lipid mediators (137). With an increased production of anti-inflammatory lipid mediators including resolvins and protectins, the low-grade inflammation associated with the insulin-resistant state may be effectively overcome and insulin sensitivity improved.

N-3 PUFA consumption has been associated with reductions in circulating TGs. For many years, obesity and insulin resistance have been positively associated with circulating TG and FA levels (74,92,93,100), although this finding is now somewhat contentious (141). Moreover, following acute lipid emulsion and heparin infusions, circulating FA concentrations are increased with concomitant declines in insulin sensitivity (101). Thus, strategies to reduce circulating fat levels may be effective targets for reducing T2D risk / prevalence. Concentrations of circulating TGs are reduced with consumption of 'practical' n-3 FA doses in patients with hypertriglyceridemia (142,143) and healthy controls (143). Moreover, associative epidemiological (21) and experimental (reviewed in (144)) data lend support to the insulin-sensitising effects of n-3 PUFA intake. Although in T2D, supplementing with varying FO doses does not improve glycaemic control (145). The mechanistic action of n-3 PUFA-mediated TG lowering effects is likely related to increased basal fat oxidation (146) and altered lipoprotein metabolism (144); perhaps regulated by the PPAR family of transcription factors (23). Thus, several mechanisms may contribute to reductions in TGs with FO intake.

Studies investigating the influence of n-3 PUFA intake on insulin sensitivity markers within the context of short-term lipid and / or energy supply are limited. However,

some preliminary indications exist regarding the potential benefits of n-3 PUFA intake during energy surplus. In a crossover study by Turvey *et al.* (147), oxidative CHO enzyme activities were increased with HF overfeeding, but attenuated with n-3 PUFA supplementation in the absence of changes in glucose / insulin profiles. In another study of crossover design, Faeh and colleagues (148) demonstrated no insulin-sensitising effect of FO when combined with fructose and energy oversupply. However, fractional hepatic *de novo* lipogenesis was reduced by ~20 % with FO (although not significant). Taken together, some FO-mediated protective mechanisms may exist, that although not translated into preservation of insulin sensitivity, may be beneficial for long-term metabolic disease risk. Although by no means conclusive at this stage, such dietary manipulation warrants consideration when we consider typical Western lipid-rich dietary habits.

*1.5. Epigenetics - Impact of epigenetics on insulin signalling*

Development of T2D is underpinned by both genetic predisposition and environmental influence. The influence of our environment, including global changes in food availability and consequent energy overconsumption and increasing sedentary behaviours, has been discussed in detail throughout this chapter. However, the role of genetics, and in particular epigenetics, requires further attention.

Genetic familial studies conducted in the 1980s by Claude Bouchard and colleagues revealed that genetic heritability accounted for ~25 % of the variance in body fat percentage and absolute fat mass between individuals (149,150). Moreover, the correlation coefficients following appropriate corrections between monozygotic

twins for fat mass gains following 22 d energy excess, and body mass gains following 84 d energy excess, indicated a moderate contribution of genetics to this response (r = 0.65 and 0.51, respectively) (151,152). Whereas these values undoubtedly highlight the significant contribution of genetic predisposition to metabolic-stress mediated body and fat mass gains, a significant proportion of unaccounted variance exists. Environmental influences such as long-term dietary patterns and habitual physical activity levels are obvious confounding factors. However, more recently, epigenetics also has been touted as a possible explanation for the variation in individual responses to various stimuli.

The term epigenetics was coined in the late 1930s when Professor Conrad Waddington proposed the term based on developmental theory and the Greek word, *epigenesis* in relation to embryonic differentiation (153,154). For the next 50 years, biologists with interests in genetics and development arguably studied epigenetic mechanisms by means of investigating various processes that were difficult to interpret with regard to genetics alone but had clear heritable traits (154). However, the lack of clarity regarding the essence of epigenetics, and the associated possible interpretations, meant that for many years several potential mechanisms went undocumented or were not considered within the study of epigenetics (154). Arguably, it was not until publication of a paper in 1987 by Professor Robin Holliday (155) that the study of epigenetics emerged as we understand it today (156).

Many definitions of epigenetics exist that are largely indicative of the study of epigenetics spanning several scientific disciplines. However, probably the most widely accepted definition refers to the study of epigenetics as heritable changes in

phenotype or gene expression that occur in the absence of changes in the DNA sequence (*e.g.* (29)). Epigenetic regulatory mechanisms include DNA methylation, histone modification and miRNA regulation (157). However, for the purposes of this thesis, the focus will be on miRNA regulation.

### *1.5.1. miRNAs*

Completion of the human genome project lead to the realisation that the vast majority of our genome sequence contains non-coding material (158). Evolutionary conservation of these non-coding sections suggests a level of functional relevance. MiRNAs are short-stranded, non-coding RNA that have received a large amount of research attention in recent years following their discovery in 1993 (159) and in light of the outcomes from the human genome project. Although the exact cellular functions of the majority of miRNAs are largely unknown, it is estimated that miRNAs may target up to a third of all genes, and that each miRNA may act on up to 200 messenger RNA (mRNA) (160). Thus, it seems clear that non-coding material has functional relevance and may be involved in the adaptive regulation of gene expression.

MiRNA biogenesis and processing has been described in detail elsewhere (28,30,31) and is depicted in **Figure 1.3**. The process of miRNA biogenesis begins within the nucleus of the cell as long nucleotide sequence primary-miRNAs (pri-miRNAs) that have been transcribed from miRNA genes by RNA polymerase II or III (30). These pri-miRNA transcripts are cleaved by the Microprocessor complex, involving Drosha and its binding partner DGCR8, into ~70 nucleotide hairpin-structure miRNA precursors

(pre-miRNA) (28). Exportin 5 transports the pre-miRNAs from the nucleus into the cytoplasm where Dicer and its binding partner, transactivation-responsive RNA-binding protein (TRBP), cleaves the pre-miRNAs into short (~22 nucleotide) imperfect double-stranded RNA duplexes (161). Each duplex consists of the mature miRNA and a complementary strand. The mature miRNA strand (and in rare cases the complementary strand) is incorporated into the RNA-induced silencing complex (RISC) for action on target mRNAs (161).

MiRNAs incorporated within the RISC alter gene expression via two mechanisms; *i*) inhibition of translation initiation of nascent mRNA transcripts and / or *ii*) degradation of mRNA (31). The resultant effect of one or both of these processes is reduced expression of genes encoded by the targeted mRNA transcripts. However, in a handful of cases miRNAs activate mRNAs rather than repressing their translation (162,163). Thus, depending on the gene target, an increase in miRNA levels can increase or decrease the relative protein expression and thus increase or decrease flux through the pathway. These regulatory functions by miRNAs are integral for 'normal' whole-body metabolic regulation (32). Altered levels of miRNAs have been shown in a variety of disease states (33,164,165), lending support to the notion that miRNAs may be useful biomarkers of disease and are likely important in health maintenance and adaptations to diet / nutrition.

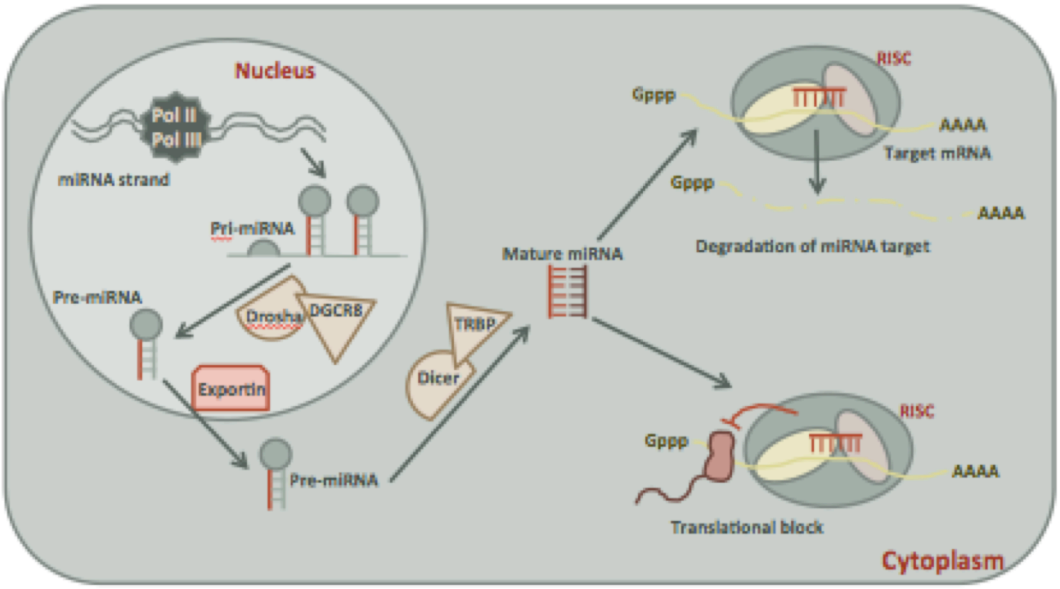



**Figure 1.3** Process of miRNA biogenesis within the cell, adapted from (288). miRNA biogenesis begins within the nucleus of the cell. Processing of the pri-miRNA and pre-miRNA strands in the nucleus is followed by exportation of the pre-miRNA strand into the cytoplasm via Exportin. Once within the cytoplasm, the mature miRNA strand is synthesised. Upon binding of the mature miRNA strand to a mRNA strand, the mRNA is degraded or its translation halted. This action results in reduced protein expression of the gene encoded by the mRNA strand.

MiRNAs exert their function on mRNA transcripts within tissue. However, in 2008, miRNAs were discovered at stable levels within the circulation (166,167). Their presence within plasma is considered possible due to incorporation into secreted exosomes, co-localisation with RISC or via binding to lipoproteins; preventing degradation by RNases (32,168–170). Rather than non-specific release from tissue, miRNAs are actively secreted into the circulation (171,172). Not all tissue miRNAs exist in plasma and miRNAs present within secreted exosomes exist in different proportions to those in their cell type of origin (171). Moreover, plasma miRNAs are now considered as communicators, rather than merely passive markers, of cellular processes and thus may have regulatory roles in normal physiology and disease

mechanisms (32). The utility of plasma miRNAs as biomarkers of disease is enhanced by their stability in the circulation and ease of sampling and analytical processing.

Considering the multi-tissue integrated nature of metabolic disease pathology, the notion of a blood-based effector regulating this co-ordinated response is an intriguing one. Much like studies into tissue miRNA regulation, studies investigating plasma miRNAs have revealed altered levels in disease states (*e.g.* (33,164,166)). To date, no studies have directly compared tissue miRNA levels to plasma miRNA levels. However, whether plasma miRNAs can be used as *meaningful* biomarkers of disease may be largely influenced by their association with tissue miRNA patterns of change / perturbations. Thus, comparisons of different tissue types are warranted.

Given the mechanistic action of epigenetic signatures on gene expression, the study of epigenetics has application across all biological disciplines and is now being studied within various contexts as a means to better understand whole-body metabolic regulation. One emerging area of research within epigenetics is that concerning the interaction between nutrition and the epigenome (173). This interaction appears to be reciprocal in nature. Nutrition can alter the epigenome, either transiently or over the lifespan, but in turn, the epigenome regulates gene expression of proteins involved in nutrient metabolism (173,174). Dietary fats have been implicated within the study of nutrigenomics (173), although existing scientific evidence is limited.

Fats, and particularly lipoproteins including LDL and HDL, have been widely attributed to the transport of extracellular miRNAs (169,170,175). Moreover, nutrigenomics has advanced to consideration for how diet-derived exogenous

miRNAs, termed xenomiRs, can contribute to the plasma miRNA pool and alter circulating miRNA homeostasis (175,176). An increase in dietary lipid intake may enhance the number of vehicles for miRNA transport, leading to altered miRNA kinetics entering and leaving the cell. Moreover, miRNAs present within animal and plant matter can enter the bloodstream following digestion, and assimilate with the existing circulating miRNA pool (175). Both of these processes will alter plasma miRNA levels and are important considerations for biomarker studies. The notion of dietary transfer of genetic material has the potential to completely alter the way we view the field at present; however, replication studies (177,178) have thus far failed to support the initial theories (175) and findings (176).

Whereas the concept of dietary miRNA transfer is still in its infancy and requires further research attention, the association between miRNA dysregulation and metabolic disease is well established. In cultured cell lines and rodent models of obesity and T2D, miRNAs are differentially expressed compared to controls (reviewed in (179–181)). Data from human models of obesity and T2D are consistent with this observation. The first large-scale gene-chip study comparing miRNA expression in human skeletal muscle of T2D and controls demonstrated differential expression of a third of the detected miRNAs (n = 62) between groups (182). These miRNA data were associated with a targeted regulation of genes involved in relevant signalling pathways (182). Two more recent reports detailing circulating miRNA levels in T2D compared to controls also demonstrate altered regulation with metabolic disease. Ortega and colleagues (164) profiled a panel of plasma miRNAs in males with T2D or normal-glucose tolerance in the basal and insulin-stimulated states and reported significant differences in miRNA levels between groups that were

modulated by insulin stimulation and some of which independently explained up to 50 % of the variance in fasting plasma glucose concentrations. Analogous findings are described by Wang *et al.* (183) with different basal circulating levels of miR-24 and miR-29b measured between males with T2D and controls. Despite these clear indications that miRNAs are associated with obesity and T2D in humans, the miRNA response to a short-term period of HFEE in humans is unknown.

There are some preliminary indications that n-3 PUFAs, like obesity / T2D, may impact miRNA profiles. The n-3 PUFA, DHA, has been shown to alter the expression of miR-192 and miR-30c in enterocytes (184), and the expression of miR-33a and miR-122 in dyslipidemic obese rodents (185). Moreover, n-3 PUFAs appear to target a number of miRNAs; altering their regulation of cancerous colon (165) and breast (186) tumours. Inflammation-related dysregulation of miRNAs (187,188) also may be an area in which n-3 PUFAs have mechanistic function, particularly within the context of obesity-related, low-grade inflammation. Taken together, these data suggest potential for n-3 PUFA dietary consumption and short-term responses to HFEE to be regulated, in part, by altered miRNA profiles.

### *1.6. Limitations of existing research*

It is clear that insulin action is impaired in states of chronic lipid oversupply and insulin resistance. However, the conclusions drawn from existing research outlined in this chapter are equivocal regarding the impact of short-term HFEE on insulin sensitivity. Moreover, the clinical relevance of n-3 PUFA ingestion for the human insulin-resistant state requires clarity. Therefore, to address these uncertainties, investigations into the impact of diet-induced maladaptation and potential

amelioration by dietary n-3 PUFAs are warranted. Specifically, investigation of the impact of increasing dietary intake of FO within the context of elevated lipid supply on markers of insulin sensitivity is of interest, and has application in an age where energy and lipid excess is increasingly common. Modest modifications to current dietary patterns have the potential to improve metabolic health and slow the alarming rate of T2D development.

A switch in research focus to that of individual variation and tailored nutrition, in combination with a more common blanket approach, may better facilitate effective dietary intake modifications within the mass population. Investigation of epigenetic signatures of metabolic disease has the potential to highlight key biomarkers of metabolic disease risk that may be used to predict an individual's risk of developing T2D. This information can be used to implement strategies with a view to counteracting such maladaptation and protecting 'at risk' individuals. It is currently unknown whether changes in miRNA levels of skeletal muscle and plasma are observed following a period of HFEE in humans.

### *1.6.1 Aims and objectives*

Overall, the aim of this thesis is to address current knowledge gaps in the literature regarding the metabolic, molecular and epigenetic responses to short-term HFEE, as a proxy for the early maladaptation leading to T2D. Specifically, we aim to investigate the impact of short-term lipid and energy excess on whole-body glucose metabolism, skeletal muscle lipid metabolism and epigenetic regulation within skeletal muscle and plasma. Moreover, the influence of n-3 PUFA ingestion on these parameters will

be investigated. These aims will be achieved by the successful completion of the following objectives:

i) To determine the whole-body responses to a well-controlled short-term (6 d) period of HFEE, with and without FO, in humans.

ii) To determine the subcellular molecular mechanisms of short-term (6 d) diet-induced maladaptation in humans, and their mediation by FO.

iii) To determine the epigenetic responses to short-term (6 d) HFEE, with and without FO, in humans.

**CHAPTER 2** Whole-body insulin sensitivity is not clearly altered following consumption of a six day, high-fat, energy excess diet, with or without dietary fish oils, in healthy males.

**Wardle SL**, Macnaughton LS, McGlory C, Witard OCW, Ferrando AA, Galloway SR, Moran CN, Tipton KD.

*2.0. Abstract*

We aimed to investigate the whole-body responses to consumption of a 6 d high-fat, hyperenergetic diet using dual-glucose stable isotopic tracer methodology. Twenty young, healthy males were matched to 1 of 2 groups; high-fat control (HF-C) or high-fat fish oil (HF-FO; n = 10 per group). All participants consumed a high-fat, energy excess diet for 6 d (HFEE; 150 % of total energy, 60% FAT, 25% CHO, 15% PRO) with 10 % of fats consumed as fish oil (FO) in HF-FO. Participants attended the laboratory following an overnight fast, immediately before and after 6 d HFEE, for two identical infusion trials. During the infusion trials, a primed continuous [6,6-$^{2}H_2$] glucose infusion and an oral glucose tolerance test (OGTT; 73 g glucose + 2 g [U-$^{13}$C] glucose) were used to assess plasma glucose kinetics. Blood samples were drawn at 10 min intervals during the OGTT. Insulin sensitivity, measured using a number of indices, was unaltered by HFEE in either group (HF-C: -2 ± 13 %; HF-FO: 8 ± 8 % (Matsuda)) despite fat mass gains in both groups (3 ± 1 % (pooled groups)). A significant increase in circulating n-3 PUFAs was observed post-HFEE in HF-FO only (107 ± 10 %; $p < 0.01$). However, HFEE did not significantly alter plasma glucose kinetics (Ra: 209.3 ± 16.6 to 204.4 ± 17.4; Rd: 264.2 ± 20.2 to 273.3 ± 20.2 mg·min$^{-1}$·kg$^{-1}$:120 min (pooled groups)) or insulin concentrations (42930 ± 4130 to 43931 ± 3907 pmol·L$^{-1}$ (pooled groups)) over the OGTT, irrespective of group. Short-term HFEE does not clearly alter insulin sensitivity group means in a cohort of young, healthy males. However, a large degree of individual variation was evident in all of our measures. Evaluation with inferential statistics indicated a possible (62 %) chance of FO consumption during short-term HFEE being beneficial for insulin sensitivity (Matsuda) change. We suggest that careful attention should be afforded to individual characteristics in future investigations of short-term HFEE.

*2.1. Introduction*

Dietary energy and fat overconsumption leading to body mass gain is associated with the development of obesity and insulin resistance (189,190). High-fat, high-energy diets have become increasingly common, particularly in Western, well-developed countries, and are thought to be a leading contributor to T2D (191). Investigation of the early, adaptive responses to fat and energy excess is important to improve our understanding of the ensuing maladaptations that will lead to obesity, insulin resistance and T2D. Enhanced comprehension of T2D progression also will allow for more targeted lifestyle interventions designed to tackle these early maladaptations, with the aim of preventing, or slowing, insulin resistance development.

Excess energy, high-fat diets of varying duration have been used previously as a model to investigate the mechanisms leading to diet-induced insulin resistance (*e.g.* (19,98,99,103)). Insulin sensitivity has been shown to decrease following short-term episodes of high-fat overfeeding (98,103,109). However, discrepant findings exist with some studies showing no influence of overfeeding periods on insulin sensitivity (19,98,99). Thus, the impact of short-term overfeeding on whole-body insulin sensitivity remains unclear.

The use of different experimental protocols and the saturated / unsaturated nature of consumed fat may explain discrepant findings between studies. Previous overfeeding protocols have varied in the degree of nutrient excess, macronutrient contributions and duration of dietary intervention. Most studies have implemented an energy excess of 40-50 % (19,98,99,108,109). However, the contribution from each macronutrient varied considerably, with percentage energy from fat (30-65 %),

carbohydrate (23-50 %) and protein (7.5-15 %) different between these studies. The type of fat consumed was mainly saturated (SFA) (103) or equal proportions of the three main classes of fat (SFA, monounsaturated (MUFA), polyunsaturated (PUFA)) (19,99). Consumption of additional energy as PUFA compared to equivalent consumption of SFA led to reduced gains in fat mass (192). These data suggest a potential beneficial effect of replacing additional SFA intake with PUFA intake on fat mass gains. However, the independent influence of reducing SFA *vs.* increasing PUFA on fat mass gains cannot be teased from these findings. Nonetheless, whether the type of fat consumed within overfeeding models has an impact on fat / body mass gains and associated changes in insulin sensitivity warrants consideration.

Altering the type of dietary fat consumed, *e.g.,* replacement of SFA with PUFA, may be an efficacious lifestyle strategy with potential to prevent or slow the decline in insulin sensitivity that is associated with chronic dietary energy excess. SFA often contribute most significantly to total fats when high-fat, high-energy diets are consumed habitually, and typically, high SFA consumption is related to the development of obesity and T2D (191). However, this notion is under increasing scrutiny. Consumption of n-3 PUFAs, commonly found in the oil of cold-water, pelagic fish (FO), has been suggested to have clinical relevance in the reduction of insulin resistance (193). Although, it must be acknowledged that findings from human studies also are equivocal in this regard (*e.g.* (194)). N-3 PUFAs are thought to improve insulin sensitivity through a number of mechanisms, including, increased fat oxidation (195), anti-inflammatory properties (196), and altered membrane composition leading to improved signal transduction (144). Therefore, increasing dietary intake of n-3 PUFAs may be a viable strategy for ameliorating insulin resistance.

Our aims for this study were twofold. First, we aimed to investigate the impact of increasing energy intake by an additional 50 %, with 60 % of energy from fat for 6 d, on whole-body insulin sensitivity, and associated metabolic mechanisms, in a group of healthy young male volunteers. Second, we aimed to investigate the impact of replacing 10 % of dietary fats with dietary FO during high-fat, energy excess (HFEE) on whole-body insulin sensitivity and associated metabolic mechanisms. The duration (6 d) and degree (150 %) of overfeeding and percentage contribution to total energy of fat (60 %) and FO (6 %) were selected following critical review of the literature (presented in **Table 1.1**). Our aims were to try and induce insulin resistance with HFEE and to provide sufficient FO to have functional, measurable, mechanistic effects, *e.g.*, incorporation into the plasma membrane. Thus, we hypothesised that 6 d HFEE would reduce whole-body insulin sensitivity and that this effect would be ameliorated with the substitution of 10 % of total fats with dietary FO.

*2.2. Methods*

*2.2.1. Participants and ethical approval*

Twenty healthy males who did not meet current UK government recommendations for physical activity were recruited from the University of Stirling and surrounding area to participate in this investigation (**Table 2.1**). Following health screening, participants were excluded if they consumed a diet high in FO sources or took FO supplements, had a fasting blood glucose of > 6.1 mmol·$L^{-1}$, or met or exceeded the recommended physical activity guidelines for health (30 min·$d^{-1}$, 5 x·$wk^{-1}$).

Ethical approval was obtained from the NHS East of Scotland Research Ethics Service (REC 2) and the study was conducted in accordance with the Declaration of Helsinki (197). Having had the study purpose and procedures explained in lay terms, all participants provided written, informed consent.

**Table 2.1** Participant characteristics.

| Parameter | HF-C | HF-FO |
|---|---|---|
| **Age (y)** | 21.8 ± 3.4 | 22.9 ± 3.7 |
| **Body mass (kg)** | 72.4 ± 7.7 | 70.1 ± 7.9 |
| **BMI (kg·$m^{-2}$)** | 21.8 ± 2.0 | 21.3 ± 2.0 |
| **Physical activity (x·$wk^{-1}$)** | 1.1 ± 0.4 | 1.2 ± 0.4 |
| **Body fat (%)** | 20.1 ± 1.5 | 21.3 ± 2.1 |

Data are presented by group (HF-C and HF-FO; n=10 per group). Values are means ± SD, no significant differences between groups (t-test).

*2.2.2. Study design*

Using a parallel study design, participants were matched to either a high-fat control (HF-C; n = 10) or a high-fat fish oil (HF-FO; n = 10) group based on age, body mass,

habitual physical activity and habitual energy intake. Prior to baseline testing, a 3 d baseline diet, designed to reflect individual habitual energy and proportional macronutrient intake, was consumed by all participants. For baseline testing (infusion trial 1, pre-HFEE), participants arrived at the laboratory following an overnight fast. A dual energy x-ray absorptiometry (DXA) scan was conducted followed by collection of a basal blood sample and skeletal muscle biopsy. Thereafter, participants completed an oral glucose tolerance test (OGTT) using dual-glucose stable isotopic tracer methodology with regular blood sampling to assess plasma glucose kinetics and insulin sensitivity. Following the OGTT, a final skeletal muscle biopsy was obtained.

Following baseline testing, participants consumed an experimental diet for 6 d, designed to represent an extreme Western diet. The diet comprised 150 % of habitual energy intake with 60 % of energy from fat, 25 % from carbohydrate and 15% from protein. The only exception was that for HF-FO, 10% of fats were provided as dietary FO. A second testing day (infusion trial 2, post-HFEE) was conducted on the morning following day 6 of HFEE. All procedures were identical to those conducted pre-HFEE. **Figure 2.1A** provides a schematic of the study design.

*2.2.3. Preliminary testing*

**Blood sampling**

A single, basal blood sample was collected in an EDTA vacutainer (Becton, Dickinson & Company, NJ, USA) from each participant prior to testing to ensure that fasting blood glucose concentrations were within the normal, healthy range (< 6.1 mmol·$L^{-1}$). Blood samples were centrifuged at 3500 rpm for 15 min at 4 °C to separate the

plasma fraction. An automated Aries ILab benchtop analyser (Instrumentation Laboratories, MA, US) was used to assess plasma glucose concentration on the same day.

**Body mass**

During preliminary testing and at the start of each of the two infusion trials, nude body mass was recorded to the nearest 0.1 kg using a digital scale. Body mass recorded at the start of each infusion trial was used to accurately calculate the infusion pump rate to achieve the desired infusion rate, and to assess changes in total body mass resulting from 6 d HFEE.

*2.2.4. Diet and physical activity control*

**Dietary control**

Dietary intake was controlled for a consecutive 9 d testing period. Participants were required to complete a 3 d weighed food and physical activity diary, that was representative of their weekly habits, to include 2 weekdays and 1 weekend day. Food diaries were analysed using dietary analysis software (Microdiet, Downlee Systems Ltd, UK) for average daily energy intake and macronutrient contributions. All participants completed a food preference questionnaire to ensure no foods were provided that were not tolerated by participants and to match dietary habits as closely as possible to aid compliance.

Habitual average daily energy intake data were used to devise both the baseline and experimental diets. All baseline diets matched the total average daily energy intake and macronutrient composition of individuals' habitual diets as recorded in the 3 d

food diaries. A standard template was used as a basis for devising baseline diets. However, given the individual variation in foods consumed between participants, an attempt was made to provide some of the foods indicated by each participant in their 3 d food diaries. Providing similar foods served to aid participant compliance and also to try and maintain habitual eating as much as possible. The food given on baseline feeding day 1 was repeated for baseline feeding day 3, with a different range of foods provided on baseline feeding day 2. Following consumption of the baseline diets, each participant consumed a 6 d experimental diet that comprised 150 % of their habitual energy intake, with 60 % of energy from fat, 25 % from carbohydrate and 15 % from protein. HF-FO had 10 % of total fats in the diet (6 % total energy) replaced by dietary FO whereas HF-C received the 60 % fat from a mixture of fat sources that did not include FO or foods highly enriched with n-3 PUFAs. Experimental diets were devised from an initial template that was altered depending on the required energy intake. The diets provided on experimental day 1-3 were repeated for experimental day 4-6. Dietary FO was provided by means of oily fish consumption from a fish meal *e.g.*, salmon / mackerel fillets, fish (salmon) oil (Smartfish Nutrition, Norway; **Appendix 2.1**) added to yoghurt based smoothies and omega-3 enriched (salmon oil) juices (Smartfish Nutrition, Norway; **Appendix 2.2**). Placebo drinks without any oil were given to individuals in HF-C (Smartfish Nutrition, Norway).

All food (both baseline and experimental diets) was provided in daily food bags for participants to consume away from the laboratory. Participants were instructed to eat all of the food and drink provided and not to consume anything other than that provided during the experiment. In the event that participants were unable to consume everything provided in their food bag, they were asked to return the

remainder of the items so that energy intake could be recalculated accordingly. None of the participants complained of any intolerances or difficulties in consuming the diets.

**Physical activity control**

During health screening, participants were asked to record the total average number of hours spent engaged in physical activity per week, as well as the specific activities that they engaged in on more than two occasions per week. Physical activity also was recorded over the same 3 d period as the weighed food record. Participants were encouraged to maintain their habitual activities of daily living but to refrain from any additional physical activity for the 48 h preceding both infusion trials (pre- and post-HFEE).

*2.2.5. Testing days (infusion trials)*

The two infusion trials, conducted on the morning following consumption of the food provided for day 3 of the baseline diet (day 4; infusion trial 1 (pre-HFEE)) and the morning following consumption of the food provided for day 6 of the experimental diet (day 10; infusion trial 2 (post-HFEE)), were identical in all respects. For a schematic of the infusion trials see **Figure 2.1B**. All trials were conducted in the laboratories of the Health and Exercise Sciences Research Group, University of Stirling.

Participants arrived at the laboratory between 0600 and 0800 following ~10 h overnight fast. Dietary compliance was confirmed verbally prior to beginning the trial and nude body mass was recorded. Next, body composition was analysed by DXA

(LUNAR iDXA; GE Healthcare Systems, UK). For each participant, the same researcher conducted both DXA scans (pre- and post-HFEE) and a standardised protocol of body positioning (198) was implemented for use between researchers.

All subsequent procedures were conducted with participants lying on a bed in the supine position. First, a catheter was inserted into a forearm vein for collection of a basal venous blood sample followed by a primed (13.5 μmol·kg$^{-1}$) continuous 3 h infusion (0.350 μmol·kg$^{-1}$·min$^{-1}$) of [6,6-$^{2}H_2$] glucose (Cambridge Isotope Laboratories, MA, USA) using an Alaris PK infusion pump (CareFusion, CA, USA). During the first hour of infusion, another catheter was inserted into the contralateral arm for repeated venous blood sampling. A venous blood sample was taken at –30 min (30 min into infusion / 30 min pre-glucose drink; all timings were based on consumption of the glucose drink) and a resting skeletal muscle biopsy was obtained. For the biopsy, a 5 mm Bergström needle was inserted through a pre-prepared anaesthetised (2 % Lidocaine) incision in the skin and fascia, into the muscle belly of the *vastus lateralis* and a sample removed under manual suction. Muscle biopsy samples were immediately rinsed using ice-cold saline (0.9 %), any visible fat or connective tissue removed and blotted before weighing and freezing in liquid nitrogen prior to storage at -80 °C pending analysis. One hour into the infusion (0 min), participants consumed a sugar drink (75 g glucose given in a dextrose solution (73 g glucose (80.3 g dextrose when corrected for crystallisation (Myprotein, Cheshire, UK) + 2 g [U-$^{13}$C] glucose in 300 mL water))) to start the OGTT. Fluid intake was restricted throughout the trial to only the OGTT drink. Throughout the 2 h that followed glucose ingestion, blood samples were drawn every 10 min, and at 120 min (3 h post infusion) a final skeletal muscle biopsy was obtained. Both biopsies (0 and

120 min) were removed from the same leg. For infusion trial 2 (post-HFEE), biopsies were obtained from the contralateral limb. The order of the first leg to be biopsied was counterbalanced between groups to eliminate any potential for order effects. Upon completion of the first infusion trial, participants were fed the breakfast from the first food bag of their experimental HFEE diet (Day 4) and allowed to leave the laboratory with the rest of their food for that day and the following day.

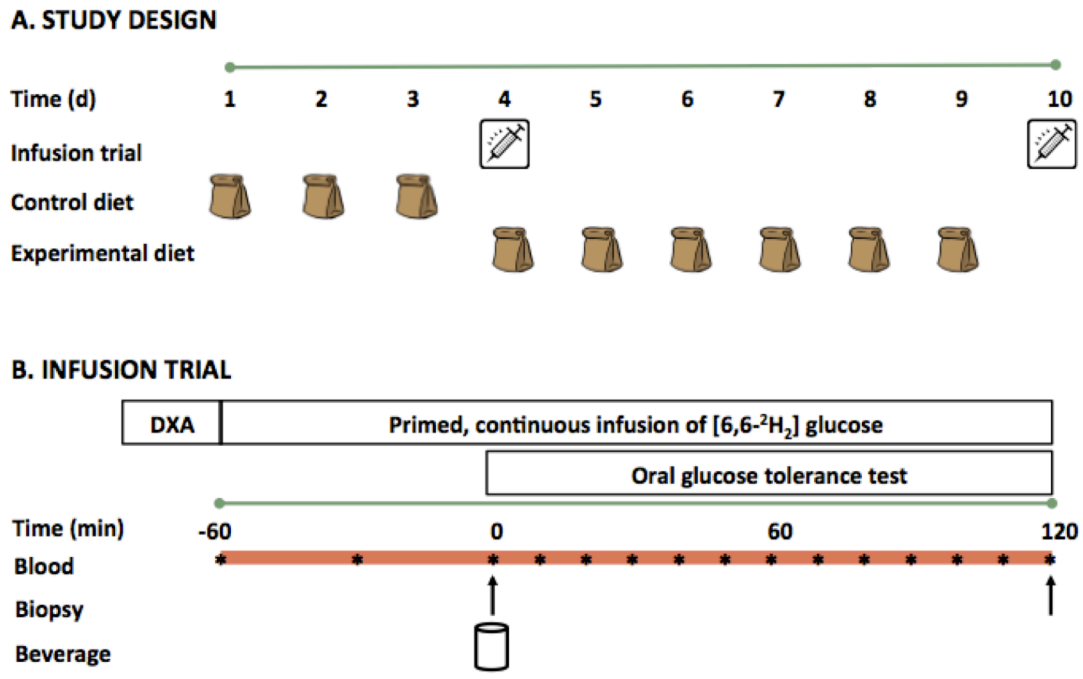



**Figure 2.1** Study design (A) and infusion trial (B) schematic. Both groups (HF-C and HF-FO) completed all aspects depicted, with the only difference between groups being the composition of fat provided in the experimental diet.

*2.2.6. Blood sampling and plasma analysis*

Blood samples were collected in EDTA vacutainers (Becton, Dickinson & Company, NJ, USA) and centrifuged at 3500 rpm for 15 min at 4 °C. Post-centrifugation, the supernatant plasma fraction was removed and aliquotted into Eppendorf tubes for storage at -80 °C pending analysis. An ILab Aries benchtop analyser with associated

reagents (Instrumentation Laboratories, MA, USA) was used to measure plasma glucose concentrations at 10 min intervals throughout the OGTT (0 – 120 min). Insulin concentrations were measured at the same time-points by ELISA according to the manufacturer's instructions (Demeditic (DE2935), Germany). Plasma enrichments of [6,6-$^{2}H_2$] glucose from the infusion and [U-$^{13}$C] glucose from the glucose drink were measured by GCMS following preparation of pentacetate derivatives using 2:1 acetic anhydride:pyridine (v/v). Plasma glucose enrichments were quantified using GCMS with chemical ionisation. Ions were selectively monitored at mass to charge ratios of 331.1 (M+0), 332.1 (M+1), 333.1 (M+2), 334.1 (M+3) and 337.1 (M+6) based on the masses of the labelled isotopes, and potential for carbon recycling. Plasma glucose enrichments for each ion were expressed relative to enrichments at 331.1 (M+0). A skew correction factor was applied to the U-$^{13}$C enrichments (337.1; M+6) to account for differences in the relative distribution of the mass spectra between the tracer (U-$^{13}$C) and tracee (unlabelled glucose). The GC System (7890A; Agilent Technologies, CA, USA) was interfaced with a 5975C MS inert XL EI/CI MSD with triple-axis detector. Methane gas was used for positive ionisation, with helium as the carrier gas. Chemstation software (Agilent Technologies, CA, USA) was used for data analysis.

*2.2.7. Blood fatty acid composition analysis*

Fatty acid methyl esters (FAME) were extracted from whole blood lipids using the Rapid Omega Test (199). Briefly, blood was spotted onto Whatman 903 cards and allowed to dry for 2 h. Automated processing isolated fatty acid methyl esters (FAME) from the dried blood spot before FAME were passed through a silica clean-up column,

eluted and re-suspended in isohexane prior to gas-liquid chromatography (GLC) analysis.

FAME were separated by GLC using a ThermoFisher Trace GC 2000 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m x 0.32 x 0.25 mm i.d.; Phenomenex, Macclesfield, UK). Hydrogen was used as carrier gas with on-column injection. Individual methyl esters were identified from previously published data (200) and the Chromcard for Windows (version 2.00) computer software (Thermoquest Italia S.p.A., Milan, Italy) was used for data collection and processing.

*2.2.8. Calculations*

**Insulin sensitivity**

Insulin sensitivity (or resistance) was estimated using plasma glucose and insulin concentrations in the basal state and / or during the OGTT within the calculations of a number of metrics outlined below. FPG = fasting plasma glucose, FPI = fasting plasma insulin.

**Matsuda insulin sensitivity index** (**ISI;** (201))

10000 / SQRT ((FPG ($mg \cdot dL^{-1}$) *FPI ($\mu U \cdot mL^{-1}$)) * (Mean OGTT Insulin * Mean OGTT Glucose ($mg \cdot dL^{-1}$)))

**HOMA-IR** (202)

(FPG ($mmol \cdot L^{-1}$) * FPI ($\mu U \cdot mL^{-1}$)) / 22.5

**HOMA-β** (202)

(20 * FPI ($\mu U \cdot mL^{-1}$)) / (FPG ($mmol \cdot L^{-1}$) - 3.5)

**Hepatic insulin resistance index** (203)

FPG ($mmol \cdot L^{-1}$) * FPI ($\mu U \cdot mL^{-1}$)

**Hepatic insulin sensitivity index** (204)

1 / (FPG ($mmol \cdot L^{-1}$) * FPI ($\mu U \cdot mL^{-1}$))

**Plasma glucose kinetics**

Total rates of glucose appearance (Ra) and disappearance (Rd) were calculated from plasma enrichments of [6,6-$^{2}H_2$] using the modified single pool non-steady state equations of Steele *et al*. (205). Exogenous (from the OGTT drink) and endogenous (inferred hepatic glucose output) Ra were calculated using the dual-glucose tracer enrichments (*i.e.* the plasma kinetics of the infused [6,6-$^{2}H_2$] and the U-$^{13}C$ tracer added to the OGTT drink (with known U-$^{13}C$ glucose enrichments of the ingested OGTT glucose drink)). A pool volume (pV) of 40 $mL \cdot kg^{-1}$ was used in the Steele equation (205) as this pV most reflects the plasma pool in the period during which steady state is altered, and minimises large fluctuations in calculated tracer enrichments during non-steady state conditions (206).

For the following equations: F = infusion rate (0.350 $\mu mol \cdot kg^{-1} \cdot min^{-1}$), pV = pool fraction of the total extracellular glucose pool (40 $mL \cdot kg^{-1}$), C = glucose concentration, E = glucose enrichment, t = time. E2 and E1 correspond to the enrichments at time-points t2 and t1, respectively.

**Total Ra (mg·min$^{-1}$·kg$^{-1}$)**

(F - ((pV * (C2 + C1) / 2) * ((E2-E1) / (t2-t1)))) / ((E2+E1) / 2)

**Exogenous Ra (mg·min$^{-1}$·kg$^{-1}$)**

% contribution of [6,6-$^{2}H_2$] glucose to total glucose concentration * Total Ra

**Endogenous Ra (mg·min$^{-1}$·kg$^{-1}$)**

Total Ra – exogenous Ra

**Total Rd (mg·min$^{-1}$·kg$^{-1}$)**

Total Ra – pV * ((C2-C1) / (t2-t1))

*2.2.9. Statistical analysis*

Power calculations for the study design were conducted using G*Power 3 (207) based on means and standard deviations of basal plasma glucose changes (4.59 ± 0.46 to 5.15 ± 0.40 mmol·L$^{-1}$) reported by Brøns *et al*. (19). An n of 8 per group offered 80 % power ($\alpha = 0.05$) to detect changes in basal plasma glucose by ANOVA.

All data were tested for normality using the Ryan Joiner test with Minitab software (version 16; Minitab, State College, PA). In the case of non-normally distributed data, Box-Cox transformations were applied before testing for normality a second time. In the instance that data remained non-normally distributed following Box-Cox transformation, outliers were removed using the median absolute deviation method (208), and normality confirmed. Thus, all statistical analyses were performed on

normally distributed data. A two-way repeated measures ANOVA (IBM SPSS Statistics 19, NY, USA) was used to assess differences between groups (HF-C *vs.* HF-FO; between-subject factor), over time (pre- and post-HFEE; within-subject factor). In the case of a significant interaction effect by two-way ANOVA, paired t-tests were conducted to assess differences over time within each group alone. Area under the plasma glucose and insulin curves (AUC) during the OGTT were calculated using GraphPad Prism version 4 (GraphPad Software Inc., CA, USA) with baseline set at individual basal values (0 min). Evaluation of inferential statistics was performed using the resource of Hopkins (209). Statistical significance was assumed at the level of $p < 0.05$. Values are presented as mean ± standard error of the mean (SEM) unless stated otherwise.

### *2.3. Results*

#### *2.3.1. Dietary analysis*

Dietary intake data are presented in **Table 2.2**. Baseline diets were not significantly different between groups. N-3 PUFA and sugar intake were significantly different between groups during HFEE.

**Table 2.2** Macronutrient composition of 3 d baseline diets and 6 d experimental diets.

| | **Baseline diets** | | **Experimental diets** | |
|---|---|---|---|---|
| | **HF-C** | **HF-FO** | **HF-C** | **HF-FO** |
| **Energy** (kcal) | 2614 ± 180 | 2443 ± 132 | 3919 ± 237 | 3658 ± 203 |
| **Fat** (g) | 104 ± 9 | 87 ± 5 | 261 ± 18 | 245 ± 14 |
| **Carbohydrate** (g) | 323 ± 22 | 325 ± 33 | 263 ± 18 | 244 ± 13 |
| **Protein** (g) | 116 ± 13 | 108 ± 7 | 146 ± 10 | 137 ± 8 |
| **SFA** (g) | 42 ± 3 | 33 ± 3 | 106 ± 7 | 100 ± 5 |
| **MUFA** (g) | 31 ± 4 | 24 ± 2 | 74 ± 4 | 69 ± 4 |
| **PUFA** (g) | 10 ± 2 | 11 ± 2 | 31 ± 2 | 38 ± 3 |
| **n-3 PUFA** (g) | | | 2 ± 0 | 25 ± 2 * |
| **Sugars** (g) | 119 ± 12 | 133 ± 19 | 121 ± 7 | 77 ± 2 * |

Data displayed by group (HF-C and HF-FO; n = 10 per group). Values are means ± SEM (values displayed as 0 were < 0.01), * significantly different from HF-C for equivalent condition, $p < 0.05$ (ANOVA).

*2.3.2. Body composition*

Changes in body composition following HFEE by DXA are presented in **Table 2.3.** All measures presented were significantly increased post-HFEE irrespective of group.

**Table 2.3** Body composition measurement by DXA.

| | **HF-C** | | **HF-FO** | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Total body mass** (kg) | 71.74 ± 3.60 | 72.25 ± 3.65 | 69.65 ± 2.32 | 70.67 ± 2.29 |
| **Total fat mass** (kg) | 13.87 ± 1.11 | 14.16 ± 1.09 | 14.40 ± 1.65 | 14.85 ± 1.61 |
| **Limb fat mass** (kg) | 6.94 ± 0.49 | 7.01 ± 0.49 | 7.40 ± 0.76 | 7.66 ± 0.77 |
| **Trunk fat mass** (kg) | 6.07 ± 0.72 | 6.27 ± 0.70 | 6.12 ± 0.90 | 6.29 ± 0.85 |
| **Android fat** (%) | 16.89 ± 2.38 | 17.88 ± 2.29 | 17.63 ± 2.83 | 18.22 ± 2.72 |
| **Total lean mass** (kg) | 55.30 ± 3.03 | 55.55 ± 3.07 | 52.89 ± 1.98 | 53.44 ± 1.85 |

Data presented pre- and post-HFEE, for both groups (HF-C and HF-FO; n=10 per group). Values are means ± SEM. All measures were significantly altered by HFEE, irrespective of group ($p < 0.05$ by ANOVA).

*2.3.3. Plasma glucose*

Plasma glucose curves for HF-C and HF-FO are presented in **Figure 2.2A and B**, respectively. Basal plasma glucose concentrations confirm participants were insulin sensitive at the start of the study with no significant differences between groups. Following 6 d HFEE, basal plasma glucose levels were comparable to pre-HFEE, with no differences between groups. Neither peak plasma glucose concentrations nor the corresponding time-point were significantly altered following 6 d HFEE or between groups.

The plasma glucose AUC over the course of the OGTT (120 min) was unaltered by 6 d HFEE with no significant difference between groups (**Figure 2.2C**). The AUCs for 0-30 and 0-60 min also were unaltered by HFEE and not statistically different between groups (data not shown). However, the mean change in AUCs across all time-brackets (*i.e.* 0-30, 0-60, 0-120) ranged from 11-52 % for HF-C, but between -6 and -12 % for HF-FO.

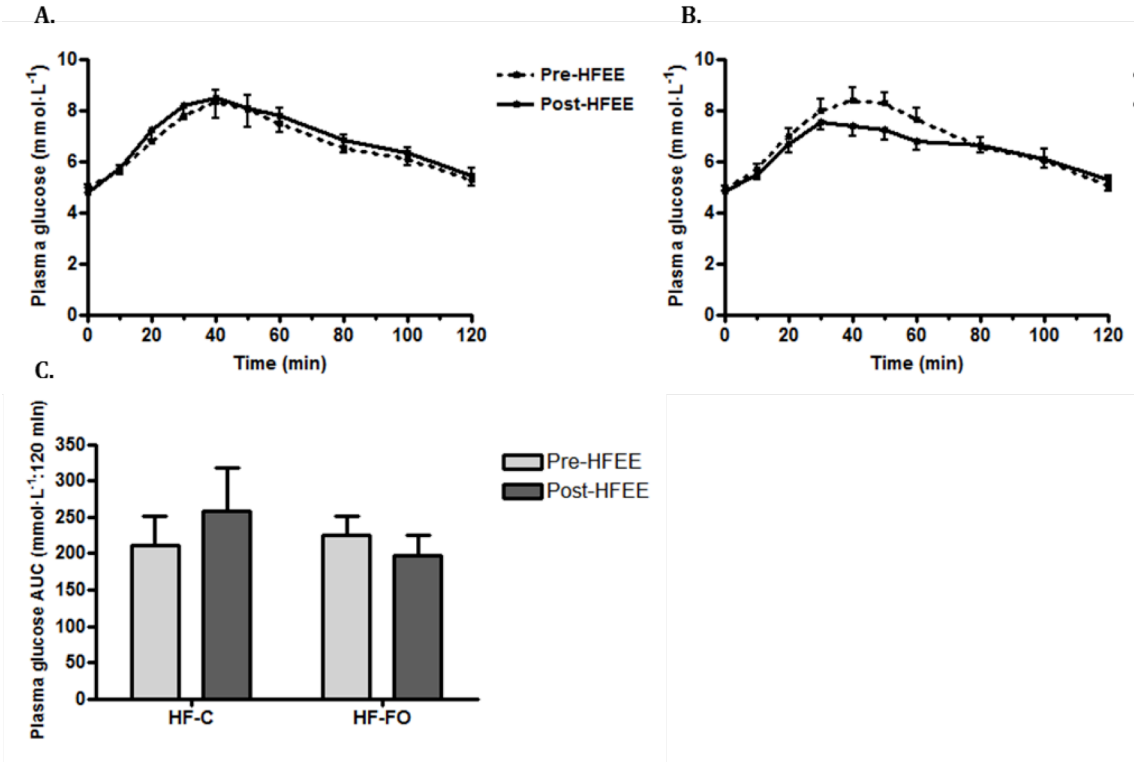



**Figure 2.2** Plasma glucose concentrations for HF-C (A) and HF-FO (B) with incremental area under the curve (C) during the OGTT (0-120 min), pre- and post-HFEE. Data are expressed as means ± SEM, n=10 in each group.

### *2.3.4. Plasma insulin*

Plasma insulin curves for HF-C and HF-FO are presented in **Figure 2.3A and B**, respectively. Basal plasma insulin concentrations were within the insulin sensitive range for both groups, pre- and post-HFEE, with no differences over the course of HFEE. Peak plasma insulin concentrations and their corresponding time-point were unaltered by HFEE and did not significantly differ between groups.

The plasma insulin AUC over the 120 min duration of the OGTT was unaltered by HFEE with no significant difference between groups (**Figure 2.3C**). The AUC for the first 30 and 60 min of the OGTT were unaltered by HFEE in either group (data not

shown) but the mean change ranged from 17-28 % for HF-C and from 3-8 % for HF-FO.

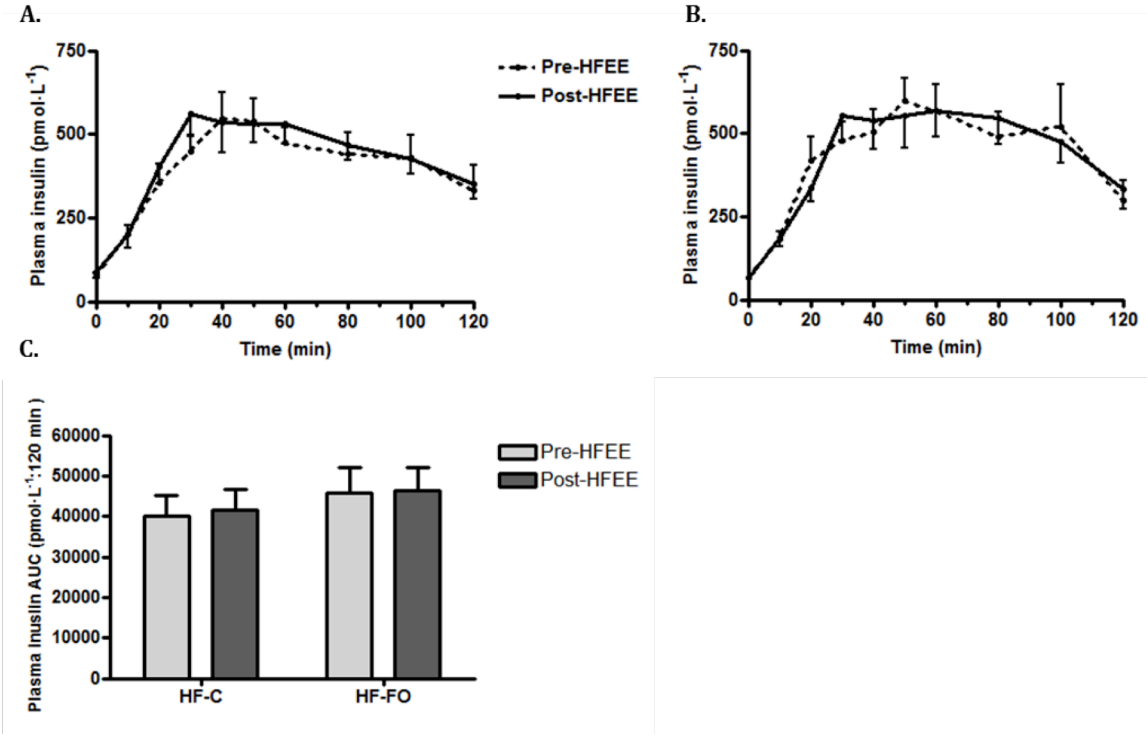



**Figure 2.3** Plasma insulin concentrations for HF-C (A) and HF-FO (B) with incremental area under the curve (C) during the OGTT (0-120 min), pre- and post-HFEE. Data are expressed as means ± SEM, n=10 in each group.

### *2.3.5. Plasma glucose enrichments*

The AUCs over the entire OGTT for total rate of appearance (Ra) of infused [6,6-$^{2}H_2$] glucose and ingested [U-$^{13}$C] glucose, exogenous (ingested glucose) and endogenous Ra (glucose production) alone, and total rate of glucose disappearance (Rd) are presented in **Table 2.4**. Peak plasma glucose Ra (total, exogenous and endogenous) and Rd with their respective time-points are presented in **Tables 2.5** and **2.6**, respectively.

**Table 2.4** Plasma stable isotope glucose AUCs.

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Total Ra** | 209.9 ± 21.5 | 195.6 ± 20.0 | 208.7 ± 25.3 | 213.3 ± 28.2 |
| **Exogenous Ra** | 318.2 ± 21.1 | 310.4 ± 14.7 | 349.9 ± 22.6 | 352.0 ± 27.9 |
| **Endogenous Ra** | 113.0 ± 8.2 | 118.4 ± 10.8 | 143.1 ± 12.6 | 139.0 ± 6.7 |
| **Total Rd** | 255.1 ± 28.1 | 278.2 ± 24.6 | 273.3 ± 28.7 | 283.2 ± 32.1 |

Data are presented in $mg \cdot min^{-1} \cdot kg^{-1}$: 120 min, measured pre- and post-HFEE, for HF-C and HF-FO. Values are means ± SEM, n = 10 per group. No statistically significant differences were observed by ANOVA.

**Table 2.5** Peak plasma stable isotope glucose appearance / disappearance.

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Total Ra** | 6.47 ± 0.33 | 6.51 ± 0.26 | 7.11 ± 0.38 | 6.98 ± 0.43 |
| **Exogenous Ra** | 4.56 ± 0.30 | 4.82 ± 0.28 | 5.17 ± 0.36 | 5.35 ± 0.36 |
| **Endogenous Ra** | 3.55 ± 0.13 | 3.47 ± 0.11 | 3.77 ± 0.15 | 3.67 ± 0.09 |
| **Total Rd** | 6.69 ± 0.38 | 6.63 ± 0.28 | 7.34 ± 0.48 | 7.18 ± 0.50 |

Data are presented in $mg \cdot min^{-1} \cdot kg^{-1}$, measured pre- and post-HFEE, for HF-C and HF-FO. Values are means ± SEM, n = 10 per group. No statistically significant differences were observed by ANOVA.

**Table 2.6** Time points corresponding to peak plasma glucose appearance / disappearance (displayed in **Table 2.5**).

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Total Ra** | 83 ± 9 | 95 ± 11 | 84 ± 9 | 100 ± 6 |
| **Exogenous Ra** | 105 ± 7 | 116 ± 4 * | 95 ± 8 | 115 ± 4 * |
| **Endogenous Ra** | 15 ± 2 | 13 ± 2 | 13 ± 2 | 11 ± 1 |
| **Total Rd** | 77 ± 6 | 87 ± 8 | 85 ± 8 | 97 ± 6 |

Data are presented in min, measured pre- and post-HFEE, for HF-C and HF-FO. Values are means ± SEM, n = 10 per group. * significantly different from pre-HFEE: main effect of time ($p < 0.05$) by ANOVA.

*2.3.6. Insulin sensitivity*

Insulin sensitivity, assessed using a number of indices, was not significantly altered by HFEE and did not statistically differ between groups (**Table 2.7**). Plots in **Figure 2.4** demonstrate the individual variation present in two representative indices, Matsuda ISI and HOMA-IR. Evaluation of Matsuda insulin sensitivity with inferential statistics indicated a possible (62 %) chance of a beneficial effect of FO consumption during HFEE (0.32 ± 0.68 (90 % CL)). Similar evaluation of HOMA-IR insulin resistance with inferential statistics also indicated a possible (71 %) chance of a beneficial effect of consuming FO during HFEE (-0.46 ± 0.84 (90 % CL)). However, the mechanistic inference of HFEE for both insulin sensitivity indices was classified as unclear, with more data required.

**Table 2.7** Insulin sensitivity indices assessed using plasma glucose and insulin values during the OGTT.

| | **HF-C** | | **HF-FO** | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Matsuda-ISI** | 3.96 ± 0.62 | 3.59 ± 0.50 | 3.81 ± 0.39 | 3.94 ± 0.36 |
| **HOMA-IR** | 2.80 ± 0.39 | 3.20 ± 0.69 | 2.45 ± 0.24 | 2.35 ± 0.21 |
| **HOMA-β** | 177.94 ± 24.05 | 218.22 ± 32.35 | 162.16 ± 12.76 | 163.65 ± 10.27 |
| **Hepatic-IR index** | 63.07 ± 8.74 | 71.89 ± 15.58 | 55.02 ± 5.38 | 52.88 ± 4.82 |
| **Hepatic-IS index** | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |

Data are displayed pre- and post-HFEE, for both groups (HF-C and HF-FO; n=10 per group). Values are means ± SEM (values displayed as 0.00 were < 0.001). No statistically significant differences were observed by ANOVA.

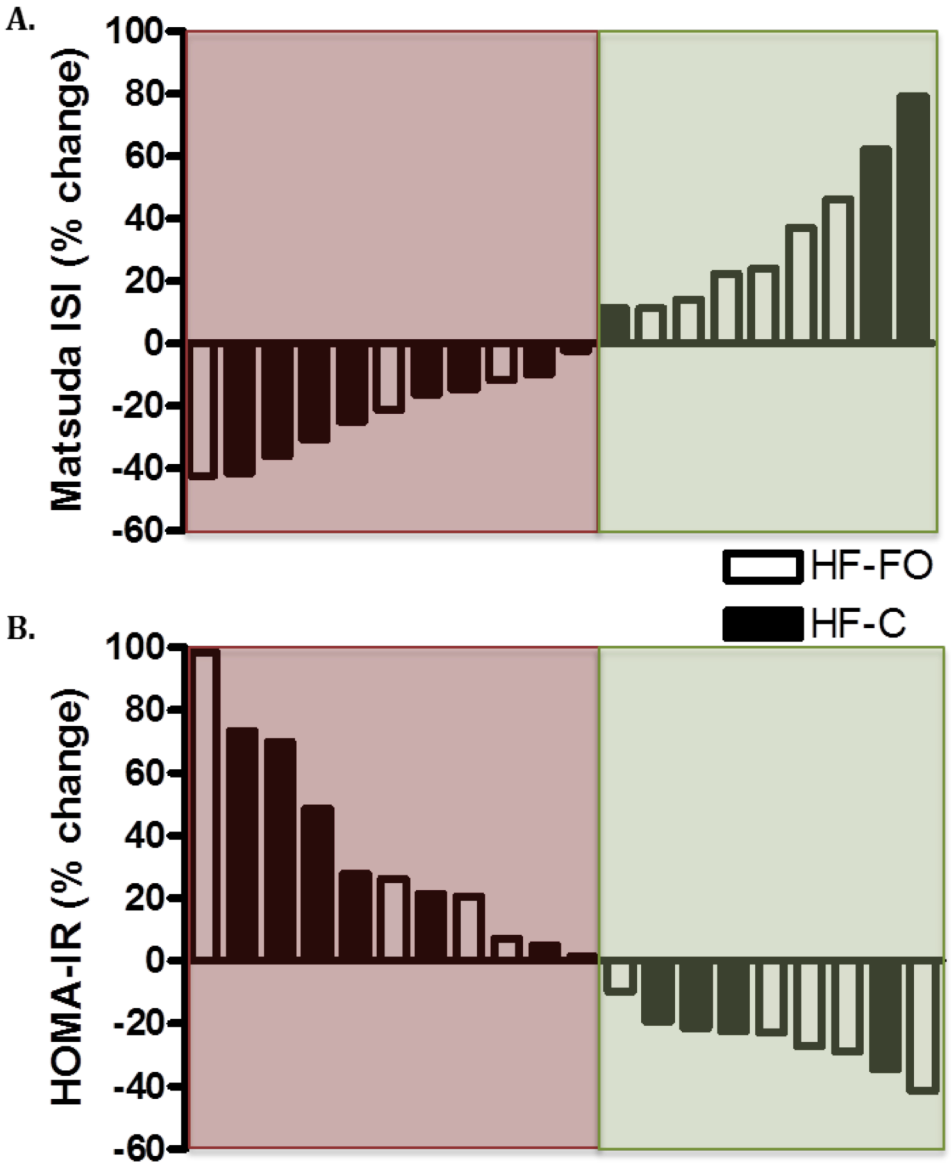



**Figure 2.4** Individual Matsuda (A) and HOMA-IR (B) responses to HFEE. The bars covered by a red shaded box indicate negative insulin sensitivity responses to HFEE, whereas the bars within a green shaded box represent positive a positive change in insulin sensitivity following HFEE. Data are expressed as individual values, n=10 in each group.

*2.3.7. Blood lipids*

A full blood FA profile is presented in **Appendix 2.3**. Total n-3 PUFAs (when expressed as a percentage of total FAs) were significantly increased in HF-FO

following HFEE, with no change in HF-C (**Figure 2.5**). Increased levels of EPA (0.57 ± 0.04 to 3.95 ± 0.38) and DHA (2.51 ± 0.14 to 4.21 ± 0.18) contributed most notably to the increases observed in total n-3 PUFAs in HF-FO. The AA:EPA ratio was significantly decreased in HF-FO over time (20.14 ± 1.57 to 3.01 ± 0.34) with no change in HF-C.

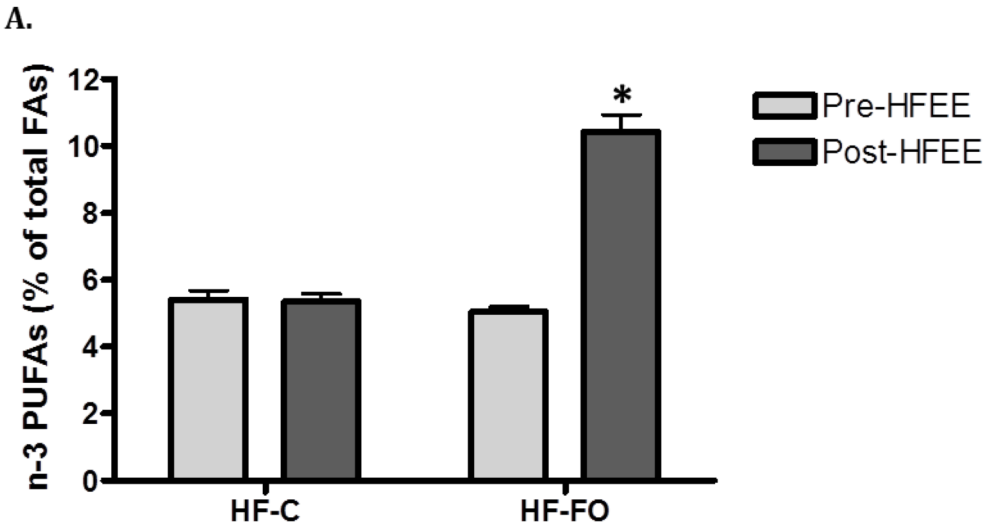



**Figure 2.5** n-3 PUFA composition of whole blood (n = 10 per group) pre- and post-HFEE for both HF-C and HF-FO, as measured by GLC. Data are expressed as means ± SEM, * significantly different from pre-HFEE for its respective group, $p < 0.01$ (t-test).

*2.4. Discussion*

We aimed to investigate various whole-body glucose metabolism responses to a 6 d period of HFEE, and to determine whether replacing 10 % of dietary fats with FO could protect against any diet-induced insulin resistance. Contrary to our hypothesis, we report that 6 d HFEE did not significantly alter whole-body insulin sensitivity group means in our participant cohort of young, healthy, inactive males - irrespective of FO consumption.

In our hands, 6 d HFEE does not clearly alter insulin sensitivity group means irrespective of FO consumption. Whereas it is clear that long-term HFEE does lead to declining insulin sensitivity (105,112), studies designed to investigate short-term responses to HFEE report equivocal findings. Thus, our finding that 6 d HFEE does not lead to overall group perturbations in whole-body insulin sensitivity is consistent with some reports (*e.g.* (19,98,99)), but is in contrast to other investigations (*e.g.* (103,109,112)). Understanding the conflicting results of these studies, including ours, is hampered by the use of different study populations and different experimental protocols. The primary differences in experimental protocol between studies include the methodology used to assess insulin action / sensitivity, and the dietary challenge including macronutrient composition and the extent / duration of energy excess. Future attempts to develop a reproducible model of diet-induced insulin resistance will need to standardise some of these factors. However, arguably more significant to our understanding of human physiological responses to short-term HFEE is the large degree of variation endemic in the majority of these studies including ours. Such individual variation in the response to a given stimulus highlights the influence of

one's metabolic phenotype, encompassing pre-disposition and post-transcriptional / translational regulation, for metabolic control and adaptation.

Outcome measures of glucose metabolism and insulin sensitivity have differed between previous studies. We employed stable isotope metabolic tracers to investigate plasma glucose kinetics following consumption of an oral glucose bolus. Despite no significant changes in the rates of glucose appearance (both endogenous and exogenous) and disappearance, the time corresponding to peak exogenous glucose appearance was significantly delayed following HFEE. Potential explanations for this finding relate to alterations in digestion and absorption processes of the ingested glucose bolus. Levels of gut incretin proteins including gastric inhibitory peptide (GIP) have previously been shown to increase following HFEE (19) and may be a potential mechanism regulating our finding of delayed peak exogenous glucose appearance.

There are a number of factors that influence the insulin sensitivity response to short-term periods of HFEE. Sex-specific differences influence substrate metabolism (210) and thus may influence the insulin sensitivity response to HFEE. When we consider that the insulin sensitivity response of our all-male cohort and other comparable single-sex male cohorts (19,99) did not significantly change following short-term HFEE, it is possible that female sex is a risk factor for maladaptive responses to short-term HFEE. This proposal is supported by experimental data from mixed-sex cohorts where detrimental effects of overfeeding are evident in females but not males (98)(112). It is important to acknowledge that the influence of sex on the insulin sensitivity response to overfeeding may be confounded by fat mass. The insulin

sensitivity response to short-term overfeeding has been shown to differ between lean and reduced-obese females (98). When we consider that females typically possess a greater proportion of their body mass as fat compared to males (210) it is possible that fat mass may be confounding our interpretation of sex-mediated differences in the insulin sensitivity response to overfeeding. Therefore, the independent effects of fat mass, between and within sexes, on insulin sensitivity change should be investigated in future studies.

Habitual participant activity levels are variable between studies and may have a role in the insulin sensitivity response to HFEE. An exclusion criterion was applied in the current study based on activity levels that met current UK government recommendations (no participant in our study exercised more than 2 $x \cdot wk^{-1}$), and in the study of Brøns *et al*. (19) based on > 10 $h \cdot wk^{-1}$. Both of these exclusion criteria permitted a degree of physical activity, and habitual activity levels reflected in cultural norms (*i.e.* Danish volunteers likely will have higher habitual levels of activity than UK counterparts) also may alter this characterisation of physical activity. However, although Brøns *et al*. (19) likely studied a more active cohort than was studied in the present investigation, participants in both studies would not be considered regular exercisers. Whereas Brøns *et al*. (19) reported a change in hepatic insulin sensitivity, but not peripheral insulin sensitivity, following HFEE, we report no change in either parameter (inferred from HOMA-IR and Matsuda indices). Other studies that used active participant cohorts have demonstrated declining whole-body insulin sensitivity following short-term HFEE (107,109,115). Thus, the insulin sensitivity response to HFEE may be mediated by participant physical activity status, with greater perturbations towards the insulin-resistant state observed in more

physically active populations. However, significant declines in insulin sensitivity also have been reported following HFEE in sedentary individuals (108,112). Thus, the influence of physical activity on the HFEE-mediated insulin sensitivity response is not completely clear.

The influence of habitual physical activity levels on the insulin sensitivity response to HFEE may relate to, and / or be explained by, metabolic flexibility. Metabolic flexibility is the ability to adjust fuel oxidation to fuel availability (211). Factors indicative of exercise adaptation including skeletal muscle fibre type composition and mitochondrial oxidative capacity can impact metabolic flexibility (211). Although the mechanisms are not currently known, metabolic flexibility and insulin sensitivity do appear to be linked (211,212). With short-term, HFEE models, metabolic perturbations (*e.g.* altered glucose handling) are observed at 3 d (103,112) arguably due to the metabolic stress placed upon the body. Yet, most studies, including ours, fail to show clear decrements in insulin sensitivity ~ 5-7 d (19,99), although this finding is equivocal (109,115). The metabolic health / flexibility of the individuals may enable the body to respond to the initial metabolic stress and adjust fuel oxidation accordingly over the subsequent days to adapt to the demands. Indeed, following a 7 d high-fat diet, fat oxidation was increased to match fat intake by day 7, but fat oxidation was not increased to match the demands at day 2 (213). This timescale of metabolic events also is supported by previous research examining the 24 h respiratory quotient over the course of a 7 d high-fat (isocaloric) diet. These data show that it is not until day 5 that RQ is reduced from ~0.88/0.89 (Day 1) to ~0.80 and plateaus thereafter (211). Moreover, day 5 is the time-point at which the greatest gap in RQ between metabolically flexible *vs.* metabolically inflexible individuals exists

(211). Unfortunately, in the present investigation, we did not measure RQ or basal metabolic rate and thus cannot infer changes in metabolic fuel oxidation over the course of the feeding intervention. However, based on our participants' body mass, insulin sensitivity and age, we would expect our participants to be largely metabolically flexible, and thus anticipate a shift towards fat oxidation during the 6 d feeding period. By increasing fat oxidation, fat accumulation, and its associated impact on insulin sensitivity, will be reduced. Therefore, metabolic flexibility and the duration of energy excess are likely important factors in determining the insulin sensitivity response to HFEE.

The time course of maladaptation from the earliest signs of defective insulin action through to the diagnosis of insulin resistance and manifest T2D remains to be established. However, even with consideration for the timescale of insulin sensitivity change and the metabolic health of participants, alterations in markers of whole-body insulin resistance may be observed that precede measurable changes in whole-body insulin sensitivity. In the present study, significant increases in fat mass within the trunk, and specifically android, regions were observed. Increases in fat mass have been widely associated with T2D development (13) yet we failed to show any decrements in insulin sensitivity at the same time point. Following a 2 wk period of overeating, fat mass was significantly increased concomitant with reduced insulin sensitivity (105). Therefore, fat mass gains may precede the development of insulin sensitivity. Brøns *et al.*, (19) also did not observe any change in whole-body insulin sensitivity following a feeding intervention largely analogous to the one in the present investigation. However, they did report a significant increase in basal hepatic glucose production using isotope tracer methodology (19). In the present

investigation, the insulin sensitivity of the hepatocyte, inferred from the rate of endogenous glucose production, was not significantly altered despite using similar stable isotope methodology. However, a 15 % mean increase in HOMA-IR, indicative of hepatic insulin resistance, was measured in the HF-C group and for some individuals (5/10 had an increase of >20%) may suggest early maladaptation of a similar nature to those documented in the study by Brøns and co-workers (19). Moreover, as with HOMA-IR, although a large inherent variability existed in the response, a mean 17 % increase in fasting plasma insulin levels in the HF-C group was observed. Evaluation of individual values demonstrated a basal plasma insulin increase of more than 20 % in 5 out of 10 participants. These increases in the fasting plasma insulin response may explain, in part, the maintained insulin-mediated suppression of hepatic glucose output following HFEE. Development of insulin resistance is thought to be preceded by an increase in basal plasma insulin levels, which are increased in order to maintain euglycaemia (8). It is therefore possible that some of our participants, particularly those in HF-C, were showing early signs of maladaptation despite the absence of clear group changes in insulin sensitivity.

A novel feature of our investigation was the inclusion of a second experimental participant group (HF-FO). HF-FO consumed a diet identical in the degree of energy excess and macronutrient contributions to HF-C, but with the provision of FO at an amount equivalent to 10 % of the total consumed dietary lipids. This FO dose was chosen to elicit functional changes in fatty acid composition of the plasma membrane. Previous studies have provided n-3 PUFAs at a proportion as high as 15 % of total FAs (147). The purpose of the HF-FO group was to ascertain whether the nature of consumed fat altered the metabolic and molecular responses to HFEE. During a 7 wk

intervention, elevated fat intake in the form of PUFA (albeit n-6) resulted in less visceral fat accumulation (a marker of hepatic insulin resistance) than equivalent intakes of SFA (192). Thus, markers of insulin sensitivity and metabolic maladaptation may be differentially altered by the type of consumed dietary fat. Evaluation of inferential statistics and the mean whole-body responses to HFEE of a number of indices suggest some possible benefits of FO consumption in the present investigation. Circulating levels of n-3 PUFAs, and in particular the most metabolically active species, EPA and DHA, were significantly increased following HFEE in the group consuming FO (HF-FO). These data indicate adequate FO dosing for detecting changes in whole blood lipids, and suggest dietary compliance to the feeding intervention with expected differences between groups. However, changes in the composition of plasma membranes require investigation before functional effects of FO intake can be assumed. Nonetheless, evaluation of Matsuda and HOMA-IR insulin sensitivity indices with inferential statistics indicated a possible (62 %) / (71 %) chance, respectively, of a beneficial effect of FO consumption during HFEE. Considering these inferences and the large degree of variation observed in our data we wish to highlight individual variation and suggest that investigation of mechanisms relating to gene expression / activation may facilitate our understanding of the underlying individual regulation of glucose metabolism.

In summary, 6 d HFEE leads to significant gains in total body mass and fat mass, largely consistent with the degree of energy oversupply. Moreover, FO consumption at a dose of 10 % of total fats within our model of energy excess results in elevated n-3 PUFA content of whole blood, suggesting adequate dosing and timing for lipid turnover. However, these findings were not translated into overall group

perturbations in whole-body insulin sensitivity in response to an oral glucose load in our healthy male volunteers. Thus, whereas some individuals did respond robustly to the dietary intervention, in our hands, 6 d HFEE with 50 % energy excess is not a complete model for investigating diet-induced whole-body insulin resistance in healthy, young, inactive males.

Researchers wishing to design a reproducible model of diet-induced insulin resistance in future should consider a time course investigation with multiple repeated measurements before, and extending past, 6 d of HFEE and / or study different populations. Particular attention should be afforded to participant sex and metabolic flexibility, including habitual activity levels, of the cohort. To enhance the chances of inducing insulin resistance during short-term HFEE, separate groups of metabolically inflexible males and females, notably sedentary and overweight, should be used. However, there also is an argument for studying a highly active cohort and restricting physical activity during HFEE so that the change in metabolic stress is maximised. Nevertheless, it must be emphasised that mechanistic perturbations at the muscle level were not investigated in the current investigation and may precede measurable changes in insulin sensitivity. In this case, 6 d HFEE may be a useful model for investigating the underlying skeletal muscle-mediated mechanisms that lead to the development of whole-body insulin resistance in the long-term.

**CHAPTER 3** Skeletal muscle ceramide content is increased in young, healthy males following 6 days high-fat energy excess in the absence of changes in mitochondrial enzyme activity.

**Wardle SL**, McGlory C, Macnaughton LS, Witard OCW, Whitfield PD, Hamilton DL, Galloway SR, Moran CN, Tipton KD.

*3.0. Abstract*

We aimed to investigate the lipid-mediated, skeletal muscle (mal)adaptations to a 6 d period of lipid and energy excess (HFEE). Twenty, young, healthy males were overfed (150 % habitual energy intake; 60 % fat, 25 % carbohydrate, 15 % protein) for 6 d. Two identical trials were conducted immediately prior to, and following, HFEE. Basal blood samples collected during each trial were used to assess plasma lipids, adiponectin and TNF-α. Basal skeletal muscle biopsies from each trial were used to assess phospholipid profiles, ceramide levels, and the activity of AMPK α2 and mitochondrial enzymes (citrate synthase and β-HAD). Skeletal muscle PKB activity was measured in both the basal and insulin-stimulated (2 h) state. Plasma levels of TG and FA were significantly reduced following HFEE, irrespective of group (-27 ± 5 % (TG), -27 ± 9 % (FA)), whereas HDL was significantly increased in HF-FO only (19 ± 5 %). Total skeletal muscle ceramide levels were increased following HFEE (HF-C: 10.18 ± 2.29 to 14.49 ± 2.88; HF-FO: 6.87 ± 0.79 to 9.70 ± 1.75 nmol·mg$^{-1}$, p = 0.04) without change in activities of citrate synthase or β-HAD. AMPK α2 activity was differentially regulated between groups; HF-FO had increased AMPK α2 activity following HFEE (4.08 ± 0.57 to 5.26 ± 0.73 μM·mg$^{-1}$), whereas HF-C had reduced AMPK α2 activity following HFEE (4.55 ± 0.72 to 3.78 ± 0.87 μM·mg$^{-1}$, interaction: p = 0.03). The insulin-stimulated rise in PKB was greater pre-HFEE compared to post-HFEE for HF-C (106 ± 38 *vs.* 95 ± 48 %) with no change in HF-FO. HFEE for 6 d is characterised by lipid-mediated perturbations at the skeletal muscle level, some of which are modified by FO consumption, and which collectively may help inform the metabolic basis for insulin resistance development.

*3.1. Introduction*

**Chapter 2** provided an overview of the whole-body responses to 6 d HFEE in the context of glucose metabolism and insulin action. The principle finding was that insulin sensitivity group means were not significantly altered following 6 d HFEE. However, a large degree of variability around the mean change was observed for insulin sensitivity and other measures. Thus, the impact of different dietary practises on metabolic responses may be greater for some individuals compared to others. Moreover, the underlying regulation of insulin sensitivity may be maladaptive at the skeletal muscle level before whole-body diet-induced insulin resistance occurs. This subcellular regulation within skeletal muscle may relate to perceived individual differences and / or be explained, in part, by the role of fats and their intermediates, present in the circulation and intramuscularly.

Insulin sensitivity is characterised by insulin action on skeletal muscle, liver and adipose tissue. Impairments in whole-body insulin sensitivity are therefore largely reflective of defects in one or more of these tissues (42,214). Insulin sensitivity is regulated by many factors. Ectopic fat deposition, *i.e.,* the storage of fat in non-adipose tissue (usually due to FA spillover from adipose tissue) is one adaptation resulting from fat oversupply that negatively impacts insulin signalling / sensitivity (215). An inability to upregulate fat oxidation in the presence of fat oversupply can lead to elevated tissue levels of TGs and other lipid moieties including DAGs, LcFA-CoAs and ceramides (78). Accumulation of these lipid moieties, particularly in insulin sensitive tissues such as skeletal muscle and liver, can negatively impact insulin signalling by inhibiting the phosphorylation of insulin signalling intermediates (78). Therefore, increases in TGs, DAGs, LcFA-CoAs and ceramides all have been touted as

characteristic of the metabolic syndrome (77). Investigation of the impact of short-term periods of HFEE on intramuscular fat accumulation, in the absence of measurable changes in whole-body insulin sensitivity, may aid our understanding of insulin resistance development.

Skeletal muscle ceramide content is altered with insulin resistance and affects insulin signalling. Ceramide synthesis is driven by palmitate availability, ER stress and inflammation, often indicated by elevated TNF-α levels (84). In contrast, the lipid mediator adiponectin stimulates ceramide degradation (84). Increased synthesis of ceramides within *in vitro* cultured myotubes has been attributed to increased palmitate availability and associates with impaired PKB phosphorylation and subsequent glucose transport in a dose-dependent manner (87). Ceramides also have been implicated in human T2D pathology (77,90,91) with increased intramuscular levels in obese, insulin-resistant individuals compared to lean counterparts (72). These findings occurred in combination with reduced insulin signalling via decreased phosphorylation of $PKB^{Ser473}$ and $PKB^{Thr308}$ in the obese, insulin-resistant cohort (72). Thus, skeletal muscle ceramide levels, PKB phosphorylation and insulin action all appear to be reciprocally regulated within the context of manifest insulin resistance. However, whether skeletal muscle ceramide levels are altered prior to the onset of insulin resistance following short-term, HFEE in humans is currently unknown.

The normal counter-response to increased fat supply and accumulation is to increase fat oxidation. Given that fat oxidation occurs within the mitochondrial matrix, FA transport into, and / or the activity of enzymes within, the mitochondrial matrix, are both factors that will regulate the rate of fat oxidation. Moreover, the density and size

of mitochondria will impact fat oxidation. Fat oxidation is reduced in T2D compared to healthy counterparts (216,217) despite comparable intrinsic mitochondrial oxidative capacities (216). However, fat oxidation is limited by a decrease in the density of mitochondria within insulin-resistant skeletal muscle tissue (216). Reduced mitochondrial content, by default, will reduce total mitochondrial enzyme activity and impact subsequent fat oxidation.

Mitochondrial fat oxidation is largely regulated by AMP-activated protein kinase (AMPK); a metabolic regulator of energy balance (218). AMPK regulates energy balance by increasing flux through ATP-producing pathways and reducing flux through ATP-consuming pathways (218). Pharmacological activation of AMPK has been shown to increase FA oxidation (219,220) whereas reduced AMPK activity is associated with decreased FA oxidation within skeletal muscle (220,221). Thus, investigations of AMPK activity and mitochondrial enzyme activity following a period of short-term, HFEE may aid our understanding of the regulation of fat oxidation in these contexts.

Rates of fat oxidation and fat accumulation may be affected by the nature of consumed fat. Research has highlighted a TG-lowering effect of n-3 PUFA intake (142,143), and a positive association between the n-3 PUFA composition of skeletal muscle membranes and insulin sensitivity (21). Moreover, ceramide synthesis is regulated by SFA availability (84) in a dose-dependent manner (87). Therefore, reductions in SFA intake and / or increased n-3 PUFA consumption may reduce TG concentrations and ceramide synthesis, and improve insulin sensitivity. Thus, the

influence of n-3 PUFA intake on lipid-mediated responses to HFEE at the skeletal muscle level warrants attention.

Our aims were two-fold. First, we aimed to investigate the impact of 6 d HFEE on circulating lipid / lipid mediator levels, skeletal muscle ceramide levels, and the relationship between any changes in ceramide levels and PKB, AMPK or mitochondrial enzyme activity. Second, we aimed to investigate the influence of replacing 10 % of dietary fats with fish oil (FO) on these (mal)adaptive responses. We hypothesised that, 1) 6 d HFEE would result in elevated skeletal muscle ceramide levels, 2) changes in ceramide levels would associate with changes in PKB, AMPK and mitochondrial enzyme activity, and 3) dietary FO consumption would ameliorate the increase in ceramide levels and associated mechanisms.

*3.2. Methods*

*3.2.1. Ethical approval*

Ethical approval was obtained from the NHS East of Scotland Research Ethics Service (REC 2) and the study was conducted in accordance with the Declaration of Helsinki (197). Having had the study purpose and procedures explained in lay terms, all participants provided written informed consent.

*3.2.2. Participants and study design*

Participants and study design have been described in detail in **Chapter 2**. Briefly, twenty, healthy males were recruited from the University of Stirling and surrounding area to participate in the investigation. Participants were excluded if they consumed a diet high in fish oil sources or took fish oil supplements, had a fasting blood glucose of > 6.1 $mmol \cdot L^{-1}$, or met the recommended physical activity guidelines for health (30 $min \cdot d^{-1}$, 5 $x \cdot wk^{-1}$).

In a randomised, parallel study design, participants were matched to either a high-fat control (HF-C; n = 10) or a high-fat, fish oil (HF-FO; n = 10) group based on age, body mass, physical activity and habitual energy intake. Two identical testing days (**Figure 2.1B (Chapter 2)**) included basal blood sampling and collection of two skeletal muscle biopsies per trial. One skeletal muscle biopsy was obtained in the basal state and the other in the insulin-stimulated state, 2 h after glucose feeding. A 3 d baseline diet was designed to reflect individual habitual energy and macronutrient intake, and was consumed by all participants prior to baseline testing (infusion trial 1; pre-HFEE). Between infusion trials 1 and 2 (*i.e.*, pre- and post-HFEE), all participants

consumed a high fat, high energy diet (150 % of habitual energy intake with 60 % of energy from fat, 25 % from carbohydrate and 15% from protein) for 6 d, with the only exception that HF-FO had 10 % of dietary fats replaced by fish oil.

*3.2.3. Blood and skeletal muscle processing*

Blood and skeletal muscle processing have been described in detail in **Chapter 2**. Briefly, blood samples (n = 15) were collected in EDTA vacutainers (Becton, Dickinson & Company, NJ, USA) and spun at 3500 rpm for 15 min at 4 °C. Post-spin, the supernatant plasma fraction was removed and aliquotted into Eppendorf tubes for storage at -80 °C pending analysis.

For the biopsy, a 5 mm Bergström needle was inserted through a pre-prepared anaesthetised (2 % Lidocaine) incision in the skin and fascia, into the muscle belly of the vastus lateralis and a sample removed under manual suction. Muscle biopsy samples were immediately rinsed using ice-cold saline (0.9 %), any visible fat or connective tissue removed. Next, samples were blotted and weighed before freezing in liquid nitrogen prior to storage at -80 °C pending analysis. Both biopsies (basal (0 min) and insulin-stimulated (120 min)) were removed from the same leg. For infusion trial 2 (post-HFEE), biopsies were obtained from the contralateral limb. The order of the first leg to be biopsied was counterbalanced between groups to eliminate any potential for order effects.

*3.2.4. Plasma analyses*

Plasma triglyceride (TG), fatty acid (FA), total cholesterol, low density lipoprotein-associated cholesterol (LDL) levels and high density lipoprotein-associated cholesterol (HDL) were assessed from blood samples taken in the basal state (-60 min) pre- and post-HFEE using an ILab Aries benchtop analyser and associated reagents (Instrumentation Laboratories, MA, US). Plasma levels of TNF-α and adiponectin were measured in the same samples using commercially available ELISA kits (RayBiotech, Inc., GA) according to the manufacturer's instructions. Gen5 software (BioTek, Winooski, VT, USA) was used for ELISA data collection.

*3.2.5. Skeletal muscle ceramide analyses*

Total lipids were extracted from skeletal muscle homogenates according to the method proposed by Folch *et al.* (222) with non-lipid impurities removed by washing with 0.88% (w/v) KCl. A 250 µL aliquot of muscle homogenate was extracted with 6 mL chloroform / methanol (2:1 (v:v)) containing 8 nmol of 17:0 ceramide (Avanti Polar Lipids Inc., Alabaster, AL, USA) as an internal standard. Ceramides were isolated using solid-phase extraction chromatography (100 mg, 3 mL silica columns, Biotage, Uppsala, Sweden). The glycosphingolipid (ceramide) fraction was eluted in 5 mL acetone:methanol (9:1) and collected in glass tubes prior to storage at -80 °C pending analysis. The ceramide eluate was removed from storage at -80 °C, dried down under $O_2$ free $N_2$ and re-suspended in 250 µL methanol containing 5 mM ammonium formate, and transferred into autosampler vials for analysis. Ceramide analysis was performed by liquid chromatography-dual mass spectrometry (LC-MS/MS) on a triple quadrupole tandem mass spectrometer (TSQ Quantum Ultra, Thermofisher,

Hemel Hempstead, UK) in multiple reaction monitoring (MRM) mode with a Kinetex C8 LC column (100 x 2.1mm x 2.6µm, Phenomenex, Macclesfield, UK). Quantification was achieved by relating the peak areas of the muscle ceramides to the peak area of the 17:0 internal standard.

*3.2.6 Skeletal muscle mitochondrial enzyme assays*

Skeletal muscle samples (5-12 mg) were homogenised in 100 µl of homogenising solution (1.36 g of 0.1 M $KH_2PO_4$ + 50 mg of BSA in 80 mL $ddH_2O$; pH adjustment to 7.3 with KOH before adding additional $ddH_2O$ to achieve a final volume of 100 mL) per 1 mg wet weight muscle using an automated homogeniser with 1.4 mm (green) ceramide beads (MagNaLyser, Roche, Germany). Samples were homogenised twice at 7000 rpm for 10 s, separated by 1 min on ice. Following homogenisation, all samples were centrifuged at 14800 rpm at 4 °C for 20 s to reduce foam. Homogenates were snap-frozen in liquid nitrogen before performing two freeze / thaw cycles. Following the final thaw, homogenates (300 µL) were transferred into individual glass vials (cuvettes) for analysis and positioned in the sample rotor according to the work list schedule on an ILab Aries benchtop analyser (Instrumentation Laboratories, MA, US). 25 µL of muscle homogenate was used for each reaction. The work list schedule and homogenate sample were the same for both the citrate synthase and β-HAD reactions.

**Citrate synthase**

225 µL of reagent 1 (TRIS buffer (pH 8.3; 7.5 mL), DTNB (1.25 mL), acetyl-CoA (2 mL) and Triton X-100 (10%; 500 µL)) and the muscle homogenate (10 µL) were combined initially, before next adding 15 µL of 10 mM oxaloacetate (reagent 2) to start the

reaction. Readings were taken at 450 nm at selected intervals (0, 39, 52, 65, 78, 91, 104, 130, 156, 182 195, 234, 260 s) over a 5 min period.

**Beta(3)-Hydroxyacyl-CoA Dehydrogenase (β-HAD)**

215 µL of reagent 1 (TRIS-HCl buffer (pH 7.0; 2.5 mL), EDTA (0.5 mL), NADH (2.5 mL) and dd$H_2O$ (19.5 mL)) and 5 µL of reagent 2 (Triton x-100 (10%)) were combined with the muscle homogenate (25 µL) for an initial 5 min incubation period before taking a slope measurement at 340 nm for 2 min to record steady state. Next, 5 µL of 5 mM acetoacetyl-CoA (reagent 3) was added to start the reaction and the decrease in NADH was measured. Readings were taken at 450 nm at selected intervals (0, 39, 52, 65, 78, 91, 104, 130 s) over a 2.5 min period.

**Calculations**

**Citrate synthase and β-HAD activity (µmol·g$^{-1}$·min$^{-1}$):**

((Absorbance·min$^{-1}$ (sample) – absorbance·min$^{-1}$ (blank) * total volume) / (external coefficient * sample volume)) * dilution factor

Where:

Absorbance·min$^{-1}$ (blank) = 0.0010

Total volume = 250 µl

Dilution factor = 101

Citrate synthase external coefficient (412 nm) = 13.6 µmol·cm$^{-2}$

Citrate synthase sample volume (muscle homogenate) = 10 µL

β-HAD external coefficient (412 nm) = 6.22 µmol·cm$^{-3}$

β -HAD synthase sample volume (muscle homogenate) = 25 µL

*3.2.7. Skeletal muscle [γ-$^{32}$P] ATP kinase activity assays*

**PKB**

Skeletal muscle samples (30-60 mg) were homogenised in 10 times (v/w) PKB lysis buffer using a Dounce homogeniser. The PKB lysis buffer included 40 µL SBT1 (1:1000), 40 µL β-mercaptoethanol, 40 µL PMSF (1:1000), 40 µL benzamidine (1:1000) and 200 µL NaOV (1:200). Homogenised lysates were stored on dry ice prior to being thawed and centrifuged at 14800 rpm for 30 min at 4 °C to pellet debris. The supernatant fraction was removed and diluted 1 in 5 with dd$H_2O$.

Next, a protein content assay was performed to establish the amount of protein in each sample. A standard curve was made using BSA and dd$H_2O$ with protein contents ranging from 0 to 10 µg. Samples (1 µL + 5 µL dd$H_2O$) and standards (5 µL) were loaded in to their respective well followed by 25 µL protein assay reagent A (1:50) and 200 µL protein assay reagent B (Bio-Rad Laboratories Ltd., Hertfordshire, UK (for both reagents)). Plates were incubated for 10 min at room temperature before reading at 750 nm using a microtiter plate reader and Gen5 software (BioTek, VT, USA). Sample protein contents were calculated from the standard curve, and adjusted with dd$H_2O$ so that all samples had identical protein contents per µL of sample.

Samples of known, and equal, protein content were immunoprecipitated at 4 °C for 2 h in PKB lysis buffer (50 mM TrisHCl (pH 7.5), 0.1 mM EGTA, 1 mM EDTA, 1 % (v/v) Triton X-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1 % β- mercaptoethanol, 1 mM $Na_3(OV)_4$) with protease inhibitors (PMSF, DTT, SBT1, Benzamidine, NaOV). Immune complexes were formed and precipitated in the presence of protein G sepharose (70 %, 2.5 µL per immunoprecipitation). Immune complexes were washed

twice with an assay specific high salt wash (PKB lysis buffer + 0.5 M NaCl) with a spin at 2000 rpm for 1 min at 4 °C and aspiration of the supernatant in between steps. A third wash was performed using TRIS assay buffer (50 mM TrisHCl (pH 7.4), 0.03 % Brij35, 0.1 % β- mercaptoethanol). Immune-bead complexes were re-suspended in 10 µL TRIS assay buffer, before being made up to a final reaction volume of 50 µL with a hot assay mix that was added to each sample at 20 s intervals. The hot assay mix comprised ATP-$MgCl_2$ (100 µM ATP + 10 mM $MgCl_2$), γ-$^{32}$P ATP (panPKB; 0.50 x $10^6$ cpm·nmoL$^{-1}$ specific activity) and 'crosstide' (GRPRTSSFAEG at 30 µM), a synthetic peptide substrate for PKB. After 20 min on a shaker (150 rpm; Stuart SSM1, Bibby Scientific, Staffordshire, UK) assays were stopped at the same 20 s intervals as they were started by spotting on squares of P81 chromatography paper (GE Healthcare, Buckinghamshire, UK) and immersing in 75 mM phosphoric acid. The assay-spotted paper squares were washed a further 3 times in 75 mM phosphoric acid for 5 min at a time before a final 5 min wash in acetone. Next, all paper squares were dried and individually immersed in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies Ltd., Chesterfield, UK). Radioactivity of the samples was quantified by scintillation counting (United Technologies Packard 2200CA TriCarb liquid scintillation analyser) in nmoL·mg$^{-1}$·min$^{-1}$. For data analysis, all values were background subtracted using 'blank' samples which were immunoprecipitated in the absence of any peptide substrate (crosstide).

**AMPK α2**

Samples with known protein contents (as described for PKB**)** were used for AMPK α2 assays. The assay procedure was the same for AMPK α2 as the assay procedure outlined for PKB, with the exception that samples were immunoprecipitated at 4 °C

overnight, the final wash step was performed using HEPES assay buffer (50 mM HEPES (pH 7.4), 1 mM DTT, 0.02 % Brj35) and immune-bead complexes were re-suspended in 20 µL of HEPES assay buffer prior to the addition of hot assay mix. The hot assay mix comprised ATP-$MgCl_2$ (200 µM ATP + 50 mM $MgCl_2$), γ-$^{32}$P ATP (panAMPK; 0.25 x $10^6$ cpm·nmoL$^{-1}$ specific activity) and the synthetic peptide substrate, 'AMARA' (AMARRAASAAALARRR at 200 µM). The homogenisation buffer comprised 50 mM TrisHCl (pH 7.25), 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg·mL$^{-1}$ soya bean trypsin inhibitor, and 1 % (v/v) Triton X-100.

*3.2.8. Skeletal muscle phospholipid analysis*

Total lipids were extracted from skeletal muscle homogenates according to the method proposed by Folch *et al.* (222) with non-lipid impurities removed by washing with 0.88 % (w/v) KCl. Phospholipids were isolated using solid-phase extraction chromatography (silica columns) and eluted in 5 mL 100 % methanol prior to processing for GCMS analysis as described previously (128). Specifically, phospholipid fractions were removed from -80 °C and allowed to equilibrate to room temperature. Eluates were dried down under $N_2$ prior to re-suspension in 1 mL chloroform:methanol (2:1) and transfer into pre-weighed 1.7 mL glass bottles. Solutes were dried again under $N_2$ and further by leaving in a vacuum desiccator overnight. The following morning the lipid containing glass bottles were weighed and a record of the weight made. Chloroform:methanol (2:1) + BHT (0.5 mL) was added to each bottle before flushing with $N_2$ and capping for overnight storage at -80 °C. The following morning, the lipid solute was transferred to a 15 mL Quickfit test tube and 17:0 standard was added at 10 % of the lipid mass. The organic solvent was removed

under $N_2$ followed by addition of the methylating reagent (1 % (v/v) sulphuric acid in methanol) and a 5 s vortex. Tubes were flushed with $N_2$ and a stopper added in combination with a piece of tissue to prevent any movement of the stopper upon heating. Heating involved an overnight incubation (minimum 16 h) of the tubes in a hot block set to 50 °C. The following morning the tubes were cooled to room temperature, the tissue removed and 2 mL of 2 % KHCO3 followed by 5 mL isohexane:diethyl ether (1:1, v/v) + 0.01 % (w/v) BHT were added. Contents were mixed by gently inverting the tubes, removing the stopper briefly to release gas and then re-applying the stopper and shaking vigorously. Next, tubes were centrifuged (with the stoppers removed) at 1500 rpm for 2 min. The upper organic layer was transferred to a clean 15 mL Quickfit test tube. A further 5 mL isohexane:diethyl ether (1:1, v/v) (no BHT) was added to the original tube and mixed and centrifuged in the same manner as previously described before again transferring the upper layer to the new 15 mL Quickfit test tube. The new solvent was evaporated under $N_2$, re-suspended in 1 mL isohexane and vortexed for 5 s, before transferring in to 2 mL autosampler vials, flushing with $N_2$ and storing at -20 °C prior to GLC analysis. FAME were separated and analysed in the same manner as described in '*Blood lipid analysis*' (**Chapter 2**).

*3.2.9. Statistical analyses*

All data were tested for normality using the Ryan Joiner test with Minitab software (version 16; Minitab, State College, PA). In the case of non-normally distributed data, Box-Cox transformations were applied before testing for normality a second time. In the instance that data remained non-normally distributed following Box-Cox transformation, outliers were removed using the median absolute deviation method

(208), and normality confirmed. Thus, all statistical analyses were performed on normally distributed data. A two-way repeated measures ANOVA (IBM SPSS Statistics 19, NY, USA) was used to assess differences between groups (HF-C *vs.* HF-FO; between-subject factor), over time (pre- and post-HFEE; within-subject factor). In the case of a significant interaction effect by two-way ANOVA, paired t-tests were conducted to assess differences over time within each group alone. Statistical significance was assumed at the level of $p < 0.05$. Values are presented as mean ± standard error of the mean (SEM) unless stated otherwise.

*3.3. Results*

*3.3.1. Plasma lipids*

Plasma lipid and adipokine levels are presented in **Table 3.1**. Levels of plasma TG and FA were significantly decreased following HFEE in both groups. HDL-associated cholesterol was significantly increased following HFEE, with a significant group interaction effect ($p = 0.02$). Follow-up paired t-tests revealed a significant effect of HFEE in HF-FO only ($p < 0.01$). Total cholesterol, LDL-associated cholesterol, adiponectin ($p = 0.09$, main effect of time) and TNF-α were not significantly different over time or between groups.

**Table 3.1** Fasting plasma lipid levels.

| | **HF-C** | | **HF-FO** | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **TG** | 0.80 ± 0.09 | 0.62 ± 0.06* | 0.76 ± 0.06 | 0.48 ± 0.03* |
| **FA** | 0.66 ± 0.06 | 0.48 ± 0.06* | 0.66 ± 0.06 | 0.39 ± 0.06* |
| **Total cholesterol** | 4.05 ± 0.26 | 3.86 ± 0.22 | 3.81 ± 0.26 | 3.98 ± 0.26 |
| **LDL** | 2.92 ± 0.24 | 2.73 ± 0.20 | 2.69 ± 0.24 | 2.67 ± 0.26 |
| **HDL** | 1.30 ± 0.05 | 1.36 ± 0.07*† | 1.32 ± 0.05 | 1.56 ± 0.05*†# |
| **Adiponectin** | 383.1 ± 27.8 | 387.9 ± 32.4 | 426.3 ± 23.8 | 458.4 ± 21.1 |
| **TNF-α** | 3.13 ± 0.37 | 3.48 ± 0.56 | 3.08 ± 0.38 | 3.17 ± 0.49 |

Data presented are pre- and post-HFEE, for both groups (HF-C and HF-FO; n=10 per group). Values are means ± SEM. * significantly different from pre-HFEE (main effect of time), † interaction effect (both by ANOVA), # significantly different from pre-HFEE for the respective group (t-test), all $p < 0.05$. TG, FA, total cholesterol, LDL and HDL are all expressed in $mmol \cdot L^{-1}$, adiponectin in $pg \cdot \mu L^{-1}$ and TNF-α in $pg \cdot mL^{-1}$.

*3.3.2. Skeletal muscle ceramides*

Total basal skeletal muscle ceramide levels were increased by 69 % (± 32) following HFEE, irrespective of group (**Figure 3.1A**). Statistical analysis of individual species

revealed significant increases in the species C18:1, C22:1, C23:0 and C24:1 (all $p < 0.05$; Figure **3.1B**), again irrespective of group.

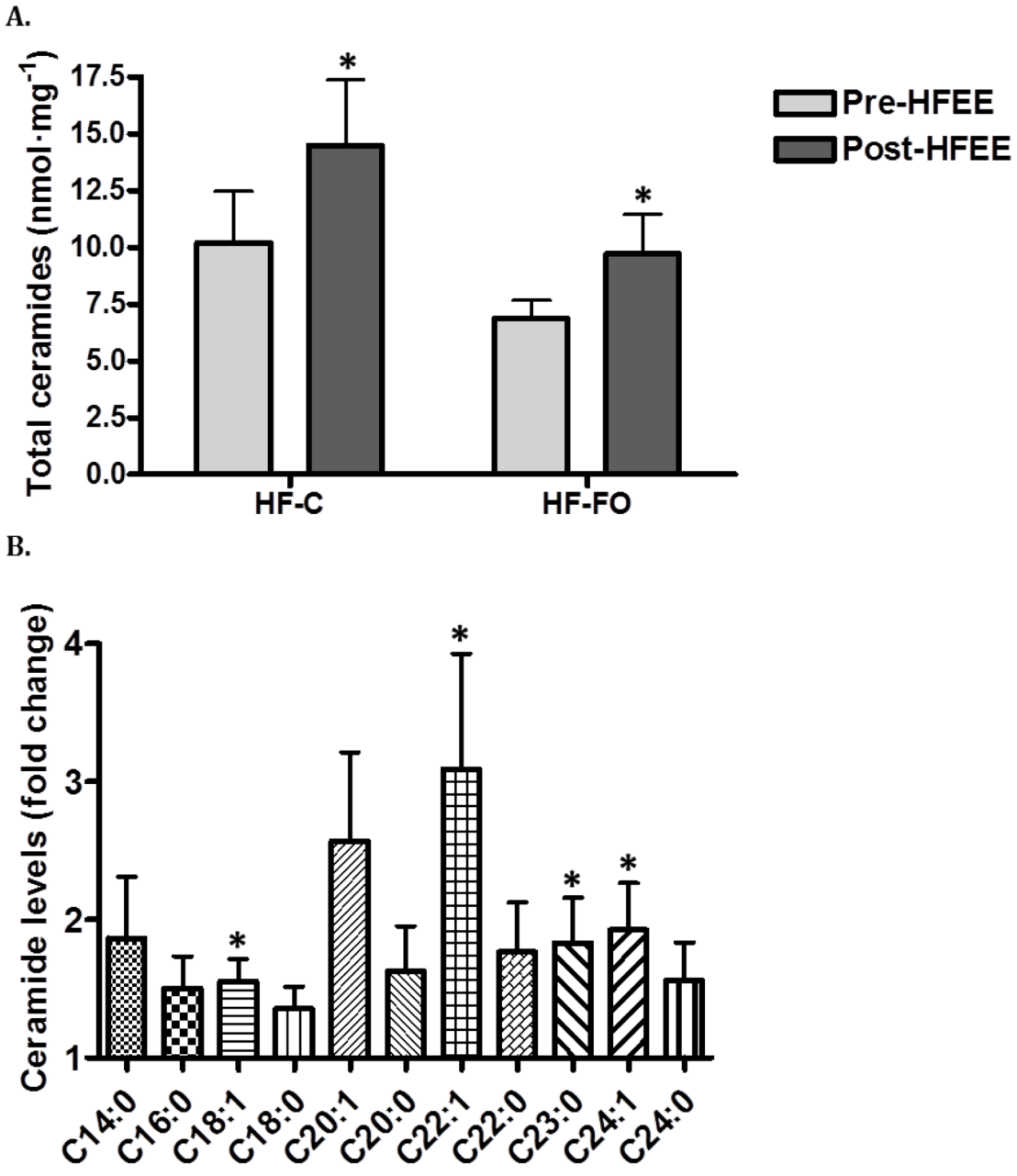



**Figure 3.1** Total skeletal muscle ceramide levels (A) were increased following HFEE irrespective of group. All measured individual ceramide species contributed to the change in total ceramide species but only certain species were significantly altered individually over the course of HFEE (B). In (B), data from both groups (HF-C and HF-FO) have been pooled together for data presentation. All bars are means ± SEM, * significantly different from pre-HFEE, $p < 0.05$ by ANOVA.

*3.3.3. PKB activity*

PKB activity was significantly greater in the insulin-stimulated state (2 h) than the basal state (0 h), irrespective of group or infusion trial (*i.e.* pre- or post-HFEE; **Figure 3.2**). Moreover, a significant interaction effect was present between group (HF-C *vs.* HF-FO) and infusion trial (pre-HFEE *vs.* post-HFEE), suggesting a differential PKB response to the feeding intervention between groups (**Figure 3.2**). Group differences tended to exist regardless of HFEE or OGTT time-point ($p = 0.053$).

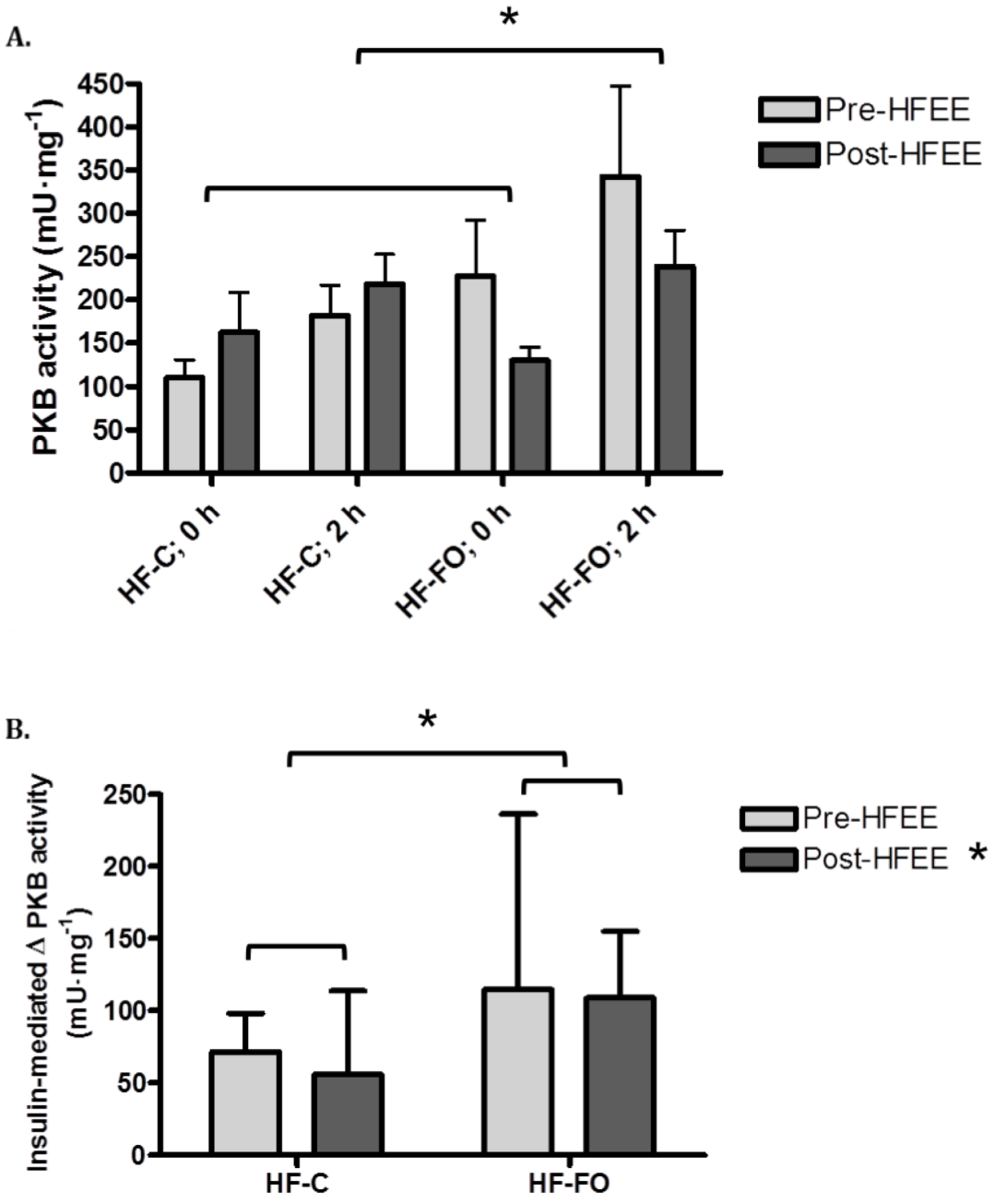



**Figure 3.2** PKB activity in the basal (0 h) and insulin stimulated states (2 h) pre- and post-HFEE for both HF-C and HF-FO (A). Insulin-stimulated change in PKB activity (B) for HF-C and HF-FO, pre- and post-HFEE. Data are expressed as means ± SEM, n=9 per group. Group*HFEE interaction; $p = 0.033$ (*), PKB activity at 2 h was significantly different from 0 h, irrespective of group ($p < 0.01$ (*)).

*3.3.4. AMPK α2*

Basal (0 min) skeletal muscle AMPK α2 activity was significantly decreased following HFEE in HF-C but increased in HF-FO (**Figure 3.4**; interaction effect: p = 0.03). Follow-up paired t-tests revealed a significant effect of HFEE in HF-FO only (p = 0.04 and 0.28 for HF-FO and HF-C, respectively).

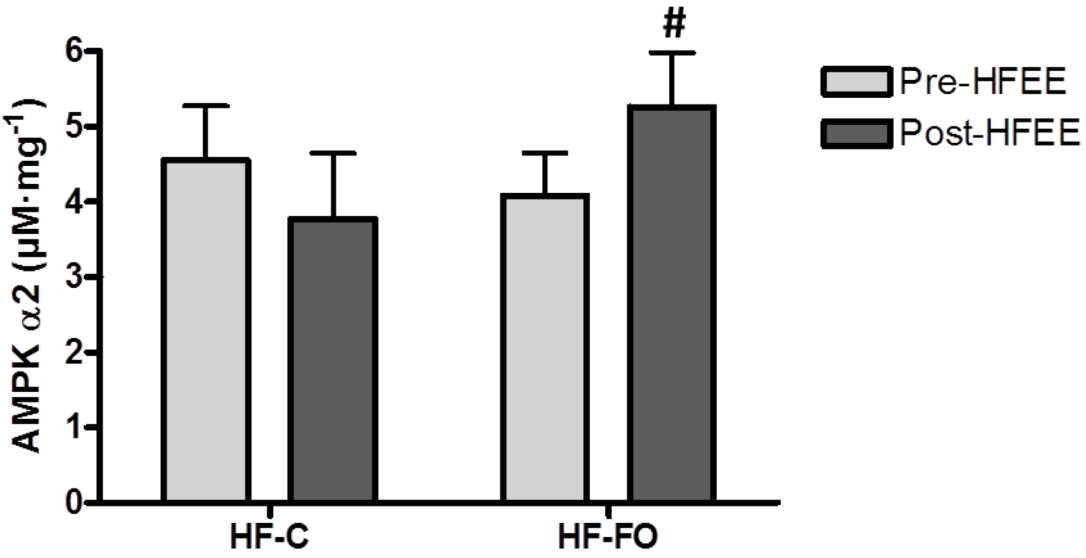



**Figure 3.3** Basal skeletal muscle AMPK α2 activity pre- and post-HFEE for both HF-C and HF-FO. Values are means ± SEM, n=9 per group. # significantly different from pre-HFEE for the respective group, $p < 0.05$ by t-test.

*3.3.5. Mitochondrial enzyme activities*

Citrate synthase and β-HAD activity in the basal state (0 min) was unaltered by HFEE in either group (**Table 3.2**).

**Table 3.2** Mitochondrial enzyme activities.

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **Citrate synthase** | 29.65 ± 3.14 | 27.51 ± 3.09 | 30.69 ± 1.90 | 27.78 ± 2.96 |
| **β-HAD** | 13.57 ± 1.31 | 14.44 ± 0.94 | 14.67 ± 0.62 | 14.07 ± 0.92 |

Data are presented pre- and post-HFEE, for both groups (HF-C and HF-FO; n=9 per group). Values are means ± SEM ($\mu mol \cdot g^{-1} \cdot min^{-1}$). No significant differences between groups or over time ($p > 0.05$ by ANOVA).

*3.3.6. Fatty acid profiling*

A full skeletal muscle phospholipid profile is presented in **Appendix 3.1**. Total SFA, total MUFA and total n-6 PUFAs were unaltered by HFEE and not significantly different between groups. A trend existed for an increase in total n-3 PUFAs in the phospholipid membrane of HF-FO following HFEE by t-test ($p = 0.06$) with no change in HF-C ($p = 0.74$). Specifically, of the n-3 PUFAs increased in HF-FO, EPA was significantly increased ($p = 0.01$), however there was no change in DHA ($p = 0.26$) by t-test. Similar to blood lipids (see section 2.3.7, **Chapter 2**), the AA:EPA ratio was significantly decreased in HF-FO ($p = 0.04$) over time with no change in HF-C ($p = 0.76$).

*3.4. Discussion*

Consistent with our hypothesis, we report a significant increase in basal levels of total, and certain individual, skeletal muscle ceramide species following 6 d HFEE. However, contrary to our hypothesis, this change occurred irrespective of dietary FO consumption. Moreover, the observed increase in ceramide levels occurred in the absence of any changes in basal mitochondrial enzyme (citrate synthase and β-HAD) activity. However, circulating TG and FA levels were reduced following HFEE. These observations may suggest an increase in fat uptake by skeletal muscle, without any change in skeletal muscle fat oxidation. AMPK α2 activity, a key regulator of fat oxidation, was increased post-HFEE when FO was consumed but decreased in the absence of FO consumption. Some FO consumption-mediated differences also were observed with PKB activity. Insulin-stimulated PKB activity was maintained at pre-HFEE levels following HFEE when FO was consumed yet reduced following HFEE in the absence of FO consumption. Together, these findings demonstrate early (mal)adaptive responses to HFEE at the lipid level that occur in the period preceding clear development of whole-body insulin resistance, and some of which are differentially mediated by FO intake.

Increased skeletal muscle ceramide levels have been consistently associated with obesity and the metabolic syndrome (77,90,91). Yet, to the best of our knowledge, this is the first investigation to detail changes in skeletal muscle ceramide levels in response to short-term, HFEE in the absence of insulin resistance. Following a longer (28 d) high-fat overfeeding intervention, Heilbronn and colleagues (108) reported significant increases in circulating total ceramide levels in sedentary, but otherwise healthy individuals; concomitant with impaired insulin sensitivity. Moreover,

significant increases in certain individual ceramide species were observed (C22:0, C24:0), but these were not the same species as those that were increased in the present investigation (C18:1, C22:1, C23:0, C24:1). The reason why levels of different species were altered between studies (particularly saturated *vs.* unsaturated fat differences) is unknown, but may reflect study design variances including the tissue sampled, type of dietary fat consumed, duration of overfeeding and / or insulin sensitivity of the participants. Based on current data from our study and others, we propose that changes in ceramide levels may precede the development of insulin resistance, with elevated levels maintained throughout insulin resistance development (108) and metabolic disease pathology (72).

Ceramides impair insulin sensitivity via their inhibitory effects on PKB. Specifically, ceramides activate PP2A and PKC isoforms, both of which dephosphorylate PKB (84). Inhibition of, or at least decreased, PKB activity will reduce downstream glucose uptake and hence PKB is an important control point for glucose homeostasis and insulin sensitivity. Although we failed to detect any overall mean changes in insulin sensitivity following HFEE, ceramide action may be otherwise examined by investigating changes in PKB activity. Elevated PKB activity was measured in the insulin-stimulated state compared to the basal state in all participants before and after HFEE. Whereas an insulin-stimulated rise in PKB is expected due to increased insulin signalling flux, the temporal response to oral glucose ingestion and its relationship with insulin sensitivity is currently unknown. A greater insulin-mediated PKB response at 2 h post-glucose ingestion is commonly considered as indicative of insulin *sensitivity*, whereby greater signalling flux results in elevated PKB activation (*e.g.* (223)). However, one also could argue that greater PKB activation 2 h post

glucose consumption is a sign of insulin *resistance*. The sensitivity of the metabolic system may be impaired if signalling proteins, including PKB, remain active after blood glucose levels have returned to baseline levels as evidenced in the present investigation. Future investigations of the temporal relationship between PKB and insulin signalling are required to better address these complexities.

PKB activity and insulin signalling may be modified by dietary fat consumption. Whereas the HFEE-mediated rise in ceramide levels occurred irrespective of FO consumption, the increase in insulin-stimulated PKB activity was maintained with FO consumption, but reduced in the absence of FO consumption, during HFEE. The reasons for these discrepancies are not immediately clear. However, observed changes in the phospholipid membrane, including a significant incorporation of EPA, and a tendency for total n-3 PUFA incorporation with FO consumption may relate to PKB and insulin signalling. Increased n-3 PUFA composition of the phospholipid membrane has been shown to positively associate with insulin sensitivity (21) and improve signalling kinetics (134,135). Thus, taken together, these data support the notion that changes in subcellular skeletal muscle-specific mechanisms occur in the early phases of HFEE, are perhaps differentially mediated by FO ingestion, and likely precede whole-body changes in insulin sensitivity.

The balance between ceramide synthesis and degradation within skeletal muscle will determine the amount of ceramide within skeletal muscle, and regulate any changes following a given stimulus (84). Fat delivery to tissue, fat transport across the plasma membrane and stimuli including SFA availability and activity of serine palmitoyltransferase (SPT) and associated enzymes, drive ceramide synthesis (84). In

contrast, the transport of lipids across the mitochondrial membrane, rate of fat oxidation, adiponectin concentrations and ceramidase activity are key determinants of ceramide degradation (224). Thus, investigation of the factors involved in these processes will facilitate understanding of the regulation of ceramide levels in tissue.

FA availability in muscle is determined, in part, by the delivery and uptake of fats from the plasma pool into muscle. Decreased circulating levels of TGs and FAs, as measured in the present investigation, may reflect a lipid shift from plasma into tissue. This lipid shift will result in increased adipose tissue mass and an increased lipid environment within tissue such as skeletal muscle, particularly when adipose tissue expansion becomes saturated (215). Adipose tissue spillover of FA, resulting in ectopic fat deposition, is thought to result in accumulation of toxic lipid metabolites including ceramides, and drive insulin resistance (215). Given our lack of change in insulin sensitivity, it is possible that ceramide accumulation has to be of great enough magnitude before the insulin resistant response is observed. A time course of ceramide accumulation mapped to insulin sensitivity change is required in future investigations to better understand development of these maladaptations.

Alternative explanations for the observed increase in ceramide levels may include a different stimulus for ceramide synthesis, for example, inflammation and other hormonal changes. Obesity and T2D are widely associated with low-grade inflammation (137) and it is this pro-inflammatory environment that, when uncontrolled, is considered highly detrimental for cellular function (225). TNF-α is one candidate inflammatory biomarker that has been shown to activate sphingomyelinase (SMase) and drive ceramide synthesis in a cell-free system

(85,226). Levels of circulating TNF-α, were not altered by HFEE at 6 d in the present study despite increases in skeletal muscle ceramide levels. However, it must be acknowledged that TNF-α was not measured in tissue and measurement of a solitary blood-based inflammatory marker is not as convincing as measurement of a panel of inflammatory proteins in tissue. Although the purported anti-inflammatory effects associated with FO consumption (137) were not apparent from the TNF- α measures in the current study, it is difficult to completely disregard these anti-inflammatory properties in the absence of altered inflammation in HF-C. Instead, the temporal resolution of an inflammatory response may offer clearer insight into such regulation.

The 'normal' counter-response to compensate for increased fat availability, storage and synthesis would be a shift towards oxidative fat metabolism. Changes in mitochondrial enzyme activities and / or the regulation of mitochondrial lipid oxidation may reflect lipid changes within skeletal muscle. AMPK α2 activity is widely considered to be important for fat oxidation (218,219,224). Activation of AMPK leads to increased CPT-1 activity via ACC-mediated malonyl-CoA suppression (224). Based on this mechanism, an increase in AMPK α2 activity, as seen with HFEE and FO consumption, will enhance fat oxidation, whereas a decrease in AMPK α2 activity (HF-C) will inhibit fat oxidation at the level of fat transport across the mitochondrial membrane. Following high-fat feeding in mice (227) and rats (228,229), AMPK activity was decreased, concomitant with impaired glucose tolerance, possibly due to reduced lipid oxidation. However, when we relate these observations to our increases in ceramide levels, which occurred irrespective of dietary FO consumption, the divergent group-responses of AMPK α2 activity are less clear. The mechanisms by which AMPK regulates energy balance are diverse in nature (217,230) and may be

related to adaptations distinct from CPT-1 and fat oxidation. Basal activity levels of two mitochondrial enzymes, citrate synthase and β-HAD, were not significantly altered following HFEE in the current study. These findings are consistent with a previous report by Samocha-Bonet and colleagues (112) who observed no changes in citrate synthase or β-HAD activity levels following 3 d or 28 d overfeeding. Finally, adiponectin, an adipose tissue derived cytokine, has been shown to increase ceramidase activity resulting in ceramide degradation to sphingosine (85). However, in the current investigation, no changes in plasma adiponectin concentrations were observed following HFEE, suggesting a lack of stimulus for ceramidase activity. Thus, taken together, these data suggest that fat oxidation and ceramide degradation remained relatively stable during the overfeeding period, and, instead, alternative mechanisms of ceramide regulation need to be explored in future investigations.

Finally, our initial hypotheses centring on possible associations between ceramide levels and PKB, AMPK α2 or mitochondrial enzyme activities were largely unsubstantiated. However, basal PKB activity pre-HFEE did significantly predict around ~19 % of the variance in the mean change in total ceramides over the course of HFEE. Whereas we cannot establish a cause and effect relationship from these data, it is interesting that higher basal PKB activity was associated with a greater increase in ceramide accumulation following HFEE. This finding perhaps infers that increased basal PKB activity is indicative of insulin *resistance* rather than insulin *sensitivity* although further work is necessary before this suggestion can be given credibility. Possible explanations for the lack of association between other seemingly associated parameters may relate to the temporal development of maladaptation in each of the measures and / or potential unaccounted covariate factors, *e.g.*, enzyme activity and /

or total content / activation of substrate proteins. Unquestionably, further work is required to better characterise these subcellular adaptations, and their associated mechanisms, if we are to better understand the pathology underlying short-term HFEE.

In conclusion, we present data to suggest that some lipid-mediated subcellular events result from HFEE that precede the clear development of whole-body insulin resistance. Circulating TG and FA concentrations were decreased, skeletal muscle ceramide levels increased, and basal mitochondrial enzyme activities were unchanged by HFEE, irrespective of FO consumption. However, FO consumption during HFEE differentially altered basal AMPK α2 activity and insulin-stimulated PKB activity compared to HFEE without FO intake. One potential explanation for the group-divergence in these findings is changes in the phospholipid membrane following FO ingestion. Alterations in the docking efficiency of transporter proteins in the plasma membrane, altered nutrient kinetics and signalling from membrane composition changes all may contribute to these groups discrepancies. Considering all findings were in the context of statistically unaltered insulin sensitivity, further work is required to elucidate the mechanisms of lipid regulation in the high-fat overfed state, as well as a time course of change in ceramide levels with declining whole-body insulin sensitivity.

**CHAPTER 4** Distinct subsets of skeletal muscle and plasma miRNAs are altered following short-term high-fat energy excess and / or following oral glucose consumption in healthy males.

**Wardle SL**, Macnaughton LS, McGlory C, Witard OCW, Galloway SR, Tipton KD, Moran CN.

*4.0. Abstract*

MiRNAs regulate gene expression within the cell. However, miRNAs also exist at stable levels within plasma. Changes in tissue and plasma miRNA levels have been associated with metabolic disease development. However, the impact of short-term high-fat energy excess (HFEE) and / or glucose consumption on plasma and skeletal muscle miRNA levels in humans is currently unknown. We investigated changes (basal and following oral glucose consumption) in miRNA levels following 6 d HFEE (150 % habitual energy intake; 60 % of energy from fat) in non-active healthy males (n=20). Ten of twenty participants consumed 10 % of total fats from fish oil (FO) sources. Levels of a number of miRNAs were significantly altered by HFEE in skeletal muscle (miR-106b-5p, miR-214-3p, miR-215) but not plasma. Certain miRNAs were responsive to the OGTT including miR-145-5p (plasma), and miR-193a-5p, miR-206 (skeletal muscle). No miRNAs in either tissue type were regulated by group alone; however, several group interactions existed with HFEE and / or the OGTT (miR-7-5p, miR-27a-3p (plasma), miR-18a-5p, miR-145-5p, miR-214-3p (skeletal muscle)). Basal levels of miR-145-5p (plasma) and miR-204-5p (skeletal muscle) significantly predicted 16-23 % of the change in HFEE-mediated insulin sensitivity. Certain miRNAs may be useful markers, and predictors, of the high-fat overfed state (with or without FO), the response to oral glucose consumption and / or the combined influence of these. However, clear associations between plasma and skeletal muscle levels of these miRNAs are lacking, suggesting that investigation of these plasma miRNAs within the context of HFEE may not inform functional outcomes within skeletal muscle, but may better relate to other tissue types.

*4.1. Introduction*

Various metabolic and molecular responses to 6 d HFEE have been presented in **Chapters 2 and 3**. These (mal)adaptive responses to nutrition and energy balance are driven by the regulation of gene expression. Whereas such (mal)adaptive responses are reasonably well characterised at the genome level, regulation by the epigenome is less well understood. Epigenetic control mechanisms, including miRNAs, and their regulation of gene expression have stimulated research interest since the discovery of miRNAs two decades ago (159).

The importance of miRNA regulation within tissue and the circulation has been investigated previously. MiRNAs exist in both tissue and the circulation, although their mechanism of action is confined to tissue. Given that skeletal muscle accounts for ~75 % of non-oxidative glucose disposal in the postprandial state (9,45), measurements of gene expression and post-translational modification in skeletal muscle are often made in studies investigating insulin sensitivity / resistance (*e.g.* (72)). However, post-transcriptional regulation, and in particular miRNA regulation, of gene expression has been less well studied within the context of insulin sensitivity. Only in the past 5 years, or so, has the role of miRNAs within T2D pathology been highlighted (231–236). Some researchers now have even suggested that T2D may be considered a miRNA-related disease (179). Thus, the impact of skeletal muscle miRNA regulation in insulin resistance pathology is apparent.

Investigation of skeletal muscle miRNA regulation provides insight into the underlying mechanism of insulin resistance. MiRNA profiling of skeletal muscle in T2D was initially confined to cell culture and rodent models of metabolic disease (*e.g.*

(237–240)). However, in recent years, reports from human studies have been published (*e.g.* (241,242)). These human studies largely provide descriptive / associative data, rather than data from interventions. However, these studies do present associations with insulin signalling genes (242) and glucose / insulin concentrations (241). These associations suggest potential for skeletal muscle miRNAs to be involved in the regulation of insulin signalling and glucose homeostasis.

Adipose tissue, liver and pancreatic β cells, like skeletal muscle, are involved in the regulation of energy excess and T2D pathology (38). Considering (mal)adaptive responses to energy excess span several tissue types, plasma miRNAs may be involved in this whole-body response - essentially communicating between insulin-responsive tissues (164). MiRNAs have been implicated in cell-cell communication (32) and are actively secreted into the circulation, rather than released in a non-specific manner, from tissue (171,172). For these reasons, plasma miRNAs may be useful biomarkers of a variety of adaptations.

Since the discovery of plasma miRNAs in 2008 (166,167), studies have investigated plasma miRNA levels in a variety of disease states, including T2D (33,164). However, much like the skeletal muscle miRNA studies, these data have been largely static in nature (*i.e.,* assessment of baseline levels). Nonetheless, these reports do lend support to the notion of altered miRNA regulation in diseased states. For example, analysis of data from a large (n = 822), prospective, population-based study (243), revealed lower plasma levels of miR-126 in individuals with T2D, but also in individuals with subsequent T2D (determined from 10 y follow-up samples). Indeed, miR-126 was a significant predictor of the T2D state (243). Moreover, a significant increase in the

levels of a battery of miRNAs involved in insulin biosynthesis and secretion was noted in T2D patients compared to controls (244). Although, in contrast to the findings with miR-126 (243), there was no significant difference between levels of these miRNAs in individuals with pre-diabetes *vs.* controls (244). These data suggest that different miRNAs may be involved in the regulation of gene expression along the continuum between initial development of insulin resistance and manifest T2D.

The involvement of plasma miRNAs in the regulation of skeletal muscle adaptation is now being considered (245). However, to the best of our knowledge, the association between miRNA levels in skeletal muscle and plasma has not been previously investigated. The usefulness and validity of miRNA biomarkers for understanding the underlying mechanisms of miRNA regulation and insulin resistance would be enhanced if they were highly related to functional tissue changes. Therefore, measurement of plasma miRNA levels in combination with tissue miRNA levels is warranted to facilitate mechanistic understanding of miRNA, and subsequent gene, regulation.

Our aims were three-fold. First, we aimed to investigate miRNA levels of both plasma and skeletal muscle samples in our healthy male volunteers subject to short-term, HFEE, with and without FO consumption. In addition to investigating the influence of HFEE on basal miRNA levels, we aimed to characterise the miRNA response to an oral glucose load, and to investigate whether fluctuations in miRNA levels during the course of an OGTT were altered by diet. Finally, we aimed to investigate the degree of association between plasma and skeletal muscle miRNA levels, and between these miRNA levels and various measures presented in **Chapters 2 and 3**. We hypothesised

that plasma and skeletal muscle miRNA levels would be altered by HFEE (both basal and the OGTT response) and that some associations would exist between miRNA levels in different sample types and other metabolic / molecular measures.

*4.2. Methods*

*4.2.1. Plasma miRNA analysis*

**RNA extraction**

Total RNA was extracted from plasma samples using commercially available RNA isolation kits (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. The starting plasma volume was 200 µL and the recovered RNA was eluted in 50 µL of RNase free water. Complementary DNA (cDNA) was synthesised using the Qiagen miScript RT II kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions in a final volume of 20 µL. A quality control (QC) plate array (Qiagen Ltd., West Sussex, UK) was performed to confirm cDNA synthesis efficiency (data not shown) prior to further analysis.

**Plasma plate arrays**

Plasma plate arrays (Qiagen Ltd., West Sussex, UK) were prepared using SYBR Green mastermix (Qiagen Ltd., West Sussex, UK) in a final reaction volume of 15 µL. The final reaction volume comprised 7.5 µL SYBR Green, 1.5 µL 10x miScript universal primer, 5.45 µL RNase free water and 0.55 µL cDNA. In total, 12 plasma array plates were prepared using pooled samples from each experimental condition (HF-C; pre- and post-HFEE, and HF-FO; pre- and post-HFEE). Three plates for each condition were used to calculate cycle threshold (Ct) values in triplicate. Real-time quantitative polymerase chain reactions (qRT-PCR) were performed on the Roche LightCycler480 (Roche, Basal, Switzerland). Reactions involved an initial 10 min incubation at 95 °C followed by 45 amplification cycles of 95 °C for 10 s and 60 °C for 1 min with a ramp rate of 1.6 $°C \cdot s^{-1}$ and an optical read at the end of each cycle. Reactions were completed with a melting curve for quality control to ensure that only a single

amplicon was present in each reaction. Baseline subtracted Ct values were imported into SABiosciences software (246) to analyse the amplification data.

The plate array data (**Appendices 4.1 and 4.2**) were used to inform subsequent plasma qPCR analysis of candidate miRNA assays. In addition to using the recommended SNORD genes as internal controls, two other methods of selecting the best reference genes were used. First, Normfinder software (247) was used to detect the most stable gene, or set of genes, within the samples, and second, the average Ct value for each plate was used as a reference. Using all of these methods, the SNORDS, miR-25-3p and miR-27a-3p were all deemed suitable reference genes, and were individually used within $2^{-\Delta\Delta Ct}$ calculations (248), along with HF-C, pre-HFEE as the control group. Establishing the appropriate reference gene is crucial to account for variation in reaction components and ensure that any measured changes are indicative of the intervention rather than changes in experimental factors (249). The miRNAs that were significantly different in the post-HFEE pools and / or between groups were taken forward into analysis with individual primer assays in individual samples. These selected miRNAs included; miR-7-5p, miR-15-5p, miR-18a-5p, miR-22-3p, miR-25-3p (reference gene for plasma assays), miR-27a-3p (reference gene for skeletal muscle assays), miR-30d-5p, miR-93-5p, miR-100-5p, miR-106b-5p, miR-126-3p, miR-145-5p, miR-193a-5p, miR-196a-5p, miR-204-5p, miR-214-3p, miR-215 and miR-885a-5p. Additionally, the myomiRs, miR-1 and miR-206, were measured in skeletal muscle samples only.

**Individual primer assays**

The same RNA eluates used for the plasma plate arrays were used for all subsequent plasma miRNA experiments. However, for the individual assays, cDNA was synthesised using 2 µL 5x reaction buffer, 5 µL nuclease free water, 1 µL enzyme mix and 2 µL template RNA (Exiqon, Vedbaek, Denmark). cDNA synthesis reactions were incubated at 42 °C for 60 min, heated to 95 °C for 5 min and then cooled to 4 °C prior to storage at -20 °C pending further analysis. Samples were diluted 1 in 40 with RNase free water and transferred to deep-well plates for long term storage at -20 °C.

Commercially available qRT-PCR primer assays (Exiqon, Vedbaek, Denmark) were used for miRNA analysis, with selection of target miRNAs determined from the plate array analysis (see, **Plasma plate arrays**). SYBR Green reactions (10 µL) comprised 5 µL PCR mastermix, 1 µL PCR primer mix and 4 µL cDNA dilution (Exiqon, Vedbaek, Denmark). MiRNA levels were measured in triplicate. Starting with an initial 1 in 2 dilution, a standard curve (1 in 4 dilution series, using a pooled sample consisting of cDNA from all participants in both groups) was included on each plate to ensure all assays were in the linear range. qRT-PCR were performed in the same manner as described for **Plasma plate arrays**, and similarly with a melting curve for quality control to ensure that only a single amplicon was present in each reaction. All Ct values were within the linear range of the standard curve. Ct outliers were removed using the median absolute deviation method (also known as the modified Z-score (208)) with the maximum acceptable threshold set at 3.5. Control samples (pooled cDNA from each participant) were included on each plate to allow standardisation of the Ct values for experiments that spanned several plates.

**Reference gene selection**

Normfinder software (247) was used to determine which single gene or group of genes was at the most stable level within the samples. For plasma samples, miR-25-3p alone was found to be most stable over time and across both groups; for skeletal muscle samples the most stable gene was miR-27a-3p. This consistency in reference gene stability across both methods of miRNA analysis (*i.e.* plate arrays and individual primer assays) gives us confidence in our selection of reference genes. These genes were therefore used to determine the relative expression of the target genes within each sample type (*i.e.* plasma / muscle), calculated using the $2^{-\Delta\Delta Ct}$ method described previously with HF-C, pre-HFEE as the control group.

*4.2.2. Skeletal muscle miRNA analysis*

Skeletal muscle samples (~20 mg) were homogenised, and RNA extracted, using the Qiagen AllPrep DNA/RNA/miRNA universal kit (Qiagen, Ltd., West Sussex, UK) according to the manufacturers' instructions. Prior to performing cDNA synthesis, all RNA samples were adjusted to a concentration of $5ng \cdot \mu L^{-1}$ with nuclease free water. cDNA synthesis reactions were performed using the same reagents and reagent volumes as those outlined for plasma samples (see, **Individual primer assays**). cDNA samples were diluted 1 in 80 using nuclease free water and then followed the same processing and qRT-PCR procedure as plasma samples (see, **Individual primer assays**).

*4.2.3. miRNA target prediction analysis: miRSystem*

Given no miRNA-mRNA analyses were performed in the current investigation we used the web-based tool, miRSystem to predict gene targets of our differentially regulated miRNAs. All differentially regulated miRNAs were entered into the model simultaneously (per tissue type and per regulatory subset (*i.e.* HFEE / OGTT)). Separate models were used for each subset of regulation and each tissue type. This analysis was conducted to ascertain whether common mRNA targets were shared to a significant extent by these miRNAs.

*4.2.4. Statistical analysis*

Power calculations for the study design have been discussed in **Chapter 2**. Separate power calculations were not performed for the miRNA analysis as no prior data existed relating to changes in miRNA levels following short-term HFEE.

Normality of logged miRNA data was assessed using the Ryan Joiner test (Minitab version 16; Minitab, State College, PA). In the case of non-normal distributions, Box-Cox transformations were performed on miRNA fold-change data, and the data transformed accordingly, before re-testing for normality. All samples were normally distributed before conducting statistical analyses. Between-group differences in miRNA levels pre- and post-HFEE and at various time-points during the OGTT were determined by ANOVA (2 x 3 x 2 mixed design (plasma) / 2 x 2 x 2 mixed design (muscle); IBM SPSS Statistics 19, Chicago, IL, USA). *Post-hoc* tests were not conducted due to only having 2 groups in the model (HF-C *vs.* HF-FO). Regression analyses were

conducted between plasma and skeletal muscle miRNA levels, and between miRNA levels from both tissue types and Matsuda insulin sensitivity data presented in **Chapter 2** using Minitab software (version 16; Minitab, State College, PA).

*4.3. Results*

*4.3.1. Plasma miRNAs (individual assays)*

Eighteen skeletal muscle miRNAs were measured in both HF-C and HF-FO groups, pre- and post-HFEE, at 0 and 2 h OGTT time-points, as well as the time-point during the OGTT that corresponded to peak plasma insulin levels. However, only 13 of these measured miRNAs were consistently detected in plasma, thus the other 5 (miR-193a-5p, miR-196a-5p, miR-204-5p, miR-214-3p, miR-885-5p) were removed from subsequent analyses. **Table 4.1** shows mean relative levels for each of the detectable test miRNAs (n=12) standardised to the reference gene (miR-25-3p) and to baseline levels in the HF-C group.

**HFEE-responsive miRNAs**

There were no statistically significant changes in plasma levels of any miRNAs in response to the 6 d period of HFEE. There was no interactive effect of group on the plasma miRNA response to HFEE.

**OGTT-responsive miRNAs**

Transient changes in miR-145-5p were observed over the course of the OGTT, irrespective of group ($p = 0.02$; **Figure 4.1C**). Specifically, peak miRNA levels were observed at the time-point corresponding to peak insulin levels, with fairly similar lower levels at 0 and 2 h.

**Interactive HFEE and OGTT-responsive miRNAs**

Two miRNAs (miR-7-5p and miR-27a-3p) were significantly altered ($p < 0.05$; **Figure 4.1A-B**) over the course of the OGTT in a different manner following HFEE compared

to pre-HFEE. Post-HFEE, levels of both miRNAs were highest at 0 h. For miR-7-5p, levels at the time-point corresponding to peak plasma insulin were the lowest of the three measures, whereas for miR-27a-3p, the lowest levels were observed at 2 h. Pre-HFEE, both miRNAs responded differently to the OGTT. Levels of miR-7-5p increased throughout the OGTT so that the lowest levels were observed at 0 h, intermediate levels at the time-point corresponding to peak plasma insulin, and the highest levels at 2 h. For miR-27a-3p, the lowest levels were observed at the time-point corresponding to peak plasma insulin levels, with the highest levels at 2 h, and intermediate levels at 0 h.

**Group-responsive miRNAs**

No miRNAs were significantly altered by group in the absence of any interaction with the within-subject factors (*i.e.* HFEE and / or OGTT).

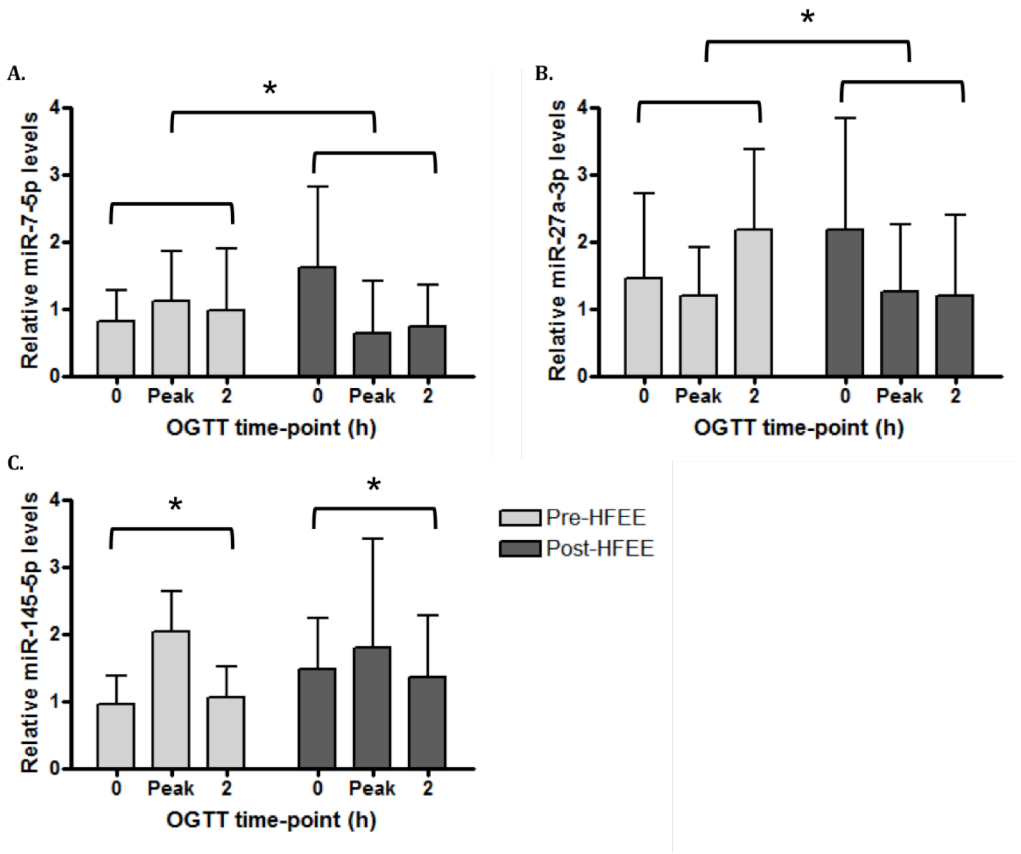



**Figure 4.1** Plasma levels of miR-7-5p (A), miR-27a-3p (B) and miR-145-5p (C). Plots A and B show HFEE x OGTT-responsive miRNAs, whereas miR-145-5p in plot C is OGTT-responsive only; * $p < 0.05$ by ANOVA. Values are means ± 95 % CIs (backtransformed data), groups (HF-C and HF-FO (n=10 per group)) have been pooled for data presentation.

**Table 4.1** Plasma miRNA levels.

| | HF-C | | | | | | HF-FO | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pre-HFEE | | | Post-HFEE | | | Pre-HFEE | | | Post-HFEE | | |
| | Basal | Peak | 2 h | Basal | Peak | 2 h | Basal | Peak | 2 h | Basal | Peak | 2 h |
| **miR-7-5p** | **1.00** (0.58-1.73) | **1.18** (0.63-2.20) | **0.52** (0.17-1.57) | **1.25** (0.48-3.26) | **0.41** (0.10-1.66) | **0.68** (0.26-1.78) | **0.66** (0.32-1.36) | **1.07** (0.47-2.43) | **1.85** (1.13-3.03) | **2.08** (1.18-3.66) | **0.98** (0.45-2.15) | **0.81** (0.38-1.71) |
| **miR-15-5p** | **1.00** (0.54-1.85) | **1.82** (0.99-3.35) | **1.47** (1.00-2.15) | **1.53** (1.12-2.08) | **1.66** (0.94-2.91) | **1.54** (0.83-2.82) | **1.79** (0.86-3.70) | **2.13** (1.09-4.16) | **2.29** (1.40-3.75) | **1.97** (0.96-4.07) | **1.45** (0.72-2.91) | **1.30** (0.75-2.23) |
| **miR-18a-5p** | **1.00** (0.71-1.41) | **1.49** (0.95-2.34) | **1.13** (0.72-1.76) | **1.22** (0.84-1.77) | **1.25** (0.65-2.39) | **1.02** (0.66-1.57) | **1.00** (0.64-1.57) | **1.61** (1.01-2.58) | **1.25** (0.77-2.02) | **2.11** (1.40-3.20) | **1.39** (0.79-2.46) | **1.45** (0.87-2.42) |
| **miR-22-3p** | **1.00** (0.68-1.46) | **1.19** (0.71-2.01) | **1.80** (1.23-2.64) | **1.61** (1.09-2.37) | **1.25** (0.77-2.04) | **1.08** (0.71-1.64) | **1.15** (0.69-1.92) | **1.24** (0.73-2.09) | **1.48** (0.83-2.63) | **1.41** (0.90-2.23) | **1.24** (0.51-3.02) | **1.22** (0.82-1.82) |
| **miR-27a-3p** | **1.00** (0.44-2.25) | **1.17** (0.59-2.32) | **1.86** (1.04-3.33) | **2.24** (0.85-5.90) | **1.47** (0.57-3.79) | **1.17** (0.50-2.73) | **2.14** (0.86-5.34) | **1.20** (0.59-2.42) | **2.52** (1.27-4.98) | **2.12** (1.12-4.02) | **1.06** (0.51-2.22) | **1.22** (0.39-3.83) |
| **miR-30d-5p** | **1.00** (0.33-3.01) | **0.79** (0.29-2.19) | **1.05** (0.36-3.07) | **1.30** (0.49-3.50) | **0.77** (0.17-3.38) | **0.63** (0.19-2.05) | **0.93** (0.39-2.23) | **0.56** (0.20-1.60) | **0.90** (0.32-2.56) | **1.39** (0.38-5.11) | **1.01** (0.25-4.02) | **0.76** (0.26-2.24) |
| **miR-93-5p** | **1.00** (0.60-1.67) | **1.84** (1.23-2.74) | **0.83** (0.37-1.86) | **1.39** (1.00-1.93) | **1.27** (0.86-1.89) | **1.16** (0.83-1.61) | **1.22** (0.86-1.73) | **1.66** (1.24-2.22) | **1.02** (0.60-1.73) | **1.31** (0.86-1.99) | **1.14** (0.70-1.86) | **1.29** (0.88-1.89) |
| **miR-100-5p** | **1.00** (0.61-1.65) | **0.93** (0.64-1.35) | **0.93** (0.59-1.47) | **0.84** (0.53-1.33) | **0.65** (0.40-1.05) | **0.78** (0.41-1.51) | **0.82** (0.46-1.45) | **0.92** (0.52-1.65) | **0.85** (0.31-2.33) | **0.90** (0.50-1.62) | **1.13** (0.69-1.85) | **0.80** (0.28-2.24) |
| **miR-106b-5p** | **1.00** (0.68-1.48) | **1.48** (0.89-2.47) | **1.08** (0.69-1.71) | **1.48** (1.07-2.05) | **1.49** (0.94-2.37) | **0.95** (0.52-1.74) | **1.06** (0.61-1.85) | **1.91** (1.18-3.08) | **1.16** (0.68-1.98) | **2.09** (1.27-3.44) | **1.90** (1.11-3.27) | **1.58** (0.88-2.82) |
| **miR-126-3p** | **1.00** (0.58-1.72) | **2.04** (1.36-3.04) | **1.24** (0.78-1.98) | **1.72** (1.22-2.42) | **1.62** (0.68-3.88) | **0.97** (0.70-1.34) | **1.19** (0.63-2.25) | **1.52** (0.85-2.71) | **1.62** (0.77-3.41) | **1.68** (1.10-2.57) | **1.46** (0.80-2.68) | **1.59** (0.76-3.31) |
| **miR-145-5p** | **1.00** (0.56-1.79) | **2.31** (1.53-3.48) | **1.11** (0.70-1.77) | **2.03** (1.00-4.14) | **2.14** (0.66-7.00) | **1.24** (0.80-1.93) | **0.93** (0.64-1.36) | **1.79** (1.29-2.46) | **0.99** (0.54-1.81) | **1.08** (0.77-1.54) | **1.50** (0.85-2.64) | **1.49** (0.57-3.88) |
| **miR-215** | **1.00** (0.71-1.41) | **1.24** (0.93-1.65) | **0.72** (0.36-1.45) | **1.18** (0.79-1.77) | **0.89** (0.57-1.39) | **0.55** (0.28-1.06) | **0.95** (0.58-1.57) | **1.41** (0.86-2.32) | **1.41** (0.96-2.09) | **1.01** (0.49-2.06) | **0.82** (0.57-1.18) | **0.82** (0.45-1.49) |

Plasma miRNA levels measured at 3 time-points during the OGTT (Basal = 0 h (pre-OGTT), Peak = time-point corresponding to peak plasma insulin concentration, 2 h = 2 h post-OGTT) pre- and post-HFEE, for both groups (HF-C and HF-FO; n=10 per group). Values are backtransformed means (95 % CIs). MiRNAs that were significantly ($p < 0.05$) altered by HFEE, OGTT or group are displayed in **Figure 4.1**.

**Table 4.2** Skeletal muscle miRNA levels.

| | HF-C | | | | HF-FO | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-HFEE | | Post-HFEE | | Pre-HFEE | | Post-HFEE | |
| | Basal (0 h) | 2 h | Basal (0 h) | 2 h | Basal (0 h) | 2 h | Basal (0 h) | 2 h |
| **miR-1** | **1.00** (0.61-1.65) | **0.92** (0.65-1.31) | **1.02** (0.55-1.88) | **0.80** (0.62-1.02) | **0.94** (0.60-1.46) | **1.12** (0.69-1.82) | **1.07** (0.72-1.59) | **0.61** (0.44-0.86) |
| **miR-7-5p** | **1.00** (0.26-3.80) | **1.85** (0.92-3.70) | **2.42** (1.26-4.63) | **1.70** (0.67-4.30) | **1.13** (0.59-2.15) | **2.29** (1.02-5.13) | **2.53** (1.89-3.38) | **1.50** (0.63-3.53) |
| **miR-15-5p** | **1.00** (0.58-1.72) | **0.73** (0.39-1.37) | **0.95** (0.71-1.26) | **0.93** (0.51-1.68) | **0.94** (0.67-1.32) | **1.28** (0.68-2.42) | **1.18** (0.73-1.92) | **1.05** (0.73-1.52) |
| **miR-18a-5p** | **1.00** (0.60-1.68) | **0.76** (0.42-1.37) | **0.86** (0.34-2.16) | **1.40** (0.83-2.38) | **0.65** (0.26-1.61) | **1.00** (0.49-2.07) | **1.35** (0.43-4.27) | **0.64** (0.27-1.51) |
| **miR-22-3p** | **1.00** (0.59-1.71) | **1.10** (0.92-1.32) | **1.41** (1.05-1.88) | **0.93** (0.65-1.34) | **1.10** (0.73-1.64) | **1.21** (0.92-1.60) | **1.18** (0.98-1.43) | **1.10** (0.75-1.62) |
| **miR-25-3p** | **1.00** (0.52-1.93) | **1.31** (0.88-1.95) | **1.32** (0.90-1.92) | **1.65** (1.19-2.30) | **1.00** (0.49-2.01) | **1.85** (1.19-2.87) | **1.18** (0.63-2.22) | **1.79** (1.22-2.61) |
| **miR-30d-5p** | **1.00** (0.64-1.57) | **1.63** (1.03-2.57) | **1.24** (0.72-2.12) | **0.97** (0.58-1.61) | **1.18** (0.76-1.83) | **1.47** (1.11-1.95) | **1.40** (0.92-2.14) | **1.47** (0.91-2.38) |
| **miR-93-5p** | **1.00** (0.65-1.54) | **1.24** (0.82-1.89) | **1.47** (0.95-2.26) | **1.46** (0.88-2.43) | **0.96** (0.53-1.73) | **1.65** (1.14-2.40) | **1.43** (0.95-2.16) | **1.04** (0.68-1.59) |
| **miR-100-5p** | **1.00** (0.56-1.78) | **0.87** (0.55-1.38) | **1.33** (1.08-1.65) | **1.01** (0.59-1.72) | **1.32** (0.91-1.93) | **1.34** (0.81-2.21) | **1.21** (0.80-1.82) | **1.52** (0.77-3.01) |
| **miR-106b-5p** | **1.00** (0.68-1.47) | **0.65** (0.45-0.95) | **1.42** (0.97-2.08) | **0.78** (0.58-1.05) | **0.72** (0.60-0.87) | **0.96** (0.70-1.31) | **1.36** (0.89-2.08) | **0.91** (0.53-1.57) |
| **miR-126-3p** | **1.00** (0.49-2.04) | **1.53** (0.92-2.53) | **1.41** (0.88-2.27) | **1.31** (0.81-2.12) | **1.53** (0.81-2.91) | **1.78** (1.09-2.92) | **1.43** (0.81-2.51) | **1.64** (0.93-2.88) |
| **miR-145-5p** | **1.00** (0.54-1.86) | **1.12** (0.60-1.20) | **1.62** (1.03-2.55) | **1.63** (0.90-2.97) | **1.52** (0.75-3.08) | **1.74** (1.06-2.87) | **1.54** (0.68-3.49) | **1.28** (0.69-2.37) |
| **miR-193a-5p** | **1.00** (0.48-2.06) | **1.85** (1.07-3.18) | **1.08** (0.64-1.82) | **1.79** (1.36-2.36) | **1.07** (0.56-2.04) | **2.29** (1.35-3.90) | **0.88** (0.62-1.25) | **1.68** (1.10-2.56) |
| **miR-196a-5p** | **1.00** (0.44-2.29) | **1.58** (1.02-2.45) | **1.45** (0.56-3.73) | **1.53** (0.91-2.57) | **1.60** (0.86-2.97) | **1.81** (0.96-3.42) | **1.14** (0.73-1.79) | **1.63** (0.97-2.75) |
| **miR-204-5p** | **1.00** (0.50-2.01) | **0.73** (0.29-1.84) | **1.37** (0.63-2.97) | **0.85** (0.20-3.52) | **1.00** (0.39-2.51) | **1.89** (0.66-5.42) | **1.20** (0.67-2.12) | **0.45** (0.05-3.83) |
| **miR-206** | **1.00** (0.59-1.70) | **2.30** (1.59-3.31) | **1.46** (0.73-2.95) | **1.72** (1.00-2.94) | **1.28** (0.65-2.54) | **2.22** (1.83-2.70) | **1.39** (0.71-2.73) | **1.68** (1.02-2.77) |
| **miR-214-3p** | **1.00** (0.54-1.85) | **2.12** (1.37-3.28) | **1.05** (0.67-1.66) | **1.57** (0.89-2.77) | **1.00** (0.40-2.51) | **2.27** (1.79-2.89) | **1.30** (0.60-2.83) | **1.02** (0.58-1.77) |
| **miR-215** | **1.00** (0.21-4.78) | **0.74** (0.17-3.30) | **1.39** (0.32-6.03) | **1.07** (0.25-4.62) | **0.37** (0.08-1.66) | **1.32** (0.27-6.56) | **1.17** (0.28-4.82) | **1.18** (0.29-4.77) |
| **miR-885-5p** | **1.00** (0.46-2.19) | **1.74** (1.23-2.46) | **1.63** (0.65-4.09) | **1.19** (0.73-1.92) | **1.02** (0.60-1.74) | **1.67** (1.20-2.31) | **1.27** (0.75-2.16) | **2.16** (1.48-3.16) |

Data presented from 2 time-points during the OGTT (Basal = 0 h (pre-OGTT), 2 h = 2 h post-OGTT) pre- and post-HFEE, for both groups (HF-C and HF-FO; n=9 per group). Values are backtransformed means (95 % CIs). MiRNAs that were significantly ($p < 0.05$) altered by HFEE, OGTT or group are displayed in **Figure 4.2 and 4.3**.

*4.3.2. Skeletal muscle miRNAs (individual assays)*

From the initial plate array analysis, twenty of these miRNAs were measured in skeletal muscle samples from both HF-C and HF-FO groups, pre- and post-HFEE, at 0 and 2 h OGTT time-points. **Table 4.2** shows mean relative levels for each of the test miRNAs (n = 19) standardised to the reference gene (miR-27a-3p) and to baseline (0 h) levels in the HF-C group.

**HFEE-responsive miRNAs**

Three of the measured skeletal muscle miRNAs were significantly altered by HFEE, irrespective of group. These miRNAs included miR-106b-5p and miR-215, levels of which were higher post-HFEE, and miR-214-3p which was reduced following HFEE ($p < 0.05$; **Figure 4.2A-C**). One miRNA (miR-145-5p) was significantly altered following 6 d HFEE, but differently between the groups ($p < 0.05$ (interaction); **Figure 4.2D**). miR-145-5p levels in HF-C were increased with HFEE, yet decreased following HFEE in HF-FO.

**OGTT-responsive miRNAs**

Levels of two of the measured skeletal muscle miRNAs (miR-193a-5p and miR-206) were significantly higher at 2 h of the OGTT compared to pre-OGTT (0 h; $p < 0.05$; **Figure 4.3A-B**).

**Interactive HFEE and OGTT-responsive miRNAs**

Skeletal muscle levels of miR-214-3p were significantly altered over the course of the OGTT in a different manner following HFEE compared to pre-HFEE ($p = 0.02$; **Figure 4.3C**). The magnitude of the increase at 2 h pre-HFEE was much greater than the

negligible increases at 2 h post-HFEE. Adding group into this interaction to create a 3-way interaction (group x OGTT x HFEE) resulted in one miRNA, miR-18a-5p, being significantly altered ($p < 0.01$); **Figure 4.3D**). Levels of miR-18a-5p were increased at 2 h pre-HFEE, yet reduced at 2 h post-HFEE in HF-FO, whereas the opposite pattern was true for HF-C.

### Group-responsive miRNAs

Like plasma, no miRNAs were significantly altered by group in the absence of any interaction with the within-subject factors (*i.e.* HFEE and / or OGTT).

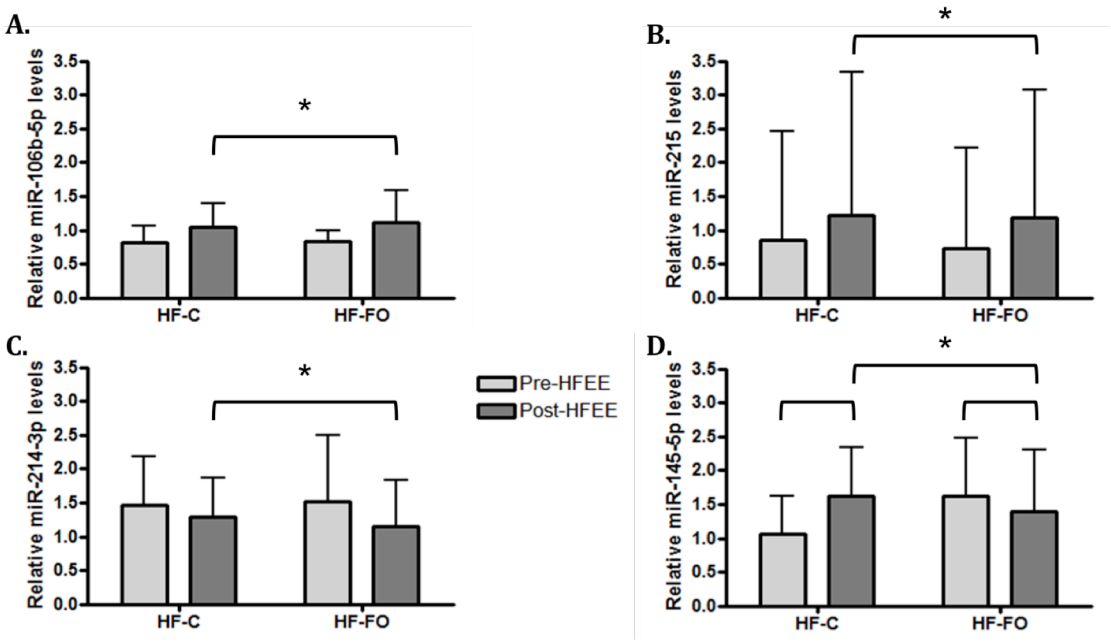



**Figure 4.2** Skeletal muscle levels (collapsed OGTT time-points) of miR-106b-5p (A), miR-215 (B), miR-214-3p (C) and miR-145-5p (D). Plots A, B and C show HFEE-responsive miRNAs, whereas miR-145-5p in plot D is HFEE x Group-responsive; * $p < 0.05$ by ANOVA. Values are means ± 95 % CIs (backtransformed data), n = 9 per group.

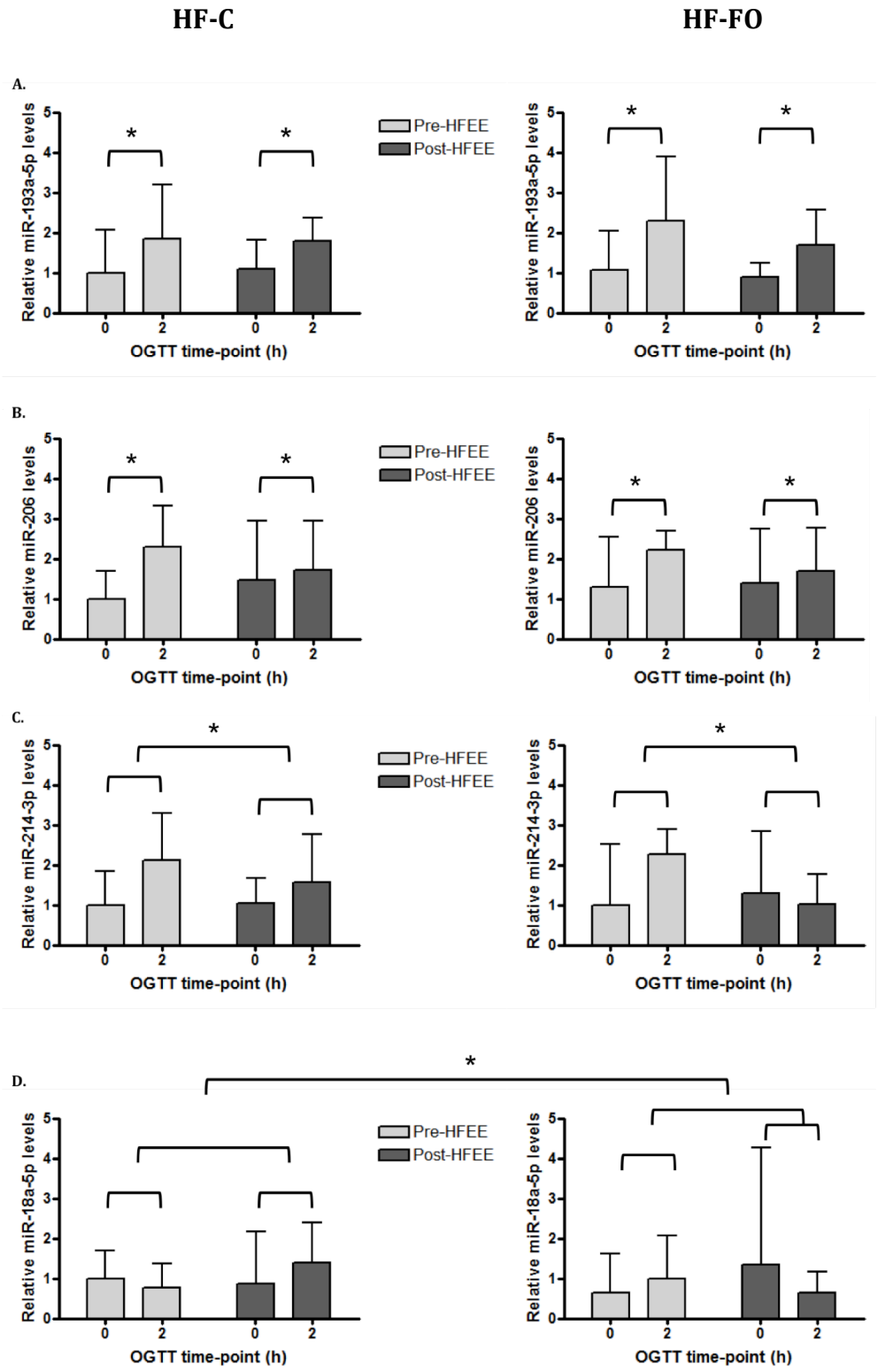



**Figure 4.3** Skeletal muscle levels of miR-193a-5p (A), miR-206 (B), miR-214-3p (C) and miR-18a-5p (D). Plots on the left-hand side are from HF-C, whereas all plots on the right-hand side are from HF-FO (n = 9 per group). Plots A and B present OGTT-responsive miRNAs, whereas miR-214-3p in plot C is HFEE x OGTT-responsive and miR-18a-5p in plot D is HFEE x OGTT x Group-responsive; * $p < 0.05$ by ANOVA. Values are means ± 95 % CIs (backtransformed data).

*4.3.3. Correlations*

**Skeletal muscle vs. plasma miRNAs**

Skeletal muscle levels of miR-7-5p, miR-93-5p and miR-100-5p explained between 11 and 16 % of the variance around the mean plasma levels of the corresponding miRNA. However, these associations were not statistically significant. With the exception of miR-30d-5p, which explained 5 % of the variance, skeletal muscle levels of all other miRNAs explained less than 1 % of the variance in plasma miRNA levels ($p > 0.39$; **Appendix 4.3**).

**Plasma miRNAs vs. Matsuda ISI**

Basal plasma miR-145-5p levels pre-HFEE significantly predicted 16 % of the variance in mean changes in insulin sensitivity (Matsuda) over the course of HFEE ($p = 0.04$). Plasma levels of miR-27a-3p, miR-30d-5p, miR-106b-5p and miR-126-3p predicted between 11 and 12 % of the variance in mean changes in insulin sensitivity, but not to a statistically significant extent. All other plasma miRNAs predicted less than 8 %, and most less than 2 %, of the variance in mean changes in insulin sensitivity ($p > 0.12$; **Appendix 4.3**).

**Skeletal muscle miRNAs vs. Matsuda ISI**

Skeletal muscle basal levels of miR-204-5p, pre-HFEE significantly predicted 23 % of the variance in mean changes in insulin sensitivity (Matsuda) over the course of HFEE ($p = 0.03$). Skeletal muscle levels of miR-22-3p, miR-193a-5p and miR-214-3p predicted between 11 and 16 % of the variance in mean changes in insulin sensitivity but these associations were not statistically significant. All other skeletal muscle

miRNAs predicted less than 10 %, and most less than 3 %, of the variance in mean changes in insulin sensitivity ($p > 0.11$; **Appendix 4.3**).

**miRNA target prediction analysis: miRSystem**

Predictions using miRSystem indicated that plasma OGTT-responsive (miR-7-5p, miR-27a-3p, miR-145-5p) and skeletal muscle HFEE- (miR-106b-5p, miR-214-3p, miR-215) and OGTT-responsive (miR-193a-5p, miR-206, miR-214-3p) miRNAs target mRNAs involved in a total of 765, 779 and 762 pathways across all integrated databases respectively (data not shown). The top 10 pathways targeted by these responsive miRNA clusters are shown in **Tables 4.3 - 4.5**.

**Table 4.3** miRSystem pathway analysis of relative plasma OGTT-responsive miRNA levels (presented in **Figure 1**).

| Database | Pathway | Total union targets (of total genes) in the pathway | Score |
|---|---|---|---|
| Kegg | Axon guidance | 43 (129) | 7.911 |
| Reactome | Axon guidance | 69 (266) | 7.351 |
| Reactome | Developmental biology | 101 (494) | 7.157 |
| Kegg | MAPK signalling pathway | 65 (272) | 5.771 |
| Kegg | Focal adhesion | 50 (199) | 5.749 |
| Kegg | ErbB signalling pathway | 28 (87) | 5.329 |
| Kegg | Regulation of actin cytoskeleton | 50 (213) | 5.148 |
| Reactome | L1CAM interactions | 30 (94) | 4.847 |
| Kegg | Neurotrophin signalling pathway | 35 (127) | 4.678 |
| Kegg | Pathways in cancer | 61 (325) | 4.676 |

Union targets refer to genes targeted by all 3 responsive miRNAs. Score is deduced from the sum of the weight of its miRNA multiplied by its enrichment, minus the log (p value) from the predicted gene targets. The weight for one miRNA is calculated by dividing its absolute expression value by the absolute sum of the expression values for all input miRNAs (250).

**Table 4.4** miRSystem pathway analysis of relative skeletal muscle HFEE-responsive miRNA levels (presented in **Figure 2A-C**).

| Database | Pathway | Total union targets (of total genes) in the pathway | Score |
|---|---|---|---|
| Reactome | Axon guidance | 95 (266) | 5.304 |
| Reactome | Signalling by NGF | 130 (494) | 4.564 |
| Kegg | Pathways in cancer | 51 (129) | 4.192 |
| Reactome | Haemostasis | 122 (467) | 4.088 |
| Reactome | Developmental biology | 93 (325) | 3.901 |
| Kegg | Axon guidance | 72 (201) | 3.762 |
| Reactome | Cyclin-D-associated events in G1 | 68 (221) | 3.734 |
| Reactome | G1 phase | 41 (94) | 3.591 |
| Reactome | Rho-GTPase cycle | 78 (272) | 3.318 |
| Reactome | Signalling by Rho-GTPases | 47 (136) | 3.204 |

Union targets refer to genes targeted by all 3 responsive miRNAs (excluding miR-145-5p which was HFEE*Group responsive). Score is deduced from the sum of the weight of its miRNA multiplied by its enrichment, minus the log (p value) from the predicted gene targets. The weight for one miRNA is calculated by dividing its absolute expression value by the absolute sum of the expression values for all input miRNAs (250).

**Table 4.5** miRSystem pathway analysis of relative skeletal muscle OGTT-responsive miRNA levels (presented in **Figure 3A-C**).

| Database | Pathway | Total union targets (of total genes) in the pathway | Score |
|---|---|---|---|
| Reactome | Axon guidance | 50 (266) | 4.930 |
| Reactome | Hemostasis | 68 (467) | 4.273 |
| Reactome | Signalling by NGF | 42 (221) | 3.795 |
| Reactome | Developmental biology | 65 (494) | 3.510 |
| Reactome | Platelet activation signalling and aggregation | 35 (205) | 3.232 |
| Reactome | NGF signalling via TrkA from the plasma membrane | 29 (136) | 3.090 |
| Kegg | Axon guidance | 25 (129) | 3.061 |
| Kegg | Pathways in cancer | 46 (325) | 3.059 |
| Kegg | Bacterial invasion of epithelial cells | 19 (70) | 2.844 |
| Reactome | Neuronal system | 39 (289) | 2.805 |

Union targets refer to genes targeted by all 3 responsive miRNAs (excluding miR-18a-5p which was HFEE*OGTT*Group responsive). Score is deduced from the sum of the weight of its miRNA multiplied by its enrichment, minus the log (p value) from the predicted gene targets. The weight for one miRNA is calculated by dividing its absolute expression value by the absolute sum of the expression values for all input miRNAs (250).

*4.4. Discussion*

We report significant differences in both plasma and skeletal muscle miRNA levels in response to oral glucose consumption and / or following HFEE, with some of these perturbations being modified by FO consumption. Basal, pre-HFEE plasma miRNA levels predicted up to 15 % of the variance in mean basal skeletal muscle miRNA levels. However, miRNA levels in these two tissue types were not significantly associated. Instead, basal, pre-HFEE levels of miR-145-5p and miR-204-5p in plasma and skeletal muscle, respectively, were significantly associated with the HFEE-mediated change in insulin sensitivity (Matsuda). These data suggest that levels of certain miRNAs in plasma and skeletal muscle transiently respond to oral glucose consumption, are HFEE-responsive and may be useful predictors of the insulin sensitivity change following short-term HFEE.

Levels of certain miRNA species have been investigated, within the context of insulin resistance / T2D, in both plasma and skeletal muscle tissue previously (33,164,182). Moreover, previous research has detailed changes in miRNA levels between individuals with pre-diabetes and manifest T2D (243). However, to our knowledge, miRNA levels have not been investigated in response to short-term HFEE in humans. We demonstrate clear subsets of miRNA regulation with miRNAs responding most to HFEE, oral glucose consumption or a combination of the two, with FO consumption mediating these responses in some instances. Only miRNAs sampled in skeletal muscle, rather than plasma, were shown to be HFEE-responsive in the current study. These HFEE-responsive miRNAs included miR-106b-5p, miR-145-5p, miR-214-3p and miR-215. Thus, these miRNAs may regulate specific aspects of the metabolic response to HFEE in skeletal muscle.

The control of glucose production is a critical component of glucose homeostasis. Failure to reduce glucose production during postprandial periods is associated with poor glucose control and impaired insulin sensitivity (50). The regulation of gluconeogenesis in primary hepatocytes has been attributed to miR-214 (251). MiR-214 overexpression was associated with suppressed gluconeogenesis whereas elevated glucose production was evidenced following miR-214 silencing (251). Levels of skeletal muscle miR-214-3p were reduced following HFEE, irrespective of FO consumption, in the current investigation. Based on the mechanism described, this reduction in miR-214-3p would be associated with an upregulation of gluconeogenesis. Levels of miR-214-3p also were altered by HFEE over the course of the OGTT. Specifically, miR-214-3p levels were altered in a different manner 2 h post-glucose consumption following HFEE. Post-HFEE miR-214-3p levels were largely comparable at both OGTT time-points (0 and 2 h) whereas miR-214-3p levels were increased at 2 h pre-HFEE. This blunted rise in miR-214-3p levels at 2 h post-HFEE may reflect a more insulin-resistant state if the relationship detailed between miR-214 and gluconeogenesis in primary hepatocytes also exists in humans and miRNAs are involved in tissue cross-talk. According to this mechanism, reduced miR-214-3p levels would correspond with an increase in endogenous glucose production (251). However, increasing endogenous glucose production in the presence of exogenous glucose availability would suggest a reduced sensitivity of insulin action. Given that increased basal endogenous glucose production is associated with insulin resistance, our findings of suppressed miR-214-3p levels may suggest early maladaptation to HFEE at the level of epigenetic regulation.

Insulin resistance in other experimental models also has been associated with changes in miRNA levels. Some consistent changes are evident between HFEE-responsive miRNAs in the present study and miRNA changes in other experimental models. Adipose tissue expression of miR-106b was positively associated with insulin resistance in women with polycystic ovary syndrome or non-polycystic ovary syndrome insulin resistance (252). Although our miRNA measures were sampled within skeletal muscle, if the same mechanism were to apply as in adipose tissue, the post-HFEE increase in miR-106b-5p may, like miR-214-3p, reflect early maladaptation to HFEE. This contention is further supported by work in C2C12 myocytes (238,253) in which miR-106b overexpression resulted in insulin resistance and mitochondrial dysfunction (238) whereas miR-106b silencing improved insulin sensitivity (253). Finally, miR-106b also has been negatively related to brown adipocyte differentiation (254). Given the role of brown adipose tissue in thermoregulation and energy balance (255), the association between miR-106b and brown adipocyte differentiation may be further indication of early HFEE-mediated maladaptation that precedes clear development of insulin resistance. Thus, taken together evidence is beginning to emerge linking epigenetic regulation to short-term HFEE, pre-insulin resistance and manifest insulin resistance.

Changes in the insulin-stimulated response to oral glucose consumption can reflect metabolic control. Transient changes in miRNA levels over the 2 h following consumption of an oral glucose bolus were observed in the present investigation and may offer insight into metabolic perturbations over the same time-frame. In contrast to HFEE-responsive miRNAs, OGTT-responsive miRNAs were observed in both plasma and skeletal muscle. However, there was no crossover between tissue types.

In plasma, levels of miR-145-5p largely reflected the temporal plasma insulin curve with peak miRNA levels (of the 3 measured time-points) corresponding with peak plasma insulin levels, and 2 h levels back to 0 h baseline levels. Although no data exist relating miR-145-5p levels to glucose provision, increased levels of miR-145 have been observed in mouse models of obesity, and mice deficient in the miR-143/145 cluster were protected from obesity-related insulin resistance (256). However, in the present study, we observed strong negative relationships ($r = -0.70$) between basal plasma miR-145 levels and the degree of fat mass gain in HF-C. These discrepancies between studies may indicate different mechanisms between obesity and short-term high-fat feeding and / or reflect the differences in models, *i.e.*, mouse *vs.* human samples. Further research from the mouse model demonstrated that in an insulin-stimulated state, overexpression of miR-145 did not alter blood glucose concentrations or hepatic insulin sensitivity (256). Thus, although some preliminary links to insulin action have been established in mouse models, the mechanisms regulating our observed changes in miR-145-5p levels over the course of an OGTT are elusive and further research is warranted in this regard.

Plasma miRNA levels may reflect regulation in various tissue types, particularly with regards to rates of endocytosis into, and exocytosis from, tissue. Temporal plasma patterns of miR-7-5p and miR-27a-3p during the course of the OGTT did not follow plasma insulin profiles in the same manner as miR-145-5p and were modified by HFEE. Paralogues of miR-7 have been shown to regulate pancreatic β-cell function / differentiation in cell culture and rodent models (257–259). Moreover, in mouse myoblasts, IRS1 expression, insulin-stimulated PKB phosphorylation, and glucose uptake were reduced with miR-7a overexpression (258). Overexpression of miR-27

paralogues in goat mammary gland epithelial cells (260) and rat hepatocytes (261) resulted in greater accumulation of fat droplets, TG, FA (particularly SFA) content, and hepatic fibrosis, respectively. Therefore, although studied in different models, some indications exist that these miRNAs may be involved in HFEE-relevant mechanisms including the response to oral glucose consumption.

The regulation of skeletal muscle miRNAs and plasma miRNAs are not completely synergistic in certain models. To our knowledge, no previous studies exist comparing skeletal muscle miRNA regulation to plasma miRNA regulation in the same participants. In the current investigation, we had an additional plasma sampling time-point that corresponded to peak plasma insulin levels that we did not have in skeletal muscle. However, we sampled both tissue types before (0 h) and after (2 h) consumption of the oral glucose bolus. Therefore, we were able to directly compare changes in the same miRNAs, in the same participants, in each tissue type, at these two time-points. Changes in miRNA levels were observed in both tissue types. However, different miRNAs were altered in plasma compared to skeletal muscle. Skeletal muscle levels of miR-193a-5p and miR-206 were both elevated at 2 h post-glucose consumption compared to 0 h. A previous study sampling peritoneal mesenchymal cells cultured in a high glucose medium, demonstrated that miR-193 was significantly increased and positively correlated with the duration of glucose exposure (262). Moreover, although not in the context of glucose consumption, miR-193 sampled from human subcutaneous adipose tissue has been shown to negatively relate to BMI following appropriate age and sex corrections (263). Thus, in combination with our data, miR-193 may be responsive to glucose concentrations and / or aspects of HFEE. The skeletal muscle-specific miRNA, miR-206, has been

mostly studied within the context of anabolic stimuli rather than glucose uptake. However, in a previous study, investigation of skeletal muscle miR-206 levels in humans following oral essential amino acid ingestion, a known stimulus for insulin secretion, were not altered over time (264). These discrepancies between studies may be explained by the prior resistance exercise performed in the study by Drummond and colleagues (264), compared to basal measurements in the present investigation and / or the stimulus of glucose *vs.* amino acids. Further research is undoubtedly required to better characterise the miR-206 response to oral glucose consumption and insulin action.

Changes in plasma miRNA levels are likely governed by rates of cell endocytosis and exocytosis. In the present investigation we performed regression analyses to investigate the association between miRNA levels in plasma and skeletal muscle. Although there was a tendency for plasma levels of miR-7-5p, miR-93-5p and miR-100-5p to associate with corresponding skeletal muscle levels, none of the associations were statistically significant. The lack of association between plasma and skeletal muscle miRNA levels may reflect disparate time-courses of action and / or contribution to / from the pool from other tissues. Thus, whereas levels of our measured plasma miRNAs may not reflect functional change within skeletal muscle, they may instead reflect functional change within other tissue types. Investigation of plasma miRNA levels in combination with sampling in other tissues is therefore warranted to better characterise plasma miRNA biomarker applicability.

The utility of miRNAs as biomarkers of given states is dependent also on their association with phenotypic measures, such as those presented in **Chapters 2 and 3**

of the current thesis. We chose to assess the association between miRNA levels in both tissue types and whole-body insulin sensitivity (Matsuda). Considering this association is useful based on our lack of statistically significant mean group change in insulin sensitivity. If a given miRNA is to be a useful biomarker of insulin resistance / HFEE maladaptation then we would expect a correlation to exist between miRNA levels and insulin sensitivity change. Basal levels of miR-145-5p and miR-204-5p in plasma and skeletal muscle, respectively, significantly associated with the mean whole-body insulin sensitivity response to 6 d HFEE. Changes in miR-145-5p levels have been associated with consumption of HF diets in fish (265) and rats (266) and thus may offer some preliminary insight into the relationship between basal miR-145-5p levels and insulin sensitivity change. Levels of miR-204-5p sampled in adipose tissue of mice were reduced following consumption of a HF diet (267) yet higher levels were observed in adipose tissue of a species of pig with large proportional fat mass compared to highly lean pigs (268). Despite these contrasting findings, the association between both miR-145-5p and miR-204-5p and HF diets in various models seems to fit with our data based on our insulin sensitivity change being mediated by HFEE. It would seem logical that these miRNAs would therefore associate with changes in mRNA of genes involved in the regulation of insulin sensitivity. Unquestionably, further work that induces insulin-resistance via dietary means is required to replicate these findings before either miRNA can be considered predictive of insulin resistance development.

Functional links between miRNAs and gene targets also can be established using bioinformatics programs such as miRSystem. However, when performing these analyses it is important to acknowledge that miRNAs target up to 400 genes

(160,269). Therefore, without performing direct miRNA-mRNA experiments we cannot be sure of the gene the miRNA is acting on. Future work will benefit from synergistically investigating miRNA and mRNA levels within tissue to better characterise miRNA function. Nonetheless, bioinformatics analysis at least enables us to predict mRNA targets of the altered miRNAs and consider whether changes in these genes are consistent with HFEE- and / or group-mediated phenotypic changes (for phenotype data see **Chapters 2 and 3**). Predicted pathways targeted by our differentially regulated plasma miRNAs included axon guidance, developmental biology, MAPK signalling, regulation of the actin cytoskeleton, focal adhesion, ErbB signalling, L1CAM interactions, neurotrophin signalling and cancer. Some pathways, including axon guidance, developmental biology and cancer, were consistent between plasma and skeletal muscle miRNAs. However, other predicted pathways targeted by skeletal muscle miRNAs included haemostasis, signalling by NGF (alone and via TrkA from the plasma membrane), platelet activation signalling and aggregation, bacterial invasion of epithelial cells, neuronal system, Rho-GTPase-related processes and G1-phase-related processes. Some of these pathways, in both tissues, share common functionalities and have been linked to insulin signalling. For example, Rho-GTPase function is associated with actin remodelling (270) and focal adhesion (271), and axon guidance is mediated NGF / Trk and neuronal signalling (272). Moreover, silencing of focal adhesion kinase in mice is associated with insulin resistance (273), actin remodelling alters MAPK signalling which in turn regulates insulin secretion in β-cell lines (274) and platelet activation is associated with diabetes (275) and hyperglycaemia in human blood preparations (276). Thus, it is logical that these pathways may be involved in the HFEE and / or insulin-mediated glucose responses.

However, exact mechanisms of action cannot be determined from this limited analysis and further work is required to corroborate these findings and associations.

In conclusion, levels of certain miRNAs in both skeletal muscle and plasma are altered by oral glucose consumption and / or HFEE. Moreover, miR-145-5p (plasma) and miR-204-5p (skeletal muscle) were significantly associated with the HFEE-mediated change in whole-body insulin sensitivity. Taken together, these data suggest a role for miRNAs in the regulation of insulin sensitivity. Future work is required to investigate the relationship between observed changes in plasma and skeletal muscle miRNA levels and target gene expression in skeletal muscle and other relevant insulin-responsive tissues.

# **CHAPTER 5** General discussion and concluding remarks

The overall aim of this thesis was to add experimental evidence to the current knowledge gaps regarding the metabolic, molecular and epigenetic responses to short-term HFEE. Also, we aimed to consider the impact of n-3 PUFA ingestion within the same short-term HFEE context on these metabolic, molecular and epigenetic responses. These two primary aims were achieved by successful completion of the following objectives:

i) To determine the whole-body responses to a well-controlled, short-term (6 d) period of HFEE, with and without FO, in humans (**Chapters 2 and 3**).

ii) To determine the subcellular molecular mechanisms of short-term (6 d) diet-induced maladaptation in humans, and their mediation by FO (**Chapters 2 and 3**).

iii) To determine the epigenetic responses to short-term (6 d) HFEE, with and without FO, in humans (**Chapter 4**).

The principal findings of this programme of work have been discussed in detail throughout the three experimental chapters and were primarily fivefold:

i) Consumption of HFEE for 6 d did not significantly alter overall insulin sensitivity in young healthy male participants, irrespective of whether FO was consumed as 10 % of total fats.

ii) Skeletal muscle levels of certain long and very long chain ceramide species were increased following 6 d HFEE, irrespective of whether FO was consumed at 10 % of total fats.

iii) Skeletal muscle AMPK α2 activity was increased following HFEE with FO consumption, but unchanged following HFEE without FO consumption.

iv) Certain miRNA levels in plasma and skeletal muscle were clearly identified as HFEE-responsive, OGTT-responsive or a combination of the two, with FO consumption mediating the response in some instances.

v) Plasma levels of miR-145-5p and skeletal muscle levels of miR-204-5p were significantly associated with the HFEE-mediated change in insulin sensitivity.

From these principal findings it is clear that the *a priori* hypotheses were largely unsupported by the experimental data generated, particularly with regard to overall group perturbations in insulin sensitivity. However, reflecting on the fact, that in the most part, our bodies are able to adapt and deal with a whole host of different metabolic stresses placed upon them, this ability is arguably as remarkable as it is essential. Imagining a scenario whereby excess or limited energy provision, altered exercise loads, varying temperature exposures, and so forth, are immediately of such detriment to our whole-body function that we cease to adapt, appears logically flawed. Thus, in some ways we may *expect* our volunteers to have the metabolic flexibility to be able to adapt to the subjected short-term HFEE. The highly regulated metabolic system that controls our whole-body function has to be adaptive in order to allow retained function in a variety of states. Unquestionably, the range in which we are able to operate and function is very tight for many processes but the fact that a *range* exists is key to this concept. The preceding hypotheses centred on the notion

that short-term HFEE would reduce insulin sensitivity. Yet, although chronic excess energy consumption is undeniably a primary risk factor for development of obesity, insulin resistance and T2D, the response to short-term episodes of HFEE is likely dependent on a host of factors specific to the individual. Whereas it is questionable that such feeding periods could ever be considered beneficial for an individual, the metabolic flexibility of the *Homo sapiens* is likely an inherent and significant trait, integral for the lifespan and evolution.

At a group level, 6 d HFEE did not result in significant perturbations in insulin sensitivity. Given the experimental design, it is not possible to offer any information on the time course of insulin sensitivity change. Intuitively, and based on the discussed literature throughout this thesis, decrements in insulin sensitivity may have been observed prior to, and / or extending past, our 6 d (post-HFEE) testing time point. Despite no significant alterations in insulin sensitivity following 6 d HFEE, some of our measures were significantly altered at 6 d. Levels of circulating FFAs and TGs were significantly lower, and skeletal muscle ceramides significantly higher post-HFEE. Blood and phospholipid membrane fatty acid composition was significantly altered in favour of n-3 PUFAs following FO intake. Fasting AMPK α2 activity was significantly increased following 6 d HFEE with FO but unaltered in the absence of FO. Significant gains in fat mass, particularly in the central trunk region, were observed at 6 d. Finally, significant alterations in the levels of plasma and skeletal muscle miRNAs were observed after 6 d HFEE, and in some instances predicted a proportion of the HFEE-mediated insulin sensitivity change, Taken together, these findings suggest that early maladaptive responses at the tissue level precede while body insulin sensitivity

change. The utility of these measures as potential markers of susceptibility to developing diet-induced insulin resistance should be explored further.

Herein, the final discussion points have been divided into three aspects; the first will discuss the concept of energy balance and in particular the role of energy expenditure in this equation. Second, individual variation within the context of HFEE will be discussed, before finally concluding with discussion of the central limitations of this thesis and suggestions for future research.

*5.1. Energy balance – The role of energy expenditure*

At this stage, the 'gluttony *vs.* sloth' debate raised at the advent of this thesis is worth reconsideration. Whereas it is undeniable that excess energy provision and consumption has contributed to the remarkable escalation of T2D prevalence, particularly in the Western world, it remains naïve to afford no attention to the counter-factor in the energy balance conundrum. Accumulating evidence suggests that habitual activity levels may be a key factor in the response to short-term periods of HFEE.

Expanding on discussions from **Chapter 2**, the insulin sensitivity response to HFEE is affected by prior exercise and / or the habitual activity status of the participant cohort. Hagobian & Braun (107) investigated the impact of performing a single bout of moderate intensity endurance exercise (treadmill or cycle ergometer; corresponding to 25 % of participants' estimated daily energy expenditure) on insulin sensitivity during a period of energy excess. Crucially, energy intake was

increased to match the energy expenditure induced by exercise, in order to maintain the same energy surplus (768 ± 203 kcal·d$^{-1}$) participants were consuming for the 3 d prior to the exercising day. Thus, total energy consumed was greater on the exercised day; however, net energy balance was equal over the energy surplus *vs.* energy surplus + exercise days. The principal findings from this study were that insulin sensitivity was reduced by 3 d overfeeding and inactivity in otherwise habitually active individuals. However, performing a single endurance exercise bout combined with the same degree of energy surplus did not restore insulin sensitivity.

Using a similar study design, Walhin and colleagues (115) studied active males over a 7 d period in which they were overfed by 50 % more energy than their habitual consumption. However, one group of participants simultaneously reduced their daily step count to < 4000 steps·d$^{-1}$ while the other group performed moderate intensity treadmill running (45 min) each day. Similar to Hagobian & Braun (107), exercising participants consumed additional energy to account for the energy expended during the exercise bout so that an energy surplus was maintained. In contrast to the findings of Hagobian & Braun (107), the exercising, unlike the non-exercising, group were able to maintain their insulin sensitivity at pre-intervention levels despite consuming a 50 % energy surplus (115). The results from this study could be interpreted as reflecting the beneficial effects of daily exercise during periods of energy excess, or the detrimental effects of reducing physical activity to below 4000 steps·d$^{-1}$ in combination with energy excess. Considering the discrepancies between study findings, these data may better support the latter contention. Further support for this notion is evident from a study conducted by Krogh-Madsen and colleagues (277). In this study, participants who habitually consumed > 2000 kcal·d$^{-1}$ were

randomly assigned to either maintain their physical activity at 10000 steps·d$^{-1}$ or to drop their step count to 1500 steps·d$^{-1}$ for a 2 wk period. Following the 2 wk intervention, a host of markers indicative of impaired insulin sensitivity including poor glycemic control and increased endogenous glucose production were evident in the reduced step count group compared to the participants who maintained recommended levels of habitual physical activity (277). Thus, reduced physical activity may be more detrimental for insulin sensitivity than the energy excess *per se*. Had we used accelerometers in the current study, we may have been able to offer information on the relationship between physical activity and insulin sensitivity change in our cohort. However, given our measure of physical activity at the pre-screening stage as times per wk, we lacked the measurement resolution to be able to tease these data from our findings. More work is justified to investigate the (in)dependent effects of physical activity and energy intake on insulin sensitivity, and future models of diet-induced insulin resistance should accurately measure both aspects of energy balance.

### *5.2. Individual variation*

The defining conceptual characteristic of this programme of work has to be that of individual variation. Admittedly, as is the case here, the individual variation inherent in each measured response is often considered more closely in instances of non-significant group means. However, despite this increased scrutiny in such instances, it is irrefutable that variation is seen in every measure, in every scientific study conducted. Instead, significant mean differences often mask the need to consider such

variation due to greater measurement consistency, rather than significance equating to no measured variation.

The individual variation in each measured response is likely meaningful. The finding that some individuals were able to effectively adapt to the increased fat and energy load placed upon them, whereas others were placed under metabolic stress in response to the same load is an intriguing concept. The correlations presented in **Chapter 4** between levels of certain plasma and skeletal muscle miRNAs and insulin sensitivity responses shed light on the potential for epigenetic mechanisms to, in part, explain differential individual responses to the same stimulus.

Variability in any given measured response is a result of measurement noise and / or individual differences. We aimed to reduce measurement noise by giving participants a 3 d baseline diet with prohibited alcohol intake and exercise during this period. Whereas these control measures will not completely eradicate the measurement variation, a tight pre-experimental control can significantly improve the degree of measurement error. Analytical measurement errors also need to be considered. For example, Doctoral work by Dr Solomon (University of Birmingham) demonstrated the test, re-test reliability of the Matsuda insulin sensitivity index, calculated from an OGTT, was around 5 %. This error of measurement is within the same range as the highly-regarded 'gold-standard' methodology of the euglycemic-hyperinsulinemic clamp (43). Using stable isotopic tracers in the present investigation provided added sensitivity in the measurement of glucose metabolism, particularly with regards to understanding endogenous and exogenous contributions to the total glucose pool. Given the sensitivity of the method, the variability in our measured responses,

particularly in terms of glucose metabolism and insulin sensitivity is likely driven in the most part by individual variance.

Each individual response to any given stimulus is governed by the combined influence of genetic predisposition, environmental influence and epigenetic control. However, the degree to which each of these factors explains the variance in any given response is dependent on the response itself. Whereas some phenotypic responses are largely heritable, others also have a large heritable component but are more variable, with a greater influence from environmental cues. Studies by Bouchard and colleagues (151,152) demonstrated a ~25 % heritability component in the fat mass change response to long-term overfeeding. Therefore, the majority of variance in the response to overfeeding is likely from environmental and other, for example, epigenetic, differences. Given the number of intertwined factors to consider within the study of HFEE, it is of little surprise that such a large degree of variation is observed in our measures. However, in order to truly understand maladaptive responses to HFEE and insulin resistance development, future studies of this nature need to consider the mechanisms governing individual responses to HFEE.

The inherent relative risk of developing T2D in scenarios of HFEE is more difficult to determine when the degree of heritability within each measured response is reduced. Replication of miRNA studies is required if miRNA levels are to be useful biomarkers of insulin resistance development, and plasma miRNA profiling, given its ease of sampling, will hold the key to biomarker utility. Hopefully, future research will benefit from studies of this nature and enhance the validity of T2D prognosis in the long-term.

*5.3. Thesis limitations and future directions*

The data presented within this thesis add important and novel information to the literature that will facilitate the advancement of knowledge within the field. However, a number of limitations exist that warrant consideration.

As detailed in the first part of this discussion, energy expenditure is an important consideration for complete understanding of energy balance. All of our participants failed to meet current government recommendations for exercise, and were asked to maintain their low habitual activities of daily living without engaging in any additional exercise throughout the study period. However, we did not measure energy expenditure during the study and thus cannot be sure that energy expenditure was not enhanced during HFEE. Subconscious manipulation of physical activity levels when in energy excess (278), the thermogenic effect of food (279–281), and non-shivering thermogenesis (282) all may have contributed to increasing the energy expenditure arm of the equation, thus reducing the significant intended skew towards a positive energy balance. Moreover, rather than solely extending time spent engaging in physical activity, reducing periods of sedentary time is important for maintaining / improving metabolic health (283,284). Thus, our participants, who were mostly students, would likely have shorter sedentary periods due to moving between classes than perhaps other populations. In future, continuous diurnal monitoring of energy expenditure using an activity monitor would be recommended to enable estimates of energy expenditure, physical activity and sedentary time to be made. Moreover, investigation of the impact of varying habitual activity levels on the

metabolic, molecular and epigenetic responses to HFEE would be of interest to better understand the role of physical activity on metabolic flexibility.

Second, all conclusions were made in the context of statistically unaltered insulin sensitivity. Insulin sensitivity was a dependent variable in the present study, and thus not a controlled variable. However, the study design and ensuing data are limited by the absence of clear changes in insulin sensitivity. The true value of FO consumption for metabolic disease risk within the context of HFEE cannot easily be discerned from these data.

Unquestionably, when we consider the percentage change of certain markers of insulin sensitivity in the current body of work, the concept of physiological *vs.* statistical significance is raised, and forms the basis for an interesting discussion on statistical principles. However, given the ambiguity in interpreting the data, establishing a time-course of declining insulin sensitivity in response to HFEE would undoubtedly facilitate the study of short-term overfeeding. Whereas a consistent, reproducible time-course may be confounded by the individual variability of any given response, attempts to at least establish key phases of insulin resistance development would be fruitful. Future study designs would be able to have greater focus on the specific question to be addressed by establishing a time-course of events following HFEE. For example, if subcellular modifications, as suggested in the present thesis, do indeed precede the development of whole-body insulin resistance, then interventions designed to target these mechanisms may elect a time-course of study that is shorter in duration than would otherwise be chosen to study mechanisms of whole-body adaptation.

Establishing a dose / time-course relationship for FO intake, in addition to a time-course of insulin sensitivity change also would aid future research in this field. Currently, the optimal dose and duration of FO supplementation for an optimal, functional cell membrane composition is not established. Moreover, the difference in dose and incorporation time for capsule / drink-based FO supplementation *vs.* dietary FO intake is not known and may add validity to this area of study. Likewise, a caveat of many of the studies designed to investigate the impact of FO supplementation / intake is that often a daily dosing strategy is implemented. Given that current government recommendations are set at 2 portions of oily fish per week (285), investigations designed to replicate more habitual consumption patterns when using dietary intake strategies are warranted.

The final methodological consideration in relation to insulin sensitivity measures relates to the use of continuous glucose monitoring to provide further information on the diurnal control of blood glucose levels. Importantly, during waking hours, individuals with T2D are often in a near-continual postprandial state with regard to their circulating glucose levels (286). Thus, measurements that are confined to an acute, post-glucose consumption time-period often will not reflect the wider impairments regarding declining insulin sensitivity. Whereas it is not possible to comment on the 24 h blood glucose control of our participants in the current study, use of continuous glucose monitoring methodology would facilitate these investigations in future studies of this nature.

Finally, all participants in the current study were non-active young males. The influence of physical activity and sex on insulin sensitivity has been discussed previously. However, it is worth re-emphasising that the age, sex and habitual physical activity status of the participant cohort limits our ability to generalise findings across other populations. Future studies should investigate the independent and inter-related impact of age, sex, physical activity and race on the insulin sensitivity response to short-term HFEE. Understanding the role of individual characteristics will facilitate development of tailored interventions to counter-act insulin resistance for specific populations.

### *5.4. Final thoughts*

Despite these clear limitations of this programme of work, the data generated provide some novel and interesting insights to our understanding of the metabolic, molecular and epigenetic regulation of the whole-body and subcellular responses of young healthy males to a short-term period of HFEE. One of the most consistent outcomes of scientific studies is that further questions are raised, and the present thesis is no exception. In particular, building on the points made during evaluation of the limitations of this thesis, the following research questions warrant further investigation.

i) What is the temporal insulin sensitivity response to HFEE?
ii) What are the temporal, subcellular muscle / liver / adipose tissue-specific responses to HFEE?

iii) How does population mediate the metabolic, molecular and epigenetic responses to HFEE?

iv) How does habitual activity status mediate the metabolic, molecular and epigenetic responses to HFEE?

v) Is there a dose-response relationship between fat / energy intake and insulin sensitivity?

vi) Is there a dose-response relationship between n-3 PUFA intake and insulin sensitivity within the context of HFEE?

Considered together, these future study ideas could be organised in four phases. The aim of **Phase 1** would be to establish a time-course of insulin sensitivity change in response to HFEE. Initially, this aim would be achieved via an OGTT performed on day 3, 5, 7, 14 and 28 of HFEE, with plasma glucose and insulin concentrations, taken during the OGTT, used to calculate whole-body insulin sensitivity (Matsuda index). These selected days of sampling would enable the temporal fluctuations in metabolic flexibility to be studied, and in order to maintain the diet on each sampling day, the 75 g glucose bolus could be accounted for within the daily dietary intake for that day. The second aspect of **Phase 1** would relate to the participant population. If, as we anticipate, the metabolic flexibility of an individual alters the insulin sensitivity response to HFEE then establishing a time-course of insulin sensitivity change in different participant cohorts is required. Example cohorts should range from highly physically active individuals to sedentary individuals with T2D, include both sexes, and consider a range of ages / ethnic origins. Only when several parallel experiments in different participant cohorts have been conducted will we have the necessary information to begin devising short-term experimental models of diet-induced insulin

resistance for specific populations.

Once a time-course of insulin sensitivity change has been established in several cohorts, more advanced / invasive techniques including dual isotopic glucose tracers and skeletal muscle / adipose tissue biopsies can be incorporated into the study design based on the point at which insulin sensitivity is compromised. Thus, **Phase 2** would be designed to investigate the metabolic, molecular and epigenetic mechanisms of diet-induced insulin resistance. Investigating the three primary insulin-responsive tissues, skeletal muscle, liver and adipose tissue, using isotope tracers and biopsy sampling would facilitate holistic study in this area.

**Phase 3** of the study programme would be the stage at which investigation of the impact of fish oil consumption during HFEE on insulin sensitivity would be incorporated. Rather than dietary manipulation during the study period, perhaps a better method would be to *pre-load* participants by providing FO supplements for a 4 wk period preceding HFEE. Based on previous data from our laboratory (128), this time-period of supplementation would facilitate remodelling of the skeletal muscle phospholipid membrane. Thus, if phospholipid membrane composition is an important functional change for preserving, or improving, insulin signalling, a 4 wk period of FO supplementation at 5 $g \cdot d^{-1}$ would achieve the desired effect (at least in healthy males). Using this methodology also would have the additional benefit of being able to feed both groups (HFEE with, and without, FO) exactly the same experimental diet (relative to habitual energy intake). Having just one experimental diet would relieve any inconsistencies between the diets consumed by the two groups, such as sugar intake, that were apparent in the present investigation.

Finally, the first three phases have been designed with the assumption that only one experimental diet and FO dose will be tested. **Phase 4**, which could be implemented in concert with each of the 3 previous phases, relates to dose-response work. As indicated previously, both HFEE and FO dose could be manipulated. Specifically, different dietary energy and fat compositions could be investigated to establish any dose-relationship of energy and / or fat on the insulin sensitivity response, and / or different FO supplementation doses could be used during the pre-load. Dose-response work has the added advantage that the minimum change required to still achieve an almost maximal response can be opted for in future studies of this nature to minimise the severity of change and enhance compliance / validity. Taken together, the result of this four-phase period of work would be greater, and more complete, understanding of the underlying metabolic, molecular and epigenetic responses to short-term HFEE. Enhanced understanding of insulin resistance development, and the potential strategies that may be used to alter this development, can only be beneficial in the global pursuit of T2D prevention.

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# APPENDICES

Denomega
Nutritional Oils

OMEGA 360°
The Complete Choice

Denomega Nutritional Oils AS
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Date: 25.03.2009

MS-136 rev. 3

Product Specification I

# Omega-360™ Pure 22

**Description**

Omega-360™ Pure 22; a marine omega-3 oil with excellent sensory characteristics with no odor or taste. Omega-360™ Pure 22 can be used as an ingredient in most food applications. It can replace all or part of the fat in an application containing fat, or it can be incorporated into a food product that normally does not contain fat.

**Country of Origin**

Omega-360™ Pure 22 is sourced and manufactured in Norway. Antioxidants are sourced primarily within EU, North and South America.

**Purity Guarantee**

Specifications

| | | |
|---|---|---|
| Free fatty acids (FFA) % | | Max. 0,2 |
| Totox (2xPV+AV) | | Max. 5 |
| | | |
| EPA + DHA (area%) | Sum EPA+DHA | Min. 22 |
| Total omega-3 fatty acids (area%) | | Min. 28 |

GRAS

Omega-360™ Pure 22 is Generally Recognized As Safe (GRAS) for use as a food ingredient.

Environmental Toxins

Omega-360™ Pure 22 meets current regulatory requirements.

**Vitamin content**

Contains natural low levels of vitamin A and vitamin $D_3$.

- Vitamin A: Typically 19 - 40 µg/g
- Vitamin $D_3$: Typically 0,01 - 0,03 µg/g

**Antioxidants**

Omega-360™ Pure 22 is stabilized with commercial food antioxidant systems.

Denomega
Nutritional Oils

OMEGA 360°
The Complete Choice

**Packaging**

9 kg Bag-In-Box. Other packaging available on request.

**Storage and Shelf Life**

Omega-360™ Pure 22 is a premium quality product which is sensitive to oxidative degradation. Omega-360™ Pure 22 can be stored 12 months frozen (-18ºC) when originally sealed. Once package is opened, use immediately; product cannot be re-used once opened.

The product can be stored chilled for a limited time period dependent upon the application. Please contact us for details.

**General Guidelines for Use**

Omega-360™ Pure 22 is typically added up to 5% in final food or beverage product. The dosage depends on specific application, typical consumer habits of servings per day and positioning of final product. Please contact us directly for further guidance.

**Quality System**

Denomega has established and implemented a Quality System to ensure that Denomega's products are safe and in compliance with national and international laws such as Food Legislation, HACCP and current Good Manufacturing Practices (cGMPs). Two vital elements of the quality system are (1) ensuring sustainable sources of crude marine oils and (2) traceability throughout the entire supply chain of the marine oils.

**GMO**

Omega-360™ Pure 22 does not contain any GMOs according to EU Regulations 1829/2003 and 1830/2003.

**Dietary Suitabilty**

| | |
|---|---|
| Kosher | Omega-360™ Pure 22 is Kosher Certified. |
| Halal | Omega-360™ Pure 22 contains no ingredients of alcohol or pork origin. |
| Vegetarian | Omega-360™ Pure 22 is not suitable for vegetarians. |
| Vegan | Omega-360™ Pure 22 is not suitable for vegans. |
| Organic | Omega-360™ Pure 22 meets requirements for use in organic products in the US. |

**Irradiation**

Omega-360™ Pure 22 has not been irradiated during any steps of the production process, ref. EU Regulation 2/1999.

**BSE/TSE**

Omega-360™ Pure 22 is free from other animal bi-product derivatives and Foot and Mouth Disease.


# SMARTFISH NUTRIFRIEND 2000

## LAVT OKSIDERT DHA & EPA

**Smartfish NutriFriend 2000 er en næringsdrikk for spesielle medisinske formål, og er velegnet for personer som har behov for høye doseringer med ferske omega-3 fettsyrer EPA og DHA. NutriFriend 2000 er derfor velegnet til personer med hjerte- og kar sykdom.**

**Nutrifriend 2000 er en næringsdrikk med ca 85% juice fra bær og frukter og har en frisk og god smak.**

**Næringsverdi per 200 ml**

| | |
|---|---|
| Omega 3 | 2500 mg |
| DHA | 1000 mg |
| EPA | 1000 mg |
| Proteiner | 5 g |
| Vitamin D3 | 10 µg |

| Næringsinnhold | 100 ml | 200 ml |
|---|---|---|
| Energi | 95 kcal | 190 kcal |
| Proteiner | 2,5 g | 5 g |
| Hvorav laktose | 0,006 g | 0,012 g |
| Karbohydrater | 11 g | 22 g |
| Fett | 4,75 g | 9,5 g |
| Mettet | 0,875 g | 1,75 g |
| Enumettet | 2,1 g | 4,2 g |
| Flerumettet | 1,75 g | 3,5 g |
| Omega 3 | 1250 mg | 2500 mg |
| DHA | 500 mg | 1000 mg |
| EPA | 500 mg | 1000 mg |
| Øvrige fettsyrer er fra fiskeoljer | | |
| Vitamin D3 | 5 µg | 10 µg |

| | |
|---|---|
| Gluten: | Gluten fri |
| Protein kilde: | Myseproteinisolat |
| Fett kilde: | Fiskeolje fra laks/ørret/torsk |
| Karbohydrat kilde: | Fruktose og glukose |

**Ingredienser:** Juice fra konsentrat av eple, pære, granateple, og aronia, fiskeolje, myseproteinisolat, pektin, aroma fra appelsin og klementin, rosmarinekstrakt, naturlige tokoferoler, vitamin D3 og soya lecitin.

**Ikke tilsatt:** Sukker, søtningsmiddel, salt, kunstige fargestoffer eller konserveringsmidler

**Anbefalt dosering:** Som forskrevet av lege eller 1-3 enheter per dag. Egnet for eldre, voksne og kan med varsomhet benyttes av barn fra 3 år. Det anbefales 1-2 gram marine omega-3 fettsyrer per dag som primær- og sekundær prevensjon mot hjerte- og karsykdom. Det tilsvarer 1 stk NutriFriend 2000 per dag.

For å ha triglyseridsenkende effekt anbefales 3-4 gram marine omega-3 fettsyrer per dag. Dette tilsvarer 2-3 enheter NutriFriend 2000 per dag.

**Forsiktighetsregler:** Må ikke konsumeres av personer med melkeproteinallergi eller melkeproteinintoleranse. Inneholder myseproteinisolat fra melk.

**Anvendelsesområde:** SMARTFISH NutriFriend 2000 er en næringsdrikk velegnet for personer som har behov for høye doseringer med de ferske omega-3 fettsyrene, EPA og DHA, og vitamin D3. NutriFriend 2000 er velegnet til personer med hjerte- og karsykdom. EPA og DHA har gunstig effekt på fettstoffene i blodet, og kan dermed virke forebyggende mot hjerte- og karsykdom. Blant annet har høye doser marine omega-3 fettsyrer vist å ha triglyseridsenkende effekt. Et økt inntakt av marine omega-3 fettsyrer har også vist å kunne beskytte mot nye tilfeller av hjerteinfarkt hos hjertesyke.

NutriFriend 2000 kan også anbefales til pasienter med inflammatoriske sykdommer som revmatisk sykdom og KOLS i samråd med klinisk ernæringsfysiolog/lege.

**NutriFriend 2000** er en tilskuddsernæringsdrikk. Den egner seg ikke som eneste næringskilde, men i kombinasjon med vanlig mat eller andre komplette næringsdrikker.

**Forpakning:** Kartonger av 200 ml. Selges enkeltvis eller i brett av 18 stk.

**Bruksanvisning:** Serveres avkjølt, ristes før bruk.

**Oppbevaring:** Oppbevares kjølig eller i romtemperatur, utenfor direkte sollys (1-25 °C). Åpnet kartong er holdbar inntil 1 døgn i kjøleskap.

**Produksjon og GMO:** Fiskeoljen er fra avskjær på norsk oppdrettsfisk, fullt sporbar, sertifisert GMP* og Kosher. Smartfish inneholder ikke og er ikke fremstilt av GMO**.

**Regulatorisk status:** Næringsmiddel for spesielle medisinske formål

*Good Manufacturing practice
**Genmodifiserte organismer

**Smartfish AS:** Smartfish leder utviklingen med marint omega-3. Selskapet har et omfattende program for kliniske studier. Http://no.smartfish.no/kliniske-studier



Produsent: Smartfish AS, Gaustadalléen 21, 0349 Oslo
Tel: +47 22519880. Fax: +4722519881

SMARTFISH®

Appendix 2.3 **Blood lipid profiling by GLC.**

| | **HF-C** | | **HF-FO** | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **14:00** | 0.57 ± 0.04 | 0.66 ± 0.03 | 0.57 ± 0.04 | 0.67 ± 0.03 |
| **15:00** | 0.20 ± 0.01 | 0.24 ± 0.01 | 0.18 ± 0.01 | 0.24 ± 0.01 |
| **16:00** | 20.97 ± 0.22 | 20.69 ± 0.14 | 20.80 ± 0.24 | 20.44 ± 0.26 |
| **18:00** | 11.42 ± 0.07 | 11.51 ± 0.10 | 11.44 ± 0.22 | 12.03 ± 0.18 |
| **20:00** | 0.25 ± 0.01 | 0.26 ± 0.01 | 0.24 ± 0.01 | 0.25 ± 0.01 |
| **22:00** | 0.76 ± 0.03 | 0.80 ± 0.03 | 0.72 ± 0.02 | 0.76 ± 0.03 |
| **24:00** | 1.13 ± 0.02 | 1.24 ± 0.14 | 1.13 ± 0.04 | 1.12 ± 0.03 |
| Total SFA | **35.30 ± 0.29** | **35.38 ± 0.30** | **35.09 ± 0.42** | **35.51 ± 0.32** |
| **16:1n-9** | 0.27 ± 0.01 | 0.24 ± 0.01 | 0.26 ± 0.02 | 0.21 ± 0.01 |
| **16:1n-7** | 1.04 ± 0.08 | 0.77 ± 0.05 | 1.15 ± 0.11 | 0.80 ± 0.07 |
| **18:1n-9** | 17.29 ± 0.43 | 15.15 ± 0.48 | 16.61 ± 0.46 | 13.44 ± 0.29 |
| **18:1n-7** | 1.64 ± 0.04 | 1.44 ± 0.04 | 1.57 ± 0.08 | 1.42 ± 0.04 |
| **20:1n-9** | 0.25 ± 0.01 | 0.23 ± 0.01 | 0.24 ± 0.01 | 0.30 ± 0.01 |
| **24:1n-9** | 1.46 ± 0.05 | 1.43 ± 0.04 | 1.33 ± 0.05 | 1.43 ± 0.05 |
| Total MUFA | **21.95 ± 0.46** | **19.25 ± 0.55** | **21.16 ± 0.61** | **17.61 ± 0.37** |
| **18:2n-6** | 18.78 ± 0.82 | 21.97 ± 0.59 | 19.91 ± 0.69 | 18.30 ± 0.32 |
| **18:3n-6** | 0.22 ± 0.02 | 0.21 ± 0.02 | 0.22 ± 0.04 | 0.13 ± 0.01 |
| **20:2n-6** | 0.22 ± 0.01 | 0.22 ± 0.01 | 0.26 ± 0.01 | 0.25 ± 0.01 |
| **20:3n-6** | 1.73 ± 0.12 | 1.50 ± 0.08 | 1.71 ± 0.14 | 1.28 ± 0.09 |
| **20:4n-6** | 10.96 ± 0.34 | 10.73 ± 0.33 | 10.93 ± 0.36 | 10.77 ± 0.29 |
| **22:4n-6** | 1.51 ± 0.05 | 1.46 ± 0.04 | 1.60 ± 0.07 | 1.51 ± 0.08 |
| **22:5n-6** | 0.32 ± 0.02 | 0.30 ± 0.01 | 0.35 ± 0.03 | 0.32 ± 0.02 |
| Total n-6 PUFA | **33.74 ± 0.55** | **36.39 ± 0.36** | **34.97 ± 0.58** | **32.54 ± 0.34** |
| **18:3n-3** | 0.51 ± 0.03 | 0.53 ± 0.03 | 0.51 ± 0.04 | 0.55 ± 0.02 |
| **20:4n-3** | 0.08 ± 0.01 | 0.07 ± 0.00 | 0.12 ± 0.02 | 0.16 ± 0.02 |
| **20:5n-3** | 0.69 ± 0.06 | 0.73 ± 0.05 | 0.57 ± 0.04 | 3.95 ± 0.38 |
| **22:5n-3** | 1.57 ± 0.06 | 1.50 ± 0.06 | 1.40 ± 0.06 | 1.56 ± 0.05 |
| **22:6n-3** | 2.58 ± 0.18 | 2.55 ± 0.15 | 2.51 ± 0.14 | 4.21 ± 0.18 |
| Total n-3 PUFA | **5.41 ± 0.26** | **5.35 ± 0.23** | **5.04 ± 0.16** | **10.43 ± 0.51** |
| **16:0DMA** | 1.26 ± 0.03 | 1.30 ± 0.04 | 1.33 ± 0.03 | 1.42 ± 0.02 |
| **18:0DMA** | 1.76 ± 0.04 | 1.75 ± 0.05 | 1.83 ± 0.06 | 1.88 ± 0.07 |
| **18:1DMA** | 0.58 ± 0.02 | 0.58 ± 0.02 | 0.57 ± 0.02 | 0.61 ± 0.03 |
| Total DMA | **3.60 ± 0.07** | **3.63 ± 0.10** | **3.74 ± 0.08** | **3.92 ± 0.08** |
| 20:4n-6/20:5n-3 | **16.66 ± 1.17** | **15.23 ± 0.89** | **20.14 ± 1.57** | **3.01 ± 0.34** |
| % n-3HUFA/Total HUFA | **24.87 ± 0.87** | **25.35 ± 0.75** | **23.46 ± 0.84** | **40.96 ± 1.49** |

**All individual fatty acids are expressed as a percentage of total fatty acids. Values are means ± SEM (n=10 per group).**

Appendix 3.1 **Skeletal muscle lipid profiling by GCMS.**

| | **HF-C** | | **HF-FO** | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| **14:0** | 0.78 ± 0.12 | 0.76 ± 0.11 | 0.82 ± 0.08 | 1.18 ± 0.16 |
| **15:0** | 0.67 ± 0.13 | 0.67 ± 0.13 | 0.56 ± 0.04 | 0.79 ± 0.09 |
| **16:0** | 20.41 ± 0.99 | 20.73 ± 0.86 | 20.91 ± 0.66 | 21.16 ± 0.77 |
| **18:0** | 13.18 ± 0.80 | 12.74 ± 0.83 | 13.32 ± 0.74 | 12.96 ± 0.69 |
| **20:0** | 0.14 ± 0.01 | 0.12 ± 0.01 | 0.16 ± 0.02 | 0.18 ± 0.04 |
| **22:0** | 0.23 ± 0.04 | 0.20 ± 0.02 | 0.24 ± 0.03 | 0.25 ± 0.04 |
| **24:0** | 0.20 ± 0.04 | 0.20 ± 0.03 | 0.22 ± 0.02 | 0.20 ± 0.04 |
| Total SFA | **35.61 ± 0.61** | **35.42 ± 0.31** | **36.23 ± 0.91** | **36.71 ± 0.89** |
| **16:1n-9** | 0.57 ± 0.27 | 0.29 ± 0.04 | 0.39 ± 0.07 | 0.40 ± 0.07 |
| **16:1n-7** | 0.59 ± 0.15 | 0.60 ± 0.12 | 0.81 ± 0.14 | 0.85 ± 0.25 |
| **18:1n-9** | 8.09 ± 0.37 | 7.70 ± 0.36 | 8.25 ± 0.42 | 8.20 ± 0.50 |
| **18:1n-7** | 1.83 ± 0.08 | 1.75 ± 0.05 | 1.80 ± 0.11 | 1.88 ± 0.12 |
| **20:1n-11** | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.05 ± 0.03 | 0.10 ± 0.04 |
| **20:1n-9** | 0.24 ± 0.08 | 0.29 ± 0.10 | 0.42 ± 0.19 | 0.68 ± 0.20 |
| **22:1n-11** | 0.24 ± 0.12 | 0.30 ± 0.15 | 0.32 ± 0.15 | 0.60 ± 0.26 |
| **22:1n-9** | 0.12 ± 0.02 | 0.10 ± 0.03 | 0.16 ± 0.03 | 0.18 ± 0.04 |
| **24:1n-9** | 0.48 ± 0.10 | 0.43 ± 0.05 | 0.56 ± 0.07 | 0.66 ± 0.15 |
| Total MUFA | **12.21 ± 0.63** | **11.51 ± 0.72** | **12.76 ± 0.99** | **13.54 ± 1.34** |
| **18:2n-6** | 29.44 ± 0.82 | 30.72 ± 1.25 | 28.60 ± 1.04 | 26.69 ± 1.38 |
| **18:3n-6** | 0.17 ± 0.03 | 0.13 ± 0.01 | 0.18 ± 0.02 | 0.16 ± 0.02 |
| **20:2n-6** | 0.12 ± 0.01 | 0.12 ± 0.01 | 0.16 ± 0.03 | 0.18 ± 0.03 |
| **20:3n-6** | 1.53 ± 0.10 | 1.49 ± 0.10 | 1.46 ± 0.09 | 1.34 ± 0.13 |
| **20:4n-6** | 14.04 ± 0.83 | 13.69 ± 0.75 | 13.79 ± 0.63 | 12.67 ± 0.65 |
| **22:4n-6** | 0.74 ± 0.06 | 0.74 ± 0.03 | 0.69 ± 0.06 | 0.58 ± 0.05 |
| **22:5n-6** | 0.41 ± 0.03 | 0.35 ± 0.03 | 0.41 ± 0.05 | 0.34 ± 0.04 |
| Total n-6 PUFA | **46.44 ± 1.10** | **47.25 ± 1.29** | **45.29 ± 1.60** | **41.96 ± 2.02** |
| **18:3n-3** | 0.36 ± 0.03 | 0.37 ± 0.02 | 0.34 ± 0.02 | 0.39 ± 0.04 |
| **18:4n-3** | 0.08 ± 0.04 | 0.13 ± 0.07 | 0.10 ± 0.06 | 0.10 ± 0.10 |
| **20:4n-3** | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.07 ± 0.02 | 0.11 ± 0.03 |
| **20:5n-3** | 1.05 ± 0.15 | 1.08 ± 0.17 | 1.01 ± 0.15 | 1.87 ± 0.32 |
| **22:5n-3** | 1.86 ± 0.09 | 1.87 ± 0.09 | 1.61 ± 0.22 | 1.91 ± 0.10 |
| **22:6n-3** | 2.32 ± 0.20 | 2.31 ± 0.25 | 2.59 ± 0.38 | 3.41 ± 0.59 |
| Total n-3 PUFA | **5.73 ± 0.47** | **5.83 ± 0.54** | **5.73 ± 0.51** | **7.79 ± 1.07** |
| 20:4n-6/20:5n-3 | 16.22 ± 2.65 | 15.71 ± 2.69 | 16.83 ± 3.20 | 8.37 ± 1.34 |
| % n-3HUFA/Total HUFA | 24.05 ± 2.10 | 24.44 ± 1.88 | 24.29 ± 1.98 | 32.04 ± 3.36 |
| 20:5n-3 + 22:6n-3 | 3.37 ± 0.34 | 3.39 ± 0.41 | 3.61 ± 0.50 | 5.29 ± 0.88 |

**All individual fatty acids are expressed as a percentage of total fatty acids. Values are means ± SEM (n=9 per group).**

Appendix 4.1 **Plate array plasma miRNA levels.**

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| miR-1 | **1.00** (0.36-2.79) | **3.60** (0.02-528.52) | **0.23** (0.01-4.22) | **0.07** (0.01-0.60) |
| miR-7-5p | **1.00** (0.12-8.62) | **0.37** (0.15-0.94) | **0.95** (0.10-9.34) | **0.13** (0.01-1.14) |
| miR-15-5p | **1.00** (0.61-1.63) | **1.74** (1.12-2.69) | **1.28** (0.33-4.95) | **4.30** (1.04-17.72) |
| miR-18a-5p | **1.00** (0.51-1.98) | **1.95** (0.64-5.94) | **1.05** (0.24-4.62) | **4.17** (0.35-49.52) |
| miR-22-3p | **1.00** (0.58-1.73) | **2.20** (0.70-6.96) | **2.82** (0.42-19.13) | **2.47** (0.52-11.78) |
| miR-25-3p | **1.00** (0.28-3.52) | **0.50** (0.27-0.95) | **0.66** (0.20-2.18) | **0.48** (0.23-1.01) |
| miR-30d-5p | **1.00** (0.45-2.20) | **2.75** (1.98-3.83) | **0.58** (0.53-0.64) | **2.26** (1.10-4.62) |
| miR-93-5p | **1.00** (0.16-6.25) | **0.67** (0.24-1.89) | **1.57** (0.43-5.78) | **1.30** (0.58-2.90) |
| miR-100-5p | **1.00** (0.25-3.98) | **6.61** (1.11-39.15) | **0.60** (0.21-1.69) | **0.63** (0.04-10.39) |
| miR-106b-5p | **1.00** (0.31-3.24) | **1.60** (0.45-5.71) | **1.66** (0.44-6.23) | **3.98** (0.09-178.17) |
| miR-126-3p | **1.00** (0.35-2.85) | **13.59** (3.77-49.01) | **0.79** (0.48-1.32) | **2.80** (0.35-22.36) |
| miR-145-5p | **1.00** (0.65-1.53) | **5.25** (2.44-11.28) | **2.40** (0.69-8.32) | **5.62** (0.22-140.90) |
| miR-193a-5p | **1.00** (0.22-4.62) | **0.34** (0.10-1.10) | **0.34** (0.03-3.92) | **0.11** (0.01-1.50) |
| miR-196a-5p | **1.00** (0.01-79.59) | **0.01** (0.00-0.12) | **0.49** (0.00-2050.39) | **0.01** (0.00-0.27) |
| miR-204-5p | **1.00** (0.11-9.23) | **3.26** (0.43-24.67) | **0.44** (0.33-0.57) | **626.13** (0.45-872997.06) |
| miR-206 | **1.00** (0.00-1832.15) | **0.91** (0.00-720.22) | **0.02** (0.00-17760.97) | **0.00** (0.00-0.21) |
| miR-214-3p | **1.00** (0.52-1.94) | **32.61** (1.81-586.08) | **140.17** (0.05-414860.75) | **63.1** (0.58-6892.87) |
| miR-215 | **1.00** (0.10-9.53) | **0.23** (0.06-0.99) | **0.03** (0.00-2.05) | **34.94** (0.02-75581.03) |
| miR-885-5p | **1.00** (0.06-16.30) | **0.75** (0.160-3.40) | **0.60** (0.04-8.37) | **0.01** (0.00-1.44) |

**Data presented pre- and post-HFEE, for both groups (HF-C and HF-FO; n=3 for each condition (pooled miRNA from 10 participants in each condition). Triplicates were achieved by creating 3 pools of sample miRNA (two pools included miRNA from 3 participants and one pool consisted of miRNA from 4 participants). The displayed data are the miRNAs that were flagged for follow-up based on a t-test p value < 0.1 and a fold change <0.8 or >1.25, when miR-27a-3p was used as a control gene with HF-C, pre-HFEE as the calibrator within $2^{-\Delta\Delta Ct}$ calculations. Values are backtransformed means (95 % CIs).**

Appendix 4.2 **Plate array plasma miRNA levels.**

| | HF-C | | HF-FO | |
|---|---|---|---|---|
| | **Pre-HFEE** | **Post-HFEE** | **Pre-HFEE** | **Post-HFEE** |
| miR-1 | **1.00** (0.15-6.52) | **7.19** (0.04-1378.39) | **0.34** (0.01-12.99) | **0.15** (0.02-1.47) |
| miR-7-5p | **1.00** (0.40-2.53) | **0.74** (0.49-1.11) | **1.45** (0.25-8.31) | **0.26** (0.04-1.60) |
| miR-15-5p | **1.00** (0.43-2.30) | **3.47** (1.78-6.74) | **1.93** (0.34-10.95) | **8.98** (3.12-25.88) |
| miR-18a-5p | **1.00** (0.47-2.14) | **3.89** (1.18-12.78) | **1.58** (1.13-2.22) | **8.71** (0.72-105.06) |
| miR-22-3p | **1.00** (0.38-2.64) | **4.40** (1.99-9.74) | **4.27** (1.72-10.56) | **5.17** (1.86-14.33) |
| miR-27a-3p | **1.00** (0.28-3.52) | **2.00** (1.06-3.77) | **1.51** (0.46-5.01) | **2.09** (0.99-4.43) |
| miR-30d-5p | **1.00** (0.14-7.19) | **5.50** (4.04-7.48) | **0.88** (0.29-2.66) | **4.71** (1.75-12.69) |
| miR-93-5p | **1.00** (0.43-2.30) | **1.33** (0.58-3.04) | **2.38** (1.41-4.02) | **2.71** (1.66-4.42) |
| miR-100-5p | **1.00** (0.09-11.52) | **13.18** (2.72-63.78) | **0.91** (0.41-2.00) | **1.32** (0.10-18.08) |
| miR-106b-5p | **1.00** (0.30-3.36) | **3.19** (0.91-11.21) | **2.51** (2.20-2.86) | **8.32** (0.21-324.91) |
| miR-126-3p | **1.00** (0.22-4.53) | **27.12** (4.97-148.01) | **1.20** (0.32-4.52) | **5.84** (0.79-43.00) |
| miR-145-5p | **1.00** (0.20-5.08) | **10.47** (3.41-32.19) | **3.63** (1.18-11.13) | **11.75** (0.61-226.76) |
| miR-193a-5p | **1.00** (0.30-3.34) | **0.68** (0.26-1.78) | **0.52** (0.15-1.81) | **0.23** (0.03-1.60) |
| miR-196a-5p | **1.00** (0.01-82.17) | **0.02** (0.01-0.08) | **0.74** (0.00-939.51) | **0.01** (0.00-0.55) |
| miR-204-5p | **1.00** (0.15-6.66) | **6.51** (1.62-26.2) | **0.66** (0.16-2.73) | **1308.18** (0.60-2857499.80) |
| miR-206 | **1.00** (0.00-6156.49) | **1.59** (0.01-458.63) | **0.04** (0.00-154264.74) | **0.01** (0.00-0.33) |
| miR-214-3p | **1.00** (0.18-5.66) | **65.06** (6.74-628.13) | **212.16** (0.20-229449.89) | **144.54** (4.68-4462.72) |
| miR-215 | **1.00** (0.04-28.13) | **0.47** (0.06-3.74) | **0.05** (0.00-1.09) | **73.00** (0.06-87041.97) |
| miR-885-5p | **1.00** (0.20-5.04) | **1.49** (0.23-9.83) | **0.91** (0.17-4.94) | **0.03** (0.00-1.45) |

**Data presented pre- and post-HFEE, for both groups (HF-C and HF-FO; n=3 for each condition (pooled miRNA from 10 participants in each condition). Triplicates were achieved by creating 3 pools of sample miRNA (two pools included miRNA from 3 participants and one pool consisted of miRNA from 4 participants). The displayed data are the miRNAs that were flagged for follow-up based on a t-test p value < 0.1 and a fold change <0.8 or >1.25, when miR-25-3p was used as a control gene with HF-C, pre-HFEE as the calibrator within $2^{-\Delta\Delta Ct}$ calculations. Values are backtransformed means (95 % CIs).**

Appendix 4.3 **Plasma vs. skeletal muscle correlations.**

| | **Plasma *vs.* skeletal muscle miRNA levels** | **Plasma miRNA levels *vs.* Matsuda ISI change** | **Skeletal muscle miRNA levels *vs.* Matsuda ISI change** |
|---|---|---|---|
| miR-1 | - | - | **< 0.001** (0.602) |
| miR-7-5p | **11.00** (0.097) | **< 0.001** (0.677) | **< 0.001** (0.851) |
| miR-15-5p | **< 0.001** (0.60) | **7.90** (0.123) | **< 0.001** (0.798) |
| miR-18a-5p | **< 0.001** (0.394) | **< 0.001** (0.349) | **< 0.001** (0.349) |
| miR-22-3p | **< 0.001** (0.653) | **< 0.001** (0.980) | **15.80** (0.058) |
| miR-25-3p | - | - | **10.20** (0.106) |
| miR-27a-3p | - | **11.70** (0.077) | - |
| miR-30d-5p | **4.80** (0.192) | **11.30** (0.081) | **< 0.001** (0.811) |
| miR-93-5p | **14.20** (0.069) | **1.70** (0.263) | **< 0.001** (0.465) |
| miR-100-5p | **15.40** (0.06) | **< 0.001** (0.489) | **< 0.001** (0.408) |
| miR-106b-5p | **< 0.001** (0.792) | **12.00** (0.074) | **< 0.001** (0.937) |
| miR-126-3p | **< 0.001** (0.570) | **11.90** (0.075) | **< 0.001** (0.470) |
| miR-145-5p | **< 0.001** (0.496) | **16.20** (0.044) | **< 0.001** (0.506) |
| miR-193a-5p | - | - | **16.00** (0.056) |
| miR-196a-5p | - | - | **< 0.001** (0.917) |
| miR-204-5p | - | - | **22.80** (0.026) |
| miR-206 | - | - | **2.90** (0.238) |
| miR-214-3p | - | - | **11.30** (0.094) |
| miR-215 | **< 0.001** (0.642) | **< 0.001** (0.727) | **7.60** (0.149) |
| miR-885-5p | - | - | **2.80** (0.240) |

**Data presented are $R^2$ adjusted values (p values). All miRNA samples were basal pre-HFEE and mixed groups. The Matsuda ISI was calculated as the percentage difference between pre- and post-HFEE values.**

CROSSTALK

## Comments on the CrossTalk proposal and opposing view: The dominant mechanism causing disuse muscle atrophy is decreased protein synthesis/proteolysis

## Integrating the regulation of muscle protein synthesis and degradation?

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It is with great interest that we read the cross-talk on the proposed roles that changes in protein synthesis and protein degradation play in disuse skeletal muscle atrophy (Phillips & McGlory, 2014; Reid *et al.* 2014). However, rather than adopting an 'either/or' position on this topic, we believe that a more integrated standpoint is warranted. For example, recent evidence suggests that cells/tissues can simultaneously coordinate changes in both protein synthesis and protein degradation, including in skeletal muscle (Baehr *et al.* 2014; Zhang *et al.* 2014). Moreover, recent evidence suggests that, under certain conditions, an increase in protein degradation could lead to a decrease in protein synthesis. For instance, the eukaryotic initiation factor subunit 3f (eIF3f), which plays a crucial role in protein synthesis, is targeted for degradation by the E3 ligase atrogin-1, and preventing the atrogin-1-induced poly-ubiquitination of eIF3f provides protection against starvation-induced muscle atrophy (Csibi *et al.* 2009, 2010). Similarly, myostatin-induced myotube atrophy is not only associated with an increase in the expression of components of the ubiquitin proteasome system, but also an increase in the degradation of various translation initiation and elongation factors, ribosomal proteins, and a decrease in the rate of protein synthesis (Lokireddy *et al.* 2011, 2012). While these data are derived from cell culture and animal models, we must consider the possibility that similar mechanisms may also operate in human models of disuse atrophy, whereby an increase in proteolysis could play a significant role in the decrease in protein synthesis.

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### Additional information

#### Competing interests

None declared.

## The dominant cause of disuse atrophy: a debate hindered by fallacy of evidence?

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Although both viewpoints deliver articulate arguments, to us, the notion of any singular mechanism *per se* dominating muscle atrophy responses to disuse is both premature and most likely overly simplistic. As is the case with many adaptive processes, disuse atrophy (as with hypertrophy!) follows a temporal pattern, i.e. with greater changes dominating initial phases (Wall *et al.* 2014), thereafter abating (Adams *et al.* 2003). To suggest synthesis *or* breakdown is dominant disregards the dynamic nature of protein balance and the likely scenario of each process contributing to different extents at different time-points. To us, this is as much an issue as opposing views being a cacophony 'of mice and men' (though the present authors agree with Phillips & McGlory this may explain some existing discordances). Additionally, whilst Reid *et al.* (2014) justly state: 'the dominant mechanism of disuse atrophy is not known', this does not preclude one transpiring – with appropriate time course studies. Moreover, suggestions: 'rates of proteolysis *vs.* synthesis cannot be quantified using existing methods nor can differences in these rates be measured directly', are misleading. There are ways to quantify synthesis and breakdown *rates* concomitantly in muscle, *in vivo,* using combinations of stable isotope tracers. For example, A–V balance: synthesis/breakdown, net-balance (Greenhaff *et al.* 2008); pulse-chase: fractional breakdown rates (Zhang *et al.* 1996); fractional synthesis rates via amino acid tracers or $D_2O$ (Wilkinson *et al.* 2014). Yes, such techniques require mass-spectrometry know-how, blood/muscle sampling and temporal resolution, but surely represent the best means to arrive at *bona fide* answers to these longstanding questions.



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### Additional information

#### Competing interests

None declared.

## Disagreements move science forward


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As is often the case in an academic debate, here the affirmative and negative present arguments that do not effectively counter the alternatively presented hypothesis. This is not a critique of either group; instead we propose it is methodological in nature.

One aspect the authors have not considered in their arguments is the effect of time. We have previously reported a temporal effect in the hypertrophic response in healthy individuals following resistance training, with an acute degradative response followed by increased pro-synthesis activity (Elliott *et al.* 2012), and our current work is examining this phenomenon during muscle atrophy. Granted, this is a different research model, but it is of interest to note. We would suggest therefore that perhaps both hypotheses are correct. At this time, the temporal nature of the disuse atrophy has yet to be adequately considered.

This disparity between results that report molecular changes and those that report isotope-based results drives an artificial controversy. Natural sciences often reach such impasses when different approaches conclude differing hypotheses. We propose that a careful modelling of changes in both isotope measures of protein balance and molecular markers of pathway activity, as a function of time, will produce a clearer picture of the mechanistic control of muscle mass. Careful critique of each hypothesis will lead to the rejection of one, or the combination of the two, as suggested above. At the risk of cliché, more research is clearly needed.


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#### Competing interests

None declared.

## Redox regulation of protein turnover during unloading-induced atrophy


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The debate articulated by Drs Phillips and McGlory (Phillips & McGlory, 2014*a*,*b*) *vs.* Drs Reid, Judge and Bodine (Reid *et al.* 2014*a*,*b*) regarding the importance of decreased protein synthesis *vs.* increased protein degradation in response to unloading/disuse is a fascinating one indeed. Skeletal muscle is a highly dynamic tissue with robust protein turnover that not only produces force and power for joint motion, but also serves as a substrate source of amino acids. Amino acid demand from skeletal muscle is enhanced for protein needs when nutrient sources are insufficient, common in our hunter–gatherer ancestors, or when injury and illness occur (Wolfe, 2006). Further, teleological arguments suggest that reduction in protein synthesis due to disuse would save energy and substrate resources.

While the mechanisms that alter protein turnover and atrophy with unloading are not fully understood, pro-oxidant (Dodd *et al.* 2010; Min *et al.* 2011) and pro-inflammatory (Judge *et al.* 2007) signalling appear to be important effectors of atrophy and fibre-type switch. Disuse increases the prevalence of oxidized proteins (Lawler *et al.* 2014). Oxidized amino acids could 'tag' proteins for degradation, particularly when levels of chaperone heat shock proteins are low (Senf *et al.* 2008). Accumulation and cross-linking of partially oxidized proteins could lead to cellular toxicity, thus providing impetus for rapid removal. Recently, reactive oxygen species (ROS) have recently been linked to (a) translocation of nNOS$\mu$ and downstream activation of FoxO3a with unloading (Lawler *et al.* 2014), and (b) suppression of Akt phosphorylation and anabolic signalling (Rahman *et al.* 2014). Future research should focus on prospective mechanisms that tie together unloading-induced responses in protein synthesis with proteolysis.


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#### Competing interests

None declared.

## Timing is of the essence

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Abbreviations: MPS, muscle protein synthesis; MPB, muscle protein breakdown.

We appreciated the scientific debate raised in the CrossTalk series regarding the physiological mechanisms underpinning muscle disuse atrophy (Phillips & McGlory, 2014). As stated by Phillips & McGlory, the combined efforts of multiple research groups over the past three decades have unequivocally shown a decline in post-absorptive and post-prandial muscle protein synthesis (MPS) rates accompanying uncomplicated, prolonged (≥2 weeks) disuse in humans (e.g. Gibson *et al.* 1987; Ferrando *et al.* 1996; Glover *et al.* 2008; Wall *et al.* 2013*b*). However, comparable data of how disuse affects muscle protein breakdown (MPB) in humans are simply not yet available. We have recently shown that as little as 5 days of disuse already leads to considerable muscle atrophy (Wall *et al.* 2013*a*), an impact that is unlikely attributed solely to a decline in MPS. However, to date, no *in vivo* human data of how MPB or MPS are affected during this first week of disuse have been generated.

Calculating expected muscle loss based on changes in MPS (and comparing this with observed muscle loss) as a means to indirectly infer changes (or lack thereof) in MPB is a tantalizing but challenging approach. Though relative changes in MPS or muscle mass can be assessed accurately in human disuse studies, the precise numerical value of both parameters are subject to variance depending upon methodological choices. Indirect evidence from humans concerning molecular pathways involved in proteolysis suggests that MPB rises rapidly and transiently at the onset of disuse, which may represent a mechanism to initiate the atrophy process (Wall *et al.* 2013*a*). Consequently, the appropriateness of potential countermeasures for disuse atrophy may be dependent upon the speed at which they are implemented. In this way, clinically and scientifically, timing may really be of the essence!

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### Additional information

#### Competing interests

None declared.

## Both proteolysis and decreased protein synthesis are important in causing disuse muscle atrophy

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Skeletal muscle forms the bulk of body weight and accounts for ∼50% of body mass in young adult males. In addition to its main functions of postural control and the powering of movement, skeletal muscle is important in the maintenance of body shape, structure and composition. It is also the main store of proteins in the body. As a result, skeletal muscle mass is highly regulated and is maintained by the fine balance between anabolism (protein synthesis) and catabolism (protein degradation). Both processes are closely coupled and are regulated by an intricate intracellular signalling pathway controlled by the protein kinase AKT (also known as protein kinase B) (Nader, 2005). Thus, anabolic signals such as resistive exercise, growth factors such as insulin-like growth factor (IGF) 1 and hormones such as male sex steroids lead to its activation (= phosphorylation) and in turn this leads to increased protein synthesis. In contrast, catabolic signals such as disuse, lack of weight bearing and pro-inflammatory cytokines, e.g. tumour necrosis factor (TNF) $\alpha$ lead to its inactivation. The inactivation of AKT is accompanied by decreased protein synthesis and the activation of the ubiquitin proteasome system. Activation of this system leads to increased protein proteolysis. Consequently, it is impossible to separate decreased protein synthesis from increased proteolysis. As a result, we suggest that disuse atrophy arises from a combination of both mechanisms and none is more dominant than the other.

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#### Competing interests

None declared.

## Love and marriage go together like a horse and carriage

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Both propositions fail to recognise that while the magnitude of change in protein synthesis after feeding and/or exercise is large, muscle growth and hypertrophy are slow temporal processes requiring multiple bouts of stimuli. This is particularly evident when compared with rapid muscle loss following even acute disuse. Therefore, protein degradation is perhaps a more sensitive process despite smaller fluctuations in magnitude compared with adaptive physiological responses underpinned by protein synthesis. It is worth reiterating that for muscle atrophy following disuse to occur, complex macromolecular protein structures must be dismantled and ultimately degraded. The question therefore seems not to be which is the dominant mechanism, but rather, after disuse what are the temporal regulators driving physiological change; which process comes first and informs the other; and finally, which is a more sensitive process. Paradoxically, it is worth noting that protein degradation is an important adaptive process for the post-loading response, culminating in early remodelling and repair to enable later hypertrophy. Therefore future research should extend the work that has already been undertaken regarding the cross-talk between signalling mechanisms of synthesis and degradation, which are inextricably linked, for example pro-synthetic Akt inhibition of pro-degradative FOXO and vice versa (Sandri *et al.* 2004; Edström *et al.* 2006), or the duality of function of p38 MAPK (first described by Gillespie *et al.* 2009). Therefore, we suggest degradation and synthesis orchestrate gains or losses in tandem; as the old saying goes, 'love and marriage go together like a horse and carriage/you cannot have one without the other'.

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### Additional information

#### Competing interests

None declared.

## Muscle atrophy – a balanced approach

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While wary of sitting on the fence it seems to me that the dichotomy between breakdown and synthesis as 'causing' muscle atrophy is a false one. More than that, surely this presumed conflict is sterile as it does not lead to new insights for therapies. Growth or atrophy arises from an altered signal of muscle activity, which generally, but is not necessarily, a consequence of actual muscle activity. A more holistic approach asks, 'What processes are required to maintain adequate function in response to that signal?' Atrophy would follow a loss of this maintenance signal.

It is clearly the balance between synthesis and breakdown that determines muscle size. Both synthesis and breakdown may increase or decrease in response to stimulus or disuse, and changes in one may regulate changes in the other. It is the resulting balance, however, that will determine whether a muscle grows or atrophies. Reid *et al.* indicate that measuring this balance biochemically is not practical but clearly the outcome – size – can be measured using imaging. A more fundamental question is what controls that balance, as it is here that interventions are more likely to be fruitful. Control requires a signal, a monitoring process and a mechanism that takes the monitored signal and converts it into a response, ideally with a small amount of feedback. Identifying and being able to monitor/adjust this mechanism would enable intervention at a high level. Setting synthesis against breakdown is at the lowest level and risks generating heat rather than light.

### Additional information

#### Competing interests

None declared.

## Time-course studies hold the key to establishing the dominant mechanism of disuse skeletal muscle atrophy in humans

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This topical CrossTalk debate presents two opposing viewpoints concerning the predominant mechanism that governs 'uncomplicated' disuse skeletal muscle atrophy in humans. Consistent with other experts (Wall & van Loon, 2013), these viewpoints agree that both a suppression of muscle protein synthesis (MPS) and acceleration of muscle protein breakdown (MPB) likely explain the more rapid muscle loss during early ($<$5 days) compared with late ($>$10 days) phases of disuse ($\sim$1.2 *vs.* 0.5% day$^{-1}$). As such, to fully elucidate the predominant mechanism underpinning disuse muscle atrophy, it is necessary to characterize transient changes in muscle protein metabolism over a prolonged time-course of disuse. This challenging prospect will be facilitated by recent developments in the application of deuterium oxide ($^2H_2O$) isotope tracer methodology. The validation of oral $^2H_2O$ isotope tracer protocols allows for the

dynamic *in vivo* measurement of integrated rates of myofibrillar MPS (MacDonald *et al.* 2013; Wilkinson *et al.* 2014) and MPB (Holm *et al.* 2013) over acute (hours), intermediate (days) and chronic (weeks) time periods. Therefore, moving forward, future time-course studies (Brocca *et al.* 2012) should apply $^2H_2O$ tracer methodology to measure temporal changes in MPS and MPB during early (<5 days), intermediate (5–10 days) and prolonged (>10 days) phases of muscle disuse. These data will help establish the predominant mechanism that drives muscle atrophy during early through to later phases of disuse. Moreover, these important data will help inform targeted interventions (exercise, pharmaceutical, nutritional) for ameliorating human muscle atrophy and the associated hypodynamia over a prolonged time-course of muscle disuse.

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### Additional information

#### Competing interests

None declared.

## There is more to skeletal muscle disuse than a dichotomy between decreased protein synthesis or increased protein breakdown


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The topic of this crosstalk is important. Too much time has been spent on trying to simplify the driving factor of human disuse to a dichotomy between decreased protein synthesis or increased protein breakdown, digging trenches between human research and research based on animal data. Few scientists argue about the importance of a decrease in protein synthesis, yet it is increasingly difficult to overlook the emerging bulk of data demonstrating that the regulation of human muscle disuse is far more complex. Specifically, in human research we have previously overlooked the importance of investigating the very early phase of disuse/unloading (1–5 days) where the atrophy response is most strongly manifested and consequently important information has therefore been unnoticed. However, a growing number of studies show evidence of an early rise in atrogenes during human disuse (deBoer *et al.* 2007; Tesch *et al.* 2008; Abadi *et al.* 2009; Suetta *et al.* 2012) with time-course patterns similar to what have previously been demonstrated in the murine model (Sacheck *et al.* 2007).

Another important point that has been overlooked in human research for years is the age-specific way human disuse atrophy seems to be regulated (Suetta *et al.* 2012) and equally important also the phase of muscle re-growth with subsequent reloading (Suetta *et al.* 2013). Notably, this influence of ageing on muscle mass homeostasis is well documented in various animal models. Importantly, however, many links remain missing in the puzzle of human muscle plasticity, which from our point of view can be achieved only through a close interaction between human and animal research.

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### Additional information

#### Competing interests

None declared.

## The dominant mechanism causing disuse muscle atrophy is proteolysis


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One may start to address whether enhanced muscle proteolysis or protein synthesis is the dominant mechanism of disuse-induced muscle atrophy (Phillips & McGlory, 2014; Reid *et al.* 2014) with a thought-experiment.

The second law of thermodynamics implies that any complex structure left on its own is subject to decay. In buildings, for instance, broken light bulbs need replacement, preferably at a rate that matches the rate of breakdown. If replacement stops entirely the building will slowly get darker. However, one could also actively remove light bulbs (apoptosis and the proteasome require ATP) from an unused room to ensure availability in other, utilised rooms. The latter seems to occur during denervation-induced atrophy where the abundance of apoptotic and proteasome components (Sacheck *et al.* 2007) and protein breakdown (Goldspink, 1976) are increased transiently during the period of rapid atrophy. Much of the denervation-induced atrophy was prevented by inhibition of the proteasome (Beehler *et al.* 2006). Since denervation-induced atrophy is faster and more pronounced than in many other models of disuse atrophy, it will elicit qualitatively similar, but quantitatively more pronounced, changes in protein synthesis and breakdown. Rodent models are extremely helpful in this context, as the pattern of disuse-induced atrophy is similar to, but because of the higher metabolic rate much faster than, in humans. In agreement with Reid *et al.* (2014) we suggest that an increased rate of protein breakdown is the predominant factor of disuse-induced atrophy; however, potential effects of a reduced rate of protein synthesis should not be ignored.

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### Additional information

#### Competing interests

None declared.

## Caught in the crossfire?


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Whereas we appreciate that the purpose of the CrossTalk article series is to compare and contrast two opposing viewpoints on a given topic, the desire to promote one's view at the expense of all else can often undermine the value of the scientific debate. In the current set of articles, the proposal and opposing view correctly acknowledge the integral contributions of both suppressed skeletal muscle protein synthesis (MPS) and elevated skeletal muscle protein breakdown (MPB) to the atrophied state, yet both articles by default offer little consideration to the integrated action of these two processes. The complexity of skeletal muscle atrophy, and indeed physiology, is such that the determination of either party to establish a dominant mechanism appears futile when we consider all the contributing factors and mechanisms at play. Instead, we suggest that disuse atrophy should be studied using a holistic approach where value is given to MPS, MPB and their underlying regulatory mechanisms. Although the simultaneous measurement of MPS and MPB during disuse atrophy in humans is not currently possible, advances in the techniques used to accurately measure *in vivo* MPB acutely (Tuvdendorj *et al.* 2013) and over extended time periods (Holm *et al.* 2013) will facilitate such holistic study when implemented in combination with existing MPS (Burd *et al.* 2011; Wilkinson *et al.* 2014) and signalling protein activation (McGlory *et al.* 2014) measures. Validation of these techniques for use in humans remains ongoing, yet collective efforts towards the goal of such an approach will greatly enhance our existing knowledge base of disuse atrophy and provide a raised platform for stimulating interesting and beneficial discussions for the scientific community.

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### Additional information

#### Competing interests

None declared.

## Either or is digital code, a bit of both is human metabolism


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We are perplexed by the heat of the debate regarding the relative contributions of muscle protein synthesis (MPS) and breakdown (MPB) to the disuse-induced loss of muscle mass. Phillips and McGlory argue that disuse-induced muscle atrophy is almost entirely due to a reduced rate of MPS. Meanwhile, Reid, Judge and Bodine argue that MPB is the dominant mechanism and essential for disuse atrophy. Neither have definitive evidence to support their cases.

Phillips and McGlory point to a lot of data demonstrating a disuse-induced decrease in MPS *in vivo* in human subjects but do they have a case for disuse-induced changes in MPS only? We contend that they do not, largely because nothing exists until we

measure it. As such, MPB during muscle disuse was measured in only one study including seven young men (Symons *et al.* 2009) and it was found that prolonged bed rest did not significantly alter it. Phillips and McGlory use this as the case in point to dismiss any contribution of MPB to disuse atrophy – conceivably a bit premature. They further argue that the calculated rate of muscle loss based on average measured MPS rates closely (but evidently not entirely) matches the measured average loss of muscle volume or cross sectional area assessed by using MRI or CT. Thus, setting aside any uncertainties of such comparison for the sake of the argument, there could be significant contribution of accelerated MPB.

Reid, Judge and Bodine focus their arguments largely on changes in cellular factors involved in regulating proteolysis in non-human models. There is also (although sometimes conflicting) evidence for cellular adaptations indicative of temporarily upregulated proteolysis in human muscle (e.g. Jones *et al.* 2004; Urso *et al.* 2006; Suetta *et al.* 2012) but the relationship between these measures and actual rates of MPB is still uncertain. Furthermore, there is nothing to suggest that MPB is essential (i.e. required) for disuse atrophy; theoretically it is certainly possible for it to occur simply as a result of the well-established disuse-induced reduction in MPS.

Since metabolic adaptations are usually multifactorial and complex, it is conceivable that muscle disuse affects both MPS and MPB to varying degrees over time. To find out to what extent alterations in each contribute to disuse atrophy will require a comprehensive (incl. fasted and fed state) and longitudinal (early/short-term *vs.* late/prolonged) simultaneous assessment of both MPS and MPB rates.

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## Additional information

### Competing interests

None declared.

# Ubiquitin-dependent proteolysis regulates muscle protein synthesis as well as breakdown


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As to the big concern that the dominant mechanism remains unknown in disuse skeletal muscle atrophy, our answer is that proteolysis is essential for the disuse atrophy. Unloading mechanical stress, such as microgravity, directly induced skeletal muscle atrophy (Vandenburgh *et al.* 1999), indicating that skeletal muscle cells can sense the unloading stress without hormonal and neural signalling. In the response of skeletal muscle cells to unloading conditions, muscle protein synthesis (MPS) is closely associated with muscle protein breakdown (MPB) like bone remodelling. In bone remodelling, removing old bone (bone resorption) by osteoclasts is the initiation at the remodelling site and is followed by bone formation by osteoblasts to repair damaged bone. Given that disuse atrophy is an adaptive consequence against unloading stress, we reason that removing damaged protein by MPB is the initiation.

In addition, disuse atrophy underlies the resistance of skeletal muscle cells to myotrophic IGF-1 signalling (Sandri *et al.* 2004; Stitt *et al.* 2004). We previously reported that ubiquitin ligase Cbl-b is a negative regulator for IGF-1 signalling during muscle atrophy caused by unloading (Nakao *et al.* 2009). The mechanism of Cbl-b-induced muscle atrophy is unique; it does not appear to involve the degradation of structural components of the muscle, rather it impairs muscle growth signals in response to unloading conditions. Unloading induced Cbl-b expression very quickly (less than 3 h) in skeletal muscle cells, and the IGF-1-mediated cell growth via MPS of rodent or human myocytes was not suppressed unless there was Cbl-b induction. On the basis of these findings, we propose that proteolysis, especially ubiquitin-dependent proteolysis, is the initiation for the disruption of the MPS and MPB balance.

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## Motion to dismiss: insufficient evidence to mount a case against protein synthesis as the dominant mechanism causing disuse muscle atrophy in the elderly

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The well-argued positions in this CrossTalk debate (Phillips & McGlory, 2014; Reid *et al.* 2014) clearly indicate that reduced muscle protein synthesis (MPS) and elevated muscle proteolysis are *both* contributing to muscle atrophy during disuse. Concluding which is the dominant mechanism based on current evidence is difficult due to technical limitations, particularly in the direct measurement of muscle proteolysis, but also as a function of the time point of sampling, e.g. acute *vs.* chronic phases of disuse, and the population examined. Defining these mechanisms is key to translation of research into appropriate treatment. Consider the case of the elderly, a population likely to encounter periods of both acute and chronic disuse. Basal rates of MPS in the postabsorptive state are similar following 7 days of bed rest compared with pre-bed rest values in an elderly cohort, despite ∼4% loss of muscle mass (Drummond *et al.* 2012). A key regulator of muscle mass is the postprandial regulation of MPS/MPB and we know that MPS is reduced by 40% in the postprandial period following acute bed rest, which potentially accounts for this loss (Drummond *et al.* 2012). However, there are currently no data directly measuring disuse-associated changes in proteolysis in elderly individuals in either the acute or chronic phase. Thus, until the technical challenges are addressed and current markers of muscle proteolysis are embraced (e.g. MAFbx, MuRF-1, FOXO3a) it is difficult to argue a case against, in the elderly at least, decreased protein synthesis as the dominant mechanism in disuse atrophy.

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## The relative role of decreased protein synthesis and increased proteolysis in causing disuse muscle atrophy varies through models, muscles and species and depends on the duration of unloading

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Our works on disuse atrophy in mice slow (Cannavino *et al.* 2014) and fast (Cannavino *et al.* 2015) muscle using hind-limb unloading indicated that both an increase of proteolysis and a decrease in protein synthesis come into play early in determining muscle atrophy and to a different extent. In the early stages of disuse, atrophy in fast muscle was caused by proteolysis only, while, in slow muscle, both activation of proteolysis and reduction of protein synthesis played a role. Furthermore, the latter studies showed a good correlation between *in vitro* parameters (catabolic and synthetic markers) and the degree of muscle atrophy. In fact, preventing catabolism activation, mass was completely preserved in fast muscle and only partially in slow muscle, where synthetic parameter down-regulation persisted.

Although caution is a must when extrapolating data from animal studies to humans because of differences between muscles, species and experimental models (Pellegrino *et al.* 2011), both proteolysis and decreased protein synthesis should still be considered likely candidates in humans too.

Indeed, an early and transient induction of proteolytic markers similar to that observed in mice (Cannavino *et al.* 2014, 2015) was observed in humans (Abadi *et al.* 2009; Gustafsson *et al.* 2010). Moreover, based on our studies in mice, it appears that no activation of MurF-1 and atrogin-1 at later stages of disuse (Brocca *et al.* 2012) does not necessarily mean proteolysis plays a minor role. Therefore, we believe that the contribution of proteolysis cannot be excluded, unless the irrelevance of such a process is experimentally proved. The doubt could be dissipated if, after preventing the early induction of catabolic markers in humans, the experimental variation of muscle mass remained unaffected.

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### Additional information

#### Competing interests

None declared.

## 'Measure what is measurable, and make measurable what is not so' (Galileo Galilei)

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Following years of research in muscle protein metabolism, there is strong evidence that muscle protein synthesis (MPS) is the dominant factor in simple disuse atrophy. Particularly, the theory that a decrease in MPS during this process is based on several studies involving *in vitro*, *ex vivo* as well as *in vivo* measurements of MPS (Mallinson *et al.* 2013). Conversely, even though the theory that proteolysis is the dominant mechanism underlying disuse atrophy is supported by reliable animal models, a lack in translational research makes it less credible.

However, either to confirm that MPS is the dominant mechanism during disuse atrophy in humans or to verify whether instead proteolysis is the main determinant, further research in the field is warranted.

Particularly there is a necessity to translate findings to an *in vivo* setting, using accurate direct dynamic labelling techniques able to compatibly assess MPS and proteolysis in humans. Indeed while MPS is currently determined by reliable methods, proteolysis falls behind. Recently deuterated water, a validated technique used to assess MPS (Gasier *et al.* 2010; Wilkinson *et al.* 2014), has been proposed to also measure MPB, thus allowing comparable measurements of both aspects of muscle protein turnover (Wolfe, 2011; Holm *et al.* 2013).

Therefore the development of new techniques and approaches will help researchers to link data coming from gene expression, signalling pathway and protein metabolism and thus better understanding the main mechanism lying behind disuse atrophy.

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### Additional information

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None declared.

## Changes in protein degradation and synthesis are both critical in muscle atrophy

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Surprisingly, Phillips & McGlory (2014) disregard the extensive evidence that increased protein breakdown is also critically important in atrophy (Mitch & Goldberg, 1996; Glass, 2010; Reid *et al.* 2014; Cohen *et al.* 2015). Pulse-chase studies in rats first showed that atrophy induced by denervation or glucocorticoids involved accelerated proteolysis (Goldberg, 1969), and once methods were developed to measure precisely rates of degradation and synthesis in isolated muscles (Fulks *et al.* 1975; Tischler *et al.* 1982), we demonstrated that degradation rises with denervation (Furuno *et al.* 1990), disuse (Tischler *et al.* 1997), fasting (Li & Goldberg, 1976), cancer (Baracos *et al.* 1995) and acidosis (Mitch *et al.* 1994). At present, no method exists to measure accurately degradation rates in human muscles. Because proteolysis cannot be measured accurately in humans, one cannot conclude that it does not increase. Also, it is invalid to predict changes in muscle mass from measurements of overall synthesis, since individual cell proteins turnover at widely different rates, and their rates of transcription (Sacheck *et al.* 2007), synthesis (Li & Goldberg, 1976; Furuno *et al.* 1990), and degradation (Li & Goldberg, 1976; Furuno *et al.* 1990; Cohen *et al.* 2009, 2012) vary as atrophy progresses.

Phillips & McGlory (2014) dismiss 'as biased' conclusions from isolated rodent muscles. However, such preparations behave linearly for hours and are still the only method to precisely measure turnover rates. Such studies first demonstrated the ability of insulin and amino acids to suppress proteolysis (Fulks *et al.* 1975; Tischler *et al.* 1982), later confirmed in humans, and enabled the discovery of the critical adaptations during atrophy: activation of the ubiquitin–proteasome pathway (Medina *et al.* 1995; Wing *et al.* 1995) through atrogene induction (Jagoe *et al.* 2002; Lecker *et al.* 2004; Sacheck *et al.* 2007). Phillips & McGlory (2014) accept our finding (Jagoe *et al.* 2002; Lecker *et al.* 2004; Reid *et al.* 2014) that a common transcriptional programme enhances muscle proteolysis in disease states but state incorrectly that similar changes do not occur with disuse. In fact, we demonstrated that denervation and pure disuse induce the same atrophy-related changes as fasting and disease (Sacheck *et al.* 2007).

In human and animal cells, contractile activity, nutrients, and insulin activate the PI3K–AKT–mTOR pathway (Glass *et al.* 2010; Cohen *et al.* 2015), which promotes translation and inhibits proteolysis by suppressing autophagy (Efeyan *et al.* 2015)

and inactivating FoxO-mediated expression of genes for ubiquitination (Sandri *et al.* 2004; Cohen *et al.* 2015) and autophagy (Mammucari *et al.* 2007; Zhao *et al.* 2007). Thus, overall rates of protein degradation and synthesis change coordinately.

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## Additional information

### Competing interests

None declared.

PLOS | ONE

RESEARCH ARTICLE

# Plasma MicroRNA Levels Differ between Endurance and Strength Athletes

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## Abstract

### Aim

MicroRNAs (miRNAs) are stable in the circulation and are likely to function in inter-organ communication during a variety of metabolic responses that involve changes in gene expression, including exercise training. However, it is unknown whether differences in circulating-miRNA (c-miRNA) levels are characteristic of training modality.

### Methods

We investigated whether levels of candidate c-miRNAs differ between elite male athletes of two different training modalities (n = 10 per group) - endurance (END) and strength (STR) - and between these groups and untrained controls (CON; n = 10). Fasted, non-exercised, morning plasma samples were analysed for 14 c-miRNAs (miR-1, miR-16-2, miR-20a-1, miR-21, miR-93, miR-103a, miR-133a, miR-146a, miR-192, miR-206, miR-221, miR-222, miR-451, miR-499). Moreover, we investigated whether c-miRNA levels were associated with quantitative performance-related phenotypes within and between groups.

### Results

miR-222 was present at different levels in the three participant groups ($p = 0.028$) with the highest levels being observed in END and the lowest in STR. A number of other c-miRNAs were present at higher levels in END than in STR (relative to STR, ± 1 SEM; miR-222: 1.94 fold (1.73-2.18), $p = 0.011$; miR-21: 1.56 fold (1.39-1.74), $p = 0.013$; miR-146a: 1.50 fold (1.38-1.64), $p = 0.019$; miR-221: 1.51 fold (1.34-1.70), $p = 0.026$). Regression analyses revealed several associations between candidate c-miRNA levels and strength-related performance measures before and after adjustment for muscle or fat mass, but not following adjustment for group.

### Conclusion

Certain c-miRNAs (miR-222, miR-21, miR-146a and miR-221) differ between endurance- and resistance-trained athletes and thus have potential as useful biomarkers of exercise

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training and / or play a role in exercise mode-specific training adaptations. However, levels of these c-miRNAs are probably unrelated to muscle bulk or fat reserves.

## Introduction

The health benefits of regular exercise are well recognised [1]. However, the physiological adaptations that occur in response to exercise training differ according to genetic predisposition [2] and exercise modality [3,4]. These differential responses are driven by a variety of mechanisms including changes in gene expression and protein abundance [5], yet the specific mechanisms governing these molecular modifications remain poorly understood.

Recent research has centred on epigenetic control mechanisms, including microRNAs (miRNAs), and their role in regulating gene expression. miRNAs are small, non-coding ribonucleic acids (RNAs) that play a vital role in regulation of gene expression. A major part of their role is in post-transcriptional regulation through direct binding to target messenger RNAs (mRNAs), and it is estimated that at least 30% of the human gene complement is regulated by miRNAs [6]. Each miRNA binds to multiple mRNAs, and each mRNA is regulated by multiple miRNAs, providing a highly sensitive reciprocal level of regulation [7].

Differences in skeletal muscle phenotype have been associated with differences in miRNA expression levels and with genetic differences in miRNA binding sites. In Texel sheep, regulation of skeletal muscle hypertrophy has been associated with variation in a region of the *Myostatin* gene containing binding sites for muscle-specific miRNAs [8]. In humans, levels of miR-378 in skeletal muscle were significantly correlated with lean muscle mass gains following a 12 week resistance exercise training intervention [9]. In addition, levels of several other miRNAs in skeletal muscle have been shown to change following acute endurance exercise and following endurance exercise training interventions [10]. Thus, miRNAs are associated with skeletal muscle adaptation to exercise training and in combination with underlying genetic differences, may be involved in the differential phenotypic response to resistance and endurance type exercise.

miRNAs are found not only in tissues (*e.g.* skeletal muscle) but also in a variety of body fluids including plasma [11,12]. Outside the cell, RNases act to rapidly degrade RNA [13]; however, miRNAs are thought to be stable within the circulation primarily through incorporation into secreted exosomes, but also through co-localisation with the RNA-induced silencing complex (RISC) or via binding to high-density lipoproteins (HDL) [14]. Additionally, miRNAs appear to be actively secreted into the circulation since exosomes contain only a subset of miRNAs, present in proportions different to those in their cell type of origin [15]. Recent studies have suggested that circulating miRNAs (c-miRNAs) are involved in cell-cell communication and thus may have regulatory roles in normal physiology and in disease mechanisms [14]. c-miRNAs are remarkably stable in the circulation, easy to sample, and can be readily extracted and measured using standard qRT-PCR techniques [11]. For these reasons, the study of c-miRNAs may yield useful insight into the mechanisms of adaptation to exercise.

The mechanisms by which exercise adaptation is effected and coordinated across multiple organ systems remain somewhat unclear. Training is known to alter the health and function of not only the exercised skeletal muscle but also a number of other organs resulting in a whole body adaptive response [16]. c-miRNAs may be acting as the orchestrators of this multi-site response. Changes in c-miRNA levels have been demonstrated following a programme of endurance training [17,18] and in response to an acute endurance [17–19] or resistance [20] exercise

bout. Post-exercise changes in c-miRNA levels also have been shown to differ between concentric and eccentric endurance exercise modalities [21]. Thus, the capacity for miRNAs to respond to the exercised and trained state, along with differential c-miRNA responses to exercise of differing modality, renders c-miRNAs potential biomarkers of specific exercise responses.

We aimed to examine whether there are differences in c-miRNA profiles between males involved in different long-term training regimes, and to determine whether c-miRNAs are specific markers of particular training modes. Such differences may form part of the basis for differences in physiological adaptation between individuals using different training modes. First, we investigated whether levels of candidate c-miRNAs differed between individuals at two ends of the training specificity spectrum, elite endurance (END) and strength (STR) athletes, and in comparison to age-matched, untrained controls (CON). In addition, since endurance and strength athletes differ phenotypically to such an extent, we conducted secondary analysis comparing c-miRNA levels in the two athlete groups alone. Finally, we investigated the association between c-miRNA levels and quantitative physiological variables indicative of strength or endurance training. We hypothesised that levels of the selected c-miRNAs, which have previously been shown to be exercise-responsive, would differ between the two athlete populations and also that c-miRNA levels would associate with respective quantitative variables.

## Materials and Methods

### Ethics statement

Independent ethical approval was obtained from the Lithuanian Bioethics Committee and all procedures were in accordance with the Declaration of Helsinki (2008). All participants gave full written informed consent prior to study commencement.

### Participant characteristics

Participant characteristics are summarised in Table 1. Participants were selected from a larger cohort of male athletes and controls recruited to a genetic association study (GeLA cohort) conducted at the Lithuanian Sports University and for whom plasma samples were available. The study sample comprised strength athletes (STR; n = 10), endurance athletes (END; n = 10) and age-matched non-exercising controls (CON; n = 10). Athlete participants were selected from a larger pool after phenotyping for athletic performance. The 10 strength athletes with the best performance across several strength / power tests (isokinetic peak torque of arms and legs, handgrip strength and counter-movement jump height) were selected. For the endurance subgroup, the 10 age-matched athletes with the largest relative maximal oxygen uptake (treadmill) were selected (for values see Table 1; for methods see S1 Methods). All athletes trained for 13 h per week on average, and competed regionally, nationally or internationally. Endurance athletes were involved in sports such as distance running and orienteering (sports that mostly require use of the legs), whereas strength athletes were involved in sports such as weightlifting and combat sports (which require a more equal contribution of arm and leg muscles). Concentric and eccentric exercise components were likely similar between training types. Control participants were age-matched to the athlete groups but otherwise selected randomly from the control group in the genetic association study, and did not compete in any competitive sport, or partake in any organised physical activity on more than two occasions per week.

### Sampling

Participants visited the laboratory in a fasted state and at least 12 h post-exercise. Venous blood samples (10 mL) were collected from all study participants in EDTA vacutainers and

**Table 1. Participant characteristics by group.**

| | CON | STR | END |
|---|---|---|---|
| n | 10 | 10 | 10 |
| Age (years) | 24.0 ± 2.8 | 22.2 ± 2.1 | 22.6 ± 3.7 |
| Body mass (kg) | 79.3 ± 15.6 | 84.8 ± 10.2 | 70.6 ± 7.2 † |
| Muscle mass (MM; kg) | 41.8 ± 7.4 | 50.7 ± 6.7 * | 42.8 ± 4.3 † |
| Fat mass (FM; kg) | 24.3 ± 9.6 | 19.7 ± 4.2 | 13.9 ± 1.2 *† |
| BMI ($kg \cdot m^{-2}$) | 24.4 ± 3.1 | 25.6 ± 2.6 | 22.2 ± 2.5 † |
| $VO_2$ max ($mL \cdot kg^{-1} \cdot min^{-1}$) | 43.5 ± 2.4 | 50.4 ± 6.5 * | 66.9 ± 4.7 *† |
| $VO_2$ max ($mL \cdot kg^{-0.75} \cdot min^{-1}$) | 129.2 ± 7.0 | 152.8 ± 5.8* | 189.1 ± 6.3 *† |
| Wingate fatigue index ($W \cdot s^{-1}$) | 6.5 ± 1.2 | 6.1 ± 0.7 | 4.8 ± 1.5 *† |
| Isokinetic knee extension peak torque (Nm; $30° \cdot s^{-1}$) | 507.4 ± 122.9 | 605.6 ± 121.5 | 459.3 ± 54.2 † |
| Isokinetic elbow extension peak torque (Nm; $30° \cdot s^{-1}$) | 115.4 ± 22.3 | 152.6 ± 46.5 * | 94.2 ± 12.9 *† |
| Handgrip strength (kg) | 121.4 ± 11.9 | 148.2 ± 14.6 * | 111.7 ± 14.3 *† |
| Counter-movement jump height (cm) | 36.1 ± 5.8 | 42.6 ± 6.3 * | 37.7 ± 5.5 |

All values are mean ± SD. With the exception of age, all measures are significantly different by ANOVA ($p < 0.05$).

* significantly different from CON by t-test;

† significantly different from STR by t-test ($p < 0.05$). $VO_2$ max ($mL \cdot kg^{-0.75} \cdot min^{-1}$) has been included for comparison to other studies.

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centrifuged at 3000 rpm for 15 min at room temperature for plasma separation. Plasma was immediately aliquoted into 1.5 mL microcentrifuge tubes for storage at -80°C pending subsequent analysis.

On the other visits to the laboratories over the period of several days to 3 weeks, a large variety of phenotype measurements were collected. These measurements included anthropometric characteristics, tests of handgrip strength, isokinetic dynamometry, pull-ups, vertical jumps, an agility shuttle run, a 30 m sprint, a Wingate test, and a continuous ramp-up spiroergometric treadmill test to exhaustion. Details of the testing procedures and protocols are provided in S1 Methods. Fat mass (FM) was calculated from the sum of 4 skinfold measurements (triceps, biceps, subscapular and suprailiac) using the body fat percentage calculation of Durnin & Womersley [22]. Muscle mass (MM) was estimated using standardised height and skinfold-corrected girth measurements of the forearm, calf and thigh within the equation of Martin *et al.* [23].

## RNA extraction and complementary DNA (cDNA) synthesis

All molecular and statistical analyses were conducted at the University of Stirling. RNA was extracted from plasma samples using the miRNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions with a starting volume of 200 μL. From the resulting RNA eluate, cDNA was synthesised using the miScript II RT Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions using 12 μl template RNA. cDNA reactions were diluted from 20 μL to 250 μL with double-distilled water and loaded into deep well plates for storage at -20°C until further analysis.

## miRNA analysis

Commercially available quantitative polymerase chain reaction (qRT-PCR) primer assays (Qiagen Ltd., West Sussex, UK) were used for miRNA analysis (target miRNAs: miR-1, miR-20a-1, miR-21, miR-133a, miR-146a, miR-206, miR-221, miR-222, miR-499; control miRNAs:

miR-16-2, miR-93, miR-103a, miR-192, miR-451). The candidate c-miRNAs measured in the present study were selected due to their observed upregulation at rest in response to endurance rowing training [17], for their status as myomiRs [24] or because we initially believed them to be suitable for use as control genes [25]. miRNA levels were measured in triplicate, using the synthesised cDNA, with the miScript SYBR Green PCR kit (Qiagen Ltd., West Sussex, UK). A standard curve (1 in 10 dilution series starting with a 1 in 10 dilution, using a pooled sample consisting of cDNA from all participants in all three groups) was included on each plate to assess whether all assays were in the linear range. The final 15 μL reaction volume comprised: 2x SYBR Green mix (7.5 μL), 10x miScript universal primer (1.5 μL), 10x miScript primer assay (1.5 μL) and cDNA dilution (4.5 μL).

Real-time quantitative polymerase chain reactions (qRT-PCR) were run on the Quantica real-time thermal cycler system (Techne, Bibby Scientific Ltd., Staffordshire, UK). An initial activation step (95°C; 15 min) preceded 40 cycles of denaturation (94°C; 15 s), annealing (55°C; 30 s) and elongation / extension (70°C; 30 s). Reactions were completed with a melting curve for quality control to ensure that only a single amplicon was present in each reaction. All cycle threshold (Ct) values were within the linear range of the standard curve. Ct outliers were removed using the median absolute deviation method (also known as the modified Z-score; [26]) with the maximum acceptable threshold set at 3.5. Control samples (pooled cDNA from each participant) were included on each plate to allow standardisation of the Ct values for experiments that spanned several plates.

Five control assays were selected (see above for miRNA controls list) based on previous reports showing expression stability within plasma [25]. Normfinder software [27] was used to determine which single gene or group of genes was at the most stable level within the samples. miR-93 alone was found to be most stable across the three groups and was therefore used to determine the relative expression of the target genes, calculated using the $2^{-\Delta\Delta Ct}$ method [28]. Consistent with miR-93 being the most stable miRNA between groups, data from other reports indicate that the other 4 miRNAs may not have been good candidates for control genes due to their association with other stimuli including cancer [29]. Thus, these 4 miRNAs were added to the set analysed as candidate genes.

## miRNA target prediction

Pathways targeted by our candidate miRNAs were predicted using the web-based tool, miRSystem [30]. miRSystem integrates several databases (Kegg, Biocarta, Pathway Interaction Database, Reactome and GO molecular function) to enable prediction of gene targets and targeted pathways.

## Statistical analysis

**Power calculations.** Power calculations for the study design were conducted using G*Power3 [31] based on means and standard deviations of relative c-miRNA levels reported by Baggish *et al.* [17]. Power calculations were also performed on a number of physiological measures from the current study to ensure sample sizes were adequate for detecting between-group differences in these physiological variables. An n of 10 offered 80% power to detect between-group differences in mean miRNA levels of 0.60 SDs by ANOVA, $\alpha = 0.05$. The smallest significant between-group difference reported by Baggish *et al.* [17] was 1.33 SDs. In all analyses described below, we have adopted the principle that because there is prior evidence for associations between levels of the c-miRNAs tested and performance-related phenotypes, an $\alpha$ of $p = 0.05$ is appropriate for each test.

**c-miRNA analysis by participant group.** Statistical analysis was conducted using Minitab software (version 16; Minitab, State College, PA). Box-Cox transformations were applied to non-normally distributed data and normal distributions were confirmed using the Ryan-Joiner test. All statistical tests were conducted on normally distributed data. Between-group differences in miRNA levels for STR, CON and END subgroups were determined by one-way ANOVA. In the case of statistical significance by ANOVA, Tukey's *post-hoc* tests were implemented. Direct comparisons of miRNA levels between the two athlete groups only were conducted by means of Student's t-tests.

**Association of c-miRNA expression with performance-related phenotypes.** Regression analyses (GLM) were conducted between c-miRNA levels and quantitative phenotypes related to strength or endurance exercise. Following initial regression analyses between levels of each candidate c-miRNA and each phenotypic measure, we ran models incorporating group (*i.e.* STR, CON, END) and tissue mass (fat mass (FM) and muscle mass (MM)) as predictive variables. Adjusting for tissue mass is important as some c-miRNA levels are correlated with tissue mass measures, and cellular miRNA pools in muscle and adipose tissue could be influencing circulating levels; this potentially confounding factor therefore needs to be accounted for. Adjusting for group further accounts for unmeasured intrinsic differences between the different types of athletes that could also be confounding uncorrected associations. All values are expressed as mean ± standard error of the mean (SEM) unless stated otherwise.

## Results

### c-miRNA analysis by group

Circulating levels of 14 miRNAs were measured in the athlete and control groups. Table 2 shows mean relative levels for each group for each of the test miRNAs, standardised to the reference gene (miR-93) and to levels in the control (CON) group. One-way ANOVA revealed a statistically significant difference ($p = 0.028$; Fig 1A) in miR-222 levels between the three

**Table 2. Levels of all measured c-miRNAs.**

| c-miRNA | CON | | | STR | | | END | | | ANOVA p value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | 95% CI | SEM | Mean | 95% CI | SEM | Mean | 95% CI | SEM | |
| **miR-1** | 1 | 0.32–3.11 | 0.65–2.08 | 0.84 | 0.42–1.67 | 0.63–1.26 | 1.17 | 0.33–4.18 | 0.74–2.71 | 0.910 |
| **miR-16-2** | 1 | 0.51–1.96 | 0.75–1.49 | 1.3 | 0.55–3.07 | 0.92–2.20 | 0.76 | 0.30–1.93 | 0.53–1.36 | 0.675 |
| **miR-20a-1** | 1 | 0.44–2.27 | 0.71–1.65 | 1.03 | 0.43–2.42 | 0.72–1.74 | 0.62 | 0.25–1.50 | 0.43–1.07 | 0.657 |
| **miR-21** | 1 | 0.73–1.37 | 0.86–1.19 | 0.77 | 0.61–0.96 | 0.69–0.87 | 1.20 † | 0.96–1.48 | 1.08–1.34 | 0.074 |
| **miR-103a** | 1 | 0.74–1.35 | 0.87–1.18 | 0.8 | 0.62–1.03 | 0.71–0.92 | 1.14 | 0.82–1.59 | 0.98–1.37 | 0.251 |
| **miR-133a** | UD | - | - | UD | - | - | UD | - | - | - |
| **miR-146a** | 1 | 0.76–1.32 | 0.88–1.16 | 0.75 | 0.57–0.97 | 0.66–0.86 | 1.12 † | 0.95–1.32 | 1.03–1.22 | 0.069 |
| **miR-192** | 1 | 0.65–1.53 | 0.82–1.27 | 1.15 | 0.65–2.03 | 0.89–1.60 | 0.88 | 0.38–2.05 | 0.62–1.48 | 0.845 |
| **miR-206** | 1 | 0.11–9.18 | 0.55–5.17 | 1.54 | 0.48–4.95 | 1.00–3.28 | 0.28 | 0.05–1.52 | 0.16–0.91 | 0.392 |
| **miR-221** | 1 | 0.71–1.41 | 0.85–1.21 | 0.9 | 0.71–1.14 | 0.80–1.02 | 1.36 † | 1.08–1.71 | 1.22–1.54 | 0.119 |
| **miR-222** | 1 | 0.73–1.37 | 0.86–1.19 | 0.66 | 0.44–1.00 | 0.55–0.83 | 1.29 † | 1.03–1.62 | 1.16–1.46 | 0.028 * |
| **miR-451** | 1 | 0.57–1.74 | 0.78–1.38 | 1.34 | 0.77–2.33 | 1.05–1.85 | 0.70 | 0.38–1.29 | 0.54–1.00 | 0.307 |
| **miR-499** | 1 | 0.47–2.13 | 0.73–1.58 | 0.53 | 0.25–1.11 | 0.39–0.83 | 0.47 | 0.13–1.68 | 0.30–1.09 | 0.485 |

Mean values are expressed relative to CON and all measurements have been standardised relative to levels of miR-93 as a control. UD = Undetectable.

* significantly different between the three groups (One way ANOVA; $p < 0.05$);

† significantly different from STR by t-test ($p < 0.05$).

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groups, with levels nominally being higher than CON in the endurance (END) group and lower than CON in the strength (STR) group. The *post-hoc* Tukey's test revealed a significant difference between STR and END ($p = 0.011$).

Plasma levels of all other c-miRNAs were not significantly different between groups by ANOVA ($p > 0.05$; Table 2). However, as we expected to find differences between elite athletes undergoing very different training regimes, we also conducted t-tests comparing the athlete groups only. This secondary analysis revealed higher plasma levels of three other miRNAs in END than in STR: miR-21 ($p = 0.013$), miR-146a ($p = 0.019$) and miR-221 ($p = 0.026$) (Fig 1B–1D), with CON intermediate in mean level, as found for miR-222.

## Association of c-miRNA expression with performance-related phenotypes

The differences in circulating levels of a subset of the miRNAs tested could reflect a response of various aspects of cellular physiology to different training regimes. Thus, we investigated whether levels of the four miRNAs showing differences between the athlete groups were correlated with values for performance in a range of tests that are indicative of the physiological adaptation to different training regimes. We investigated these correlations with and without adjustment for athlete group and anthropometric measures (FM and MM) in the regression models. These corrections enabled us to account for inherent training / phenotypic differences between the groups and to control for differences in body composition between groups. Differences in muscle bulk or fat reserves may influence levels of c-miRNAs either via rates of production or via rates of uptake by these tissues, both of which have an important influence on capacity for, or the adaptation to, exercise. The characteristics of participants in each group for these measures are summarised in S1 Table and results of the regression models are summarised in S2–S5 Tables.

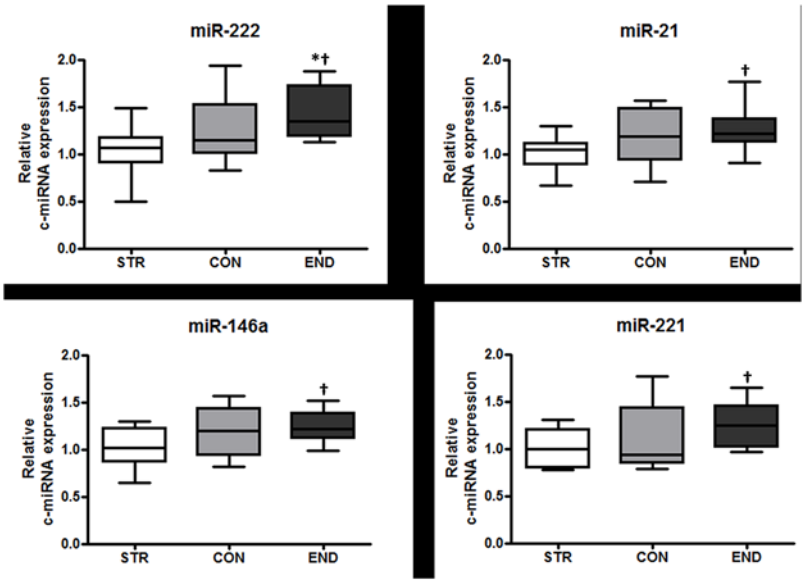


**Fig 1. Relative expression levels of miR-222 (A), -21 (B), -146a (C), and -221 (D) in STR, CON and END.** Box plots depict the range (upper and lower whiskers), median (centre line) and interquartile range (edge of boxes). * significantly different between all groups (One way ANOVA; $p < 0.05$); † significantly different from STR (t-test; $p < 0.05$).

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Plasma miR-222 levels (S2 Table) were positively correlated with height, body mass, muscle mass and fat mass after adjustment for group ($p < 0.05$). Also, plasma miR-222 levels were positively correlated with a strength-related performance measure, isokinetic leg flexion peak torque at various contraction velocities, after adjustment for group ($p = 0.018$ to $0.043$), but not in the basic model, nor after adjustment for MM or FM. None of the other performance measures were correlated with levels of this c-miRNA.

Plasma miR-21 levels (S3 Table) were not associated with anthropometric parameters, but were negatively correlated with a subset of strength / power and endurance-related measures in some models. These strength-related associations were true for handgrip strength in the unadjusted model ($p = 0.039$) and for some velocities of isokinetic leg and arm extension peak torque in the unadjusted and MM-adjusted models (p ranges from 0.015 to 0.044). A similar negative correlation was seen with an anaerobic endurance parameter, the Wingate fatigue index, in the unadjusted and MM-adjusted models ($p < 0.045$).

Plasma miR-146a levels (S4 Table) were positively correlated with height in the group-adjusted ($p = 0.023$), MM-adjusted ($p < 0.001$) and FM-adjusted ($p = 0.016$) models and negatively correlated with BMI in the MM-adjusted ($p = 0.010$) and FM-adjusted ($p = 0.005$) models. Levels of miR-146a were not associated with endurance- or power-related measurements, but were negatively correlated with a subset of strength-related measures in some models. These strength-related associations were true for handgrip strength in the unadjusted, and both MM- and FM-adjusted, models ($p < 0.027$), and for most measures of isokinetic arm flexion and extension peak torque in the unadjusted, and both MM- and FM-adjusted, models (p ranges from 0.002 to 0.045). No significant associations (other than for height) were observed following adjustment for group.

Plasma miR-221 levels (S5 Table) were positively associated with height after MM- or FM-adjustment ($p < 0.038$) and negatively correlated with BMI after adjustment for MM ($p = 0.016$). Levels of this c-miRNA were not associated with endurance- or power-related measurements, but were negatively associated with some strength-related measures. These strength-related negative associations included handgrip strength in the unadjusted and FM-adjusted models ($p < 0.024$) and particularly in the MM-adjusted model ($p = 0.001$). Moreover, most measures of isokinetic arm flexion and extension peak torque in the MM-adjusted models (p ranges from 0.008 to 0.046) also were negatively associated with plasma miR-221 levels. Again, no significant associations were observed following adjustment for group.

It should be noted that a number of myomiRs (miR-1, -133a, -206, -499) were assessed in the present investigation; however, they are found at very low levels in the circulation, which affected measurement consistency in our analysis. Such measurement inconsistencies also have been noted in some reports [18,32] in the context of c-miRNAs and we would recommend a pre-amplification step when investigating these miRNAs in future.

## Predicted miRNA targets

Given the differences between the athlete groups in a subset of the miRNAs tested (miR-21, miR-146a, miR-221 and miR-222) and the association between levels of these c-miRNAs and a subset of performance parameters, we conducted an analysis to ascertain whether common mRNA targets were shared to a significant extent by these c-miRNAs. Predictions using miRSystem indicated that these four miRNAs, when added into the model simultaneously, target mRNAs contained in a total of 736 pathways across all integrated databases (data not shown). The top 8 pathways targeted by all four miRNAs together (arbitrarily selected based on a score $> 3.0$) are shown in Table 3.

**Table 3. miRSystem pathway analysis.**

| Database | Pathway | Total union targets (of total genes in the pathway) | Score |
|---|---|---|---|
| Pathway interaction database | Direct p53 effectors | 31 (of 137) | 4.430 |
| Pathway interaction database | PDGFR-β signalling | 26 (of 126) | 3.862 |
| Pathway interaction database | c-myb transcription factor network | 17 (of 81) | 3.511 |
| Go molecular function (Tier 2) | Protein binding transcription factor activity | 45 (of 369) | 3.445 |
| Kegg | TGF-β signalling | 17 (of 84) | 3.347 |
| Kegg | Pathways in cancer | 43 (of 325) | 3.310 |
| Kegg | MAPK signalling | 36 (of 272) | 3.167 |
| Pathway interaction database | SHP2 signalling | 14 (of 54) | 3.143 |

Union targets refers to genes targeted by all 4 miRNAs; score is deduced from the sum of the weight of its miRNA multiplied by its enrichment, minus the log (p value) from the predicted gene targets. The weight for one miRNA is calculated by dividing its absolute expression value by the absolute sum of the expression values for all input miRNAs [30].

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## Discussion

We report a significant difference in circulating miR-222 levels between male endurance athletes, strength athletes and age-matched untrained controls. Moreover, in endurance athletes, plasma levels of miR-21, miR-146a and miR-221, as well as miR-222, were significantly higher than in strength athletes. Levels of these c-miRNAs were significantly associated with performance parameters indicative of training mode, such as isokinetic peak torque and $VO_2$max, particularly in tissue-corrected models. To the best of our knowledge this study is the first to investigate levels of c-miRNAs in distinct athlete groups and in comparison to untrained controls in a single study, and also is the first to offer a comprehensive range of quantitative phenotypic variables for comparative assessment in the same individuals.

Data from previous investigations of the candidate c-miRNAs and their direction of change in association with exercise adaptation have been somewhat inconsistent. Moreover, the potential for differential regulation by endurance and resistance exercise has not previously been addressed in a single study. In response to both an acute, single-bout endurance exercise test and a 12 wk period of longitudinal endurance exercise training, Baggish *et al.* [17] reported an increase of the same four c-miRNAs found to be higher in endurance athletes here (miR-21, -146a, -221 and -222). In another study, plasma miR-146a and -221 levels were reduced 3 days after performing an acute bout of resistance exercise [20], consistent with our findings in strength trained athletes, although longitudinal data to relate acute effects to sustained training responses are lacking. These data suggest that levels of particular c-miRNAs may change in opposite directions following endurance *vs*. resistance exercise. However, other studies have reported conflicting results. Some studies report no change in circulating levels of miR-21 and -222 following acute resistance exercise [20]; others report lower circulating levels of miR-21, and -222 in individuals with high (151.2 $mL \cdot kg^{-0.75} \cdot min^{-1}$) compared to low (104.2 $mL \cdot kg^{-0.75} \cdot min^{-1}$) $VO_2$max [33]; or a reduction in miR-21 in previously trained males after 12 wk of further endurance exercise training [18]. However, our endurance athletes have an even higher $VO_2$max (see Table 1) and may not be directly comparable to these aforementioned studies. These contrasting findings may suggest an increased responsiveness of these c-miRNAs to uncontrolled environmental influences and consideration should be given to subtle differences in subgroup selection, study design and participant physiology.

Athletes training and competing in diverse exercise modes display clear phenotypic differences. Endurance athletes are typically lighter, leaner, have higher cardiorespiratory fitness,

and are less strong and less powerful than their resistance-trained counterparts. These differences are underlain by genetic predisposition and training mode, and these influences combine and interact in complicated ways, being mediated largely through epigenetic regulation. By investigating associations between levels of our differentially regulated c-miRNAs and quantitative phenotypic variables we can better understand this epigenetic regulation of phenotype. A strength of our study was that we took the important additional step of adjusting for the type of athlete / training mode in our statistical analyses, which has been done only rarely in previous studies. We found that correcting for group had the clearest influence on associations between levels of our candidate c-miRNAs and physiological characteristics. This finding may indicate that the unmeasured intrinsic differences (or other unmeasured environmental differences, such as subtle differences in diet) between different kinds of athlete, or athletes on different training regimes, have a role in driving the uncorrected associations and justify the adjustment for group. Furthermore, we also adjusted for two measures of tissue mass, which, again, has been done only rarely in previous studies. It can be hypothesised that tissue mass may correlate with the pool size of c-miRNAs available for endocytosis / exocytosis. However, the fact that we observed associations in the models corrected for tissue mass suggests that between-group differences in c-miRNA levels are likely unrelated to tissue mass, and hence does not provide support for the 'pool' hypothesis. At this stage it is possible only to speculate about alternative explanations, but possible factors explaining the between-group differences include training characteristics, innate contractile properties of the muscle, and habitual dietary differences. Further work is required to better define the relationship between changes in c-miRNA levels and performance / health-related phenotypes.

Aerobic capacity is a hallmark of the endurance trained state. In the present study, the endurance-trained cohort had significantly greater relative $VO_2$max than the strength-trained cohort. However, $VO_2$max was not significantly associated with levels of any of the candidate miRNAs, in either the uncorrected or group- / tissue mass-corrected models. We did note that miR-21 was significantly inversely associated with Wingate fatigue index, a measure derived from the Wingate test of anaerobic endurance capacity [34], in the uncorrected (r = -0.39) and muscle mass-corrected (r = -0.38) models, but the other mi-RNAs showed no association with this measure. Thus, with the potential exception of miR-21, differences in c-miRNA levels between groups are not involved in differences in aerobic or anaerobic endurance capacity between, or within, groups. This conclusion is therefore consistent with findings from Bye *et al.* [33] who reported a lack of association between $VO_2$max and miR-222 and only a weak association (r = -0.20) between endurance capacity ($VO_2$max) and miR-21.

Strength and power phenotypes are characteristic of resistance-trained individuals. Thus, whether markers of strength and / or power performance (which were significantly higher in our strength-trained cohort compared to the endurance-trained athletes) were significantly associated with c-miRNA levels in uncorrected and group- / tissue mass-corrected models was investigated. In the uncorrected model, total handgrip strength was significantly negatively associated with circulating levels of miR-21, -146a and -221. We had expected these associations to exist in the absence of any corrections because of the selection criteria we applied for inclusion in each group. However, upon correction for group, these associations are no longer apparent; suggesting that the variation in c-miRNA levels within groups does not associate with the variation in total handgrip strength within groups. In this regard, candidate c-miRNA levels do not associate with strength *per se*; rather c-miRNA levels must associate with other between-group differences that are explanatory for the variation. Differences in fat mass may explain some of the variation in plasma miR-21 levels with regards to strength phenotypes, but neither fat mass nor muscle mass explains the variation in levels of any other c-miRNA. In fact, correction for group appears to be the only correction factor that alters the associations relative

to the uncorrected model for all differentially regulated c-miRNAs. For miR-21, -146a and-221, negative associations with several markers of strength, most notably isokinetic peak torque at various contraction velocities, are apparent in the uncorrected and / or muscle mass-corrected models. For miR-21 and -221 in particular, it is likely that individuals with lower levels of these c-miRNAs are stronger per unit of muscle mass (*i.e.* possess greater specific force). The only c-miRNA to differ significantly between all groups (including controls), miR-222, is not significantly associated with any parameter in the uncorrected or tissue mass-corrected models. With group accounted for, miR-222 significantly associates with some anthropometric and strength measures. However, the association between miR-222 and isokinetic peak torque at faster flexion velocities is actually positive in nature. This direction of association is in contrast to previously reported associations and to what we would expect from the case-control analysis. These findings suggest that, rather than training mode, other between-group factors; for example, dietary habits, physical size and skeletal muscle fibre type differences, may be having a greater effect on miR-222 level. Although the lack of clear associations between miR-222 and performance phenotypes is disappointing following the results from the case-control analyses, this disconnect highlights the value of including quantitative phenotype measures in study designs. Since miRNAs have inhibitory actions on gene expression, the lower levels of candidate c-miRNAs observed in our strength cohort would suggest removal of suppression of gene expression. Considering the miRSystem pathway analysis demonstrated that the associated set of miRNAs target genes were involved in skeletal muscle remodelling, our conclusion would be that remodelling processes governed by the products of these genes may be involved in strength training-related muscle adaptation.

Although no tissue was collected for gene expression analysis of our c-miRNA targets, bioinformatic analyses with miRSystem [30] at least allows us to predict the mRNA targets of the candidate miRNAs investigated in our study and consider whether these are consistent with the observed phenotypic differences between groups. Predicted pathways targeted by our differentially regulated miRNAs included signalling pathways involved in cell growth and inflammation. Specifically, platelet-derived growth factor receptor (PDGF-R), transforming growth factor beta (TGF-β) and mitogen-activated protein kinase (MAPK) signalling pathways as well as transcription factor networks (c-Myb transcription factor network, protein binding transcription factor activity) were identified. PDGF-R signalling has been implicated in blood vessel growth via recruitment of vascular smooth muscle cells [35] and appears to be regulated by miR-146a in particular [36]. TGF-β also is involved in cell growth and control of inflammation [37] while MAPK signalling occurs in several tissues and is implicated in many diverse physiological processes including gene expression regulation and cell differentiation [38]. In the context of exercise training, well-known adaptations include enhanced angiogenesis leading to a greater microvascular volume [39], anti-inflammatory responses leading to a more optimal hormonal environment [16] and enhanced skeletal muscle cell differentiation and proliferation [40]. Thus, it is logical that these pathways may be involved in the training response although exact mechanisms of action cannot be determined from this analysis.

Despite the limited influence of correction for muscle mass or fat mass within our models, given that c-miRNAs must be released by a tissue and ultimately act on mRNAs within a tissue, it is important to consider how c-miRNAs relate to tissue mass. Within an exercise context, an obvious source of non-specific miRNA release into plasma is from muscle-damaging exercise. Banzet *et al.* [21] revealed enhanced levels of muscle-specific c-miRNAs (myomiRs; miR-1, -133a, -133b and -208) following a bout of eccentric exercise, compared to pre-exercise levels (with no change observed following concentric endurance exercise). This led the authors to conclude that changes in c-miRNA levels were driven by the damaging nature of eccentric exercise and muscle leakage in the post-exercise period (up to 6 h). However, markers of muscle

damage were not different between groups in that study, not all muscle miRNAs are present in the circulation and the direction of change of c-miRNA levels is not always consistent with non-specific release from tissue (*i.e.* increased levels in the circulation) [21,41]. Furthermore, recent reports [18,19] suggest limited associations between c-miRNA levels and muscle damage, or a failure to detect muscle-specific miRNAs following exhaustive exercise. Therefore, we believe it is unlikely that muscle leakage is the primary cause of changes in c-miRNA levels, although more work is required to confirm this belief, and to better understand other aspects of c-miRNA regulation in general.

In summary, a subset of c-miRNAs (miR-21, -146a, -221 and -222) are associated with training-related performance parameters in a manner consistent with them being involved in the whole-body adaptive response to differential forms of exercise training. Thus, these c-miRNAs may be useful biomarkers of exercise training and / or have potential roles in exercise mode-specific training adaptations. However, their levels in the circulation appear to be unrelated to having more muscle bulk or larger fat reserves (with the exception of the miR-21 relationship with differences in fat mass), and thus it is unlikely that they are limited by the amount of tissue available for the release / uptake of c-miRNAs. Nonetheless, whether the presence of c-miRNAs in the circulatory system is regulated primarily by tissue exocytosis or endocytosis still remains of interest and further research is warranted to investigate the relationship between observed differences in c-miRNA levels and target gene expression in relevant tissues within the context of exercise training.

## Supporting Information

**S1 Fig. Relative miRNA expression of targets (A), myomiRs (B) and control miRNAs (C) for each of the 3 groups (STR, CON, END).** Bars are means ± 95% confidence limits; * significantly different between all groups (One way ANOVA; $p < 0.05$); † significantly different from STR (t-test; $p < 0.05$). miR-133a was not consistently detected and thus is omitted from the myomiRs plot.
(TIF)

**S1 Methods. Organisation and methods of the study.**
(DOC)

**S1 Table. Participant characteristics of all performance-related variables.** Values are mean ± standard error of the mean (SEM). * significantly different from CON; † significantly different from STR ($p < 0.05$).
(DOCX)

**S2 Table. Regression analyses of miR-222 and performance-related variables before and after correction for group, MM or FM.** Shaded boxes denote $p < 0.05$. Coefficients represent the direction and magnitude of the response. Regression analyses were performed on z-score data.
(DOCX)

**S3 Table. Regression analyses of miR-21 and performance-related variables before and after correction for group, MM or FM.** Shaded boxes denote $p < 0.05$. Coefficients represent the direction and magnitude of the response. Regression analyses were performed on z-score data.
(DOCX)

**S4 Table. Regression analyses of miR-146a and performance-related variables before and after correction for group, MM or FM.** Shaded boxes denote $p < 0.05$. Coefficients represent

the direction and magnitude of the response. Regression analyses were performed on z-score data.
(DOCX)

**S5 Table. Regression analyses of miR-221 and performance-related variables before and after correction for group, MM or FM.** Shaded boxes denote $p < 0.05$. Coefficients represent the direction and magnitude of the response. Regression analyses were performed on z-score data.
(DOCX)

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## Author Contributions

Conceived and designed the experiments: MESB DM RHW TV CNM. Performed the experiments: SLW AK TV. Analyzed the data: SLW CNM. Contributed reagents/materials/analysis tools: AK DM RHW TV CNM. Wrote the paper: SLW MESB AK DM RHW TV CNM.

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# Commentaries on Viewpoint: What is the relationship between acute measure of muscle protein synthesis and changes in muscle mass?

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# Commentaries on Viewpoint: What is the relationship between acute measure of muscle protein synthesis and changes in muscle mass?

## COMMENTARY RESPONSE TO VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: Mitchell et al. (3) critique putative links between *acute* muscle protein synthesis (MPS) and ensuing hypertrophy after resistance exercise (RE) training (RT). Just two papers have addressed this longitudinally. The first reports that young but not older individuals exhibit *acute* (fasted *mixed* muscle MPS, 24 h post-RE) increases in MPS (2); yet hypertrophy gains, assessed by DXA and fiber area, were equal. Similarly, poor quantitative MPS linkages were reported 1–6 h post-RE (*myofibrillar* muscle, fed state) with ensuing hypertrophy (4), i.e., two study's suggesting bona fide dissociation. However, for (2), prior reports of age-related temporal differences in acute MPS responses to RE (1) and lack of intra/inter age group correlations (2) mitigate such resounding conclusions. Also, in (4), RE was under fed state conditions; this is significant because consumption of protein feeds *extends* (not *amplifies*) elevations in MPS post-RE (5). Therefore, MPS responses 1–6 h post-RE were chiefly predominated by feeding, i.e., the coupling of which to RE may not aptly reflect inter-individual variation (5), e.g., due to isolated mechano-auto/paracrine responses to RE. Yet, *acute* MPS can/does inform on group interventions for RT-induced muscle hypertrophy, signifying it a practicable, informative end-point. But, could *quantitative* relationships still exist and what defines "*acute*" post-RE MPS? Fasted/fed? Immediately (<6 h?)/later (>24?) What muscle fraction(s)? Is there an applicable "snapshot"? Without defining *acute* this is indiscernible. The authors justifiably raise potential technical, temporal, methodological confounder(s). Because hypertrophy is a heterogeneous and temporally dynamic process we hypothesize that (coupled to not isolating/fractionating/holistically capturing "*acute*" MPS) interindividual trajectories and *plateauing* hypertrophy in the face of fixed study end-points are the major barriers to defining quantitative links between *acute* MPS and RT-induced hypertrophy.

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## COMMENT VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: Mitchell et al. (4) state that acute measurements of muscle protein synthesis may not necessarily reflect the magnitude of hypertrophy during long-term training studies, which we agree with. Two points deserve further comment: *1*) magnitude of muscle protein synthesis does not necessarily equate with hypertrophy, but rather remodeling, and *2*) limitations of acute measurements are a primary reason some in the field advocate for long-term measurements of synthesis. Regarding the first point, as the authors mention, synthesis is more commonly measured than breakdown and is likely the driving factor behind phenotypic change. However, it is still important to consider that breakdown can change the outcomes. If a high rate of synthesis is accompanied by equal or slightly lower rates of degradation, one would have hypertrophy, whereas the other may not. Importantly though, both are indicative of remodeling. This concept is clear when one studies endurance exercise where there is a high degree of protein remodeling, and phenotypic change, without hypertrophy (5). Regarding our second point, we have advocated for the use of deuterium oxide to measure long-term changes in protein synthesis (1, 3) because of the limitations of acute protein synthesis measurements (4). We have even emphasized this point in a previous "Viewpoint" (2). Although acute measurements of protein synthesis have value, the overall outcome of a long-term treatment or intervention should be assessed by long-term measurement techniques to understand the integrated responses over time. We hope that others, like Mitchell et al., continue to recognize this important point.

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## THE POSTEXERCISE INCREASE IN MUSCLE PROTEIN SYNTHESIS RATE IS INDICATIVE OF SKELETAL MUSCLE RECONDITIONING RATHER THAN MUSCLE HYPERTROPHY PER SE

TO THE EDITOR: We regard the acute muscle protein synthetic response to exercise as an indicator of skeletal muscle reconditioning rather than predictive for muscle hypertrophy. For example, a single bout of endurance-type exercise also acutely increases muscle protein fractional synthetic rate (FSR) (1–3). The increase in the muscle protein synthetic response to endurance type exercise is generally not accompanied by substantial gains in muscle mass and, as such, is far from predictive for muscle hypertrophy in healthy, lean individuals. Instead, the postexercise increase in muscle protein synthesis is rather representative of muscle reconditioning, comprising muscle repair and remodeling. Consequently, the postexercise increase in muscle protein FSR should not be regarded as a marker for exercise training induced hypertrophy but rather as an indicator of skeletal muscle reconditioning, which comes in many different forms and measures. The muscle protein synthetic response to a single bout of resistance type exercise training may provide some insight in the extent of muscle hypertrophy observed during more prolonged resistance type exercise training but does not provide a quantitative estimation of hypertrophy in the individual (4).

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## COMMENT ON VIEWPOINT: WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?

TO THE EDITOR: Mitchell and colleagues (2) present a nicely balanced viewpoint regarding the discordance between acute measurements of muscle protein synthesis (MPS) and long-term muscle mass gains in response to exercise training and nutrition. Individual genetic and other physiological characteristics, as well as methodological limitations of the endpoint measures, are correctly identified as contributing factors to the hypertrophic variability inherent in these types of training studies (1). However, the practicalities of controlling long-term training studies themselves are not addressed.

Consideration is warranted for the difficulties in controlling extended-duration intervention studies to generate appropriately valid and reliable results. Changes and variability in diet, timing of exercise in relation to meal ingestion, sleep patterns, daily stress, and compliance with the training regimen will contribute to the considerable intrinsic variability of measured changes in muscle mass (3). Small differences between interventions may easily be missed when these confounding factors are coupled with the variations outlined in the Viewpoint. Acute measurements are much easier to control and differences between interventions often can be easily detected. Therefore, any disconnect between acute metabolic studies and long-term changes in muscle mass do not necessarily reflect the worth of the metabolic studies.

There is no question that acute measurements of MPS alone should not be used to predict the training response of any given individual. However, with appropriate appreciation for their limitations, these methods can play a valuable role in acquiring information to help determine appropriate training and nutrition interventions for various populations.

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### COMMENTARY RESPONSE TO VIEWPOINT: "WHAT IS THE RELATIONSHIP BETWEEN ACUTE MEASURES OF MUSCLE PROTEIN SYNTHESIS AND CHANGES IN MUSCLE MASS?"

TO THE EDITOR: The authors raise several insightful points while describing the discordance they observe between acute measures of postexercise muscle protein synthesis (MPS) and the subsequent resistance exercise (RE) training-induced muscle hypertrophy (1, 2). Although the authors suggest that intersubject variability in the MPS response to RE is a likely major contributory factor, the impact of recruiting individuals naive to the mode of RE employed cannot be overstated. Moderate (60–75% of 1-repetition maximum) noneccentric RE in unaccustomed individuals has been shown to result in myofibrillar damage, increased inflammatory signaling (5), and induction of the unfolded protein response (4), events that are thought to modulate MPS drive. Moreover, we have observed that 24 h after a single-bout of unaccustomed concentric RE, the transcriptional "program" elicited in muscle appears dependent on whether damage has purportedly occurred and varies between individuals (3). Specifically, we observed a discord in the transcriptional regulation of gene pathways associated with MPS signaling, which could in part explain the variability observed by the authors. In contrast, the transcriptional response to a repeat session of RE performed days later was consistent across volunteers and was not punctuated by increased markers of muscle damage (3). Collectively, these observations suggest that, when attempting to translate acute, exercise-induced changes in MPS to longer term gains in muscle mass, using subjects who have already undergone a period of familiarization to laboratory RE protocols may offer a more promising approach.

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### NEED FOR MEASURES OF SATELLITE CELL ACTIVATION ALONG WITH MUSCLE PROTEIN SYNTHESIS?

TO THE EDITOR: The Viewpoint by Mitchell and colleagues (4) provides a timely reminder that acute responses to exercise-nutrient interventions are not always the perfect proxy for chronic training-induced adaptations and/or changes in functional outcomes. Skeletal muscle displays remarkable plasticity with the capacity to alter the type and amount of protein in response to habitual level of contractile activity, the prevailing substrate availability, and environmental conditions (3). Such "adaptation plasticity" is common to all vertebrates but a large variation in the degree of adaptability between humans is evident, explaining the large inter-individual responses after exercise-nutrient interventions (2). The author's state that an individual's "inherited genetic predisposition, epigenetic influence, and transcriptional plasticity" are potential sources for "hypertrophic variability" after resistance training (RT). Added to that list is the possibility that satellite cell activation may underlie part of the variability in the muscle hypertrophic response. Previous work shows the acute satellite cell response to a single bout of resistance exercise is associated with the subsequent accretion of lean mass (LM) after 16 wk RT (1). Although this suggests postexercise measures of satellite cell activity could be a valid surrogate of an individual's ability to accrue LM after RT, acute measures of MPS still provide important mechanistic insight to the "anabolic" events in response to exercise-nutrient interventions. Ultimately it is clear that chronic training studies with comprehensive time-course responses of selected cellular and functional outcomes are required to provide mechanistic insight as to why training-nutrient interventions result in variable responses between individuals.

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**ARE NOT INTRACELLULAR HEAT AND NEURAL ELECTRICITY STRESSES THE CAUSE OF EXERCISE-INDUCED PROTEIN SYNTHESIS?**

TO THE EDITOR: Dr. Mitchell and colleagues (5) support their thesis for a possible lack of correlation between acute and chronic muscle protein synthesis (MPS) partly with their Refs. 14, 15, and 16. The conclusion for such a lack is drawn in all of these three references. However, while in Refs. 15 and 16 the quadriceps hypertrophy after 10 or 16 wk of resistance training (RT) was between 5 and 9.5% the same in Ref. 14 after 16 wk RT was 37–40%. In our view, the difference was because in Ref. 14 the load was progressively increased during the RT, while in Refs. 15 and 16 it was not. Qualitatively, it is the same difference as between MPS response after acute and chronic RT.

Myocytes are extremely vulnerable to overheating, because they can increase energy production more than 100-fold in less than a second. They do not have intracellular convective heat transfer because almost 80% of their water is entrapped in the myofibrils. The anaerobic nature of RT increases with the increase of loading because more energy is released during contraction, when muscle capillaries are compressed, preventing blood flow and cooling. Elevated myocyte temperature prevents protein synthesis, causes protein denaturation, and stimulates heat shock protein synthesis, which during postexercise repair in excess damaged protein (3, 4). This reasoning is supported by the similar effect produced by the blood flow restriction (2). Stimulation of protein synthesis by electric field (1) hints that excessive muscle neural electricity, triggered by unaccustomed loading, should have supportive MPS effect.

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TO THE EDITOR: Mitchell and colleagues have identified an important problem: can a single measurement of protein synthesis be extrapolated to provide changes in muscle mass(1)? Muscle mass is often used as a surrogate for muscle protein content, but although muscle weight reflects muscle mass, muscle weight includes muscle proteins, adipose tissue (especially, in aged or type II diabetes), and up to 70% water (3). For example loss of muscle mass in exercise reflects loss of water with or without loss of muscle protein. As suggested by Mitchell, although an increase in protein synthesis suggests increased muscle protein mass, no conclusion is possible without measurement of protein degradation. Infusion of L-(1–13C) leucine or L-(ring-2H5) phenylalanine has been used to measure whole body or skeletal muscle protein metabolism in humans (2). In animal models, rates of protein synthesis and degradation are often assayed *ex vivo* by measuring the rate of tyrosine incorporation into protein (protein synthesis) plus the release of tyrosine from muscle proteins (protein degradation) (4). This approach, however, does not allow both synthesis and degradation information to be gathered from the same animal. The rate of protein degradation is virtually always greater than the rate of protein synthesis regardless of whether undergoing muscle hypertrophy or atrophy. If the goal of measuring protein synthesis is to provide an index of changes in muscle protein mass, we suggest that investigators assess the distribution of the areas of myofibers in a cross section of muscle as described for assessing the effects of XIAP on muscle hypertrophy (5).

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**SIGNIFICANCE OF LONG-TERM STUDIES IN RESISTANCE TRAINING AND MUSCLE HYPERTROPHY**

TO THE EDITOR: Muscle being a postmitotic tissue is endowed with an efficient means of cell replacement to avoid muscle cell death and maintain skeletal mass. This is carried out through the dynamic balance between muscle protein synthesis and degradation (1). Muscle hypertrophy occurs when protein synthesis exceeds protein breakdown. Although the Viewpoint authors (2) agree with the importance of acute measurements in understanding mechanisms of divergent exercises and nutritional manipulations, they advocate the need for long-term studies to understand the holistic adaptations due to altered phenotype.

Muscle hypertrophy is a multifactorial process involving mechanical tension, muscle damage, and metabolic stress. In addition, many have observed numerous other factors, such as genetic predisposition, epigenetic influence, and transcriptional plasticity, age, gender, habitual physical activity, and training status, to influence the hypertrophic response to a training protocol affecting both the rate and the total gain in lean muscle mass. Hormones and cytokines, namely growth hormone, testosterone, interleukin-5, interleukin-6, etc., are also shown to play complex roles in hypertrophic process (3). Additionally, some of these hormones

have effects on immune system, bone remodeling, and extracellular fluid volume. A 20-wk-long RT revealed greater adaptability within endocrine system only in younger men (4). A discrepancy in immune responses to short-term and moderate exercise training is reported recently (5). Exercise-induced free norepinephrine concentration was reported to have effect on circulating hematopoietic stem and progenitor cell number and functionality (3). In the light of such varied complexities, it is appropriate to pursue long-term effects of exercise training on muscle hypertrophy.

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## Pattern of protein ingestion to maximise muscle protein synthesis after resistance exercise

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The maintenance of skeletal muscle mass is dependent upon the temporal and coordinated interaction between muscle/myofibrillar protein synthesis (MPS) and muscle protein breakdown (MPB). Resistance exercise (RE) alone elevates MPS and, to a lesser extent, MPB such that net muscle protein balance (NPB) remains negative. However, when RE is coupled with protein ingestion there is an accumulative effect on MPS resulting in a positive NPB (Phillips *et al.* 2005). Thus, repeated bouts of RE coupled with protein feeding is a viable strategy to maximise skeletal muscle hypertrophy and strength.

The impact of protein feeding on RE-induced increases in MPS has received much attention. One study has demonstrated that in young healthy males ∼20 g of high-quality protein is sufficient to maximise RE-induced rates of MPS over 4 h post-exercise (Moore *et al.* 2009). However, the interplay between the timing and quantity of protein consumed and subsequent anabolic responses throughout the course of a whole day is still poorly understood. In particular, there is a lack of data examining how the pattern of post-RE protein ingestion influences MPS later in the recovery phase (i.e. 4–12 h). A recent article published in *The Journal of Physiology* attempts to address this knowledge gap and in doing so provides valuable insights into how post-RE protein feeding strategies might be manipulated to optimise muscle anabolism. In an elegantly designed study, Areta *et al.* (2013) examined three groups of eight healthy, trained males. Participants performed a bout of bilateral leg extension RE followed by the consumption of 80 g of whey protein over 12 h of recovery ingested as either 8 × 10 g every 1.5 h, 4 × 20 g every 3 h or 2 × 40 g every 6 h. A stable isotope infusion was coupled with frequent skeletal muscle biopsy sampling to determine rates of MPS for 12 h post-RE. The data demonstrate that although all feeding strategies elevated MPS during the 12 h recovery period, consuming 20 g of whey protein every 3 h was the superior strategy for stimulating MPS rates. The authors concluded that these findings have the potential to maximise outcomes of resistance training designed to elicit a maximal hypertrophic response.

The data of Areta *et al.* show that manipulating the pattern of protein ingestion following RE can have a significant impact on the subsequent muscle anabolic response. The divergent feeding strategies of Areta *et al.* were used to mimic possible patterns of protein intake commonly observed in resistance-trained athletes. That is, 8 × 10 g every 1.5 h represents a 'grazing' approach, whereas 2 × 40 g every 6 h relates to the 'three square meals per day' approach. Yet, both of these strategies were inferior for stimulating MPS over 12 h of post-RE recovery compared with 4 × 20 g ingested every 3 h. However, it is important to note that this response was characterised when protein was ingested alone, and as the authors acknowledge, this finding cannot be evaluated in the context of a mixed meal. Indeed, it is commonplace to consume protein in the form of a mixed-macronutrient meal. Therefore, it is reasonable to postulate that macronutrient co-ingestion could alter intestinal transit, thus influencing amino acid absorption kinetics (Deutz *et al.* 1995) and perhaps MPS. Moreover, this study used high-quality whey protein and it remains to be seen if a similar pattern of MPS post-RE would be observed using the same feeding strategies with a slow-release protein such as casein. Such information may be valuable to individuals who choose not to (or are unable to) ingest high-quality protein in supplemental form following exercise, but instead consume whole-food protein sources.

Areta *et al.* should be highly commended for underlining the importance of not only the quantity, but particularly the pattern of post-RE protein ingestion to maximise the rate of MPS over 12 h. However, as a note of caution, their findings are limited to a healthy young male population. In this regard, recent evidence demonstrates that the elderly require more protein (40 to > 20 g) to elicit optimal increases in RE-induced rates of MPS than the young (Yang *et al.* 2012). It is therefore reasonable to consider whether the temporal influence of post-RE protein feeding on elderly muscle could be different compared to that of young. In this regard, the next logical step is to apply the model of Areta *et al.* in elderly and other populations, in whom maintenance of muscle mass is a critical determinant of longevity and quality of life. Yet, it should be acknowledged that Areta *et al.* afford data pertaining to only 12 h of recovery from RE. Hence, whether the acute responses of MPS to RE and protein feeding translate into a long-term functional response remains unknown.

The findings of Areta *et al.* will no doubt also grasp the attention of coaches and athletes alike. As such, some may cite the use of a bilateral exercise stimulus and absence of participants with large amounts of lean mass (>75 kg) as issues that preclude full applicability in a 'real-world' setting. To date, it is unclear whether exercising a greater volume of muscle mass is limiting for MPS in response to a given protein dose. Therefore, individuals with greater muscle mass or those engaged in whole-body RE training sessions may require ingestion of a greater protein dose to stimulate MPS maximally. With regard to the notion of applicability to the 'real-world' setting, it also may be significant that the participants entered the experimental trial in the fasted state. As a result the authors are unable to identify whether a pre-exercise meal would influence the MPS response to RE and various feeding strategies. This point becomes more relevant when considering the impact of insulin on MPB with regard to the true *growth* response and therefore the long-term applicability of the findings. Future studies assessing MPS and MPB in both the clinical and the athletic setting following RE and feeding are now required.

The study by Areta *et al.* also reveals novel nutrient–exercise interactions in cellular signalling. Phosphorylated mTOR$^{Ser2448}$ was ∼2- to ∼6-fold above resting values throughout the 12 h recovery period independent of protein feeding strategy. Phosphorylation of p70S6K$^{Thr389}$ was also increased above baseline, again in all feeding

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strategies. However, there was discordance between the degree of p70S6K$^{Thr389}$ phosphorylation and the MPS response. In fact, the magnitude of phosphorylated p70S6K$^{Thr389}$ displayed a $2 \times 40$ g to $> 4 \times 20$ g to $> 8 \times 10$ g pattern at 1 and 7 h post-RE. This finding is surprising given that phosphorylated p70S6K$^{Thr389}$ is a key player in protein synthesis yet it was the $4 \times 20$ g strategy that induced the most favourable influence on MPS but median impact on phosphorylated p70S6K$^{Thr389}$. However, it is important to recognise that the timing of the biopsies at 1 and 7 h coincided with a greater volume of protein consumed prior to those biopsies for the $2 \times 40$ g condition, which may explain the discordance between p70S6K$^{Thr389}$ signalling and MPS.

The common method employed to assay protein phosphorylation, a proxy of activity, in an exercise science setting, and in the present investigation, is Western blotting (WB). In contrast to the quantitative and reproducible techniques used to measure MPS, WB is a semi-quantitative method. Additionally, phosphorylated p70S6K$^{Thr389}$ is recognised as a key controller of ribosomal biogenesis. So although the phosphorylation of p70S6K$^{Thr389}$ post-RE does not correspond to the greatest acute MPS response it may in fact be leading to greater levels of ribosomal transcription. Interestingly, phosphorylation of p70S6K following RE often occurs in the nucleus, where ribosomal biogenesis commences. A caveat of the field is that no study has employed cellular fractionation techniques to reveal whether different RE and feedings strategies alter the ratio of nuclear to cytoplasmic phosphorylated p70S6K in human skeletal muscle. Hence, the lack of concordance between the MPS and signalling response in this and numerous other works emphasises the need for the development of new measures regarding readouts of ribosomal biogenesis in addition to fully quantitative methods to ascertain signalling activity following RE and nutrition.

To conclude, the study by Areta *et al.* contributes novel data to the body of literature highlighting the importance of the timing and quantity of protein consumed post-RE for muscle anabolism. By mimicking the habitual feeding strategies of many athletes engaged in resistance training, the authors move closer to bridging the gap between science and the applied setting. Future work that identifies the impact of different macronutrients consumed in combination, i.e. fat, carbohydrate, protein and fibre, on MPS in both elderly and young is warranted. Furthermore, there is growing interest in whether having greater amounts of muscle mass, or indeed exercising muscle mass involved in trainin impact RE-induced rates of MPS. Thus, future studies that examine the MPS response in individuals with large muscle mass, performing real-world RE, may provide informative data for clinical and athletic practice.

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