The Role of Adipose Tissue in the Metabolism of Fatty Acid Hydroxy Fatty Acids in Humans



Jonathan Gamwell

Radcliffe Department of Medicine

University College

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Declaration

I hereby declare that no part of this thesis has been submitted for any degree at this or any other university.

The work presented herein is entirely my own except where stated in the text and below:

- The *in vitro* work using Patient 2 preadipocytes was planned and carried out in conjunction with Dr Marijana Todorčević of OCDEM.
- The participant plasma samples and adipose tissue biopsies were collected by nurses Jane Cheeseman and Diana Mantripp of OCDEM.
- The extraction from and measurement of FAHFAs in the plasma samples of the large cohort described in Chapter 4 was carried out by Martin Riečan of the Czech Academy of Sciences

Supervision for the work was provided by Professor Leanne Hodson and Dr Katherine Pinnick and funding was provided by the Medical Research Council and University College, Oxford.

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Finally, I'm grateful to the Medical Research Council and the University College War Memorial Fund for their financial support throughout my DPhil.

The Impact of COVID-19 on my Work

The period of my DPhil straddled the lockdowns associated with the COVID-19 pandemic and my work was affected as a result of that, despite the eventual award of a three month extension. The start of participant recruitment was delayed by thirteen months, pushed back from 2020 to 2021, delaying the availability of samples to analyse and primary cells to work with *in vitro*. My access to the Department of Chemistry where I was making use of a liquid chromatography- mass spectrometry machine was rescinded for seventeen months, halting progress in protocol optimisation and sample analysis. In part, the lack of access to the machine was mitigated by collaboration with colleagues at the Czech Academy of Sciences, however, when their machine broke down, I was left without enough time to satisfactorily re-optimise the processes for the machine in Oxford. As a consequence, some work for Chapters 4 and 5 was left incomplete with not all sample types able to be analysed.

Abstract

Excess adiposity is associated with the metabolic syndrome and type 2 diabetes and hence to the rising healthcare burden of those and related diseases. Consequently, adipose tissue (AT) and its role in glycaemic control and systemic inflammation is an important area of contemporary research.

Recently, a novel family of endogenously-synthesised lipids was identified. Fatty acid-hydroxy fatty acids (FAHFAs) have been shown, with acute treatment, to reduce glycaemia in mice by a mechanism which increased translocation of the GLUT4 glucose transporter to the cell membrane of adipocytes and which involves the free fatty acid receptor 4 (FFAR4). In humans, it has been suggested that plasma levels of certain FAHFAs are lower in individuals with reduced insulin sensitivity but this was not replicated in another study. It has also been suggested that the plasma levels of FAHFAs are lower in morbidly obese people. Mechanistic studies *in vitro* with murine cells have also shown equivocal results with FAHFA treatments either inhibiting or enhancing lipolysis, a key process in adipocytes. Additionally, it is suggested that *de novo* lipogenesis (DNL), another key process, is also affected by FAHFAs. Little is known about the distribution or composition of FAHFAs in the blood and AT, nor of their synthesis and degradation.

In order to better understand FAHFA distribution in humans and to clarify their association with insulin resistance and other measures of metabolic health and phenotype, I examined plasma and adipose tissue FAHFA concentrations in healthy adults with a range of body mass index. In addition, I optimised *in vitro* adipocyte culture conditions and used both primary human cells from biopsies and immortalised preadipocytes to test the effect of FAHFAs on adipocyte function.

In vivo, I found plasma FAHFAs to be higher in men than women. There was no association between fasting plasma FAHFAs and insulin sensitivity, and some evidence that plasma FAHFAs may increase with increasing adiposity in women. Fasting plasma FAHFAs were not correlated with plasma fatty acids despite expectations that they would be regulated in a similar manner. *In vitro*, FAHFAs were found to inhibit lipolysis, as insulin does, but, in contrast to insulin, FAHFAs inhibited DNL. The capacity to synthesise FAHFAs was similar between the abdominal and gluteal adipocytes but there was a suggestion, from the AT biopsies, that the concentrations of FAHFAs may be higher in abdominal than in gluteal AT.

Overall, my work has added further to the knowledge of the distribution of FAHFAs in the body and their relationship to phenotype in humans. There were only weak relationships between plasma FAHFAs and either insulin sensitivity or adiposity and the directions of these were opposite to previous reports, suggesting that, if these associations are substantiated in vivo, then they are probably complex and multifactorial. I have uncovered some aspects of FAHFAs which differ between the sexes or between the AT depots, which should be borne in mind for future research. In examining the effect of FAHFAs in adipocytes, I have identified an effect on DNL which merits further investigation.

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Abbreviations

ALA	Alpha-linolenic Acid
ASAT	Abdominal Subcutaneous Adipose Tissue
AT	Adipose Tissue
BAT	Brown Adipose Tissue
BCAA	Branched Chain Amino Acid
BMDC	Bone Marrow Dendritic Cell
BSA	Bovine Serum Albumin
DAG	Diacylglyceride
DEXA	Dual Energy X-ray Absorptiometry
DHA	Docosa-4,7,10,13,16,19-hexaenoic acid (22:6 n3)
DNL	*De Novo* Lipogenesis
EPA	Eicosa-5,8,11,14,17-pentaenoic acid (20:5 n3)
FA	Fatty Acid
FAHFA	Fatty Acid ester of Hydroxy Fatty Acid; "-NEFA" denoting the non-esterified form and "-TAG" denoting the esterified form.
FAME	Fatty Acid Methyl Esters
FFAR	Free Fatty Acid Receptor
GC	Gas Chromatography
GC-MS	Gas Chromatography- Mass Spectrometry
GPCR	G-Protein-Coupled Receptor
GSAT	Gluteal Subcutaneous Adipose Tissue
HFA	Hydroxy Fatty Acid
HFD	High Fat Diet
KRH	Krebs Ringer Hepes (buffer)
LA	Linoleic Acid

LCFA	Long-chain fatty acid
LC-MS	Liquid Chromatography- Mass Spectrometry
LPS	Lipopolysaccharide
NEFA	Non-Esterified Fatty Acid
OA	Oleic Acid
OGTT	Oral Glucose Tolerance Test
OPLA	Oleic-Palmitoleic-Linoleic-Alphalinolenic fatty acid mix
PA	Palmitic Acid
PAHSA	Palmitic acid hydroxy stearic acid, a FAHFA
PBS	Phosphate-Buffered Saline Solution
PUFA	Polyunsaturated fatty acid
SAT	Subcutaneous Adipose Tissue
SCFA	Short-chain fatty acid
TAG	Triacylglycerol
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue

Chapter 1

Introduction

1 Introduction

1.1 Adipose Tissue and Adipose Tissue Distribution in Health and Disease

Adipose tissue (AT) is an organ, arranged in depots around the human body, with a heterogeneous population of preadipocytes, white and brown mature adipocytes, blood vessels and immune cells. White adipocytes predominate and white adipose tissue (WAT) is found in the viscera (visceral adipose tissue or VAT) and under the skin (subcutaneous adipose tissue, or SAT). While the scarce brown adipose tissue (BAT) has an energy-consuming role in non-shivering thermogenesis, WAT has a complex role in energy homeostasis, including uptake of glucose in response to insulin after feeding, supply of fatty acids (FAs) to other non-adipose tissue organs during fasting and release of hormones. AT dysfunction and dysregulation of these processes are linked with excess adiposity and aspects of the metabolic syndrome including insulin insensitivity and chronic inflammation.

The Body Mass Index (BMI) is a measure of a person's adiposity and is used as a gauge of healthy weight with cut-offs at 25 and 30 $kg.m^{-2}$ to classify "overweight" and "obese" respectively. The association between BMI and all-cause mortality is, in fact, J-shaped or tick-shaped where a low BMI correlates with a risk which decreases towards the optimal BMI of around 22 and then increases with increasing BMI (Sun *et al.*, 2019). This suggests that moderate fatness is better than very low adiposity but that excessive adiposity is deleterious to health.

Not all AT is equal, however; the risk of all-cause mortality increases even more steeply with waist-to-hip ratio (WHR), waist-to-height ratio (WHtR) and waist circumference than with BMI (Rost *et al.*, 2018), indicating that adiposity around the waist carries a greater risk of morbidity than adiposity around the hips. Assessing adipose distribution by the more sophisticated dual-energy x-ray absorptiometry (DEXA) method, an even stronger protective effect against components of the metabolic syndrome (Pinnick *et al.*, 2014) and cardiovascular disease (Vasan *et al.*, 2018) for lower-body fat have been identified. These and similar findings led to the popular notion that pear-shaped individuals, who tend to lay fat stores in their gluteofemoral depots, are healthier than apple-shaped individuals, who tend to store fat in abdominal and visceral depots.

The differences may be due to the distinctly different developmental profiles of adipocytes from the two depots ((Pinnick *et al.*, 2014); (Tchkonia *et al.*, 2007); (Gehrke *et al.*, 2013)) leading to the alternative phenotypic traits which have been observed. Abdominal subcutaneous adipose tissue (ASAT) tends to expand hypertrophically (i.e. individual adipocytes get larger), while gluteal (GSAT) tends to rely on hyperplasia (i.e. additional preadipocytes are recruited to mature into adipocytes) (Tchoukalova *et al.*, 2010) where the latter is linked to better glucose control. Gluteofemoral tissue

tends to take up FAs from liver-derived very low-density lipoproteins (VLDL) (McQuaid *et al.*, 2010) more readily than dietary FAs circulating in chylomicrons and tends to release FAs less than abdominal tissue during fasting (Martin and Jensen, 1991), meaning that excess FAs may be diverted away from visceral stores where its presence is linked to poor health. Palmitoleate, which is proposed to sensitise the insulin receptor (Cao *et al.*, 2008) and the plasma concentration of which is suggested to correlate positively with insulin sensitivity, is released in greater amounts by gluteofemoral fat than abdominal when measured by arterio-venous difference (Pinnick *et al.*, 2012).

There remain many questions over which depot function(s) lead to the difference in health outcomes or whether individuals can alter, pharmaceutically or otherwise, their AT distribution or health trajectory (although PPARγ agonists have had the effect of increasing gluteal adiposity while improving insulin sensitivity (Gerstein *et al.*, 2006)).

Since the healthcare burden associated with excess adiposity is high, with an upward trend, there is a need to better understand the role of AT and AT distribution in health and disease. One area of research is understanding the role of AT in type 2 diabetes, where glucose uptake after feeding is impaired. Recently, the role of the newly-discovered, AT-derived lipids, fatty acid esters of hydroxy fatty acids (FAHFAs), in glucose homeostasis and type 2 diabetes has been brought into the spotlight. As a novel product of adipose tissue with positive effects on metabolic health, one speculates whether differential FAHFA metabolism between the AT depots has a role to play in explaining why gluteofemoral adiposity may be beneficial.

1.2 Fatty Acid Esters of Hydroxy Fatty Acids

1.2.1 Discovery

Obese or Type 2 diabetic humans and mice have a down-regulation of the insulin-responsive glucose transporter GLUT4 in adipocytes, which is associated with reduced glucose uptake and altered AT function (Shepherd and Kahn, 1999). Herman *et al.* (2012) reported that the reduced glucose uptake associated with knocking-out GLUT4 downregulated a *de novo* fatty acid synthesis promoter gene (cHREBP) and that the gene, presumably through changes to fatty acid synthesis, is related to insulin sensitivity.

In an attempt to elucidate the reason why endogenously-synthesised FAs might be linked positively to insulin sensitivity, lipidomic screening of GLUT4-overexpressing (AG4OX) mice versus *Glut4* knockout mice, reflecting the extremes of insulin sensitivity and resistance respectively, revealed an unknown family of FAs found in significantly higher concentrations in the serum and tissues of the GLUT4-overexpressing model (Yore *et al.*, 2014). The insulin-sensitive AG4OX mice were found to

have elevated adipose *de novo* lipogenesis (DNL) resulting from elevated glucose supply and so it was proposed that this family of lipids were produced *de novo* in adipocytes. Supporting this, knocking out ChREBP (carbohydrate response element binding protein), a transcription factor regulating *Glut4*, in the AG4OX mice eliminated the difference in the concentrations of the FAs, indicating that they are made *de novo* when glucose supply is sufficient. The unknown FAs were determined to be a new class of lipids, named FAHFAs.

These findings led to the initial proposed feedback mechanism whereby glucose enters an adipocyte, activating CHREBP, which promotes the transcription of DNL genes thus increasing synthesis and the abundance of FAHFAs which then act to increase the translocation of GLUT4, allowing more glucose to enter the cell.

1.2.2 Structure and Nomenclature

Mass spectrometry revealed FAHFAs to have a common structure in which one FA is bound by its carboxyl group to a hydroxyl group on a hydroxy fatty acid (HFA). Many FA / HFA combinations have been found, Ma *et al.* (2015) have created an *in silico* library of 1089 FAHFA species, but those comprising the most common FAs in the body (e.g. palmitic, palmitoleic, oleic and stearic acid) are the most abundant and studied to date. Members of the family are named by fatty acid followed by hydroxy fatty acid and prefixed by the number of the carbon at the hydroxyl group: Figure 1-1 describes a member of the Palmitic Acid Hydroxy Stearic Acid (PAHSA) family.

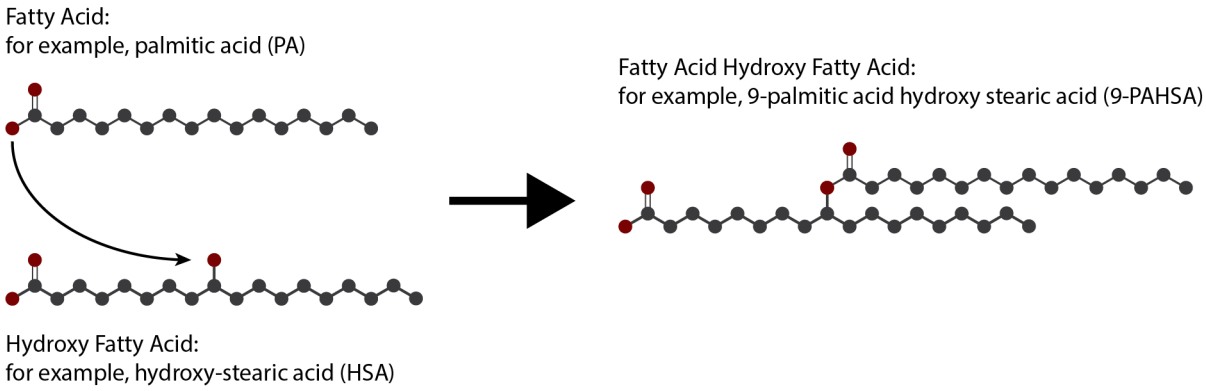


Figure 1-1: Example of the composition and naming of a FAHFA

It follows that, for each sub-family, a number of regioisomers exist depending on the location of the hydroxy bond and, in the case of PAHSAs, Yore *et al.* (2014) identified 5-, 9-, 10-, 12- and 13-PAHSA in their initial screen.

Stereoisomers also exist depending on the orientation at the hydroxy bond. Nelson *et al.* (2017) reported that R-9-PAHSA is found in greater abundance than S-9-PAHSA in the AG4OX mice, suggesting that the production is enzymatic. However, they also noted that the pancreatic hydrolase

CEL prefers the S isomer, so the result could also be interpreted as S-9-PAHSA being preferentially degraded if PAHSAs cycle through the body. The same group later reported that 13-LAHLA in oat oil is a racemic mix (Kolar *et al.*, 2019), indicating that it is either not synthesised enzymatically, there are two enzymes or there is one, non-specific enzyme.

Furthermore, different sub-families are isomers of each other; naturally PAHSAs have the same mass as SAHPAs.

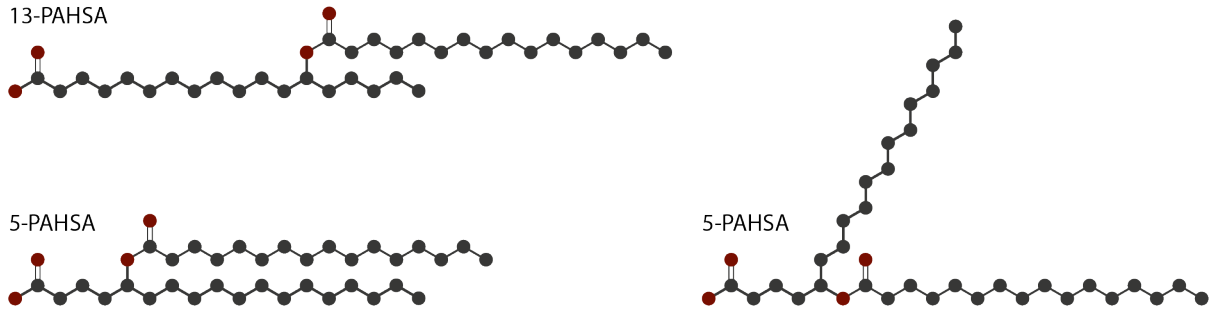


Figure 1-2: Example regio- and stereoisomers of PAHSAs.

1.2.3 Endogenous and Exogenous Sources and Tissue Distribution

Yore *et al.* (2014) observed FAHFA-synthesis in both murine adipocytes and hepatocytes and, since the amount found in human adipose tissue is high, around 10-fold more dense (Yore *et al.*, 2014), compared to the amount found in foodstuffs, it is assumed that the same synthetic activity exists in humans, at least in adipose tissue.

In the time since Yore *et al.* (2014) measured FAHFAs in their mice's chow and a handful of human foods, Liberati-Cizmek *et al.* (2019) have published a list of 18 reportedly anti-inflammatory foodstuffs and their content of 16 selected FAHFA families, revealing a broad spread but low abundance in the human diet. That range of 16 families is fairly limited and did not include, for example, any FAHFAs derived from polyunsaturated FAs (PUFAs), since these had not previously been identified and added to the list.

In humans, FAHFAs have been identified in subcutaneous WAT and plasma (Yore *et al.*, 2014) and in breastmilk (Brezinova *et al.*, 2018). In mice, they have additionally been recorded in BAT, liver, kidney and pancreas (Yore *et al.*, 2014).

While it would be interesting to expand the picture of FAHFAs in foods, it is unlikely that exogenous supplies are very important except, perhaps, for specific species where the precursors are otherwise missing from the diet. It would be more interesting to know whether the human liver also synthesises FAHFAs and how important redistribution between adipose depots, liver and other tissues is.

1.2.4 Purported Effects on Glucose Homeostasis

Given the route to discovery and the global interest in the Type 2 Diabetes epidemic, it is unsurprising that much focus has been on the role of FAHFAs in glucose homeostasis.

Yore *et al.* (2014) showed that, in insulin-resistant mice, glycaemia was reduced 30 minutes after an oral gavage of 5-PAHSA (with concomitant increases in 5-PAHSA in plasma indicating absorption of 5-PAHSA from the gut). They also showed that the area under the curve for a subsequent oral glucose tolerance test (OGTT) was lower following the 5-PAHSA oral gavage than that without 5-PAHSA feeding. Furthermore, Yore *et al.* (2014) reported a strong correlation between the glucose infusion rate during a hyperinsulinemic-euglycemic clamp and the fasting concentrations of both the total and individual isomers of non-esterified PAHSA (PAHSA-NEFA) in serum and in AT in 13 non-diabetic humans. Subjects were then divided into those with either good or poor insulin sensitivity, according to the clamp results, and significant differences between each group's PAHSA-NEFAs in serum and AT were found. However, the two groups also had significantly different BMIs and this should not have been ruled out as being linked to PAHSA-NEFA concentrations since adipose metabolic dysregulation resulting from excess adiposity may alter FAHFA metabolism separately to changes in insulin sensitivity. Kellerer *et al.* (2021) probed this question and found no differences in the circulating levels of several FAHFAs, including PAHSAs, between lean diabetics and non-diabetics but did find them to be lower in healthy obese compared to lean individuals. Similarly, in a population of elderly atherosclerotic patients, Lopez-Bascon *et al.* (2016) found one FAHFA to be higher in participants with elevated fasting glucose and one to be lower, thus putting Yore *et al's* (2014) conclusion further into doubt. So, while Kellerer may have offered an alternative explanation for Yore *et al's* (2014) results, i.e. that adiposity rather than insulin sensitivity is associated with FAHFAs, further work in this area would be useful to clear the equivocation and extend the results to a wider selection of FAHFAs.

To examine the function of FAHFAs with regard to glucose uptake, Yore *et al.* (2014) used the 3T3-L1 murine adipocyte cell line to show that the administration of 5-PAHSA alongside insulin, augmented both the insulin-stimulated and basal translocation of GLUT4 to the cell membrane, explaining the improved glucose uptake that was seen in the gavaged mice. This effect on GLUT4 translocation was attributed to the activation of the G-protein-coupled receptor 120 (GPCR120), also known as the Free Fatty Acid Receptor 4 (FFAR4). Furthermore, experiments with human pancreatic islets demonstrated that 5-PAHSA stimulated an increased release of insulin from the beta cells and experiments with STC-1 enteroendocrine cells showed that 5-PAHSA increased the release of GLP-1 which would, in turn, act on the beta cells to increase insulin release. This effect was later demonstrated by Syed *et al.* (2018) to be mediated by the activation of GPCR40 / FFAR1 in both cell types. Reinforcing this

latter point, but possibly undermining Yore *et al.'s* (2014) *in vitro* work on GLUT4 translocation after FFAR4 stimulation, Sundström *et al.* (2017) found that the effect of lowering glycaemia in mice by the use of FFAR4 agonists was heavily dependent on the stimulation of GLP-1 production.

In contrast to Yore *et al.'s* (2014) study, during their long-term feeding experiment in mice, Benlebna *et al.* (2020), found that, after 12-weeks on a diet supplemented with either 9-PAHPA or 9-OAHPA, glucose tolerance measured by OGTT was not changed. However, they did find that insulin sensitivity, measured during insulin infusion, was markedly increased. Whether insulin sensitivity was increased as a result of a long-term effect of supplementation or was due to elevated levels of plasma PAHPA or OAHPA at the time of the test is not clear, so mechanisms still need to be investigated.

Zhou *et al.* (2019) reported reduced glycaemia in chow-fed mice supplemented with 9-PAHSA as a result of decreased hepatic glucose production. The same decrease in glucose production occurred in high-fat diet-fed mice but glycaemia did not significantly change because the treatment caused a reduction in glucose clearance; an unexpected result given the other evidence (Yore *et al.*, 2014) of increased glucose uptake in adipose tissue.

Taken together, murine studies showing that FAHFA administration increases glucose clearance, improves insulin sensitivity and increases GLP-1 release are all promising with an eye to treatment of hyperglycaemia. However, whether or not FAHFA levels *in vivo* have any relevance to long-term glucose control continues to be debated, although the weight of Kellerer *et al.'s* (2021) evidence suggests not. This may be due to differences between mice and humans or to the fact that typical circulating levels of plasma FAHFAs are measured in the nano- or picomolar range while *in vitro* work has utilised micro-molar concentrations and Yore *et al.'s* (2014) murine gavage experiment increased the fasted levels (i.e. the peak levels *in vivo*) of 5-PAHSA three-fold. The long-term low concentrations *in vivo* may just not be enough to have any effect on insulin resistance despite the experimental results.

1.2.5 Purported Effects on Adipose Tissue Composition

Expansion of AT in the face of energy excess can occur through recruiting new adipocytes, hyperplasia, or by increasing the size of existing adipocytes, hypertrophy. It is well-established that the size of cells in SAT has a role to play in health and disease; for example, Lundgren *et al.* (2007) showed, in humans, that fasting glucose and glucose disposal rate were inversely correlated with cell diameter and Rýden *et al.* (2014) reported a negative correlation between cell volume and insulin sensitivity in abdominal SAT and VAT. These effects are independent of actual fat mass and are likely

caused by variation in the ability to recruit and differentiate preadipocytes (Gustafson *et al.*, 2015) with the result that existing adipocytes become larger and more dysfunctional.

Hammarstedt *et al.* (2018) found that concentrations of (non-esterified) PAHSAs in abdominal SAT correlated positively with GLUT4 protein expression and negatively with cell size, reflecting an overall positive association between PAHSAs and healthy adipose tissue. Further, they showed that adding 5- or 9-PAHSA to the culture media of differentiating 3T3-L1 cells increased expression of some adipogenic genes suggesting that PAHSAs might promote conversion of preadipocytes to adipocytes and hence hyperplasia. Song *et al.* (2016) have also shown increased adipogenesis on activation of FFAR4 by a synthetic agonist in 3T3-L1 cells while Hilgendorf *et al.* (2019) recently described in detail how the activation of FFAR4 by docosahexaenoic acid (DHA) in the cilia of preadipocytes promotes differentiation. Taken together, it would seem that FAHFAs can promote healthy AT expansion and that this may involve FFAR signalling.

As well as WAT, humans have small amounts of energy-consuming BAT, which develops by "browning" of white adipocytes. Knowing that activation of FFAR4 promotes browning of adipocytes (Quesada-Lopez *et al.*, 2016), Wang *et al.* (2018) investigated whether 9-PAHSA had this effect. In 3T3-L1 cells, 30 μM 9-PAHSA significantly increased mRNA expression of genes related to brown fat from the fourth day of differentiation. One month of feeding had similar effects on the mRNA and protein levels in WAT of the ob/ob mouse model of obesity, effects which were absent when FFAR4 was knocked down.

In summary, there is evidence that FAHFAs have the capacity to influence several aspects of adipocyte biology which could result in a healthy profile of tissue composition, enhancing their reputation as beneficial to metabolic health. Whether or not the low endogenous concentrations of FAHFAs are sufficient to have a meaningful effect is not clear.

1.2.6 Purported Effects on Lipolysis and Lipogenesis

Treatment of murine adipose tissue explants with 9-PAHSA has been shown to mimic and enhance the inhibitory effect of insulin on isoproterenol-stimulated lipolysis (Zhou *et al.*, 2019), which seems intuitive given the similar reinforcement of insulin's action on GLUT4 translocation. Indeed, activation of FFAR4 has been shown previously to inhibit lipolysis (Satapati *et al.*, 2017; Husted *et al.*, 2020).

However, Paluchova *et al.* (2020) observed that pre-incubation with insulin prevented the lipolytic effect of forskolin treatment but that pre-incubation with 5-PAHSA possibly even enhanced the lipolytic effect in 3T3-L1 cells. Similarly, where the pre-incubation preceded isoproterenol stimulation and

included a coincubation of insulin and 5-PAHSA, 5-PAHSA counteracted the anti-lipolytic effect of insulin. This contradiction remains to be resolved.

Paluchova *et al.* (2020) also examined the influence of 5-PAHSA on lipogenesis in mice. They described 5-PAHSA as having a stimulatory effect on DNL because there were many nascent 16- and 18-carbon FAs but the data could alternatively be interpreted as 5-PAHSA having an inhibitory effect on DNL steps (elongation and desaturation) beyond 18-carbon lengths, causing a build-up of the shorter chains. Additionally, the comparison of insulin- versus 5-PAHSA-induced lipogenesis in this study showed that the PAHSA effect was to put carbons into the metabolic pathway for FAs, whereas the insulin effect was to put carbons into the glycerol backbone, indicating two different roles for the signalling molecules. Taken together, this means that either the signalling cascade from FFAR4 is more complex than merely mimicking insulin or FAHFAs have other effects elsewhere at the surface or inside the cell.

1.2.7 Purported Effects on Inflammation

FFAR4 has been shown to mediate the anti-inflammatory effects of PUFA in murine macrophages (Oh *et al.*, 2010). Yore *et al.* (2014) examined the role of FAHFAs in the response of bone marrow dendritic cells (BMDCs) to the inflammatory Lipopolysaccharides (LPS) and found that the presence of 9-PAHSA reduced the usual inflammatory activation of dendritic cells in a dose-dependent manner, an effect not seen when repeated with palmitic acid. *In vivo*, the AT macrophages of high fat diet (HFD) mice, which had been gavaged with 9-PAHSA for three days, showed reduced secretion of the pro-inflammatory cytokines, TNFα and IL-1β, compared to animals that were not gavaged. The reduced secretion levels were similar to those in the control, chow-fed mice, suggesting decreased inflammation.

The same group have published several other reports investigating the anti-inflammatory effects of FAHFAs. Lee *et al.* (2016) treated mice with colitis-inducing dextran-sulphate-sodium water with and without 5- or 9-PAHSA and found that colitis symptoms were reduced (by around half) and that T-cell activation was reduced. These effects were, at least partially, dependent on FFAR4 activation. Kolar *et al.* (2019) compared the effects of 9-PAHSA and 13-LAHLA against a control on the LPS stimulation of RAW 264.7 macrophages (murine) and found that the mRNA expression of inflammatory markers iNOS, IL-1β, IL-6 and COX-2 were approximately halved by 13-LAHLA treatment. 13-LAHLA was more potent in reducing expression than either of its constituent parts, LA and 13-HLA. Syed *et al.* (2019) chronically fed the Non-Obese Diabetic strain of mice with 5- and 9-PAHSA from week 4 of life to 30 weeks and found that, in treated mice, the onset of diabetes was delayed by 3 weeks, the incidence was reduced from 82 % to 35 % and survival rate at 30 weeks increased from 22 % in

control to 78 %. In parallel, glucose-stimulated insulin release, fasting glycaemia and glucose response were improved with PAHSA treatment. Further, they measured a reduction in the infiltration of activated T-cells into the pancreata of the mice, which they reasoned to be the cause of the improved β-cell function.

Wang *et al.* (2018) showed that the FFAR4 activation by 9-PAHSA which promoted browning (see 1.2.5), also counteracted the role of LPS in activating the pro-inflammatory IκBα and NF-κB.

DHAHLA has been shown to reduce inflammatory markers in BMDCs in the presence of LPS to a similar degree as DHA and 9-PAHSA (Kuda *et al.*, 2016), which recapitulated Yore *et al.*'s (2014) work with 9-PAHSA. This anti-inflammatory effect was demonstrated not to have been merely a case of the DHAHLA breaking down to DHA and acting as DHA does alone.

Many effects of FFAR4 activation by long chain fatty acids (LCFAs), including omega-3s, and synthetic ligands have been established (Im, 2018) including the anti-inflammatory effects discussed here. FAHFAs have now been added to the list of ligands for FFAR4 and it is clear that they may have a beneficial effect *in vivo* as a treatment against inflammation in mouse models. What is lacking, however, is evidence of the effects in humans, including an understanding of the effect on adipocytes sharing the AT with macrophages, an understanding of how well typical *in vivo* concentrations of FAHFAs activate FFAR4 and whether different FAHFAs cause different responses (e.g. GPCR preference for β-arrestin-2 recruitment over G_{α} subunit release).

1.2.8 Purported Effects on the Liver

As noted in section 1.2.4, Zhou *et al.* (2019) reported reduced hepatic glucose production in mice after 8 weeks 9-PAHSA-supplemented food. This effect was the result of inhibition of the cAMP→PKA pathway. Furthermore, hepatic glucose uptake, measured as incorporation of 2-deoxyglucose in liver glycogen, increased after 9-PAHSA treatment. Together, these three observations suggest that 9-PAHSA improves hepatic insulin sensitivity. A negative consequence of improved insulin signalling might be an increase in lipogenesis and triacylglycerol (TAG) accumulation within the liver, but the investigators found no increase in hepatic TAG after 2 months of 9-PAHSA treatment. However, Benlebna *et al.* (2020) did report that long-term supplementation (12 weeks) with 9-PAHPA or 9-OAHPA caused some livers to have visible abnormalities and measurable steatosis at time of dissection. Furthermore, they found fibrosis, as measured by Sirius staining, to be up to four-fold greater, possibly due to increased insulin sensitivity.

Further negative effects on the liver were also reported by Rychtrmoc *et al.* (2020) who carried out a short-term study where mice received oral gavage of either 9-PAHSA or 9-OAHSA for three days.

Plasma levels of alanine amino transferase and aspartate amino transferase levels were slightly and significantly raised compared to control for 9-PAHSA and 9-OAHSA respectively, suggesting a change in liver function or possibly damage.

These negative findings are surprising given that Nakamoto *et al.* (2019) showed that activation of FFAR4 reduced indicators of hepatic steatosis and so there is a suggestion that FAHFAs may have effects in the liver other than as FFAR4 ligands. Certainly, these findings are potential warning signs that FAHFAs may have adverse health effects which should be investigated further before recommending FAHFAs as a diabetes treatment.

1.2.9 FAHFAs Stored in Triacylglycerol

FAHFAs also exist as components of TAG (Tan *et al.*, 2019; Paluchova *et al.*, 2020) though in what proportion and how that proportion changes with metabolic state remains unclear. Since TAG is the medium for the storage of FAs by adipocytes, incorporation into TAG allows for a reservoir of FAHFAs and Tan *et al.* (2019) reported the concentration of FAHFA-containing TAGs (FAHFA-TAG) to be 100-fold higher than free FAHFAs (FAHFA-NEFA) within adipocytes (e.g. 10-PAHSA ca. 1 $pmol.mg^{-1}$ versus 0.01 $pmol.mg^{-1}$).

If FAHFAs are synthesised and stored in FAHFA-TAG when glucose is available, then they, similarly to other FAs, should be released during fasting as a result of lipolysis. Indeed, free PAHSAs were found to be higher in the WAT of mice after fasting compared to after feeding (Yore *et al.*, 2014). Paluchova *et al.* (2020) showed that the lipolytic release of FAHFAs is dependent on adipose triglyceride lipase (ATGL), as is the release of FAs, so it would seem, superficially at least, that the life-cycle of FAHFAs into and out of storage is similar to that of FAs.

Paluchova *et al.* (2020) showed that the upregulation of DNL, which occurred in WAT during cold exposure resulted in increased FAHFA production and that feeding mice with 5-PAHSA enhanced DNL further, presumably through its stimulatory effect on glucose uptake. Furthermore, by utilising deuterium enrichment they were able to determine that 5-PAHSA resulted in less glycerol and more FAs being synthesised than in cold exposure alone. This occurred together with a general remodelling of diacylglycerol (DAG) species with more 16:0 and 18:0 being present in DAG. Using cultured 3T3-L1 cells, Paluchova *et al.* determined that the 5-PAHSA was influencing the re-esterification part of the cold exposure FA cycling, promoting the esterification of nascent shorter, saturated FAs. The mechanism(s) underpinning this effect remains to be elucidated.

An increased basal metabolic rate was found by Benlebna *et al.* (2020) in mice supplemented with FAHFAs compared to controls without any difference in weight, adiposity or food intake. It could be

speculated that the increased metabolic activity might be related to the cycling of lipolysis and re-esterification that was shown to be increased by 5-PAHSA by Paluchova *et al.* (2020).

While Yore *et al.* (2014) observed reduced FAHFA-NEFA in fasting plasma of insulin resistant individuals, it remains to be elucidated whether or not those individuals also have reduced FAHFA-TAGs, i.e. it is not known if insulin resistance might impair the synthesis or the release of FAHFAs or, in fact, both processes. It is also not known if all FAHFAs are esterified or released by ATGL equally. The ability to store FAHFAs may also increase the importance of the previously downplayed FAHFAs in food; their abundance may be low but, if they were to contain species which are hard to synthesise *in vivo*, then storing them for later use could be important if those species enhanced the potency of FAHFAs.

These findings with regard to esterification and release during fasting or cold exposure introduce a time delay linked to the feed-fast cycle which refines the proposed mechanism for FAHFA regulation of glucose control described in section 1.2.1. FAHFAs are produced in the post-prandial state when substrates (e.g. glucose and FAs) are abundant, stored in TAG and later released during fasting where they are in place to enhance the insulin response to the next meal- see Figure 1-3.

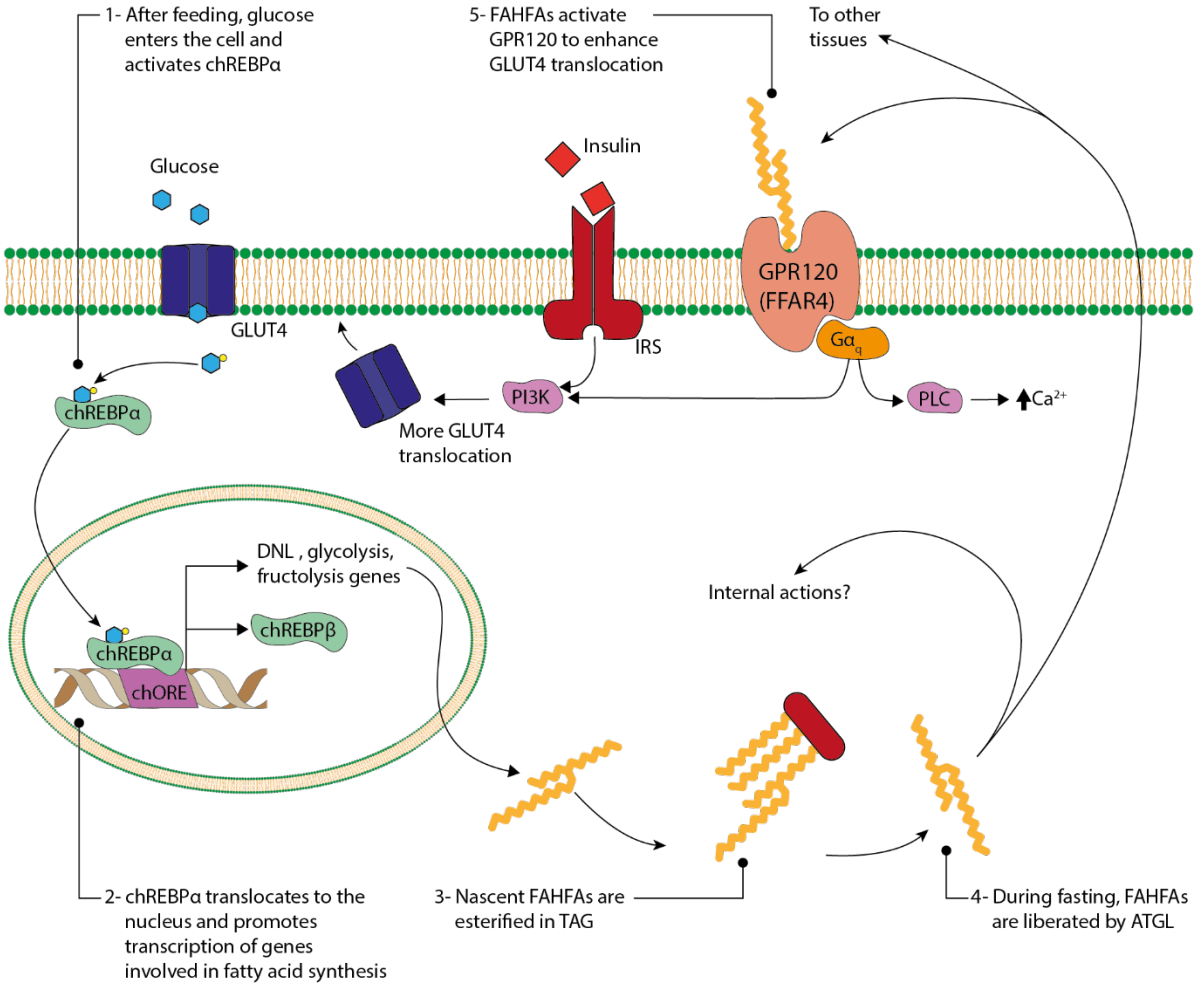


Figure 1-3: Proposed mechanism of FAHFA enhancement of glucose uptake through the feeding cycle

Glucose from a meal stimulates and provides substrate for DNL (1), a process which results in production of FAHFAs (2). FAHFAs are integrated in TAG (3) and stored until released at a later time point during fasting (4). After release, they are exported to the plasma where they have auto- and paracrine effects on glucose uptake via FFAR4 (5). Abbreviations: DNL- *de novo* lipogenesis.

1.2.10 Synthesis and Degradation

Yore *et al.* (2014) demonstrated, in mice, that FAHFAs are synthesised in both adipose and hepatic tissues. Synthesis appears to require similar general conditions as required for the synthesis of common FAs. For example, a supply of carbons, such as glucose, is obvious but also required is ATGL; where Mottillo *et al.* (2014) showed that DNL in mice is stopped by ATGL knockout, Paluchova *et al.* (2020) showed that ATGL knockout limited the accumulation of FAHFA-TAGs. In addition to DNL, the existence of FAHFAs comprising essential FAs (see section 1.2.12) demonstrates that they can also be synthesised from existing FAs.

Patel *et al.* (2021) have expanded on Paluchova's work to elucidate the role of ATGL in FAHFA synthesis and degradation. They showed that ATGL catalyses the transacylation of a FA in a TAG or

DAG to an HFA. ATGL then plays further roles in the esterification of the FAHFA into DAG/TAG and the ultimate release of it in response to lipolytic signals including starvation and circulating catecholamines, natriuretic peptide and glucagon (Yang and Mottillo, 2020). This may not be the only route to synthesis, though: AT-specific ATGL knockout mice showed much-reduced FAHFA levels in plasma and AT (69- 98 % range for different species in different fractions) but had no differences in the liver (Patel *et al.*, 2022). This leaves a question over whether the liver also utilises ATGL, has an alternative synthesis pathway or whether dietary FAHFAs are partitioned into the liver. These data might also suggest that the liver exports very few FAHFAs.

Kuda *et al.* (2018) proposed a mechanism which may partially account for the regulation of the production of HFAs, which are necessary for FAHFA synthesis. Using quantitative trait analysis to link genomic loci with variation in plasma concentrations of certain PAHSAs in mice, they identified genes involved in anti-oxidant activity and regulated by the transcription factor Nrf-2. They proposed that peroxidases generate HFAs from hyperoxidised membrane phospholipids, providing substrate for ATGL and transacylation. Oxidative stress has been reported as both a cause and a consequence of metabolic syndrome (Maslov *et al.*, 2019). In the latter case, the production of beneficial FAHFAs in response to reactive oxygen species would be plausible but in the former, it would suggest that either the stress does not produce enough FAHFAs or that FAHFAs don't protect against the syndrome. Kuda *et al.* (2018) also proposed that Nrf-2 downregulates or interferes with ChREBP's role in GLUT4 expression and translocation, thus putting an apparently-contradictory brake on the *de novo* production of FAHFAs. However, the evidence presented for Nrf-2 related gene expression and FAHFA levels in mice overexpressing Nrf-2 is not conclusive nor corroborated in humans so there is still much to be learned about that aspect of FAHFA synthesis.

To date, three hydrolases have been identified as being active in the breakdown of FAHFAs into their component fatty- and hydroxy-fatty acids: Carboxyl Ester Lipase (Kolar *et al.*, 2016), AIG1 and ADTRP (Parsons *et al.*, 2016). A whole-mouse knockout of ADTRP resulted in 5-fold increases in non-esterified 9-PAHSA and 9-OAHSA in all adipose depots, while 12/13-PAHSA was the only other FAHFA to increase, by around 2-fold, and only in BAT (Ertunc *et al.*, 2020). FAHFA-TAGs increased in BAT but not in WAT or liver. Knocking out AIG1 had no significant effect on tissue FAHFA concentrations (Ertunc *et al.*, 2020). Though a double knockout of ADTRP and AIG1 elevated plasma FAHFA levels (Ertunc *et al.*, 2020), it did not affect glycaemia in mice in the way that acute administration did (Yore *et al.*, 2014). This may be due to these hydrolases only influencing a small number of isomers, leaving other isomers free to act, or it may be that very high dosages, such as those used in the acute administration, are required to cause a significant reduction in glycaemia.

1.2.11 Methods of Measurement

Across the various laboratories working with FAHFAs, the common process for quantification involves the extraction of lipids from samples by the Folch method (Folch, Lees and Stanley, 1957) followed by separation using a solid phase extraction column before targeted detection using liquid chromatography-mass spectrometry (LC-MS). However, no standardised protocols for either the liquid chromatography or the mass spectrometry components have been established yet, with different groups trialling different techniques in an attempt to improve resolution between FAHFA species and to avoid overlap with contaminants such as ceramides, depending on their species of interest.

The extraction and separation steps are time-consuming and the development of standard libraries is expensive, so it is tempting to utilise untargeted MS methods common in metabolomics to examine biological samples more directly. It has been identified, though, that dimers of FAs can form during the LC process and, since these have the same masses as FAHFAs comprising the same pairs of FA, the dimers can confound measurement of FAHFAs (Nelson *et al.*, 2022). Consequently, targeted approaches looking at specific species compared against known standards is a robust approach for much research.

1.2.12 FAHFA Profile Variation

If health outcomes are influenced by FAHFAs, then it would be of interest to know what non-modifiable and modifiable factors influence the profile of circulating and stored FAHFAs.

In elderly women (65 to 80 years) participating in an aerobic and resistance exercise intervention study for 4 months, exercise was found to cause an increase in serum and AT PAHSA levels (Brezinova *et al.*, 2020). This observation is interesting as it suggests some form of signalling between tissues but it is not known whether this is a result of increased DNL in general or a specific upregulation of PAHSAs.

Diet has been shown to have an influence on plasma FAHFAs. Kellerer *et al.* (2021) found several FAHFA species to be higher in the plasma of omnivores than in vegans and to be higher in young men after one week of 1000 $kcal.day^{-1}$ overfeeding of saturated fats (all the FAHFAs measured included saturated fats). In WAT, Kuda *et al.* (2016) found that DHAHLAs were found in human WAT after supplementation with omega-3 PUFAs (2.8 g of EPA and DHA for 24 weeks). In my own work to optimise an LC-MS method, I have used samples from a separate fish oil supplementation study and also noted an increase in all FAHFA combinations of EPA and DHA (omega-3 PUFAs).

In mice, age had an influence on the profile of FAHFAs in visceral AT, with the number of FAHFA families increasing with age (Zhu *et al.*, 2020). The increases are not uniform; some families go up in

middle age and then down again in old age, while others increase in middle age and remain increased, suggesting a form of regulation separate from mere accumulation with time. In humans, Kellerer *et al.* (2021) found plasma concentrations of several FAHFAs to be higher in adults than in adolescents and the elderly.

Kuda *et al.'s* work on quantitative trait loci (2018) in mice revealed some insight into genes associated with FAHFA synthesis. Repeating similar work in humans would be useful.

While there are some interesting findings in this area, there are still many unknowns and opportunities for investigation in different populations to determine clear patterns and to elucidate mechanisms for variations in profiles.

1.3 Are FAHFAs Special?

This is not a question of whether the reported effects are genuine or not, but a question about whether FAHFAs are unique in achieving those effects or not.

As lipid-derived molecules which have signalling effects, FAHFAs are not unique but fall under the banner of lipokines. Other lipokines include sphingolipids, which promote insulin resistance (Chaurasia and Summers, 2015), endocannabinoids, which regulate appetite (Silvestri and Di Marzo, 2013) and PUFA which act to reduce inflammation, also through FFAR4 (Oh *et al.*, 2010).

Another lipokine candidate, derived from the DNL pathway rather than food, is palmitoleate which, like FAHFAs, has been reported to have an effect on glucose homeostasis by sensitising the insulin receptor and thus increasing glucose uptake (Cao *et al.*, 2008). However, attempts to link circulating palmitoleate concentrations to insulin sensitivity in man have been equivocal. It could be, though, that FAHFAs exert more potent effects and that the palmitoleate results have been confounded by unmeasured FAHFAs concentrations.

Since the greatest effects of FAHFAs on AT are mediated through FFAR4, it is reasonable to ask whether those same effects are also stimulated by palmitoleate and the other circulating endogenous NEFA. The efficacy of those NEFA in activating the receptor may be lower than FAHFAs (Ulven and Christiansen, 2015) but their concentrations are orders of magnitude greater. A study by Pflimlin *et al.* (2018), which failed to replicate the work of Yore *et al.'s* (2014) was criticised for several inconsistencies in its methodologies (Kuda, 2018), one of which was the use of oleate in the gavage. This was criticised because of its FFAR4 activity but, since it is the most abundant FA in the plasma NEFA pool (Hodson, Skeaff and Fielding, 2008), this can't be ignored when considering free-living humans.

FAHFAs may have metabolically-beneficial effects but disentangling their relevance from that of other FAs *in vivo* in order to declare them as something special may be challenging.

1.4 Free Fatty Acid Receptors

The four free fatty acid receptors are members of the G-protein coupled, cell membrane receptor class, briefly summarised in Figure 1-4. FFARs 1 and 4 are receptors for the LCFAs and FAHFAs, while FFARs 2 and 3 are receptors for short-chain fatty acids (SCFAs), such as those derived from the gut microbiome. Their effects are mediated by either the G_{α} subunits or by β-arrestin 2 recruitment. They are expressed in various tissues, including AT. FFAR3 has been reported in whole human AT (depot unspecified) and FFAR4 has been identified in mature human adipocytes but not preadipocytes (depot unspecified) (Gotoh *et al.*, 2007). FFAR1 (Gotoh *et al.*, 2007) and FFAR2 (Brown *et al.*, 2003) have been looked for but not found in man.

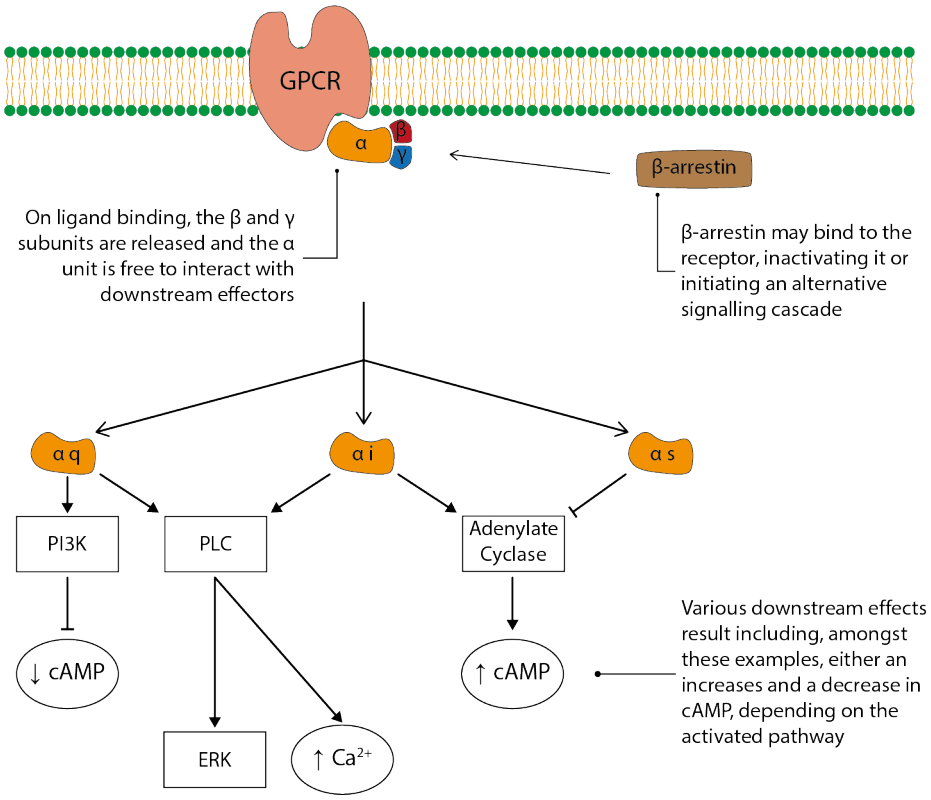


Figure 1-4: G-protein coupled receptors

The g-protein comprises the alpha, beta and gamma subunits. Upon ligand binding, conformational change allows the beta and gamma subunits to be released, leaving the alpha subunit to interact with downstream effector proteins. The alpha subunit has several forms, including the q, i and s types which have all been associated with free fatty acid receptors. Downstream effects are varied and, in some cases, contradictory depending on the type of alpha protein involved.

While several studies have examined the potency and efficacy of various fatty acids in activating FFARs 1 and 4, there are inconsistencies over the cell type, effect measured (i.e. increase in Ca^{2+} or

activation of β-arrestin 2) and pattern of expression (over-expressed by transfection or endogenous). It is difficult, therefore, to be categorical about what efficacy a particular ligand would have on receptors in human adipocytes but it seems clear that FAHFAs and PUFAs have greater efficacy than SFAs.

FFAR4 can be phosphorylated by receptor tyrosine kinases, including the insulin receptor, causing its internalisation and inactivation (Villegas-Comonfort *et al.*, 2019). Given that activation of FFAR4 mimics or potentiates the effect of insulin, this is either an unexpected contradiction or a piece of negative feedback to prevent the system from being too over-stimulated.

1.5 Thesis Aims

The aims of my thesis are to characterise the distribution, metabolism and role of FAHFAs in man and specifically to determine:

1. If, at a whole-body level, there are differences in the pattern of circulating FAHFAs which correlate with phenotypic measures including adiposity, body fat distribution, insulinaemia, glycaemia, etc.
2. If there are depot-specific differences in the synthesis and storage of FAHFAs between abdominal and gluteal adipose tissue and to elucidate relevant pathways.
3. What the effects of FAHFAs on adipose tissue function are and whether they differ between abdominal and gluteal depots.

Chapter 2

Methods

2 Methods

2.1 Human Adipose Tissue Biopsy and Preadipocyte Isolation

Participants free from known disease were recruited from the Oxford Biobank (Karpe *et al.*, 2018) and invited to the Oxford Centre for Diabetes Endocrinology and Metabolism Clinical Research Unit for the procedure and other anthropometric measures. Participants were briefed on the procedure and gave informed consent (Oxfordshire Clinical Research Ethics Committee, reference 08/H0606/107+5). Paired adipose tissue samples were collected from the subcutaneous abdominal and gluteal depots by needle aspiration (12-gauge needle) under local anaesthetic (lidocaine).

Immediately after the procedure, the contents of each syringe were placed on a sterile 250 µm gauze (Sefar) and washed with a jet of saline from a syringe and a spatula to separate blood, capillaries and other fibrous material as best as possible. A portion of the sample (approximately 100 mg) was wrapped in tin-foil, snap-frozen in liquid nitrogen and transferred to -80 °C. The remainder of the sample was then immersed in Hanks Balanced Salt Solution (HBSS) (Merck, H9394) and transferred on ice to a biological safety cabinet.

The samples were transferred to 10 mL of a sterile-filtered, 1 $mg.mL^{-1}$ solution of collagenase H (Roche, 11074032001) in HBSS and shaken in the water bath for 45 min at 37 °C and 0.5 Hz. The solution was centrifuged for 5 min at 240 ×g, the supernatant removed and the resulting pellet resuspended in HBSS. The resuspension was then filtered through a sterile 200 µm mesh (Pluriselect, 43-50200-03) and then through a sterile 100 µm mesh (Pluriselect, 43-50100-51) before centrifuging again at 240 ×g. The pellet was resuspended in 5 mL growth medium (see 2.2) and seeded in a T25 flask.

2.2 Maintenance of Cells in Culture

Both primary and immortalised preadipocytes were maintained in DMEM/F12 (Sigma, D6421) supplemented with 10 % (v/v) foetal calf serum (Labtech), 100 $U.mL^{-1}$ penicillin/ 100 $µg.mL^{-1}$ streptomycin (Invitrogen, 15140-122), 2 $mmol.L^{-1}$ L-glutamine (Invitrogen, 25030-024) and 0.25 $ng.mL^{-1}$ fibroblast growth factor (R&D Systems, 233-FB). Cells were grown in culture flasks, the medium was changed every 2 days and cells were passaged upon reaching 80-90 % confluence.

Preadipocytes were differentiated into adipocytes according to the protocols discussed in Chapter 3.

Cell stocks were tested quarterly for mycoplasma and were confirmed to be mycoplasma-free throughout the project.

2.3 Measurement of Total Intracellular TAG *In Vitro*

After removal of media from the 6-well culture plate, the cells were washed in phosphate-buffered saline (PBS) (Sigma, D1408) and then lysed with 150 µL TAG lysis buffer. The buffer is 1 % NP-40 which comprises 150 mM NaCl, 50 mM Tris-HCl and 1 % NP-40 (also known as IGEPAL-630) (Sigma, I3021).

Before further processing, the lysates were sonicated (5 s pulses for a total of 20 s at 40 kHz) and assessed for protein concentration as described in 2.6.

The lysates were boiled at ~95 °C for 30 min, vortexed and left to cool to room temperature. Next, the lysates were centrifuged at 12000 ×g for 10 min and the supernatant removed for analysis on a chemical analyser (Beckman Coulter, AU480) using the Quantlab TAG kit (Quantlab, 0018255640). The assay kit includes reagents and protocols for calibrating and quality controlling the assay by a concentration curve and known standard.

2.4 Measurement of Media Glucose or Lactate *In Vitro*

Media was sampled from the 6-well culture plates (1 mL) and analysed on a chemical analyser (Beckmann, AU480) using assay kits for glucose (Quantlab, 0018250840) or lactate (Randox, LC3980). The assay kits include reagents and protocols for calibrating and quality controlling the assay by a concentration curve and known standard.

2.5 Assessment of Stimulated Lipolysis by Measurement of Non-Esterified Fatty Acids and/or Glycerol Release *In Vitro*

Lipolysis experiments using differentiated adipocytes seeded at 200k cells per well took place in 6-well plates using a Krebs Ringer Hepes (KRH) buffer as the media for incubations. The KRH comprises NaCl (120 mM), KCl (4.8 mM), $CaCl{\cdot}2H_2O$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO4{\cdot}7H_2O$ (1.2 mM), $NaHCO_3$ (15 mM), HEPES buffer (10 mM) (Sigma H0887) and glucose (6 mM) (Sigma, G8270) with 3.5 % Bovine Serum Albumin (BSA) (Sigma, A7030). The KRH was sterile filtered and warmed prior to the experiment.

The culture media was removed and replaced with 1mL KRH and the plate incubated for 45-60 min for equilibration. The KRH was removed, discarded and replaced with 900 µL KRH and the plate incubated for 90 min. This KRH was removed and saved as the basal sample. A final 900 µL of KRH was added for the stimulation step. Where required, the relevant treatment was added and the plate incubated for 30 min before the addition of the relevant stimulant and a final incubation of 90 min (see results chapters for relevant treatments and stimulants). The KRH was collected and saved as the

stimulated sample and the cells lysed with 150 µL of either protein (cOmplete Lysis M buffer with cOmplete EDTA-free protease inhibitor and phosStop phosphatase inhibitor, Sigma, 4693132001, 4906845001, 4719964001) or TAG lysis buffer (see 2.3) as required for further analysis.

The collected KRH samples, along with a blank KRH sample were analysed on a chemical analyser (Beckmann, AU480) using assay kits for NEFA (Randox, FA115) or glycerol (Randox, GY105). The assay kits include reagents and protocols for calibrating and quality controlling the assay by a concentration curve and known standard.

2.6 Protein Quantification Assay

The protein concentration of cell lysates, whether prepared for TAG analysis or Western blotting (see 2.3 or 2.7), was determined using the Bio-Rad DC protein assay (5000111).

A protein standard was prepared by dissolving 4 $mg.mL^{-1}$ BSA (Sigma, A7030) in PBS and serially-diluting in the relevant lysis buffer. The standard and samples were pipetted in triplicate onto 96-well plates at a volume of 5 µL and the kit reagents added according to the manufacturer's instructions. After at least 15 min incubation in the dark at room temperature, absorbance was read at 650 nm on an automatic plate reader (Versamax). A standard curve and its equation were generated to allow calculation of protein concentration from the absorbance values.

2.7 Western Blotting

The lysis buffer used for all Western Blot experiments was Lysis M buffer with cOmplete EDTA-free protease inhibitor and phosStop phosphatase inhibitor (Sigma, 4693132001, 4906845001, 4719964001). Lysates were homogenised by sonication (5 s pulses for a total of 20 s at 40 kHz), centrifuged for 3 min at 12000 ×g and frozen at -80 °C as required. Protein quantities for samples were determined as described in 2.6.

Sample volumes for set protein masses (typically 20 µg for each sample in the gel, but variable dependent on sample availability and assay protein abundance), were calculated and made up to a suitable volume for the electrophoresis with deionised water and 4X Laemmli buffer (Biorad, 1610747). Typically, samples were heated to 90 °C for 10 min but transmembrane proteins were left at room temperature for 30 min following the addition of the Laemmli buffer to avoid protein aggregation caused by boiling.

Biorad Criterion stain-free, precast gels with 7.5 % acrylamide were loaded with a ladder (Jena Bioscience, Blue-Eye, PS-104) and the samples. Gels were run at 230 V in a tris-glycine-SDS running buffer in a transfer tank (Biorad) for approximately 35 min. Gels were imaged to verify protein migration

using a Biorad Chemidoc visualiser. Wet transfers with a trizma/ glycine buffer were used to transfer the separated proteins to a 0.45 µm polyvinylidene-fluoride (PVDF) membrane (Immobilon, IPVH00010). The transfers were carried out at 80 V for 45 to 60 min and the membranes imaged on the visualiser to verify protein transfer.

Membranes were blocked in blocking buffer (5 % milk powder in TBS-T (1 % tween-20 in tris-buffered saline)) for 60 min at room temperature with rocking. The blocking buffer was replaced with the primary antibody for the protein of interest, diluted in the same milk or BSA buffer. Membranes were incubated overnight at 4 °C with rocking. After removing the primary antibody buffer, the membranes were washed in TBS-T (four times ~5 min) and then incubated for 60 min at room temperature with the appropriate secondary antibody in the milk or BSA buffer. After washing in TBS-T, the membrane was incubated for 60 sec in ECL substrate (Biorad Clarity) and the protein bands imaged on the visualiser. The imaged band intensities for the protein of interest were measured using FIJI or Adobe Photoshop and normalised to those of a loading control protein. Details of the individual antibodies are given in the results chapters.

2.8 Gene Expression Assays by qPCR

Cells in 6-well plates (200k cells per well) were washed in PBS and incubated for 2 to 3 min in 500 µL Tri-Reagent (Ambion, AM9738) before scraping and freezing to -80 °C. Defrosted samples were transferred to phase lock gel tubes (5-Prime, 2302830) and 100 µL chloroform added. After centrifuging at 12000 ×g and 4 °C for 15 min the aqueous, RNA-containing phase was transferred to elution tubes with an equal volume of isopropanol added. After an overnight incubation at -20 °C, the samples were centrifuged for 30 min at 12000 ×g and 4 °C and the supernatant removed. The pellet was suspended in 80 % ethanol and centrifuged again for 30 min at 12000 ×g and 4 °C and the process repeated. After removing the second round of ethanol, the pellet was dried and resuspended in RNAse-free water for quantification using a spectrophotometer (Nanodrop, ND-1000).

Synthesis of cDNA utilised a reverse transcription kit (Life Technologies, UK, 4368813) and thermocycler (MJ Research, PTC-225) using 600 to 800 ng RNA.

cDNA was diluted at 1:40 in 10 mM tris hydrochloride for qPCR utilising Taqman assays and Fast Master Mix (Kappa Biosystems) on a Quantstudio 7 Flex (ABI) machine. All samples were analysed in triplicate using 2.7 µL dilute cDNA, 0.3 µL Taqman probe and 3 µL Master Mix.

Relative transcript expression was calculated using the $\Delta\Delta C_t$ relative quantification method (Pfaffl, 2001). The ΔC_t values of target genes were normalized to the geometric mean of the ΔC_t of housekeeper transcripts *18S*, *PGK1*, *PPIA* and *UBC* (Neville *et al.*, 2011).

2.9 Assessment of Insulin Signalling by AKT Phosphorylation *In Vitro*

On the day prior to the assay, cell media was changed to a low-glucose, insulin-free media. Insulin (25 nM) (Invitrogen, 12585-014) was added to each stimulated well while unstimulated wells were treated with vehicle (PBS). After 10 min of incubation at room temperature, the wells were washed twice with PBS and cell lysis buffer added (cOmplete Lysis-M EDTA-free (4719964001) and PhosSTOP (4906845001) phosphatase inhibitor, Roche). Lysates were homogenised by sonication (total 30 seconds) and the protein content determined using the Biorad DC assay (see 2.6). Western blots were carried out (see 2.7) to detect the relative abundance of total-AKT, phospho-AKT S473 and alpha-tubulin. Membranes were blocked in BSA before an overnight incubation in the primary antibody (Cell Signalling 4058S (1:1000) & 2920S (1:1000), Abcam 15246 (1:2000)) and a 1 h incubation in the secondary (31430 and 41360, both Invitrogen). Band intensities were normalised against the alpha-tubulin intensities.

2.10 FAHFA *In Vitro* Biosynthesis Assays

Confluent adipocytes were differentiated for 10 days in T75 flasks, lysed in buffer (cOmplete Lysis-M EDTA-free (4719964001) and PhosSTOP (4906845001) phosphatase inhibitor, Roche) and scraped.

Lysates were homogenised by sonication and divided into 100 µL samples. The control samples were boiled for 10 min at 90 °C to denature enzymes. To each sample was added 50 µL of 12-hydroxyoctadecanoic acid (Sigma, 219967) and 50 µL of palmitoyl-coenzyme-A (Sigma, P9716) before 120 min incubation at 37 °C.

The samples were then processed to extract FAHFA-NEFA as described in 2.12.

2.11 Co-Immunoprecipitation of BSA and Conjugated Fatty Acids or FAHFAs

A 10 mL, 5 % (w/v) solution of BSA (Sigma-Aldrich, A7030) was prepared and 5 mg 5-PAHSA (Sigma-Aldrich, 807761) added to achieve a BSA:FAHFA ratio of approximately 1:1.

For each sample or control, 50 µL of Dynabead Protein A solution (Invitrogen, 10001D) was added to a 1.5 mL tube in a paramagnetic rack to separate the beads and allow removal of the transport supernatant. To each tube was added 200 µL Phosphate-Buffered Saline solution (PBS) and, except the controls, 10 µg of anti-BSA IgG antibody (Invitrogen, A11133). After 10 min of mixing, the beads were separated by magnet and the supernatant removed before washing the beads with 200 µL 0.02 % TWEEN-20 in PBS. 100 µL of the BSA/ 5-PAHSA solution were added and mixed on a rocker for 10 min. The beads were washed twice with 200 µL 0.02 % TWEEN-20 in PBS and transferred to a clean tube. The supernatant was separated and removed and 20 µL 50 mM glycine at pH 2.5

(Sigma, G7126) added to elute the BSA. After mixing, the beads were separated and the eluent removed for processing.

Lipids in the eluent were extracted as 2.12 and analysed by LC-MS as 2.15.

2.12 Extraction of FAHFAs from Plasma or Lysates for Liquid Chromatography

2.12.1 Folch-based Method

The protocol for extraction of FAHFAs combines those of Kolar *et al.* (2018) and Tan *et al.* (2019). Where cell lysates are used, they are harvested in TAG lysis buffer (see 2.3).

In a 10 mL glass tube, 200 µL of sample was added to 1.3 mL PBS, 1.5 mL methanol, 3 mL chloroform and an internal standard ($^{13}C_4$ 9-PAHSA, 17725, Cayman). After mixing, the tubes were centrifuged for 10 min at 800 ×g at 4 °C to separate the phases. The solvent phase was transferred to a glass tube, dried under nitrogen and reconstituted in 200 µL chloroform.

Solid phase extraction (SPE) columns (Biotage, 3mL, 500 mg silica) were washed with 6 mL ethyl acetate and conditioned with 6 mL hexane, drawn through by vacuum. The samples in chloroform were added and left to drain through by gravity.

The non-polar lipid fraction, containing FAHFA-TAG, was eluted with 6 mL of 5 % ethyl acetate in hexane and dried under nitrogen. The polar fraction, containing FAHFA-NEFA was eluted with 4 mL ethyl acetate and dried under nitrogen. The sample was reconstituted in methanol, transferred to a glass vial and sealed, ready for analysis.

The non-polar fraction was reconstituted in 200 µL ethanol and 20 µL 1 M LiOH added to hydrolyse the TAG and liberate FAHFA-NEFA. After overnight shaking, the base was neutralised with 20 µL 2M HCl and added to a fresh PBS/ chloroform/ methanol mixture to repeat the above process, isolating the FAHFA-NEFAs.

2.12.2 Citrate Buffer Method

This method was used for samples processed in collaboration with the Kuda group at the Czech Academy of Sciences and is based on previously published methods (Kuda *et al.*, 2018).

In a 1.5 mL Eppendorf tube, 150 µL of sample was added to 350 µL citrate buffer (1 M NaCl, 100 mM trisodium citrate, pH 3.6), 1 mL ethyl acetate and the internal standard ($^{13}C_4$ 9-PAHSA, 17725, Cayman). Samples were mixed for 20 min at 30 Hz on a reciprocating/ orbital mixer at 4 °C and then centrifuged at 12000 ×g and 4 °C for 10 min. The solvent phase was removed to a 5 mL glass tube and dried down.

Solid phase extraction (SPE) columns (Thermofisher Hypersep, 10mL, 500 mg silica) were washed with 10 mL ethyl acetate and dried the day prior to the extraction. On the day, they were conditioned with 10 mL hexane, injected by syringe into the bottom of the column and left to drain out by gravity. Next, 10 mL 5 % ethyl acetate in hexane was added to the top of the column and left to drain out by gravity. The dried samples were reconstituted in 300 µL 5 % ethyl acetate in hexane and added to the columns.

The non-polar lipid fraction, containing FAHFA-TAG, was eluted with 10 mL of 5 % ethyl acetate in hexane and dried under nitrogen. The polar fraction, containing FAHFA-NEFA was eluted with 5 mL ethyl acetate and dried under nitrogen. The sample was reconstituted in methanol, transferred to a glass vial and sealed, ready for analysis.

The non-polar fraction was reconstituted in 200 µL ethanol and 20 µL 1 M LiOH added to hydrolyse the TAG and liberate FAHFA-NEFA. After overnight shaking, the base was neutralised with 20 µL 2 M HCl and added to a fresh citrate buffer and ethyl acetate mixture to repeat the above process, isolating the FAHFA-NEFAs.

2.13 Extraction of FAHFAs from Adipose Tissue for Liquid Chromatography

Working on liquid nitrogen, 50 mg of tissue was taken from each snap-frozen biopsy sample and added to 500 µL citrate buffer and 1 mL ethyl acetate to repeat the same process as detailed in 2.12.2.

2.14 Extraction of Lipids from Plasma or Lysates for Gas Chromatography

Total lipids were extracted from samples using the Folch method (Burdge *et al.*, 2000). To do this, 150 µL of sample and 350 µL 0.9 % saline were added to glass, screw-top tubes along with 10 µL internal standard (50.12 µg /20 µL 45:0 TAG), 5 mL 2:1 chloroform:methanol and 1 mL 1 M Na^+Cl^-. After 15 min rotary mixing, the tubes were centrifuged for 10 min at 800 ×g at 4 °C to separate the phases. The solvent phase was transferred to a glass methylation tube and dried under nitrogen.

Next, the lipids were derivitized by methylation to generate fatty acid methyl esters (FAMEs). To the dried sample was added 400 µL of a 10 % BHT (w/v) in toluene mixture and 800 µL 2 % H_2SO_4 in methanol. After vortexing to dissolve the samples, the tubes were placed in the water bath at 80 °C for 90 min. After cooling, the mixture was neutralised with 2 mL neutralising solution (25 mM $KHCO_3$ and 25 mM K_2CO_3 in H_2O), mixed for 15 min and centrifuged for 10 min at 800 ×g at 4 °C. Again, the solvent phase was transferred to a glass tube and dried under nitrogen. The lipid sample was reconstituted in 400 µL chloroform in glass GC vials.

2.15 Liquid Chromatography and Mass Spectrometry for Quantification of FAHFAs

2.15.1 Liquid Chromatography- Hu Method

The LC-MS machine used was a Xevo G2-XS QToF (Waters) with a C18, 1.7 µm, 100 mm UPLC column (Acquity 186002352 by Waters).

The mobile phase comprises two elution solvents, the ratio of which changes over the course of the 29-minute run as shown in Figure 2-1. Solvent A is water with 5 mM ammonium acetate and Solvent B is 3:1 acetonitrile:propan-2-ol with 2 mM ammonium acetate. The solvents are stable and stored at 4 °C for up to two months before renewing. The injection volume was 4 µL.

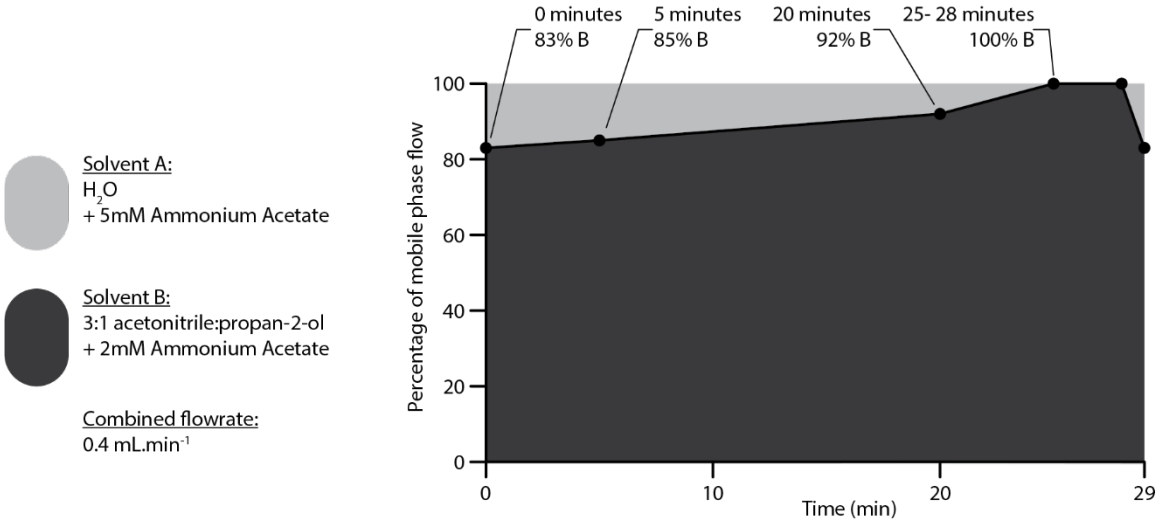


Figure 2-1: Solvent mix and flowrate profile for Hu liquid chromatography method.

Original method according to Hu *et al.* (2018).

In order to avoid timeshift in the peaks, it is necessary to equilibrate the column by running at least five blank samples at the beginning of each session. This can utilise four short cycles and one standard cycle where the short cycles go through each step in Figure 2-1 for 2 minutes, totalling an 8-minute run.

At the end of a session, the column is flushed with methanol.

2.15.2 Liquid Chromatography- Kuda Method

The LC-MS machine used was a Xevo G2-XS QToF (Waters) with a C18, 1.7 µm, 100 mm UPLC column (Acquity 186002352 by Waters).

Before a session, the column is equilibrated with 70:30 H_2O:acetonitrile at a flow of 0.2 $mL.min^{-1}$ until the pressure drop across the column stabilises.

The mobile phase comprises two elution solvents, the ratio of which changes over the course of the 29-minute run as shown in Figure 2-2. Solvent A is 70:30 H_2O:acetonitrile with 0.02 % acetic acid and Solvent B is 1:1 acetonitrile:propan-2-ol. The solvents are stable and stored at 4 °C for up to two months before renewing. The injection volume was 4 µL.

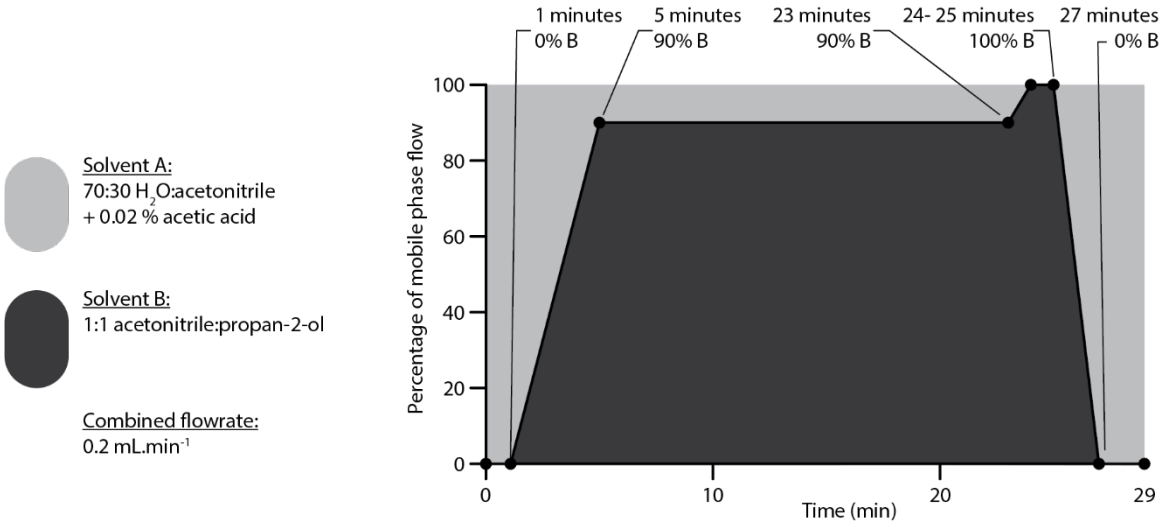


Figure 2-2: Solvent mix and flowrate profile for Kuda liquid chromatography method.

2.15.3 Mass Spectrometry

The MS method relates to the parameters applied by the MS machine to the material eluted from the column. These parameters include the ionisation of the sample, the temperature and flow of the gas after vaporisation and the voltage applied to accelerate the sample ions.

The following machine parameters were used:

- Ion mode = negative
- Collision Voltage = 10 kV
- Gas Flow = 850 $L.min^{-1}$
- Desolvation Temperature = 450 °C
- Scan time = 500 ms

For untargeted analysis of the MS data, the Progenesis software package (Waters) was used with the FAHFA database developed by the Fiehn laboratory (Ma *et al.*, 2015). For targeted analysis of specific species, the Mass Lynx 4.1 (Waters) software package was used to isolate selected masses. Elution times of the FAHFA isomers were determined from standards made up of multiple PAHSAs, PAHPAs and OAHSAs (17043, 17037, 19973, 17107, 17044, 19975, 17038- Cayman Chemicals). The heights of peaks in the samples corresponding to the elution times were normalised to the height of peaks of the internal standard $^{13}C_4$ 9-PAHSA (17725, Cayman).

2.16 Quantification of Fatty Acid Composition by Gas Chromatography

FAMEs were analysed by Gas Chromatography (GC) using an Agilent 7890 GC (Agilent, USA). The 30 m, 0.53 mm ID column uses a DB-WAX stationary phase (125-7032, Agilent) with helium as the carrier gas. Starting at 60 °C, the oven temperature ramp rate was 20 °C.min^{-1} to 200 °C, then, after a 12 min hold, 2 °C.min^{-1} 220°C, then, after a 2 min hold 1 °C.min^{-1} to 230 °C, with a final hold for 25 min. Injection volume was 5 μL. The analysis was carried out using the OpenLAB software (Agilent). Peaks for individual fatty acids were identified by comparison with the retention times of a set of external standards of known composition (RM6 (RE35027, Restek) and STDB (18919-1AMP, Merck) FAME mixes) while the quantities of each fatty acid were calculated by comparison of the area of each peak with the peak area of the internal standard.

2.17 Quantification of Stable Isotope Tracer and Tracee by Gas Chromatography- Mass Spectrometry

Total lipids were extracted from lysates of cells differentiated in the presence of heavy water using the Folch method and methylated as 2.12. Reconstituted in chloroform, the samples were analysed using Gas Chromatography-Mass Spectrometry (GC-MS) using a 6890 GC in line with a 5973 MS (Agilent, USA). The 30 m, 0.32 mm ID column uses a fused silica stationary phase (RE10224 Rtx-5, Agilent) with helium as the carrier gas. Starting at 50 °C, the oven temperature ramp rate was 25 °C.min^{-1} to 200 °C, then 10 °C.min^{-1} 230 °C, with a final hold for 10 min. The inoisation voltage was 70eV with the source at 230 °C. Injection volume was 0.2 μL. Selected ion monitoring was used to quantify the number of ions at the mass-to-charge ratios m_0, m_1 and m_2 of the fatty acid of interest.

Following the reasoning of Diraison *et al.* (1997), the fraction of fatty acids derived from DNL, F, was determined by examining the measured ratio of deuterium atoms to the total number of molecules and the expected ratio of deuterium atoms to the total number of molecules. The expected number is given by the product of the media heavy water enrichment and the maximum number of hydrogen atoms which could be incorporated in the fatty acid of interest. See Equation 1.

$$F = \frac{\left[\frac{(m_1 + m_2)}{(m_0 + m_1 + m_2)}\right]}{[\%\, enrichment \times max.deuteria]}$$

Equation 1- Fraction of fatty acid derived from DNL after culturing cells with heavy water

Following the reasoning of Cobelli *et al.* (1992), the tracer to tracee ratio (TTR) was also calculated as an indicator of DNL activity as per Equation 2.

$$TTR = \frac{(m_1 + m_2)}{m_0}$$

Equation 2- Tracer to tracee ratio

2.18 Statistics

Data were analysed in Excel and R Studio with figures prepared using the ggplot2() and ggpubr() R packages.

The normality of data sets was tested by the Shapiro-Wilks test and by visual inspection of distribution histograms and residual plots, where linear models were used. Data were transformed where this made a meaningful improvement in normality and the appropriate statistical tests chosen for the eventual normality and scedasticity.

Experimental powers were calculated using the pwr() R package with α=0.05, β=0.2 and appropriate effect sizes. Not all experiments were designed on a particular power e.g. the historical set of samples described in Chapter 4 had no opportunity to increase the N number.

The applied tests are described in the individual results chapters and include Pearson correlations, Welch's t-tests, one- and two-way ANOVAs with Tukey post-hoc tests, paired or un-paired as required.

Chapter 3

Optimisation of the Adipocyte Model

3 Optimisation of the Adipocyte Model

3.1 Introduction

All of the published work on the functional aspects of FAHFAs has been carried out in mice or with murine cell models with only observational work in humans ((Yore *et al.*, 2014) (Kellerer *et al.*, 2021) (Brezinova *et al.*, 2018) (Brezinova *et al.*, 2020)). In order to expand our understanding of the roles and metabolism of FAHFAs in man, I planned to use human adipocytes *in vitro* and it was necessary to select an appropriate cell model and ensure that the culture protocols were suitable for FAHFA research.

3.1.1 *In Vitro* Cell Models for Adipose Research

The difficulties of studying adipose metabolism *in vivo* mean that, where detailed mechanistic studies are required, *in vitro* cell models are necessary and these are routinely used for AT research.

Primary preadipocytes may be isolated from the stromal-vascular fraction of fat depots from a donor (human or animal), expanded and differentiated for experiments (Skurk and Hauner, 2012). The use of primary cells allows probing of cells from donors with different phenotypes but that same variability means that greater numbers of donors are required for statistical power. Moreover, the risk of senescence (Todorcevic *et al.*, 2017) and changes from passage to passage mean that they may become less responsive or respond differently to stimuli. The preadipocytes may also be immortalised to create a cell line which can be extensively expanded and passaged, providing an easy-to-access stock of similar cells for repeatable work. Downsides to immortalisation include that all experiments are based on the genetics of the single donor and, given the considerable genetic manipulation, there is a risk of phenotypic and hence functional changes being introduced. Furthermore, while the ability to culture cells to high passage numbers means that they are useful for a long time, multiple passaging can also introduce genetic drift, taking them further from the original phenotype.

Murine cell lines are often used in the adipose field, including the near-ubiquitous 3T3-L1, but there are obvious drawbacks including questions over transferability of conclusions from mouse to human and an inability to expose depot-specific functional differences between the abdominal and gluteo-femoral tissue that are interesting in humans and important to my research aims.

Available human preadipocyte cell lines include adipose-derived stem cells (Zuk *et al.*, 2001); (Dicker *et al.*, 2005); (Kolodziej *et al.*, 2019), primary preadipocytes (Baker *et al.*, 2017), immortalised preadipocyte cell lines (Darimont *et al.*, 2003) and Simpson-Golabi-Behmel Syndrome (SGBS) cells (Wabitsch *et al.*, 2001). Each has pros and cons but in order to study depot differences between the

abdominal and gluteofemoral subcutaneous depots, our research group has developed its own immortal cell line of preadipocytes from each depot (Todorcevic *et al.*, 2017), which retain depot-specific gene expression, the ability to accumulate lipid and to respond to catecholamines. These cells are referred to as “Patient 2” (PAT2) and were derived by virally transfecting the primary abdominal and gluteal cells of the same male donor with the human papillomavirus type 16-E7 oncoprotein gene and the human telomerase gene. During the course of my thesis work, my colleague, Dr Nellie Loh, also developed a second pair of immortalised lines from a female donor. These, the MBD5 lines, were immortalised by the same method and then further selected for adipogenic potential. The selection process involved multiple rounds of differentiating, selecting the fattest cells by buoyancy, de-differentiating them by ceiling culture and repeating. As yet, these cells remain unpublished.

For this thesis, I would investigate both the PAT2 and MBD5 cell lines for suitability for FAHFA research but it was also important that experimental results were validated in primary cells to ensure that the results remained in a wider range of phenotypes, without risk of being skewed by side effects of the immortalisation processes.

3.1.2 Protocols for Differentiating Mature Adipocytes

Whether using PAT2 or primary preadipocytes, they must be induced to differentiate into mature, lipid-laden adipocytes for study *in vitro*. The standard method of adipocyte differentiation in our research group, which takes place over the course of 10-14 days, follows common practices (Skurk and Hauner, 2012) for dosage and timing of various culture medium additives. The basis of the medium is DMEM/F12 with 17.5 mM glucose, a HEPES buffer, penicillin-streptomycin, glutamine, transferrin, pantothenate and biotin. To that is added triiodothyronine (T3, a thyroid hormone regulating the transcription of adipogenic genes), dexamethasone (a glucocorticoid which increases cAMP levels which, in turn, activates CEBPβ) and insulin (which, via the insulin receptor, increases phosphorylated protein kinase B (pAKT) which, in turn, activates PPARγ, the master regulator of differentiation). For the first four days only, this is supplemented with IBMX (which has the same effect on cAMP) and troglitazone (which directly activates PPARγ).

It would be desirable to use a culture medium which reflects the human *in vivo* environment as closely as possible, but this protocol includes elements which deviate significantly from healthy physiology. Glucose is provided as an essential energy supply but 17.5 mM (versus approximately 5 mM in blood and less, deeper in the adipose tissue) is a high concentration. We know that high concentrations of glucose affect insulin signalling in, for example, mouse adipocytes (Palacios-Ortega *et al.*, 2016) and human myoblasts (Bertacca *et al.*, 2005) and this would be detrimental. Extended incubation with insulin has also been shown to impair insulin signalling in mouse adipocytes (Nelson, Robinson and

Buse, 2002). The artificial IBMX and troglitazone are necessary to kick-start differentiation but there are examples of these being removed after 48 rather than 96 hours (Park, Ahrends and Teruel, 2012). The other factors are held at consistently high concentrations rather than oscillating as they would *in vivo*. Finally, FAs are missing altogether from the medium but these are both present and important to adipocytes *in vivo* (they are regulators of various processes including, for example, adipogenesis (Gotoh *et al.*, 2007)). A lack of FAs may artificially force DNL from other substrates, where DNL normally only accounts for, at most, 20 % of stored TAG *in vivo* (Strawford *et al.*, 2004).

3.1.3 Aims for the Model Optimisation

The parallel aims for this work were to;

1) Optimise the adipogenic differentiation media with respect to glucose concentration, hormone provision and FA provision in order to adjust the standard media towards more physiological conditions for cell culture. To gauge positive or negative effects, I would compare the adipocytes' capacity to perform functions relating to FAHFAs; DNL to produce TAG, lipolysis and insulin signalling.
2) Determine whether the PAT2 or MBD5 cells were suitable for my research and which one would be more desirable to use based on the same functional assays.

The work towards fulfilling the first aim was carried out in collaboration with Dr Marijana Todorčević.

3.2 Methods

3.2.1 General Methods

The work described in this chapter utilised the following general methods detailed in Chapter 2:

- Human Adipose Tissue Biopsy and Preadipocyte Isolation (2.1)
- Maintenance of Cells in Culture (2.2)
- Measurement of Total Intracellular TAG *In Vitro* (2.3)
- Assessment of Stimulated Lipolysis by Measurement of Non-Esterified Fatty Acids and/or Glycerol Release *In Vitro* (2.8)
- Protein Quantification Assay (2.6)
- Western Blotting (2.7)
- Gene Expression Assays by qPCR (2.8)

3.2.2 Specific Methods

Adipogenic cocktails for comparison

To examine the effects of alterations to the adipogenic cocktail in PAT2 cells, we compared three media, Conditions 1 to 3 in Figure 3-1, referred to as HG+H (17.5 mM glucose plus standard hormones), HG+FA (17.5 mM glucose plus 200 μM fatty acids) and LG+FA (5 mM glucose plus

200 μM fatty acids). HG+H represents the “standard” cocktail used by our group. The feeding pattern is shown in Figure 3-1. The mixture of FAs bound at a ratio of 7:1 to BSA was added at a final FA concentration of 200 μM. The mixture comprised 45 % oleic acid (OA), 30 % palmitic acid (PA) and 25 % linoleic acid (LA), which reflects the typical serum proportions of these, the three most abundant fatty acids in serum (Hodson, Skeaff and Fielding, 2008).

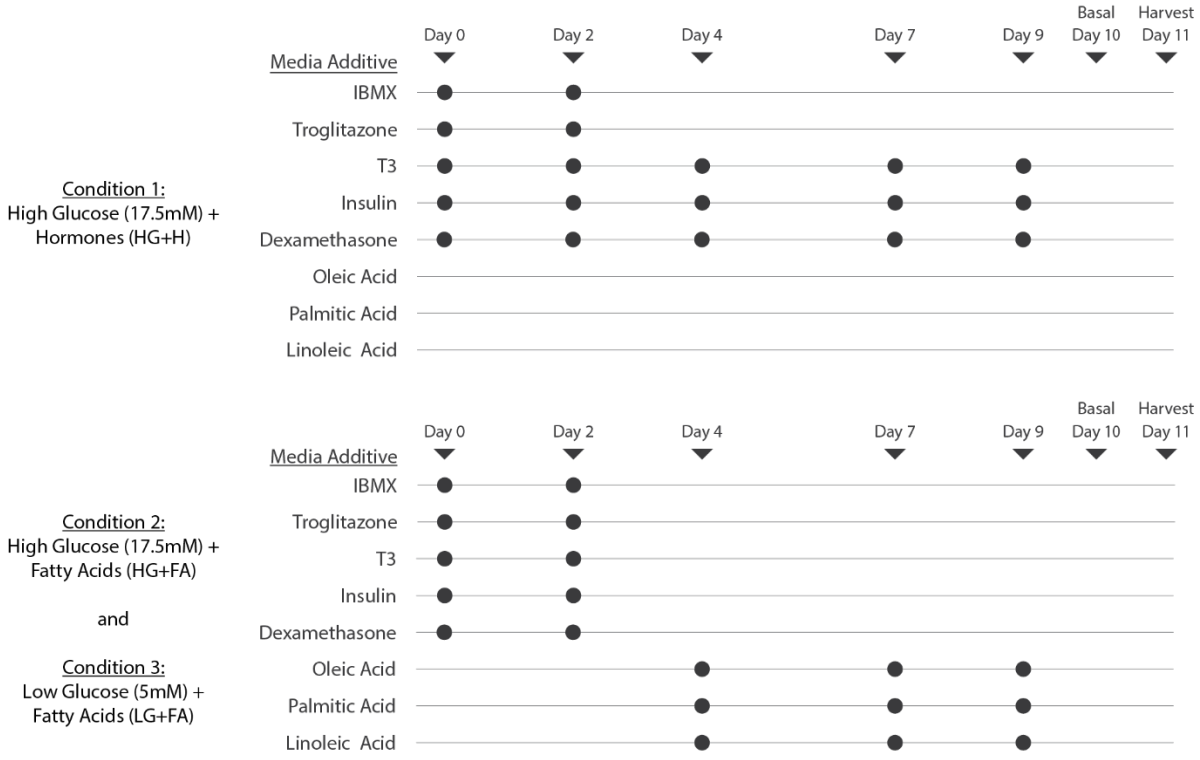


Figure 3-1: Timetable and composition of culture media to assess optimal conditions for adipocyte differentiation

mRNA Expression

The following Taqman assays were used for the mRNA expression assessment (*GENE*- product code):

- *CEBPA*- Hs0029972_m1,
- *CEBPB*- Hs00942496_s1,
- *PPARG*- Hs01115729_m1,
- *ADIPOQ*- Hs00605917_m1,
- *LEP*- Hs00174877_*m1,*
- *INSR*- Hs00961557_m1,
- *GLUT4*- Hs00168966_m1,
- *FASN*- Hs00188012_m1,
- *ELOVL6*- Hs00907564_m1,
- *SCD*- Hs00748952_m1,

- *IL6*- Hs00174131_m1,
- *DGAT2*- Hs01017541_m1,
- *PNPLA2*- Hs00386101_m1,
- *PLIN1*- Hs00160173_m1,
- *LIPE*- Hs00193510_m1,
- *18S*- Hs99999901_s1,
- *PGK1*- Hs99999906_m1,
- *PPIA*- Hs99999904_m1,
- *UBC*- Hs00824723_m1.

Western Blotting

The following antibodies were used in assessing AKT (Protein, manufacturer, product code, dilution):

- Total-AKT, Cell Signalling 2920, 1:1000,
- Phospho-AKT S-473, Cell Signalling, 4058, 1:1000,
- GLUT4, Abcam, 33780, 1:1000,
- Insulin receptor, Cell Signalling, 2025, 1:1000
- Phospho-insulin receptor, Cell Signalling, 2021, 1:800
- Alpha-tubulin, Abcam, 15246, 1:2000,
- GAPDH, Cell Signalling, 2118,1:2000.

Statistics

Welch's t-tests were used to determine differences between two conditions. Two-way ANOVAs with Tukey tests were used to identify differences between multiple variables. The alpha value was set to 0.05. Data was processed in R.

3.3 Results

3.3.1 Patient 2 Cell Line

Markers of Differentiation

The most salient endpoint marker of adipocyte differentiation is the accumulation of TAG (Skurk and Hauner, 2012). In culture, this is normally derived from the DNL pathway, critical for FAHFA synthesis, although the addition of exogenous FAs may decrease the DNL component. Figure 3-2 shows the mean accumulated TAG for each treatment in abdominal and gluteal PAT2 cells after 11 days of differentiation (n=4). There were no differences between the two cocktails containing FAs but the cells treated with high glucose and hormones accumulated significantly less TAG. This suggests that the adipocytes have more capacity to accumulate TAG than can be filled by DNL despite the abundant glucose and that the extended use of hormones is not required for TAG accumulation. The gluteal

cells accumulated significantly more TAG than the abdominal ($CI_{95\%}$ [17,185]), according to two-way ANOVA.

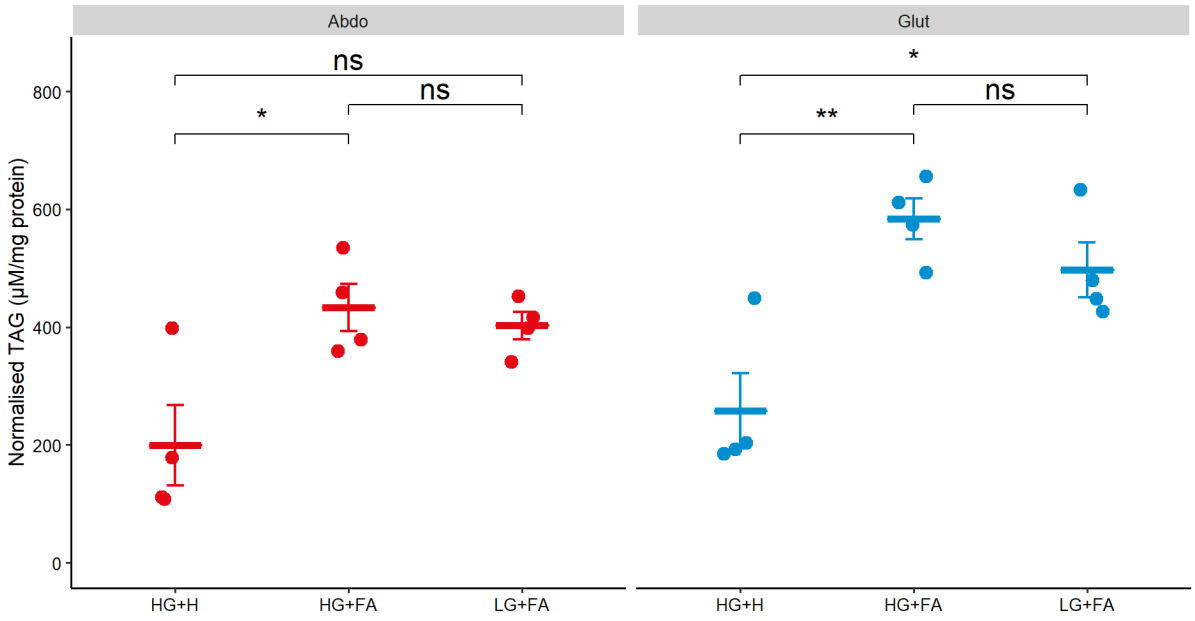


Figure 3-2: Normalised triglyceride content of Patient 2 abdominal and gluteal adipocytes after 11 days of differentiation in standard and two alternative media shows that TAG content is increased after feeding with fatty acids.

N=4. TAG, triglyceride; HG+H, High Glucose (17.5 mM) plus Hormones throughout; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids throughout. Horizontal bars are means and error bars represent SEM. * indicates p-value <0.05 and ** indicates p-value <0.01 according to Welch t-test.

To determine whether HG+FA or LG+FA cocktails had any effects on the processes of differentiation or lipogenesis which differed from the standard HG+H, mRNA was collected to assess the expression of critical adipogenic markers and transcription factors at points across the 10-day differentiation period and assessed differences using the methodology described in 2.8 and two-way ANOVAs with post-hoc Tukey tests (n=5).

Qualitatively, Figure 3-3 shows that the expression patterns are as one would expect, with *CEBPB* peaking early and *CEBPA* and *PPARG* steadily increasing. There is a tendency to dip at Day 10 and the reasons for this are not clear. As shown in Figure 3-3 and summarised in Table 3-1, significant ($p>0.05$) differences between the depots exist for the adipogenesis-regulating transcription factors *CEBPA* and *PPARG*, and the adipogenic marker hormone *LEP* where they are less expressed in abdominal cells. Considering differences between treatments, there are negligible ones; *PPARG* is lower in LG+FA than HG+H in abdominal only, while *LEP* is lower in both LG+FA and HG+FA than HG+H in gluteal cells.

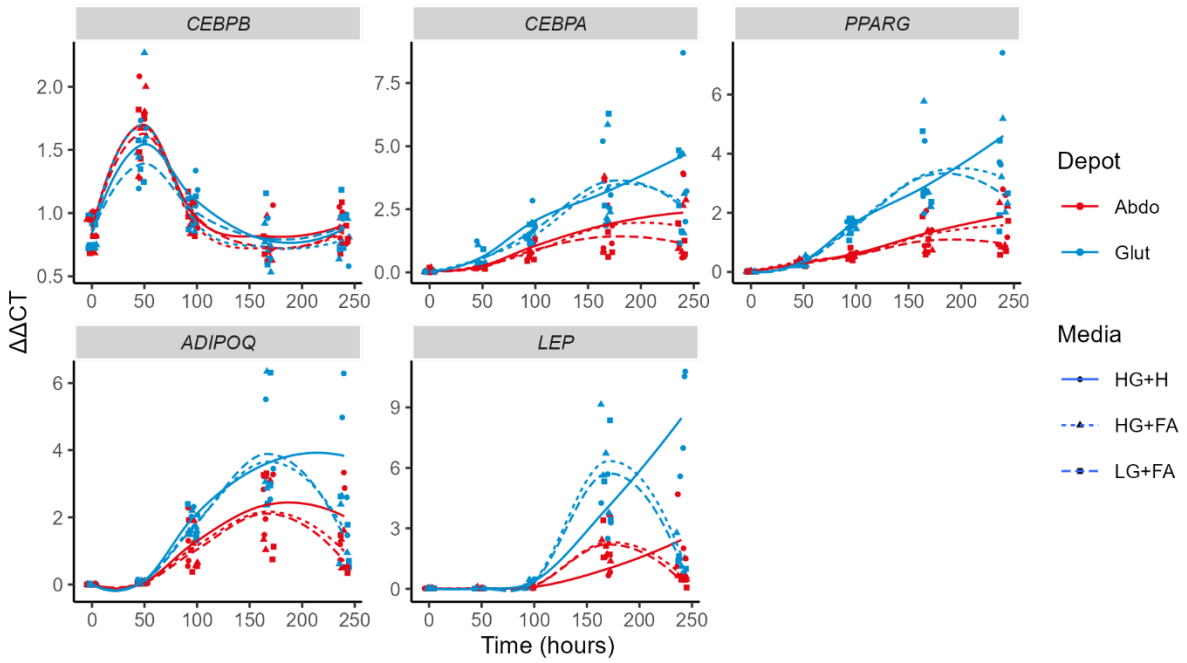


Figure 3-3: Relative expression of selected genes relating to adipocyte differentiation indicate some differences between gluteal and abdominal depots but few differences between media treatments.

HG+H, High Glucose (17.5 mM) plus Hormones throughout; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids throughout. N=5.

When considering lipogenic genes (those involved in glucose uptake and DNL), as in Figure 3-4 and Table 3-1, the two depots are similar, with only *SCD* being expressed less in abdominal cells treated with LG+FA. However, more consistent and expected differences exist between treatments where *GLUT4*, *FASN* and *ELOVL6* are less expressed in the HG+FA and LG+FA treatments while *SCD* is less expressed in the LG+FA arm only when compared to HG+H. These genes are essential to the process of DNL and to see them downregulated when FAs are supplied is logical. There are no differences in the expression of genes related to lipid handling (*DGAT2*, *PNPLA2* and *PLIN1*) despite the differences in the accumulation of TAG, suggesting that increases in size of lipid droplet don't drive upregulation of gene expression.

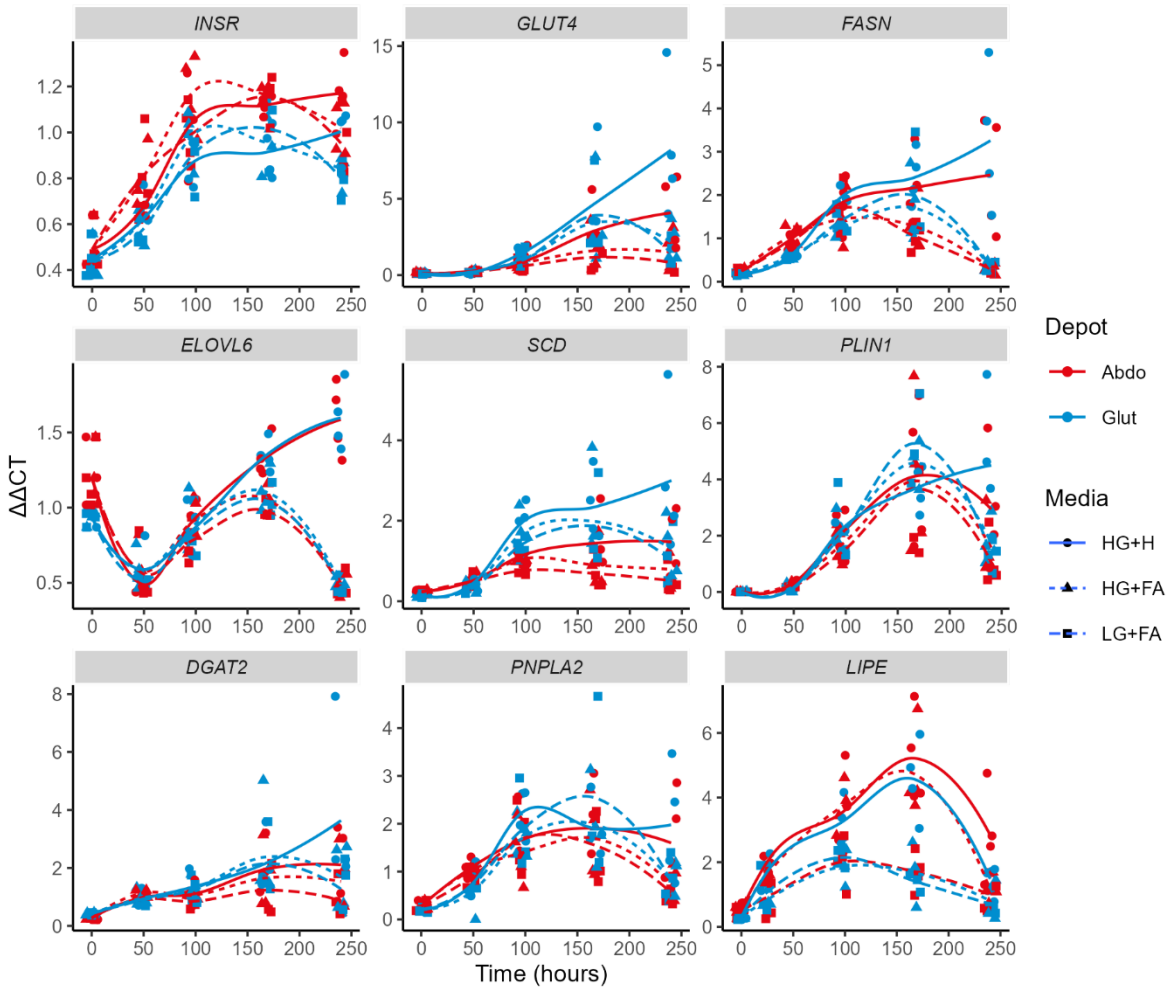


Figure 3-4: Relative expression of selected genes relating to adipocyte *de novo* lipogenesis indicate some differences between gluteal and abdominal depots but few differences between media treatments.

HG+H, High Glucose (17.5 mM) plus Hormones throughout; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids throughout. N=5.

Table 3-1: Significant differences in mRNA expression between depots and treatments in PAT2 immortalised preadipocytes from abdominal and gluteal depots differentiated for 10 days in 3 alternative media.

	Between Depot Comparison			Between Treatment Comparison					
	Abdominal v Gluteal			Abdominal			Gluteal		
Transcript	HG+H	HG+FA	LG+FA	LG+FA v HG+H	LG+FA v HG+FA	HG+FA v HG+H	LG+FA v HG+H	LG+FA v HG+FA	HG+FA v HG+H
CEPBβ	-	-	-	-	-	-	-	-	-
CEBPα	-	-	Lower p= 0.04	-	-	-	-	-	-
PPARG	Lower p= 0.04	Lower p= 0.03	Lower p= 0.01	lower p< 0.01	-	-	-	-	-
ADIPOQ	-	-	-	-	-	-	-	-	-
LEP	Lower p= 0.04	-	-	-	-	-	lower p< 0.01	-	Lower p= 0.04
INSR	-	-	-	-	-	-	-	-	-
GLUT4	-	-	-	lower p< 0.01	-	lower p= 0.02	lower p= 0.02	-	lower p= 0.03
FASN	-	-	-	lower p< 0.001	-	lower p< 0.001	lower p< 0.001	-	lower p< 0.001
ELOVL6	-	-	-	lower p< 0.001	-	lower p< 0.001	lower p< 0.001	-	lower p< 0.001
SCD	-	-	Lower p= 0.05	lower p< 0.01	-	-	lower p= 0.04	-	-
DGAT2	-	-	-	-	-	-	-	-	-
PNPLA2	-	-	-	-	-	-	-	-	-
PLIN1	-	-	-	-	-	-	-	-	-
LIPE	-	-	-	lower p< 0.001	lower p< 0.001	-	lower p< 0.001	-	lower p< 0.001

HG+H, High Glucose (17.5 mM) plus hormones throughout differentiation; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids throughout. P-values generated by ANOVA. Where no p-value is shown, no significant difference exists. N=4.

Insulin Signalling

The binding of insulin to an adipocyte's insulin receptor starts a signalling cascade which, ultimately, causes the translocation of GLUT4 and a consequent increase in glucose uptake (Saltiel and Kahn, 2001), which is essential to normal adipocyte function and, reportedly, to the synthesis of FAHFAs (Yore *et al.*, 2014). The initial action is auto-phosphorylation of the insulin receptor at Tyr1131 and an

early point on the signalling pathway is the phosphorylation of AKT at serine-473 so, to examine the integrity of insulin signalling, the lysates of sets of cells treated with either insulin or PBS for 10 minutes (see 2.6) were probed by Western Blot for differences in the ratios of pIR-Tyr1131 to total IR and pAKT-Ser473 to total AKT (n=4). Figure 3-5 and Figure 3-6 show that IR is phosphorylated to a significantly higher degree upon insulin stimulation in all groups except HG+H in both depots and HG+FA in gluteal cells, while AKT is phosphorylated in all conditions except HG+H. Moreover, the basal (vehicle-treated) levels of phosphorylation are elevated in the HG+H conditions, except with respect to pIR in abdominal cells, compared to those cells not supplied with hormones, including insulin, throughout differentiation.

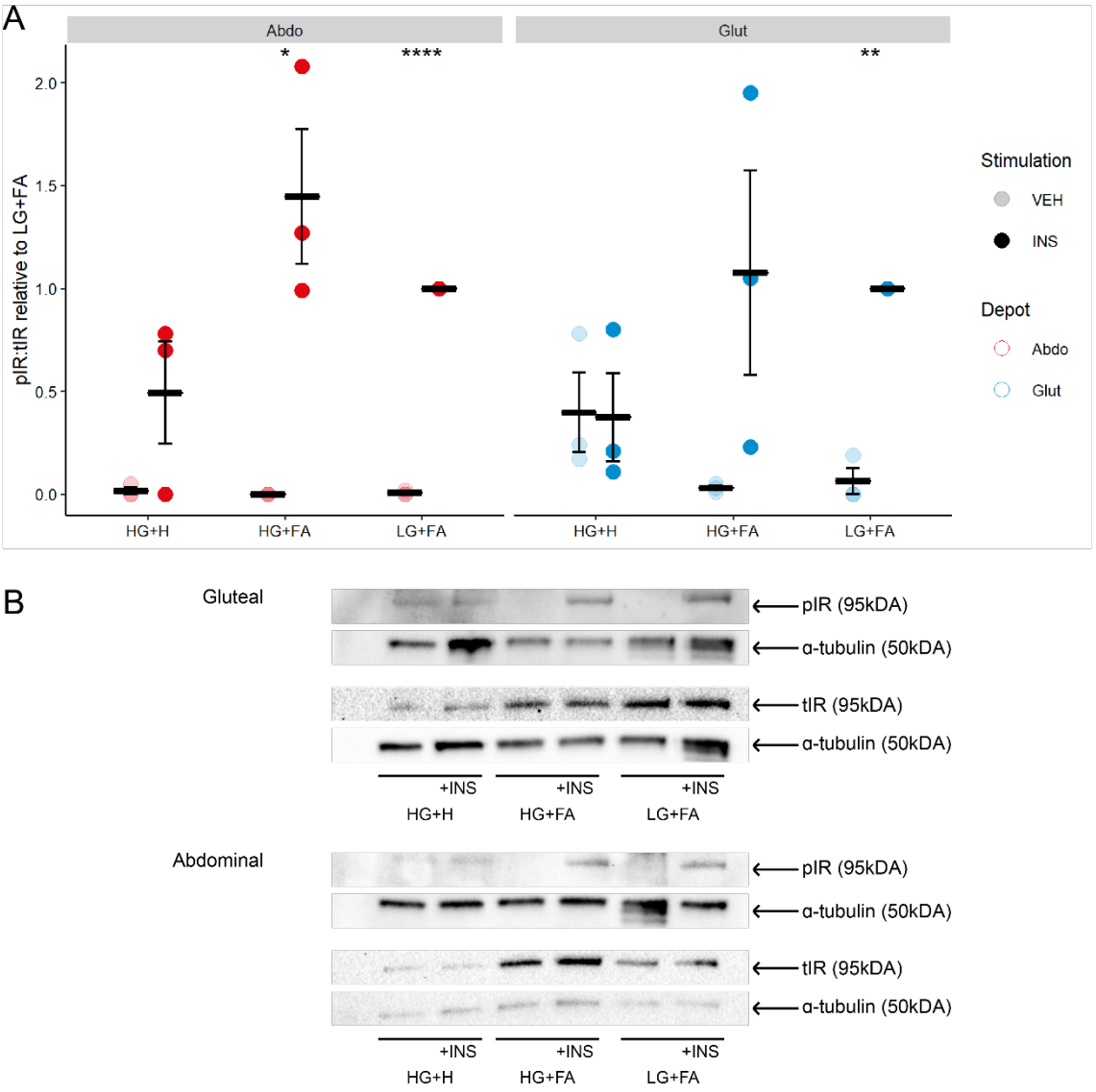


Figure 3-5: The presence of hormones throughout differentiation impairs insulin receptor phosphorylation.

A: Ratio of phospho-IR (Tyr1131) to total IR after 10 minutes incubation with either Vehicle (PBS) or Insulin (25 nM) in Patient 2 abdominal and gluteal adipocytes after 11 days of differentiation in three alternative media shows that receptor phosphorylation is blunted in high glucose with hormones. B: Representative blots showing detection of pIR, tIR and α-tubulin in abdominal and gluteal cells.

VEH, Vehicle; INS, Insulin; HG+H, High Glucose (17.5 mM) plus Hormones throughout; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids (200 µM) throughout. Error bars represent SEM. * indicates $p<0.05$ according to one-way ANOVA between basal and stimulated. N=4.

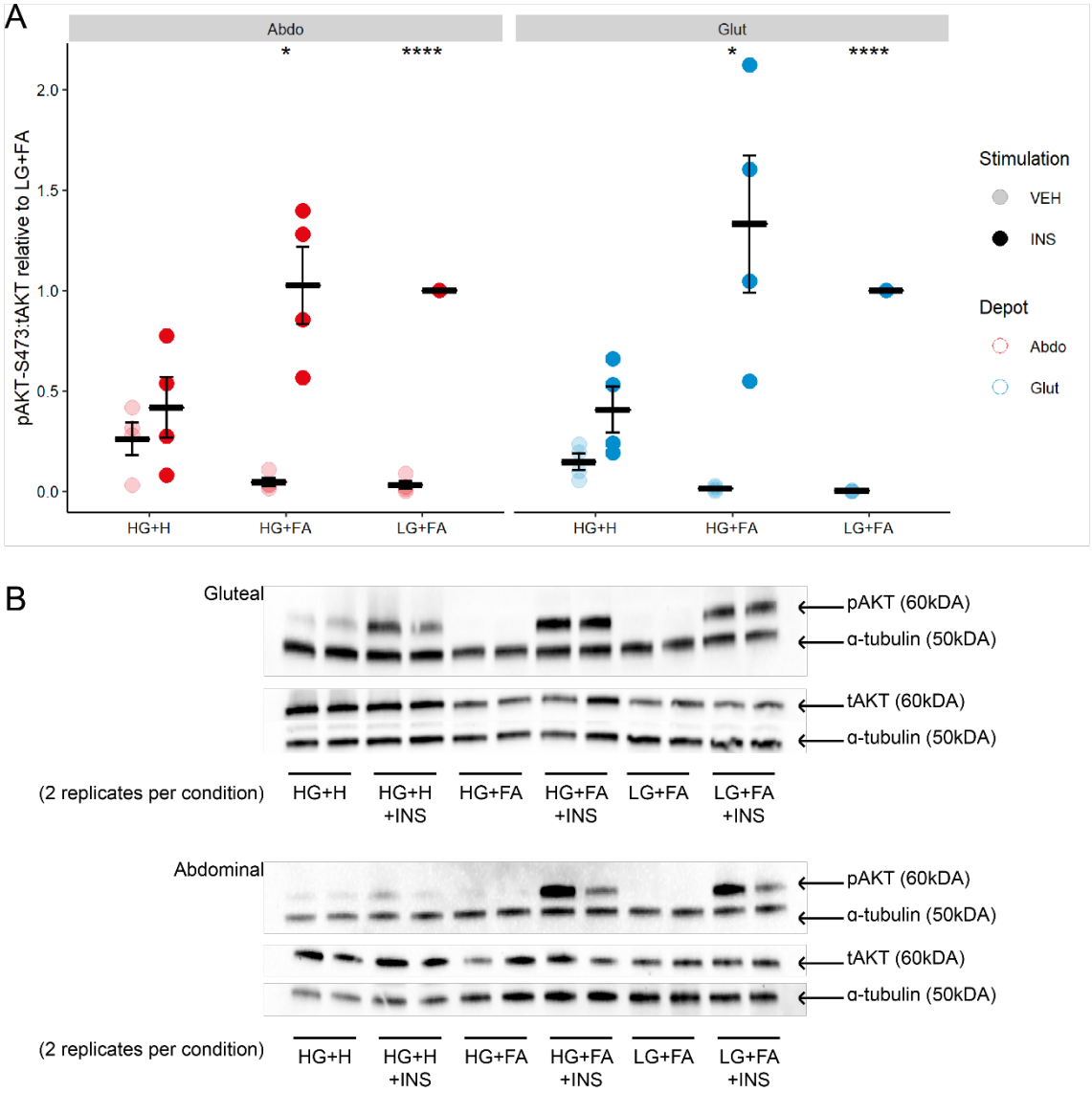


Figure 3-6: The presence of hormones throughout differentiation impairs insulin signalling.

A: Ratio of phospho-AKT (Ser437) to total AKT after 10 minutes incubation with either Vehicle (PBS) or Insulin (25 nM) in Patient 2 abdominal and gluteal adipocytes after 11 days of differentiation in three alternative media shows that insulin signalling is blunted in high glucose with hormones. B: Representative blots showing detection of pAKT, tAKT and α-tubulin in abdominal and gluteal cells.

VEH, Vehicle; INS, Insulin; HG+H, High Glucose (17.5 mM) plus Hormones throughout; HG+FA, High Glucose plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids (200 μM) throughout. Error bars represent SEM.

* indicates $p<0.05$ and **** indicates $p<0.0001$ according to one-way ANOVA between basal and stimulated. N=4.

Since the standard differentiation cocktail appears to induce insulin resistance in the PAT2 cells, I examined the same outcome in primary cells to confirm that the problem originates in the medium and not the cell line. Abdominal and gluteal primary cells were obtained from a female donor and a more limited experiment considering just the HG+H and LG+FA media and AKT phosphorylation (n=3). The biopsy, isolation and cell expansion were carried out according to 2.1.

As shown in Figure 3-7, the same pattern of elevated basal phosphorylation and blunted Ser473 phosphorylation upon stimulation was observed, indicating that the finding is not specific to the PAT2 cell line.

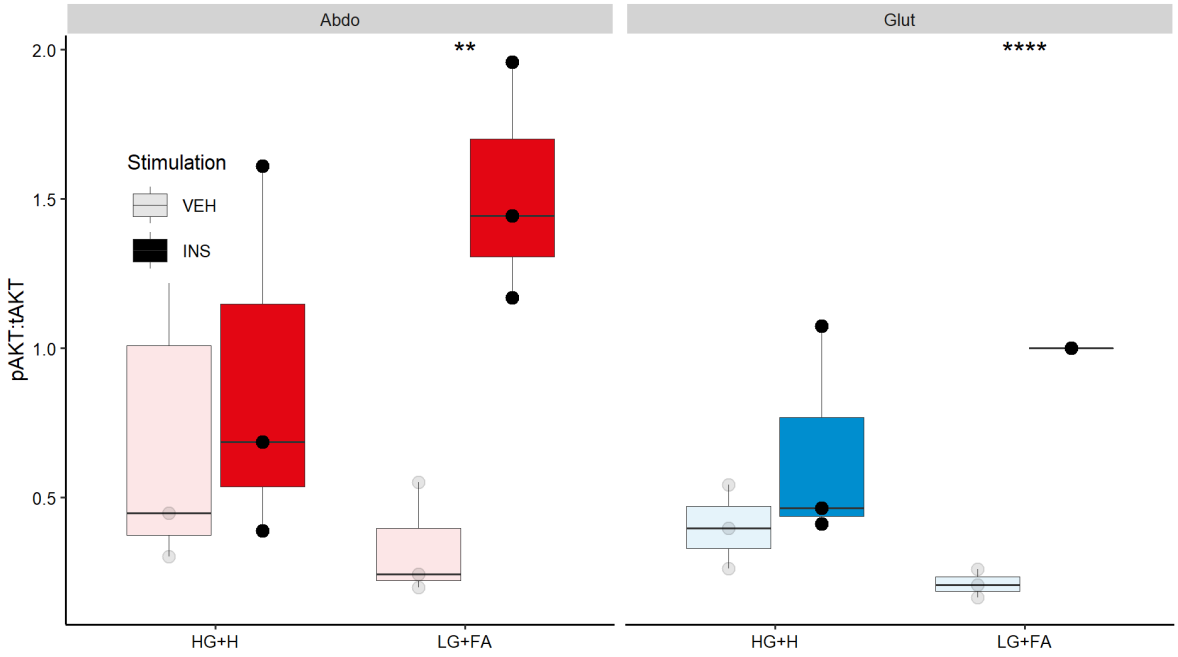


Figure 3-7: Ratio of phospho-AKT (Ser437) to total AKT after 10 minutes incubation with either Vehicle (PBS) or Insulin (25 µM) in primary abdominal and gluteal adipocytes after 11 days of differentiation in two alternative media shows that insulin signalling is blunted in high glucose with hormones.
VEH, Vehicle; INS, Insulin; HG+FA, High Glucose (17.5 mM) plus Fatty Acids throughout; LG+FA, Low Glucose (5 mM) plus Fatty Acids (200 mM) throughout. ** indicates p-value <0.01 and **** indicates p-value <0.0001 according to ANOVA. N=3.

Lipolysis

The flipside to AT storing energy after feeding is releasing it during fasting by the lipolysis of NEFA from TAG. This process is critical to getting adipose-derived FAHFAs into the circulation and has been suggested to be altered by FAHFA treatment *in vitro*. Lipolytic activity in the presence of adrenaline was measured in abdominal and gluteal PAT2 cells after 11 days of differentiation in each cocktail, following the method described in section 2.5 (n=4). In each case, the values recorded by the chemical analyser were no different before or after stimulation, and often less than zero, indicating that they were at the limit of detection. In each instance, new reagents were used in an effort to expose an experimental problem but none was identified. Other members of our research group experienced similar problems, suggesting that the PAT2 cells may not be able to perform this basic adipocyte function which would be essential to the study of FAHFAs. On this basis, I investigated lipolysis in primary adipocytes to confirm that it was a model-specific and not technical problem.

Abdominal and gluteal cells from a single female donor were assessed for lipolysis (n=3). The biopsy, isolation and cell expansion were carried out according to 2.1 and only the LG+FA media was examined.

NEFA release into the culture medium of each well was measured according to section 2.5 and normalised to the total TAG content of the well. The results shown in Figure 3-8 show that the basal levels of NEFA were virtually nil and that, after 2 h of incubation with either 10 nM adrenaline or 10 nM isoprenaline, NEFA levels were higher in both depots, albeit only significantly in the HG+H media due to an outlier set in the LG+H media. The response of the gluteal cells to adrenaline was muted, as it is expected to be, due to lesser expression of the β-adrenergic receptor (Arner *et al.*, 1990).

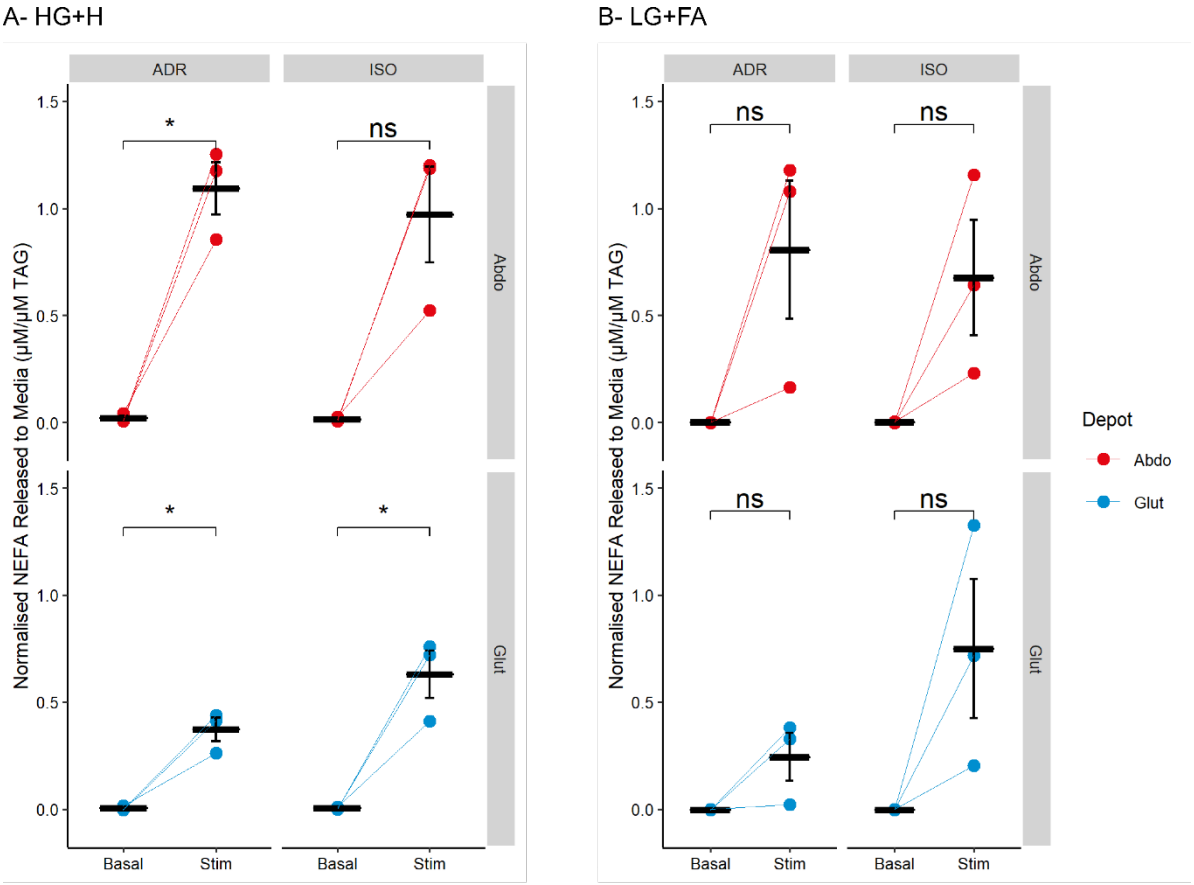


Figure 3-8: Non-esterified Fatty Acids in the media of Primary abdominal and gluteal adipocytes cultured in HG+H (Panel A) or LG+FA (Panel B) is increased after 2 hours incubation with 10 nM adrenaline or isoprenaline.

ADR, adrenaline; ISO, isoprenaline; TAG, triglyceride content. * denotes p<0.05 according to T-test. N=3.

3.3.2 The Alternative MBD5 Cell Line

Since lipolysis was a critical experiment in my plans for FAHFA research, the PAT2 cell line was not, apparently, viable for me to use due to their inability to perform stimulated lipolysis. Consequently I tested the MBD5 cells and, as part of a group effort to validate and characterise the functionality of

the cell line, I performed a series of experiments related to those which would be relevant to the study of FAHFAs.

First I made a simple check of the mRNA expression of PPAR*γ*, the master adipogenic transcription factor, and the AT hormones *ADIPOQ* and *LEPTIN* at days 4 and 10 of differentiation. As one would expect for cells which had been selected for their differentiation capacity, all three were higher at day 10 than day 4 and there were no differences between depots (data not shown).

Next, I tested the lipolytic capacity of the mature, differentiated cells, utilising isoproterenol as a reagent which similarly stimulates both adipose depots, to verify the release of NEFA and the inhibitory effect of insulin. Media NEFA increased after 90 minutes of stimulation as shown in Figure 3-9. When the change between basal and stimulated states is expressed as a fraction of that change in the control cells, it can be seen in Figure 3-10 that insulin significantly inhibits the release, as it should, and that the inhibition is similar in both depots.

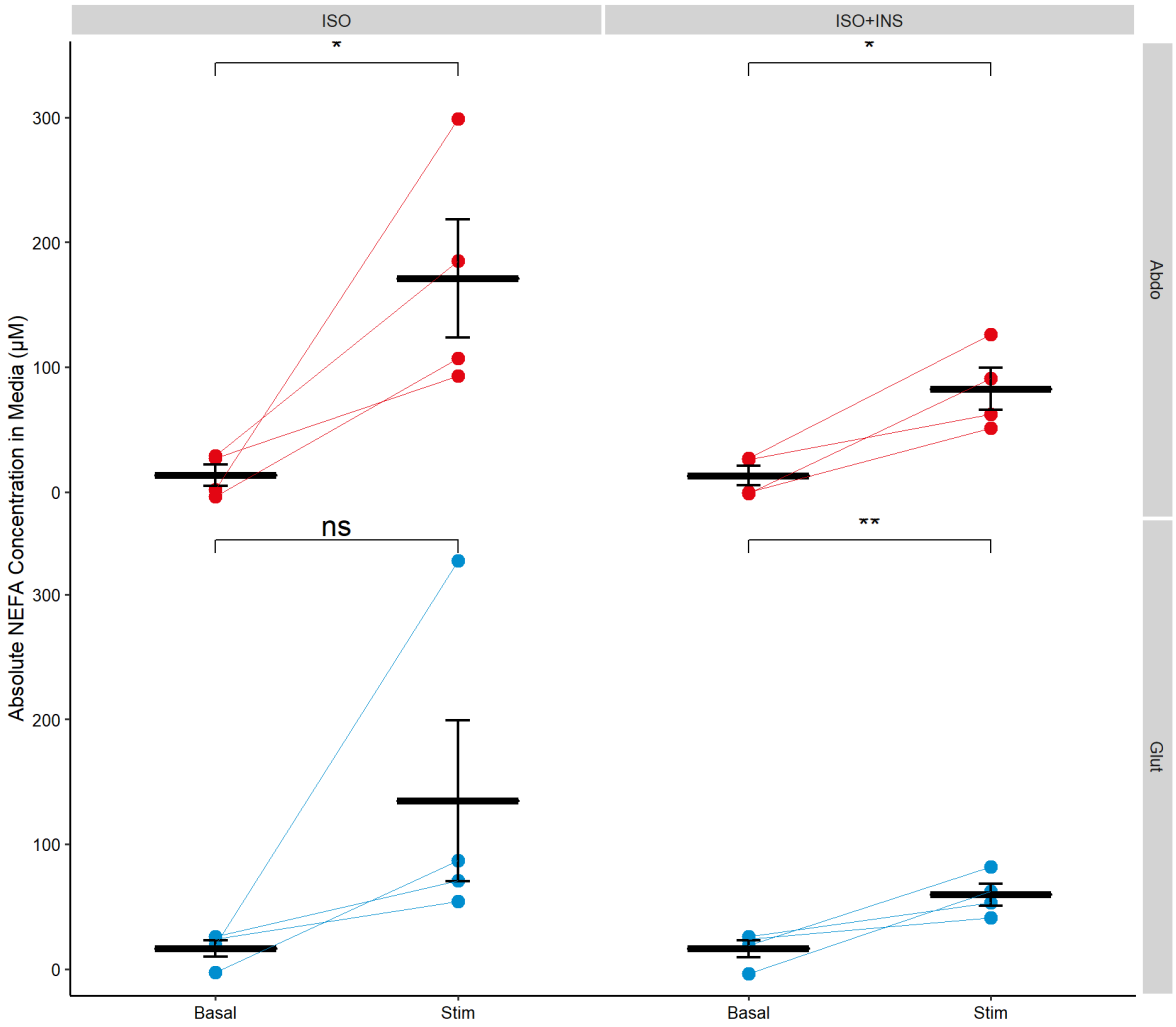


Figure 3-9: Media NEFA concentration increases upon stimulation (90 mins) of mature abdominal and gluteal MBD5 adipocytes with 10 nM isoproterenol (ISO) with and without pre-incubation (30 mins) with 120 pM insulin (INS)

Horizontal bars are means and error bars represent SEM. *** denotes p<0.05 and ** denotes p<0.01.** N=4.

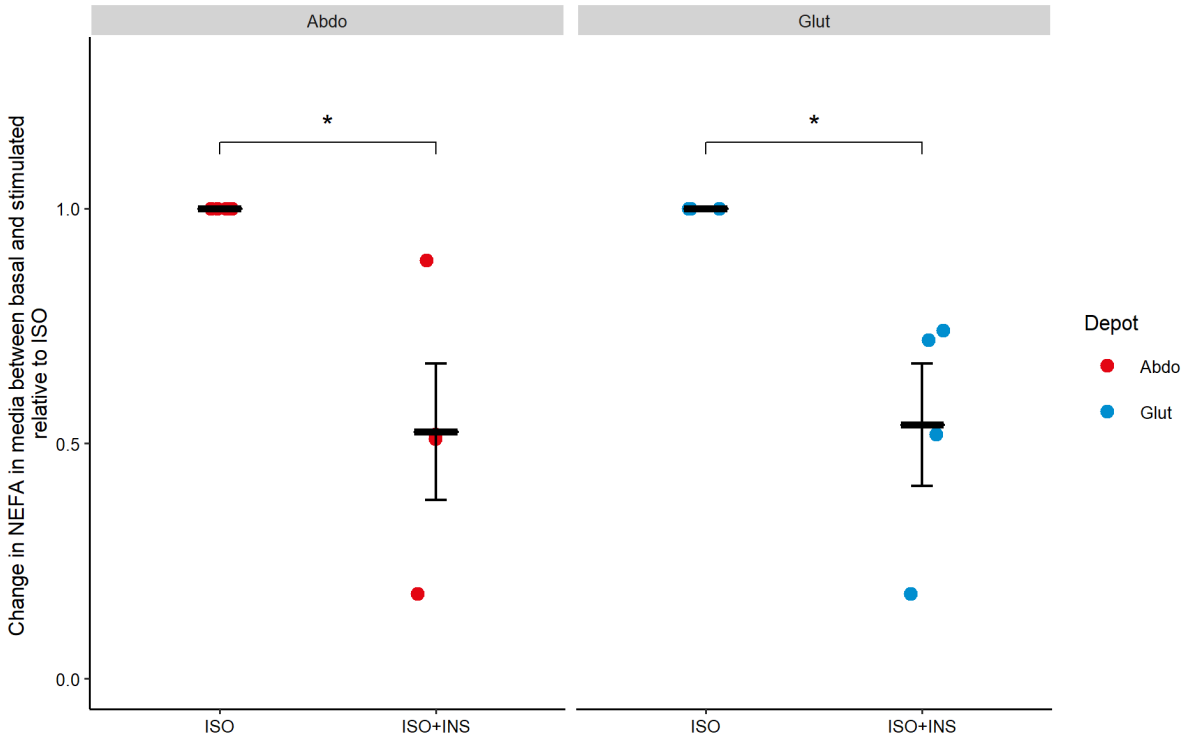


Figure 3-10: Change in media NEFA concentration upon stimulation (90 mins) with 10 nM isoproterenol (ISO) with and without pre-incubation (30 mins) with 120 pM insulin (INS) expressed relative to the ISO-only change.

Horizontal bars are means and error bars represent SEM. MBD5 cells. * indicates $p<0.05$. N=3.

3.3.3 Establishing Fatty Acid Concentrations for Culture

Having shown that the provision of FAs did not impede adipogenesis in the PAT2 cells, I used the MBD5 cells to assess adjustments to certain parameters of the FAs.

The work described up to this point utilised a fatty acid mixture used previously by our group which comprises 45 % OA, 30 % PA and 25 % LA at a final concentration of 200 µM. The DMEM/F12 contains a further 0.15 µM alpha-linolenic acid (ALA). The basis for these percentage splits is an attempt to match the circulating NEFA pool (Hodson, Skeaff and Fielding, 2008). The group has also used a slightly different mixture which reduces LA to 24 % and adds 1 % ALA and this mixture is referred to as OPLA. The addition of ALA is an attempt to ensure that the essential fatty acids which would be found in the human diet are available *in vitro* and I opted to use this mix in order to introduce uniformity across the research group.

However, I wanted to know what affect the FA concentration would have on cultured cells and I wanted to improve one aspect of the mixture that appeared sub-optimal. The FAs are complexed to BSA in solution with an FA:BSA ratio of approximately 6.6:1 but this is non-physiological, with 3:1 being reported as typical and 5:1 as being present in some disease states (Alsabeeh *et al.*, 2018). The ratio

is important and is a key factor in determining the rate of FA uptake into the cells, while the concentration of FAs may have little influence (Sorrentino *et al.*, 1989). Furthermore, the higher the ratio, the more FAs will be floating free rather than BSA-bound in the solution, potentially increasing the risk of lipotoxicity.

To examine these questions, I used the MBD5 cell line and assessed the uptake of FAs and the consequent accumulation of TAG.

Abdominal and gluteal MBD5 cells were differentiated over 10 days in either 100 or 200 μM OPLA where the OPLA was prepared at an FA:BSA ratio of either 6.6:1 or 3.3:1, with a control which lacked FAs. To measure uptake over a 48 h period the media was sampled prior to refreshing on days 6, 8 and 10 for measurement of NEFA (as 2.5) and the cells were harvested on day 10 for analysis of TAG (as 2.2).

Based on the minimal remaining NEFA in the culture medium 48 hours after each refresh, it is apparent that the MBD5 cells take up all the FAs available to them, irrespective of concentration or FA:BSA ratio (Figure 3-11). According to two-way ANOVA, all conditions resulted in a similar accumulation of TAG (Figure 3-12), including the control, which suggests that these cells will make a set amount of TAG from whatever substrate is available, utilising DNL where NEFA is not available.

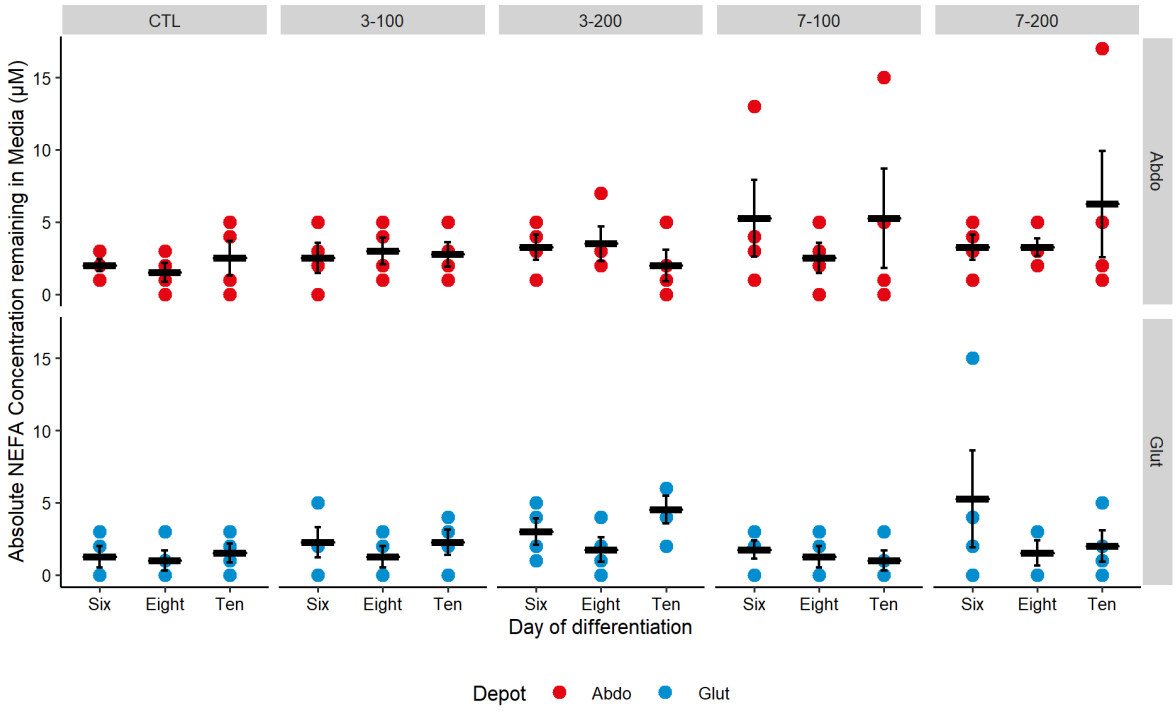


Figure 3-11: MBD5 preadipocytes take up all available NEFA within 48 hours of culture media replenishment.

Horizontal bars are means and error bars represent SEM. Y-axis shows the remaining NEFA in the media after 48 hours. There is no difference between conditions despite different starting concentrations. Media conditions- CTL: Control (glucose only), 3-100: 100 µM fatty acids at 3.3:1 ratio, 3-200: 200 µM fatty acids at 3.3:1 ratio, 7-100: 100 µM fatty acids at 6.6:1 ratio, 7-200: 200 µM fatty acids at 6.6:1 ratio. N=4.

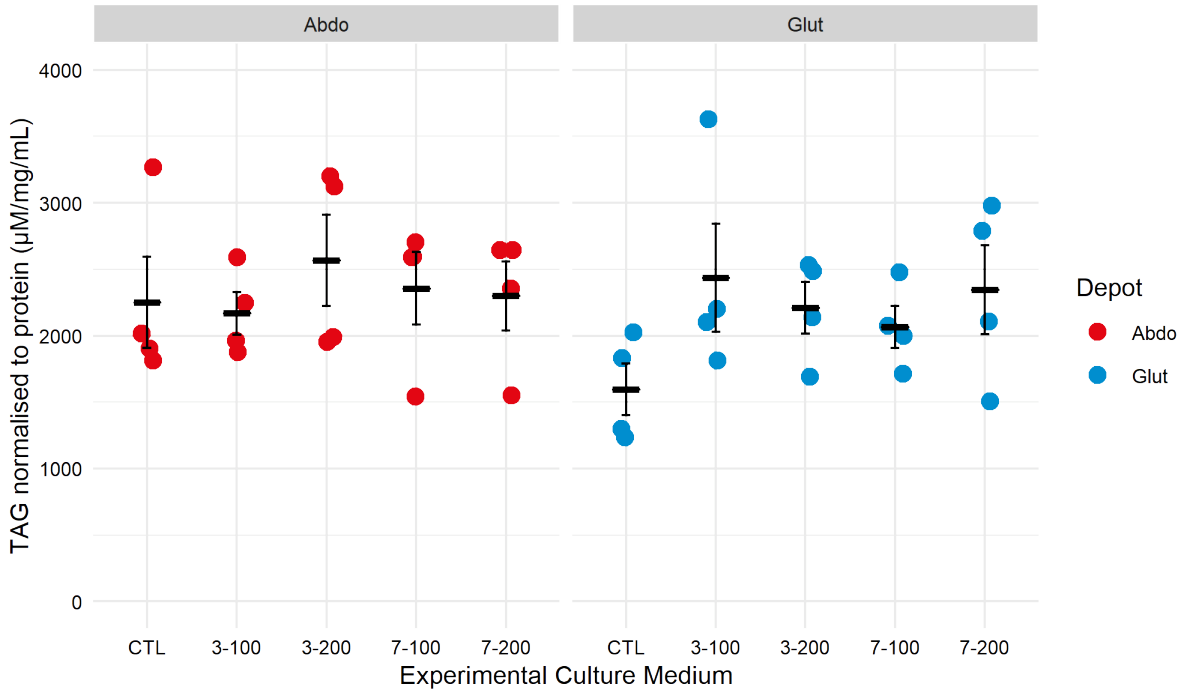


Figure 3-12: MBD5 adipocytes generate similar amounts of TAG after 10 days of differentiation irrespective of the available amount of NEFA in the culture medium.

Horizontal bars are means and error bars represent SEM. Media conditions- CTL: Control (glucose only), 3-100: 100 µM fatty acids at 3.3:1 ratio, 3-200: 200 µM fatty acids at 3.3:1 ratio, 7-100: 100 µM fatty acids at 6.6:1 ratio, 7-200: 200 µM fatty acids at 6.6:1 ratio. N=4.

Comparing Figure 3-12 with Figure 3-2, it is clear that the MBD cells accumulate a considerably greater density of TAG than the PAT2 cells and this is seen under the microscope as a greater number of differentiated cells with larger lipid droplets (Figure 3-13).

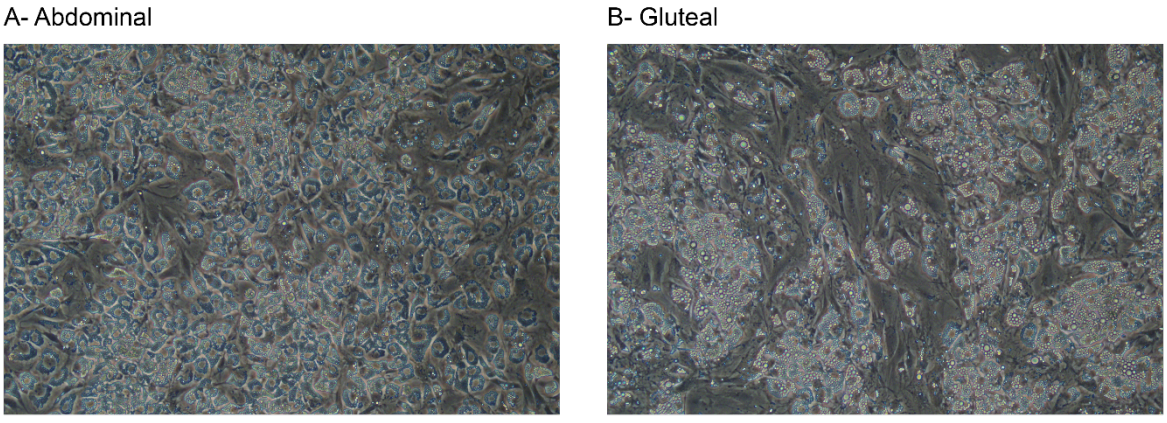


Figure 3-13: Examples of differentiated MBD5 immortalised preadipocytes, highlighting more, smaller lipid droplets in abdominal (Panel A) and fewer, larger lipid droplets in gluteal (Panel B).

Brightfield images at x100 magnification. Cells differentiated in 5 mM glucose and without exogenous fatty acids. Images taken at Day 9.

3.3.4 Understanding Glucose Uptake in the MBD5 Cell Line

Glucose uptake via GLUT4 has been implicated as critical for FAHFA synthesis and, since I had collected the media samples to measure NEFA (Figure 3-11), I also took the opportunity to measure glucose and lactate concentrations 48 h after each media change. As shown in Figure 3-14, which examines only the control cells i.e. 5 mM glucose without FAs, the abdominal cells leave less glucose from the initial 5 mM than gluteal (though the difference is not statistically-significant at Day Ten) and produce more lactate. As differentiation progresses the degree of uptake decreases.

Given that one unit of glucose could be converted to two units of lactate, the data suggest that, in the abdominal cells, around three quarters of the glucose taken up by the cells is converted to lactate by glycolysis (some small proportion may come from the 0.5 mM pyruvate in the media but that is unlikely since it is not apparent in the gluteal cells).

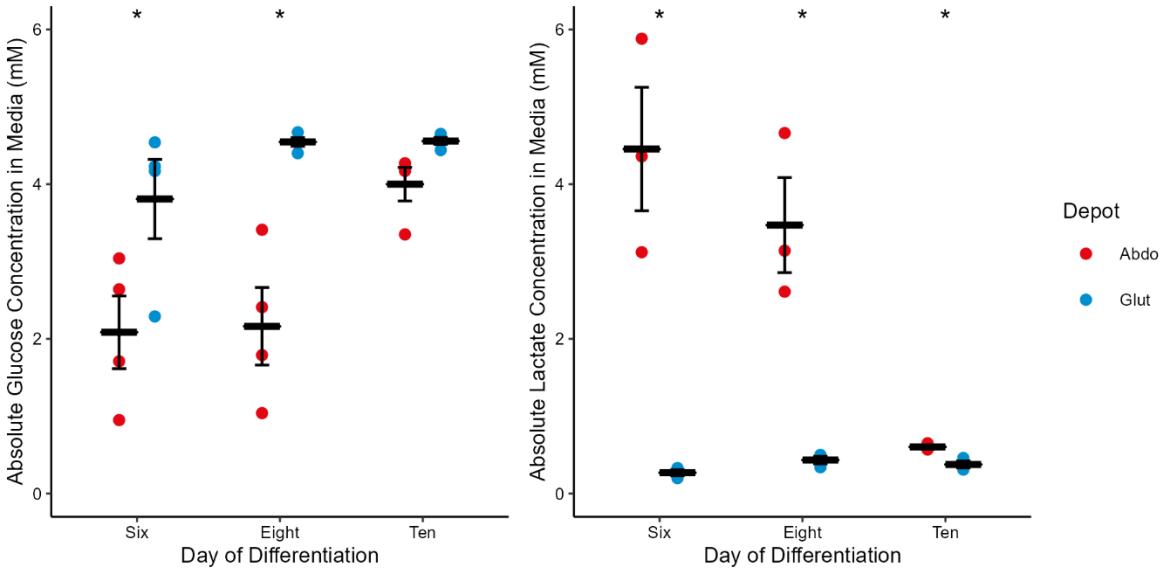


Figure 3-14: MBD5 abdominal adipocytes take up more glucose and excrete more lactate than do gluteal adipocytes in each 48 h period following a media change.

Media contained 5 mM glucose and no fatty acids. Horizontal bars are means and error bars represent SEM. * indicates $p<0.05$ according to ANOVA between depots at each day. N=4 for glucose, N=3 for lactate.

This depot-difference was not replicated in n=5 primary adipocytes (1 female, 4 males). The uptake in primary abdominal cells was marginally greater but not to a statistically-significant degree, although, since the total uptake is less than in MBD5 cells, differences may be masked and require a greater statistical power to reveal them (Figure 3-15).

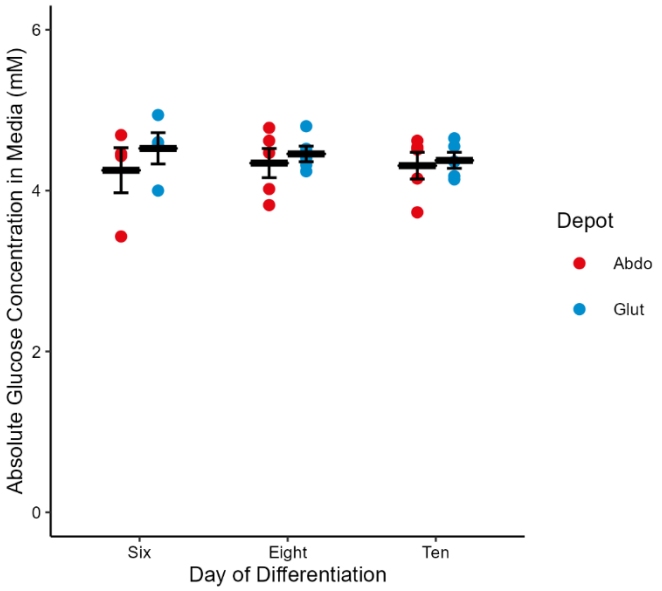


Figure 3-15: Primary abdominal and gluteal adipocytes take up similar amounts of glucose in each 48 h period following a media change

Horizontal bars are means and error bars represent SEM.. N=5 (1 female, 4 male).

This observation seems to be an oddity of the MBD5 cells which may require further investigation. From the point of view of FAHFAs, I checked that there was no difference in GLUT4 expression between the depots. To do this, I measured mRNA expression (n=5) and protein expression (n=3) in abdominal and gluteal MBD5 cells given 5 mM glucose from Days 4 to 12 of differentiation.

Although a two-way ANOVA reveals no overall difference in mRNA expression, between Days 4 and 8, the gluteal cells tend to have a higher expression, reaching statistical significance at Day 8 (Figure 3-16). Thereafter, the expression tends to be higher in abdominal cells. Effectively, the shapes of each depot's mRNA expression profile are similar but the gluteal cells reach their peak two days earlier.

To compare the two depots, a Western blot was performed using lysates from Day 10 only of both depots on the same gel (n=3). There was no difference in protein expression between the two depots as shown in Figure 3-17.

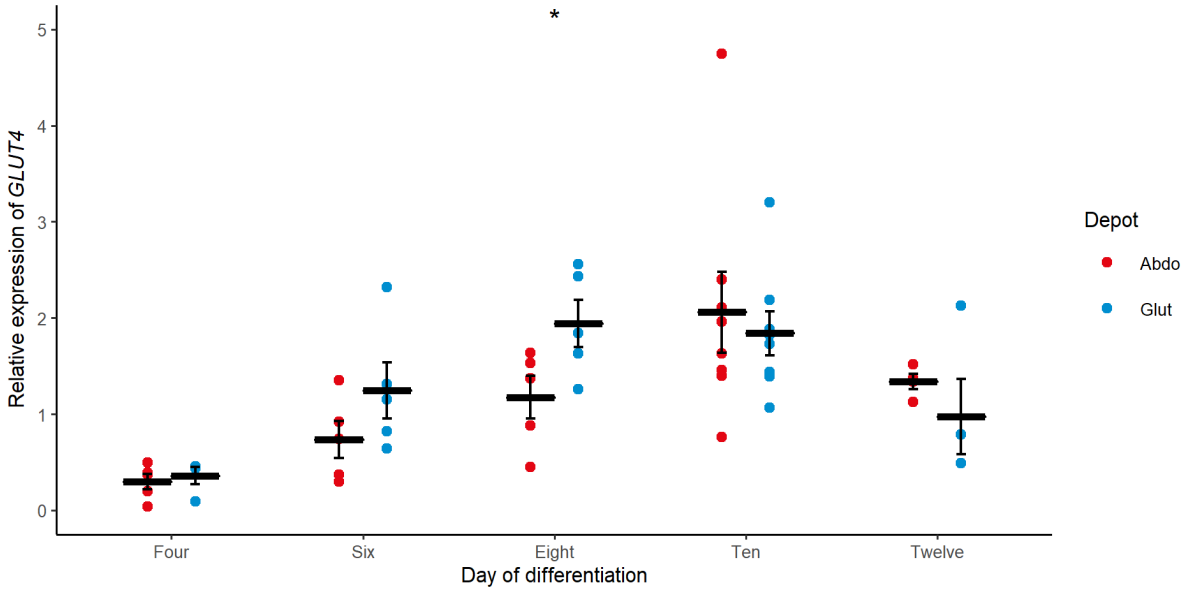


Figure 3-16: mRNA expression of *GLUT4* in abdominal and gluteal MBD5 adipocytes across the differentiation period.

N=5 for. Horizontal bars are means and error bars represent SEM. * indicates p<0.05 according to ANOVA between depots at each day.

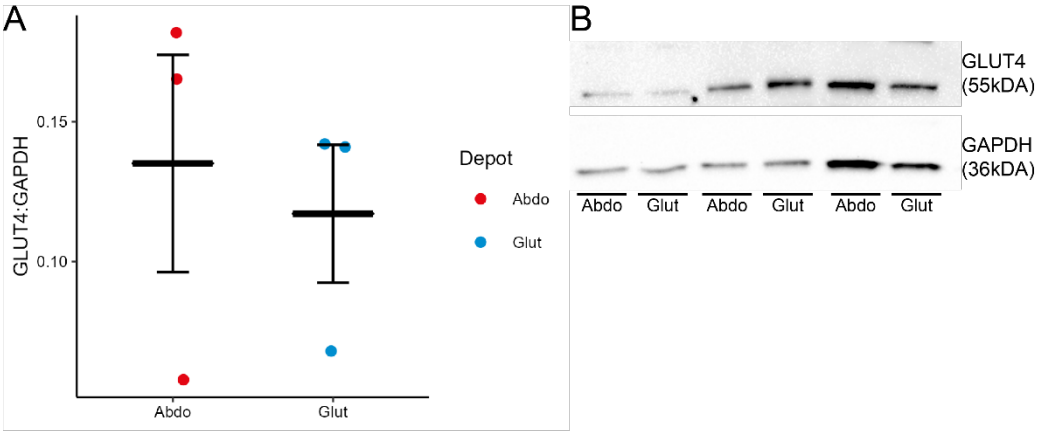


Figure 3-17: Protein expression of GLUT4 is similar in abdominal and gluteal MBD5 adipocytes at Day 10 of differentiation.

N=3. Horizontal bars are means and error bars represent SEM.

3.4 Discussion

I have examined two immortalised cell lines for use in my work to study FAHFAs and explored possible improvements to the standard protocols for adipogenic differentiation of preadipocytes. The results in this chapter confirm that there are opportunities to refine the adipogenic protocols, making them more physiological, and show that the MBD5 abdominal and gluteal cell lines are the preferred model for my work.

3.4.1 Optimising Adipocyte Culture Conditions for FAHFA *in vitro* studies

Human adipocyte cell models are important for the study of adipose biology and pathology, including FAHFA metabolism, but the variation in culture conditions, specifically the adipogenic cocktails used to differentiate preadipocytes into mature adipocytes, and the non-physiological nature of those cocktails may make the models unrepresentative of *in vivo* biology.

The aim of this work was to demonstrate that using adipogenic cocktails that were more akin to *in vivo* conditions with respect to glucose and hormone concentration were equally as successful as the common protocols in initiating differentiation and that the differentiated cells functioned as expected, i.e. with typical gene expression patterns and functioning DNL, insulin signalling and lipolysis as required for my FAHFA research.

The few observed differences in the expression of adipogenic genes in the PAT2 cells appear to be unimportant since all treatments resulted in visible adipogenesis and accumulation of TAG. We showed, therefore, that high concentrations of glucose were not necessary and that insulin, dexamethasone and T3 could be removed from the maintenance media.

Moreover, we showed that the continued presence of those hormones, as standard protocols dictate, results in the development of insulin resistance, as measured by the phosphorylation of the insulin receptor and AKT at Ser473. A blunting of AKT phosphorylation at Ser473 has been demonstrated in rat (Lu *et al.*, 2018) and mouse (Nelson, Robinson and Buse, 2002) adipocytes cultured in hyperglycaemic and hyperinsulinaemic conditions so this result is not unexpected although, to the best of my knowledge, this has not been reported in human cell models before. We did not, in the first instance, assess the phosphorylation of AKT at Thr308, nor other downstream phosphorylation events, which may have been interesting since Ser473 phosphorylation results indirectly from insulin receptor activation while activity at Thr308 is more direct. However, an undergraduate student working under my supervision did use MBD5 cells and confirmed that phosphorylation at Thr308 is also blunted by chronic insulin exposure.

We have shown that these negative effects of hormones on insulin signalling are alleviated if the hormones are removed after day 4, once their task of initiating differentiation has been completed. Consequently, I chose to use this hormone-free method for future work.

Since we did not have an experimental condition with hormones and low glucose, we could not identify any effect which high glucose might have on insulin signalling but it is worth noting the report by Nelson *et al.* (2006), whose results in murine cells indicate that this version of insulin resistance only applies if both high glucose and insulin are present. Palacios-Ortega *et al.* (2016) also reported high basal

and muted stimulated AKT phosphorylation in murine 3T3-L1 cells after 9 days of high-glucose culture. Previously to our combined work, Dr Marijana Todorčević had shown that insulin-stimulated glucose uptake was blunted after differentiation in 17.5 mM compared to 11 mM or 5 mM glucose where all media contained the standard, continuous hormones. For this reason, I chose to use 5 mM glucose for future work.

Whilst I have satisfied myself that the choice of differentiation media conditions is right for my work, there is still scope for further investigation and optimisation. Although I have used the phrase “more physiological”, that does not mean that the complete removal of T3 and insulin is actually physiological since they would circulate at low and fluctuating concentrations *in vivo*. Further work in this area could include deeper investigation into the effects of changing dosages of T3 and insulin, including the question of what is the minimum required to cause insulin resistance if that is a model which one wants to study and how far down the insulin signalling pathway are the observed negative effects propagated?

3.4.2 Characterising the MBD5 Cell Line

During the course of this work, I identified problems with the PAT2 cell line, including the poor lypolitic response. Fortunately, I was able to shift to the new MBD5 cell line which does maintain an appropriate lipolytic response, responds to insulin and behaves as we hoped in our alternative adipogenic cocktail. In fact, the MBD5 cells are particularly good at committing to differentiation, with a large proportion of the cells in the well developing fat droplets, and at generating TAG from the available substrates. This is the result of the rounds of defatting, where cells from the original, immortalised stroma-vascular fraction, which did not become fat were deselected. The MBD5 model is, therefore, less like adipose tissue than primary cells or the PAT2 cells but is an excellent model for mature white adipocytes, making them useful for me in the rest of my work.

The MBD5 cells’ ability to make TAG suggests that they will be capable of generating FAHFAs by DNL. However, the fact that glucose uptake in the gluteal cells is low in the latter stages of differentiation may prove to be a cause for concern despite the fact that they clearly perform DNL. FAHFA synthesis in mice *in vivo* was much reduced by knockout of the DNL-promoting transcription factor *ChREBP,* which suggests that the intracellular presence of glucose, which is required for *ChREBP* transcription, is critical (Yore *et al.*, 2014).

Although it is unlikely to affect the ability of glucose to activate ChREBP, of note is the observation that around three-quarters of the glucose taken up by abdominal cells was converted to lactate via glycolysis. It is known that enhanced glycolysis and reduced mitochondrial oxidation are associated

with immortalised cells (Kondoh, 2008) as they are with tumour cells, where these changes combat oxidative damage in hypoxic conditions. The MBD5 cells were immortalised with HPV-16 E7, which has been show in fibroblasts to reduce glycolysis (Mazurek *et al.*, 2001), and hTERT, which would be expected to increase glycololysis (Bagheri *et al.*, 2006). Perhaps hTERT exerts a greater effect in the MBD5 cells or perhaps pyruvate dehydrogenase activity is reduced. Future work to further characterise this aspect of the MBD5 cells could include examining how the flow of carbons from pyruvate to lactate rather than to acetyl-CoA and DNL might differ between primary and immortalised cells and whether this affects the relative contributions of other carbon sources. Our own lab has shown that media glucose (42 %), pyruvate (12 %) and glutamine (10 %) account for a total of around 65 % of the carbons in DNL-derived palmitate in primary cells (Collins *et al.*, 2011). Others have shown that branched chain amino acids (BCAAs) could contribute around 30 % when the supraphysiological concentrations of amino acids found in DMEM/F12 are used (Green *et al.*, 2016), which could be important given that the BCAA metabolic proteome differs between abdominal and gluteal depots (Raajendiran *et al.*, 2021). This work could be carried out by stable-isotope labelling of the carbon sources and mass spectrometry of the resulting lipids at the end of differentiation.

Given the proposed mode of action for FAHFAs, where glucose uptake promotes FAHFA synthesis which, in turn, improves glucose uptake, I have investigated this area because depot differences here might explain any emergent depot differences in studies examining FAHFAs. However, despite there being some uncertainty over the fate of glucose, it clearly does not affect DNL overall, so is unlikely to affect FAHFAs in my further work.

3.4.3 Fine-tuning the Provision of Fatty Acids

Having demonstrated that the addition of FAs with a composition based in physiology did not harm the cells or disrupt adipogenesis, I used both primary preadipocytes and the MBD5 model to investigate whether certain parameters of the delivery of the FAs were critical. I showed that the concentration of FAs in the media had no effect on TAG accumulation and that a physiological FA:BSA ratio of 3:1 was equally successful in allowing the cells to take up what they needed. For my further work I planned to use the OPLA mix of fatty acids at 100 μM and at a FA:BSA ratio of 3:1, except in particular circumstances. In experiments where one would want to specifically push FA-induced signalling, one might want to increase the concentration to ensure that the signalling is active and sustained for a period. Equally, in experiments where one wanted to avoid incidental signalling or other FA effect, one would choose to forego the FAs.

3.4.4 Summary

During the course of this work, I have shown convincingly that the traditional protocols for adipocyte differentiation can be modified to a cocktail which more closely resembles the *in vivo* state and that this is beneficial rather than just “no worse”. However, there is still space to develop the protocol to make it more physiological, most notably with respect to the provision of insulin.

The MBD5 cell line promises to be a good workhorse for the rest of my work and the characterisation carried out to date has been useful in highlighting differences between cells from the two adipose depots and also between MBD5 and primary cells. Further work can be done in this area, specifically with respect to any process, gene or protein that this cell line might be used to investigate since assumptions from other cell lines or primary cells may not hold. This is particularly true with respect to sex since these cells are derived from a female donor.

Chapter 4
Characterisation of FAHFAs in Humans

4 Characterisation of FAHFAs in Humans

4.1 Introduction

4.1.1 Background

FAHFAs may be important signalling molecules for good metabolic health but our understanding of their partitioning in our bodies and alterations to that partitioning in disease is limited. As noted in Chapter 1, FAHFAs have been reported in human WAT and kidney (Yore *et al.*, 2014) but it is not known if there are differences in the quantity or pattern of FAHFA species between different WAT depots or if they vary with metabolic health. They have also been reported in human breastmilk (Brezinova *et al.*, 2018) with decreased levels in obese mothers.

Yore *et al.* (2014) suggested that there is a positive correlation between all PAHSAs in plasma and insulin sensitivity but did not adjust for BMI or body fat, potential confounders given that their insulin-insensitive group was overweight and the insulin sensitive group was lean. Bascon *et al.* (2016), on the other hand, did not find correlations for PAHSAs and fasting glycaemia but did find positive correlations between both total POHPA and total PAHPA and fasting glycaemia while also, tellingly, finding a negative correlation between PAHPA and obesity.

Kellerer *et al.* (2021) undertook a cross-sectional study where they measured seven FAHFAs (9-, 10-, 12/13-PAHSA, 9-OAHSA, 9-SAHSA, 9-PAHPA) in the plasma of large groups and considered correlations with age, BMI, sex, dietary habit and diabetes status. In one healthy study group (n=60), they found most FAHFAs to be elevated in adults compared to adolescents and in leaner compared to fatter adults but no differences between sexes. In a second study group (n=36), lean controls had higher levels of all measured FAHFAs than morbidly obese subjects but, six months after bariatric surgery, those morbidly obese subjects only saw an increase in 9-OAHSA while other species remained low. Vegetarians were found to have lower levels of all but two FAHFAs compared to omnivores and, in a group of young men fed an excess of 1000 kcal in the form of whipping cream for one week, circulating FAHFAs increased. Finally, 8 obese, diabetic adults were compared with 20 obese controls and no differences in circulating FAHFAs were found. The data is quite convincing, with the take-home message that diet, weight and age are related to circulating FAHFAs but insulin sensitivity is not. However, the study is limited by the small number of FAHFAs measured, by the small number of parameters examined and by the lack of comparison of FAHFAs stored in tissue.

Given the relative paucity of corroborated human data and the lack of detailed analysis of FAHFAs relative to phenotype and confounding variables, I aimed to use a larger group of subjects to bring greater clarity to the picture of FAHFAs in human plasma and AT. I would investigate correlations

between anthropometric measurements (such as fat mass or android:gynoid fat ratio) and functional measurements (such as insulin resistance) to both circulating and WAT-stored FAHFAs. In addition to adding weight to one or other side of the question over links to insulin sensitivity, this would reveal influences of or on adipose depot differences.

To carry out my study, I utilised two sources of subjects. The first was a collation of existing plasma samples from around 200 participants in previous studies for whom we already held anthropometric and fasting plasma biochemical data. The second source was the recruitment of 40 subjects for the same panel of measurements but with the addition of abdominal and gluteal WAT biopsies to examine the pattern of stored FAHFAs.

As noted in 1.3, FAHFAs, if they are insulin-sensitising lipokines, are not the only lipids to have been claimed as such. Like FAHFAs, palmitoleate is found in the body at a greater proportion than in food and has been reported as a lipokine (Cao *et al.*, 2008). There is still not agreement in the literature that palmitoleate synthesis or release is regulated for the purpose of achieving this insulin-sensitising effect. The collated data in my study includes plasma FA composition and so this presents an opportunity to identify any correlation between either FAHFAs or palmitoleate and insulin sensitivity and to see if it is the same for both lipids.

4.1.2 Aims

My aims were to gain a better understanding of the distribution of FAHFAs in the human body and on their links to anthropometric and phenotypic measures and markers of metabolic health. Specifically, I would:

1. Measure FAHFAs in plasma and look for correlations between them and anthropometric measures and phenotypic traits.
2. Measure FAHFAs in the abdominal and gluteal adipose tissues and compare differences between depots and the plasma while looking for correlations with anthropometric measures and phenotypic traits.
3. Identify similarities with palmitoleate and determine whether or not either FAHFAs or palmitoleate are markers or predictors of insulin resistance.

4.2 Methods

4.2.1 General Methods

The work described in this chapter utilised the following general methods detailed in Chapter 2:

- Human Adipose Tissue Biopsy and Preadipocyte Isolation (2.1)
- Extraction of FAHFAs from Plasma or Lysates for Liquid Chromatography (2.12)

- Extraction of FAHFAs from Adipose Tissue for Liquid Chromatography (2.13)
- Extraction of Lipids from Plasma or Lysates for Gas Chromatography (2.14)
- Liquid Chromatography and Mass Spectrometry for Quantification of FAHFAs (2.15)
- Quantification of Fatty Acid Composition by Gas Chromatography (2.16)

4.2.2 Specific Methods

Participants

The large cohort for the plasma study included samples from seven previously published studies. All participants were non-diabetic, free from other known disease, had a BMI < 40 $kg.m^{-2}$ and were not taking any medication known to influence glucose metabolism. All gave informed consent to take part.

The smaller cohort of 39 people recruited from the Oxford Biobank for the adipose tissue study were all, free from known disease, had a BMI < 35 $kg.m^{-2}$ and were not taking any medication known to influence glucose metabolism. All gave informed consent to take part.

FAHFA Extractions and LC-MS

FAHFAs were extracted from the plasma of the large cohort and analysed by LC-MS by Martin Riečan of the Czech Academy of Sciences. Peak heights normalised to internal standard were provided to me for analysis. In this cohort, the OAHSA, PAHSA and PAHPA sub-families were measured.

FAHFAs were extracted from the plasma and adipose tissue of the small cohort and analysed by LC-MS by me. In this cohort, the OAHSA, PAHSA and PAHPA sub-families were measured.

Limitations to both these processes meant that the number of FAHFA families was restricted to those noted above rather than the broader range intended. In both cases this was due to poor sensitivity in the LC-MS machines meaning that the protocols had to be optimised to separate individual isomers of a few families, with those chosen being amongst the most abundant in human tissue.

LC-MS data processing

Peak heights for the internal standard and FAHFA species of interest were measured using the Mass Lynx 4.1 software (Waters). Samples with very low internal standard peaks, were filtered out of the analysis. The peak heights of FAHFAs were normalised to the internal standards and the resulting figure used for all further analysis.

Statistics

Welch's t-tests and one-way ANOVAs with Tukey post-hoc tests were used to determine differences between groups. Pearson correlation coefficients were calculated using the R cor() function. Since the FAHFA and palmitoleate plasma concentrations are right-skewed, transformations were explored but,

since transformations did not make the data completely normal and since the Pearson correlation is robust against non-normality (Edgell and Noon, 1984), they were left untransformed. The alpha value was set to 0.05. In the correlation studies, the Benjamini-Hochberg method was used to identify statistically-significant correlations amongst the multiple tests. A false discovery rate of 20 % was applied. Data was processed in Excel and R. Figures were generated using the ggplot2() R package.

4.3 Results

4.3.1 Cross-sectional Study of Plasma FAHFAs

A summary of the participant characteristics is shown in Table 4-1.

Table 4-1: Participant characteristics for FAHFA plasma study

Parameter	Female	Male	Difference
N	109	66	
Age (y)	47.6 ± 0.6 [26 – 63]	44.9 ± 0.7 [26 – 55]	p= 0.01
Body Mass (kg)	72.3 ± 1.1 [49 – 108]	91.5 ± 1.7 (66 - 131)	p< 0.001
BMI ($kg.m^{-2}$)	26.5 ± 0.4 [19.8 – 40]	28.3 ± 0.5 [20 – 41.3]	p< 0.01
Fat Mass (kg)	27.1 ± 0.9 [10.7 – 51.9]	26.6 ± 0.9 [8.2 – 57.5]	ns
Relative fat mass (%)	36.5 ± 0.6 [19 – 53]	28.1 ± 0.6 [11 – 45]	p< 0.001
Waist (cm)	85 ± 1.1 [69 – 112]	97.3 ± 1.3 [74 – 131]	p< 0.001
Waist:Hip	0.84 ± 0.01 [0.73 – 1.03]	0.96 ± 0.01 [0.78 – 1.16]	p< 0.001
Android:gynoid	0.39 ± 0.01 [0.12 – 0.80]	0.68 ± 0.02 [0.26 – 1.02]	p< 0.001
Plasma NEFA (μM)	711 ±36 [132 – 2389]	592 ± 38 [144 – 1603]	p= 0.03
Plasma Total Triglycerides (mM)	0.9 ±0.05 [0.29 – 4.5]	1.2 ±0.1 [0.33 – 4.4]	p=0.007
Plasma insulin ($mU.L^{-1}$)	11.97 ±0.68 [0.8– 41.3]	12.64 ±0.94 [3.9 – 38.8]	ns
Plasma Glucose (mM)	5.11 ±0.68 [3.4– 11.2]	5.26 ±0.94 [3.5 – 6.6]	ns

Data presented as mean ± SEM [range]. p-values result from Welch's t-test between sexes. Fat mess, relative fat mass and android:gynoid are derived from DEXA scans, which were completed for 86 females and 62 males.

PAHSAs and PAHPAs Circulate at Different Concentrations in Men and Women and are not regulated as NEFA

The concentrations of PAHSA and PAHPA in fasting plasma samples are summarised in Table 4-2. It is evident that concentrations of these two FAHFA families are significantly higher in men than in women, which is opposite to the observed pattern of NEFA being higher in women (Table 4-1).

Looking more closely at NEFA, we see that, in males, plasma total NEFA concentration was weakly but significantly negatively correlated (Pearson coefficients -0.24 to -0.3, $p<0.05$) with 9-PAHSA, 11-PAHSA, 12/13-PAHSA, 9-PAHPA and 12/13-PAHPA. Similar but non-significant relationships exist with 10-PAHSA and 10-PAHPA in males. The only significant correlations with individual FAs in the NEFA pool were 11-PAHPA with palmitate ($R= -0.29$, $p<0.05$) and 9-PAHSA with linoleate ($R= -0.31$, $p<0.05$). When expressing the FA composition as percentages of total NEFA, the percentage stearate in males was positively correlated with 9- and 10-PAHSA ($R= 0.31$ and 0.30 respectively, $p<0.05$), while the percentage oleate was negatively correlated with 10-PAHSA ($R= -0.28$, $p<0.05$). In females, no such relationships existed between FAs and FAHFAs but, expressed as a percentage of total NEFA, palmitate had weak, positive correlations with 9-, 10-, 11-PAHSA and 9-PAHPA ($R= 0.23$, 0.26. 0.25 and 0.26 respectively, $p<0.05$), while the percentage stearate had a weak, negative correlation ($R= -0.29$, $p<0.05$) with 12/13-PAHSA.

Table 4-2: Plasma concentrations of PAHSA- and PAHPA-NEFAs differ between men and women.

FAHFA	Female	Male	Difference
9-PAHSA	0.34 ± 0.02	0.40 ± 0.02	p= 0.03
10-PAHSA	0.36 ± 0.02	0.40 ± 0.02	ns
11-PAHSA	0.21 ± 0.02	0.25 ± 0.02	ns
12/13-PAHSA	0.25 ± 0.03	0.33 ± 0.02	p= 0.04
Total-PAHSA	1.16 ± 0.06	1.38 ± 0.09	p= 0.04
9-PAHPA	0.26 ± 0.01	0.30 ± 0.01	ns
10-PAHPA	0.17 ± 0.01	0.20 ± 0.01	p= 0.03
12/13-PAHPA	0.31 ± 0.02	0.42 ± 0.04	p= 0.01
Total-PAHPA	0.75 ± 0.04	0.92 ± 0.06	p= 0.01

Data presented as mean ± SEM. Relative concentrations measured by LC-MS and normalised to internal standard. N= 109 female, 67 male. p-values result from Welch t-tests between sexes.

Plasma FAHFAs correlate with some measures of body fatness in women but not men

Table 4-3 details the uncorrected Pearson correlation coefficients for the concentrations of FAHFAs in plasma with various anthropometric measures in the female cohort. There were several weak,

positive and significant correlations between both 12/13-PAHSA and 12/13-PAHPA and measures of body fatness including body mass, absolute fat mass, BMI and hip circumference but there was no association with android:gynoid ratio or the absolute and relative depot fat masses. BMI was positively related to 9-PAHSA. In contrast, there were no significant correlations in males. Table 4-4 shows the Pearson coefficients for both sexes combined. Most of the associations become weaker than when considering the females separately and the hip relationship vanishes. However, some become stronger and the android percentage fat mass becomes significant for 10-PAHPA.

Table 4-3: Correlation matrix between plasma FAHFAs and anthropometric measures in females.

	9-PAHSA	10-PAHSA	11-PAHSA	12/13-PAHSA	9-PAHPA	10-PAHPA	12/13-PAHPA
Age	0.10	0.09	0.09	0.07	0.12	0.16	0.08
Height	-0.01	0.03	0.05	0.08	0.00	0.02	0.06
Mass	0.14	0.05	0.07	**0.33** ***	0.09	0.14	**0.31** **
BMI	**0.26** ***	0.13	0.11	**0.40** ***	0.15	0.18	**0.38** ***
Waist	-0.05	-0.12	-0.09	0.20	-0.09	-0.03	0.18
Hip	0.02	-0.06	-0.03	**0.30** **	-0.01	0.07	**0.30** **
Waist:Hip	-0.11	-0.13	-0.14	0.00	-0.16	-0.18	-0.06
Waist:Height	-0.03	-0.10	-0.08	0.19	-0.04	-0.02	0.20
Total fat mass	0.07	-0.01	-0.03	**0.29** **	0.00	0.06	**0.25** *
Percentage fat mass	0.08	0.01	-0.03	0.19	0.00	0.07	0.17
Android fat mass	0.02	0.00	0.03	0.24	-0.07	0.14	0.23
Percentage android fat mass	-0.13	-0.12	-0.06	0.03	-0.12	0.04	0.06
Gynoid fat mass	0.07	0.08	0.05	0.19	-0.02	0.15	0.23
Percentage gynoid fat mass	0.10	0.18	0.04	-0.23	0.07	-0.09	-0.21

Values are unadjusted Pearson correlation coefficients. N=109 except for DEXA-derived fat mass data where n=86. Bold text with asterisks indicates significant after BH correction for multiple testing where * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. Blue shade highlights positive relationship and red shade highlights negative relationship.

Table 4-4: Correlation matrix between plasma FAHFAs and anthropometric measures in females and males combined.

	9-PAHSA	10-PAHSA	11-PAHSA	12/13-PAHSA	9-PAHPA	10-PAHPA	12/13-PAHPA
Age	-0.01	0.03	0.01	-0.03	0.00	0.01	-0.04
Height	0.06	0.05	0.09	0.12	0.06	0.08	0.11
Mass	**0.18 ***	0.08	0.13	**0.27 *****	**0.17 ***	**0.19 ***	**0.27 *****
BMI	**0.21 ****	0.12	0.11	**0.28 *****	**0.16 ***	**0.17 ***	**0.29 *****
Waist	0.04	-0.05	0.01	0.14	0.01	0.09	0.18
Hip	0.01	-0.07	-0.04	0.19	0.00	0.05	0.18
Waist:Hip	-0.03	-0.08	-0.05	0.04	-0.07	0.00	0.06
Waist:Height	-0.01	-0.07	-0.03	0.08	-0.01	0.04	0.12
Total fat mass	0.01	-0.01	-0.01	0.13	0.01	0.04	0.13
Percentage fat mass	-0.06	-0.03	-0.06	0.00	-0.08	-0.02	0.01
Android fat mass	0.09	0.02	0.11	0.12	0.07	0.18	0.16
Percentage android fat mass	0.08	0.03	0.13	0.10	0.09	**0.19 ***	0.18
Gynoid fat mass	-0.06	-0.02	-0.02	0.01	-0.06	0.02	-0.01
Percentage gynoid fat mass	-0.13	0.00	-0.14	**-0.20 ***	-0.12	**-0.22 ***	**-0.25 ***

Values are unadjusted Pearson correlation coefficients. Bold text with asterisks indicates significant after BH correction for multiple testing where * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. N=175 except for DEXA-derived fat mass data where n=148. Blue shade highlights positive relationship and red shade highlights negative relationship.

Based on Kellerer *et al.'s* (2021) observation of differences in plasma FAHFAs between healthy weight and morbidly obese subjects and that Yore *et al.'s* (2014) original insulin sensitive/ insensitive groups had a wide BMI difference, I split the group by sex and BMI into Healthy (BMI< 25 $kg.m^{-2}$), Overweight (25 < BMI < 30 $kg.m^{-2}$) and Obese (BMI > 30 $kg.m^{-2}$) to see if the weak correlations became stronger when comparing the extremes. There were 14, 33 and 20 males in the respective BMI groups and 45, 38 and 23 females. The results are given in Figures 4-1 to 4-4.

In both sexes, 12/13-PAHPA was lower in healthy than in overweight and obese subjects (Figure 4-2 and Figure 4-4) and this was the same for 12/13-PAHSA in females only (Figure 4-3), while healthy males had lower 12/13-PAHSA than the obese group only (Figure 4-1). There were no correlations between BMI and any FAHFAs in either sex. However, where there was a trend towards negative

correlations for all FAHFAs in overweight and obese males, there is a trend towards positive correlations for all FAHFAs in all groups of females, strengthening the suggestion of sexual dimorphism in FAHFA regulation.

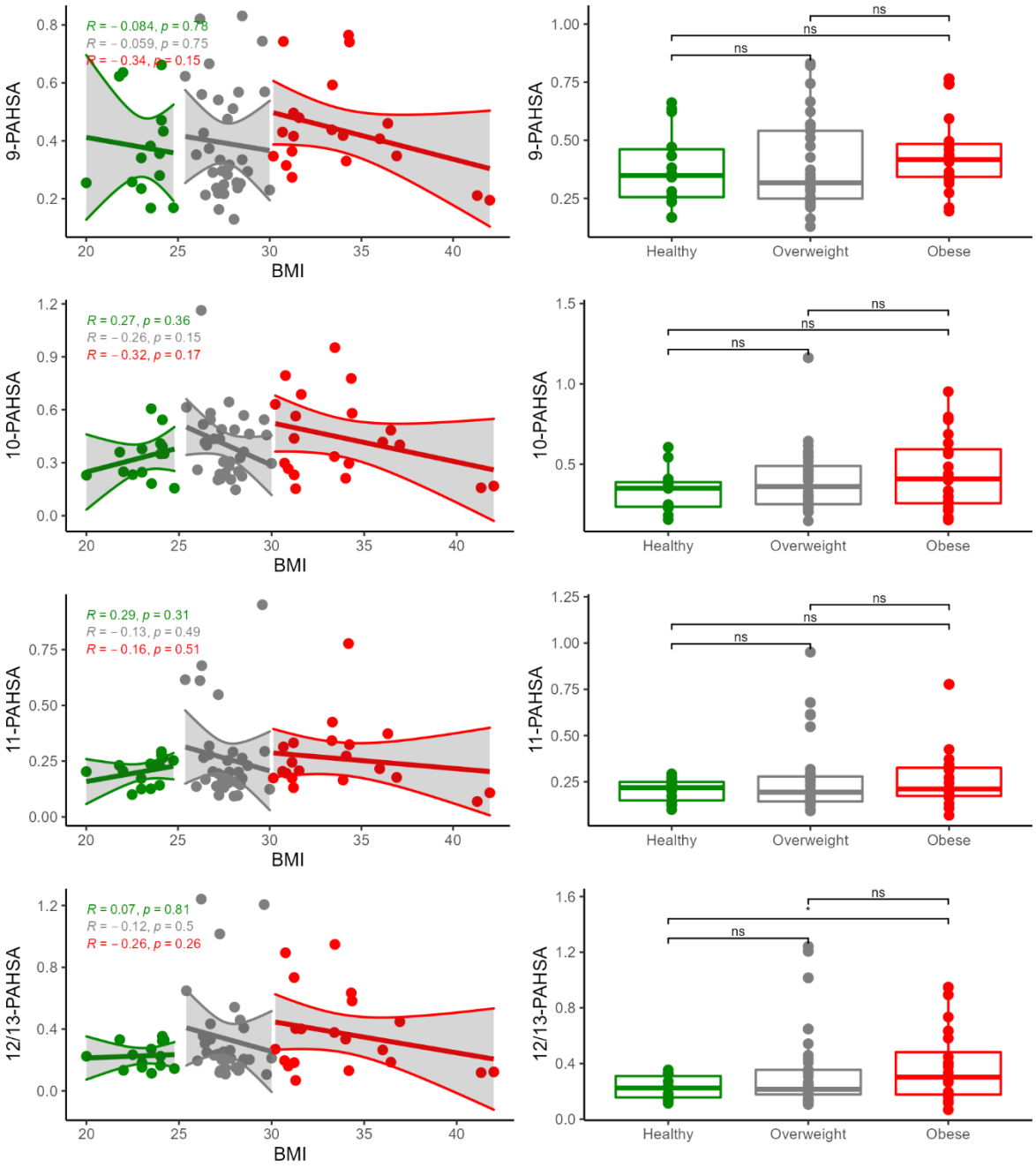


Figure 4-1: Plasma PAHSA concentrations versus BMI categories in males

Green= healthy weight (20-24.9 kg.m^{-2}), n=14. Grey= overweight (25-29.9 kg.m^{-2}), n=33. Red= obese (>30 kg.m^{-2}), n=20. R denotes Pearson correlation coefficient. * indicates p<0.05 according to one-way ANOVA.

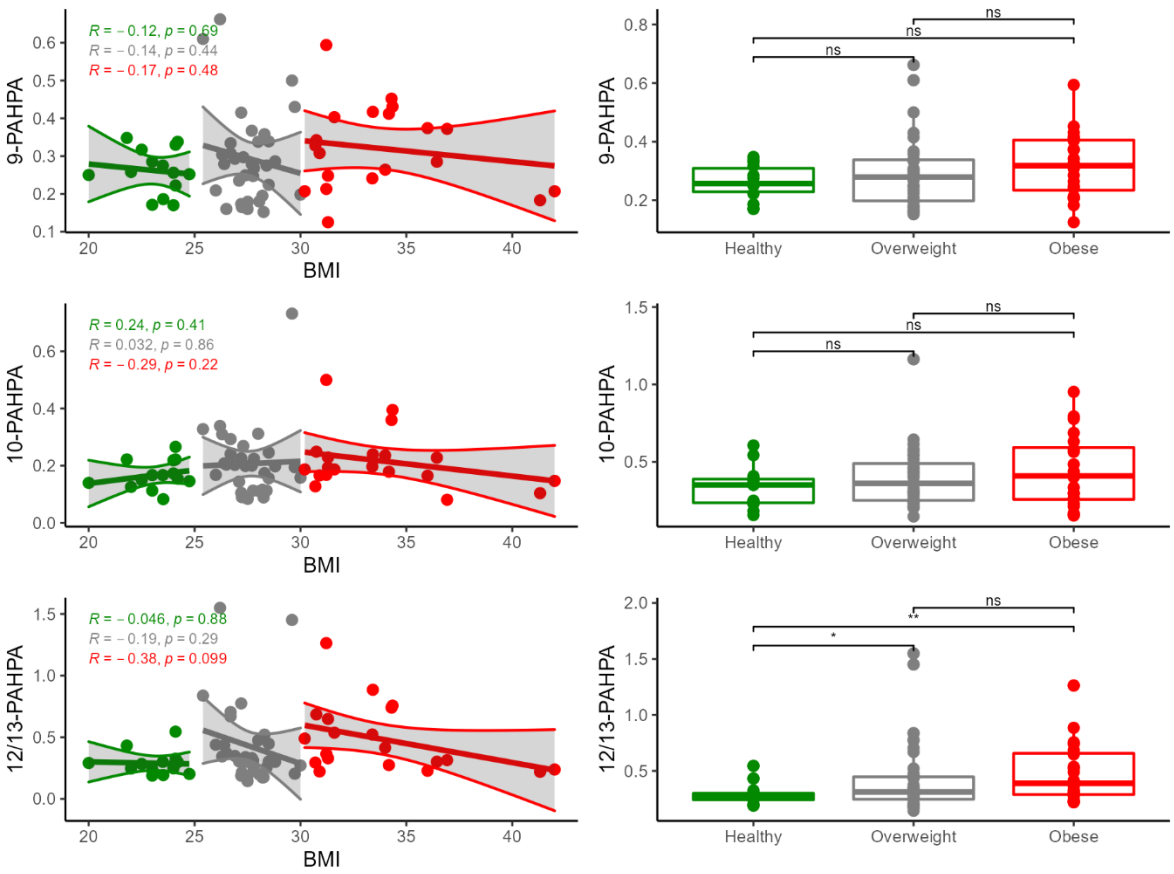


Figure 4-2: Plasma PAHPA concentrations versus BMI categories in males

Green= healthy weight (20-25 kg.m^{-2}), n=14. Grey= overweight (25-30 kg.m^{-2}), n=33. Red= obese (>30 kg.m^{-2}), n=20. R denotes Pearson correlation coefficient. * indicates p<0.05 and ** indicates p<0.01 according to one-way ANOVA.

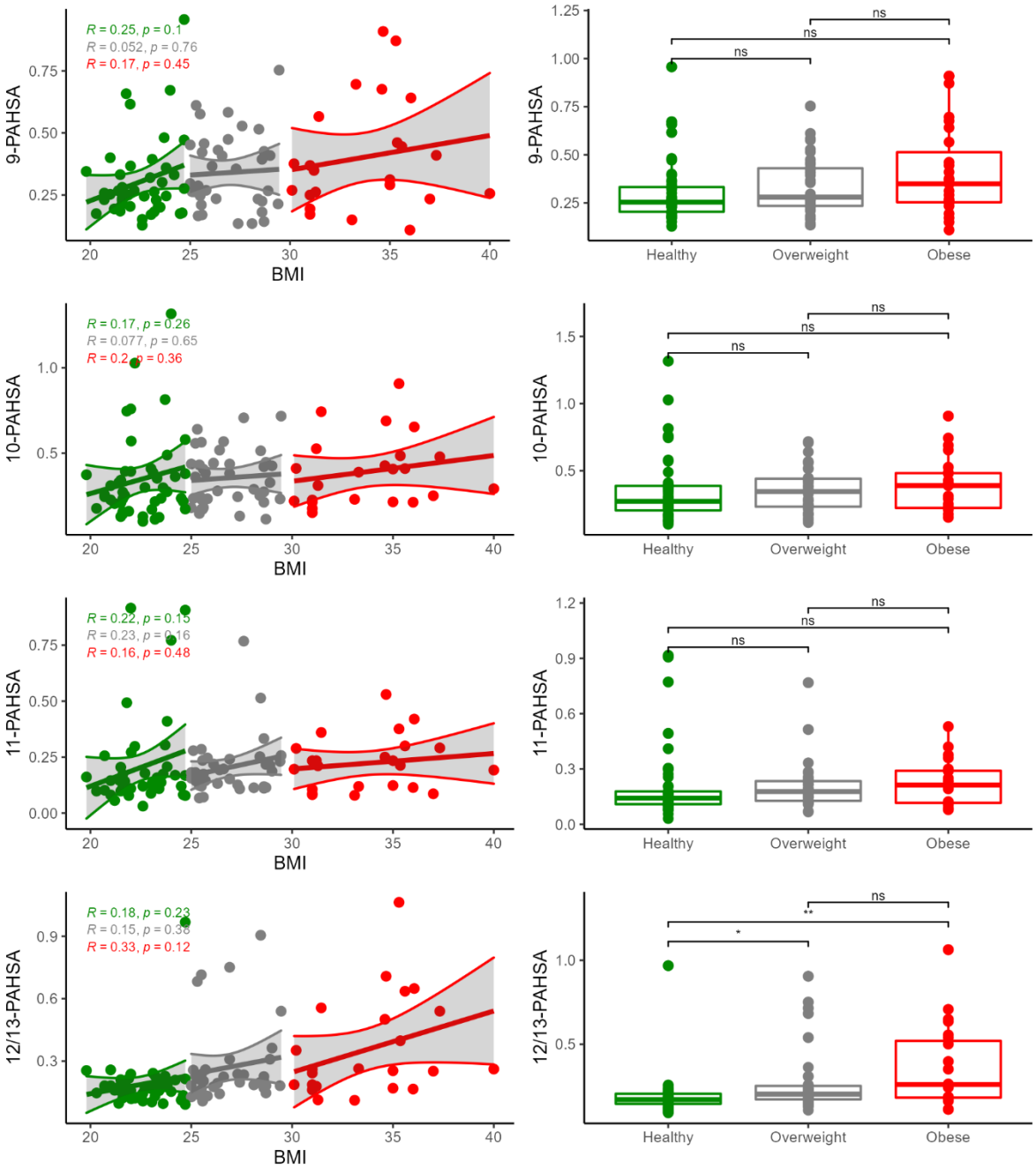


Figure 4-3: Plasma PAHSA concentrations versus BMI categories in females

Green= healthy weight (20-25 kg.m^{-2}), n=45. Grey= overweight (25-30 kg.m^{-2}), n=38. Red= obese (>30 kg.m^{-2}), 23. R denotes Pearson correlation coefficient. * indicates p<0.05 and ** indicates p<0.01 according to one-way ANOVA.

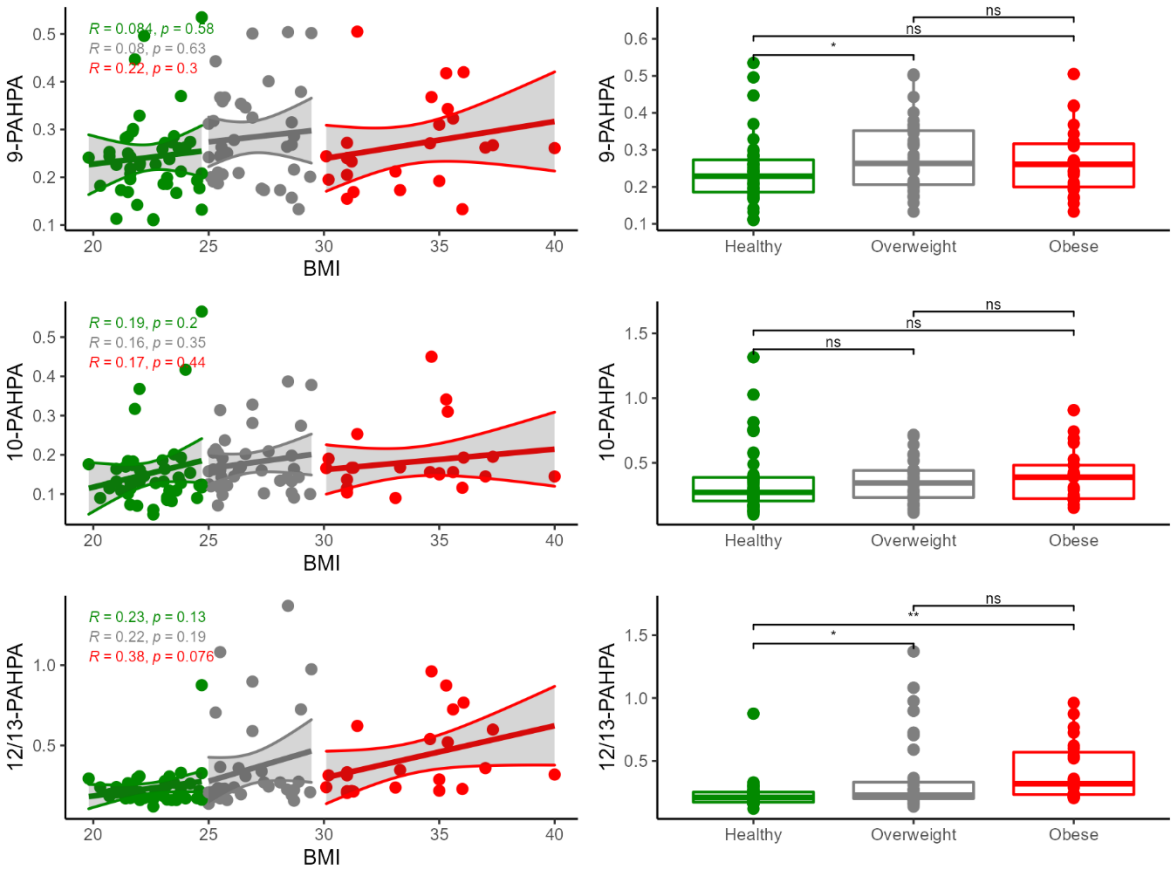


Figure 4-4: Plasma PAHPA concentrations versus BMI categories in females

Green= healthy weight (20-25 kg.m^{-2}), n=45. Grey= overweight (25-30 kg.m^{-2}), n=38. Red= obese (>30 kg.m^{-2}), 23. R denotes Pearson correlation coefficient. * indicates p<0.05 and ** indicates p<0.01 according to one-way ANOVA.

Plasma FAHFAs correlate with measures of insulin sensitivity

Although there were no associations between blood lipids (total triglycerides, total cholesterol or HDL-cholesterol) and FAHFAs, there were some correlations with fasting glucose and insulin and their associated metric, HOMA-IR.

In males, there were weak, positive correlations between fasting glucose and several FAHFAs but there were no associations between FAHFAs and either fasting insulin or HOMA-IR (Table 4-5). In females, however, there were weak, positive correlations between several FAHFAs and insulin, glucose and HOMA-IR.

Table 4-5: Correlation matrix between indicators of insulin resistance and plasma PAHSAs and PAHPAs in males.

	9-PAHSA	10-PAHSA	11-PAHSA	12/13-PAHSA	9-PAHPA	10-PAHPA	12/13-PAHPA
Insulin	-0.03	-0.01	-0.01	0.01	0.05	-0.04	-0.02
Glucose	0.20	0.31 *	**0.25** *	0.33 *	**0.29** *	0.23	**0.26** *
HOMA-IR	0.00	0.04	0.04	0.06	0.10	-0.01	0.02

Numbers are Pearson correlation coefficients. Bold text with asterisks indicates significant after BH correction for multiple testing where * denotes p-value <0.05, ** denotes p-value <0.01, *** denotes p-value<0.001. Blue shade highlights positive relationship and red shade highlights negative relationship.

Table 4-6: Correlation matrix between indicators of insulin resistance and plasma PAHSAs and PAHPAs in females.

	9-PAHSA	10-PAHSA	11-PAHSA	12/13-PAHSA	9-PAHPA	10-PAHPA	12/13-PAHPA
Insulin	**0.29** **	0.17	0.09	**0.36** ***	**0.23** **	0.18	**0.40** ***
Glucose	**0.34** ***	0.18	0.12	**0.25** **	0.02	0.18	**0.24** **
HOMA-IR	**0.37** ***	0.20 *	0.11	**0.39** ***	0.19 *	**0.21** ***	**0.42**

Numbers are Pearson correlation coefficients. Bold text with asterisks indicates significant after BH correction for multiple testing where * denotes p-value <0.05, ** denotes p-value <0.01, *** denotes p-value<0.001. Blue shade highlights positive relationship and red shade highlights negative relationship.

To tease out more detail in these correlations, I split the cohorts by fasting insulin, as a measure of long-term insulin sensitivity, using the 75th centile of plasma insulin concentration (13.3 mU.L^{-1}) as the cut-off between subjects classified as normo- and hyper-insulinaemic (NI and HI) (Hodson *et al.*, 2007). In addition to giving two groups for direct comparison, if FAHFAs are specifically upregulated with increasing insulin resistance, then one might expect a positive correlation between FAHFAs and fasting insulin in the HI group but not necessarily in the NI or amalgamated groups. The characteristics of the resulting groups are given in Table 4-7 and, in addition to higher fasting insulin, the HI groups had higher fasting glucose. Some differences in fatness and fat distribution were also evident; the HI groups had higher body mass, relative fat mass and waist to hip ratio while the male HI group also had higher android:gynoid ratio. In men only, all PAHSAs and PAHPAs were higher in the HI group.

Table 4-7: Characteristics of the normo- and hyper-insulinaemic groups for plasma FAHFA comparisons.

	Female		Male	
Parameter	**NI**	**HI**	**NI**	**HI**
N	83	26	48	18
Age (y)	47.8 ± 0.9 [26 - 62]	46.8 ± 1.4 [34 - 63]	44.5 ± 0.9 [26 - 55]	45.8 ± 1.3 [36 - 54]
BMI ($kg.m^{-2}$)	24.9 ± 0.4 [19.8 – 35.3]	31.3† ± 1.0 [21.5 - 40]	26.7 ± 0.4 [20.0 – 31.3]	32.7† ± 1.0 [23.0 – 41.3]
Relative Fat Mass (%)	34.5 ± 0.8 [19 – 51]	42.3† ± 0.1 [22 - 53]	25.7 ± 1.0 [11.0 – 40.0]	34.0† ± 1.4 [20.0 – 44]
Waist:Hip	0.84 ± 0.01 [0.73 – 1.03]	0.89† ± 0.02 [0.78 – 0.98]	0.92 ± 0.02 [0.78 – 1.04]	1.02† ± 0.04 [0.87 – 1.16]
Android:Gynoid	0.4 ± 0.02 [0.12 – 0.8]	0.39 ± 0.07 [0.24 – 0.56]	0.66 ± 0.03 [0.26 – 0.97]	0.82† ± 0.05 [0.64 – 1.02]
Fasting Insulin ($mU.L^{-1}$)	8.9 ± 0.32 [0.8 – 13.1]	21.9† ± 1.5 [13.4– 41.3]	8.9 ± 0.4 [3.9 – 13.2]	22.7† ± 1.8 [13.5 – 38.8
Fasting Glucose (mM)	4.9 ± 0.1 [3.4 – 6.2]	5.7† ± 0.3 [4.5– 11.2]	5.2 ± 0.1 [3.5 – 6.6]	5.5† ± 0.1 [4.8 – 6.6]
9-PAHSA	0.33 ± 0.02 [0.13 – 0.96]	0.34 ± 0.03 [0.11 – 0.91]	0.36 ± 0.02 [0.13 – 0.68]	0.51 ± 0.05† [0.26 – 1.38]
10-PAHSA	0.35 ± 0.02 [0.06 – 1.32]	0.34 ± 0.03 [0.10 – 0.91]	0.34 ± 0.02 [0.15 – 0.63]	0.49 ± 0.05† [0.21 – 1.16]
11-PAHSA	0.20 ± 0.02 [0.06 – 0.92]	0.20 ± 0.02 [0.03 – 0.77]	0.22 ± 0.02 [0.09 – 0.62]	0.35 ± 0.06† [0.14 – 1.14]
12/13-PAHSA	0.21 ± 0.02 [0.08 – 0.97]	0.26 ± 0.03 [0.09 – 1.06]	0.26 ± 0.03 [0.07 – 1.02]	0.42 ± 0.08† [0.10 – 1.24]
9-PAHPA	0.25 ± 0.01 [0.11 – 0.54]	0.26 ± 0.01 [0.11 – 0.51]	0.26 ± 0.01 [0.13 – 0.61]	0.36 ± 0.03† [0.17 – 0.77]
10-PAHPA	0.16 ± 0.01 [0.06 – 0.57]	0.16 ± 0.01 [0.05 – 0.45]	0.18 ± 0.01 [0.08 – 0.33]	0.24 ± 0.03† [0.12 – 0.73]
12/13-PAHPA	0.26 ± 0.02 [0.12 – 0.90]	0.31 ± 0.04 [0.15 – 1.08]	0.35 ± 0.03 [0.14 – 0.84]	0.56 ± 0.10† [0.17 – 1.91]

Data presented as mean ± SEM [range]. Insulin group cut-off at 75th centile of whole group. NI: normo-insulinaemic. HI: hyper-insulinaemic. † indicates significantly different to NI.

The insulin and PAHSA data were plotted against each other in Figure 4-5. In HI females, there were moderate, positive and significant correlations between fasting insulin and 9-, 10- and 11-PAHSA while significance was not reached for the positive correlation with 12/13-PAHSA. In HI males, significance was not reached for any PAHSA but there were trends for weak to moderate negative correlations, making them opposite to females. The NI groups do not show any associations with fasting insulin.

Figure 4-6 shows the same plots for PAHPAs. Again, the HI females and males showed weak, positive and weak, negative trends respectively but only for 10-PAHPA in males is the relationship significant. The NI groups do not show any associations with fasting insulin.

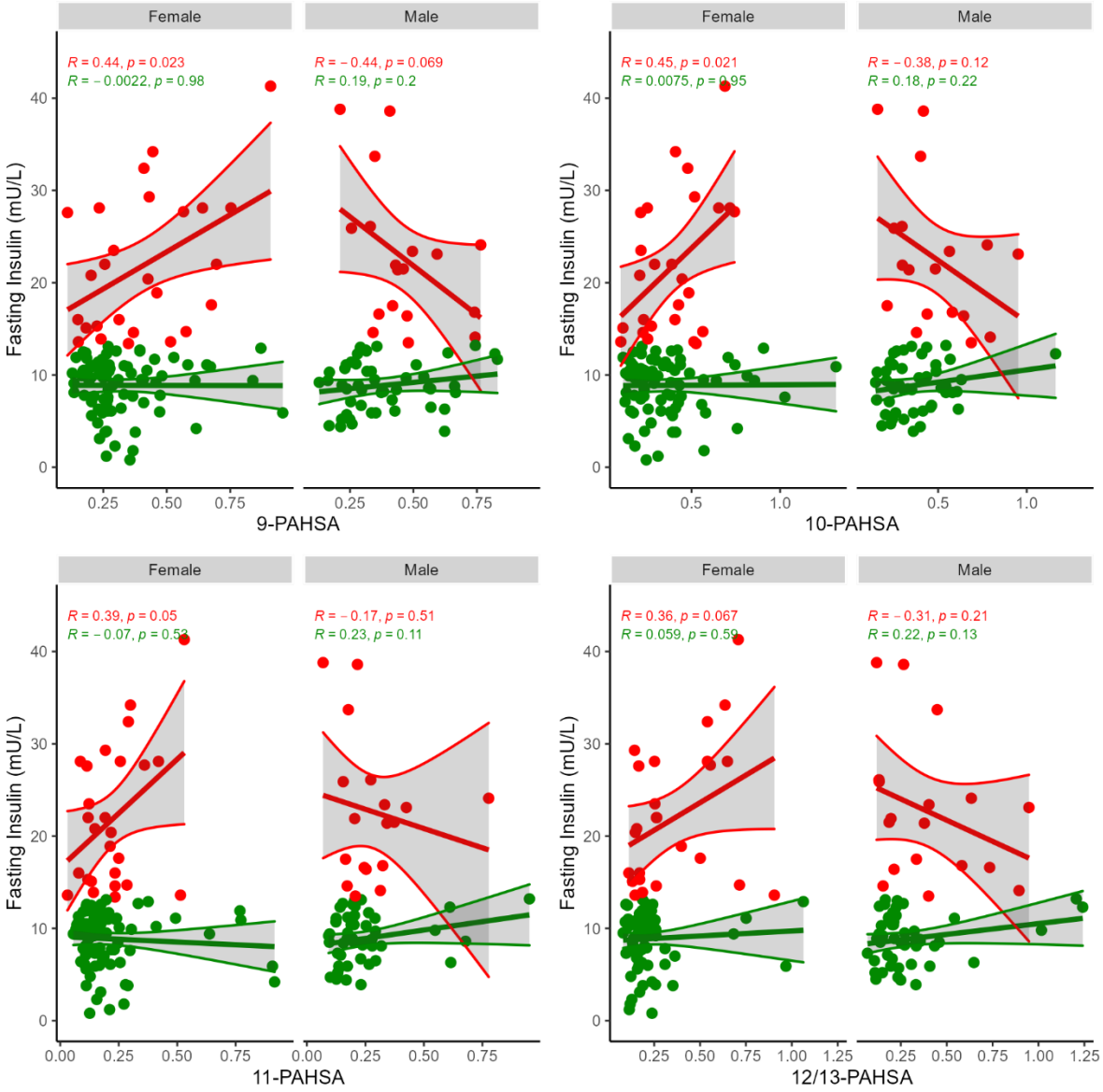


Figure 4-5: Correlations between plasma PAHSAs and fasting insulin in female and male subjects stratified by insulinaemia display sexual dimorphism.

Red: hyperinsulinaemic, green: normoinsulinaemic. R is the Pearson correlation coefficient

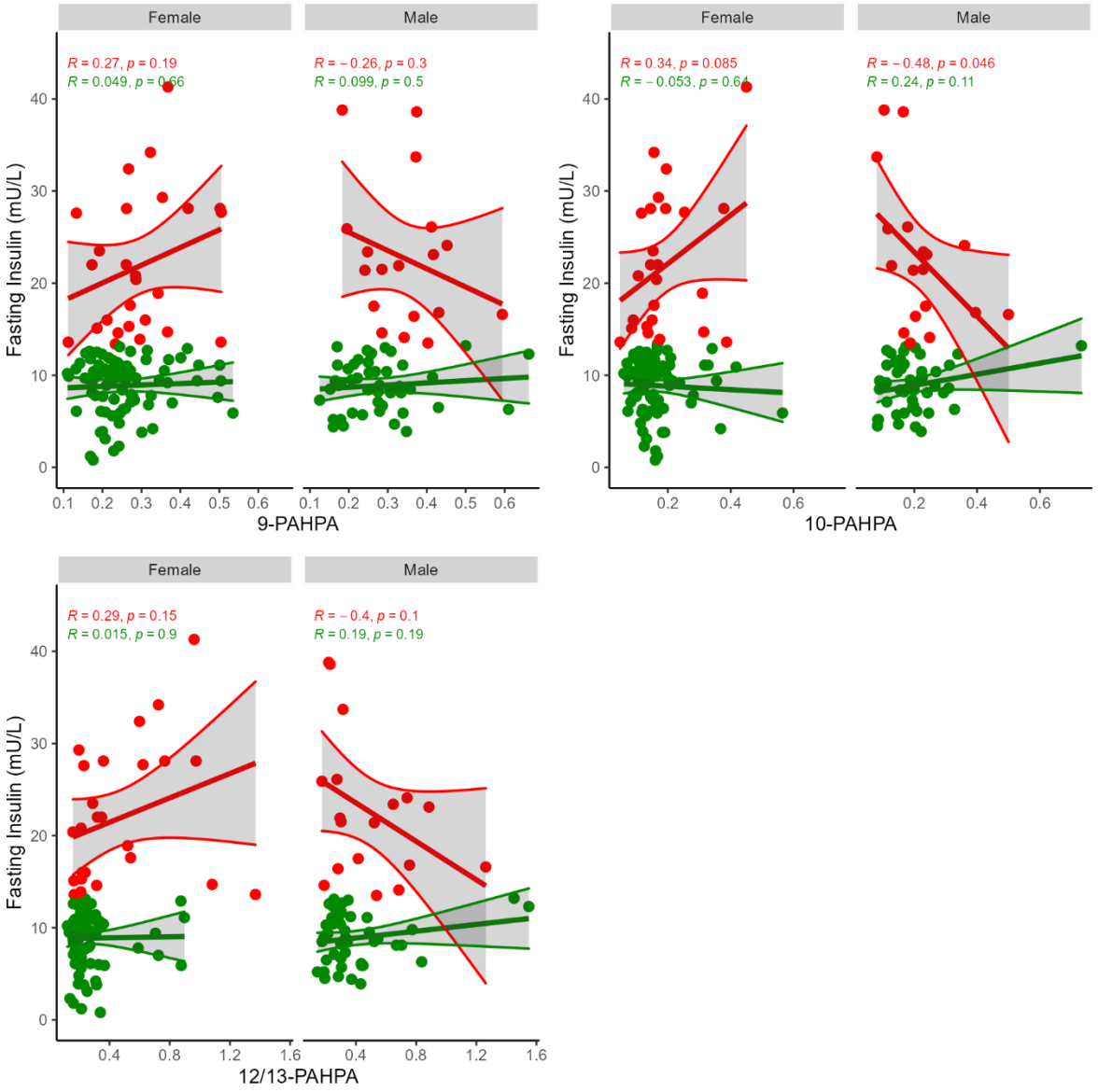


Figure 4-6: Correlations between plasma PAHPAs and fasting insulin in female and male subjects stratified by insulinaemia display sexual dimorphism.

Red: hyperinsulinaemic, green: normoinsulinaemic. R is the Pearson correlation coefficient

The fasting insulin of one of the female participants was an outlier (more than 150 % of the interquartile range greater than the group median) and so the analysis was repeated with that participant excluded. In this case the positive direction of the associations described in Figure 4-5 and Figure 4-6 remained but significance in the correlations was lost.

Since there are some weak relationships between the measured FAHFAs and BMI and since fasting insulin is moderately and strongly correlated with BMI in the HI groups (data not shown), I repeated the analysis with fasting insulin adjusted for BMI. This adjustment had the effect of further decreasing the significance of the relationships in the female HI group and increasing the significance values in

the male HI group but did not alter the patterns of the relationships between FAHFAs and fasting insulin (data not shown).

Men and women have different adipose tissue distribution but this does not explain the opposite trends in FAHFAs

As with the general population (Tichet *et al.*, 1993), the men in this cohort had a higher Waist:Hip (i.e. greater abdominal adiposity) than women (0.95 v. 0.84, $p<0.001$). However, this is unlikely to be a contributing factor to the apparently opposite regulation of plasma FAHFAs between the sexes, since there were no correlations between FAHFAs and Waist:Hip, android:gynoid, and absolute or relative android and gynoid fat masses (Table 4-3 & Table 4-4).

Pre- and post-menopausal women have similar circulating FAHFAs

Within our large cohort, we had a subset of female participants who had been defined as pre- or post-menopausal (n=30 pre-, n=28 post-menopause) according to circulating hormone levels. I found no effect of menopause status on circulating PAHSA or PAHPA concentrations (data not shown).

4.3.2 Cross-sectional Study of Adipose Tissue FAHFAs and Phenotypic Measurements

The intention with this piece of work was to measure the FAHFAs present in whole abdominal and gluteal AT in their FAHFA-NEFA and FAHFA-TAG form. Due to complications with extraction of the FAHFA-TAG and the mass-spectrometry of the FAHFA-NEFA, the data is less complete than intended. With regard to the FAHFA-TAG, the issue was with the hydrolysis step of the extraction which separates the individual FAHFA-NEFAs and FAs from the glycerol backbone. Trouble-shooting with a simple TAG, tri-pentadecaenoic acid, performing the hydrolysis and then creating FAMEs with the product for analysis by GC (2.16), I determined that I could recover around one third of the tri-pentadecaenoic acid that I had started with. This rate of recovery was probably too small to observe FAHFAs from an already-small tissue pool and helps to explain, in part, why I was not able to successfully apply the method to measuring FAHFAs in TAG. Given more time, I would attempt to optimise the hydrolysis step, which must be robust enough to dissociate the FAHFAs from the glycerol but not the fatty acid from the hydroxy-fatty acid. With regard to the FAHFA-NEFA, OAHSAs were the only family from the FAHFA-NEFA samples which I could robustly identify. Nevertheless, there are some interesting observations which could direct future study.

The participant characteristics are given in Table 4-8.

Table 4-8: Participant characteristics

Parameter	Female	Male	Difference
N	20	19	
Age (y)	48.2 ± 1.7 [38 – 60]	48.3 ± 1.9 [38 – 58]	ns
Body Mass (kg)	75.6 ± 3.2 [54 – 108]	82.7 ± 2.3 (70 - 112)	ns
BMI ($kg.m^{-2}$)	27 ± 1.0 [21.3 – 38.1]	25.7 ± 0.5 [22.3 – 31.1]	ns
Fat Mass (kg)	28.9 ± 2.7 [13.7 – 56.5]	21.8 ± 1.5 [12.8 – 41.1]	p= 0.03
Relative fat mass (%)	37.0 ± 1.7 [21 – 52]	26.1 ± 1.3 [17 – 37]	p< 0.001
Waist (cm)	87 ± 2.9 [64 – 112]	92.8 ± 1.8 [80 – 114]	ns
Waist:Hip	0.82 ± 0.02 [0.70 – 0.94]	0.92 ± 0.01 [0.84 – 1.04]	p< 0.001
Android:gynoid	0.42 ± 0.03 [0.121 – 0.75]	0.65 ± 0.04 [0.30 – 0.96]	p< 0.001
Fasting Plasma NEFA (μM)	422 ±25 [232 – 618]	362 ± 35 (177 – 864)	ns
Fasting Plasma Total Triglycerides (mM)	1.1 ±0.14 [0.41 – 3.0]	1.3 ±0.13 [0.62 – 2.9]	ns
Fasting Plasma Insulin ($mU.L^{-1}$)	5.8 ±1.0 [1.3– 15.8]	5.0 ±0.6 [0.8 – 12.1]	ns
Fasting Plasma Glucose (mM)	4.9 ±0.1 [4.2– 5.6]	5.1 ±0.1 [4.3 – 5.9]	ns

Data presented as mean ± SEM [range]. p-values result from Welch's t-test between sexes. N.B. the fat mass, relative fat mass and android:gynoid are derived from DEXA scans.

In women, all OAHSA-NEFA except 10-OAHSA were lower in gluteal than abdominal tissue (Figure 4-7). In men, only 8-, 9- and 12/13-OAHSA were lower in gluteal compared to abdominal tissue. In men, no individual OAHSA-NEFA were correlated with the same OAHSA-NEFA in gluteal tissue. In women, no OAHSA-NEFA other than 5-OAHSA ($R=0.80$, $p<0.05$) were correlated between adipose depots. Together, these preliminary data suggest that there are differences in OAHSA metabolism between specific SAT depots.

Further to this, in men, 10-OAHSA in plasma was negatively correlated ($R= -0.64$, $p<0.05$) and 12/13-OAHSA in plasma positively correlated ($R=0.81$, $p<0.05$) with the same OAHSA-NEFAs in abdominal tissue. In women, there were no correlations between OAHSAs in plasma and either adipose depot. This suggests that the circulating pool of OAHSAs was not related to the adipose NEFA pool.

Examining the correlations between the tissue OAHSAs and the various phenotypic measurements revealed only a few significant correlations. In women, though, 5-, 8-, 9, 10, and 12/13-OAHSA in gluteal tissue were all negatively correlated with plasma glucose (-0.37 <R< -0.76, p<0.05), 11-OAHSA was the opposite (R=0.56, p<0.05) being positively associated with plasma glucose. The women's plasma OAHSAs are not correlated with glucose. In men, 11-OAHSA in gluteal tissue was positively correlated with total plasma NEFA and the individual NEFA species (0.47 <R< 0.81, p<0.05).

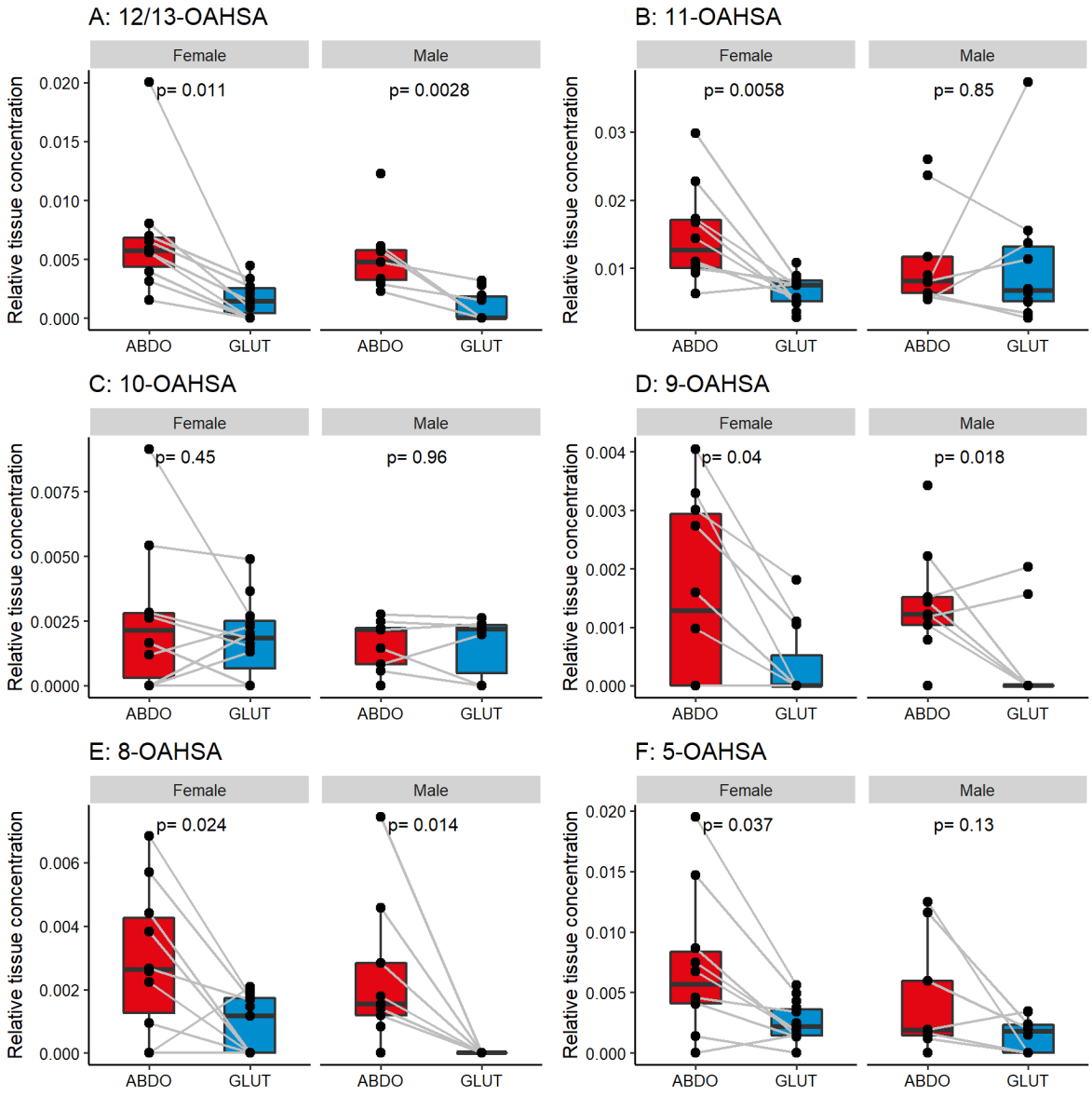


Figure 4-7: Several OAHSA-NEFAs are higher in gluteal than in abdominal adipose tissue.

Values obtained by normalising to internal standard and tissue sample mass. Connecting lines indicate paired samples from the same participant where pairs exist. P values derived from paired t-test between depots.

4.3.3 Comparing Plasma FAHFAs to Palmitoleate

In order to determine whether or not the concentration of palmitoleate in plasma shares similar patterns to plasma FAHFAs which might suggest that both experience similar regulation, I examined our dataset in a similar manner with the absolute and relative concentrations of palmitoleate as the key variables, where "relative" means the absolute concentration as a fraction of total plasma NEFA.

Participant characteristics

The characteristics of the participants in the group comparing FAHFAs with palmitoleate are given in Table 4-9.

Table 4-9: Participant characteristics for palmitoleate plasma study

Parameter	Female	Male	Difference
N	119	91	
Age (y)	48.4 ± 0.6 [26 – 63]	45.5 ± 0.7 [26 – 59]	p< 0.01
Mass (kg)	71.3 ± 1.1 [49.0 – 107.1]	88.1 ± 1.2 (65.8 – 121.1)	p< 0.001
BMI ($kg.m^{-2}$)	26.0 ± 0.3 [19.8 – 36.7]	27.6 ± 0.3 [21.2 – 34.6]	p< 0.01
Fat Mass (kg)	26.5 ± 0.9 [10.7 – 51.5]	25.2 ± 0.9 [8.2 – 52.6]	ns
Relative fat mass (%)	36.5 ± 0.7 [19 – 51]	28.2 ± 0.7 [11 – 49]	p< 0.001
Waist (cm)	85.4 ± 1.0 [64 – 112]	96.4 ± 1.1 [77 – 127]	p< 0.001
Waist:Hip	0.84 ± 0.01 [0.70 – 1.03]	0.95 ± 0.01 [0.84 – 1.07]	p< 0.001
Android:gynoid	0.41 ± 0.02 [0.12 – 0.80]	0.69 ± 0.02 [0.26 – 1.03]	p< 0.001
Plasma NEFA (μM)	560 ±36 [132 – 1571]	456 ± 38 (144 – 1276)	p< 0.001

Data presented as mean ± SEM [range]. p-values result from Welch's t-test between sexes

Palmitoleate does not correlate with plasma FAHFAs

Neither absolute nor relative plasma palmitoleate concentrations showed any significant correlation with any of the PAHSAs or PAHPAs. Palmitoleate concentration was tightly linked to NEFA concentration and so this follows the fact that the PAHSAs and PAHPAs are not regulated in a similar manner to NEFA (see section 4.3.1).

Women have higher concentrations of circulating palmitoleate and the relative concentration increases with absolute concentration

In line with other reports (Ramos, Bush and Jensen, 2020), I found women to have higher levels of circulating palmitoleate, a pattern similar to overall total NEFA but opposite to FAHFAs, which were higher in men. There were weak, positive and significant correlations between relative palmitoleate and total NEFA in both men (R=0.23) and women (R=0.32) , which was similar to the observations of Ramos *et al.* (2020).

Table 4-10: Absolute and relative plasma concentrations of palmitoleate differ between men and women.

		Female	Male	Difference
Absolute concentration of POA	mean ± SEM (μM)	23.8 ± 1.3	14.0 ± 0.9	p< 0.001
Relative concentration of POA	mean ± SEM	0.041 ± 0.001	0.030 ± 0.001	p< 0.001

Concentrations measured by gas chromatography and normalised to internal standard. N= 119 female, 91 male. p-values result from Welch t-tests between sexes.

Palmitoleate does not correlate with measures of insulin sensitivity

Subjects were divided by fasting insulin using the 75th centile (12.6 mU.L^{-1}) as the cutoff between normo- and hyper-insulinaemic (NI and HI, respectively) subjects. The characteristics of the groups are given in Table 4-11.

Three measures of palmitoleate, i) the absolute concentration, ii) the relative concentration and, iii) the SCD ratio (ratio of 16:1 to 16:0) were compared with three measures of insulin sensitivity: i) fasting plasma insulin, ii) fasting plasma glucose and, iii) HOMA-IR (Figures 4-8 to 4-10). There were no significant correlations between the measures of palmitoleate and the measures of insulin sensitivity in any group. However, in most cases, the gradient of the linear regression line for the HI group was more positive than the NI group, suggesting a trend of upregulation of palmitoleate synthesis and release with decreasing insulin sensitivity.

Table 4-11: Participant characteristics for the hyper- and normo-insulinaemic groups in the palmitoleate study.

	Female		Male	
Parameter	**NI**	**HI**	**NI**	**HI**
N	90	27	65	26
Age	48.1 ± 0.9 [26 - 62]	48.4 ± 1.5 [34 - 63]	43.3 ± 0.9 [26 - 59]	46.3† ± 1.1 [27 - 50]
BMI	25.0 ± 0.3 [19.8 - 31.8]	29.0† ± 0.9 [21.5 - 36.7]	26.6 ± 0.3 [21.2 - 34.6]	29.9† ± 0.5 [24.8 - 34.3]
Relative Fat Mass	34.7 ± 0.7 [19 - 50]	42.1† ± 1.0 [33 - 51]	26.5 ± 0.8 [11.0 - 45.0]	32.2† ± 1.1 [21.0 - 49.0]
Waist:Hip	0.83 ± 0.01 [0.70 - 1.03]	0.88† ± 0.01 [0.79 - 0.98]	0.93 ± 0.01 [0.84 - 1.05]	0.97† ± 0.01 [0.86 - 1.07]
Android:Gynoid	0.4 ± 0.02 [0.12 - 0.8]	0.50† ± 0.03 [0.24 - 0.67]	0.65 ± 0.02 [0.26 - 0.97]	0.8† ± 0.03 [0.45 - 1.03]
Fasting Insulin	8.0 ± 0.33 [0.8 - 12.5]	17.8† ± 1.3 [12.6 - 41.3]	7.6 ± 0.3 [0.8 - 12.3]	17.2† ± 0.8 [12.6 - 26.1]
Fasting Glucose	4.9 ± 0.1 [3.4 - 6.2]	5.6† ± 0.2 [4.5 - 11.2]	5.1 ± 0.1 [3.5 - 6.4]	5.6† ± 0.1 [5.0 - 6.6]

Data presented as mean ± SEM [range]. Insulin group cut-off at 75th centile of whole group. NI: normo-insulinaemic. HI: hyper-insulinaemic. † indicates significantly different to NI.

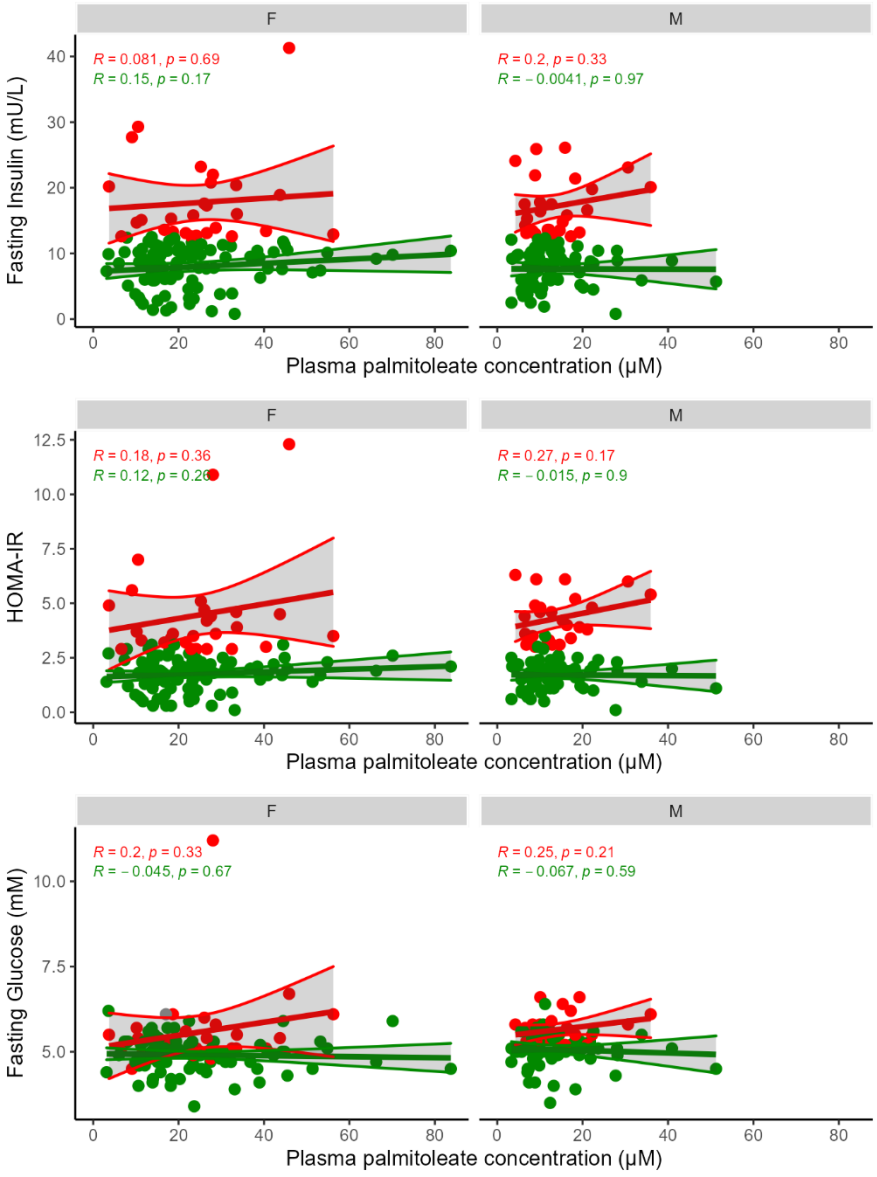


Figure 4-8: Correlations between absolute plasma palmitoleate concentration and measures of insulin sensitivity.

Red: hyper-insulinaemic, green: normo- insulinaemic. R is the Pearson correlation coefficient

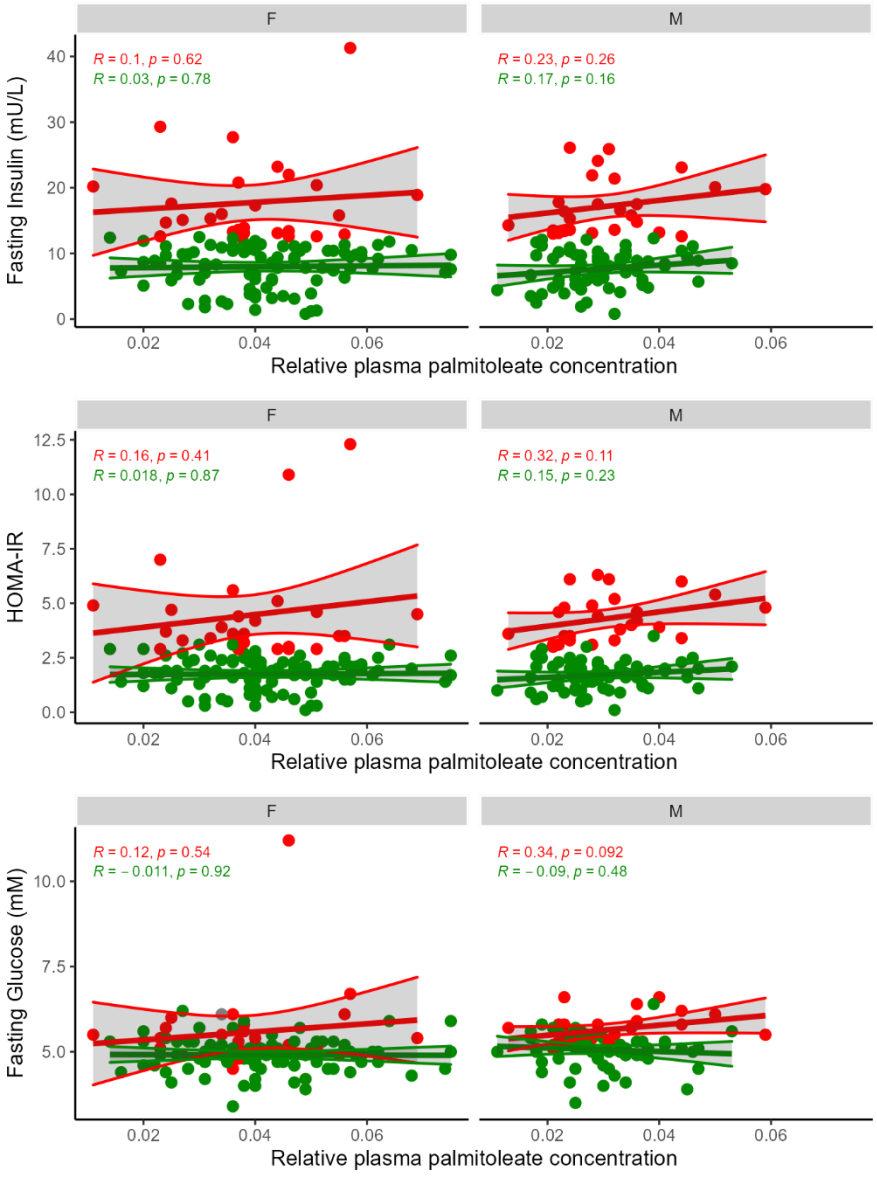


Figure 4-9: Correlations between relative plasma palmitoleate concentration and measures of insulin sensitivity.

Red: hyper-insulinaemic, green: normo-insulinaemic. R is the Pearson correlation coefficient

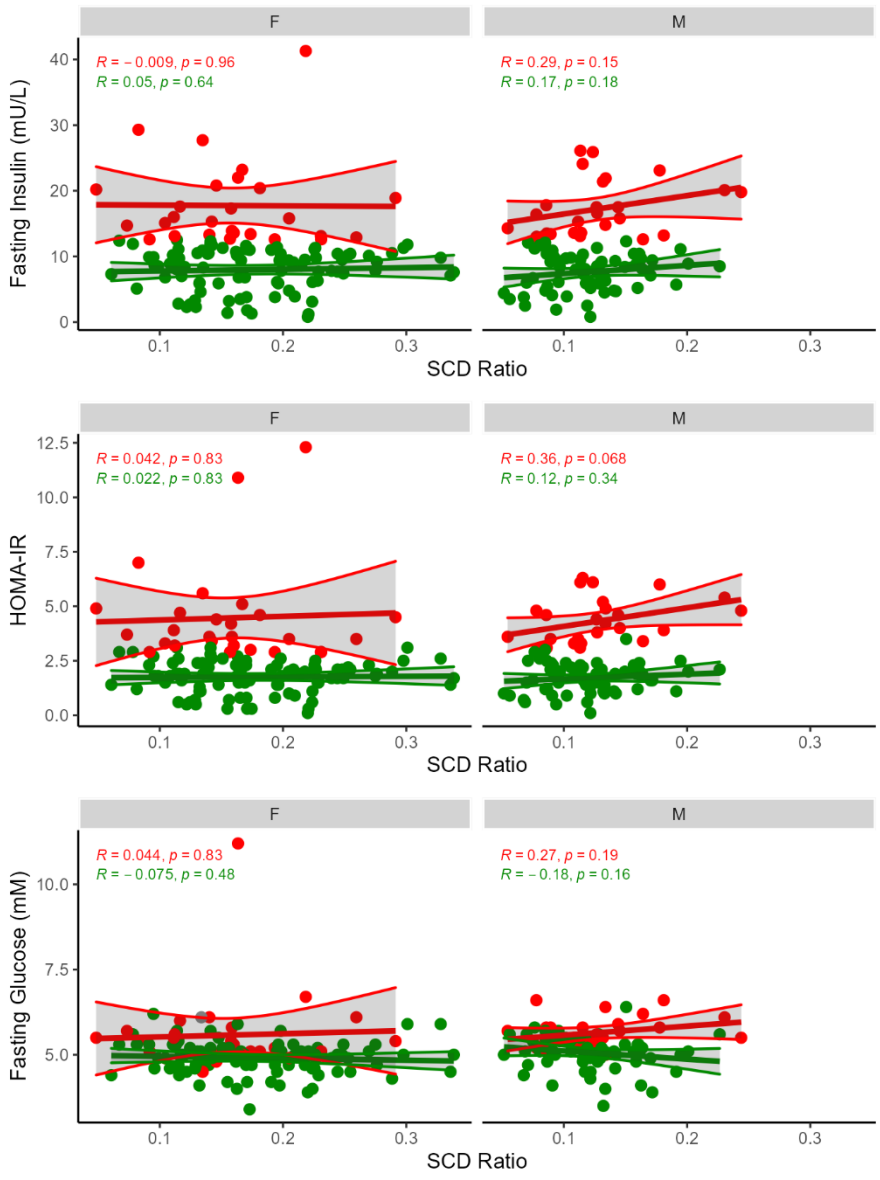


Figure 4-10: Correlations between SCD ratio and measures of insulin sensitivity.

Red: hyper-insulinaemic, green: normo-insulinaemic. R is the Pearson correlation coefficient

4.4 Discussion

Data for the distribution of FAHFAs in the plasma and AT of humans is limited and conclusions about the relationships between circulating FAHFAs and measures of insulin sensitivity are equivocal. I aimed to expand what is known about FAHFAs in humans, particularly in adipose tissue, and to clarify the associations with insulin sensitivity.

FAHFA-NEFAs are not like NEFA in plasma

I found circulating PAHSAs and PAHPAs to be higher in men than in women. Kellerer *et al.* (2021) did not make this same observation when comparing 30 women against 30 men. The two studies did not

look at exactly the same panel of FAHFAs but there is some overlap. Given the equivocation, it is difficult to conclude that the observed sex difference is a real effect which carries through to all FAHFA species or not. However, if true, it hints at some unexpected regulation of FAHFA metabolism; one might presume that FAHFAs would follow a similar pattern to NEFA in plasma but NEFA are higher in women.

Further to that, in men, most of the measured PAHSAs and PAHPAs were negatively correlated with plasma NEFA, while the trends were not significant in women. This suggests that either the release from TAG or the removal by degradation or reuptake of FAHFA-NEFAs are handled differently to those of ordinary FAs, at least in men. The little that is known about release of FAHFAs from TAG, i.e. that ATGL is the key player as it is for FAs, would suggest that release is not the differentiating step. However, I did observe that the OAHSA-NEFAs in the AT samples did not correlate with the OAHSA-NEFA in the plasma so it may be that FAHFAs are released from TAG but do not make it out of the adipocyte in the same way as the FAs. Results in Section 5.3.3 highlight unexpected patterns of release, and there is certainly more to be investigated there to understand the process of FAHFAs leaving the adipocyte. As knowledge is sparse regarding FAHFA degradation or reuptake by AT it is challenging to form a plausible hypothesis around those pathways and how they might operate over a prolonged, overnight fast to create the observed contrast between FAHFA-NEFAs and NEFA. Whatever the regulatory process is which explains this, it is likely to be complex since it is not just the case that FAHFAs don't follow FAs but that they are apparently regulated in the opposite direction.

One of the limitations of this work is that, due to complications in the extraction and analysis of FAHFAs, we have not been able to examine a wide panel of FAHFAs in each stage of the lifecycle (i.e. stored, released early in a fast and released late in a fast) to be able to better answer or hypothesise about the observed divergence between FAHFAs and FAs. However, one of the strengths is the size of the large cohort from which the plasma was drawn and so the observation that PAHSAs and PAHPAs are regulated oppositely to NEFA in fasted plasma is robust enough to encourage more work to unpick it. *In vitro* work to examine the fate of FAHFAs during lipolytic conditions would be useful to clarify both the release from TAG and the export from the adipocyte. *In vivo* work looking at the rates of appearance or clearance from the blood post-prandially and then across a period of fasting would be useful in identifying whether our post-overnight fast samples are representative of the conditions during the period of fasting preceding the measurement of insulin sensitivity.

FAHFAs are not strongly associated with insulin resistance and there are sexual dimporphisms

Yore *et al.* (2014) claimed that insulin sensitive mice and humans had higher circulating PAHSAs, implying that high PAHSA levels were protective with respect to insulin resistance. In the humans, this may actually just have been a case of overweight people having lower PAHSAs and coincidently having lower insulin sensitivity, since Kellerer *et al*. (2021) later found obese people to have lower PAHSAs, and BMI-matched diabetics and non-diabetics to have similar PAHSAs.

I reasoned that, if insulin resistance caused a reduction in plasma FAHFAs, then normo-insulinaemic people would have higher FAHFAs. Alternatively, if FAHFA production or release was upregulated in response to insulin resistance, as a means to maintain glucose uptake, then normo-insulinaemic people would have lower FAHFAs and the plasma concentration of FAHFAs would be positively correlated with fasting insulin.

In men, I found all of the plasma PAHSAs and PAHPAs to be higher in the HI compared to NI participants. However, there was a trend for an inverse relationship between PAHSAs and PAHPAs and insulin insensitivity, albeit this was only significant for one, 10-PAHPA. It is possible that FAHFA plasma concentrations are increased in response to increasing insulin insensitivity but that the increase becomes dysregulated after a certain time; it would be of interest to examine this in a longitudinal study.

In women, though, the HI and NI groups had similar plasma PAHSA and PAHPA levels and, contrary to the males, there were trends for positive associations in the HI group between PAHSAs and PAHPAs and increasing insulin insensitivity. Although these trends were not significant after the removal of an outlier, the direction remained opposite to the male HI participants and suggests another sexual dimorphism; not only do men have higher concentrations of FAHFAs in plasma but FAHFAs display opposing associations with insulin sensitivity between the sexes which may point to differences in regulation/metabolism of FAHFA between men and women.

Plasma FAHFAs are not lower in obese people

Although Kellerer *et al*. (2021) found total plasma PAHSAs and 9-PAHPA to be lower in morbidly obese compared to lean subjects, I did not find this to be the case. When correlating the PAHSAs and PAHPAs with BMI, body mass or fat mass, the trends tended to be weakly positive but significant associations were only observed in women. There were only significant between-group differences between healthy-weight and obese participants when looking at 12/13-PAHSA and 12/13-PAHPA (in both men and women). It may be that my data differ from Kellerer et al.'s (2021) because their obese

participants were waiting for bariatric surgery and had BMI> 40 $kg.m^{-2}$, whereas my participants were healthy, with BMIs < 35 $kg.m^{-2}$. My data are also different to Yore *et al.'s* (2014) despite the BMI ranges of our groups being similar and this can't be explained by the differences in insulin sensitivity between our groupings, given my findings above. In favour of my results is that the cohort is larger, with a broad spectrum of BMI's and without any specific disease state, so I believe that the data are a good representation of a European population.

FAHFA concentrations may not be the same between adipose tissue depots

In most of the six OAHSA-NEFAs examined, the concentrations were lower in the gluteal AT compared to abdominal and this was most pronounced for females. These OAHSAs may be mostly made up of those FAHFAs recently liberated from TAG with some coming from re-uptake from the plasma. Unfortunately, I was not able to measure the FAHFAs stored in TAG which would be required to confirm the result and its significance.

The lower concentrations of OAHSA-NEFA in gluteal AT may not reflect the OAHSA-TAG content of the tissue since gluteal AT is less responsive to lipolysis during fasting (Martin and Jensen, 1991) and so, even if both depots had the same OAHSA-TAG concentration, they may not have the same OAHSA-NEFA concentration due to differences in the lipolysis of that stored OAHSA-TAG. However, if the OAHSA-NEFA concentration is proportional to OAHSA-TAG content, then the fact that the gluteal tissue takes up more recirculated FAs from VLDL while the abdominal tissue takes up more dietary fat (McQuaid *et al.*, 2010), may be a cause of the observed depot difference; however more would need to also be known about synthesis to fully understand the differences. I have shown in Section 5.3.1 that both depots, , are capable of similar synthesis rates of 12-PAHSA when provided excess substrate, so differences between substrate availability in the depots may be important to creating this difference in OAHSA-NEFA concentrations between the depots, assuming that the synthesis of other FAHFAs is similar to 12-PAHSA.

The differences that I have presented in this chapter in FAHFA concentrations between the gluteal and abdominal depots suggest that it is important to consider both SAT depots when studying FAHFA metabolism. For example, if only abdominal AT was biopsied for analysis, as is common in AT research, then one might overestimate the OAHSAs in the body or underestimate other FAHFAs which may be more abundant in gluteal tissue.

FAHFAs are not regulated like palmitoleate

I found FAHFAs not to be correlated with palmitoleate, which follows the pattern of FAHFAs being negatively correlated with NEFA. As with other reports (Ramos, Bush and Jensen, 2020), I found

palmitoleate to be higher in women than in men and not to be associated with insulin sensitivity. This latter result adds to the data that suggests that palmitoleate isn't relevant to insulin sensitivity, while there remain several reports which suggest that it is. Common to those is that the correlations between fasting palmitoleate and measures of insulin resistance are not very strong and, I speculate, that while palmitoleate may have a positive role to play in control of glycaemia, it is just one actor in a field of many other actors (including FAHFAs and FAs that can bind to FFAR4) and, consequently, the effect of any one of them is challenging to differentiate *in vivo*.

4.4.1 Summary

Having set out to generate a more comprehensive picture of the distribution of FAHFAs in plasma and SAT depots in humans and of their relationship to other health markers, I have made some interesting observations whilst being disappointed that technical difficulties have limited the number of FAHFA families that I have been able to examine.

An unexpected but significant finding which merits further investigation is that FAHFAs, despite being stored in TAG and released from there by ATGL, just like FAs, are not positively correlated with FAs in fasting plasma, suggesting alternative regulation of release or turnover. The factors relating to FAHFA handling are likely to be complex and understanding whether or not this is specific to the timing of the fasted samples or persists across the feeding/ fasting cycle would be a important to define.

The recurring theme of my data is that the metabolism of FAHFAs is different between men and women. This difference may not be the result of sex hormones, since I saw no differences in circulating levels between pre- and post-menopausal women. The differences mean that male and female subjects should be considered separately and the donor's sex must be considered when interpreting future *in vitro* work.

Similarly, the data suggest that there may be differences in FAHFA metabolism between the SAT depots and this needs to be investigated more deeply, with the depots being examined separately.

Chapter 5
FAHFA Synthesis, Storage, Release and Transport

5 FAHFA Synthesis, Storage, Release and Transport

5.1 Introduction

5.1.1 Background

It has been suggested that FAHFAs are beneficial to health, metabolic or otherwise, but, if they were to find a use as a pharmacological agent or if there was a desire to enhance endogenous synthesis, there is a need to better understand the FAHFA lifecycle.

The understanding of FAHFA synthesis has been progressed recently by Paluchova *et al.* (2020) and Patel *et al.* (2022) who found that, in mice, ATGL was critical for the synthesis of FAHFAs in AT, where it catalyses the esterification of the fatty and hydroxy-fatty acids- see 1.2.10. It is not confirmed, though, whether ATGL is critical in humans, nor is it known whether ATGL is the sole transacetylase capable of fulfilling the role or if it is also the key enzyme in liver tissue.

It is well established that FAHFAs are stored in di- and triglycerides (see 1.2.9) and that ATGL is also responsible for that incorporation. However, given the range of FAHFA size and structure, it is conceivable that some are preferentially incorporated into TAG but the compositional profiles of FAHFAs in TAG and free FAHFAs in plasma have not been compared.

Although the release of FAHFAs from their storage depots has not been directly studied, it has been seen that lipolytic conditions (including fasting and cold exposure) promote an increase in plasma FAHFA concentrations (Paluchova *et al.*, 2020; Yore *et al.*, 2014).

Although several facts about FAHFA synthesis in mice have been determined, when viewing the story from a human perspective, much still needs to be confirmed. Moreover, when considering the importance of body fat distribution in human metabolic health, it will be important to uncover any differences in synthesis and handling which may occur in the different AT depots.

Finally, it remains unclear how FAHFAs are transported in the blood; something unstudied but critical for FAHFAs to be moved from adipose or hepatic tissue to their various potential points of action. The poor solubility of NEFAs in aqueous solutions means that the vast majority of them in plasma are bound to albumin (van der Vusse, 2009). The even poorer solubility of FAHFAs means that there must be a similar transport mechanism but, while it is a logical assumption, it is not known if albumin performs this task as well. Esterified FAs are also transported as TAG in lipoprotein particles and FAHFAs may also be present there.

5.1.2 Aims

The aims of this work were to better characterise FAHFAs in humans and the individual SAT depots by, specifically:

1) Determining if there is a difference in the synthetic capacity of abdominal and gluteal adipocytes and to elucidate the reasons for any eventual difference,
2) Measuring the compositional profiles of FAHFAs in abdominal and gluteal tissues,
3) Confirming that there is no preference for exporting particular FAHFAs to the blood,
4) Confirming that albumin has the capacity to transport FAHFA-NEFA and to explore whether or not the liver exports FAHFA-TAGs in lipoproteins.

5.2 Methods

5.2.1 General Methods

The work described in this chapter utilised the following general methods detailed in Chapter 2:

- Human Adipose Tissue Biopsy and Preadipocyte Isolation (2.1)
- Maintenance of Cells in Culture (2.2)
- Protein Quantification Assay (2.6)
- Gene Expression Assays by qPCR (2.8)
- FAHFA *In Vitro* Biosynthesis Assays (2.10)
- Co-Immunoprecipitation of BSA and Conjugated Fatty Acids or FAHFAs (2.11)
- Extraction of FAHFAs from Plasma or Lysates for Liquid Chromatography (2.12)
- Extraction of FAHFAs from Adipose Tissue for Liquid Chromatography (2.13)
- Liquid Chromatography and Mass Spectrometry for Quantification of FAHFAs (2.15)

5.2.2 Specific Methods

mRNA Expression

MBD5 preadipocytes were differentiated over the course of 12 days with samples collected on days 6, 8, 10 and 12. The following Taqman assays were used for the mRNA expression assessment (*GENE*- product code):

- *PNPLA2 (ATGL)*- Hs00386101_m1

Plasma Samples

The plasma samples for the A-V difference study are described in 4.2.2 and additional samples were received from Dr Konstantinos Manolopoulos (University of Birmingham). All had been stored at -80 °C.

FAHFA Extractions and LC-MS

As described in Chapter 4 the requirement to optimise the protocols to separate individual isomers of a few families, due to the poor sensitivity of the LC-MS machine, meant that only PAHSAs, PAHPAs and OAHSAs could be measured with confidence and this was a limitation to the aims of the study.

Analysis of Mass Spectrometry Data

Data were viewed and analysed using MassLynx 4.1 (Waters). Relevant peaks were identified by comparison to an artificial standard. Peak area was generated automatically and normalised to the peak area of an internal standard (5-PAHSA, Cayman 17404).

Statistics

Data were processed in Excel and R. Differences between two conditions were assessed by Welch's t-tests. Differences between two conditions over a course of time were analysed by two-way ANOVA. Figures were produced in R using the ggplot2() package.

5.3 Results

5.3.1 Synthesis of FAHFAs by Abdominal and Gluteal Adipocytes *in vitro*

LC-MS analysis of differentiated MBD5 cell lysates, incubated for 2 h with the substrates of 12-PAHSA (Figure 5-1) showed that there were no differences in the synthetic capacity of abdominal and gluteal cells (Figure 5-2). Furthermore, synthesis depends on the machinery of mature adipocytes, since undifferentiated preadipocytes synthesise virtually nil (Figure 5-3).

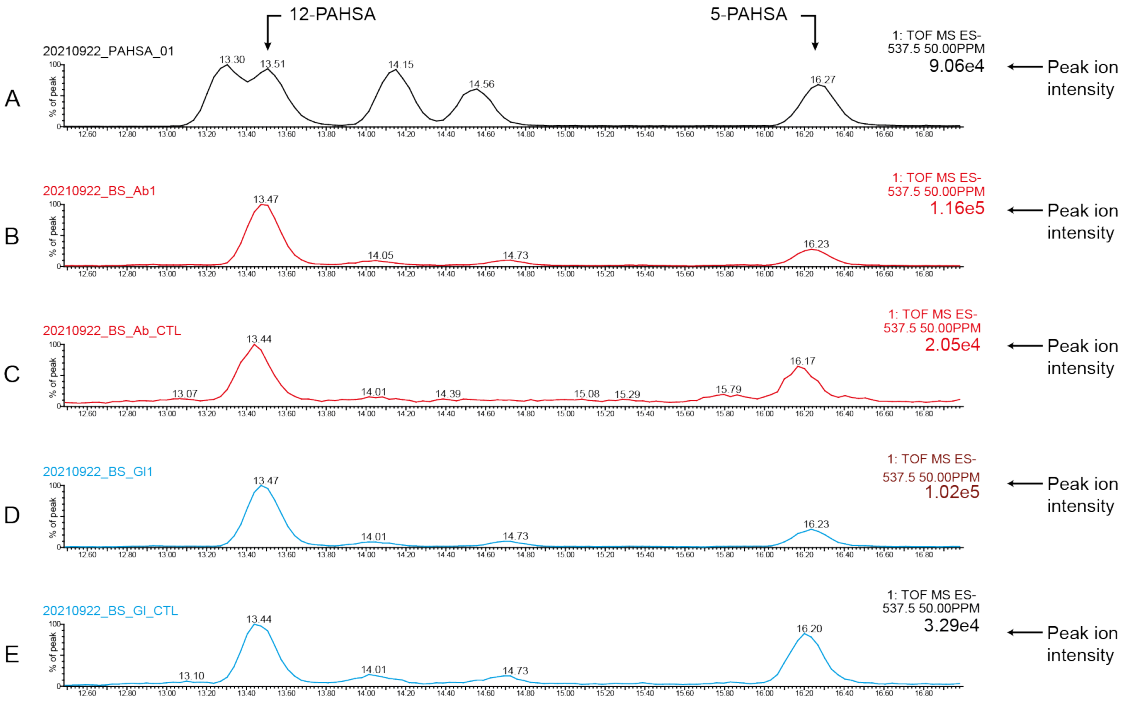


Figure 5-1: Representative trace from LC-MS analysis examining differences in FAHFA synthetic capacity between differentiated abdominal and gluteal MBD5 adipocytes.

Cells were differentiated for 10 days prior to lysis and 2 h incubation with 12-hydroxyoctadecanoic acid and palmitoyl-coenzyme-A. Control lysates were boiled for 10 minutes prior to incubation. Trace A- PAHSA standard. Trace B- abdominal cell lysate. Trace C- boiled abdominal cell lysate. Trace D- gluteal cell lysate. Trace E- boiled gluteal cell lysate.

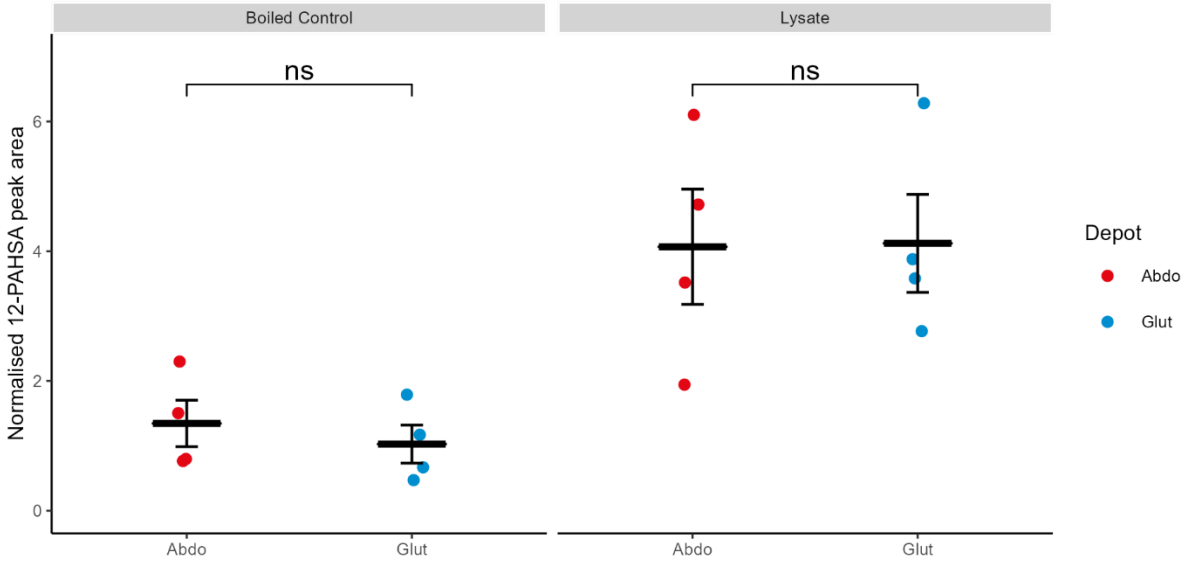


Figure 5-2: Differentiated abdominal and gluteal MBD5 adipocytes synthesise similar amounts of 12-PAHSA given the same substrate.

Horizontal bars are means and error bars represent SEM. ns indicates p>0.05 according to Welch t-test between depots. Cells were differentiated for 10 days prior to lysis and 2 h incubation with 12-hydroxyoctadecanoic acid and palmitoyl-coenzyme-A. Control lysates were boiled for 10 minutes prior to incubation. 12-PAHSA peak area normalised to that of 5-PAHSA standard. N=4 for each depot.

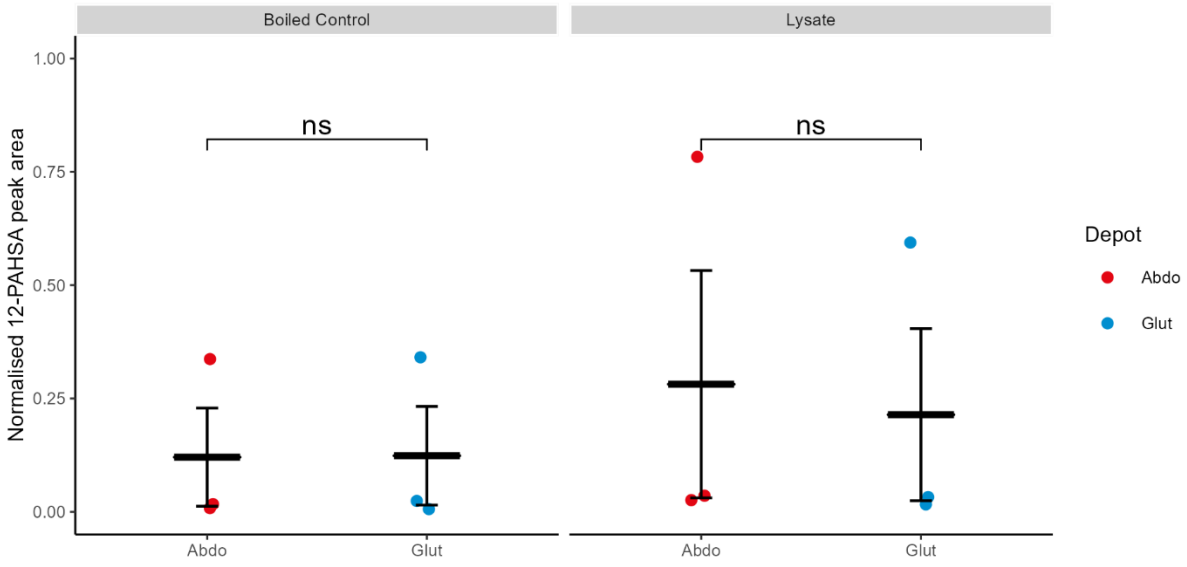


Figure 5-3: Undifferentiated abdominal and gluteal MBD5 preadipocytes do not synthesise 12-PAHSA given excess substrate.

Horizontal bars are means and error bars represent SEM. ns indicates p>0.05 according to Welch t-test between depots. Lysates were incubated for 2 h with 12-hydroxyoctadecanoic acid and palmitoyl-coenzyme-A. Controls were boiled for 10 minutes prior to incubation. N=3 for each depot.

Given the recent findings regarding ATGL and its proposed role in FAHFA synthesis, I measured the expression of *PNPLA2* (the ATGL gene) mRNA across differentiation in abdominal and gluteal MBD5 cells and found that, although there were depot-differences in mRNA expression of *PNPLA2* at Day 10, there were no overall differences between the two depots over the course of differentiation (Figure 5-4). These observations fit with the observed lack of difference in synthetic capacity between the depots. In a more limited data set of primary cells, *PNPLA2* expression was not different between abdominal and gluteal adipocytes at day 12 of differentiation (Figure 5-5).

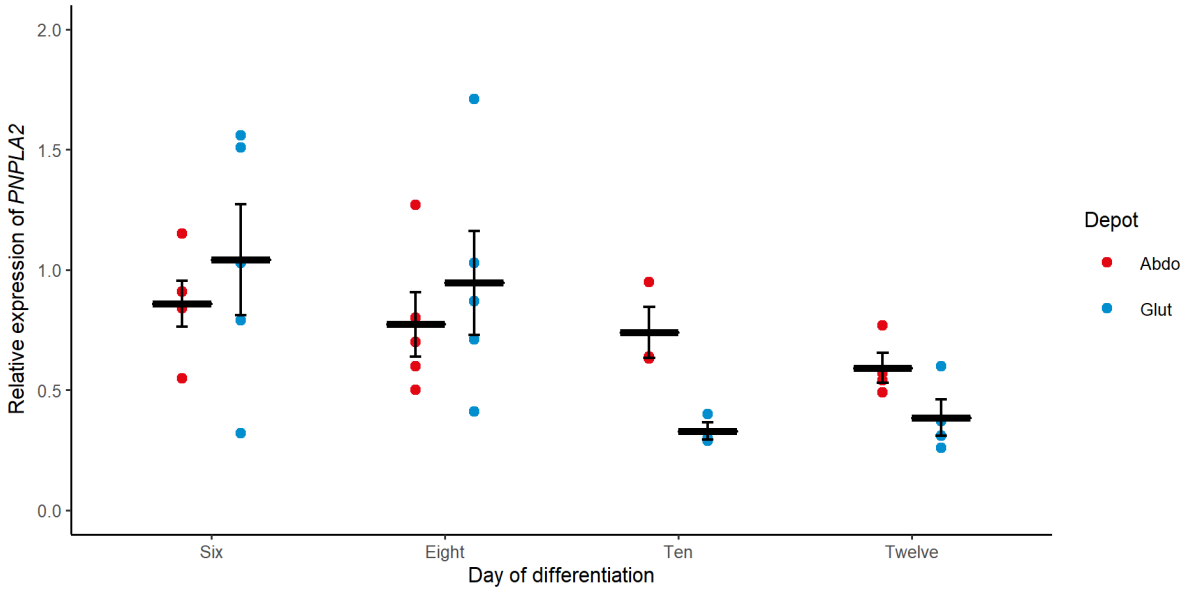


Figure 5-4: *PNPLA2* (ATGL) expression over the course of differentiation is not different between abdominal and gluteal MBD5 adipocytes.

Horizontal bars are means and error bars represent SEM. N=5 (3 at Day 10).

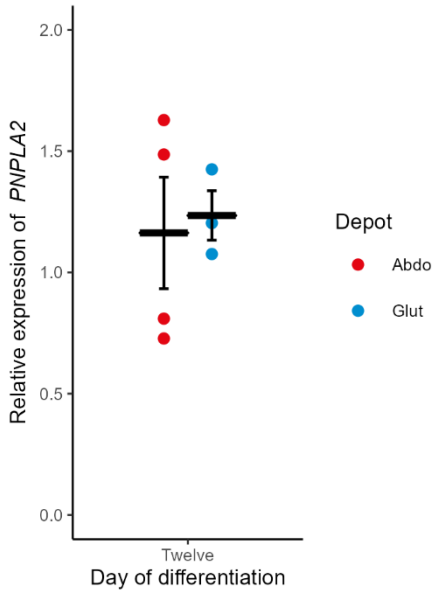


Figure 5-5: *PNPLA2* (ATGL) expression at day 12 of differentiation is not different between abdominal and gluteal primary adipocytes.

Data presented as means (horizontal bars) and SEM. N=4 (1 female)

5.3.2 Transport of Non-esterified FAHFAs

In order to determine whether or not FAHFAs do bind to albumin, I carried out co-immunoprecipitations of a solution of BSA and 5-PAHSA, using an anti-BSA antibody, and examined the eventual BSA eluent for the presence of 5-PAHSA by LC-MS.

I observed that 5-PAHSA binds to BSA and was absent in the control samples which lacked the antibody which binds the BSA to the magnetic beads. The chromatogram for one of the three experimental sets demonstrating this is shown in Figure 5-6. Trace A has a clear, single peak at the same time point as the 5-PAHSA in the standard (Trace C). Trace B is a control sample and although it had a peak as the same time point, the magnitude was considerably reduced (circa two thousand times smaller). It is likely that this peak is residual 5-PAHSA which was not completely washed from the beads during the co-immunoprecipitation.

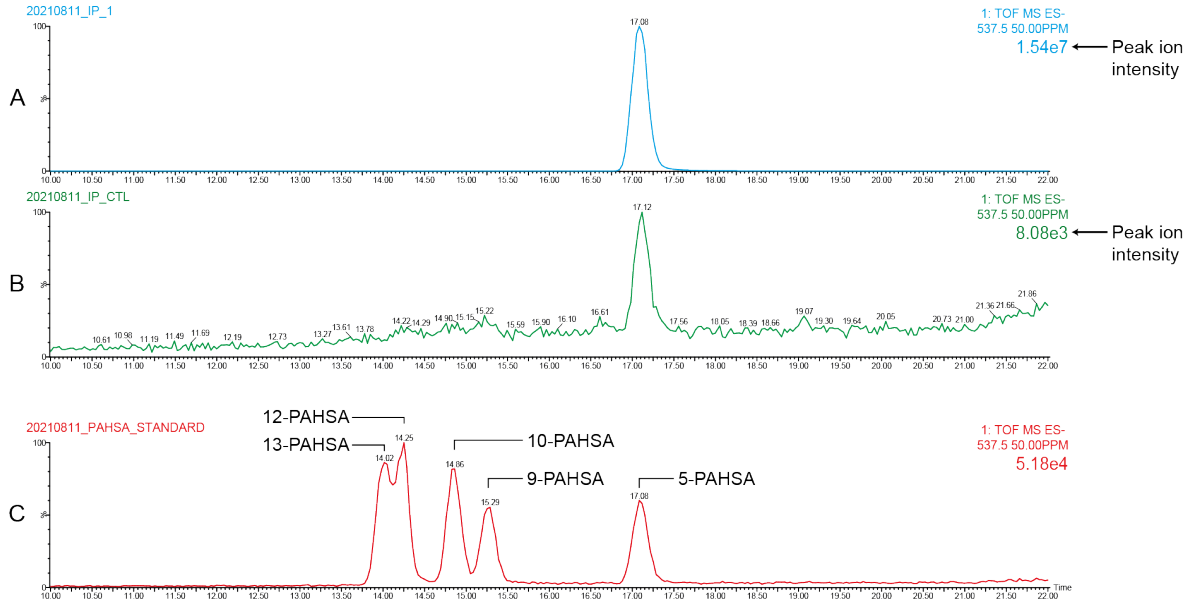


Figure 5-6 LC-MS detects that 5-PAHSA binds to bovine serum albumin (BSA) in co-precipitated samples.

Trace A shows a single peak in the sample at 17.08 min and with an intensity of $1.54x10^7$. Trace B shows a peak in the control sample at the same time with an intensity 4 orders of magnitude less at $8.08x10^3$. Trace C is a standard made up of five PAHSA species including 5-PAHSA at 17.08 min. x-axis shows time in minutes, y-axis shows relative ion intensity

5.3.3 Arterio-Venous Difference Studies Reveal Unexpected Patterns of Uptake and Release

To investigate the pattern of release of FAHFAs from AT, I examined the samples from 26 of the participants (12 female) described in section 4.3.1 for whom we had arterial and venous plasma samples, where the cannulated vein was the epigastric, draining the abdominal SAT. Panel A of Figure 5-7, shows that total NEFA concentration was approximately two-fold higher in the venous plasma than the arterial, in line with what has previously been reported in the fasting state (Frayn and Humphreys, 2012). This is the result of lipolysis releasing NEFA from adipose TAG. In contrast, 9-PAHSA, was found to be significantly lower in the venous compared to arterial plasma of women, suggesting uptake by the adipose tissue (Figure 5-7, Panel B). For all the PAHSAs and PAHPAs, with

the exception of 9-PAHPA, the mean values of the venous plasma were lower, although not significantly, than the arterial values, suggesting no net release.

In order to see if this phenomenon of 9-PAHSA uptake was also present in the gluteal SAT depot, I utilised the fasting plasma samples of five male participants provided by Dr Konstantinos Manolopoulos (University of Birmingham), which included venous plasma from both the abdominal and gluteofemoral depots and matched arterial plasma. I measured PAHSAs in these samples and found that neither the abdominal nor gluteal venous concentrations to be different to the arterial (Figure 5-8, Panels A to C), indicating no net release but also no uptake as with 9-PAHSA in the larger cohort. In the gluteal vein, 10-PAHSA was higher than the abdominal vein (Figure 5-8, Panel B). There were no differences between the respective plasma fractions for OAHSAs.

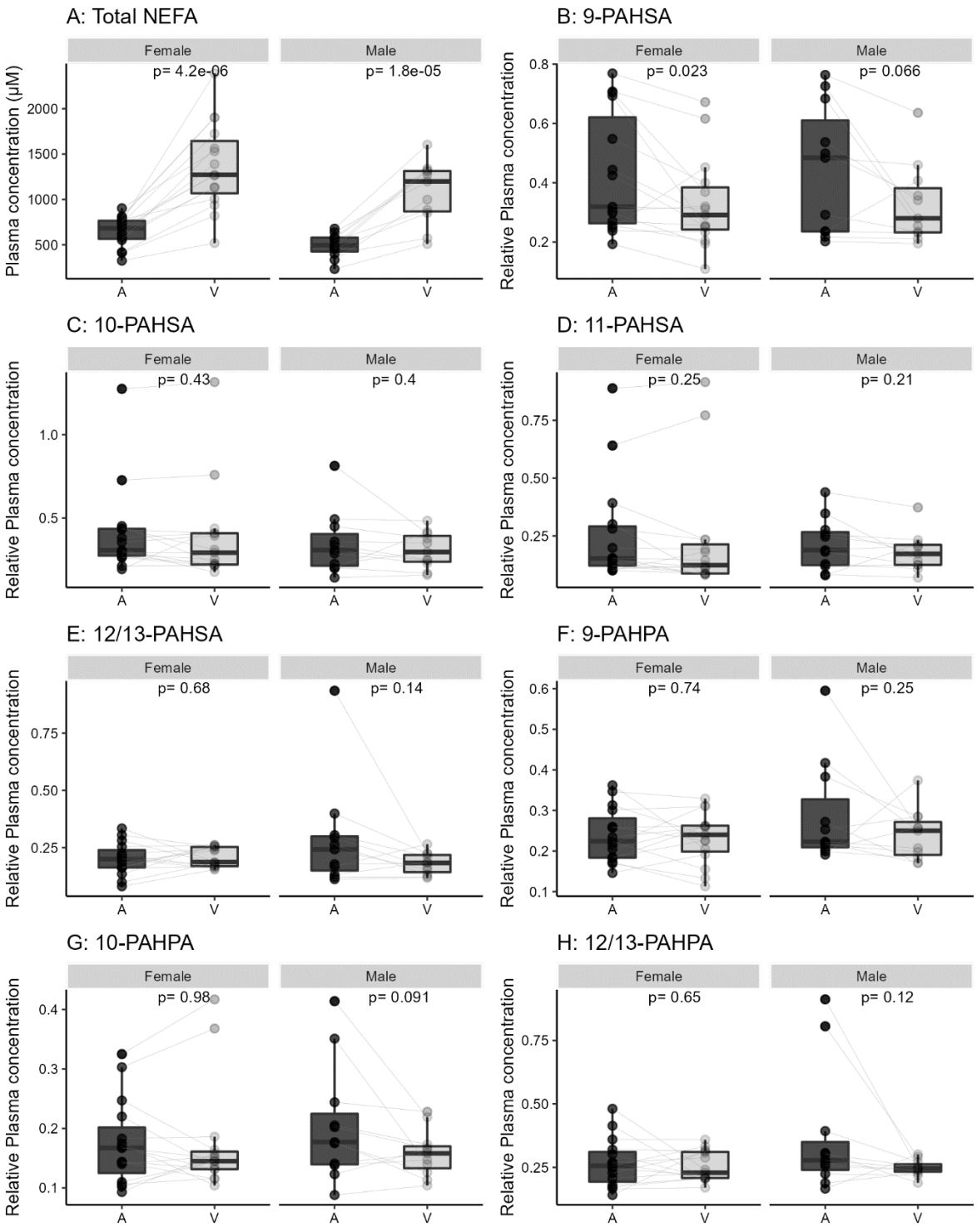


Figure 5-7: Arterial and venous concentrations of PAHSAs, PAHPAs and NEFA.

FAHFA concentrations expressed as spectrographic peak height relative to peak height of internal standard. Connecting lines indicate paired samples. p-values derived from paired Welch's t-tests. A: arterial. V: venous. N=26.

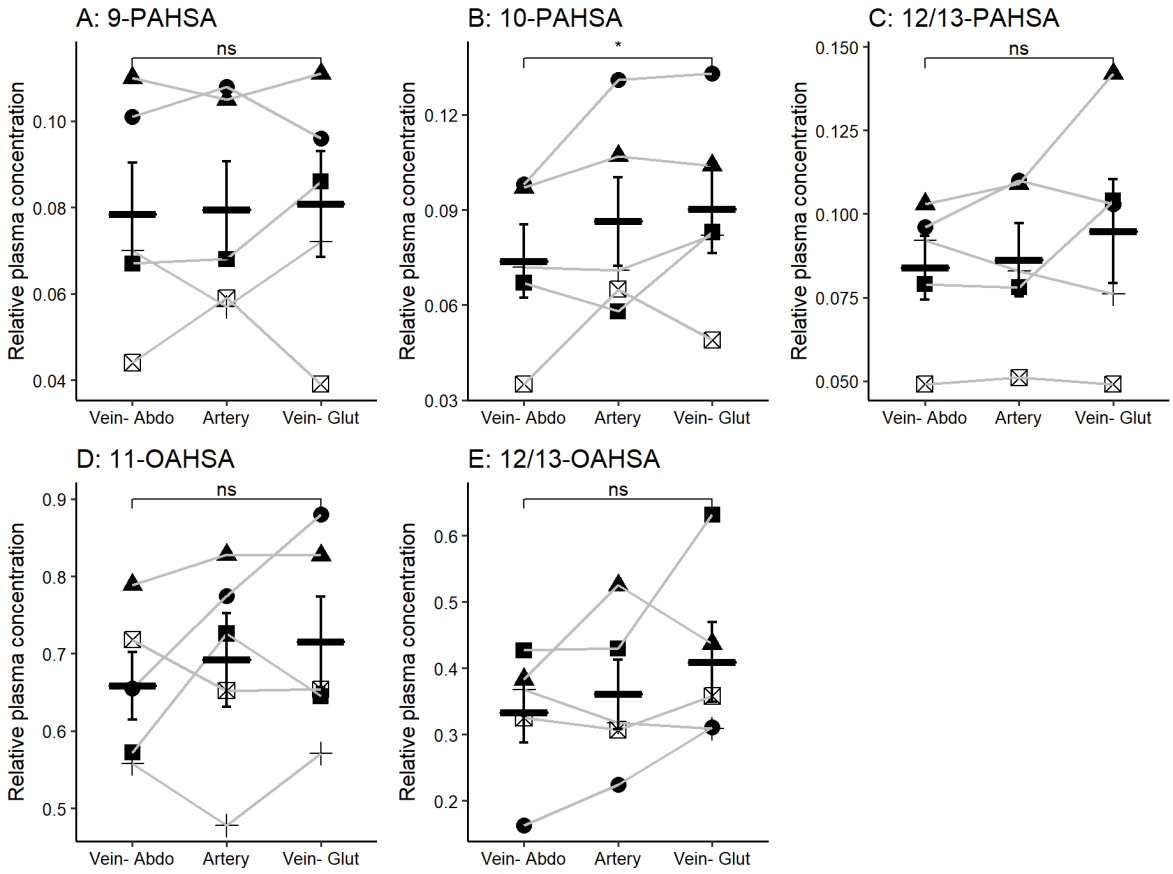


Figure 5-8: Abdominal and gluteal arterial and venous concentrations of PAHSAs and OAHSAs

FAHFA concentrations expressed as spectrographic peak height relative to peak height of internal standard. Connecting lines indicate paired samples. Horizontal bars represent the mean and error bars the SEM. p-values derived from one-way ANOVA. N=5

5.4 Discussion

Having a clearer insight in to the lifecycle of FAHFAs in humans is important since, to date, the only work in humans has been observational, with mechanistic studies limited to mice. I set out to identify differences in the synthesis, storage and release of FAHFAs from the abdominal and gluteal SAT depots and to confirm that albumin is capable of transporting FAHFAs in the blood.

I found that there were no differences in synthesis between depots, that albumin can bind FAHFAs and that the release of FAHFAs during fasting in humans is not regulated in the same way that the release of NEFA is. Due to technical complications, I was not able to examine the profile of FAHFAs stored in the two depots.

5.4.1 FAHFA Synthesis

My results suggest that abdominal and gluteal adipocytes have a similar capacity to synthesise FAHFAs *in vitro* given excess substrate of fatty and hydroxy-fatty acids. This synthesis is suggested

to be undertaken by ATGL (Patel *et al.*, 2022) and I found that the expression of the ATGL-encoding gene, *PNPLA2*, was also not different between depots in the MBD5 cells when aggregated over the course of differentiation. This mRNA expression might be expected to reflect protein expression and hence enzyme activity at the end of differentiation. Although more limited, a small set of male-dominated primary adipocyte data also suggested no difference in *PNPLA2* expression between depots, like the female MBD5s. Further work confirming the role of ATGL in FAHFA metabolism in humans, replicating Patel et al.'s (2022) murine studies where ATGL is knocked out, inhibited or overexpressed may be useful to confirm if it is indeed the primary actor in transacylation of fatty acids and hydroxy-fatty acids and whether ATGL (dys)function results in metabolic defects which may be linked to FAHFAs.

In their report, Patel et al. (2022) point to research that ATGL is downregulated in insulin resistant overweight or obese subjects, suggesting that the insulin resistance may result from lower FAHFA synthesis (Jocken *et al.*, 2007). At the same time, they explain that their own ATGL-KO mice are not insulin resistant because the lack of ATGL means less lipolysis and hence less glycerol availability for hepatic gluconeogenesis. Whilst either argument is plausible, together they are in contradiction. Jocken's data could be explained by the fact that the insulin resistant subjects have high circulating insulin and insulin inhibits ATGL transcription and activity (Kim *et al.*, 2006), which might lead one to predict that insulin resistant subjects have low FAHFAs, which matches Yore *et al.* (2014) but contradicts Kellerer *et al.* (2021) and my own work in Chapter 4. There seems to be a need to draw these threads together into a single, human, study measuring FAHFAs and ATGL in insulin sensitive and insensitive, obese subjects to get a clear picture of what effect reduced ATGL might actually have on circulating FAHFAs.

This work has not touched on the source of the HFAs required by ATGL to synthesise FAHFAs. Although Kuda *et al.* (2018) proposed a method which may explain the source of some of the HFAs, (see section 1.2.10) there remain opportunities to expand on that work.

5.4.2 FAHFA Storage and Release

Since different FAHFAs may have different affinities for the FFARs, it is important to understand which FAHFAs humans store in SAT and if they are released uniformly or with particular preferences. To date this has not been measured in humans. Furthermore, it would be useful to know if there are differences between the abdominal and gluteal SAT depots since differences in that may partly explain differences in health outcomes for people with different body fat distribution.

With regards to the release of FAHFAs, the best method to examine this is using arterio-venous sampling to compare the concentration of FAHFAs in the artery entering the adipose tissue and in the vein leaving the adipose tissue. Due the complexities of carrying out these procedures, I relied on historical samples from past studies which were slightly limited in their number. Nonetheless, I found that the release of FAHFAs from SAT in fasting humans does not follow the same pattern as that of NEFA. In our abdominal samples (n=26), 9-PAHSA appears to be taken up by abdominal SAT at the time when NEFA are being released. The other PAHSA and PAHPA species behave equivocally with no significant differences between the arterial and venous concentrations. The 9-PAHSA result was not replicated in a smaller (n=5) collection of samples analysed at a later date on a different machine but, again, there was no obvious release of the examined PAHSAs and OAHSAs from either SAT depot. This is unexpected since fasting, for example, has been shown to increase FAHFA levels in mice (Yore *et al.*, 2014) but it does match the data described in Chapter 4. It is not clear as to whether or not this is due to a difference between mice and humans, to the concentrations and release rates being too low to detect differences or to a fundamentally different mode of regulation for FAHFAs compared to NEFA. Longer studies with fasting baseline, test meal and postprandial measures at intervals would be useful in order to get a clearer idea of how plasma levels vary with insulin and glucagon.

With regards to storage, my aim had been to measure the FAHFA-TAG in the matched abdominal and gluteal tissue samples of the study participants in order to identify differences in the compositional profile of stored FAHFAs. However, as a result of the time constraints imposed by COVID-19, I was not able to successfully optimise the hydrolysis of FAHFA-TAG for the reasons described in 4.3.2 and thus could not complete that part of the study. The samples are stored and wait for future analysis.

5.4.3 FAHFA Transport in the Blood

I have demonstrated that non-esterified FAHFAs can be bound to albumin and thus can be transported in the blood in the same way as NEFAs. Albumin has seven NEFA binding sites but different NEFA have different binding affinities and the occupation of a site can cause conformational changes to albumin which preclude the use of some of the remaining sites (van der Vusse, 2009). It would be useful to know the affinities of the various FAHFAs to albumin and their effect on the carrying capacity of albumin in order to further understand how much FAHFA can be transported in blood, since this may present an upper limit which could make it impossible to achieve a concentration significant enough to have the pharmacological effects which might be desired.

Since FAHFAs can also be synthesised in liver and transported there from AT, it could be assumed that they are esterified into TAG and exported from the liver as part of a very low density lipoprotein

(VLDL). I had hoped to confirm this by mass spectrometry of FAHFAs extracted from the VLDL fraction of plasma using the TAG hydrolysis method described in 2.12 but this proved challenging for the reasons outlined in 4.3.2 and I was unable to detect traces in the final sample.

5.4.4 Summary

I have found that synthesis does not appear to be different between the abdominal and gluteal SAT depots *in vivo,* which extends the previous mouse data into humans. I have also demonstrated that if exported to the blood, FAHFAs can be carried by albumin but I was not able to investigate the presence of FAHFAs in liver-derived VLDL.

By using arterio-venous difference measurements, I have identified that FAHFAs may not follow the same regulation of release as NEFA during fasting as previously thought (Figure 1-3). The evidence is not categorical but strongly suggests behaviour which warrants further investigation. If their release is regulated by some other mechanism than simple lipolysis, as NEFA, then this needs to be understood in order to properly understand how FAHFAs might influence glycaemia, for example.

Chapter 6
The Action of FAHFAs Via Free Fatty Acid Receptors

6 The Actions of FAHFAs via Adipose Free Fatty Acid Receptors

6.1 Introduction

FAHFAs have been shown to exert some of their effects through binding and activating the cell-surface free fatty acid receptors, FFAR1 and FFAR4. In this chapter, I would explore the AT depot-specific expression of the receptors and the effects of FAHFA agonism of the receptors on lipolytic and lipogenic processes in adipocytes.

6.1.1 Free Fatty Acid Receptors

The four free fatty acid receptors (FFARs) are cell membrane-bound G-protein-coupled receptors (GPCRs) whose agonists are FAs and FAHFAs. FFARs 2 and 3 are activated by SCFAs and are, thus, unlikely to be relevant to the study of FAHFAs but both FFAR1 (sometimes referred to as GPR40) and FFAR4 (sometimes referred to as GPR120) have been reported as being agonised by FAHFAs (Yore *et al.*, 2014; Syed *et al.*, 2018).

As members of the large GPCR family, the FFARs have the capacity to interact with four groups of G_{α}-protein subunits, with different signalling outcomes, depending on which G_{α} subunit is involved. Additionally, GPCRs can bind β-arrestin which can act to internalise and thus desensitise the receptor and also facilitate alternative signalling pathways (see Figure 1-4). In their recent review, Kimura *et al.* (2020) identified a list of studies which had identified FFAR1 as binding with $G_{\alpha q}$, $G_{\alpha i}$, $G_{\alpha s}$ proteins and β-arrestin and identified FFAR4 as binding with the $G_{\alpha q}$ protein and β-arrestin in various tissues. This means that the FFARs have the potential to modulate cell function by increasing intracellular calcium ($G_{\alpha q}$), decreasing intracellular cAMP ($G_{\alpha i}$), increasing intracellular cAMP ($G_{\alpha s}$), activating ERKs ($G_{\alpha q}$), activating Akt ($G_{\alpha q}$) or interfering with TAK1-mediate inflammation (β-arrestin). Few of these FFAR-linked pathways have been demonstrated in action in adipocytes.

Via the G_{α}-mediated pathways, several roles have been identified for the FFARs in different tissues including, for example:

- On the tongue, dietary fats are detected by FFAR4 (Galindo *et al.*, 2012)
- In the gut, dietary fats activate FFAR1 trigger the release of GLP-1 which, in turn, causes insulin release from pancreatic beta cells (Syed *et al.*, 2018; Sundstrom *et al.*, 2017)
- In the lungs, FFAR4 activation leads to relaxation of smooth muscle and relief of inflammatory symptoms (Prihandoko *et al.*, 2020)
- In adipose tissue macrophages, FFAR4 agonists promote anti-inflammatory M2 macrophages over the inflammatory M1 type (Oh *et al.*, 2010), and
- In WAT, FFAR4 mediates glucose uptake through GLUT4 (Oh *et al.*, 2010)

These examples could all be considered beneficial to health and the FFARs 1 and 4 have been considered for pharmacological targeting, particularly with the aim of reducing blood glucose. Natural agonists such as omega-3s from fish oil have been used experimentally but, as noted by Oh *et al.* (2014), the volume of fish oil consumption required for significant effects is impractical in a normal diet. Several synthetic agonists and antagonists have been developed as alternatives (Milligan *et al.*, 2017) but, to date, no clinical trial has been completed with a suitable drug for human use. These synthetic drugs are, however, useful for *in vitro* work investigating the interaction of FAHFAs and FFARs.

6.1.2 Free Fatty Acid Receptors in Human Adipose Tissue

FFAR4 is known to be expressed in mature human adipocytes (Gotoh *et al.*, 2007) and a small study (n=6 healthy-weight males) showed that mRNA expression was not significantly higher in subcutaneous abdominal than in gluteal tissue (Amisten *et al.*, 2015). FFAR1 mRNA expression has been investigated in man but found to be absent in pre- and mature adipocytes (Gotoh *et al.*, 2007). FFAR3 has been reported in whole human AT but the depot was not specified, while FFAR2 was absent from that same depot (Brown *et al.*, 2003).

FFAR4 expression may be altered by obesity but results in humans are contradictory. Rodriguez-Pacheco *et al.* (2016) found higher mRNA and protein expression in VAT of lean compared to obese fasted subjects but expression in SAT was not associated with obesity status. They also showed that expression was increased in isolated primary visceral adipocytes from lean subjects when exposed to various FAs but only by linolenic acid in visceral adipocytes from obese subjects (linoleic acid was also the most potent FA in the adipocytes from lean subjects). In contrast, Ichimura *et al.* (2012) found obese subjects to have greater FFAR4 mRNA expression in SAT and omental tissue. In mice, lean animals have higher expression than obese (Satapati *et al.*, 2017).

The contrast between Ichimura *et al.*'s results and Rodriguez-Pacheco *et al.*'s may be due to their fasting status, since this is not reported for Ichimura *et al.*'s subjects, but it is not clear whether that would explain it fully. Rodriguez-Pacheco *et al.*'s fasted subjects had higher plasma NEFA and lower *FFAR4* in VAT while their VAT preadipocytes received exogenous NEFA for 24 h, to simulate the elevated NEFA levels in fasting, and that resulted in higher FFAR4 expression. Furthermore Rodriguez-Pacheco *et al.* fed subjects a meal, which ought to depress NEFA levels in the blood but they didn't report a measurement, and found higher expression of *FFAR4* in peripheral blood mononuclear cells.

Taken together, there is not a convincing picture that *FFAR4* expression is altered by obesity and, although it seems that circulating NEFA may play a role in regulation, it is not clear what that is in white adipocytes from either the abdominal or gluteal depots. This deserves closer attention, particularly with respect to differences between the SAT depots.

6.1.3 Effect of FAHFAs on Adipose Tissue

With acute treatment *in vitro*, FAHFAs have been shown to mimic insulin in increasing GLUT4 translocation in murine cells (Yore *et al.*, 2014) and inhibiting lipolysis (Zhou *et al.*, 2019) in murine AT explants. A similar anti-lipolytic effect of a synthetic FFAR4 agonist has been reported in mice (Satapati *et al.*, 2017) while both synthetic and lipid (omega-3s) agonists have had insulin-sensitising effects in mice *in vivo* and promoted GLUT4 translocation in murine cells *in vitro* (Oh *et al.*, 2010). Oh *et al.* (2010) showed that knocking down $G_{\alpha q}$ prevented the GLUT4 effect and, since $G_{\alpha q}$ would also be responsible for lipolysis inhibition, this may explain the anti-lipolytic effects as well. However, Paluchova *et al.* (2020) found that 5-PAHSA enhanced lipolysis in murine cells *in vitro*, which runs counter to the previous findings. This might suggest that the $G_{\alpha s}$ pathway, which would enhance lipolysis, might have been activated by 5-PAHSA in their experiment but this is unclear.

De novo lipogenesis is another function of adipose tissue, is upregulated by insulin, and is important to the synthesis of FAHFAs. Paluchova *et al.'s* (2020) data also suggested that there may be some effect on aspects of DNL with chronic 5-PAHSA treatment; possibly with reductions in elongation.

Taken together, it seems that FAHFAs have the capacity to influence important processes in adipose tissue, although the results relating to lipolysis are conflicting and little is known about DNL. Furthermore, since all work to date has taken place in mice, there is a need to develop our knowledge in man.

6.1.4 Aims

My aims for this chapter were to:

1. Determine the expression of the FFARs, particularly FFAR4, in SAT and the MBD5 cell line and to elucidate any differences between abdominal and gluteal depots,
2. Determine whether the expression of FFARs *in vitro* is modulated by the presence of exogenous NEFA and whether this modulation is depot specific,
3. Clarify the effect of FAHFAs on lipolysis in human adipocytes *in vitro*,
4. Investigate the effect of FAHFAs on DNL in human adipocytes.

6.2 Methods

6.2.1 General Methods

The work described in this chapter utilised the following general methods detailed in Chapter 2:

- Human Adipose Tissue Biopsy and Preadipocyte Isolation (2.1)
- Maintenance of Cells in Culture (2.2)
- Assessment of Stimulated Lipolysis by Measurement of Non-Esterified Fatty Acids and/or Glycerol Release *In Vitro* (2.5)
- Protein Quantification Assay (2.6)
- Western Blotting (2.7)
- Gene Expression Assays by qPCR (2.8)
- Extraction of Lipids from Plasma or Lysates for Gas Chromatography (2.14)
- Quantification of Stable Isotope Tracer and Tracee by Gas Chromatography- Mass Spectrometry (2.17)

6.2.2 Specific Methods

FFAR Expression

Abdominal and gluteal MBD5 preadipocytes (n=5) were differentiated for 12 days in 5 mM glucose and either 200 µM or 0 µM OPLA. At days 4, 6, 8, 10 and 12, cells were harvested for mRNA as described in section 2.8 and protein as described in 2.7. Abdominal and gluteal primary cells (n=8, 3F / 5M) received the same treatment but were harvested at days 4 and 12 only.

qPCR was carried out using the following probes:

- *FFAR4*- Hs00699184_m1,
- *18S*- Hs99999901_s1,
- *PGK1*- Hs99999906_m1,
- *PPIA*- Hs99999904_m1,
- *UBC*- Hs00824723_m1.

Western blotting was carried out using the following primary antibodies:

- FFAR4- PA550973 (Life Technologies)
- A-Tubulin- ab15246 (Abcam)

Effect of FAHFAs on Lipolysis

Abdominal and gluteal MBD5 preadipocytes were differentiated for 10 days in 5 mM glucose with no fatty acids and a lipolysis experiment carried out described in 2.5. In addition to 90 min isoprenaline treatment, the cells received a 30 min pre-incubation with either 40 µM 5-PAHSA, 10 µM TUG891 (a selective FFAR4 agonist), 120 pM insulin or combinations thereof. The difference between the

concentration of NEFA released to the media in the basal and stimulated states was calculated to yield the change in NEFA release upon stimulation.

Effect of FAHFAs on de novo Lipogenesis

Abdominal and gluteal MBD5 preadipocytes were differentiated for 10 days in 5 mM glucose with no fatty acids but supplemented with 10 % $^{2}H_2O$. From day 4 onwards, the medium was supplemented with either 40 µM 5-PAHSA, 5 µM Compound C or 40 µM OPLA. At day 10, the cells were harvested in NP40 lysis buffer for extraction of lipids according to 2.14 and then analysis of tracer incorporation by GCMS (2.17) and fatty acid composition by GC (2.16).

Statistics

Data were processed in Excel and R. Differences between two conditions were assessed by Welch's t-tests. Differences between two conditions over a course of time were analysed by two-way ANOVA. Figures were produced in R using the ggplot2() package.

6.3 Results

6.3.1 FFAR mRNA Expression in Adipose Tissue

Thanks to the work of various colleagues, I was able to examine the expression of FFARs as measured by RNAseq from several previous studies.

In raw RNAseq data from male and female AT samples collected by Dr Todorčević, it was evident that *FFARs* 1 and 3 were not expressed in mature adipocytes, while *FFARs* 2 and 4 were. *FFARs* 1 and 3 were, however, expressed to a degree in the stromal-vascular fraction. After normalising and processing, *FFAR4* expression in mature abdominal adipocytes is double that in gluteal cells while *FFAR2* expression is around 50 % higher in mature abdominal adipocytes. These patterns repeat in samples of whole tissue but, in samples from the stroma-vascular fraction, FFAR2 is 50 % higher in the gluteal compared to abdominal.

In RNAseq data from the AT samples of age-, BMI- and android:gynoid- matched males and females collected by Dr Matt Neville and analysed by Daniel Phillips, *FFAR1* was not detected. In men, *FFAR3* was expressed more highly in abdominal SAT than gluteal. In women, *FFAR2* was higher in abdominal SAT but not significantly while *FFAR3* and *FFAR4* were higher in abdominal. *FFAR3* and *FFAR4* were expressed more highly in women than men in both AT depots. However, given that FFAR3 was only found in the stromal-vascular fraction and not mature adipocytes in the first dataset, these differences are probably not relevant to adipocyte function.

These data confirm the expectation that *FFAR1* is not expressed in AT and therefore not relevant to studies of FAHFAs in AT but also, contrary to previous reports, that *FFAR4* may be more highly expressed in abdominal AT. If this higher gene expression in abdominal AT carries through to protein expression then it may result in differential effects of FAHFAs in the two depots.

6.3.2 *FFAR4* mRNA Expression in Differentiating Preadipocytes

FFAR4 expression in abdominal and gluteal MBD5 cells cultured with (FA) and without (CTL) OPLA is compared in Figure 6-1, Panel A. By ANOVA, *FFAR4* was shown to be more highly expressed in abdominal cells compared to gluteal (Table 6-1) although this seems to be stronger when FAs are not present. The MBD5 cells therefore match the tissue RNAseq data.

When comparing the control and OPLA-supplemented cells within each depot (Figure 6-1B) it can be seen that the presence of FAs depresses the expression of *FFAR4* in both depots (Table 6-1).

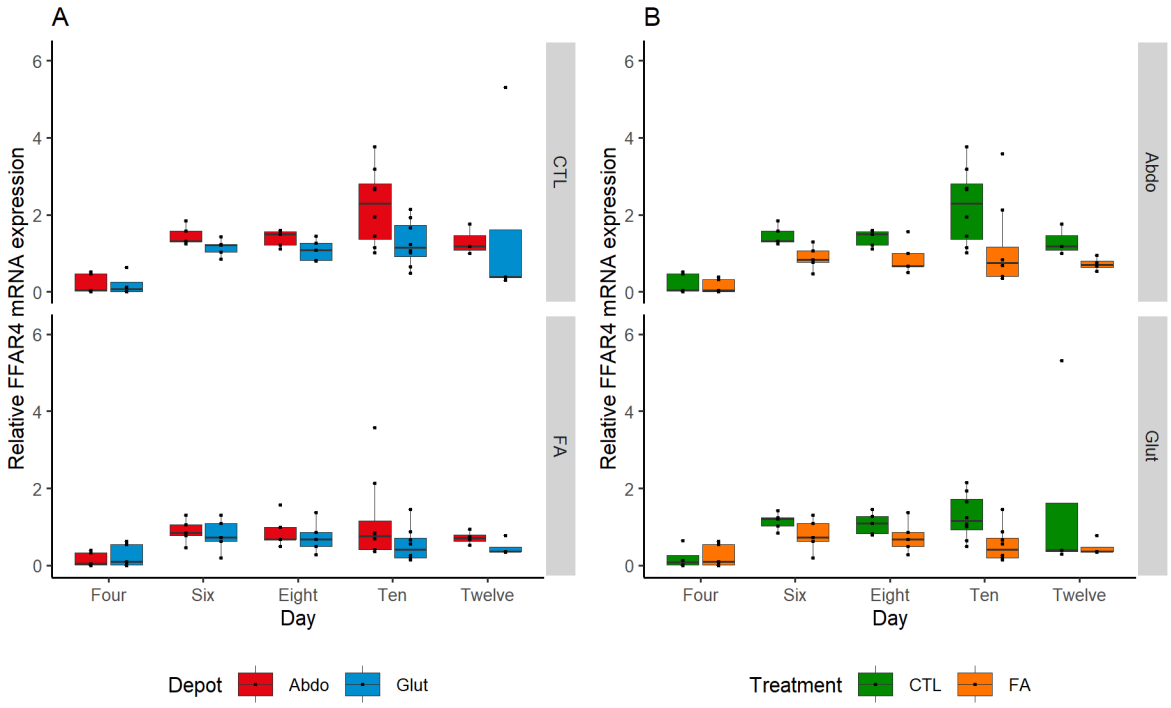


Figure 6-1: Expression of Free Fatty Acid Receptor 4 mRNA is different between abdominal and gluteal MBD5 cells (Panel A) and is altered by the presence of fatty acids in the culture medium (Panel B).

CTL: Control, 5 mM glucose. FA: Fatty acids, 5 mM glucose plus 100 µM OPLA fatty acid mix. N=4.

Table 6-1: The effect of depot and treatment on the expression of FFARs

Receptor	Depot difference	Treatment difference	Day effect
FFAR4	Abdo > Glut p= 0.03	CTL > FA p< 0.001	p<0.001

Depot and Treatment difference p-values from two-way ANOVA with post-hoc Tukey tests. Day effect p-value from two-way ANOVA.

The between-depot differences observed at days 4 and 12 in the MBD5 cells are not replicated in the primary cells from 8 donors (3 female, 5 male) (Figure 6-2). Since the primary cells mature more slowly, day 12 may be more comparable to day 8 or 10 in the MBD5 cells but, again, the primary day 12 data does not replicate the depot differences seen in MBD5 on those days. No effect of depot, treatment, sex or day were found by ANOVA. That expression does not change between days 4 and 12 is strange, given what is seen in the MBD5 cells, but this may be due to the relatively fewer mature adipocytes and relatively more numerous non-adipocytes from the stroma-vascular fraction in the primary samples.

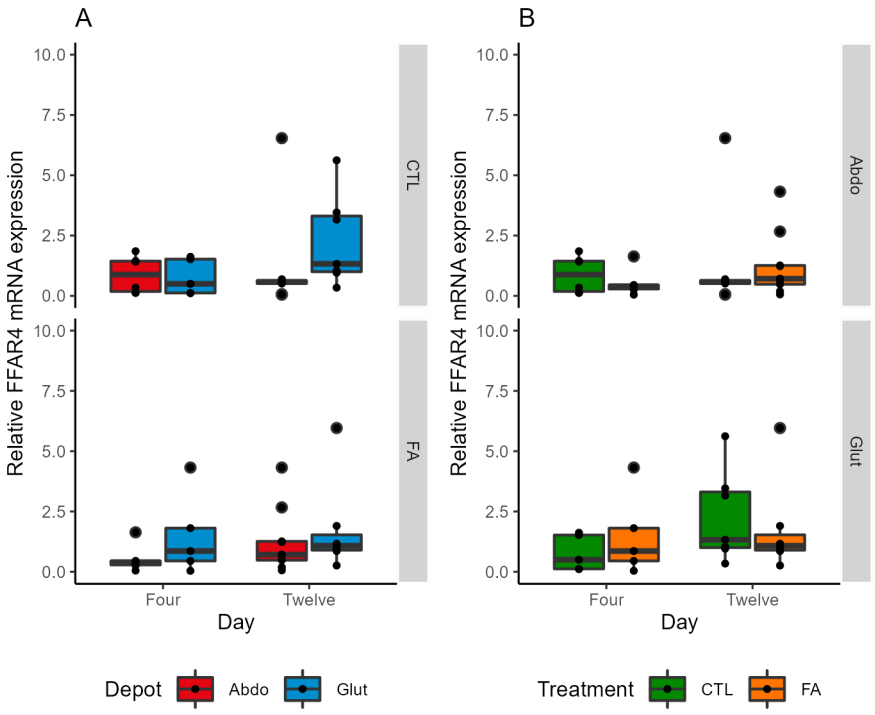


Figure 6-2: Expression of Free Fatty Acid Receptor 4 mRNA is not different between abdominal and gluteal primary cells (Panel A) and is not modulated by FAs in the culture medium (Panel B).

CTL: Control, 5 mM glucose. FA: Fatty acids, 5 mM glucose plus 100 µM OPLA fatty acid mix. N=8 (3F / 5M)

6.3.3 FFAR Protein Expression in Differentiating Preadipocytes

To see if these patterns of mRNA expression translate into functionally significant differences in protein abundance, I differentiated abdominal and gluteal MBD5 cells over the course of 12 days with or without 100 μM OPLA (n=3). Lysates were collected and probed by western blot with antibodies against FFAR4.

The FFAR4 antibody identified bands at two weights which corresponded to a light splice variant (~26 kDa) and, potentially a dimeric form of the protein (~68kDA) compared to the anticipated 42 kDa weight. It may be that the proteins were able to retain these multimers through the process of blotting because, as 7-transmembrane proteins, a gentle method of reduction was used in preparing the samples in order to avoid protein aggregation. However, the presence of multimerization was inconsistent and, across 5 experimental sets, there did not seem to be patterns according to either time, treatment or depot. It is uncertain whether this is natural variation in protein expression and structure, or if the variation is introduced during sample denaturing. Increasing the reducing time or the concentration of Laemmli buffer did not have any effect on the outcome. Example blots are given in Figure 6-3. I did not analyse these quantitatively and, due to the variability, could draw no conclusions qualitatively.

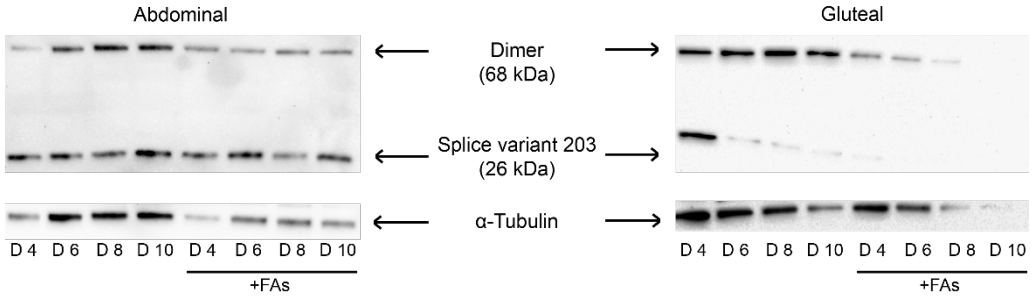


Figure 6-3: Protein expression of FFAR4 across the course of adipocyte differentiation.

Example blots from one experimental set. D= Day.

6.3.4 FAHFAs Influence Isoprenaline-Stimulated Lipolysis

To test the effect of 5-PAHSA on lipolysis, abdominal and gluteal MBD5 preadipocytes were differentiated for 10 days prior to isoprenaline-stimulated lipolysis experiments (n=5). There were no differences between the abdominal and gluteal depots in any treatment and so the data were combined for further analysis. Insulin alone, acting as a positive control to strongly inhibit lipolysis, reduced the basal to stimulated change in NEFA release to 22 % of that with isoprenaline alone (Figure 6-4). The addition of either TUG891 or 5-PAHSA to insulin made negligible further reductions. 5-PAHSA alone reduced the change in release of NEFA (to 80 % of isoprenaline), but this was not

significant, while TUG891 was more potent, reducing the change to 70 %. The difference between TUG891 and 5-PAHSA was not significant (Figure 6-4).

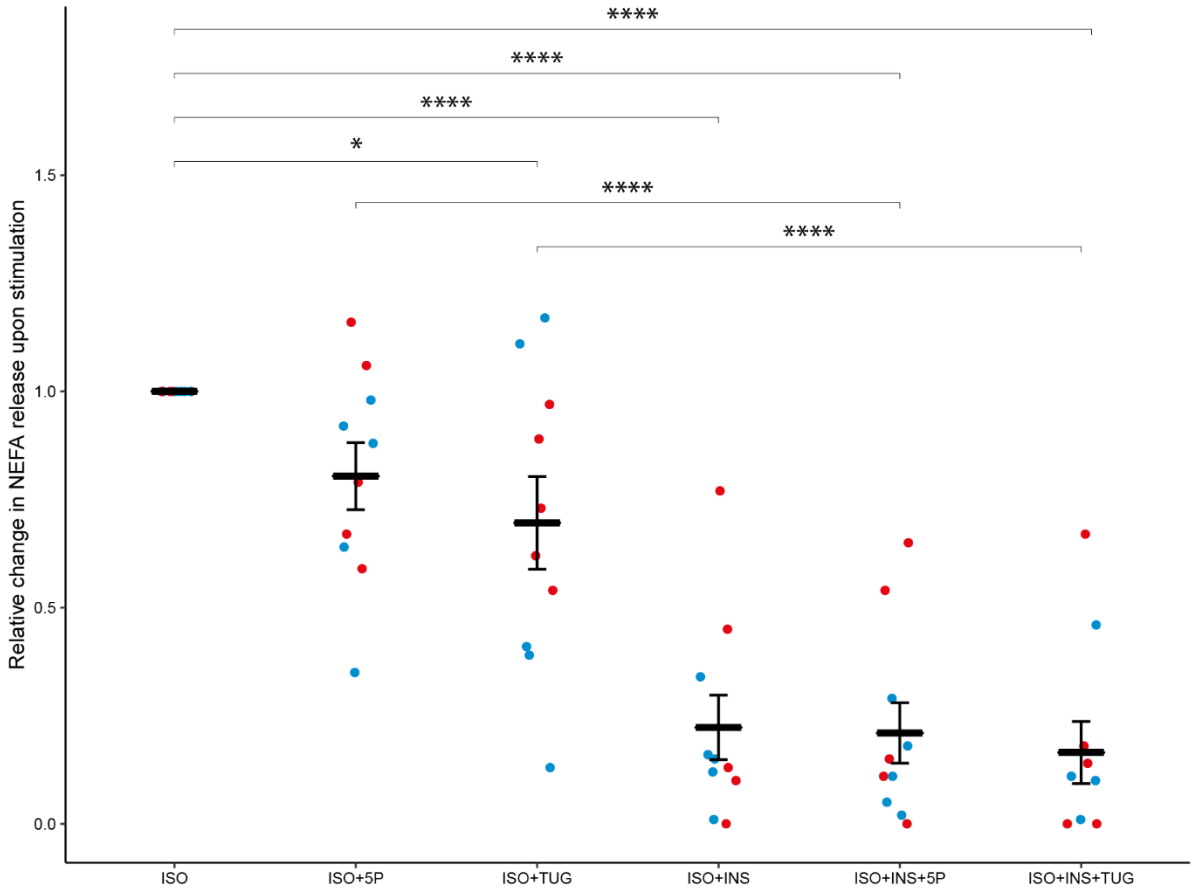


Figure 6-4: 5-PAHSA or synthetic FFAR4 agonist TUG891 reduce isoprenaline-stimulated lipolysis

Change in NEFA measured between basal and stimulated states. Stimulation by 90 min incubation with 10 nM isoprenaline (ISO) and 30 min preincubation with 40 µM 5-PAHSA (5P), 10 µM TUG891 (TUG) or 120 pM insulin (INS). * and **** denote p-adj<0.05 and p-adj<0.00001 according to ANOVA with post-hoc Tukey tests. Blue=gluteal, red=abdominal. N=5 for each depot.

6.3.5 FAHFAs Inhibit *De Novo* Lipogenesis

Abdominal and gluteal MBD5 preadipocytes were differentiated for 10 days (n=4). Alongside 5-PAHSA, an OPLA treatment was used since exogenous FAs would be expected to downregulate DNL and an AMPK inhibitor (Compound C) was considered because AMPK may be a mediator of the FA suppression of DNL (Ceddia, 2013). Following GC-MS analysis, the fraction of palmitate derived from DNL was calculated and this is shown in Figure 6-5. The contribution of DNL-derived palmitate to the palmitate pool was significantly reduced by 5-PAHSA; 0.69 compared to the control's 0.87 in gluteal cells and 0.66 compared to 0.86 in abdominal cells. It may be expected that the presence of exogenous FAs would inhibit DNL (and for the palmitate in the mix to reduce the fraction) but the

reductions in DNL in both depots were negligible in response to OPLA. The AMPK inhibitor Compound C also had no effect on 5-PAHSA's suppression of lipolysis (Figure 6-5).

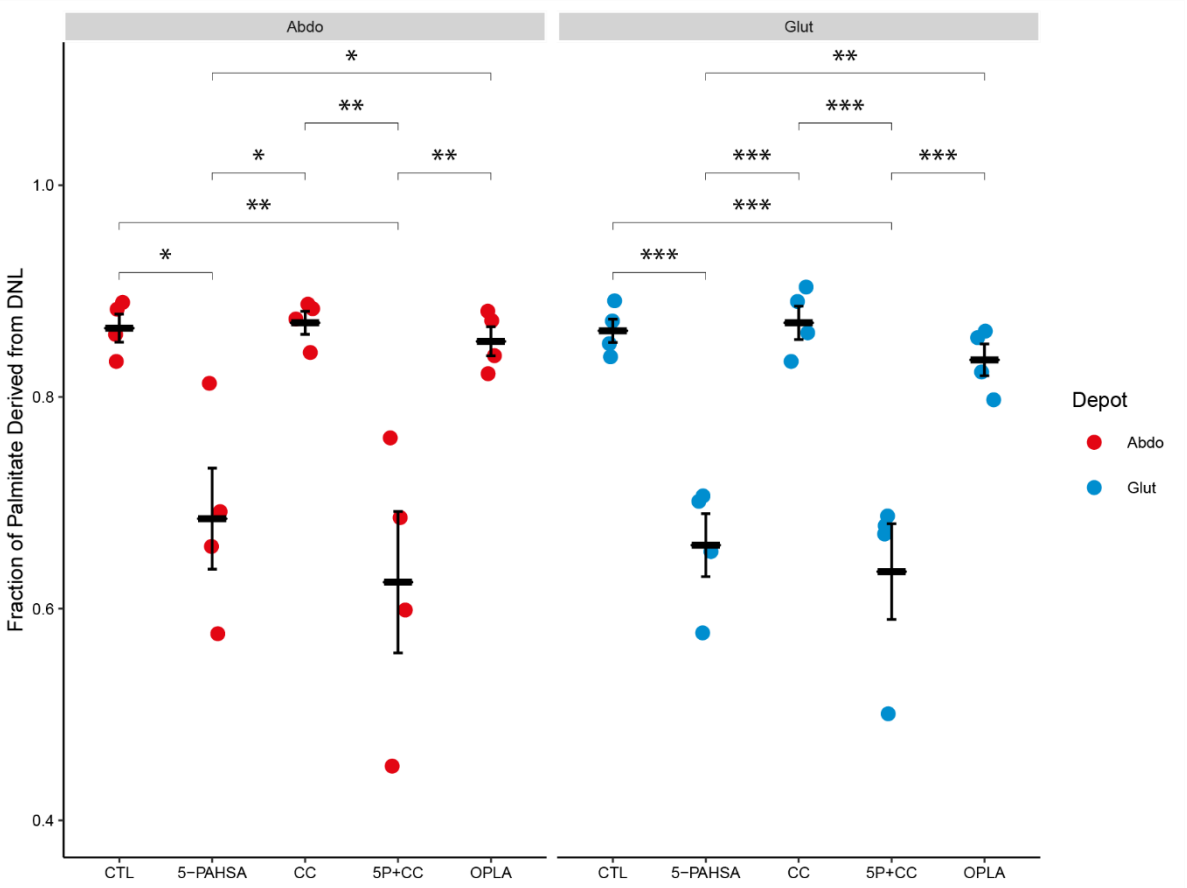


Figure 6-5: 5-PAHSA inhibits *de novo* lipogenesis of palmitate

Deuterium enrichment measured by GC-MS after 10 days of differentiation including 6 days of treatment with heavy water and either 40 μM 5-PAHSA, 5 μM Compound C (CC) or 40 μM OPLA. CTL = 5 mM glucose. *, ** and *** denote p-adj<0.05, p-adj<0.01 and p-adj<0.001 respectively according to ANOVA with post-hoc Tukey tests.

The control and 5-PAHSA-treated cells were assessed for FA composition by GC and the summary for the five principal FAs is given in Figure 6-6. Treatment with 5-PAHSA yielded a significantly higher fraction of palmitate than control in the gluteal cells. The difference was not significant in abdominal cells. The increase in gluteal palmitate with 5-PAHSA treatment appears to lead to a (non-significant) reduction in palmitoleate, the desaturated product of palmitate, and vaccenate, the elongation product of palmitoleate. Similar but less obvious trends also occurred with stearate and its desaturation product oleate. These changes led to reduction in the C16 to C16:1 ratio, a possible indicator of SCD activity; from 1.33 in the abdominal control cells to 1.01 in the %-PAHSA-treated abdominal cells and, similarly from 1.2 to 0.85 in gluteal cells. The same reductions exist in the C18 to C18:1 ratios which fell from 10.48 to 7.33 in the abdominal cells and from 10.39 to 7.03 in the gluteal cells.

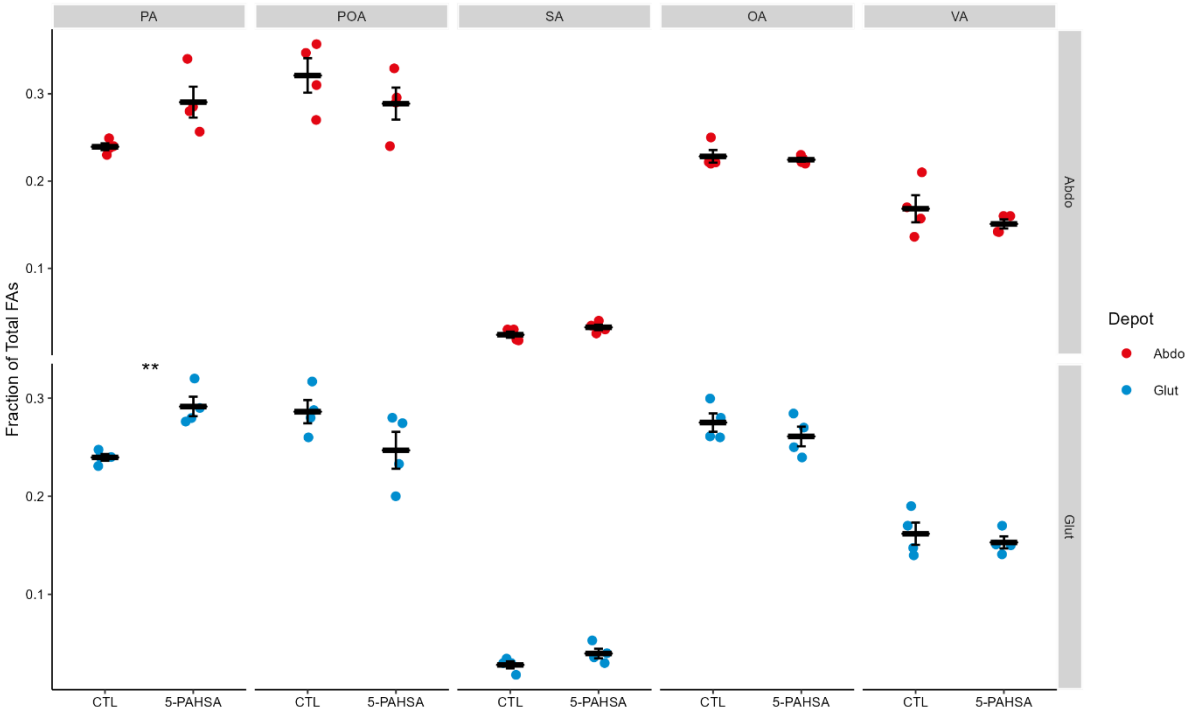


Figure 6-6: 5-PAHSA alters the fatty acid composition of adipocytes by apparently reducing desaturation

Total FA composition measured after 10 days of differentiation including 6 days of treatment with 40 μM 5-PAHSA. CTL= 5 mM glucose. PA= palmitate. POA=palmitoleate. SA= stearate. OA=oleate. VA=vaccinate. ** denotes p<0.01 according to Welch's t-test.

To see if the effects of 5-PAHSA on aspects of the DNL pathway occurred through the alteration of gene expression, I examined mRNA expression of a panel of DNL-related genes. As shown in Figure 6-7, 5-PAHSA treatment significantly reduced mRNA expression in all measured genes with the exceptions of *ELOVL1* in both depots, *FASN* in abdominal cells and *ELOVL3* in gluteal cells.

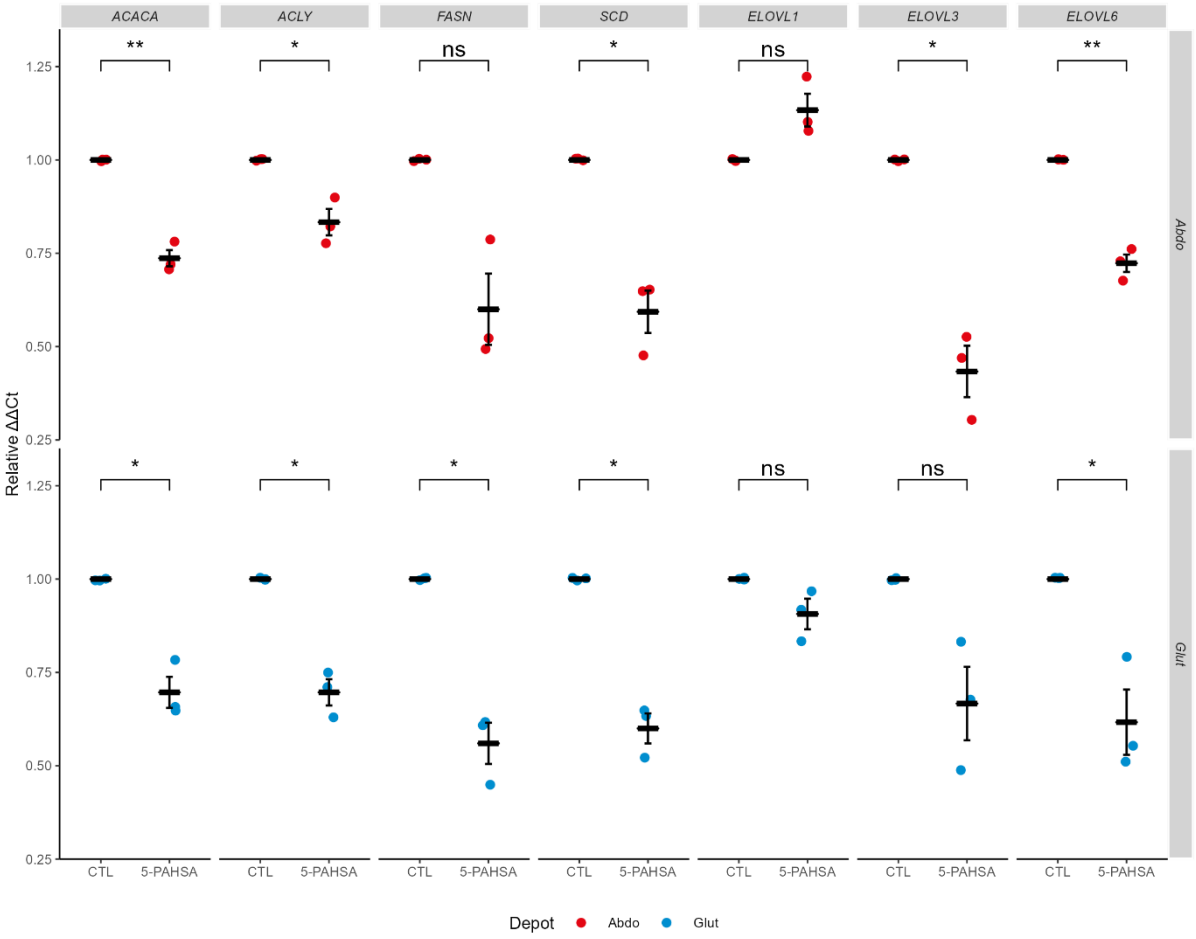


Figure 6-7: Relative gene expression of DNL genes reduced by 5-PAHSA treatment

Relative mRNA expression measured at day 10 of differentiation including 6 days of treatment with 40 μM 5-PAHSA. CTL= 5 mM glucose. * denotes $p<0.05$ according to Welch's t-test.

6.3.6 FFAR Signalling Pathways to Inhibit Lipolysis

Since FFAR4 might operate through several different α-subunits of the g-protein, I was interested in determining which subunits might be responsible for the signalling which resulted in depression of lipolysis. Often this type of work is done by over-expressing the receptor in an alternative cell line (like Chinese Hamster Ovary cells) and measuring changes in downstream effectors (like cAMP) but I was interested to know if this could be assessed in the adipocytes using a functional output such as lipolysis.

To this end, I piloted a lipolysis experiment as above, pre-incubating with TUG891 (FFAR4 agonist) and combinations of Pertussis Toxin (αi inhibitor) and YM254890 (αq inhibitor) before isoprenaline stimulation. Unfortunately, I could not detect any modulation of lipolysis with the inhibitors, so I did not pursue this functional assay further.

6.4 Discussion

Since FAHFAs have been reported to act through the FFARs and to have the capacity to modulate the adipocyte functions of lipogenesis and lipolysis in mice, I wanted to explore these findings further in humans. I first aimed to characterise the expression of FFARs in human abdominal and gluteal adipocytes, finding *FFAR4* mRNA to be present and more highly expressed in abdominal cells. I was not able to reliably measure FFAR4 protein to confirm if this follows through to protein expression. I then clarified a point of contention, that 5-PAHSA, like insulin, reduces lipolysis in human cells and found that chronic 5-PAHSA treatment, unlike insulin, impairs DNL and desaturation of FAs.

6.4.1 FFAR Gene Expression

At the mRNA level in the MBD5 cell line, FFAR4, which is a LCFA receptor, was expressed more highly in abdominal than in gluteal cells. There has been debate about the impact of FAs on expression of FFAR4 but, in this model, the presence of exogenous FAs in the differentiation medium suppressed the expression of FFAR4. This is a logical response to attenuate the increasing signal from excess ligand and thus leave the system sensitive to fluctuations.

In primary cells from male and female donors, these expression differences were not apparent. This may be due to the reduced scope of the experiment (sampling at two timepoints, rather than six) or because the majority of the MBD5 cells differentiate into mature adipocytes while the very mixed population of the SVF cells from the primary biopsies contained considerably fewer adipocytes and more of other cells which may not express the FFARs. Since the research interest is in what health benefits might result from FFAR agonists such as FAHFAs or omega-3s, it is plausible that the MBD5 data is valid in predicting how agonists may affect the target cells in the SAT depots differently.

Other researchers have studied mRNA expression of FFARs in SAT, often focussing on the difference in lean and obese and usually only examining the abdominal SAT. Amisten *et al.* (2015) did look at both SAT depots and detected low expression of FFAR4 mRNA in biopsies of 6 males with no differences between depots. To an extent, these data match my own primary expression data, which differs from the MBD5 data, possibly for the reasons outlined above or because of a sexual dimorphism; it would be of interest to examine a male version of the MBD5 cells.

Rodriguez-Pacheco *et al.* (2014) found no difference in expression of FFAR4 in abdominal SAT between lean and obese individuals while Ichimura *et al.* (2012) found expression to be considerably higher in obese subjects. Participants in both groups of the former study had low waist:hip ratios (i.e a propensity towards gluteofemoral fat) but this measurement was not reported in the latter study so it cannot be ruled out that they had more abdominal fat. Perhaps if Rodriguez-Pacheco *et al.* had

looked at the gluteal depot, they would have found an increase in mRNA expression in the obese group (as they did in the VAT of that group) and perhaps if Ichimura *et al.* had looked at the gluteal depot, they would have found no difference between lean and obese. My data highlight that the SAT depots within the human body are not the same and that mature adipocytes express more FFAR4 mRNA than immature (compare days 4 and 12), so depots showing more expansion and adipocyte recruitment would be expected to have higher expression.

Overall, I have highlighted that it is important to examine both of these SAT depots if one is studying this receptor or its agonists, since interesting findings may be missed by only examining the abdominal depot.

6.4.2 FFAR Protein Expression

Agonists act on proteins and not mRNA so it is important to determine if the identified differences in mRNA expression persist through translation. My western blot data for FFAR4 was inconsistent and did not confirm the same depot differences as seen with the mRNA expression data, but, assuming the antibody is specific, it does suggest oligomerisation of the receptors which may be an important feature of their activation or inactivation (Vilardaga *et al.*, 2010).

Further work in this area, perhaps utilising an alternative methodology, such as mass spectrometry, would be useful to clarify if the differences in mRNA expression are consequential.

6.4.3 Lipolysis and Lipogenesis

The opposing reports that lipolysis was either reduced (Zhou *et al.*, 2019) or enhanced (Paluchova *et al.*, 2020) by 9- and 5-PAHSA respectively in murine models are curious. Since 5-PAHSA mimics insulin in causing GLUT4 translocation, it could be predicted that it would mimic insulin in reducing lipolysis. I found that 5-PAHSA and the FFAR4 agonist TUG891 did both reduce lipolysis, although the 5-PAHSA result was not significant due to greater variation.

With the logic that 5-PAHSA would again mimic insulin in enhancing lipogenesis, I measured the incorporation of heavy water from the differentiation medium into palmitate at the end of 10 days of differentiation to gauge the contribution of DNL to the pool of palmitate. Surprisingly, I found the presence of 5-PAHSA reduced the DNL-derived fraction of palmitate. Exogenous FAs also suppress lipogenesis by activation of AMPK and it may be that 5-PAHSA mimicked FAs more closely than it mimicked insulin. However, OPLA treatment made no noticeable difference to the rate of DNL and the use of an AMPK inhibitor did not limit 5-PAHSA's inhibition of DNL, suggesting that it operates through a different pathway.

In addition to reducing DNL, 5-PAHSA also appears to inhibit the desaturation enzyme, SCD, since the cells treated with 5-PAHSA contained a higher proportion of palmitate and stearate and lower proportions of their desaturation products, palmitoleate and oleate, than the control cells, as evidenced by shifts in the ratios of 16:0 to 16:1 and 18:0 to 18:1. The addition of exogenous omega-3 FAs (EPA, DPA and DHA), which are agonists of FFARs 4, to culture media has been shown to suppress *SCD* gene transcription in murine 3T3-L1 cells (Barber, Sinclair and Cameron-Smith, 2013). In humans, Telle-Hansen *et al.* (2012) found that increasing the intake of salmon (a source of omega-3s) resulted in a decreased ratio of 18:0 to 18:1 (a measure of the whole body activity of SCD activity) in plasma triglycerides suggesting a reduction in SCD activity. Finally, in unpublished data from the group, we found that the 16:0 to 16:1 ratio is decreased in the gluteal but not abdominal AT after an intervention where participants were supplemented with the n-3 fatty acids, EPA and DHA (as ethyl esters) for 8 weeks. Taken together in combination with the gene expression data, where FFAR 4 is higher in abdominal than gluteal cells, this suggests that dietary lipids, which are agonists of FFAR4, may cause a reduction in SCD activity in AT with a greater effect in the abdominal depot. Both positive and negative health outcomes have been inconclusively linked to SCD activity but causal directionality is unclear (Hodson and Fielding, 2013) so whether or not this effect is a good or a bad thing for abdominal tissue remains to be elucidated.

The studies here did not examine the dose response of the effects of 5-PAHSA on lipolysis and lipogenesis, which would be of interest since the 40 μM used here to align with he previously published but conflicting studies is higher than physiological. That the evidence noted above suggests that n-3 fatty acids at physiological levels may be having similar effects suggests that physiological levels of 5-PAHSA might also be able to achieve these, but it is uncertain.

6.4.4 Summary

I have shown depot differences in the gene expression of FFAR in mature human adipocytes which may not be so obvious in studies which have examined whole tissue but which may be important when the function of mature cells is the point of interest. Future work on these receptors and their agonists should consider both SAT depots since it is clear that the abdominal biopsy-only approach could miss much. The gene expression differences did not materialise in the protein data but the interpretation of that was made difficult by oligomerisation and inconsistencies in that.

Lipolysis was reduced by 5-PAHSA, in agreement with one research group and in opposition to another. This result was, in a sense, predictable, since 5-PAHSA mimics insulin in GLUT4 translocation and would be expected to mimic insulin in suppressing lipolysis. However, that

predictable logic did not hold for DNL, which is enhanced by insulin but which I found to be suppressed by 5-PAHSA. As a next step it would be important to know if different FAHFAs replicate these opposing results and to investigate the GPCR signalling pathways to identify where this divergence might take place.

When considering the significance of my results and when planning and executing future work, it is worth bearing in mind that at least 163 GPCRs have their mRNA expressed in adipose tissue and are agonised by at least 180 endogenous ligands (Amisten *et al.*, 2015). Consequently, the FFARs and their lipid ligands do not impact in isolation on the various signalling pathways and this must be taken into account when extrapolating from a cell-culture dish to a whole body.

Chapter 7
Discussion and Future Directions

7 Discussion and Future Directions

7.1 Background

The recently-discovered class of lipids, FAHFAs, have been proposed to act as lipokines, able to reduce glycaemia by stimulating GLUT4 translocation in adipocytes and by stimulating GLP-1 secretion from enteroendocrine cells via pathways through the g-protein-coupled fatty acid receptors FFAR1 and FFAR4 (Yore *et al.*, 2014; Syed *et al.*, 2018; Sundstrom *et al.*, 2017). Furthermore, they have been shown in murine models to reduce inflammatory processes in immune cells (Kolar *et al.*, 2019). These findings make FAHFAs a potentially interesting proposition for treating aspects of the metabolic syndrome, which include poor glycaemic control and chronic inflammation.

To date, the majority of research into the systemic effects and underlying mechanisms of action of FAHFAs has been carried out in murine models, and observational data in humans is equivocal. For example, Yore *et al.* (2014) reported that PAHSAs (a subfamily of FAHFAs) were lower in the plasma of fasted subjects with reduced insulin sensitivity but Kellerer *et al.* (2021) found no difference in plasma PAHSAs between diabetic and non-diabetic subjects and, instead identified that morbidly obese subjects had lower plasma FAHFAs than lean subjects. Neither was able to offer a direction of causality but both might be questioned; Yore *et al.* because the two groups were not matched for BMI and Kellerer *et al.* for using bariatric subjects with BMI>40 $kg.m^{-2}$ who were, therefore, not representative of the wider overweight and obese population. As these studies were small, there was a need to investigate these aspects of FAHFA metabolism in more detail in a larger, more representative cohort in humans.

The underlying mechanistic actions of FAHFAs in adipose tissue have only been demonstrated in mice and, again, some findings are equivocal. Yore *et al.* (2014) demonstrated the insulin-mimicking effect of FAHFAs to increase glucose uptake through initiating GLUT4 translocation but, where Zhou *et al.* (2019) demonstrated the insulin-mimicking effect of FAFAHAs to inhibit stimulated lipolysis, Paluchova *et al.* (2020) found FAHFAs to enhance lipolysis. Again, there was a need to replicate these findings in human adipocytes and to resolve the conflicting reports.

Furthermore, very little is known about the lifecycle of FAHFAs. It has become clear that synthesis from the two substrates (an FA and an HFA) is handled by ATGL, which also adds and removes FAHFAs to and from TAG, and there has been a proposal of where the substrates may originate (Kuda *et al.*, 2018) but there is very little clarity over regulation of the processes. There is also little evidence as to what controls the release of FAHFAs from or reuptake into adipose tissue other than the assumption that they follow the same patterns as ordinary FAs being released during fasting. Another

assumption is that, in systemic circulation, FAHFAs are bound to albumin for transport in the blood but this was unconfirmed. Finally, while a few hydrolases have been shown to be capable of degrading FAHFAs, they have not been shown to be capable of degrading all species of FAHFA and so another gap in their lifecycle remains to be filled and more work is required to identify all of the hydrolases and their specificities.

7.2 Thesis summary

In order to address some of the gaps in the knowledge of FAHFAs in humans, the overall aims of my thesis were to: i) determine the distribution and composition of FAHFAs in the plasma and specific subcutaneous adipose tissue depots of humans, ii) look for associations between these patterns and markers of metabolic health and, iii) to examine the mechanistic effects of FAHFAs in adipose tissue. I achieved this by undertaking observational studies in human participants and *in vitro* cellular studies using both immortalised and primary adipocytes derived from both the abdominal and gluteal adipose depots. I hypothesised that, if FAHFAs were beneficial to metabolic health, then some difference in FAHFA metabolism between the depots may partially explain why gluteofemoral adiposity is protective against metabolic ill health, while abdominal adiposity is not.

I began my work by looking, in collaboration with my colleagues, at the composition of the adipocyte differentiation cocktail and at a new abdominal and gluteal preadipocyte cell line (MBD5) with the aim of establishing a robust *in vitro* workhorse, capable of performing the adipocyte functions necessary for my work. I determined that the standard cocktail induced insulin resistance in cultured cells and that this could be avoided by reducing the use of hormones in the latter part of differentiation. I also found that adipocytes did not require high concentrations of glucose to survive and they were not perturbed by the application of exogenous FAs, which allowed *in vivo* conditions to better mimicked.

The cross-sectional human studies showed that, in men, 6 of the 9 FAHFA species examined were higher in subjects in the upper tertile of fasting plasma insulin concentrations compared to the rest of the cohort. This was not the case in women where there were no differences. This is in contrast to the original claim by Yore *et al.* (2014) that higher concentrations of FAHFAs are protective against insulin resistance but might be explained as FAHFA synthesis and release being upregulated in response to increasing insulin resistance. However, examining the correlation trends, this does not seem to be the case for men although it may be for women. It is not clear why sexual dimorphism exists but it may be that there is a positive regulation of FAHFAs in response to increasing insulin insensitivity up to a plateau and that men reach that plateau earlier. I found no suggestion that plasma FAHFA concentrations decreased with increasing BMI, as observed by Kellerer *et al.* (2021), but rather I observed that some FAHFAs were higher in obese compared to lean subjects. This may be due to

the lack of participants with extreme adiposity in my study, which Kellerer *et al.* (2021) had in theirs and might indicate that in morbid obesity or metabolic distress, there is some dysregulation that occurs to reduce circulating FAHFAs.

An important observation of the cross-sectional study is that plasma FAHFAs after an overnight fast are regulated oppositely to plasma NEFAs; that is, when NEFA is high, FAHFAs are low. This may be because FAHFAs are, unexpectedly, not liberated from adipocyte TAG and released in the same way as NEFA, or it may be because, overnight, the concentrations of NEFA somehow influence the rates of FAHFA degradation or uptake of FAHFAs to other tissues. There is still much to be elucidated about this aspect of the FAHFA lifecycle in humans.

In vitro, I confirmed that albumin can bind FAHFAs to carry them in the blood, although there remains a question as to the affinity of different FAHFAs for albumin and whether low affinities might result in limits to the carrying capacity of blood.

I also showed, *in vitro*, that both abdominal and gluteal adipocytes (but not immature preadipocytes) have the same capacity to synthesise FAHFAs in the presence of excess substrate. However, *in vivo*, in adipose tissue, I measured one family of free FAHFAs, not bound in TAG, and these were found at higher concentrations in abdominal than in gluteal tissue. Although the assumption that the free FAHFAs reflect the stored FAHFAs, this still needs to be shown, and the difference in depots suggests that either the substrate supply, the release from or the reuptake to the adipose tissue differs between the depots. A small study measuring the FAHFAs in plasma from the femoral artery and veins draining gluteal and abdominal SAT did not indicate that the uptake or release was different between depots.

FAHFAs act as ligands for FFARs 1 and 4; FFAR4 is found in adipose tissue. Although mRNA expression of FFAR4 was slightly higher in differentiating abdominal compared to gluteal cells in our cell line, this was not the case in primary adipocytes from men and women. I found that acute administration of 5-PAHSA or a synthetic FFAR4 agonist reduced stimulated lipolysis, as insulin did, to a similar degree in both depots of our cell line. However, I also found that chronic administration of 5-PAHSA across the course of differentiation resulted in a downregulation of DNL, which is contrary to the effect of insulin. This effect may be a negative feedback loop to control FAHFA synthesis but it suggests more than one pathway of action for FAHFAs and there remain opportunities to examine that in greater depth.

7.3 Limitations of the work

A major limitation of this thesis is the restricted number of FAHFA species examined by LC-MS in the studies outlined in Chapters 4 and 5. Given that some of my results counter previously-published

studies, which also reported limited ranges of FAHFAs, it would have been ideal that this, larger, study would have reported a much broader dataset and potentially highlighted differences between families and species.

Where I have reported on the effects of 5-PAHSA treatment on adipocytes *in vitro* in Chapter 6, I would have liked to examine more closely the dose-response but lacked the time and resources to do so. The 40 μM which has become standard across several publications is much higher than physiological levels and so it will be important to understand the physiological relevance of the reported responses.

Similarly, in those functional experiments, I would have liked to confirm that the observed effects of 5-PAHSA were solely through FFAR4 by, for example, inducible knockdown of the receptor but this was, again, beyond the limits of time and resource.

The functional experiments also made use of only 5-PAHSA, since the ground had been set by previous studies which had used this and to which I was comparing. However, there is a possibility that other FAHFA species may have different signalling effects than those observed here.

Finally, the in vitro experiments were carried out using the MBD5 cell line which is of female origin and, when using primary cells from both genders, the results are not always similar. It was not possible to discern if any effects are sex specific, although I described in Chapter 4 that the circulating levels of FAHFAs are sex specific, or whether differences result from the fact that the MBD5 cells are selected for their capacity to differentiate where the primary cell cultures differentiate less well.

7.4 Future directions

Although this work has answered some questions relating to the role of AT in regulating and acting in response to plasma FAHFAs, the full picture of FAHFA metabolism is far from complete. A priority for future research must be to complete those pieces of characterisation which I was unable to do due to technical complications, namely, the measurement of FAHFAs in TAG in AT and a broader palette of FAHFA families. This would give a more complete picture of the distribution in humans and a starting point to investigate further questions arising from this thesis.

Central to those questions is that of how plasma FAHFAs are regulated and why the regulation appears to differ from that of plasma NEFA. A timecourse observational study of participants pre-and post-prandially would allow plasma FAHFAs and NEFAs to be tracked against each other through the cycle from overnight fast, through the response to feeding and back into fasting again, in order to identify a point of divergence.

A further question for *in vitro* investigations is that of the signalling pathways which result in the apparently contradictory findings that FAHFAs reduce both lipogenesis and its counterpart, lipolysis. If both of these outcomes result from FFAR4 agonism by FAHFAs, then the downstream signalling pathways should be looked at in detail to understand how and what the metabolic implications might be of having both pathways activated concurrently.

7.5 Conclusion

In this thesis work, I have used adipocyte models and observational studies to study both improvements in adipocyte culture and aspects of FAHFA metabolism and action.

To the adipocyte field in general, I have contributed an exemplar of culture conditions which more closely mimic physiology and which avoid unintentional insulin resistance. Some of this work has since been published (Gamwell *et al.*, 2023) and attracted interest from the field.

In the FAHFA field, the controversy over the effect of FAHFA treatment on lipolysis may have been closed by this work but the same cannot be said of the equivocation on the link between circulating FAHFAs and obesity and an unexpected alteration of DNL by FAHFA treatment has been uncovered. Many questions remain over the lifecycle of FAHFAs in humans and their relevance at physiological concentrations in a milieu shared with other FAs and more work to answer these questions may follow from this thesis.

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