University of Southampton

FACULTY OF MEDICINE

Human Development and Health

Adipose tissue inflammation in obesity and the influence of marine long chain polyunsaturated omega-3 fatty acids

by

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Abstract

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Obesity is an excess of adipose tissue and is linked with increased inflammation that enhances risk of type-2 diabetes and cardiovascular disease. Despite this knowledge, a comprehensive overview of the inflammatory state of subcutaneous white adipose tissue (scWAT), which is the main pool for excess dietary lipid storage and plays a major role in whole body endocrine processes, is not reported for humans, particularly in those in which metabolic syndrome is yet to manifest. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been widely examined for their anti-inflammatory effects including modulating gene expression and secretion of systemic inflammatory markers.

The research described in this thesis explores scWAT fatty acid (FA) and lipid metabolite composition, the whole tissue transcriptome, protein expression, enzyme activity, and tissue morphology in metabolically healthy obese individuals in comparison to normal weight individuals to provide a comprehensive overview of the inflammatory and metabolic state of the tissue in this condition. Furthermore, it explores the anti-inflammatory potential of a 12-week fish oil (FO) intervention on these tissue parameters.

Metabolically healthy obesity (MHO) was associated with an altered FA composition and lipid metabolite profile of scWAT. There was a lower proportion of saturated fatty acids, a higher proportion of monounsaturated fatty acids (MUFA), a higher proportion of arachidonic acid (AA) and concentrations of respective oxylipins and COX-2 protein, lower concentrations of DHA oxylipins, and an alteration of the endocannabinoid system (ECS). MHO was further associated with an altered

transcriptome suggestive of enhanced inflammation, immune response, tissue remodelling and expansion, altered lipid and carbohydrate metabolism, and lipid mobilization. This was concordant with tissue morphology which showed evidence of adipocyte hypertrophy and the presence of macrophages arranged in crown like structures.

Chronic supplementation with EPA+DHA increased concentrations of sWAT omega-3 FAs and derived oxylipins and decreased AA oxylipins, with an effect on the ECS predominantly in normal weight individuals. EPA+DHA modulated the scWAT transcriptome suggesting promotion of tissue remodelling and down regulation of cell differentiation and the chronic inflammatory response, but to a lesser extent in MHO than in normal weight individuals. This lesser effect in MHO may be explained by several processes observed to be altered in MHO at study entry including mobilization and metabolism of EPA and DHA, in addition to greater proportions of omega-6 PUFA which persisted after 12-week FO intervention. The ratio of omega-6: omega-3 FA is of importance and therefore, greater concentrations of EPA and DHA may be required in these individuals to alter this ratio and subsequent oxylipin synthesis and transcriptome modulation.

These data suggest MHO is associated with enhanced sWAT inflammation in the context of tissue expansion, remodelling, and alteration to lipid metabolism and signalling. Furthermore, the data suggest that sWAT from metabolically healthy obese individuals without metabolic syndrome maintains some degree of normal function in that it is not fibrotic and is sensitive to dietary lipid manipulation. EPA+DHA modulated synthesis of EPA, DHA and AA derived oxylipins and the transcriptome but resistance to these effects, particularly on the ECS, was exhibited in MHO.

By Helena Fisk

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Research Thesis: Declaration of Authorship

Print name: HELENA LUCY FISK

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I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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Definitions and Abbreviations

(m/z), mass-to-charge ratio;

11,12-DHET, 11,12-dihydroxy-eisoatrienoic acid;

11,12,15-HETrE, 11,12,15-hydroxy-eicosatrienoic acid;

11-dh-TXB3, 11-dehydro-thromboxane B3;

11-HDoHE, 11-hydroxy-docosahexaenoic acid;

11-HETE, 11-hydroxy-eicosatetraenoic acid;

1-20:4-glycerol, 1-arachidonoyl-glycerol;

12,13-EpOME, 12,13-epoxy-octadecenoic acid;

12-HEPE, 12-hydroxy-eicosapentaenoic acid;

12-HETE, 12-hydroxy-eicosatetraenoic acid;

12-HHT, 12-hydroxy-heptadecatrenoic acid;

13-HODE, 13-hydroxy-octadecadienoic acid

13-oxo-ODE, 13-oxo-octadecadienoic acid;

14_0_EA, myristoyl-ethanolamide;

14-15-DHET, 14-15-dihydroxyeisoatrienoic acid;

14-HDoHE, 14-hydroxy-docosahexaenoic acid;

15-HETrE, 15-hydroxy-eicosatrienoic acid

16_1_EA, palmitoyl-ethanolamide;

17, 18-DiHETE, 17, 18-dihydroxyeicosatetraenoic acid;

18_1_EA, oleoyl-ethanolamide;

18_2_EA, linoleoyl-ethanolamide;

18_3_EA, alpha linolenoyl-ethanolamide;

2_16_0-glycerol, 2-palmitoyl-glycerol;

20_4_EA, arachidonoyl-ethanolamide (synonym: anandamide);

20_5_EA, eicosapentaenoyl-ethanolamide;

20_COOH_AA, 20-COOH-arachidonic acid;

20_COOH_LTB4, 20-COOH-leukotriene B4;

2_16:1-glycerol, 2-palmitoleoyl-ethanolamide;

2_18:1-glycerol, 2-oleoyl-ethanolamide;

22_6_EA, docosahexaenoyl-ethanolamide;

2_20_5-glycerol, 2-eicosapentaenoyl-glycerol;

2_22_5-glycerol, 2-docosapentaenoyl-glycerol;

2_AG, 2-arachidonoyl glycerol;

4-HDoHE, 4-hydroxy-docosahexaenoic acid;

5-HETE, 5-hydroxyeicosatetraenoic acid;

8-HDoHE, 8-hydroxy-docosahexaenoic acid;

9,10-EpOME, 9,10-epoxy-octadecenoic acid;

9-HETE, 9-hydroxyeicosatetraenoic acid;

9-HpODE, 9-hydroperoxy-octadecadienoic acid;

9-oxo-ODE, 9-oxo-octadecadienoic acid;

AA, arachidonic acid;

ACC, acetyl Co-A carboxylase;

ACP, acyl carrier protein;

Acyl CoA, acyl coenzyme-A;

ADD1, adipocyte determination and differentiation factor-1;

AEA, anandamide;

Ag, antigen;

ALA, alpha-linolenic acid;

AMPK, adenosine monophosphate-activated protein kinase;

Ang-1, angiopoietin-1;

Ang-1, angiopoietin-2;

Akt, protein kinase B;

AT, adipose tissue;

ATGL, adipocyte triglyceride lipase;

BAT, brown adipose tissue;

bHLH, basic helix-loop-helix;

BMI, body mass index;

C18, carbon 18;

cAMP, cyclic adenosine monophosphate;

CB1, cannabinoid receptor-1, CNR1;

CCL18, macrophage inflammatory protein-4;

cDNA, complementary DNA;

CEBP, CCAAT/ enhancer binding protein;

CGI-58, comparative gene identification-58 protein;

CIITA, class II transactivator;

CLS, crown like structures

CO, corn oil;

COX, cyclooxygenase;

Cps, counts per second;

CVD, cardiovascular disease;

D5D, delta-5 desaturase’

D6D, delta-6 desaturase;

DA, dalton;

DACT, dishevelled binding antagonist of beta catenin, dapper protein

DAGL, diacyglycerol lipase;

DC, dendritic cell;

DEPC, diethylpyrocarbonate;

DGAT, diacylglycerol acyltransferase;

DGLA, dihomo-gamma-linolenic acid

DHA, docosahexaenoic acid;

Dhk-PGD2, dihydro-keto-prostaglandin D2;

DiHDPA, dihydroxy-docosapentaenoic acid;

DiHETE, dihydroxy-docosapentaenoic acid;

DiHOME, dihydroxy-octadaecenoic acid;

Dnase, deoxyribonuclease;

DPA, docosapentaenoic acid;

DRV, daily recommended value;

EC, endocannabinoid;

ECM, extracellular matrix;

EET, epoxy-eicosatetraenoic acids;

EGFL6, epidermal growth factor like domain multiple-6;

EPA, eicosapentaenoic acid;

EpDPA, epoxy-docosapentaenoic acid;

EPEA, eicosapentaenoyl-ethanolamide;

EpOME, epoxy-octadecaenoic acid;

ER, endoplasmic reticulum;

FA, fatty acid;

FAAH, fatty acid amid hydrolase;

FABP, fatty acid binding protein;

FABPc, cytosolic fatty acid binding protein;

FABPpm, plasma membrane fatty acid binding protein;

FAMEs, fatty acid methyl esters;

FAS, fatty acid synthetase;

FAT/CD36, fatty acid translocase;

FATP, fatty acid transport protein;

FATP1, fatty acid transport protein-1; synonym ACSVL1, acyl co-A synthetase

FC, fold change;

FDR, false discovery rate;

FFAs, free fatty acids;

FID, flame ionisation detector;

FO, fish oil;

GC, gas chromatography;

GGCR, glucagon receptor;

GLA, gamma-linolenic acid;

GLM, general linear model;

GLUT, glucose transporter;

GM-CSF, granulocyte-macrophage colony stimulating factor;

GWAS, genome wide association studies;

HDPA, hydroxy-docosapentaenoic acid

HDHA, hydroxydocosahexaenoic acid (synonym HDoHE);

HDoHE, hydroxydocosahexaenoic acid (synonym HDHA);

HDL-C, high density lipoprotein cholesterol;

HEPE, hydroxy-eicosapentaenoic acid;

HETE, hydroxy-eicosatetraenoic acid;

HFD, high fat diet;

HIF1-α, hypoxia inducible factor 1-alpha;

HODE, hydroxy-octadecadienoic acid;

HOMA-IR, homeostatic model assessment of insulin resistance;

HpDHA, hydroperoxy-docosahexaenoic acid;

HpEPE, hydroperoxy-eicosapentaenoic acid;

HpETE, hydroperoxy-eicosatetraenoic acid;

HpODE, hydroperoxy-octadecadienoic acid;

HSL, hormone sensitive lipase;

HX, hepoxilin;

ICAM-1, intracellular adhesion molecule-1;

IFN-γ, interferon-gamma;

IL, interleukin;

IκB, inhibitor of nuclear factor kappa B,

IR, insulin receptor;

IRS-1, insulin receptor substrate-1;

LA, linoleic acid;

LC PUFA, long chain polyunsaturated fatty acid;

LC n-3 PUFA, long chain omega-3 polyunsaturated fatty acid;

LCFA, long chain fatty acid;

LDL-C, low density lipoprotein cholesterol;

LOX, lipoxygenase;

LPL, lipoprotein lipase;

LPS, lipopolysaccharide;

LTE4, leukotriene E4;

LT, leukotriene;

LXB4, lipoxin B4;

LX, lipoxin;

MaR, maresin;

MCA, multichannel analyser;

MCP-1, monocyte chemoattractant protein-1 (CCL3);

MetS, metabolic syndrome ;

MGLL, monoacylglycerol lipase;

MHC, major histocompatibility complex;

MHO, metabolically healthy obese

MIP1α, macrophage inflammatory protein-1α, synonym CCL3;

MMP, matrix metalloproteinase;

MRM, multiple reaction monitoring;

MUFA, monounsaturated fatty acid;

MUO, metabolically unhealthy obese;

n-3, omega-3;

n-6, omega-6;

NAPE-PLD, n-acyl phosphatidyl ethanolamide – phospholipase-D;

NAFLD, non-alcoholic fatty liver disease;

NEFA, non-esterified fatty acid;

NF-κB, nuclear factor kappa light chain enhancer of activated B cells;

NK, natural killer;

NLRP3, NOD like receptor 3;

NTC, non-template control;

OEA, oleoylethanolamide

PDs, protectins;

PEA, palmitoylethanolamide

PI3-k, phosphoinositide 3-kinase;

PG, prostaglandin;

PGC1α, PPAR-γ coactivator 1α;

PKA, protein kinase A;

PL, phospholipid;

PMN, polymorphonuclear cells

PPAR-γ, peroxisome proliferator activated receptor-gamma;

Pref-1, preadipocyte differentiation factor-1;

PUFA, polyunsaturated fatty acid;

Q1, quadrupole 1;

Q2, quadrupole 2;

Q3, quadrupole 3;

qRT-PCR, quantitative real-time reverse transcription- polymerase chain reaction;

RANTES, CCL5;

RIN, RNA integrity;

RNase, ribonuclease;

RNA-Seq, RNA-sequencing

Rv, resolvin;

RvD2, resolvin D2;

RvE1, resolvin E1;

RvE3, resolvin E3;

RXR, retinoid X receptor;

SACN, scientific advisory committee on nutrition;

scWAT, subcutaneous white adipose tissue;

SCD, stearoyl-CoA-desaturase.

Sec, seconds;

SFA, saturated fatty acid;

SPE, solid phase extraction;

SPM, specialised pro-resolving mediators

SREBP, sterol regulatory element binding protein;

SREBPc1/ADD1, protein sterol regulatory element binding protein-c1 / adipocyte determination and differentiation factor-1;

SVC, stromal vascular cell

T2DM, type-2 diabetes;

TC, total cholesterol;

TG, triglyceride, triacylglycerols;

Th, T-helper;

TIE, receptor tyrosine kinase with immunoglobulin and epidermal growth factors homology domains;

TLR, toll like receptor;

TNF-α, tumour necrosis factor-alpha;

T-reg, T-regulatory;

TREM1, triggering receptor expressed of myeloid cells;

TX, thromboxane;

UPLC, ultra-pure liquid chromatography;

UPLC/MS-MS, ultra-pure liquid chromatography tandem mass spectrometry;

VCAM-1, vascular cell adhesion molecule-1;

VEGF, vascular endothelial growth factor;

VLCFA, very long chain fatty acid;

VLDL, very low density lipoprotein;

vWAT, visceral white adipose tissue

WAT, white adipose tissue;

WTCRF, welcome trust clinical research facility;

Chapter 1 Introduction

Low-grade chronic Inflammation is a feature of obesity and has been related to manifestations of metabolic diseases such as insulin resistance, type 2 diabetes (T2DM), and cardiovascular disease (CVD) (British Heart Foundation, 2018, National Statistics and NHS Digital, 2017 , Public Health England, 2014). White adipose tissue (WAT) is the main storage pool for excess lipid and has a role in maintaining whole body energy homeostasis through the uptake and release of fatty acids (FA), hormones such as leptin, and the secretion of signalling molecules such as cytokines and lipid metabolites. Homeostatic processes become dysregulated in obesity resulting in a state of low-grade chronic inflammation and the development of metabolic disease (Hotamisligil, 2006, Baker et al., 2011, Vachharajani and Granger, 2009). However, despite knowledge of subcutaneous WAT (scWAT) as an endocrine organ and contributor to this state of inflammation in obesity, investigations into the inflammatory state of scWAT in humans with obesity, and particularly in early stages of obesity, remain limited.

There is positive association between the consumption of marine derived long chain omega-3 (n-3) polyunsaturated fatty acids (LC n-3 PUFA) from oily ("fatty") fish and long-term health benefits, a mechanism of which includes the control of, and perhaps the resolution of, inflammation (Calder, 2018). LC n-3 PUFA may have anti-inflammatory actions in scWAT and may ameliorate obesity-associated scWAT inflammation. Evidence for the effects of LC n-3 PUFA in human scWAT is currently limited and inconclusive.

The research in this thesis aims to identify in greater detail differences in the inflammatory state of scWAT from normal weight and metabolically healthy obese (MHO) individuals and to investigate whether LC n-3 PUFA have an anti-inflammatory effect in human scWAT. To provide a background to this research, this chapter will describe WAT function under homeostatic conditions and in obesity, inflammation in the context of obesity, and the role of LC n-3 PUFA in inflammation.

1.1 Fatty acids

1.1.1 Structure and nomenclature

FAs are naturally occurring and are made up of a hydrocarbon chain with a methyl group at one end and a carboxyl group at the other. The carboxyl group can readily form ester links with alcohol groups to form acylglycerols such as triacylglycerols (TG), phospholipids (PL), and cholesteryl esters (Burdge and Calder, 2015). The hydrocarbon chain length can vary up to 30

carbons and this chain can contain double bonds. If a FA contains no double bonds it is described as a saturated fatty acid (SFA), if it has one double bond it is described as a monounsaturated fatty acid (MUFA) and if it contains two or more double bonds it is described as a polyunsaturated fatty acid (PUFA). The carbon at the methyl terminal of the chain is called the omega (ω or n) carbon (Gurr et al., 2002). Two groups of PUFA, n-3 and omega-6 (n-6), include FAs described as essential as they cannot be synthesized *de novo* in mammals. The common name of the essential n-3 PUFA is α-linolenic acid (ALA) and the common name of the essential n-6 PUFA is linoleic acid (LA). FAs can also be described using a shorthand nomenclature which is more commonly used than systematic names. This shorthand nomenclature describes a FA by the number of carbons in the hydrocarbon chain and the number and position of double bonds occurring from the methyl group end of the chain. Using this system, ALA is described as 18:3n-3 (as the hydrocarbon chain is 18 carbons long with 3 double bonds, the first of which found on the third carbon at the methyl end of the chain), and LA is described as 18:2n-6 (as the hydrocarbon chain is 18 carbons long with two double bonds, the first of which is found on the sixth carbon at the methyl end) (Burdge and Calder, 2015). Table 1.1 displays the common names, shorthand nomenclature, and structure of FAs commonly reported in humans.

Common name	Shorthand Nomenclature	Structure
Myristic acid	14:0	$CH_3(CH_2)_{12}COOH$
Palmitic acid	16:0	$CH_3(CH_2)_{14}COOH$
Palmitoleic acid	16:1n-7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Stearic acid	18:0	$CH_3(CH_2)_{16}COOH$
Oleic acid	18:1n-9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Vaccenic acid	18:1n-7	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
Linoleic acid	18:2n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
γ-linolenic acid	18:3n-6	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2(CH_2)_6COOH$
α-linolenic acid	18:3n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2(CH_2)_6COOH$
Arachidic acid	20:0	$CH_3(CH_2)_{18}COOH$
Gondoic acid	20:1n-9	$CH_3(CH_2)_7CH=CH(CH_2)_9COOH$
Eicosadienoic acid	20:2n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_9COOH$
Dihomo-γ linolenic acid	20:3n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_6COOH$
Arachidonic acid	20:4n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
Eicosapentaenoic acid	20:5n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
Docosapentaenoic acid	22:5n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$
Docosahexaenoic acid	22:6n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$

Table 1.1 Nomenclature and structure of fatty acids commonly reported in humans.

1.1.2 Metabolism and biosynthesis of FAs

FAs can be obtained through the diet or be synthesised *de novo* or from other FAs. FAs occur in the diet predominantly in the form of TG; these must be digested and the products absorbed before TG are reformed and released into the bloodstream as components of chylomicrons. TG are also released from the liver into the bloodstream as components of very low density lipoproteins (VLDL). FAs are also found in PL and cholesteryl esters in the diet, and PL and cholesteryl esters also circulate in the bloodstream as components of lipoproteins. TG are stored mainly in WAT. Non-esterified fatty acids (NEFAs) are also found in the bloodstream but at higher levels in a fasting state. NEFAs are released from WAT as a result of TG hydrolysis. The composition of FAs within each of these circulating lipid pools can reflect dietary FA intake but is also influenced by FA and complex lipid (e.g. TG) metabolism.

Dietary TG first undergo partial hydrolysis in the mouth by lingual lipase with further hydrolysis in the small intestine by pancreatic lipases to release NEFAs and 2-monoglycerides. Pancreatic phospholipase A_2 releases NEFAs and lysophospholipids from PL (Phan and Tso, 2001). Cholesteryl esters are hydrolysed into free cholesterol and NEFAs; NEFAs, monoglycerides and lysophospholipids are absorbed by the enterocytes in the intestinal wall (Nelson and Ackman, 1988, Mu and Hoy, 2004). Here they undergo re-esterification into TG which combine with apolipoproteins such as ApoB-48, ApoAI, ApoAII and ApoE to form chylomicrons (Summers et al., 2000). Chylomicrons are secreted into the lacteals where they join the lymph and are then secreted into the systemic circulation (Phan and Tso, 2001). In the circulation, chylomicron TG is hydrolysed by lipoprotein lipase (LPL) to release NEFAs, making them available for uptake into peripheral tissues such as muscle, liver and WAT (Figure 1.1) (Phan and Tso, 2001, Rutkowski et al., 2009).

FAs have various roles in the body including: undergoing β-oxidation to release energy (as ATP) by most aerobic tissues, incorporation into storage pools such as TG from where they can be later utilised for energy in times of need, and importantly contributing to cell membrane composition as components of PL (Calder, 2015a). Cell membranes comprise of PL which are one or two FAs linked to a phosphoglycerol backbone. The composition of cell membranes can be influenced by dietary FA intake, as well as by metabolism, cellular stimulation and other factors such as genetics (Calder, 2015a).

Changes in cell membrane FA composition can alter membrane fluidity which influences the activity of membrane proteins such as receptors, ion channels and enzymes. Within the cell membrane there are structures of specific protein, lipid and FA composition known as

microdomains or lipid rafts that have a major role in the initiation of signalling pathways (Yaqoob, 2009, Calder, 2015b). Changes in the FA composition of non-raft regions can modify the lipid rafts and alter signalling pathways such as those involved in inflammation through modulation of transcription factors such as nuclear factor-κ B (NF-κB) and peroxisome proliferator activated receptor-γ (PPAR-γ) (Calder, 2015a). Through this mechanism, FAs can affect gene expression (Yaqoob, 2009, Calder, 2015b), and can alter synthesis of inflammatory FA metabolites such as eicosanoids and docosanoids (Calder, 2015a).

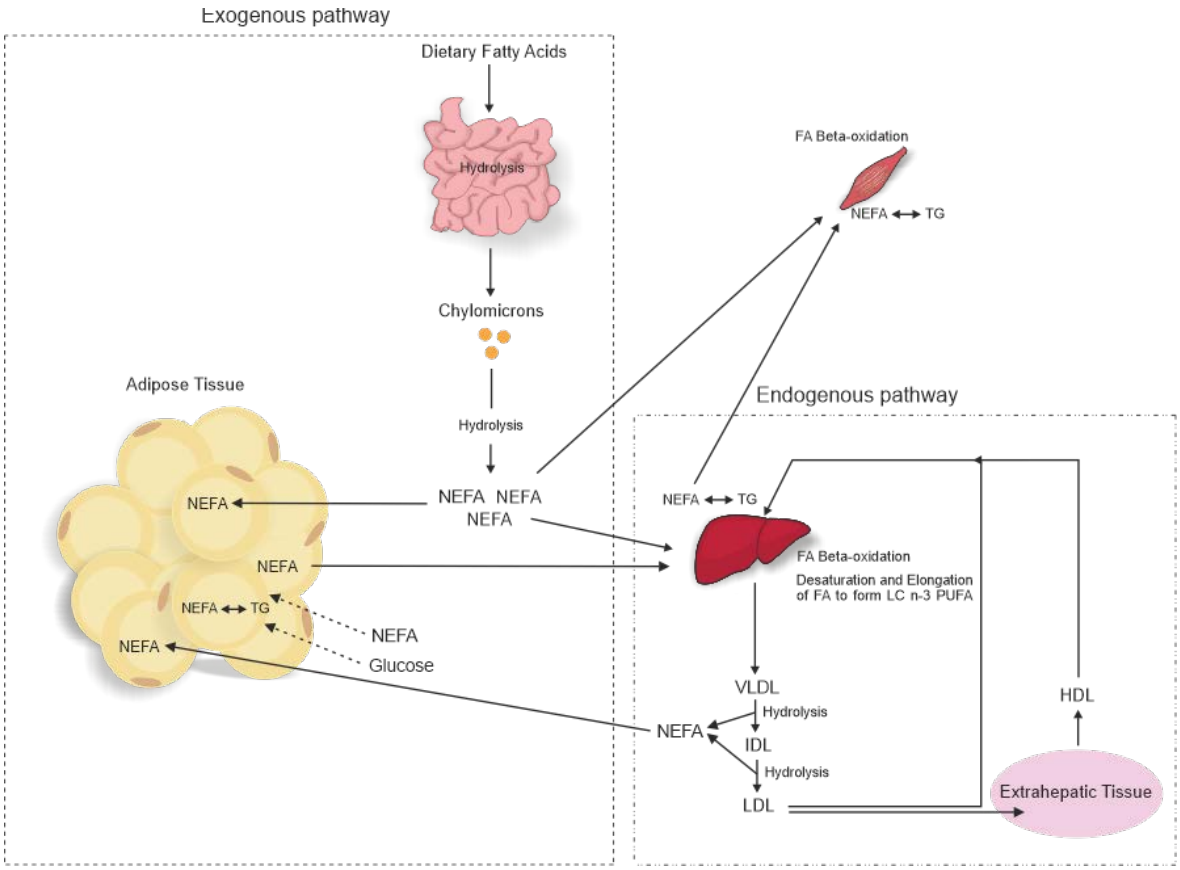


Figure 1.1 Metabolism of dietary fatty acids and incorporation into peripheral tissues.

FAs can also be synthesised *de novo* from carbohydrate (CHO) and protein. Acetyl-coenzyme A (acetyl-CoA) formed within the mitochondria from the metabolism of amino acids and glucose, combined with oxaloacetate from the citric acid cycle, forms citrate which is transported across the mitochondrial membrane into the cytoplasm. Here it is cleaved to produce acetyl-coA and oxaloacetate (Gurr et al., 2002). Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, which is then converted to malonyl-ACP with the addition of acyl carrier protein (ACP). FAs are then synthesised through a series of steps catalysed by the enzyme fatty acid

synthase (FAS). The hydrocarbon chain of the FA is built up by carbon atoms donated from malonyl-ACP and undergoes dehydration and reduction by acetyl-ACP to form butyryl-ACP. Butyryl-ACP undergoes further dehydration and reduction to form a six-carbon acyl-ACP. The cycle is repeated up to seven times resulting in the formation of a sixteen-carbon-palmitoyl-ACP; this is then hydrolysed to form palmitic acid (16:0). Palmitic acid can be elongated to produce the eighteen-carbon FA stearic acid (18:0) by enzymes termed elongases. A number of FAs are the substrates of these enzymes and so can be elongated into longer chain FAs (Arab, 2003, Arteburn et al., 2006).

A further group of enzymes termed desaturases catalyse the insertion of double bonds into the FA hydrocarbon chain to form MUFA and PUFA (Arab, 2003, Arteburn et al., 2006). Metabolism of SFA into MUFA requires delta-9 desaturase. For example, stearic acid (18:0) is converted to oleic acid (18:1n-9) by delta-9 desaturase. In animals, LA and ALA cannot be synthesised, but this occurs readily in plants. Firstly, delta-12 desaturase can convert oleic acid to LA and then delta-15 desaturase can convert LA to ALA. Although animals cannot synthesise LA and ALA, they can metabolise these two essential FAs further. This requires the activity of delta-5 desaturase (D5D), delta-6 desaturase (D6D) and elongase 5 and 2 enzymes (Arteburn et al., 2006, Hodson et al., 2008, Baker et al., 2016, Sibbons et al., 2018). This metabolism occurs predominantly in the liver with elongation and desaturation resulting in arachidonic acid (AA, 20:4n-6) formation from LA, and eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3). and docosahexaenoic acid (DHA, 22:6n03), from ALA (Arteburn et al., 2006, Hodson et al., 2008, Kopecky et al., 2009, Vezeridis et al., 2011) (Figure 1.2).The metabolism of FAs can be influenced by hormones and by the type of dietary FA which can regulate the activity of desaturases (Arab, 2003, Vezeridis et al., 2011). The conversion of ALA to EPA and DHA in humans is considered to be inefficient as approximately 15-35% of ALA is oxidised and only around 1-9% is successfully converted to DHA in men and women respectively (Arteburn et al., 2006, Baker et al., 2016). Therefore, to increase blood, cell and tissue levels of LC n-3 PUFA, it is suggested that the consumption of preformed EPA and DHA needs to be increased as opposed to the shorter chain precursor FAs.

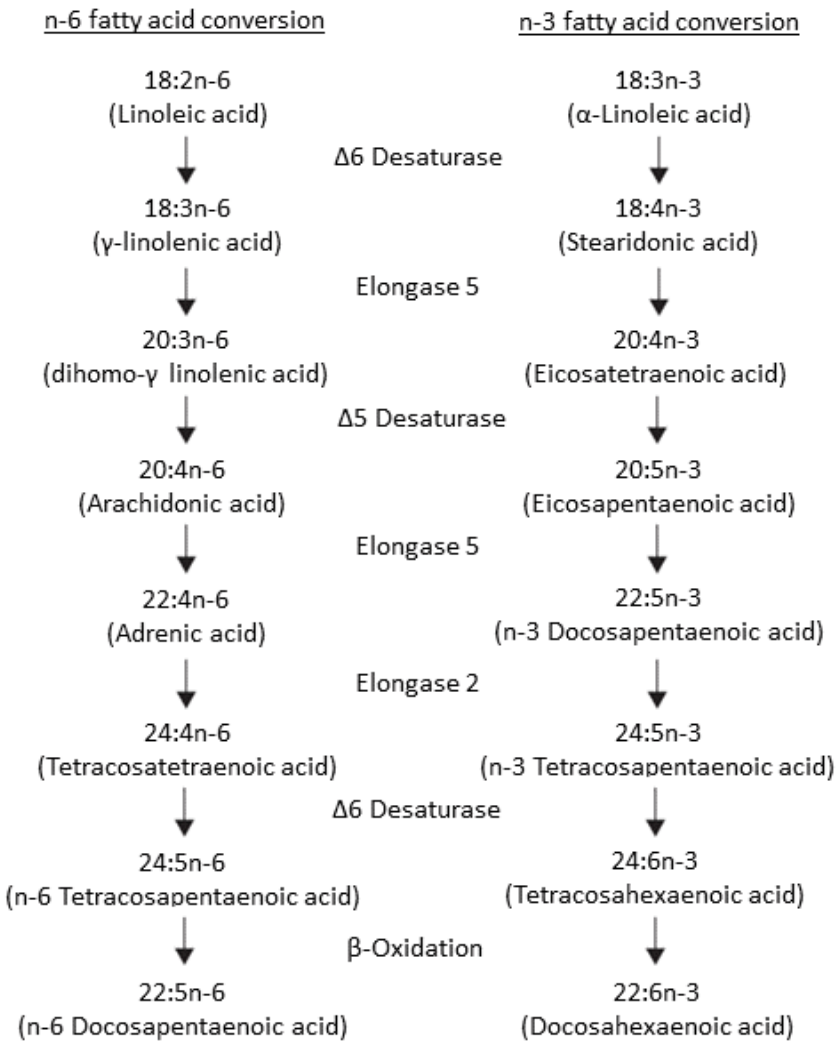


Figure 1.2 The elongation and desaturation pathway of essential fatty acids, linoleic and α-linolenic acid, resulting in formation of AA, EPA, DPA and DHA.

1.1.3 Dietary sources of saturated, monounsaturated, and polyunsaturated fatty acids, and their intakes in the UK

As described above, some FAs are essential, meaning humans cannot synthesize them and therefore they must be obtained from the diet. The essential n-6 PUFA LA can be obtained through consumption of cooking oils such as safflower oil (in which LA contributes 75% of the total FAs) and soybean oil (of which LA contributes 50-60% of the total FAs). The essential n-3 PUFA, ALA can be obtained through consumption of flaxseed or flaxseed oil (in which ALA contributes >50% of the total FAs), and as a lesser component of soybean oil (of which ALA contributes 10% of the total FAs), rapeseed oil and some nuts (e.g. walnuts). The most

predominantly consumed PUFA in the western diet is LA, followed by ALA; in the UK, the consumption of LA has increased to an average of around 11-12 g/day. This increase has been seen in many Western countries as people moved from animal fats like butter and lard to plant fats like margarines and vegetable oils. The consumption of ALA varies between 0.5-2.3 g/day in the western diet (Baker et al., 2016).

Changes in food processing, cooking methods, and use of plant oils as opposed to animal fats have had an influence on the profile of FA intake from the diet. In the UK, the Scientific Advisory Committee on Nutrition (SACN) reports daily n-6 PUFA intakes in UK adults to contribute 4.8% of total dietary energy consumed. SACN further reports SFA intake to contribute 12.1% of total daily energy in UK adults which is above the daily recommended value (DRV) of 10% (SACN, 2018). The most predominant sources of SFA consumed in the UK are animal products such as meat and dairy, including cheese, and cereal-based products such as cakes, biscuits, puddings and pastries (SACN, 2018). Further to this, SACN report MUFA intake in UK adults to meet the DRV at 12% of total daily energy intake, but that n-3 PUFA DRVs are not met, with n-3 PUFA contributing only 1% to total daily energy intake in UK adults (SACN, 2018). Meat from fish, particularly oily or 'fatty' fish, is a good source of LC n-3 PUFA. Thus EPA, DPA and DHA can be obtained through the diet through consumption of oily fish such as mackerel, salmon, herring, sardines and kippers. SACN recommends consumption of two portions (140 g each) of fish per week, of which one should be oily to obtain the equivalent of 0.45 g of LC n-3 PUFA a day (SACN, 2004). Intakes of LC n-3 PUFA in most people are well below this.

1.1.4 Transport of fatty acids into adipose tissue

NEFAs released from metabolism of circulating lipoproteins (e.g. chylomicrons) can be transported from the circulation into the WAT, which acts as a safe store for excess lipid. Within WAT, FAs are re-esterified into TG, the main storage form of lipid. TG within WAT form lipid droplets. The incorporation of NEFAs into WAT for storage requires the transport of the FAs across the plasma membrane of adipocytes. This requires the following steps: dissociation from albumin, diffusion through the outer aqueous phase, insertion into the outer leaflet of the cell membrane, translocation from the outer to the inner leaflet of the cell membrane, and dissociation from the inner leaflet of the cell membrane into the inner aqueous phase. A number of proteins are known to play a role in this FA transport; however, many of their exact functions are still currently unknown. Plasma membrane fatty acid binding protein (FABPpm), cytosolic FABP (FABPc), FA translocase (FAT/CD36), fatty acyl co-A synthetases FATP and ASC, and caveolin-1 are involved in the transport of FAs out of the circulation and into adipocytes (Glatz et al., 2010, Thompson et al., 2010) (Figure 1.3).

Caveolin-1 is expressed in adipocytes comprising 25% of the adipocyte membrane and forming specialised microdomains in the PL membrane by clustering lipid raft domains (Thompson et al., 2010). Caveolin-1 has been shown to bind FAs and have a role in moving the lipid droplet from the PL membrane in response to FA influx. It is suspected that FAs accumulate in the caveolae membrane leading to membrane asymmetry and subsequent budding to form vesicles of lipid (Thompson et al., 2010). In addition to expression on adipocytes, caveolin-1 is expressed on immune cells and bacterial lipopolysaccharide (LPS) stimulation of macrophages increases the expression of caveolin-1 mRNA; therefore, there may be an increase of caveolin-1 in pro-inflammatory conditions associated with obesity (Lei and Morrison, 2000).

CD36 is associated with lipid microdomains on the cell surface as well as being localised on the endoplasmic reticulum (ER), intracellular vesicles and mitochondria. It plays a role in facilitated diffusion of FAs across the membrane and has been shown to be important for the transfer of long chain FAs (LCFAs) (Glatz et al., 2010, Thompson et al., 2010). The level of CD36 mRNA is shown to increase in obesity, and under the influence of peroxisome proliferator activated receptor-gamma (PPAR-γ) activators; further to this, CD36 translocation to the cell membrane is stimulated by insulin (Glatz et al., 2010, Thompson et al., 2010).

Translocation of FATP1, which is efficient at transporting LCFAs and very-LCFAs (VLCFAs), to the plasma membrane is also stimulated in response to insulin signalling and it is suggested that FATP1 transports LCFAs coupled to metabolism through a mechanism termed vectoral acylation. In this process, FATP1 translocates to disordered non-lipid raft regions of the membrane from which it may then transport FAs from the inner leaflet of the membrane and esterify them to form acyl Co-A, creating a FA gradient across the membrane. ACS1 further converts transported FAs into FA acyl-CoA esters within the cytoplasm; once converted to acyl-CoA esters, FAs can then be incorporated into PL or TG, be elongated and/or desaturated, or undergo β-oxidation (Watkins, 1997, Watkins, 2008). In the scenario of lipid storage, FA acyl-CoA can be re-esterified into TG by the action of diacylglycerol acyltransferase (DGAT) enzymes -1 and -2; as TG accumulates within lipid droplets in the cytoplasm which form and bud from the ER membrane (Figure 1.3).

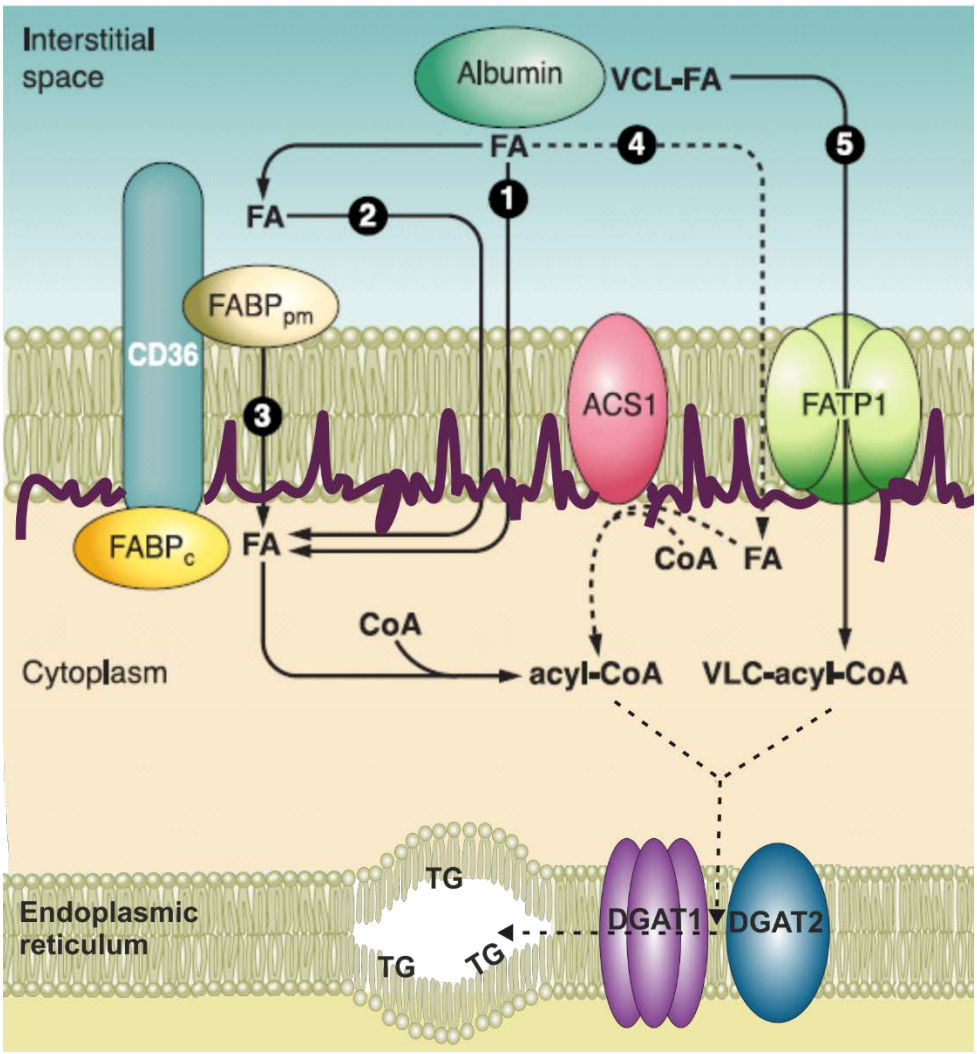


Figure 1.3 Routes of FA transport into WAT at caveolin rich domains of the plasma membrane.

1. FAs may dissociate from albumin and diffuse passively across the plasma membrane
2. FAs may be transported across the plasma membrane by facilitated diffusion aided by transport proteins FABPpm or CD36
3. FAs may be transported across the plasma membrane by CD36 then bound by FABPc before entering metabolic signalling pathways
4. A minority of FAs may be transported by FATP1 and converted to acyl co-A esters by the action of ACS1
5. Very long chain FAs (VLCFAs) are preferentially transported by FATP1 and converted to VLC-acyl Co-A esters
6. FA acyl-CoA and VLC FA acyl-CoA can be re-esterified into TG by the action of DGAT1 and DGAT2

Abbreviations: ACS1, fatty acyl co-A synthetase-1; CD36; fatty acid translocase; DGAT1, diacylglycerol acyltransferase-1; DGAT2, diacylglycerol acyltransferase-2; FA, fatty acid; FABPc, cytosolic fatty acid binding protein; FABPpm, plasma membrane fatty acid binding protein; FATP1, fatty acid transport protein-1 / very long change fatty acyl co-A synthetase-1; TG, triglyceride.

Adapted from (Yen et al., 2008, Glatz et al., 2010)

1.2 Adipose tissue

1.2.1 Adipose tissue physiology

1.2.1.1 Adipose tissue composition

AT is largely composed of fat storing cells termed adipocytes and is approximately 70% lipid. In addition, immune/inflammatory and endothelial cells, as well as adipocyte precursors are present and are collectively termed stromal vascular cells (SVCs) (Masoodi et al., 2014, Raclot and Oudart, 2000, Kopecky et al., 2009). AT is described as either subcutaneous or visceral (omental), depending upon its location, and is categorized into two types based upon its cellular composition. Brown adipose tissue (BAT) is comprised of mainly multilocular brown adipocytes, which contain many lipid droplets and mitochondria, whereas WAT comprises single lipid droplet containing adipocytes described as being unilocular (Masoodi et al., 2014, Raclot and Oudart, 2000). Beiging of the AT is also observed in which brown adipocytes appear in classical WAT defined depots (Chait. A and den Hartigh. L J, 2020).

1.2.1.2 Energy regulation

An important role of the WAT is maintaining energy homeostasis which includes the safe storage of excess lipid in the form of TG and the release of stored lipid for energy in times of need such as caloric restriction (Soula et al., 2015). Dietary and *de novo* synthesised FAs are transported to the AT as described in section 1.1.4, and are re-esterified to TG, which accumulate in the ER PL bilayer that eventually buds off to form a lipid droplet within the adipocyte (Ducharme and Bickel, 2008, Soula et al., 2015). This packaging of TG into lipid droplets is a mechanism for regulating lipid availability as substrate for lipid signalling metabolites or to be utilised for energy (Ducharme and Bickel, 2008). The lipid droplets are surrounded by a PL monolayer derived from the membrane bilayer which acts to stabilise the droplet. Lipid droplets of mature adipocytes are further coated in perilipin, a protein with a role in regulation of the hydrolysis of TG within the droplet (Ducharme and Bickel, 2008, Tansey et al., 2004, Duncan et al., 2007). There are two perilipins comprising this layer, A and B, with a greater contribution of perilipin A (Tansey et al., 2004, Duncan et al., 2007). Perilipin A regulates energy release through the ability to block hormone sensitive lipase (HSL) mediated lipolysis; however, upon phosphorylation by protein kinase A (PKA), it promotes interactions between HSL and the lipid droplet surface and subsequent TG hydrolysis (Tansey et al., 2004, Ducharme and Bickel, 2008, Duncan et al., 2007).

WAT lipolysis can occur via catecholamine binding of G protein coupled receptors (such as adrenaline binding to β-adrenergic receptors) on the adipocyte membrane, triggering signalling pathways resulting in subsequent generation of cyclic adenosine monophosphate (cAMP) (Duncan et al., 2007, Tansey et al., 2004, Ducharme and Bickel, 2008) (Figure 1.4). This sequentially activates PKA leading to phosphorylation of HSL and perilipin A, which mediates the ability of HSL to interact with the lipid droplet surface, promoting the hydrolysis of TG to release FAs (Tansey et al., 2004, Ducharme and Bickel, 2008, Duncan et al., 2007) (Figure 1.4).

TG is first hydrolysed by adipocyte triglyceride lipase (ATGL), activated by perilipin bound comparative gene identification-58 protein (CGI-58) that upon lipolytic stimulus is released from the lipid droplet surface and exerts its pro TG hydrolytic effects (Ducharme and Bickel, 2008, Rutkowski et al., 2015, Duncan et al., 2007). This first rate-limiting step of TG hydrolysis within the adipocyte lipid droplet results in DAG which is subsequently hydrolysed by HSL to form monoacylglycerol (MAG). MAG is finally hydrolysed by monoacylglycerol lipase (MGLL) resulting in glycerol and a free fatty acid (FFA) (Soula et al., 2015, Ducharme and Bickel, 2008, Rutkowski et al., 2015, Duncan et al., 2007). The FFAs are then transported out of the adipocytes via FABP and FATP action and can be utilized by other tissues for energy (Duncan et al., 2007).

In normal circumstances, lipolysis occurs in the fasting state whereby FAs are released from the WAT based upon their chain length and degree of saturation with chain lengths between 16-20 carbon atoms long with 4-5 double bonds exhibiting the greatest mobilisation (Raclot, 2003). In the fed state, the secretion of insulin has a major role in adipocytes to control the storage vs mobilisation of FAs (Lan et al., 2019). Insulin suppresses lipolysis by activating phosphoinositol-3 kinase signalling resulting in activation of phosphodiesterase-3B and the conversion of cAMP to 5'-AMP, inhibiting cAMP mediated activation of the lipolysis cascade (Morigny et al., 2016). Insulin also differentially regulates HSL isoform transcription and post transcriptionally affects HSL phosphorylation by stimulating PKA. Furthermore, insulin indirectly controls HSL expression by regulating the activity of growth hormone (Lan et al., 2019).

In obesity, the rate of lipolysis is reported to be significantly altered, contributed to by increased cytokine production, dysregulation of adipose hormones (adipokines), and the onset of insulin resistance (Magda M.I. Hennes et al., 1996, Zhang et al., 2002, Greenberg and Obin, 2006, Guilherme et al., 2008, Bézaire. V et al., 2009, Choi et al., 2010) (Table 1.2). Further to this, the rate of re-esterification of FFAs into TG differs between normal weight and obese individuals (Guilherme et al., 2008). In obese individuals, characteristically enlarged adipocytes exhibit reduced re-esterification of FFAs; in conjunction with enhanced lipolysis of TG, subsequently

greater concentrations of FFAs appear in the circulation of these individuals (Greenberg and Obin, 2006, Zhang et al., 2002, Guilherme et al., 2008).

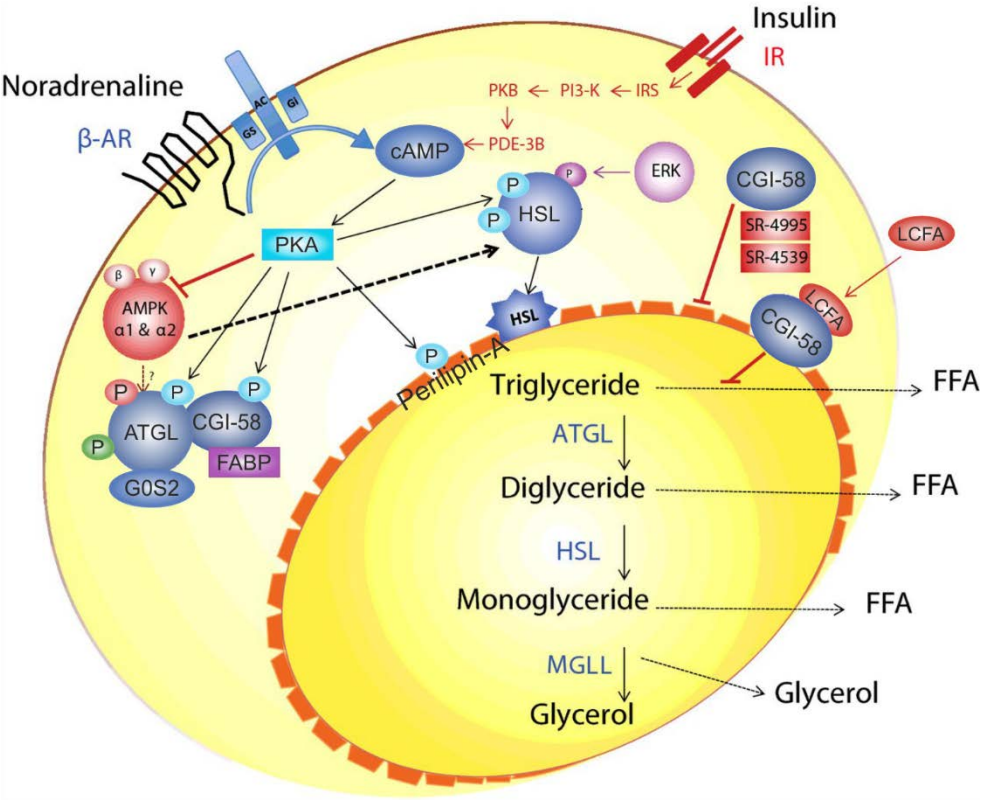


Figure 1.4 Regulation of adipocyte lipolysis.

Red pathways denote inhibitory responses; black pathways denote stimulatory responses; PKA phosphorylation sites are in blue.

Abbreviations: AC, adenylyl cyclase; FABP, fatty acid binding protein; AMPK 5', adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; ATGL, adipose triglyceride lipase; β-AR, β-adrenergic receptor; CGI-58, comparative gene identification 58. cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol lipase; ERK, extracellular signal-regulated kinase; FFA, free fatty acid; G0S2, switch gene-2; GI, inhibitory GTP-binding protein; HSL, hormone sensitive lipase; IRS, insulin receptor substrate; LCFA, long-chain fatty acid; MAG, monoacylglycerol; MGLL, monoacylglycerol lipase; P, phosphate; PDE-3B, phosphodiesterase-3B; PI3-K, phosphoinositide-3 kinase; PKA, protein kinase-A;

Adapted from (Raajendiran et al., 2016).

Mediator	Regulation in obesity	Action	Reference
Leptin	Increased secretion	Promotes lipolysis	(Hosogai et al., 2007)
Resistin	Increased secretion	Promotes lipolysis	(Trujillo and Scherer, 2006).
Adiponectin	Decreased secretion	Inhibits glucose and FFA transport	(Achari and Jain, 2017)
IL-6	Increased secretion	Promotes lipolysis	(Lafontan and Langin, 2009)
TNF-α	Increased secretion	Promotes lipolysis	(Zhang et al., 2002)
IFN-γ	Increased secretion	Promotes lipolysis	(Deng et al., 2013)

Table 1.2 Regulation of adipokines and cytokines in obesity, and consequence on the regulation of lipolysis.

1.2.1.3 Association of adipose tissue fatty acids with diet

A similarity between dietary FA composition and that of the WAT has been demonstrated (Marckmann et al., 1995, Hodson et al., 2008); however, incorporation of *de novo* synthesised SFA and MUFA means WAT composition may not always reflect that of the diet (Summers et al., 2000). Further to this, preferential mobilisation of FAs has been observed and FA release into the plasma is not proportional to the amount present in the WAT but is influenced by FA structure (Connor et al., 1996, Summers et al., 2000). It has been reported that the more unsaturated a fatty acid is, the greater the rate of its mobilisation during lipolysis, but this rate decreases the longer the fatty acid carbon chain is (Summers et al., 2000, Connor et al., 1996).

1.2.2 Adipose tissue expansion in obesity

As lipid in excess to energy need is accumulated, the plasticity and expandability of WAT is utilised. This is observed in the development of obesity in which existing adipocytes expand, termed hypertrophy, and new adipocytes are recruited through the differentiation of preadipocytes, termed hyperplasia, to accommodate the excess lipid (Jo et al., 2009, Drolet et al., 2008).

1.2.2.1 Adipose hyperplasia

Exogenous stimuli such as glucocorticoids, insulin, or lipophilic ligand binding, can trigger adipocyte hyperplasia (Morrison and Farmer, 2000, Otto and Lane, 2005, MacDougald and Lane, 1995, Kliewer et al., 1997, Rosen et al., 2002). The chronological activation of transcription factors and expression of genes promotes the acquisition of an adipocyte phenotype in which fibroblast

like preadipocytes differentiate into mature adipocytes with endocrine function (Gregoire et al., 1998, Gregoire, 2001, Kersten, 2002, Morrison and Farmer, 2000, Sarjeant and Stephens, 2012, Li et al., 2005, Rosen et al., 2002). For cells to be committed to an adipocyte phenotype they must withdraw from the cell cycle and enter growth arrest; in this phase, cells express a high concentration of preadipocyte factor-1 (Pref-1) which maintains the cells in the arrested state (Gregoire, 2001, Gregoire et al., 1998, Otto and Lane, 2005). Therefore, for differentiation into mature adipocytes to occur, Pref-1 must be inhibited (Otto and Lane, 2005, Gregoire, 2001, Gregoire et al., 1998). Inhibition of Pref-1 precedes an increase in the expression of CCAAT/enhancer binding proteins (CEBP) β and δ which mediate the expression of PPAR-γ and its heterodimer formation with the retinoid X receptor (RXR), allowing functional DNA binding (Morrison and Farmer, 2000, Otto and Lane, 2005).

Predominantly expressed in WAT, PPAR-γ1 and PPAR-γ2 isoforms have a role in adipocyte differentiation through inducing gene transcription via binding of small sequence elements in the promoter regions of pro-adipogenic genes (Kersten, 2002, Gregoire et al., 1998, Morrison and Farmer, 2000, Birsoy et al., 2008). For preadipocytes to continue through differentiation they must re-enter the cell cycle and undergo further rounds of cell division known as mitotic clonal expansion (Gregoire, 2001, Gregoire et al., 1998). During this clonal expansion, the expression of CEBP-β and C/EBP-δ decreases, and the expression of CEBP-α and PPAR-γ increases along with expression of the basic helix-loop-helix (bHLH) protein sterol regulatory element binding protein-c1 / adipocyte determination and differentiation factor-1 (SREBPc1/ADD1) (Gregoire, 2001, Gregoire et al., 1998, Tang et al., 2003). SREBPc1/ADD1 is capable of up regulating PPAR-γ and is itself able to trans-activate lipogenic adipocyte specific genes required for successful adipocyte differentiation (Gregoire et al., 1998, Morrison and Farmer, 2000, Rosen et al., 2002). C/EBP-α and PPAR-γ cross regulate one another to maintain their expression during this expansion phase and trans-activate adipocyte specific genes in the absence of CEBP-β and CEBP-δ (Morrison and Farmer, 2000, Rosen et al., 2002). Such genes include adipose specific FABP, CD36, perilipin, LPL and glucose transporter type 4 (GLUT4) which induce lipid accumulation and a spherical adipocyte morphology (Otto and Lane, 2005, Gregoire et al., 1998, Tamori et al., 2002).

Many morphological changes occur during mitotic clonal expansion as a result of alterations in extracellular matrix (ECM) components and rearrangement of cytoskeletal networks, including decreased expression of actin and tubulin components (Gregoire, 2001, Gregoire et al., 1998, Rodríguez Fernández and Ben-Ze'ev, 1989, Spiegelman and Farmer, 1982, Smas et al., 1998, MacDougald and Lane, 1995). These structural changes are required for communication between the ECM and pro-adipogenic transcription factors for preadipocytic ECM differentiation into the

basal lamina network phenotypic of fully differentiated adipocytes (Spiegelman and Farmer, 1982, Rodríguez Fernández and Ben-Ze'ev, 1989).

Adipose hyperplasia and adipogenesis in response to nutrient surplus are also under the control of Wnt/ β-catenin signalling (Sethi and Vidal-Puig, 2010). The Wnt/ β-catenin/T-cell factor dependent pathway inhibits adipogenic and lipogenic transcription factors such as PPARγ and CEBPα, in part modulated by the scaffold protein dishevelled binding antagonist of beta catenin (DACT) Dapper-1, and therefore inhibits adipogenesis. In normal 'healthy' WAT, Wnt signalling and DACT1 expression are high, therefore inhibiting adipogenesis. Both Wnt and DACT1 ligands are nutritionally regulated and their expression is down regulated in response to nutrient surplus allowing increased expression of PPARγ and CEBPα, subsequently promoting adipogenesis to accommodate the need for enhanced TG storage (Sethi and Vidal-Puig, 2010).

1.2.2.2 Adipose hypertrophy

Adipocyte hypertrophy sees excess lipid stored in existing adipocytes, which expand to accommodate the increasing supply of TG. As described in sections 1.1.2 and 1.1.4, dietary TG from chylomicrons and VLDL particles is broken down into NEFAs, which along with *de novo* synthesised NEFAs, are transported into the WAT and are re-esterified within the ER phospholipid membrane of adipocytes (Rutkowski et al., 2015, Walther and Farese, 2012, Thiam et al., 2013). This membrane is not suitable for the storage of large quantities of TG and an emulsion of lipid droplets forms in the cytoplasm (Thiam et al., 2013).

The expansion of the WAT is under tight regulation of transcription factors, gene expression, and many inflammatory signalling mediators such as cytokines and lipid metabolites, which have a role in lipogenesis and lipolysis, and therefore energy regulation, as well as in the remodelling of the surrounding microenvironment (Drolet et al., 2008, Jo et al., 2009). This remodelling of the WAT involves the degradation of pre-existing ECM, the formation of new blood vessels to vascularise the expanding tissue (angiogenesis), and the formation of new extracellular structures.

1.2.2.3 Hypoxia and adipose remodelling

Decreased oxygen supply can occur in expanding WAT due to reduced blood flow and increased demand of oxygen to diffuse over larger distances as the tissue increases in size (Lee et al., 2010, Halberg et al., 2009, Pasarica et al., 2009b). This state of oxygen depletion is termed hypoxia and results in the accumulation of hypoxia inducing factor-1α (HIF1-α). In normoxic conditions, HIF1-α is rapidly degraded by oxygen dependent hydroxylation of HIF1-α proline residues and subsequent proteasomal degradation (Halberg et al., 2009). However, in abundance, HIF1-α translocates to the nucleus where through binding to hypoxia response elements, it stimulates

the expression of a range of genes involved in angiogenesis, glycolysis, and erythropoiesis (Halberg et al., 2009, Hosogai et al., 2007, Wang et al., 2007, Sun et al., 2013, Tahergorabi and Khazaei, 2013). Through this transcriptional action, HIF1-α is able to elicit a fibrotic response in hypoxic AT by inducing the expression of many ECM components such as collagens (Sun et al., 2013, Pasarica et al., 2009a).

WAT ECM is comprised richly of collagen VI and the expression of the COL6-3a protein is seen to increase in hypoxic conditions and with increasing body mass index (BMI) (Pasarica et al., 2009a). Adipocytes within a restrictive fibrotic ECM lose function and necrotise triggering infiltration of lymphocytes, neutrophils and macrophages, creating a pro-inflammatory environment (Sun et al., 2013). Adipose vascularity is not only important in supplying oxygen to the expanding tissue but also in supplying growth factors and cytokines required by adipocytes for proper physiological function, supplying bone marrow derived stem cells capable of differentiation into pre-adipocytes, adipocytes and further vascular cells, and to facilitate the removal of metabolic waste products (Cao, 2010). A mechanism to counteract the disconnection between the expanding WAT and the vasculature is angiogenesis.

HIF1-α and PPAR-γ coactivator 1α (PGC1α) activation of pro-angiogenic factors such as angiopoietin-2 (Ang-2) and vascular endothelial growth factor (VEGF)-α, promotes the sprouting of new blood vessels to re-supply the expanding tissue (Adair and Montani, 2011, Corvera and Gealekman, 2014, Arany et al., 2008). Expression of VEGF-A initiates the proliferation of endothelial cells, which migrate through the adipose ECM creating sprouting capillaries in the direction of VEGF-A stimulus. The vascular basement membrane and adipose ECM are degraded by the action of matrix metalloproteinases (MMPs) which weaken endothelial cell-cell interactions allowing the detachment of pericytes and protrusion of endothelial cells from pre-existing blood vessels to form sprouting capillaries (Rundhaug, 2005, Corvera and Gealekman, 2014, Adair and Montani, 2011). When two sprouting capillaries meet at the source of VEGF-A, they fuse to create a continuous lumen, stabilised by pericytes and the formation of a new basement membrane, through which blood and oxygen can flow (Corvera and Gealekman, 2014, Adair and Montani, 2011, Rundhaug, 2005).

Angiopoeitin-1 (Ang-1) and Ang-2 act through the receptor tyrosine kinase with immunoglobulin and epidermal growth factors homology domains (TIE)-2 (Voros et al., 2005, Dallabrida et al., 2003). Ang-1 phosphorylates this receptor to promote the stabilisation of formed vessels through recruitment of pericytes and stimulating endothelial cell-mural cell interactions. This in turn reduces the permeability of the newly formed vessels, reducing immune cell extravasation (Dallabrida et al., 2003, Voros et al., 2005, Tahergorabi and Khazaei, 2013). Ang-2 is an antagonist

for TIE-2 and may have a role in the initial steps of vessel formation as it leads to disruption of endothelial cell-pericyte interactions (Voros et al., 2005, Dallabrida et al., 2003, Distler et al., 2003). However, this is also a crucial step in blood vessel regression suggesting Ang-2 has both angiogenic and angiostatic properties (Distler et al., 2003, Voros et al., 2005). Indeed, in the presence of other pro-angiogenic factors such as VEGF, Ang-2 induces the migration and proliferation of endothelial cells; however, in the absence of such stimulants, Ang-2 causes regression of blood vessels and endothelial apoptosis (Distler et al., 2003).

The plasticity of WAT vascularity is dependent on the balance between pro- and anti-angiogenic factors. It has been demonstrated in both murine and human studies that obesity is associated with an imbalance of pro-angiogenic factors in which increased VEGF, MMP-2 and -9, and the proangiogenic factors angiogen and angiopoietin have been reported in obese humans and are seen to be positively correlated with BMI (Silha et al., 2005, Chavey et al., 2003)

1.3 Inflammation

1.3.1 Overview of inflammatory response

Inflammation is a protective response which involves cellular activation and the secretion of chemical mediators in the context of immune defence and tissue repair (Bessesen et al., 2015). The accumulation of immune cells and release of chemical mediators systemically and at the site of damage, results in the presentation of the classic signs of inflammation which are redness, heat, swelling, pain, and ultimately loss of function. The activation of an inflammatory or immune response increases vascular permeability enhancing blood flow and extravasation of immune cells to the site of damage, resulting in the activation of immune cells and subsequent secretion of pro-inflammatory mediators which have actions in further immune cell recruitment, tissue remodelling and repair, and metabolic signalling. An inflammatory response may arise from response to pathogen attack or metabolic stress.

In normal circumstances, inflammation is self-limiting and controlled by negative feedback mechanisms that inhibit pro-inflammatory signalling, promote secretion of pro-resolving mediators and other anti-inflammatory mediators such as certain cytokines, and promote the shedding of inflammatory mediator receptors inhibiting cell response to inflammatory signals (Serhan and Savill, 2005, Serhan et al., 2015, Calder, 2017). Failure to resolve inflammation results in sustained increased concentrations of chemical mediators and inflammatory cells in the circulation and at the site of inflammation (Serhan and Savill, 2005, Johnson et al., 2012). This is described as a state of chronic inflammation and is observed in the onset of, and during obesity

(Johnson et al., 2012, Chen et al., 2014, Masoodi et al., 2014, Bessesen et al., 2015) and in this context is often described as metabolic, or meta, flammation as it increases the risk of developing further metabolic disease such as insulin resistance and diabetes (Masoodi et al., 2014, Choe et al., 2016, Gerlini et al., 2018).

1.3.2 Metabolic inflammation

Obesity is categorised by an increase in fat mass with an increased contribution of AT to total body weight (Kopecky et al., 2009); according to World Health Organization an individual is considered to be overweight if their body mass index (BMI) is between 25.0 and 29.9 kg/m^2, and obese if their BMI is greater than 30 kg/m^2 (WHO, 2015).

The prevalence of obesity has tripled in the last decades with 39% of the world's adult population classified as overweight, and 13% as obese in 2016 (WHO, 2015). Obesity is considered to be one of the greatest public health challenges and in the UK alone, approximately 620,000 individuals were admitted to hospital with a health problem associated with obesity between 2016-2017 (National Statistics and NHS Digital, 2018). Obesity increases the risk of developing diseases such as CVD, non-alcoholic fatty liver disease (NAFLD),T2DM and some cancers (British Heart Foundation, 2018, National Statistics and NHS Digital, 2017 , Public Health England, 2014). Increased risk of such diseases arises through the manifestation of metabolic disturbances and altered endocrine functions associated with obesity and low-grade chronic inflammation.

Inflammation sustained in response to metabolic stress, such as in obesity, differs greatly from that from pathogen attack in that it manifests gradually over time, described as low-grade chronic inflammation. This inflammation arises from nutrient excess and metabolic tissue inflammatory mediators resulting in the activation of pro-inflammatory signalling cascades. These include binding and activation of NF-κB, activation of IL-1β and potentially the inflammasome, immune cell recruitment, the secretion of pro-inflammatory mediators including lipid metabolites, chemokines and cytokines, and altered insulin sensitivity (Hotamisligil, 2006, Baker et al., 2011, Vachharajani and Granger, 2009).

In most healthy normal weight individuals, excess lipid is stored in defined WAT depots; these include upper (abdominal) and lower (gluteo-femoral) scWAT, and visceral/ omental (intraabdominal) WAT (vWAT) depots (Chait. A and den Hartigh. L J, 2020). In situations of chronic excess lipid intake such as obesity or in conditions such as lipodystrophy, the plasticity of scWAT is compromised, and excess lipid accumulates ectopically in areas in which TG storage is not a primary function. This can increase susceptibility to comorbidities such as atherosclerosis and T2DM (Chait. A and den Hartigh. L J, 2020). This ectopic accumulation of fat is usually in the

visceral cavity and includes accumulation in the liver (intrahepatic), around major blood vessels (perivascular), between the heart and pericardium (epicardial), over the intestines, liver and stomach (omental, often grouped with scWAT as 'abdominal' fat), and surrounding the kidneys (retroperitoneal, often grouped with omental to define 'vWAT') (Chait. A and den Hartigh. L J, 2020).

Ectopic fat occurring in and around major glucose regulatory organs such as the liver, skeletal muscle and pancreas can interfere with insulin signalling, reduce glucose uptake, and may result in insulin resistance and increase risk of developing T2DM (Rutkowski et al., 2015). An excessive amount of vWAT is also considered to be ectopic and is associated with features of metabolic syndrome (MetS) and risk of cardiovascular complications (Chait. A and den Hartigh. L J, 2020). Approximately 60% of ectopic TG in the liver observed in patients with NAFLD derives from FFA released from WAT (Donnelly et al., 2005). The liver is a major target of inflammatory signalling molecules released from the WAT, contributing to hepatic lipid accumulation, steatosis, and increased risk of hepatic insulin resistance (Kabir et al., 2005, Björntorp, 1990). In addition to increased liver fat, approximately 2/3 of obese individuals have excessive pancreatic fat which is associated with beta-cell dysfunction and T2DM (Uygun et al., 2015). Furthermore, obesity and T2DM (Yafei et al., 2019) are also associated with fat accumulation in the heart which is a strong predictor of CVD (Rabkin, 2014).

vWAT and scWAT depots are both correlated with multiple features of MetS but risk markers associated with vWAT are significantly stronger (Fox. C S et al., 2007, Kim et al., 2014). Greater rates of lipolysis and inflammatory changes are observed in vWAT in obesity and are more strongly associated with systemic inflammation, T2DM, and CVD than scWAT (Schlecht et al., 2016, Verboven et al., 2018, Chait. A and den Hartigh. L J, 2020). Individuals who accumulate fat mainly in scWAT depots, which are designed for safe storage of neutral TG, tend to have lower levels of inflammation and do not present with insulin resistance (Chait. A and den Hartigh. L J, 2020). These individuals are defined as MHO individuals. Those presenting with insulin resistance, systemic inflammation, and other features of MetS are defined as metabolically unhealthy obese (MUO) individuals (Kim et al., 2014, Chait. A and den Hartigh. L J, 2020). Despite stronger associations between metabolic complication and vWAT measures, the role of scWAT in the development of obesity related metabolic complications should not be overlooked as it is a significantly larger depot than vWAT (Thompson. D et al., 2012). In terms of experimental investigation, scWAT is easier to obtain than vWAT and may be more easily manipulated through lifestyle changes: scWAT has exhibited anti-inflammatory responses to LC n-3 PUFA intervention not observed in vWAT (Itariu et al., 2012). A summary of metabolic and inflammatory features of vWAT, scWAT and BAT associated with T2DM and CVD is detailed in Table 1.3.

		Associated with T2DM		Associated with CVD
Intrabdominal vWAT	⇑⇑	TNF-α, IL-6, MCP-1, Serum amyloid-A	⇑⇑	TNF-α, IL-6, MCP-1, Serum amyloid-A
	⇑⇑	Inflammatory macrophages	⇑⇑	Inflammatory macrophages
	⇓	Adiponectin, omentin	⇓	Adiponectin, omentin
	⇑	Leptin, Resistin	⇑	Leptin, Resistin
	⇑	Th1 and cytotoxic T cells	⇑⇑	FFA
	⇓	iNKT cells		
	⇑⇑	FFA		
	⇑	ECM		
	⇑	Insulin resistance		
scWAT	⇑	TNF-α, IL-6, MCP-1, Serum amyloid-A	⇑	TNF-α, IL-6, MCP-1, Serum amyloid-A
	⇑	Inflammatory macrophages	⇑	Inflammatory macrophages
	⇓⇓	Adiponectin, omentin	⇓⇓	Adiponectin, omentin
	⇑	ECM	⇑	ECM
	⇓	Adipose plasticity	⇓	Adipose plasticity
BAT	⇓	Mass - metabolic function	⇓	Mass - metabolic function
	⇑	TNF-α	⇑	TNF-α
	⇑	Macrophages	⇑	Macrophages

Table 1.3 Metabolic and inflammatory features of vWAT, scWAT and BAT associated with type-2 diabetes and cardiovascular disease (Chait. A and den Hartigh. L J, 2020).

The mechanisms by which WAT inflammation occurs and influences metabolic processes are described in further detail below.

1.3.2.1 Obesity associated metabolic inflammation

As previously described, WAT is implicated in the secretion of many pro- and anti-inflammatory mediators such as hormones, acute phase proteins, cytokines, chemokines and growth factors (Bessesen et al., 2015, Camargo et al., 2014, Chait. A and den Hartigh. L J, 2020) in order to maintain homeostatic conditions, including insulin sensitivity and energy regulation and in response to conditions of stress such as hypoxia or lipotoxicity (Masoodi et al., 2014). As described, an increase in WAT is observed in the development of obesity and the 'normal' WAT endocrine functions become dysregulated (Jo et al., 2009, Drolet et al., 2008).

These signalling molecules are predominantly synthesized and released from WAT resident immune cells in the SVC population, of which sub-populations are altered during and in the onset of obesity and its associated inflammation (Bertola et al., 2012, Lynch et al., 2012, Stefanovic-Racic et al., 2012, Winer et al., 2014, Boutens et al., 2018, Lu et al., 2019).

WAT SVCs include endothelial cells and immune cells including neutrophils, lymphocytes, monocytes, dendritic cells (DCs) and macrophages. These cells are implicated in immune response, inflammatory processes, and immunometabolism (Ferrante, 2013, Schipper et al., 2012).

1.3.2.2 Adipose tissue immune cell infiltration

Under normal (non-inflammatory) conditions, the rate of immune cell infiltration, particularly of monocytes, into WAT is low (Murphy K et al., 2008). However, in response to an inflammatory stimulus, adhesion and chemotaxis of immune cells, and ultimately infiltration to the site of inflammation, is enhanced (Murphy K et al., 2008). The infiltration of immune cells out of the circulation and into peripheral tissues such as the WAT is promoted by an upregulation in the expression and secretion of adhesion molecules. Adhesion molecules, such as vascular cell adhesion protein-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and P-and E-selectins, are involved in the recruitment of leukocytes such as neutrophils and monocytes to sites of inflammation. Cell recruitment occurs by the tethering and rolling of neutrophils via interaction of E-selectin on the endothelium surface and binding site receptors on the neutrophils, which brings the neutrophil into close proximity of the vascular endothelium (Golias et al., 2007). This allows adhesion and migration of the neutrophil via interaction between integrins on the neutrophil cell surface and ICAM-1 on the endothelial surface (Golias et al., 2007). Further to this, monocyte chemoattractant protein 1 (MCP-1, CCL3) secretion promotes the adhesion and migration of monocytes into the WAT.

Monocytes can be identified by the cell surface markers CD14 and CD16; classical monocytes are $CD14^{++}CD16^{-}$, have phagocytic properties, and account for 85% of total blood monocytes (Ziegler-Heitbrock et al., 2010). 5% of the monocyte population are termed intermediate monocytes displaying $CD14^{++}CD16^{+}$, and 10% are classed as non-classical monocytes as they display $CD14^{+}CD16^{++}$ and have inflammatory properties upon activation (Ziegler-Heitbrock et al., 2010, Mukherjee et al., 2015). Further to this, non-classical monocytes express major histocompatibility molecule (MHC) class II (MHCII), therefore displaying properties of antigen (Ag) presentation (Lee et al., 2017). MHCII activation results in T-helper (Th) -1 cell activation and subsequent secretion of Th1 derived cytokines such as interferon-gamma (IFN-γ), interleukin (IL)-2 and tumour necrosis factor-alpha (TNF-α). Monocytes are capable of differentiation into DCs and macrophages depending on the inflammatory state of the surrounding environment. Under conditions where there are high levels of IFN-γ, IL-6, or IL-10, monocytes differentiate into macrophages (Delneste et al., 2003, Chomarat P et al., 2000), and under conditions of high granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-4, monocytes differentiate into DCs (Chapuis F et al., 1997).

1.3.2.3 Immune cell polarisation

Under normal physiological conditions, macrophages perform homeostatic roles and aid tissue repair. In healthy WAT, macrophages have a role in remodelling the tissue environment to aid

WAT hypertrophy and angiogenesis in response to nutritional surplus as well as lipid buffering, and removal of cells undergoing apoptosis. These macrophages are termed 'alternatively activated M2' macrophages and they produce anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 (Masoodi et al., 2014, Bessesen et al., 2015, Thomas and Apovian, 2017).

Enhanced infiltration of macrophages into the WAT is associated with obesity and can be stimulated by a state of hypoxia in the tissue caused by hypertrophy and necrosis of adipocytes, as well as by increased secretion of MCP-1 from the adipocytes (Bessesen et al., 2015, Masoodi et al., 2014, Kopecky et al., 2009). This infiltration is initially part of 'normal' homeostatic function as macrophages clear adipocytes undergoing apoptosis and harmful debris and help regulate adipose remodelling. However, obesity is not only associated with an increase in number of macrophages, but also a change in polarization of macrophages in which there is a switch to 'classically activated M1' macrophages which are observed to aggregate around dying cells in formations termed crown like structures and to secrete pro-inflammatory cytokines such as TNF-α, and IL-6 that further exacerbate AT inflammation (Masoodi et al., 2014) (Figure 1.5) .

Changes in macrophage polarisation in obesity can occur due to disruption of immune cell homeostasis. In obesity, an increase in the concentration of pro-inflammatory $CD4^+$ (Fabbrini et al., 2013, Duffaut et al., 2009) and $CD8^+$ T cells (Duffaut et al., 2009), and a decrease in anti-inflammatory Th2 cells, results in an increase in IFN-γ production (Figure 1.5). Leptin signalling, which is also observed to be increased in obesity, is also capable of inducing IFN-γ secretion and therefore, macrophage polarisation towards the M1 phenotype and lipolysis (Deng et al., 2013). Further to the actions of IFN-γ, the activation of $CD4^+$ T cells to produce such cytokines requires the recognition of MHCII on Ag presenting cells such as macrophages (Deng et al., 2013). Therefore, macrophages may also play a role in the activation of these cells and the change in their own polarization (Deng et al., 2013).

In the WAT, macrophages and adipocytes interact to regulate the inflammatory and metabolic status of the surrounding environment. CD36 is a class B scavenger receptor expressed on a variety of cells including adipocytes and macrophages and is implicated in communication between adipocytes and macrophages (Love-Gregory and Abumrad, 2011). Its expression is positively correlated with body fat (Nisoli et al., 2000, Masuda et al., 2009) and it is proposed to mediate communication through promoting pro-inflammatory cytokine secretion (Love-Gregory and Abumrad, 2011) and is therefore associated with the M1 macrophage phenotype (Nisoli et al., 2000). Changes in macrophage phenotype and resultant cytokines alter normal adipose function; classically activated M1 macrophages secrete the pro-inflammatory cytokine TNF-α which acts on adipocytes to promote lipolysis and further pro-inflammatory cytokine secretion.

Not only can TNF-α activate the NF-κB pathway, but free SFA released during lipolysis can also activate NF-κB by acting as ligands for toll-like receptor (TLR)-4 expressed on macrophages.

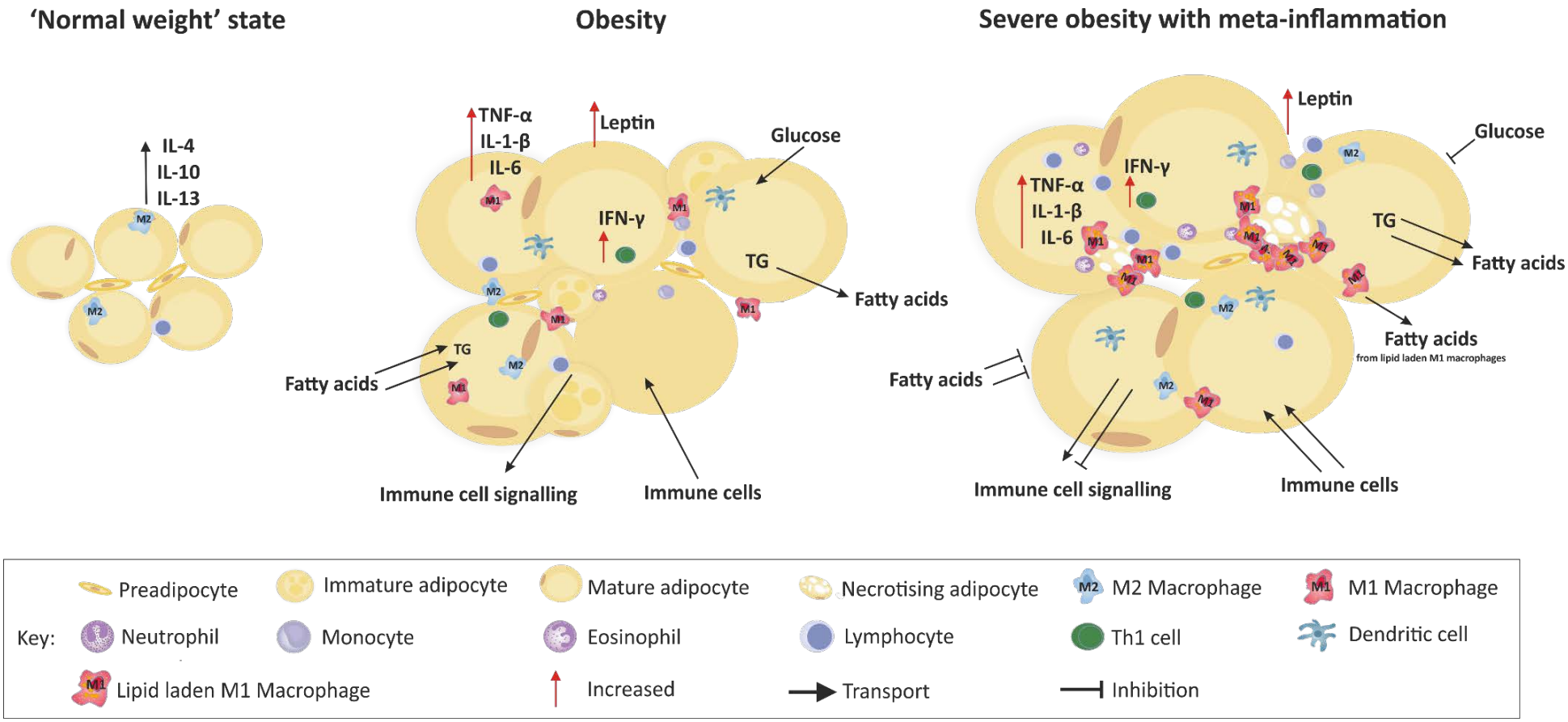


Figure 1.5 Immune cell infiltration, polarisation and signalling in the onset of obesity and associated metabolic inflammation in human subcutaneous white adipose tissue.

1.3.2.4 Cytokines and chemokines

As briefly described above, cytokines secreted by adipose resident cells are capable of influencing immune cell polarisation and cell signalling. Through such signalling, cytokines can modulate insulin sensitivity, lipid storage, recruitment of inflammatory cells, and overall metabolism and the inflammatory state of the tissue (Jr. et al., 2010, Park and Ahima, 2014, Trujillo and Scherer, 2006, Gonzalez-Periz and Claria, 2010). As suggested, cytokines can have either anti- or pro-inflammatory actions in the WAT, and secretions of cytokines are altered in the onset of obesity and metabolic disease (Park and Ahima, 2014, Pickup et al., 1997, Straczkowski et al., 2002, Bruun et al., 2003, Bruun et al., 2004, Micallef et al., 2009, Bing, 2015).

IL-6 is expressed by adipocytes and SVCs; circulatory concentrations of IL-6 are elevated in obesity, with one third secreted by adipocytes (Park and Ahima, 2014, Trujillo and Scherer, 2006). Expression of IL-6 in the AT and circulation increase with the onset of obesity and is positively correlated with insulin resistance and impaired glucose tolerance (Park and Ahima, 2014, Pickup et al., 1997). IL-6 is known to inhibit adipogenesis and has been seen experimentally *in vitro* and *in vivo* to increase levels of FFAs (Trujillo and Scherer, 2006) (Figure 1.6).

A chemokine expressed predominantly by adipose resident macrophages, and to lesser extent by adipocytes, is IL-8; IL-8 and has a role in modulating an inflammatory response through promotion of chemoattraction and adhesion (Straczkowski et al., 2002) (Figure 1.6). The concentration of IL-8 is positively correlated with BMI and percentage fat mass, with increased concentrations reported in obese individuals; however this is only significant between IL-8 secreted from non-fat adipose cells such as macrophages, and not between concentrations secreted from isolated adipocytes (Straczkowski et al., 2002, Bruun et al., 2003, Bruun et al., 2004). IL-8 secretion is also reported to be regulated by TNF-α and activated macrophage derived IL-1β; these mechanisms are thought to be main contributors to the increase in IL-8 with obesity as both TNF-α and IL-1β levels are seen to increase with the onset of obesity (Straczkowski et al., 2002, Bruun et al., 2003).

TNF-α is expressed by adipocytes and SVCs in WAT; circulating concentrations are positively correlated with insulin resistance as well as adiposity (Park and Ahima, 2014, Micallef et al., 2009). Expression of TNF-α in WAT suppresses genes involved in the uptake and storage of NEFAs and glucose, as well as genes encoding transcription factors involved in lipogenesis (Park and Ahima, 2014) (Figure 1.6). TNF-α further modulates the expression of hormones secreted by the adipocytes such as increasing leptin and decreasing adiponectin secretion (Park and Ahima, 2014). These actions of TNF-α result in increased FFA and glucose in the circulation contributing to MetS and a pro-inflammatory environment in the WAT.

IL-1β, predominantly secreted by macrophages in the WAT, is also implicated in metabolic dysregulation. Cleavage of pro-IL-1β to IL-1β by caspase-1 is controlled by the NOD like receptor 3 (NLRP3) inflammasome becoming biologically active in response to inflammatory or nutrient stimuli, such as SFA (Dinarello, 2009, Snodgrass et al., 2013). Active IL-1β is involved in potentiating and initiating immune and inflammatory responses. In 3T3-L1 adipocytes, treatment with IL-1β induced insulin resistance through decreasing the expression and translocation of glucose transport protein GLUT4 (Jager et al., 2007) (Figure 1.5). GLUT4 is an important glucose transport protein that mediates the uptake of exogenous glucose into adipocytes (Huang and Czech, 2007). This activity is insulin mediated (removal of glucose from the bloodstream occurs in response to insulin secretion resulting from an increased glycaemic load) and subsequently decreased expression of GLUT4 has been observed in insulin resistance particularly accompanied by increased IL-6 and TNF-α concentrations (Stephens et al., 1997, Rotter et al., 2003, Huang and Czech, 2007). Secretion of IL-1β from non-fat WAT cells is enhanced in obesity, suggesting involvement in associated metabolic inflammation (Bing, 2015).

A cytokine with anti-inflammatory actions expressed by macrophages in WAT is IL-10; the concentration of IL-10 is positively correlated with insulin sensitivity (Figure 1.6). Adipocytes express receptors for IL-10 and actions of IL-10 include reducing MCP-1 secretion, resolving negative effects of TNF-α on the GLUT-4 transporter and counteracting IL-6 induced insulin resistance (Gonzalez-Periz and Claria, 2010, Roher. et al., 2008).

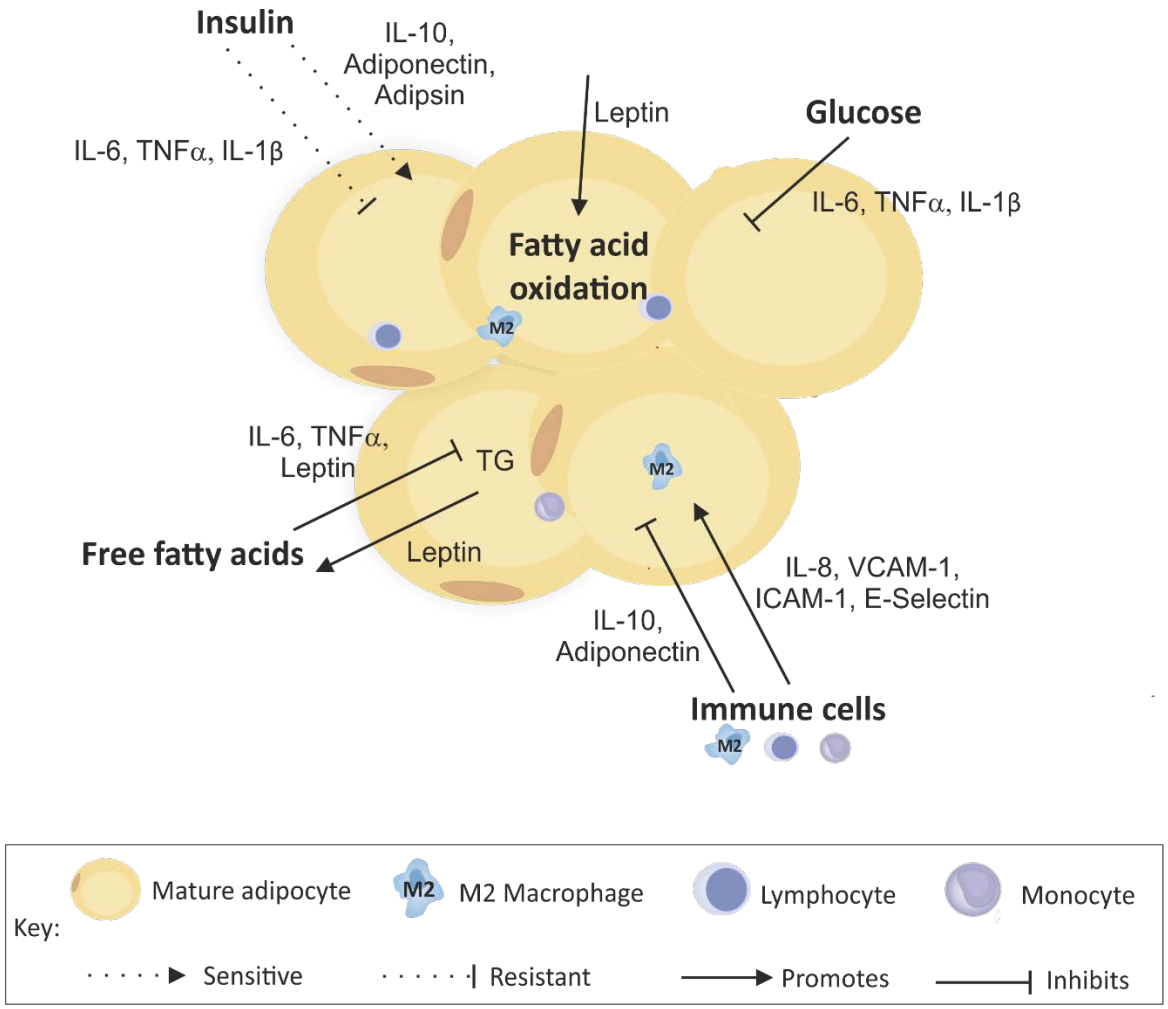


Figure 1.6 Cytokine and adipokine signalling in human white adipose tissue.

1.3.2.5 Adipokines

In addition to cytokines, a group of adipose-derived hormones termed adipokines have a role in inflammatory signalling and energy metabolism in WAT (Bessesen et al., 2015, Wiecek et al., 2002).

The leptin gene LEP, is expressed in all WAT depots with the most abundant expression in scWAT in humans and with greater expression in obese individuals (Jr. et al., 2010, Chávez, 2014). Leptin is synthesised by adipocytes and its concentration in the circulation is proportional to the amount of WAT present; therefore, providing information about the state of energy reserves (Camargo et al., 2014, Hosogai et al., 2007). Through interaction with the hypothalamus, leptin can act to decrease appetite via inhibition of 5' adenosine monophosphate activated protein kinase (AMPK) which stimulates acetyl Co-A carboxylase (ACC) in the brain resulting in suppressed food intake (Hosogai et al., 2007). Leptin can also increase energy expenditure in skeletal muscle where in

contrast to its activity in the brain, it activates AMPK therefore inhibiting ACC resulting in increased fatty acid, mainly TG, oxidation promoting lipolysis and reduced lipogenesis (Hosogai et al., 2007) (Figure 1.6). However, hyperleptinemia and leptin resistance are often seen in obese individuals which is thought to be initiated by the increase in inflammatory signalling pathways in the hypothalamus which can impair leptin receptor signalling (Trayhurn and Beattie, 2007).

Further to its role in energy regulation, leptin is implicated in immune function and is considered pro-inflammatory due to its ability to promote the release of pro-inflammatory mediators. Leptin binds to receptors expressed on monocytes and macrophages, initiating the release of pro-inflammatory cytokines including IL-12, IL-6 (Loffreda et al., 1998), and TNF-α (Trayhurn and Beattie, 2007, Loffreda et al., 1998). Leptin further interacts with immune cells promoting Th1 immune responses by increasing TNF-α and IFN-γ in memory T cells as well as activating B cells, resulting in the secretion of IL-6, IL-10 and TNF-α via the initiation of tyrosine kinase JAK2 / transcription factor STAT3 and p38 MAPK signalling pathways (Hosogai et al., 2007, Trayhurn and Beattie, 2007).

Adipsin is a serine protein first identified in murine models and later confirmed to be identical to complement factor D in humans (White et al., 1992). It plays a role in the activation of the complement system through the alternative pathway and through this, has a role in insulin secretion and β cell functioning (Lo et al., 2014) (Figure 1.6). Decreased levels of adipsin are observed in rodent obesity but reports in humans are inconsistent with increased or unchanged levels reported (Cianflone et al., 2003, Derosa et al., 2013). It is further reported from murine models that depletion of adipsin dampened high fat diet induced AT inflammation (Lo et al., 2014).

Resistin is a large polypeptide secreted predominantly by monocytes and macrophages in WAT. Its secretion is up regulated during adipocyte differentiation in the onset of obesity and is thought to contribute to obesity related metabolic complications such as lipotoxicity, dyslipidaemia and insulin resistance (Camargo et al., 2014, Park and Ahima, 2014, Trujillo and Scherer, 2006). This is through its ability to induce lipolysis via PKA activated catecholamine signalling pathways resulting in the phosphorylation of the lipid droplet proteins promoting hydrolysis of TG (Trujillo and Scherer, 2006). Subsequent release of FFAs into the circulation may result in dyslipidaemia and contribute to insulin resistance (Figure 1.6). Resistin has also been seen to interact with adiponectin and it is suggested its actions may mimic those of TNF-α, inhibiting adiponectin secretion (Trujillo and Scherer, 2006).

Adiponectin is also produced by adipocytes and unlike leptin is considered to be anti-inflammatory (Bessesen et al., 2015). In normal weight individuals the circulating levels of

adiponectin are relatively high compared to leptin, around 2-25 ug/ml, and they are inversely correlated to the amount of AT present (Bessesen et al., 2015, Camargo et al., 2014). In obese individuals there is a decrease in adiponectin concentration, which results in loss of the positive anti-inflammatory actions as well as decreased sensitivity of tissues to insulin, resulting in decreased glucose and FFA transport into WAT as well as into muscle and the liver (Park and Ahima, 2014, Micallef et al., 2009). Adiponectin acts to inhibit the phagocytic activity of macrophages as well as suppressing the production of cytokines such as TNF-α. It further inhibits endothelial cell expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin through this pro-inflammatory cytokine reduction (Bessesen et al., 2015, Camargo et al., 2014) (Figure 1.5).

1.3.2.6 Lipid metabolites

Signalling molecules derived from FAs (referred to as lipid metabolites or FA metabolites) have a role in adipose homeostasis as well as in pro- and anti-inflammatory signalling.

There are 4 main classes of FA metabolites involved in WAT signalling namely classic eicosanoids, classic docosanoids, other oxylipins and their hydroperoxy precursors, and endocannabinoids (ECs) (Figure 1.7). These metabolites can be synthesised from a range of FAs stored within WAT with those predominantly involved in pro- and anti-inflammatory signalling synthesised from AA, EPA, and DHA (Figure 1.8).

In obesity, the main source of eicosanoids in WAT is immune cells. These are likely to also produce other classes of FA metabolites; however, adipocytes are capable of synthesising prostanoids and leukotrienes (LTs), expressing eicosanoid receptors, and interacting with components of the immune system (Masoodi et al., 2014, Calder, 2011). Under pro-inflammatory conditions FA metabolites are synthesised from their respective FFA precursors, released from cell membranes by phospholipase A_2. The FFAs then undergo oxidation by lipoxygenase (LOX), cyclooxygenase (COX), or cytochrome p450 enzymes (Masoodi et al., 2014, Calder, 2017, Powell and Rokach, 2015).

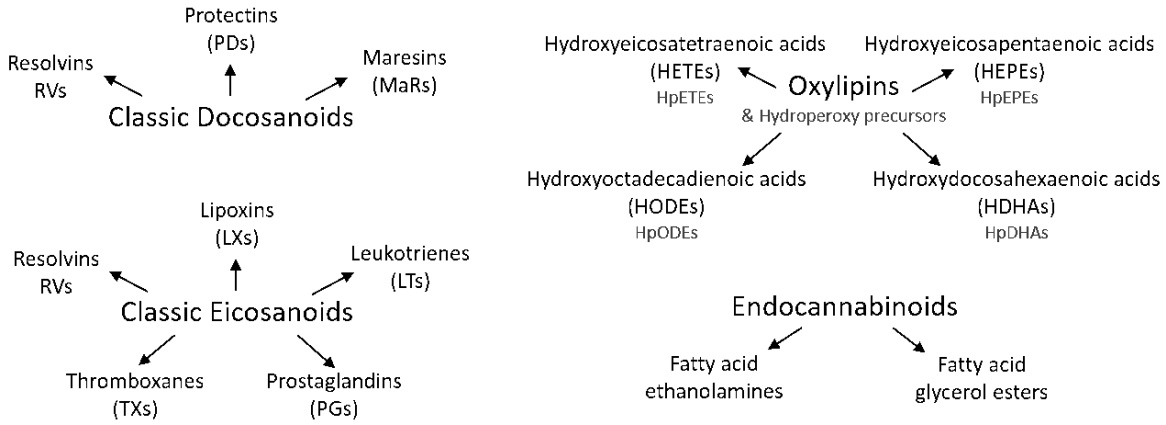


Figure 1.7 Classes of fatty acid metabolites.

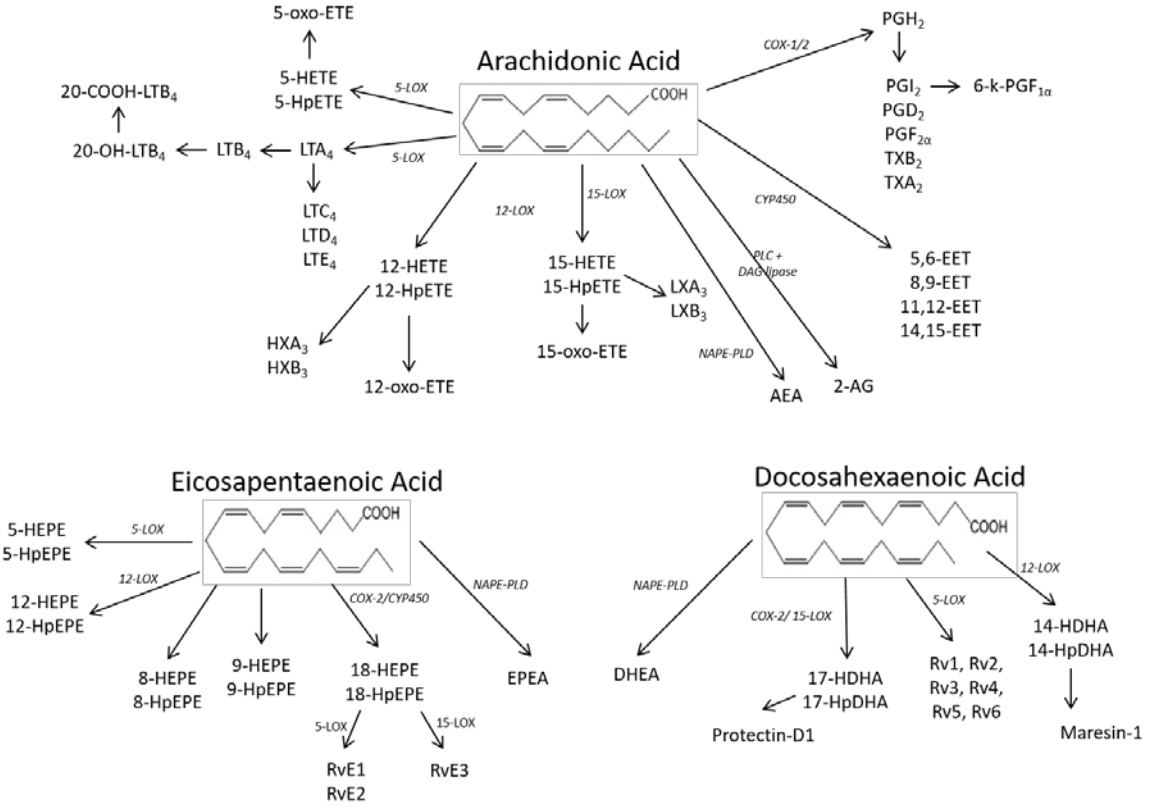


Figure 1.8 Overview of the synthesis of fatty acid metabolites from arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.

AA can be metabolised to prostaglandins (PGs), hydroxy-eicosatetraenoic acids (HETEs) and their hydroperoxy precursors, LTs, hepoxilins (HXs), thromboxanes (TXs), and AA-containing ECs (Figure 1.8). In general, these metabolites tend to have pro-inflammatory actions promoting the infiltration and activation of immune cells (Bittleman and Casale, 1995, Wright et al., 2010, Hecker. G et al., 1990), alter WAT lipid metabolism to promote lipolysis (Fain et al., 2001) or lipid accumulation, and promote WAT expansion and remodelling of the tissue environment (Balvers et al., 2010) (Table 1.3).

LC n-3 PUFA derived fatty acid metabolites include resolvins (Rvs), protectins (PDs), and maresins (MaRs), termed specialised pro-resolving mediators (SPMs). EPA is metabolised by 5-LOX to form the E-series Rvs, and DHA by 5-LOX to form the D-series Rvs. RvE1 and RvD2 have anti-inflammatory actions and have been reported to regulate the infiltration of leukocytes, increase removal of dying polymorphonuclear cells (PMNs) via macrophage phagocytosis, block IL-1 induced NF-κB activation, and decrease the expression of many pro-inflammatory cytokines including IFN-γ, TNF-α and IL-6 (Schwab et al., 2007, Croasdell et al., 2016, Claria et al., 2012, Serhan, 2014) (Table 1.3). Fatty acid metabolites and their actions in AT are detailed in Table 1.3.

Under normal physiological conditions, these actions are part of the homeostatic regulation of WAT, facilitating its response to increased nutrient intake; however, in the development of obesity, the production of pro- and anti-inflammatory FA metabolites becomes dysregulated resulting in a more pro-inflammatory environment. Changes in FA metabolite synthesis in obesity may occur through the availability of precursor FA for metabolism, i.e. increased concentration of AA, and therefore increased concentrations of AA derived metabolites, or through altered expression of the enzymes involved in the metabolism of FAs such as COX and LOX enzymes. Indeed, altered expression of COX and LOX enzymes is observed in obesity (Garcia-Alonso et al., 2016, Chan et al., 2016), in addition to altered concentrations of FA metabolites including an increase in AA derived metabolites such as HETEs (Pickens et al., 2017) and AA-containing ECs (Naughton et al., 2013, Côté et al., 2007, Gatta-Cherifi et al., 2012, Gatta-Cherifi and Cota, 2015), and lower concentrations of LC n-3 PUFA derived SPMs (Neuhofer et al., 2013). Changes in the activity of enzymes involved in the degradation of FA metabolites may also contribute to increased concentrations remaining in the tissue. Fatty acid amide hydrolase (FAAH) is an enzyme that breaks down FA ethanolamides; expression of FAAH is observed to be lower in obese individuals and may contribute to altered concentrations of WAT ECs (Bluher et al., 2006, Engeli et al., 2005).

Fatty acid	Fatty acid metabolite	Action	Studied with	Reference
AA	PGD_2	Inhibits lipolysis	3T3-L1 Adipocytes	(Wakai et al., 2017)
		Macrophage polarisation towards M2	Murine AT and Human scWAT	(Virtue et al., 2015)
AA	PGE_2	Modulates adipogenesis	Human scWAT	(Fain et al., 2001)
		Stimulates leptin release	Human scWAT	(Fain et al., 2000, Fain et al., 2001)
		Stimulates macrophage migration in lipolysis	3T3-L1 adipocytes +RAW 264.7 macrophages Murine epididymal AT	(Hu et al., 2016)
AA	$PGF_{2\alpha}$	Enhances glucose transport	3T3-L1 Adipocytes	(Chiou and Fong, 2004)
		Inhibits adipogenesis	3T3-L1 Adipocytes	(Draman et al., 2013, Taketani et al., 2014)
AA	PGJ_2	Induces macrophage inhibitory cytokine-1	Human scWAT	(Ding et al., 2009)
AA	8-HETE	Stimulates adipogenesis	3T3-L1 Adipocytes	(Yu et al., 1995)
AA	5-HETE	Increases IL-6, TNFα, and MCP-1 secretion	Rat Perigonadal AT	(Chakrabarti et al., 2009)
AA	12-HETE	Increases IL-6, TNFα, and MCP-1 secretion	Rat Perigonadal AT	(Chakrabarti et al., 2009)
		Increases AT inflammation in obesity	Human scWAT	(Dobrian et al., 2010)
AA	15-HETE	Induced adipose angiogenesis	Rat Epididymal AT	Soumya 2013
AA	EET, DHET	Modulates adipocyte differentiation Decreases TNFα and MCP-1 secretion Increases adiponectin secretion	Murine vWAT	(Burgess et al., 2012)
AA	LTB_4	Macrophage chemoattractant	Human scWAT Murine Epididymal AT	(Mothe-Satney et al., 2012) Li 2015
AA	20_4_EA, 2-AG	Stimulates adipogenesis Improves insulin sensitivity Stimulates glucose transport	3T3-L1 Adipocytes	(Karaliota et al., 2009, Bouaboula et al., 2005, Motaghedi and McGraw, 2008)
EPA	HEPEs (5, 8, 9, 12 & 18)	Stimulates PPAR-y and promotes adipogenesis	3T3-F442A Adipocytes	(Yamada et al., 2014)

EPA	RvE1	Improves insulin sensitivity	Murine blood	Pal 2020
DHA	RvD1, RvD2	Increases IL-10 and adiponectin secretion Decreases IL-1β, IL-6, MCP-1, TNFα, and leptin secretion	Murine Epididymal AT Human vWAT	(Claria et al., 2012) (Titos et al., 2016)
DHA	22_6_EA	Decreases IL-6 and MCP-1 secretion	3T3-L1 Adipocytes	(Balvers et al., 2010)
DHA	HXA3, HXB3	Stimulates adipogenesis	3T3-L1 Adipocytes	(Hallenborg et al., 2010)
Oleic acid	OEA	Impairs glucose tolerance and inhibits insulin secretion Stimulates lipolysis	Rat Epididymal AT	(Guzman et al., 2004)
Palmitic acid	PEA	Promotes macrophage class switch to M2 phenotype	Rat Epididymal AT	(Mattace Raso et al., 2014)

Table 1.4 Actions of fatty acid metabolites in adipose tissue (adapted from (Masoodi et al., 2014)).

AA, Arachidonic acid; PGD_2, Prostaglandin-D_2; PGE_2, Prostaglandin-E_2 $PGF_{2\alpha}$; Prostaglandin- $F_{2\alpha}$; PGJ_2, Prostaglandin-J_2; 8-HETE, 8-hydroxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; EET, epoxyeicosatetraenoic acid; DHET, dihydroxyeicosatrienoic acid; LTB_4, leukotriene-B_4; 20_4_EA, arachidonoylethanolamide; 2-AG; 2-arachiodonoyl glycerol; EPA, eicosapentaenoic acid; 5-HEPE, hydroxyeicosapentaenoic acid; 8-HEPE, 8-hydroxyeicosapentaenoic acid; 9-HEPE, 9-hydroxyeicosapentaenoic acid; 12-HEPE, 12- hydroxyeicosapentaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; DHA, docosahexaenoic acid; RvD1, resolvin-D1; RvD2, resolvin-D2; 22_6_EA, docosahexaenoylethanolamide; HXA3, hepoxilin-A3; HXB3, hepoxilin-B3; OEA, oleoylethanolamide; PEA, palmitoylethanolamide.; IL, interleukin, TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein.

1.4 Fatty acids and inflammation

As discussed in section 1.1.4, dietary and *de novo* synthesized FAs are transported into WAT in the form of FFAs, which undergo re-esterification into TG within adipocyte ER membranes and accumulate to form a lipid droplet. As described, the WAT includes EC and immune cells forming a SVC fraction in addition to adipocytes and their precursors. Dietary and *de-novo* synthesized FAs transported into the WAT are incorporated into the membranes of the SVCs as well as adipocytes. As outlined in section 1.1.2, changes in cell membrane composition alter membrane fluidity, which can influence the activity of receptors, enzymes and signalling molecules (Yaqoob, 2009, Calder, 2015a) (Figure 1.9). Changes in FA composition can also alter lipid raft function resulting in modulation of transcription factors and resultant gene expression (Yaqoob, 2009, Calder, 2015b). Through regulation of transcription factors such as NF-κB and PPAR-γ, FA can regulate inflammatory signal secretion (fatty acid metabolites, cytokines, chemokines and adipokines), adipocyte differentiation, fatty acid storage, and glucose metabolism (Calder, 2015a).

A mechanism by which FAs can influence the activation of NF-κB and influence inflammation is the priming and activation of the NLRP3 inflammasome (Roche, 2019). The inflammasome is a cytosolic multi-protein complex which is made up of the effector subunit, procaspase-1, and the adapter molecule, apoptosis-associated speck like protein, which contains a caspase recruitment domain (Roche, 2019). The recruitment and activation of caspase-1 to this complex is required for the activation of the NLRP3 inflammasome (Roche, 2019).

The priming and activation of the NLRP3 inflammasome is sensitive not only to metabolic and inflammatory stress signals, like those observed in the progression of obesity and related comorbidities, but also by nutritional cues such as FAs and glucose (Roche, 2019, Dewhurst-Trigg et al., 2020, Charles-Messance et al., 2020). The role of FAs in the regulation of inflammatory processes, including NRLP3 inflammasome activation are described in more detail below.

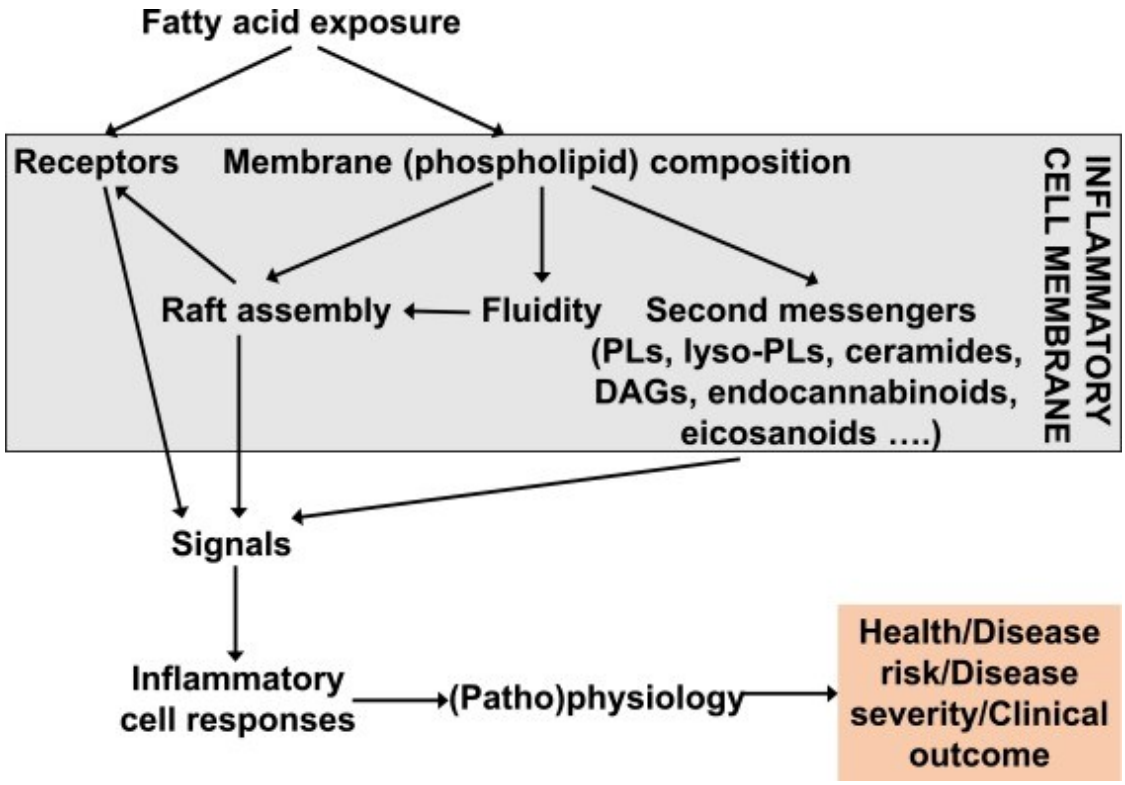


Figure 1.9 Mechanisms by which fatty acids can influence inflammatory cells and signalling. Abbreviations: PL, phospholipid; DAG, diacylglycerol.

1.4.1 Saturated fatty acids

SFA are components of cell membrane PL, which are particularly rich in palmitic and stearic acids (Calder, 2015a). SFA have been implicated in promoting inflammation through their ability to act via TLR4 (Hwang et al., 2016) and TLR2 to activate NF-κB and the NLRP3 inflammasome, promoting secretion of active IL-1β, which requires the conversion of pro- IL-1β to mature 'active' IL-1β by the inflammasome (Roche, 2019). Via these actions, SFA promote the secretion of IL-1β from dendritic cells (Reynolds et al., 2012), macrophages, and adipocytes (Mastrocola et al., 2016) to promote pro-inflammatory pathways (Wen et al., 2011, Shi et al., 2006, Roche, 2019, Dewhurst-Trigg et al., 2020, Charles-Messance et al., 2020). In addition, SFA can also reconfigure immune cell metabolism, in particular lipid metabolism in macrophages, so that the cell becomes more responsive to SFA induced inflammation and metabolic stress (Roche, 2019).

The addition of palmitic acid to macrophages and adipocytes elicits a TLR4 dependent inflammatory response in which NF-κB activation is increased along with the secretion of TNF-α (Shi et al., 2006). Lauric acid can act via TLR2 dimerization with TLR1 or TLR6 to activate NLRP3 and promote secretion of IL-1β (Fleet et al., 1992). In WAT, NF-κB activation may result in

increased secretion of pro-inflammatory cytokines in addition to TNF-α, including IL-6, and IL-1β, which can be further activated by SFA (Dinarello, 2009, Snodgrass et al., 2013). In addition, TLR4/ NF-κB activation by the SFA stearic and palmitic acid has been shown to influence the polarisation of adipose macrophages through increasing MCP-1 expression promoting the recruitment of monocytes to the tissue (Saberi et al., 2009). Lipopolysaccharide (LPS) is also capable of stimulating cells to produce inflammatory cytokines and promote a switch in macrophage polarisation towards a pro-inflammatory phenotype (M1) (Hersoug et al., 2018). Increased concentrations of plasma LPS have been associated with increased gut permeability in response to detection of increased SFA (Hersoug et al., 2018). Circulating LPS levels in obese individuals are observed to be higher than in normal weight individuals which may be due to higher proportions of SFA in the diet and less diversity in their gut microbiota (Ley et al., 2006, Boutagy et al., 2016). The pathways activated by SFA are likely to result in enhanced immune cell infiltration and dysregulation of WAT expansion and remodelling in response to nutrient excess, as well as decreased glucose tolerance and the development of insulin resistance (Trujillo and Scherer, 2006, Wen et al., 2011, McArdle et al., 2013, Park and Ahima, 2014).

1.4.2 Monounsaturated fatty acids

The most predominant MUFA in the human diet is oleic acid (18:1n-9). Found in olive oil, oleic acid is a component of the Mediterranean diet, which has been of interest in many dietary interventions (Vessby et al., 2001, SACN, 2018).

In contrast to palmitic acid, which elicits a pro-inflammatory response through NF-κB signalling, pro-inflammatory cytokines, and disrupted insulin signalling; oleic and palmitoleic acids, have opposing effects. When replacing SFA in high fat diet (HFD) fed mice, oleic acid improved insulin sensitivity and reduced secretion of active IL-1β in the AT (Finucane et al., 2015) and prevented IL-1β secretion from macrophages exposed to palmitic acid + LPS (Finucane et al., 2015). Further to this, oleic acid decreased the secretion of pro-inflammatory cytokines IL-6 and TNF-α in adipocytes from non-obese humans (Rodriguez-Pacheco et al., 2017), and increased M2 macrophage markers in mesenteric AT (Camell and Smith, 2013). Palmitoleic acid has shown to elicit anti-inflammatory actions by reducing NLRP3 inflammasome activation (Wen et al., 2011) and inhibiting NF-κB signalling (Salminen et al., 2011) through maintaining phosphorylation of AMPK . Palmitoleic acid may also influence macrophage polarisation. In bone marrow derived macrophages, incubation with palmitoleic acid reduced SFA induced M1 macrophage polarisation and NF-κB signalling (Chan et al., 2015). Increases in inflammatory gene expression and oxidative metabolism were observed which is indicative of M2 macrophage phenotype (Chan et al., 2015).

1.4.3 Polyunsaturated fatty acids

1.4.3.1 Omega-6 PUFA

LA is the most predominant n-6 PUFA in the western diet (Baker et al., 2016, Innes and Calder, 2018) and can be desaturated to gamma-linolenic acid (GLA, 18:3n-6), which can be subsequently elongated to dihomo-gamma-linolenic acid (DGLA, 20:3n-6), which can undergo desaturation to form AA (Figure 1.2)(Innes and Calder, 2018). AA can be obtained through the diet (e.g. from meat, offal and eggs) or by endogenous synthesis from LA. As previously described, AA can alter inflammatory signalling through its incorporation into cell membranes where it can elicit actions itself or as oxidised derivatives such as eicosanoids (Figure 1.9). As described in section 1.3.3.4, AA-derived metabolites are capable of eliciting a range of actions that promote pro-inflammatory responses (Table 1.3, Figure 1.8). The synthesis of PGs, HETEs, LTs and TXs from AA, results in the infiltration and activation of immune cells, subsequent pro-inflammatory cytokine secretion, altered lipid metabolism, and promotion of insulin resistance (Bittleman and Casale, 1995, Wright et al., 2010, Hecker. G et al., 1990, Fain et al., 2001). LA can also elicit pro-inflammatory actions once oxidised to hydroxyoctadecadieonic acids (HODEs) by LOX enzymes, including the promotion of endothelial cell adhesion (Vangaveti et al., 2016).

1.4.3.2 Long chain omega-3 PUFA

EPA and DHA are LC n-3 PUFA that have been widely investigated for their benefits to human health. Studies have provided evidence for lowering plasma TG, protecting against CVD, playing an important role in brain development and slowing cognitive decline in Alzheimer's disease, improving insulin sensitivity, and aiding in the resolution of inflammation (Calder, 2018, Cunnane et al., 2013, Calder, 2013, Djousse et al., 2012).

As described in section 1.1.2, the LC n-3 PUFA EPA and DHA can be synthesized to some extent from the precursor FA ALA (Arteburn et al., 2006, Hodson et al., 2008, Kopecky et al., 2009, Vezeridis et al., 2011) (Figure 1.2). This is however inefficient in humans and therefore, the primary source of EPA and DHA is through dietary intake of EPA and DHA themselves. Similarly to SFA, MUFA and n-6 PUFA, LC n-3 PUFA can be incorporated into cell membranes and alter lipid rafts and membrane fluidity, subsequently altering the activity of transcription factors, and the synthesis and secretion of inflammatory signalling molecules (Figure 1.9). These mechanisms are a major route by which EPA and DHA modulate inflammation and adipocyte differentiation as well as the metabolism of FAs (Calder, 2014, Calder, 2015b).

Like AA, EPA and DHA can elicit their actions as themselves, or as their oxidised derivatives (Calder, 2017). EPA and DHA can act as signalling molecules by binding to PPARs (Nisoli et al.,

2000) to increase the expression of genes encoding PPAR–α, which is associated with increased FA β oxidation in the liver and lipolysis, and decreases the expression of PPAR–γ, which has a role in adipogenesis (Mejia-Barradas et al., 2014, Larsen et al., 2003). Through these actions, n-3 PUFA may be capable of inhibiting the expansion of fat mass through promoting increased lipolysis and inhibition of adipogenesis. LC n-3 PUFA are also ligands for the G-protein coupled receptor (GPR) 120; this receptor has been established to be highly expressed in mature adipocytes and pro-inflammatory macrophages and is sensitive to LC n-3 PUFA, especially DHA (Oh et al., 2010). DHA stimulation of GPR120 has been shown to inhibit both TLR and TNF-α pro-inflammatory cascades as well as to improve insulin sensitivity in murine models and 3T3-L1 adipocytes by increasing GLUT4 translocation to the cell membrane (Villaret et al., 2010).

EPA and DHA have been observed to reduce the expression and secretion of IL-1β, IL-6, TNF-α, and NF-κB in LPS stimulated macrophages derived from humans (Weldon 2007), and shown to inhibit LPS induced secretion of IL-1β from macrophages and hepatocytes in LDLR deficient mice by reducing cleavage of caspase-1 and NLRP3 activation. Therefore, LC n-3 PUFA may attenuate obesity related adipose inflammation via NLRP3 pathways; however, evidence for this has not been observed in humans (Messance 2020).

As described in section 1.3.3.4, EPA and DHA can undergo oxidation to form fatty acid metabolites including the SPMs Rvs, PDs and MaRs. These signalling molecules have a role in aiding the resolution of inflammation (Gonzalez-Periz and Claria, 2010). Specifically, RvE1 and D2 have been reported to regulate the infiltration of leukocytes, increase removal of dying PMNs via macrophage phagocytosis, block IL-1β induced NF-κB activation, decrease the expression of many pro-inflammatory cytokines including IFN-γ, TNF-α and IL-6, and have insulin sensitising effects through induction of adiponectin, GLUT4 and IRS-1 (Schwab et al., 2007, Croasdell et al., 2016, Claria et al., 2012, Serhan, 2014, Gonzalez-Periz et al., 2009). Further to this, PD1 also increases adiponectin expression whilst decreasing leptin expression and pro-inflammatory cytokine secretion in explants of AT from obese mice (Claria et al., 2012).

In humans, RCTs investigating the actions of LC-n-3 PUFA on WAT inflammation and WAT physiology remain limited to 5 studies to my knowledge with evidence being inconclusive (Dewhurst-Trigg et al., 2020). A summary of these studies is detailed in Table 1.5. Spencer *et al.* observed beneficial changes in WAT inflammatory status in insulin resistant humans treated with 4 g/day of Omacor, a concentrated EPA + DHA preparation. A decrease in blood MCP-1 concentration as well as MCP-1 and CD68 mRNA expression in scWAT, along with a decrease in the number of macrophages and CLS in scWAT was observed. They concluded that the subjects with the most macrophages had the greatest response to LC n-3 PUFA intervention (Spencer et

al., 2013). Itariu *et al.* reported no change in the numbers of macrophages but a decrease in expression of the M1 phenotype macrophage marker CD40 in AT following an 8-week fish oil (FO) intervention (3.36 g EPA + DHA/day). They also reported down regulation of transforming growth factor-β (TGF-β), MCP-1 and CCL2 mRNA expression in scWAT and a trend towards a decrease in IL-6 expression and an increase in adiponectin gene expression following intervention (Itariu et al., 2012). In addition, Roberts-Lee *et al.* reported downregulation of inflammatory genes *IL-6*, *CCL2*, *CXC3L1*, and inflammasome related genes *IL-1β*, *IL-18*, and *Casp1* in gluteal adipose tissue from obese non-diabetic individuals following 8-week intervention with 4 g/d Omacor (Roberts Lee et al., 2019).

In contrast, Kratz *et al.* reported no effect of a 14 week FO intervention (3.0 g EPA + DHA/day) on AT inflammatory mediators including TNF-α, IL-6, CD14, ICAM1, PAI-1 and CCL2 (Kratz et al., 2013) and a recent study by de Mello *et al.* reported no changes to expression of inflammatory genes in the scWAT of overweight and obese adults with abnormal glucose metabolism following 12-week intervention with fatty fish (equating to 1g EPA+DHA per day), white fish, or ALA rich *Camelina sativa* oil PUFA (de Mello et al., 2019). Therefore, there is need for more comprehensive investigation of the effects of LC n-3 PUFA on a wide range of inflammatory parameters in human WAT.

Author	Cohort	Tissue	Intervention	Main findings
Spencer *et al* (2013)	Non-diabetic obese individuals with impaired glucose tolerance, impaired fasting glucose, or at least 3 features of metabolic syndrome	Abdominal scWAT	4 g/d EPA+DHA ethyl esters 12 weeks	Increase: - Decrease: scWAT MCP-1, CD68 mRNA # scWAT macrophages and CLS No change: Plasma cytokines
Itariu *et al* (2012)	Non-diabetic obese individuals scheduled for elective bariatric surgery	Abdominal scWAT and vWAT	3.36 g/d EPA+DHA ethyl esters 8 weeks	Increase: scWAT Adiponectin mRNA Decrease: scWAT TGF-β, MCP-1, CCL2, CD40 mRNA No change: # macrophages
Roberts Lee *et al* (2019)	Non-diabetic obese individuals	Gluteal scWAT	4 g/d EPA+DHA ethyl esters 8 weeks	Increase: - Decrease: scWAT IL-6, CCL2, CX3CL1, ILR7, IL-1β, IL-18, Casp1 mRNA No change: - scWAT NLRP mRNA
Kratz *et al* (2013)	Non-diabetic overweight and obese individuals	Abdominal scWAT	3 g/d EPA+DHA 14 weeks	Increase: - Decrease: - No change to scWAT TNF-α, IL-6, CD14, CCL2, ICAM1, NOS2, SAA1, ADIPOQ mRNA
De Mello *et al* (2019)	Non-diabetic overweight and obese individuals	Abdominal scWAT	1 g/d EPA+DHA from fatty fish 12 weeks	Increase: - Decrease: scWAT IL1RN mRNA No change: scWAT CCL2, ICAM1, IL-1β, IL-6, IL-10, TNF, TNFRSF1A, TNFRSF1B, TLR2, TLR4, ADIPOQ mRNA

Table 1.5 Summary of EPA+DHA interventions in humans and effects on adipose tissue inflammation

1.5 Knowledge gaps

Investigation of the effects of obesity on the FA composition and inflammatory status of scWAT is limited in humans. Reports are predominantly from blood and plasma parameters and the few investigations in human scWAT are restricted to individuals exhibiting MetS, termed metabolically unhealthy obese (MUO), which can differ greatly from those without diagnosed metabolic complications (Perreault et al., 2014, Pickens et al., 2014, Fjeldborg et al., 2014, Pickens et al., 2017). This can also be said for investigations examining the effects of LC n-3 PUFA on AT inflammation (Rossmeisl et al., 2018, Polus et al., 2016, Martinez-Fernandez et al., 2015). Those studies that have differentiated between diabetic and non-diabetic obese individuals have still not considered metabolic health beyond this diagnosis and many individuals classified as 'non-diabetic' have blood lipid profiles suggestive of metabolic complications. Further to this, comprehensive investigation of scWAT assessing multiple aspects of tissue physiology and inflammation in obesity in comparison to normal weight individuals and in response to LC n-3 PUFA are lacking. This is highlighted in a recent review by Dewhurst-Trigg *et al.* discussing current literature providing evidence for the effect of quantity and quality of dietary fat on scWAT inflammatory responses, in which the use of LC n-3 PUFA is identified to be clearly under investigated (Dewhurst-Trigg et al., 2020).

Therefore, the objective of this study is to examine multiple aspects of human scWAT physiology and inflammation in normal weight individuals and in MHO individuals to provide a comprehensive evaluation of 'physiological' scWAT and changes in non-insulin resistant 'healthy' obesity, and in response to LC n-3 PUFA. This investigation will provide new insight into the progression of adipose dysfunction in these early stages of obesity and identify novel features in these individuals which may have potential as targets for therapeutic intervention to generate further hypotheses for more tailored future investigations.

Based on the limited current knowledge and the purpose of the investigations, the following hypotheses will be tested:

- scWAT obtained from MHO individuals will exhibit a higher level of inflammation in comparison to scWAT from normal weight individuals. This will be evaluated using multiple WAT parameters in which it is hypothesised that there will be:
    - Higher proportion of SFA and lower proportion of EPA and DHA in scWAT from MHO individuals in comparison to normal weight individuals
    - Higher concentrations of pro-inflammatory mediators, and expression and activity of enzymes involved in their pathways in scWAT in MHO individuals in comparison to normal weight individuals

- Increased expression of pro-inflammatory genes and decreased expression of anti-inflammatory genes in scWAT from MHO individuals in comparison to normal weight individuals
- Enlarged adipocytes and increased levels of fibrosis in scWAT from MHO individuals in comparison to normal weight individuals
- Increased infiltration of macrophages and presence of crown like structures in scWAT from MHO individuals in comparison to normal weight individuals

- FO intervention for 12-weeks will increase the proportion of EPA and DHA in scWAT and will have significant anti-inflammatory effects by:
    - Altering the proportion and concentration of n-6 and n-3 FA metabolites in the scWAT to promote anti-inflammatory signalling and decrease pro-inflammatory signalling
    - Downregulating the expression of scWAT genes involved in the positive regulation of tissue inflammation
    - Decreasing the level of fibrosis in the scWAT
    - Decreasing macrophage infiltration and their accumulation in crown like structure in the scWAT

- There will be greater effects of FO intervention in MHO individuals in comparison to normal weight individuals due to the inflammatory status of the tissue at study entry.

Chapter 2 Study design and population

2.1 Study design

All procedures involving human participants were approved by the National Research Ethics Service South Central–Berkshire Research Ethics Committee (submission no. 11/SC/0384). The study is registered at www.isrctn.com (study ID: ISRCTN96712688). This research was supported by the European Commission through its Seventh Framework Programme, BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food (grant agreement no. 244995). The trial was conducted according to the principles of the Declaration of Helsinki, and all participants gave written informed consent prior to enrolment.

2.1.1 Study population

The objective of the research described in this thesis was to investigate subcutaneous white adipose tissue (scWAT) inflammation in obesity and in response to intervention with marine long chain omega-3 polyunsaturated fatty acids (n-3 LC PUFA). This research set out to address the hypotheses outlined in Chapter 1 section 1.5. These hypotheses were addressed by recruiting healthy normal weight and healthy obese individuals into a double blind placebo controlled trial in which a scWAT biopsy was collected prior to and following 12-week intervention with either fish oil (FO) or corn oil (CO) as described in section 2.2.3.

2.1.2 Sample size calculation

The following parameters were considered to determine appropriate sample size:

1. Typical distribution of variables of interest (this included blood parameters to be measured as part of wider analysis not reported in this thesis).

2. Expected response in variables of interest, determined from previous trial reports; for example, 20% reduction in the inflammatory mediator interleukin-6 (IL-6) following FO intervention.

3. Estimated drop out of participants over the 12-week intervention period; a loss of 20% was considered.

A sample size of 25 participants per group was deemed appropriate to obtain > 80% power at a 5% level of significance with consideration for 20% loss. Therefore, 121 individuals were invited onto the study with a total of 50 normal weight and 50 obese individuals recruited.

The study was powered based upon the original primary outcome of a change in plasma IL-6 concentration following FO intervention. Varying numbers of scWAT samples were available for different analyses reported in this thesis. The advice of Scott Harris, a medical statistician at the University of Southampton was sought and it was agreed that power calculations for individual analyses were not appropriate and therefore these were not made. This project had no single primary outcome, and instead, sought to investigate changes in scWAT inflammatory parameters in obesity and following FO and CO intervention to provide an overview which could be the basis for further hypothesis generation and more tailored investigations.

2.1.3 Recruitment

Participants were recruited via posters and email, and through advertisements placed in newspapers and broadcast over radio. Participants with an interest in taking part were screened by telephone interview to determine if they were likely to meet the inclusion criteria.

Inclusion and exclusion criteria were as follows:

Inclusion:

1. Male or female aged 18 to 65 years
2. BMI between 18.5 to 25 kg/m^2 (normal weight) or 30 to 40 kg/m^2 (obese)
3. If BMI is between 30 to 40 kg/m^2, waist circumference ≥ 94cm for men and ≥ 80 cm for women
4. Not consuming FO or other oil supplements
5. Not consuming more than one portion of oily fish per week
6. Being able to provide written informed consent

Exclusion criteria:

1. Male or female aged < 18 or > 65 years
2. BMI < 18.5 kg/m^2, 25.1 to 29.9 kg/m^2, or > 40 kg/m^2

3. Diagnosed metabolic disease (e.g. diabetes, CVD) or chronic gastrointestinal problems (e.g. inflammatory bowel disease, celiac disease, cancer)
4. Use of prescribed medicine to control inflammation, blood lipids or blood pressure
5. Use of FO or other oil supplements
6. Consumption of more than one portion of oily fish per week (140 g cooked)
7. Pregnant or planning to become pregnant within the study period
8. Participation in another clinical trial

Recruitment criteria were defined and recruitment completed prior to my PhD enrolment, therefore I had no input into this. Limitations of this recruitment design included lack of information on participants' ethnicity (however individuals were recruited from the Southampton area in the South Coast of England, UK), alcohol consumption, and whether they were a smoker. These factors have been identified to contribute to differences in n-3 PUFA metabolism and concentrations (Steffen et al., 2012, Sozio and Crabb, 2008 , Pawlosky et al., 2007) and so may account for some of the variation observed between individuals in the current study.

If participants met the inclusion criteria, they were sent an information sheet (Appendix A), and an appointment to visit the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital was arranged on their behalf. During this visit, inclusion/exclusion criteria were confirmed and participants willing to take part in the study provided written consent (Appendix B).

2.1.4 Study design

Study design was defined, all study visits were completed and all biological samples were collected prior to my PhD enrolment.

This study was a double blind, placebo controlled trial in which healthy males and females aged 18-65 y were recruited according to the criteria outlined above. Participants with a BMI of 18.5 to 25 kg/m^2 were classified as normal weight, and participants with a BMI of 30 to 40 kg/m^2 and waist circumference $\underline{\geq}$ 94 cm for men and ≥ 80 cm for women were classified as obese.

Participant flow through the study is depicted in Figure 2.1. Participants visited the WTCRF on 3 occasions. During the first visit, written consent was obtained and participants' exact height, weight, BMI and waist circumference were recorded to assess eligibility based on the inclusion

criteria. If participants were deemed eligible, a further two visits were arranged on their behalf. Anthropometric measurements, fasted blood and a scWAT biopsy were collected at visits 2 and 3. Following blood sampling and scWAT biopsy at visit 2, participants were randomly assigned to 12-weeks of either EPAX600 FO capsules, providing 1.80 g EPA and 0.88 g DHA in 3 capsules daily, or comparative oil capsules containing CO, providing predominantly LA and oleic acid (1.65 g and 0.81 g in 3 capsules daily, respectively). The full FA composition of the study oils is detailed in Table 2.1. Participants returned for the third and final visit following the 12-week intervention period and anthropometric measurements, fasted blood and a scWAT biopsy were collected again. Blood analyses (e.g. lipids, glucose, insulin) were conducted and reported by Carolina Paras-Chavez for her doctoral thesis (Chávez, 2014), data from these analyses are reported in this thesis to provide study entry characteristic data for all participants to assess their metabolic health. Use of blood analysis data for study entry characteristics or correlations within each experimental chapter is higlighted along with approriate methodology and reference to the original work.

Individuals were recruited based on no diagnosis of metabolic disease which included diabetes and cardiovascular events. Therefore, they were not screened based on their blood lipids, or insulin and glucose concentrations. These parameters were assessed retrospectively to investigate the metabolic health of the obese cohort as described in more detail in section 2.1.7 ‘Participant characteristics at study entry’.

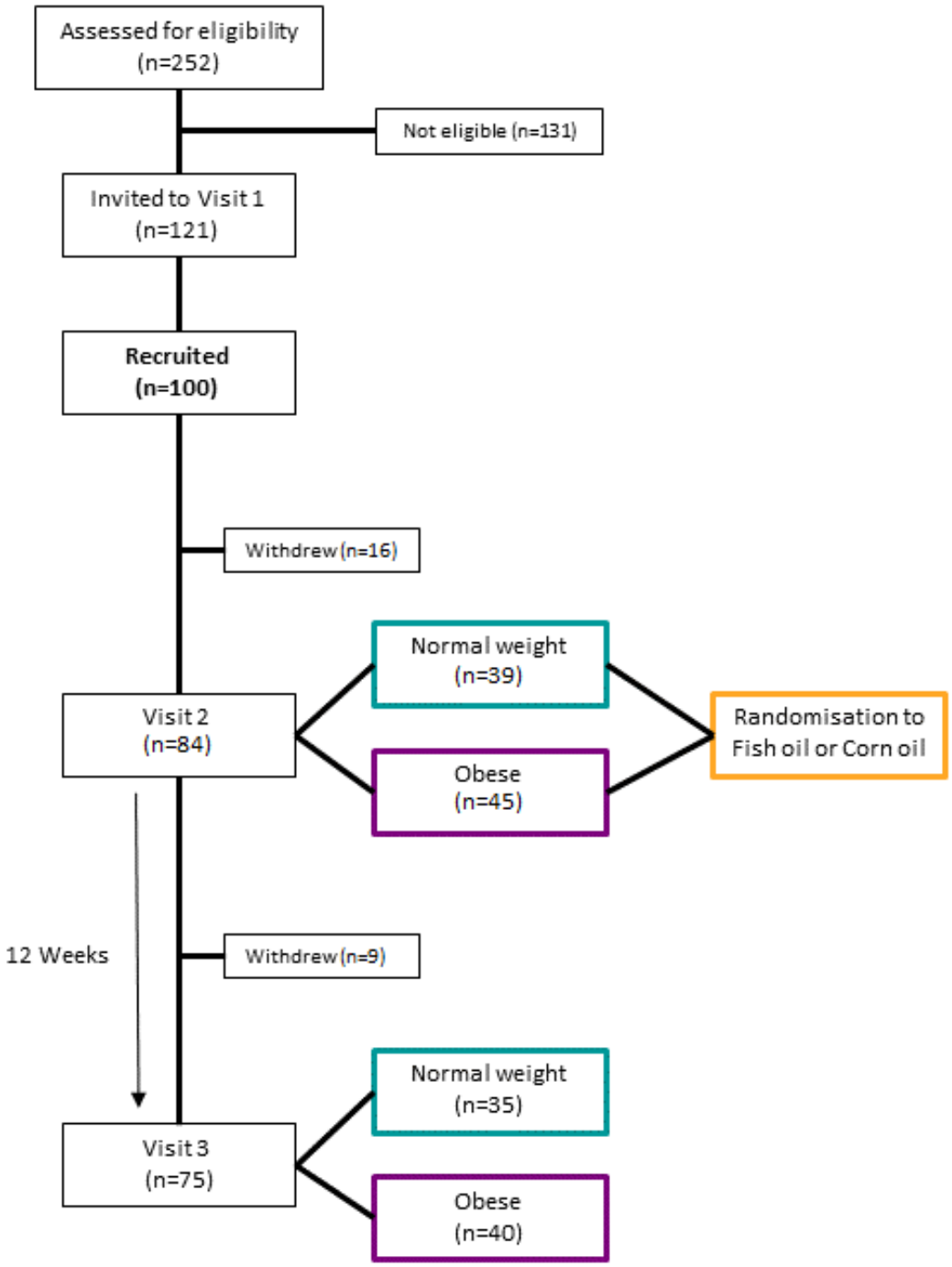


Figure 2.1 CONSORT diagram of participant inclusion and flow through the study.

CONSORT, Consolidated Standards of Reporting Trials.

		Fish oil	Corn oil
Saturated fatty acids (g)	16:00	0.04	0.11
	18:00	0.04	0.02
	Total SFA	**0.08**	**0.12**
Monounsaturated (g)	16:1n-7	0.01	0.00
	18:1n-7	0.03	0.01
	Total n-7	**0.04**	**0.01**
	18:1n-9	0.08	0.27
	20:1n-9	0.02	0.00
	Total n-9	**0.10**	**0.27**
	Total MUFA	**0.14**	**0.28**
Polyunsaturated (g)	18:2n-6	0.01	0.55
	20:4n-6	0.02	0.00
	Total n-6 PUFA	**0.03**	**0.55**
	18:3n-3	0.02	0.01
	20:4n-3	0.02	0.00
	20:5n-3	0.36	0.00
	22:5n-3	0.05	0.00
	22:6n-3	0.26	0.00
	Total n-3 PUFA	**0.71**	**0.00**
	Total PUFA	**0.77**	**0.56**
Total FA		**0.98**	**0.98**

g of fatty acid/g of oil

Table 2.1 Fatty acid composition of intervention oils (g of fatty acid/g of oil). Three g of oil were administered each day for 12-weeks.

2.1.5 Sample processing

Sample collection and storage occurred prior to my PhD enrolment. Processing and storage of blood and adipose samples was performed by Dr Carolina Paras Chavez.

Fasted blood samples collected into lithium heparin vacutainers were processed to analyse blood lipids, glucose, and insulin as described by Dr Carolina Paras-Chavez in her doctoral thesis. Data from these analyses are reported for participant characteristics and as correlations with adipose tissue measurements (Chávez, 2014).

Fasted plasma samples collected into lithium heparin vacutainers were processed to analyse a range of cytokines and adipokines as described by Dr Carolina Paras Chavez in her doctoral thesis (Chávez, 2014). Data from analyses of interleukin (IL)-6 and -10, tumour necrosis factor alpha

(TNF-α), vascular cell adhesion molecule-1 (VCAM-1), C-reactive protein (CRP), adiponectin and leptin are reported as correlations with adipose tissue measurements (Chapter 7).

Fasted abdominal scWAT biopsies were collected via surgical removal under local anaesthetic (1% lidocaine) to provide approx. 1 g of intact tissue. The scWAT was immediately divided into 5 aliquots averaging 200 mg and stored as detailed in Figure 2.2. scWAT intended for immunohistochemistry analyses was formalin fixed in 4 mL of 10% neutral buffered formalin and stored at room temperature. scWAT intended for RNA analysis was stored in 4 mL of RNAlater (Sigma, St. Louis, Missouri, United States) and stored for 24 hours between 2-4°C and then at -20°C until use. scWAT samples intended for FA, lipid metabolite and protein analyses were wrapped in foil, placed in cryovials and snap frozen in liquid nitrogen.

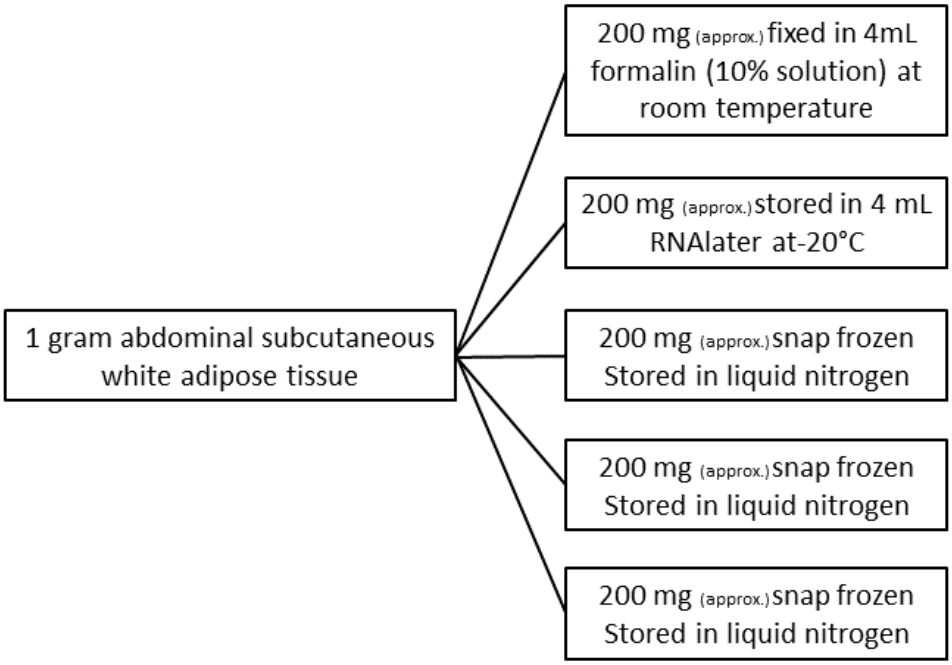


Figure 2.2 Storage and processing of white adipose tissue biopsies.

2.1.6 Anthropometry

Anthropometric measurements were made by research nurses at the WTCRF.

Height was measured using a Seca stadiometer (Seca, Hamburg, Germany); the participant removed their shoes, stood upright against the stadiometer, took a deep breath in and held it whilst the headboard of the stadiometer was lowered to the upper point of the head and compressed against the participant's hair. The participant's height was then recorded to the nearest mm.

Weight and body composition measurements were made using digital impedance apparatus (TANITA BC-418). The participant removed their shoes and socks, heavy clothing and any personal

items upon their body. The participants' age, sex, and height were input into the TANITA system and the participant then proceeded to stand upon the metal TANITA platform. The heels of their bare feet were placed on the posterior electrodes and the front of their foot placed on the anterior electrodes. Participants held the TANITA handles and stood still during the measurements. Participants' weight, fat mass, lean mass, and total body water were recorded. Waist and hip circumference measurements were made using a tape measure. Participants wore loose/thin clothing and stood upright with feet together in a relaxed position. Participants were then asked to raise their arms whilst a tape measure was placed around their waist between the ribs and hip bone, one centimetre under the umbilicus. The tape was kept taught and flat against the participant's abdomen during the measurement. Participants maintained this position for hip circumference measurement and the tape measure placed around the greatest area of protrusion of the buttocks.

2.2 Participant characteristics at study entry

Anthropometric and metabolic measurements taken at study entry (week-0) are reported for all participants in Table 2.2.

As expected, obese individuals had significantly greater BMI, waist circumference, hip circumference, body fat % and body fat mass than normal weight individuals (Table 2.2). They were also significantly older than normal weight individuals; due to this, the effects of age on various outcomes were investigated and controlled for if deemed significant. Obese individuals had significantly higher blood concentrations of TG, LDL-C, glucose and insulin than normal weight individuals, and obese males had higher total cholesterol concentrations than normal weight (Table 2.2). Subsequently, obese individuals had a significantly greater mean HOMA2-IR score; this score is adapted from the original HOMA-IR equation of ((glucose mmol/L) x (insulin µIU/L))/22.5, and corrects the score for variations in hepatic and peripheral glucose resistance, increases in insulin secretion curve for plasma glucose concentrations above 10 mmol/L, and for the contribution of circulating proinsulin. Interpretation of these findings indicates that, on average, the obese population fall within a 'normal range'. There were no significant differences in blood concentrations of HDL-C or NEFAs between normal weight and obese individuals.

The diagnosis of MetS is based upon having 3 or more of the following features (defined by NHS, UK):

1. High blood pressure ($\geq$ 140/90 mmHg)
2. Waist circumference above the healthy range (> 80 cm female, > 94 cm male)
3. High blood TG (>1.7 mmol/L)

4. Insulin resistance
5. An increased risk of developing clots
6. A tendency to develop swelling of body tissue (inflammation)

The obese individuals in the current study were healthy in that they did not have any current or previously diagnosed diabetes or heart complications; however, assessment of the MetS parameters provides greater insight into their metabolic health. The blood lipid parameters of obese individuals recruited into the study were within the normal ranges (with the exception of LDL-C), albeit higher than seen in the normal weight individuals. HOMA2-IR scores were above the normal range but below the level indicative of early insulin resistance. However; the waist circumference of both obese male and females recruited to the current study was above the normal range, as expected since this was an inclusion criterion. Unfortunately, blood pressure measurements were not collected, so were not available to help determine the metabolic health of these individuals. Table 2.2 details participant characteristics and 'healthy' reference ranges for adults in the UK. 96% of normal weight individuals had a waist circumference within the healthy range whereas 100% of obese individuals had a waist circumference above the healthy range. Ninety-six % of normal weight individuals and 80% of obese individuals had TG levels within the healthy range. One hundred % of normal weight individuals had glucose and insulin levels within the healthy range, and 96% and 94% of obese individuals had glucose and insulin levels within the healthy range respectively. As all obese individuals had a waist circumference above the normal range, the addition of two further features of MetS would define them as being metabolically unhealthy. Twelve obese individuals had at least one other feature of MetS which tended to be elevated TG (2 of the 12 individuals had elevated glucose and 3 had elevated insulin levels).Two obese individuals had 3 features of MetS which were an elevated waist cm, blood TG, and HOMA-IR above the normal range. Therefore, 96% of the obese cohort could be described as being metabolically healthy. This classification is to distinguish between the current study and the existing literature in which many studies in human obesity have included individuals with type-2 diabetes and non-alcoholic fatty liver disease and would therefore be described as metabolically unhealthy obese (MUO) individuals. Investigation of the inflammatory parameters within scWAT detailed in this thesis may help shed greater light on the health and metabolic status of these MHO individuals.

	Male			Female			Effect of sex		
	[1]Normal weight	[1]Obese	[2]*P*	[1]Normal weight	[1]Obese	[2]*P*	Normal weight [2]*P*	Obese [2]*P*	Healthy range
Age (years)	27.15 ± 4.12	42.39 ± 3.95	0.015	33.30 + 2.88	45.33 ± 2.02	0.001	-		-
BMI (kg/m^2)	23.57 ± 0.35	34.60 ± 1.07	≤ 0.001	21.78 ± 0.33	34.87 ± 0.45	≤ 0.001	0.001	-	18.5 - 25 kg/m^2
[3]Waist (cm)	82.51 ± 2.35	117.63 ± 3.26	≤ 0.001	72.88 ± 0.91	105.07 ± 1.80	≤ 0.001	0.002	0.003	< 94 cm Male < 80 cm Female
Hip (cm)	94.59 ± 1.27	116.74 ± 2.60	≤ 0.001	92.32 ± 1.05	118.10 ± 1.40	≤ 0.001	-	-	-
Body fat (%)	13.62 ± 1.97	31.75 ± 1.49	≤ 0.001	26.24 ± 0.81	44.77 ± 0.59	≤ 0.001	<0.001	<0.001	< 22% Male < 35% Female
Body fat mass (kg)	10.02 ± 1.54	35.59 ± 2.25	≤ 0.001	15.22 ± 0.65	42.08 ± 1.24	≤ 0.001	0.009	0.022	-
[3]TG (mmol/L)	0.83 ± 0.08	1.54 ± 0.21	0.01	0.78 ± 0.08	1.31 ± 0.13	0.002	-	-	≤ 1.7 mmol/L
NEFAs (µmol/L)	427.67 ± 65.24	560.26 ± 52.70	0.127	576.60 ± 38.52	601.60 ± 39.44	0.654	-	-	-
TC (mmol/L)	3.99 ± 0.20	5.38 ± 0.26	0.001	4.66 ± 0.24	5.31 ± 0.18	0.31	0.042	-	≤ 5.0mmol/L
HDL-C (mmol/L)	1.20 ± 0.08	1.35 ± 0.09	0.271	1.73 ± 0.06	1.52 ± 0.07	0.3	<0.001	-	≥ 1.0 mmol/L
LDL-C (mmol/L)	2.63 ± 0.17	3.72 ± 0.21	0.001	2.78 ± 0.21	3.53 ± 0.16	0.006	-	-	≤ 3.0 mmol/L
[3]Glucose (mmol/L)	4.75 ± 0.17	5.84 ± 0.41	0.036	4.72 ± 0.07	5.34 ± 0.12	≤ 0.001	-	-	≤ 7.0 mmol/L
[3]Insulin (µIU/L)	5.72 ± 1.11	14.03 ± 1.86	0.001	5.39 ± 0.49	12.67 ± 1.27	≤ 0.001	-	-	2.6-24.9 µIU/L
[34]HOMA2-IR	0.79 ± 0.18	1.85 ± 0.25	0.008	0.71 ± 0.06	1.56 ± 0.14	≤ 0.001	-	-	0.5-1.4 < 1.9
[5]Adipose-IR	2030 ± 1098	7354 ± 2655	0.004	3137 ± 2304.07	7018.42 ± 3579	0.044	-	-	

Table 2.2 Anthropometric and metabolic characteristics in normal weight and obese individuals. Measurement above the healthy range. Measurement borderline healthy range.

[1]Mean ± SD;

[2]*P* obtained from independent samples t-test analysis by comparison of obese and normal weight data split by sex;

[3] Measurement used to determine metabolic health of obese individuals

[4]HOMA2-IR = (((insulin mmol/L) x (glucose IU/L)) / 22.5) corrected for variations in hepatic and peripheral glucose resistance, increases in insulin secretion curve for plasma glucose concentrations above 10 mmol/L, and the contribution of circulation proinsulin).

[5]Adipose-IR = (NEFAs µmol/L) x (insulin µIU/L)

Chapter 3 Fatty acid composition of human subcutaneous white adipose tissue is altered in metabolically healthy obese subjects and in response to 12-week fish oil intervention

3.1 Introduction

Subcutaneous white adipose tissue (scWAT) is well described as an endocrine organ, but one of its primary purposes is to provide safe storage of lipids (Kershaw and Flier, 2004, Jensen, 2006, Masoodi et al., 2014, Bessesen et al., 2015). The composition of scWAT fatty acids (FAs) is observed to reflect that of the diet and is therefore sensitive to modulation by dietary fat manipulation (Beynen et al., 1980, Marckmann et al., 1995, Baylin et al., 2002, Hodson et al., 2008).

Non-esterified fatty acids (NEFAs) from hydrolysed chylomicrons and very low density lipoproteins (VLDL) can be taken up by the scWAT and re-esterified into triglyceride (TG) and stored within the adipocyte (Thompson et al., 2010, Walther and Farese, 2012, Rutkowski et al., 2015, Ducharme and Bickel, 2008, Thiam et al., 2013). Therefore, scWAT TG is considered to be a good biomarker of long term habitual dietary FA intake, particularly of polyunsaturated fatty acids (PUFA) (Marckmann et al., 1995, Burdge and Calder, 2006). However, FAs stored in the scWAT also reflect those generated by endogenous lipogenesis and as such, there are differences in the proportions of FAs stored in comparison to their intake (Summers et al., 2000).

FAs within scWAT can be utilised as substrate for the synthesis of inflammatory signalling molecules (Fain et al., 2001, Balvers et al., 2010, Masoodi et al., 2014) and are capable of regulating transcription factors determining expression of genes involved in lipid metabolism and inflammation (Lo et al., 1999, Lee et al., 2001, Novak et al., 2003, Zhao et al., 2004, Zapata-Gonzalez et al., 2008, Kong et al., 2010). Therefore, the composition of FAs in the scWAT is important in determining the metabolic and inflammatory status of the tissue.

Obesity is characterised by an increase in adipose mass and is accompanied by a state of chronic low-grade inflammation (Hotamisligil, 2006, Vachharajani and Granger, 2009). Altered dietary fat

intake and lifestyle factors in obesity have been shown to significantly affect the transport and metabolism of FAs (Jensen, 1998, Jensen et al., 1989, Singla et al., 2010, McQuaid et al., 2011). This may result in changes to the composition of the scWAT, which may culminate in dysregulation of adipose functions including inflammatory signalling, expansion, and whole body energy homeostasis. Despite current knowledge, examination of FA composition in obesity is predominantly limited to blood and in the context of MetS associated with obesity (Pickens et al., 2014, Camargo et al., 2014).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been extensively studied for their anti-inflammatory benefits to human health (Calder, 2017); supplementation with these FAs results in increased concentrations appearing in blood and tissue lipids (Browning et al., 2012). Storage of these FAs in the scWAT is not favoured and only small increases in the amounts of these fatty acids in scWAT are seen following supplementation (Browning et al., 2012). Altered lipid handling in obesity may influence the incorporation and storage of these FAs in the scWAT. There is evidence for differential handling of long chain omega-3 (LC n-3) PUFA in human obesity but these reports are predominantly limited to blood analyses (Micallef et al., 2009, Jensen, 1989, Thifault et al., 2013, Fisk et al., 2018).

In light of the current limitations in the existing literature, investigation of scWAT FA profile in obese individuals without diagnosed metabolic complications in comparison to normal weight individuals, and investigation of EPA and DHA incorporation into human WAT in the presence of obesity with normal metabolic parameters is of great interest, particularly with respect to attributing the effects of fish oil (FO) intervention on adipose functions. Lack of such investigations led to the development of experimental aims as outlined in section 3.2.

3.2 Aims:

The aim of the research described in this chapter was to determine the FA composition of scWAT of normal weight and obese individuals without diagnosed metabolic complications (termed metabolically healthy obese (MHO) individuals) to:

- Investigate whether metabolically healthy obesity is associated with an altered scWAT FA composition in which there are higher proportions of SFA and lower proportions of LC n-3 PUFA
- Investigate whether metabolically healthy obesity is associated with reduced responses to change in FA composition following fish oil supplementation

3.3 Methods:

Please refer to ‘Chapter 2– Study design and population’.

3.3.1 Experimental reagents and materials

See Appendix C

3.3.2 Fatty acid identification and quantification by gas chromatography

3.3.2.1 Principles of gas chromatography

Gas chromatography (GC) can be used to identify and quantify FAs in the form of volatile FA methyl esters (FAMEs) present in a wide range of samples including foods, plasma, blood, and tissues. FAMEs are created by the addition of a methyl group to the carboxylic end of the FA by reaction with a methyl donor such as methanol in the presence of a catalyst such as sulphuric acid. This reaction increases the volatility of the FAs which can therefore be heated at lower temperatures, such as 200-300°C, upon injection into the GC to result in their vaporisation (Fisk et al., 2014).

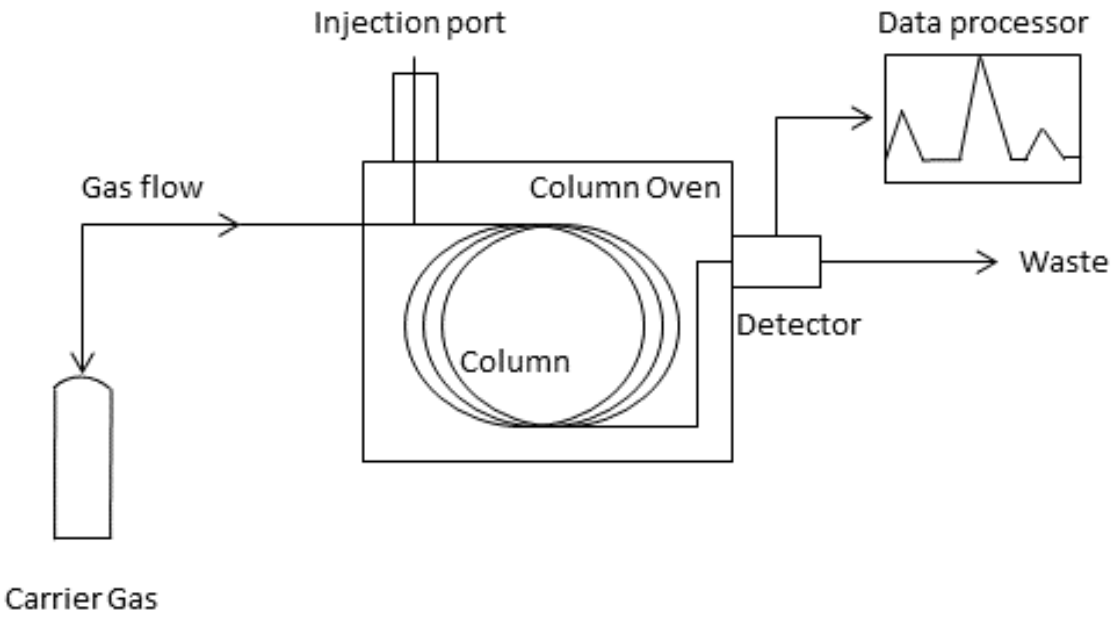


Figure 3.1 Schematic of gas chromatograph.

Adapted from (Poole, 2012).

Figure 3.1 depicts the GC system which consists of an injection port, in which the FAMEs are injected and heated, a capillary column housed inside an oven, and a detector. The injected FAMEs are transported into the capillary column by a carrier gas such as helium or hydrogen where they condense onto the cooler column lining. Differences in the interaction between

FAMEs and the column lining are also utilised in the separation of FAs. The column is then heated, and the FAMEs are separated based upon their volatility which is determined by carbon chain length and the number and position of double bonds present. The greater the number of carbon molecules, the greater the temperature required to result in vaporisation; however, as the number of double bonds increase, this temperature decreases. As the dissociated FAMEs reach the end of the column they pass through a flame ionisation detector (FID) heated at 250°C and are combusted by a hydrogen flame which generates an ion current proportional to the amount of FAME present. This information is displayed on a chromatogram in which each FAME is represented by a singular peak, the area of which corresponds to the amount of FAME present in the sample. The time taken for the FAME to dissociate from the column (the length of time the molecule is retained on the column) and reach the FID is reported as the retention time along the x-axis of the chromatogram. Unknown FAMEs can be identified by their retention time in comparison to retention times of known FAMEs in a calibration standard (Poole, 2012, Fisk et al., 2014).

3.3.2.2. Total lipid extraction and formation of FAMEs

scWAT was collected from normal weight and MHO subjects prior to and after 12 weeks of intake of FO or CO (see Chapter 2). Total lipid was extracted from 10-20 mg of frozen scWAT. Frozen scWAT was homogenised in 800 µl ice cold 0.9% sodium chloride (NaCl). 5 mL $CHCl_3$ (chloroform): CH_3OH (methanol) (2:1) containing $C_{15}H_{24}O$ (butylated hydroxytoluene, BHT) (50 mg/l) as anti-oxidant and 1 ml 1 M NaCl was added to the homogenate to promote separation of the aqueous and organic phases (Folch et al., 1956, Bligh and Dyer, 1959, Fisk et al., 2014). Samples were thoroughly vortex mixed and centrifuged at 2000 rpm (1000 g) for 10 minutes at room temperature (RT) (15-20°C). The lower phase containing total lipids was collected and dried under nitrogen gas (N_2) at 40°C (Folch et al., 1956, Bligh and Dyer, 1959, Fisk et al., 2014). The total lipid extract was re-suspended in 500 µl of toluene and FAMEs were produced by incubation with 1 mL of methanol containing 2% (v/v) H_2SO_4 (sulphuric acid) at 50°C for 2 hours. Samples were allowed to cool and were neutralised by the addition of 1 mL of neutralising solution (0.25M $KHCO_3$ (potassium bicarbonate) (25.03 g/l), 0.5M K_2CO_3 (potassium carbonate) (69.10 g/l)). 1 mL of C_6H_{14} (hexane) was then added and the sample thoroughly vortex mixed and centrifuged at 1000 rpm (250 g) for 2 min at RT. The upper hexane phase containing the FAMEs was collected and dried under N_2. Samples were re-suspended in 150 µl of dry hexane and transferred to a GC autosampler vial (Fisk et al., 2014).

3.3.2.3. GC Analysis

GC analysis was performed using a Hewlett-Packard 6890 chromatograph fitted with a SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 μm) and a FID with the following temperature protocol: Injector temperature = 300°C; initial column temperature = 115°C, hold 2 min, ramp 10°C/min to 200°C, hold 18.5 min, ramp 60°C/min to 245°C, hold 4 min; detector temperature = 300°C; and gas protocol: column = helium gas, flow rate 1.0, pressure 14.6 and velocity; detector = hydrogen gas, flow rate 40.0, air flow rate 184.0; make up gas = helium gas, flow rate 45.0. The split ratio was programmed to 50:1 (Fisk et al., 2014).

Resulting chromatograms were analysed with Agilent ChemStation software. In order to calibrate and identify FAs according to FAME retention time, two standards were used; 37 FAMEs and menhaden oil. Figure 3.2 shows a 'typical' chromatogram of a sample of human scWAT from a normal weight and an obese individual prior to FO intervention.

3.3.3 Statistical analyses

All statistical analyses were conducted using SPSS software (version 21; SPSS Inc.). The distribution of the data was assessed using the Shapiro-Wilk test and by plotting distributions of residuals obtained from general linear model (GLM) analysis of the data. Data did not conform to the normal distribution and remained not normally distributed following $\log_{10}$ transformation. Therefore, the Mann-Whitney U test was used to identify differences in FA composition of MHO individuals in comparison to normal weight individuals, and the Wilcoxon test was used on data split by BMI, selected by treatment, to compare paired post intervention data (week-12) with study entry (week-0). All data are reported as median $\pm$ interquartile range. There was no significant effect of sex on study entry FAs or their change in response to intervention and as such, the analyses are not controlled for sex ($P > 0.187$, data not shown).

MHO individuals are referred to as 'obese' in all figures and tables herein.

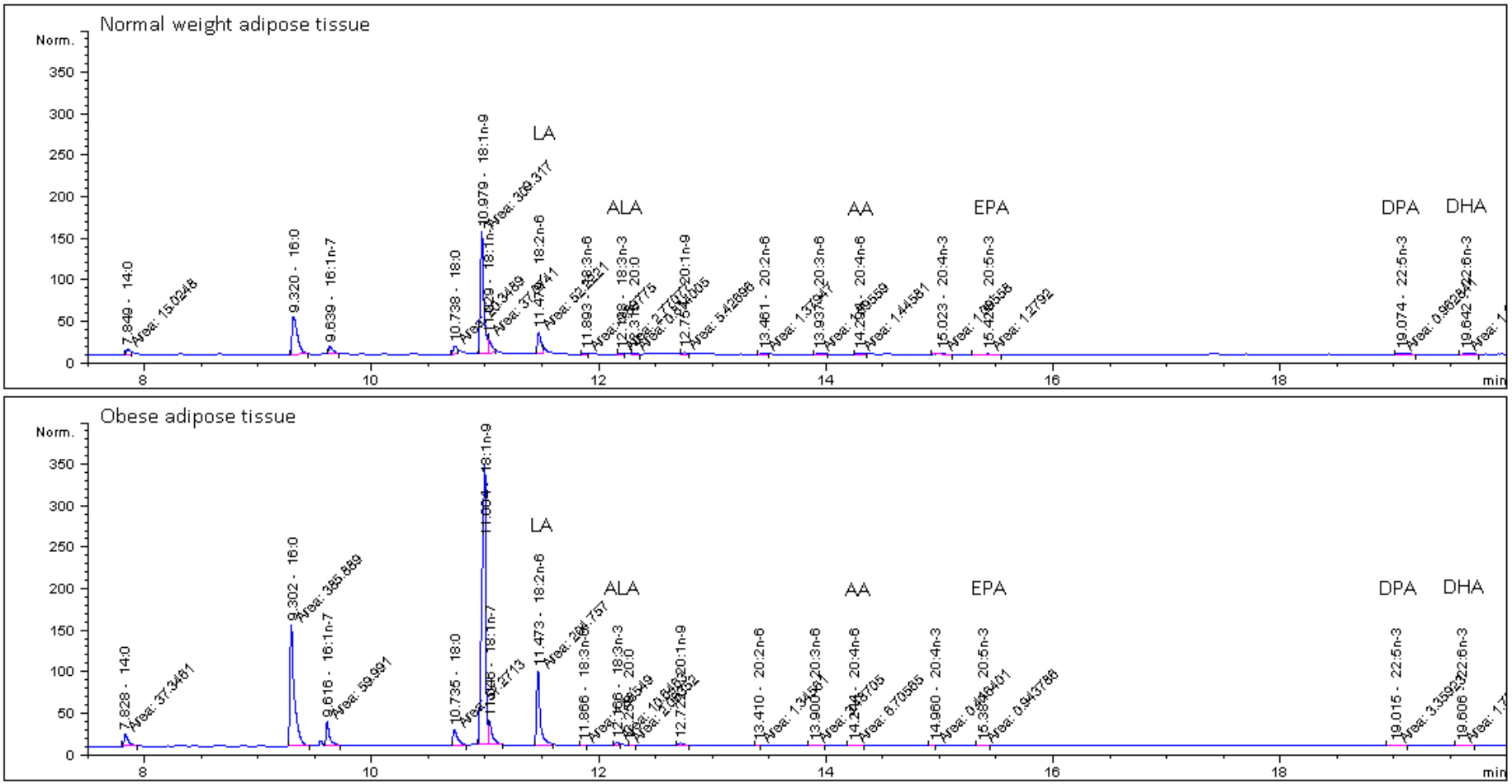


Figure 3.2 Chromatogram of a representative scWAT sample from a normal weight and metabolically healthy obese individual at study entry (week 0).

3.4 Results

3.4.1 Metabolically healthy obesity is associated with a significantly altered scWAT fatty acid composition

The most abundant FA present in human scWAT was 18:1n-9 followed by 16:0 and 18:2n-6 (LA) (Figure 3.2). The MUFA, 16:1n-7 and 18:1n-7 contributed between 2-6% of total FA, the n-6 PUFA 20:4n-6 (AA) contributed 0.3-0.6%, and the LC n-3 PUFA EPA (20:5n-3), DPA (22:5n-3), and DHA (22:6n-3) in total contributed < 1%.

In comparison with scWAT from normal weight individuals, scWAT from MHO individuals had significantly higher proportions of the MUFA 16:1n-7, 18:1n-7 and 18:1n-9, the n-6 PUFA 20:3n-6 and AA, and the n-3 PUFA EPA and DPA (Figure 3.3). Obese individuals also had significantly lower proportions of the saturated fatty acids (SFA) 14:0, 18:0, and 20:0, the MUFA 20:1n-9, and the n-3 PUFA ALA and 20:4n-3 (Figure 3.3) in comparison with normal weight individuals.

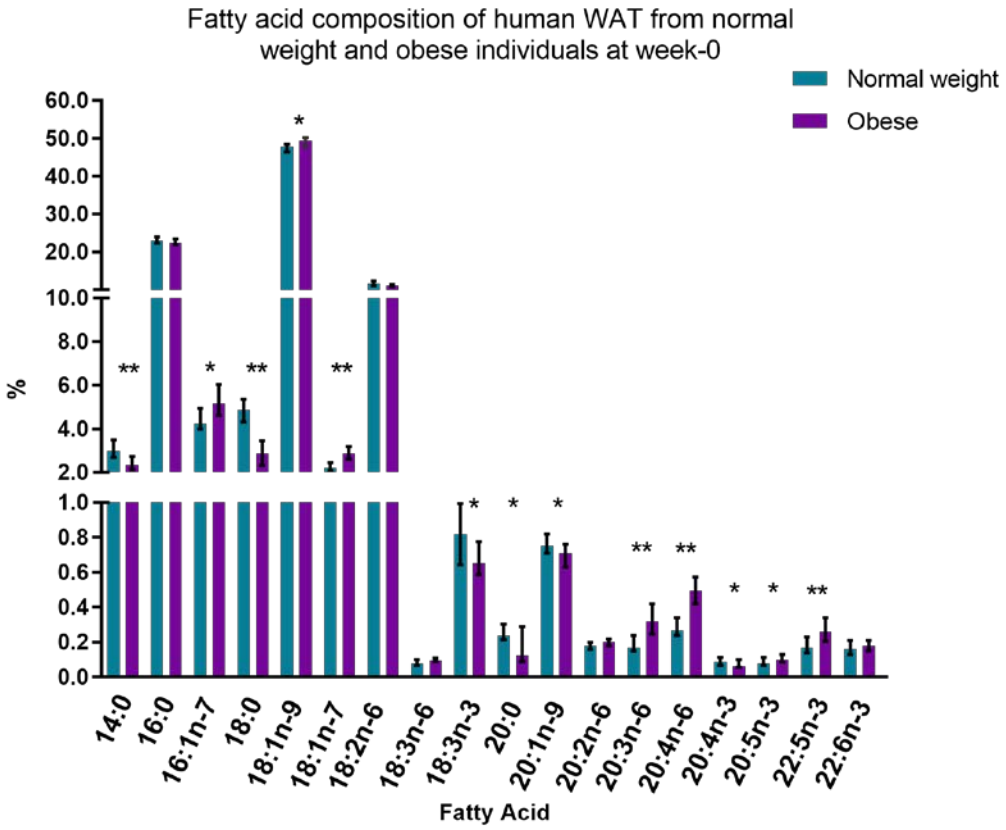


Figure 3.3 Fatty acid composition of scWAT in normal weight and metabolically healthy obese individuals at study entry (week-0).

Median (± interquartile range) scWAT from metabolically healthy obese (n = 29) compared to scWAT from normal weight (n = 38) individuals. *P* obtained from Mann-Whitney U test. * $P < 0.050$, ** $P < 0.001$. % is calculated as % of each fatty acid as a proportion of the total measured of which all are displayed in the figure.

3.4.2 12-week fish oil intervention increases the proportion of WAT EPA and DHA similarly in normal weight and MHO individuals

The effects of 12-week FO intervention on the proportion of all scWAT FAs are detailed in Appendix D.

There was no significant effect of FO intervention on the proportions of SFA, MUFA and many n-6 PUFA in either normal weight or MHO individuals (Appendix D.) but there was a trend for FO intervention to increase the proportion of 20.2n-6 in normal weight individuals only ($P = 0.056$) (Appendix D.).

12 week FO intervention significantly increased the proportion of scWAT EPA, DPA and DHA in normal weight individuals ($P = 0.006$, <0.001 and <0.001 respectively) and EPA in MHO individuals

(*P* = <0.001) (Appendix D., Figure 3.5). There was a similar increase in DHA in MHO individuals as that observed in normal weight individuals, but this exhibited only a trend for being significant (*P* = 0.067). Adjusting the data by kg of body fat resulted in a trend also for an increase in DPA in MHO subjects (*P* = 0.064). Lack of significance may reflect the greater variation observed in MHO individuals.

Despite significant incorporation of DPA occurring in normal weight individuals only, significantly higher proportions of DPA (which were evident at study entry (week-0)) were still observed in MHO individuals following FO intervention.

3.4.3 12-week corn oil intervention does not affect proportions of omega-3 or omega-6 fatty acids in scWAT

The effects of 12-week CO intervention on the proportion of all scWAT FAs are detailed in Appendix E. There were no significant effects of 12-week CO intervention on the proportion of EPA, DPA and DHA, SFA, or MUFA (Figure 3.5). There was however a trend for a significant increase in the proportion of LA in response to CO intervention in normal weight individuals only (*P* = 0.060, Appendix E).

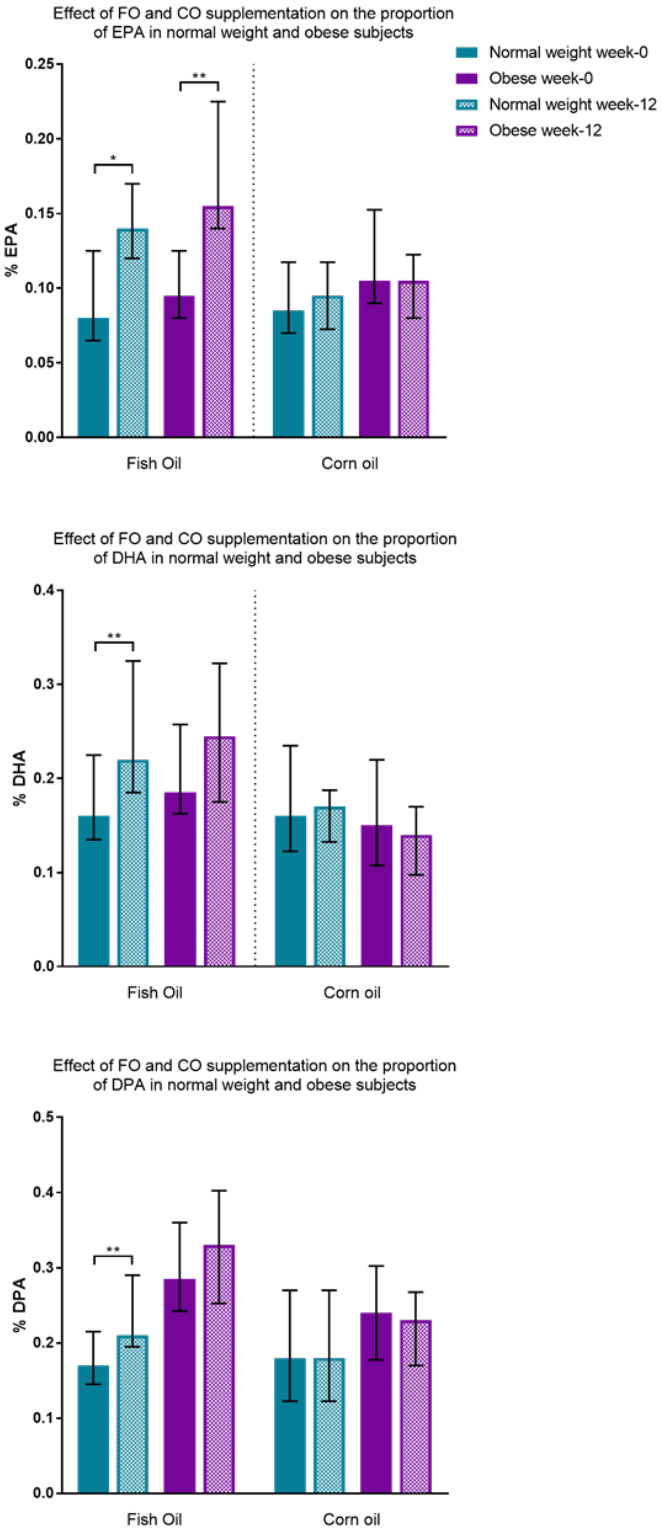


Figure 3.4 Effect of 12-week fish oil intervention on scWAT EPA, DHA, and DPA in normal weight (n= 29) and metabolically healthy obese individuals (n=38)

Median (± interquartile range), *P* obtained using a Wilcoxon model comparing week-12 vs week-0 data split by treatment and BMI group. * $P = 0.004$, ** $P < 0.001$. % is calculated as the % of EPA, DHA and DPA as a proportion of total fatty acids measured, as reported in Figure 3.3.

3.4.3 Study entry and change in scWAT LC n-3 PUFA following 12-week FO intervention are positively correlated with red blood cell LC n-3 PUFA and are significantly correlated with blood metabolic parameters

Red blood cells (RBCs) were collected and analysed by Dr Carolina Paras-Chavez and are reported in her doctoral thesis (Chávez, 2014).

Correlations between LC n-3 PUFA in scWAT and RBCs are displayed in Table 3.1, and correlations between measurements of metabolic health in blood, RBC LC n-3 PUFA, and scWAT LC n-3 PUFA are displayed in Table 3.2.

Study entry scWAT EPA and DHA were positively correlated with RBC EPA and DHA (Coefficient $\geq$ 0.37, $P \leq 0.05$, Table 3.1) indicating blood EPA and DHA may be a good marker of scWAT EPA and DHA at study entry. Change (Δ) in scWAT EPA and DHA and RBC EPA and DHA were more strongly correlated than study entry values (Coefficient $\geq$ 0.49, $P \leq 0.001$, Table 3.1) and further suggest that change in blood EPA and DHA is a good indicator of EPA and DHA response in the scWAT.

	Study entry (week-0)					
	RBC EPA[1]		**RBC DPA[1]**		**RBC DHA[1]**	
	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]
scWAT EPA[1]	0.373	0.002	-0.060	-	0.291	0.017
scWAT DPA[1]	0.485	<0.001	0.090	-	0.263	0.031
scWAT DHA[1]	0.400	0.001	-0.182	-	0.508	<0.001

	Change in response to FO intervention					
	RBC Δ EPA[1]		**RBC Δ DPA[1]**		**RBC Δ DHA[1]**	
	Coefficient[1]	*P*[1]	Coefficient[1]	*P*[1]	Coefficient[1]	*P*[1]
scWAT Δ EPA[1]	0.557	<0.001	0.452	<0.001	0.517	<0.001
scWAT Δ DPA[1]	0.376	0.002	0.254	0.038	0.282	0.021
scWAT Δ DHA[1]	0.490	<0.001	0.406	0.001	0.393	0.001

[1] Proportional data (%)

[2]Coefficient and *P* value obtained using Spearman's correlation, significance is deemed $P \leq 0.05$.

Table 3.1 Correlation between RBC and scWAT LC n-3 PUFA

Despite RBC EPA and DHA being positively correlated with scWAT EPA and DHA, there are differences in the correlation between these pools and blood metabolic parameters.

Study entry RBC EPA was positively correlated with blood LDL-cholesterol ($P = 0.004$) as was scWAT EPA which was further positively correlated with blood total TG ($P \leq 0.19$) and adipose-IR score ($P = 0.043$) (Table 3.2). Study entry RBC DPA was negatively correlated with HDL-cholesterol concentrations ($P = 0.014$), whereas scWAT DPA was positively correlated with blood LDL-cholesterol, TG, glucose, insulin, HOMA2-IR and adipose-IR score ($P \leq 0.038$) (Table 3.2). Study entry RBC DHA exhibited no correlation with any blood metabolic parameter; however scWAT DHA was positively correlated with blood LDL-cholesterol and total TG ($P \leq 0.002$, Table 3.2).

Δ RBC EPA was negatively correlated with blood total NEFAs at study entry, as was change in scWAT EPA ($P \leq 0.037$) which was also positively correlated with blood HDL-cholesterol ($P = 0.015$) (Table 3.2). Δ RBC EPA was also negatively correlated with adipose-IR score ($P = 0.025$, Table 3.4). Δ RBC DPA was not correlated with any metabolic parameter; however change in scWAT DPA was negatively correlated with blood insulin ($P = 0.001$), HOMA2-IR scores ($P = 0.002$), and adipose-IR scores ($P < 0.001$) (Table 3.2). Δ RBC and scWAT DHA were negatively correlated with total blood NEFAs ($P \leq 0.032$), and Δ scWAT DHA was also negatively correlated with total blood TG ($P = 0.021$), blood insulin ($P = 0.013$), HOMA2-IR score ($P = 0.023$), and adipose-IR score ($P = 0.003$), and positively correlated with blood HDL-cholesterol ($P = 0.008$) (Table 3.2). There was also a trend for Δ RBC DHA to be negatively correlated with adipose-IR score ($P = 0.060$, Table 3.2).

These data indicate that the response of RBC LC n-3 PUFA is a good indicator of scWAT LC n-3 PUFA response and that metabolic health is significantly associated with these responses, indicating impaired LC n-3 PUFA response with declining metabolic health. This may however only be true with intervention of 12 weeks or longer which reflects the minimum red cell turnover time. Further to this, the relationship of metabolic health with LC n-3 PUFA response is stronger for scWAT in comparison to blood and is therefore of great importance to consider when interpreting the results reported within this thesis.

Study entry (week-0)

	scWAT EPA[1]		scWAT DPA[1]		scWAT DHA[1]		RBC EPA[1]		RBC DPA[1]		RBC DHA[1]	
	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]
HDL[3]	-0.236	0.055	-0.067	-	0.013	-	-0.027	-	-0.284	0.014	-0.008	-
LDL[3]	0.286	0.019	0.553	<0.001	0.402	0.001	0.335	0.004	0.157	-	-0.030	-
Total TG[3]	0.312	0.010	0.549	<0.001	0.374	0.002	0.189	-	0.043	-	-0.022	-
Total NEFA[3]	0.140	-	0.174	-	0.160	-	-0.068	-	-0.142	-	0.029	-
Glucose[3]	0.074	-	0.305	0.012	0.054	-	0.044	-	-0.013	-	0.113	-
Insulin[3]	0.206	-	0.291	0.017	0.002	-	0.031	-	0.038	-	-0.013	-
HOMA2-IR[3]	0.170	-	0.258	0.038	-0.015	-	0.015	-	0.067	-	-0.003	-
Adipose-IR[3]	0.248	0.043	0.342	0.005	0.062	-	-0.016	-	-0.044	-	-0.007	-

Change in response to 12-week FO intervention

	scWAT EPA[1]		scWAT DPA[1]		scWAT DHA[1]		RBC EPA[1]		RBC DPA[1]		RBC DHA[1]	
	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]	Coefficient[2]	*P*[2]
HDL[3]	0.296	0.015	0.223	-	0.321	0.008	0.165	-	0.110	-	0.115	-
LDL[3]	0.031	-	-0.004	-	-0.138	-	-0.006	-	-0.033	-	-0.113	-
Total TG[3]	-0.031	-	-0.210	-	-0.282	0.021	0.028	-	-0.029	-	-0.014	-
Total NEFA[3]	-0.271	0.027	-0.170	-	-0.263	0.032	-0.242	0.037	-0.168	-	-0.258	0.026
Glucose[3]	0.081	-	-0.008	-	0.073	-	0.060	-	0.009	-	-0.020	-
Insulin[3]	-0.130	-	-0.405	0.001	-0.301	0.013	-0.183	-	-0.165	-	-0.118	-
HOMA2-IR[3]	-0.108	-	-0.375	0.002	-0.282	0.023	-0.194	-	-0.175	-	-0.111	-
Adipose-IR[3]	-0.224	0.068	-0.423	<0.001	-0.357	0.003	-0.260	0.025	-0.213	0.069	-0.220	0.060

[1] Proportional data (%)

[2]Coefficient and *P* value obtained using Spearman's correlation, significance is deemed $P \leq 0.05$.

[3] Study entry metabolic parameter. HDL (mmol/L), LDL (mmol/L), Total TG (mmol/L), Total NEFA (mmol/L), Glucose (mmol/L), Insulin (µIU/L), HOMA2-IR = (((insulin mmol/L) x (glucose IU/L)) / 22.5) corrected for variations in hepatic and peripheral glucose resistance, increases in insulin secretion curve for plasma glucose concentrations above 10 mmol/L, and the contribution of circulation proinsulin), Adipose-IR = (NEFAs mmol/L) x (insulin µIU/L).

Table 3.2 Correlations between scWAT and RBC LC n-3 PUFA and blood markers of metabolic health.

3.5 Discussion and conclusion

The current study successfully identifies and reports the proportion of 18 FAs in human scWAT (Table 3.1). Data from the current study indicate a significantly different scWAT FA composition in metabolically healthy obesity compared to normal weight but generally similar incorporation of LC n-3 PUFA in response to 12-week FO intervention. This may indicate preservation of adipose function in the early stages of obesity in which metabolic complications have not manifested as the tissue remains sensitive to dietary lipid manipulation, at least as far as EPA and DHA incorporation are concerned.

In general, the overall composition of scWAT collected from normal weight individuals is concordant with previous reports in healthy humans (Table 3.3). The most abundant FA in human scWAT is reported to be 18:1n-9, followed by 16:0 (Bouéa et al., 2000, Baylin et al., 2002, Hodson et al., 2008, Walker et al., 2014). Hodson *et al.* review FA composition data for 4258 healthy men and 3096 healthy women from 19 studies and report 18:1n-9 and 16:0 to contribute 44% and 22% of WAT total FAs respectively. In addition to those studies reviewed, Walker *et al.* report 18:1n-9 and 16:0 to contribute 46% and 23% of WAT total FA respectively (Table 3.3).

Data from the current study are concordant with these reports: 18:1n-9 contributed 48% of total scWAT FAs, with 16:0 contributing 23% of total scWAT FAs (Table 3.5). In addition to studies reviewed by Hodson *et al.*, Browning *et al.* further report low proportions of LC n-3 PUFA in human scWAT; data from the current study agree with this.

3.5.1 Fatty acid composition in metabolically healthy obese individuals

Reports of WAT FA composition in obesity are predominantly limited to the context of calorie reduction and weight loss in comparison with MHO individuals (of similar average BMI and fasting glucose measurement). The FA profile of the MHO population in the current study is similar to a previous report from such a population (Kunešová et al., 2012). The majority of FA comparisons between normal weight and MHO individuals are limited to human plasma and therefore scWAT profiles from MHO individuals in direct comparison to normal weight individuals are lacking.

The current study provides evidence to fill this knowledge gap and reports greater proportions of the MUFA 16:1n-7, 18:1n-9 and 18:1n-7, the n-6 PUFA 20:3n-6 and AA (20:4n-6), and the LC n-3

PUFA EPA (20:5n-3) and DPA (22:5n-3), along with lower proportions of the SFA 14:0, 18:0 and 20:0, and n-3 FA ALA (18:3n-3) in scWAT from MHO individuals. However, there was no significant difference in the ratio of n-3: n-6 FAs between scWAT from normal weight and MHO subjects (data not shown).

Fatty Acid (%)	Walker *et al.*[1]	Hodson *et al.*[12]	Browning *et al.*[1]	Hames *et al.*[1]	Baylin *et al.* [1]	Boue *et al.* [1](females)	Current study[1]
14:0		4%			1%	3%	3%
16:0	23%	22%			21%	22%	23%
16:1n-7	5%	7%			7%	4%	5%
18:0	4%	3%			3%	5%	5%
18:1n-9	46%	44%			43%	40%	48%
18:2n-6	13%	14%			16%	14%	12%
18:3n-3		0.8%			0.6%	0.4%	0.8%
20:3n-6		0.2%			0.3%	0.2%	0.2%
20:4n-6		0.3%			0.5%	0.4%	0.3%
20:5n-3		0%	0.2%	0.06%	0%	0%	0.1%
22:5n-3		0.1%			0.2%	0.1%	0.2%
22:6n-3		0.1%	0.2%		0.1%	0.1%	0.2%

[1]Mean proportion of total fatty acids, [2] Review of 19 studies

Table 3.3 Comparison of scWAT fatty acid composition from different studies in humans.

3.5.1.1 Attribution of differences in diet, storage, and role of metabolic health status

As reported, there is some correlation between FA intake and scWAT FA composition (Marckmann et al., 1995, Burdge and Calder, 2006), so differences in diet and types of fats, particularly LC n-3 PUFA, consumed in these two subgroups may contribute to the differences observed in WAT FA composition. Unfortunately, dietary data were not collected in this study, so attribution of dietary fat intake to differences in scWAT FAs cannot be made. However, despite reports of some correlation between diet and WAT FA composition, there is great variability in the significance and strength of associations between dietary FA intake and concentration of FAs in adipose tissue, particularly of SFA and MUFA for which a number of studies meta-analysed by Hodson *et al.* do not report significant associations (Hodson et al., 2008). Arab *et al.* further report the general low correlation between dietary intake of SFA and MUFA and their concentration in WAT (Arab, 2003) and discuss discrepancies in using food frequency questionnaires in which memory of consumption, portion size estimation, unknown recipe, information on food fortification, and unknown composition of mixed dishes may affect the scores (Arab and Akbar, 2006). SFA and MUFA can be synthesised endogenously from glucose so their appearance in the WAT will reflect this and be influenced by carbohydrate intake in addition to SFA and MUFA intake from the diet. This may be the greatest / most important contribution to differences between WAT concentration and dietary intake of SFA and MUFA with the exception of the PUFA LA and ALA which cannot be synthesized de-novo.

Differences in dietary FA intake in obese and normal weight adults are poorly reported; one Western study to note, reports dietary information for 200 Canadian adults and found no differences between type 2 diabetic obese and non-obese males but reported higher SFA intake and lower fibre intake in type 2 diabetic obese women compared to non-obese women (Jarvandi, 2011). However, under reporting of energy intake in obesity is well described and therefore assessment of FA intake by food frequency questionnaire may not be accurate.

In addition to the studies included in the meta-analysis by Hodson *et al.*, Summers *et al.* further report differences in the proportion of stored FAs in comparison to intake, suggesting differences in hydrolysis of FAs and/or de novo synthesis of FAs contributing to FA composition independent of dietary intake (Connor et al., 1996, Summers et al., 2000). Indeed as described above, the FA composition of scWAT will also reflect storage of SFA and MUFA (with the exception of the PUFA LA and ALA) generated by de novo lipogenesis which has been shown to be reduced in obesity so differences by BMI subgroup may be attributed to by this (Solinas et al., 2015). In addition to lipogenesis, further changes in FA handling are reported in obesity in which storage of dietary fat is downregulated. McQuaid *et al.* report a decrease in postprandial WAT storage of dietary FAs in

obese individuals in comparison to normal weight individuals (McQuaid et al., 2011). This study further highlights the importance of metabolic health status in obesity, as unlike reports in which the rate of lipolysis is observed to be increased in obesity and with insulin resistance (Reynisdottir et al., 1995, Tsujita et al., 1995, Ryden and Arner, 2007, Arner and Langin, 2014, Morigny et al., 2016), McQuaid *et al.* report a significant down regulation of lipolysis resulting in a normal NEFA plasma profile in MHO individuals in comparison to normal weight individuals (McQuaid et al., 2011). Therefore, differences in scWAT content of SFA and MUFA may reflect differences in dietary intake and de-novo lipogenesis, whereas differences in the content of LC n-3 PUFA and LC n-6 PUFA (AA) may reflect dietary intake, altered synthesis and/or altered mobilisation of these FAs.

Previous reports also highlight a correlation between plasma TG and adipocyte size, as well as between WAT lipolysis and circulating lipid levels, which were further correlated with BMI (Stern et al., 1973, Ryden and Arner, 2017). However, the degree of correlation between the FA composition of scWAT and that of RBC and plasma lipid fractions is under-reported and varies between lipid classes (Hodson et al., 2008). The current study provides novel evidence for the positive correlation between both study entry proportions of LC n-3 PUFA as well as change in response to FO intervention indicating RBC FA composition and response to be a good indicator of scWAT LC n-3 PUFA status (Table 3.1).

In addition to this, the current study reports associations between blood markers of metabolic health and scWAT LC n-3 PUFA at study entry and in response to FO intervention indicating the state of metabolic health is of great importance when comparing data from normal weight and MHO individuals. Such consideration is limited in the current literature, with few studies reporting the metabolic health of the obese individuals included in their studies. One example is the report of increased plasma n-6 PUFA concentrations, particularly 20:3n-6 and AA, in addition to altered concentrations of SFA and MUFA in obesity (Perreault et al., 2014) which upon further interrogation of the obese individuals' metabolic health, were only significant between metabolically unhealthy obese (MUO) individuals in comparison to 'lean' (normal weight) individuals with the only changes reported between MHO and 'lean' individuals being altered 18:2n-6 and 20:3n-6. Further to this, conflicting changes in concentrations of MUFA and n-3 PUFA are reported by Pickens *et al.* in which there was no consideration of metabolic health of the obese individuals included; therefore, it would be important to consider metabolic health status when comparing obese to normal weight individuals (Pickens et al., 2014). This is of particular

importance when reviewing literature investigating WAT biology in the context of obesity as many studies focus on MUO individuals in which insulin resistance has manifested which poses a greater influence on FA transport and metabolism (Morigny et al., 2016).

In agreement with this, the current study provides evidence for not only significant associations between metabolic health and LC n-3 PUFA at study entry, but also suggests there is impaired LC n-3 PUFA response (i.e. ability to incorporate LC n-3 PUFA) with declining metabolic health. This is of importance to consider when interpreting the results from the current study as obese individuals included are deemed to be metabolically healthy based upon their blood measurements (Table 2.2).

3.5.1.2 Attribution of differences in fatty acid metabolism

In addition to dietary intake, transport and storage, differences in reports of FA profiles in obesity may also be attributed to the activity of enzymes involved in FA metabolism, which may further be dependent on the metabolic status of the individuals. The family of desaturase enzymes are responsible for the addition of double bonds to a FA structure; D5D and D6D catalyse the addition of double bonds in the pathway of interconversion of PUFA (Lee et al., 2016), and stearoyl-CoA-desaturase (SCD) catalyses the conversion of SFA into MUFA (Dobrzyn et al., 2010, Lee et al., 2016). The activity of these enzymes has been reported using the ratio of 18:3n-6: 18:2n-6 to indicate D6D activity, 20:4n-6: 20:3n-6 to indicate D5D activity, 16:1n-7: 16:0 to indicate SCDn-7 activity, and 18:1n-9: 18:0 to indicate SCDn-9 activity (Pickens et al., 2014, Lee et al., 2016). From plasma measurements, increased D6D and SCDn-7 activity, and decreased D5D activity have been reported in obese individuals (Pickens et al., 2014).

From the current scWAT analysis, using the ratios described above to estimate enzyme activity, there was significantly increased SCDn-7 and SCDn-9 activity observed in MHO individuals ($P \leq 0.001$, Figure 3.5). This is concordant with increased proportions of 16:1n-7 and 18:1n-9, and a decreased proportion of 18:0 observed in scWAT from MHO individuals (Figure 3.3**.**). Increased activity of D6D and SCD is reported to be associated with the development of insulin resistance and MetS in which FA metabolism is dysregulated (Dobrzyn et al., 2010, Pickens et al., 2014). The current study provides further evidence for this in which SCDn-9 activity was positively correlated with blood insulin (r= 0.274, $P = 0.018$, data not shown) and adipose-IR score (r= 0.353, $P = 0.002$, data not shown) providing further evidence for the importance of metabolic status, as well as BMI/body weight parameters when considering the association of obesity with scWAT FA composition.

FA ratios as indicator of enzyme activity are widely used, but for more accurate assessment of enzyme activity, carbon labelled fatty acid studies could be performed to follow the fate of dietary 16:0, 18:0, 18:2n-6 and 20:3n-6 and assess desaturase activity.

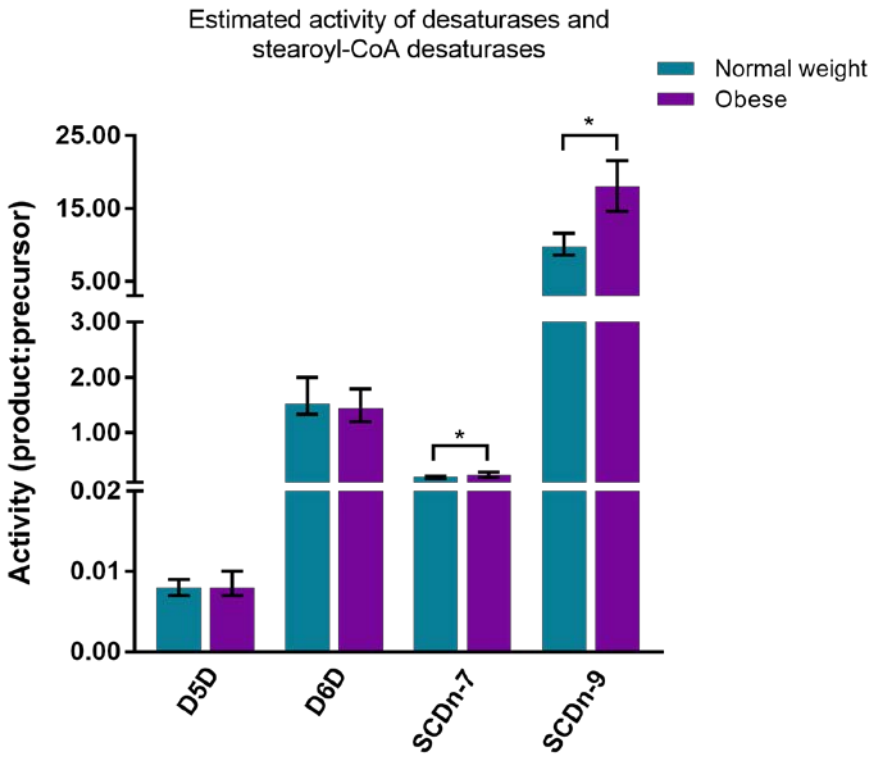


Figure 3.5 Estimated activity of desaturases and stearoyl-CoA desaturases in normal weight (n=29) and metabolically healthy obese individuals (n=38) at study entry (week-0).

Median (± interquartile range) scWAT from metabolically healthy obese compared to scWAT from normal weight. D5D, delta-5 desaturase calculated as 18:3n-6: 18:2n-6; D6D, delta-6 desaturase calculated as 20:4n-6: 20:3n-6; SCDn-7, stearoyl-CoA desaturase-7 calculated as 16:1n-7: 16:0; SCDn-9, stearoyl-CoA desaturase-9 calculated as 18:1n-9: 18:0. *P* obtained from Kruskal Wallis test comparing metabolically healthy obese with normal weight. * $P \leq 0.001$.

3.5.2 Fatty acid composition of scWAT in response to FO intervention

Previous investigations into the incorporation of EPA and DHA in to scWAT are limited; Todorcevic and Hodson's (Todorcevic and Hodson, 2016) review of analyses in scWAT report just 2 studies in human scWAT dating from 1997 and 2012, highlighting lack of comprehensive investigation in this area. Both Katan *et al.* (Katan et al., 1997) and Browning *et al.* (Browning et al., 2012) report

significant increases in scWAT EPA, DPA and DHA in response to EPA + DHA intervention with the greatest changes in DHA. However, these reports do not differentiate between normal weight and obese individuals despite recruiting individuals with a range of BMI (Katan et al., 1997, Browning et al., 2012). More recent studies which investigate EPA+DHA intervention in humans are detailed in Table 1.5 but these do not compare normal weight with obese individuals or report incorporation data for EPA and DHA as the primary focus was on inflammatory outcomes (de Mello et al., 2019, Roberts Lee et al., 2019).

The current study reports that 12-week FO intervention increased the proportion of scWAT LC n-3 PUFA. However, the increase in DHA was not significant in MHO individuals despite showing similar incorporation to normal weight individuals. Both normal weight and MHO individuals were given the same dose of FO (1.8 g of EPA + DHA/ day) with no consideration of dose: fat mass ratio, however when data were adjusted for body fat mass (kg), the increase in DHA remained non-significant in MHO individuals.

3.5.3 Conclusion and limitations

To conclude, the current study reports an altered FA composition of human scWAT in MHO. This may be due to altered dietary intake of FAs or altered metabolism (de novo lipogenesis; elongation/desaturation; lipolysis). The current study further reports FO intervention is capable of increasing scWAT LC n-3 PUFA, and that differences in significance between normal weight and MHO individuals are independent of body fat mass and may be due to variation in biological parameters such as HOMA2-IR. In agreement with this, the current study reports novel evidence for the association of scWAT LC n-3 PUFA response and markers of metabolic health.

In addition, the current study reports novel evidence for RBC LC n-3 PUFA and Δ RBC LC n-3 PUFA to be a good indicator of scWAT LC n-3 PUFA status and response to FO intervention.

These data are novel for MHO individuals in comparison to normal weight individuals and further investigation into the expression of genes involved in the handling of these lipids will provide evidence for differences in LC n-3 PUFA transport and metabolism in obesity, as well as, modulation of these by LC n-3 PUFA themselves. Such investigations are described in Chapter 5 and Chapter 6.

The composition of the scWAT is important as it influences a range of adipose functions; FAs stored within the tissue can undergo oxidation to form a range of bioactive FA metabolites that have a significant role in the regulation of inflammatory signalling, adipose expansion and whole body energy homeostasis. Linking the composition of the scWAT to FA metabolite, gene

expression and protein analyses (described in Chapter 4, Chapter 5, and Chapter 6) will provide more comprehensive evidence for the role of scWAT, and specifically, the types of FAs that are stored in the scWAT, in the development of obesity-associated scWAT inflammation.

A limitation which hinders the interpretation of the results in this chapter is lack of habitual dietary intake data. This would allow attribution of differences in dietary FAs to differences in dietary intake or suggest that these are independent of diet and more likely be due to one or more of the altered transport and metabolic pathways proposed above. In addition, lack of information regarding smoking status and alcohol consumption is a further limitation. Both have been reported to be associated with differences in n-3 PUFA metabolism and concentrations, and therefore may contribute to a proportion of the variation seen within, and the differences between, the subgroups (Steffen et al., 2012, Sozio and Crabb, 2008 , Pawlosky et al., 2007). A further limitation is that absolute quantification of FAs was not possible as internal standards were not added to the tissue homogenate. These data would allow investigation of the absolute concentration of FAs incorporated into the AT and allow further comparison of lipid handling in response to FA intervention and in obesity.

Chapter 4 Fatty acid metabolite composition of human subcutaneous white adipose tissue is altered in metabolically healthy obese subjects and in response to 12-week fish oil intervention

4.1 Introduction

Obesity is characterised by a state of chronic low-grade inflammation contributed to by altered production of many fatty acid (FA) metabolites in the circulation and in the white adipose tissue (WAT) itself.

As described in Chapter 1, section 1.3.3.4, classic eicosanoids and docosanoids, along with further oxylipins, hydroperoxy precursors, and endocannabinoids (ECs) can be synthesised from FAs and secreted by adipose resident cells to modulate pro- and anti-inflammatory signalling pathways.

Inflammation occurring in the WAT at the onset of, and during, obesity is associated with increased pro- and anti-inflammatory signalling and loss of sensitivity to many signalling molecules (Masoodi et al., 2014). Obesity has been reported to result in dysregulation of many plasma linoleic acid (LA) and arachidonic acid (AA) derived metabolites including hydroxyeicosatetraenoic acids (HETEs,) dihydroxy-HETEs (DiHETEs), epoxyoctadecaenoic acid (EpOMEs), and dihydroxy-octadeacenoic acids (diHOMEs), and plasma LC n-3 PUFA derived metabolites such as hydroxy-docosapentaenoic acids (HDPAs) (Pickens et al., 2017, Wang et al., 2017).

It has also been suggested that the EC system of the WAT becomes dysregulated during obesity; current evidence for this is inconsistent and previous studies report both lower and higher levels of classical AA-containing ECs in obesity (Annuzzi et al., 2010, Lee et al., 2009). There is slightly more conclusive evidence in plasma, where the concentration of 2-arachidonoyl glycerol (2-AG) is elevated in obesity and is positively correlated with BMI, waist circumference and intra-abdominal adiposity (Naughton et al., 2013, Côté et al., 2007, Gatta-Cherifi et al., 2012, Gatta-Cherifi and Cota, 2015). As ECs contain FAs that may be derived from the diet or from dietary precursors(e.g.

AA), the concentrations of ECs can be modulated by change in diet (Naughton et al., 2013, Balvers et al., 2012).

High fat diets (HFDs) have been observed to increase AA-containing EC concentrations and increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake is reported to modulate AA-, EPA- and DHA-containing EC concentrations in murine plasma, VAT and epididymal AT (Naughton et al., 2013, Balvers et al., 2012, Batetta et al., 2009, Kim et al., 2015), as well as in human plasma from obese males (Banni et al., 2011). The production of EPA + DHA derived metabolites often occurs at the expense of AA-derived metabolites and as such EPA + DHA supplementation has been seen to decrease AA-containing metabolites including ECs and to increase concentrations of EPA- and DHA-containing ECs, but this is again underreported for human WAT (Rossmeisl et al., 2012, Kim et al., 2015, Wood et al., 2010).

Current reports of FA metabolite concentrations in WAT are limited to *in vitro* or *in vivo* animal models, with reports in humans limited to associating plasma FA metabolites to fat mass. In addition to this, investigation of habitual levels and responses to fish oil (FO) intervention in subcutaneous WAT (scWAT) of obese individuals without metabolic complication, termed metabolically healthy obese individuals (MHO), is completely lacking. Therefore, experimental objectives with the aim to provide evidence to fill current gaps in knowledge were developed and are outlined in section 4.2.

4.2 Aims

The aim of this chapter was to profile FA metabolites in scWAT of normal weight and MHO individuals to:

- Investigate whether metabolically healthy obesity is associated with a more pro-inflammatory FA metabolite profile in scWAT
- Investigate whether FO intervention decreases pro-inflammatory metabolites and increases anti-inflammatory/pro-resolving metabolites in scWAT of normal weight and metabolically healthy obese individuals
- Investigate whether the responses to FO supplementation differ in that a greater anti-inflammatory effect is seen in metabolically healthy obesity

4.3 Methods

4.3.1 Experimental reagents and materials.

See Appendix C.

4.3.2 Extraction of lipids and fatty acid metabolites from white adipose tissue

Please refer to 'Chapter 2– Study design and population'.

4.3.2.1 Fatty acid metabolite extraction procedure

FA metabolite extraction was performed at the Prague Institute of Physiology by myself and Dr Ondrej Kuda. Protocol optimisation was performed under the supervision of Dr Kuda but due to machine faults resulting in the UPLC/MS-MS being un-usable for the remainder of my stay in Prague, the remaining sample extraction and all analysis via UPLC/MS-MS were performed by Dr Kuda. Dr Kuda also performed UPLC/MS-MS analysis of calibration standards. Raw data files of all analyses were sent to myself, and I performed all data integration to obtain peak area counts and calculation of proportional and absolute concentrations of FA metabolites. All statistical analysis was performed by myself.

Approximately 100 mg of frozen scWAT was homogenised in 650 µl chilled 100% methanol (-20°C) then stored at -80°C for 30 minutes to allow proteins in the sample to precipitate. The following deuterated internal standards (25 µg each) were added to the homogenate to aid identification of FA peaks: arachidonic acid-d8, docosahexaenoic acid-d5, eicosapentaenoic acid-d5, PGD2-d4, 15(s)-hydroxy-eicosatetraenoic aicd-d8, 12(s)-hydroxy-eicosatetraenoic aicd-d8, 5(s)-hydroxy-eicosatetraenoic aicd-d8, 6-keto-PGF1-alpha-d4, 8-iso-PGF2-alpha-d4, PGF2-d9, 15(s)-hydroxy-eicosatetraenoic acid-d8, and 8-9 dihydroxy-eicosatrienoic acid-d11 (Cayman Chemical Company, Michigan, United States). Following centrifugation of the homogenate at 12,000 x g for 10 minutes at 4°C, 650 µl of supernatant was collected and FA metabolites were isolated from this using solid phase extraction (SPE) (Dumlao et al., 2011, Kuda et al., 2016). The SPE protocol was optimised for the use of human scWAT in collaboration with the Prague Institute of Adipose Biology; increased SPE cartridge capacity and double the usual volume of solvents were required for complete isolation and elution of all FA metabolite classes.

The optimised protocol is as follows: Strata-X polymeric reversed phase 500 mg 6 ml capacity, SPE cartridges (Phenomenex, California, United States) and SPE tank needles were activated with two 6 ml washes of ethyl acetate and the cartridges were further conditioned with sequential addition and removal of 2 x 6 ml washes of chilled methanol, hexane and H_2O under vacuum ensuring the cartridge sorbent remained saturated. Chilled H_2O (3.38 ml) was added to the homogenate supernatant, vortex mixed and followed by the addition of 300 µl of chilled 0.1 mM hydrochloric

acid. This solution was immediately applied to the SPE cartridge along with 6 x 1 ml of chilled H_2O used to rinse the tube. The SPE sorbent was allowed to dry before 6 ml of hexane was added. All FA metabolites were then eluted from the column into a tube containing 10 µl of chilled 30% glycerol in methanol with the sequential addition of 6 ml chilled methyl formate and 2 ml of methanol (Dumlao et al., 2011). These were dried, dissolved in chilled mobile phase (30% acetonitrile, 60% H_2O, 10% methanol), applied to a polyvinylidene fluoride micro filter and centrifuged at 12,000 x g for 2 minutes at 4°C. The filtrate was then transferred to a 300 µl insert glass vial.

4.3.3 Analysis of fatty acid metabolites via ultra-pure liquid chromatography tandem mass spectrometry (UPLC/MS-MS)

4.3.3.1 Principle of UPLC/MS-MS

UPLC/MS-MS is a technique which exploits polarity and mass-charge ratio (m/z) to separate, identify and quantify lipids and FA metabolites (Figure 4.1).

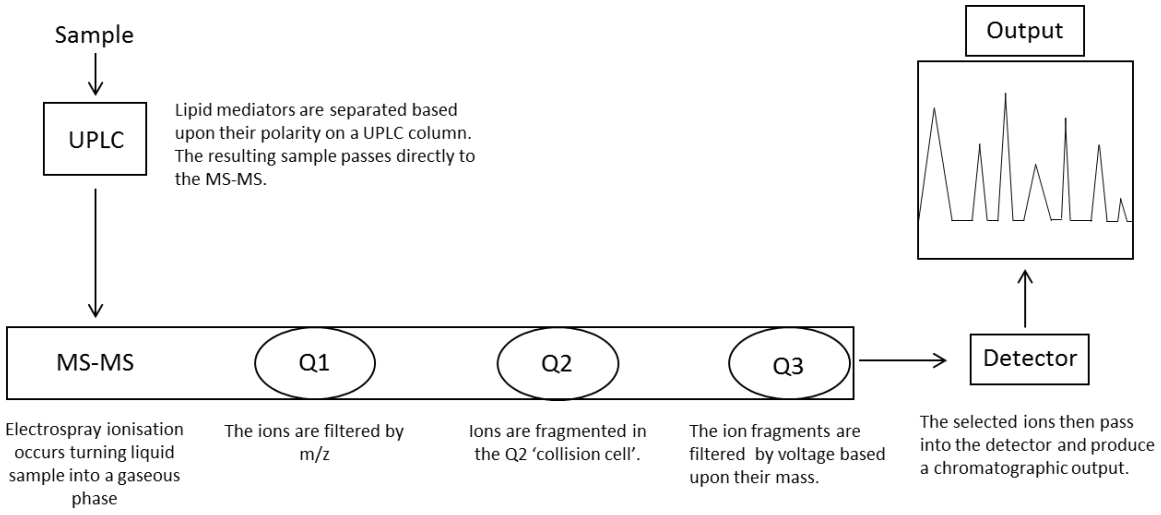


Figure 4.1 Schematic of fatty acid metabolite separation and detection using ultra-pure liquid chromatography tandem mass spectrometry (Amended from (Cazes, 2005)).

4.3.3.2 Separation and identification of fatty acid metabolite by UPLC/MS-MS procedure:

The FA metabolites were separated on a Thermo Dionex Ultimate 3000 RSLC (Dionex corporation, California, United States) liquid chromatograph fitted with a Kinetex® UPLC column 1.7 µm particle size, C18 stationary phase, 100 A pore size, 150 x 2.1 mm (Phenomenex, California, United States) with mobile phase composition A = 70% H_2O, 30% acetonitrile and 0.02% acetic acid; B = 50% acetonitrile and 50% isopropanol, and identified on an AB SCIEX SelexION QTRAP 5500 (AB

SCIEX, Massachusetts, United States) triple quadrupole mass spectrometer selecting for electrospray in both positive Scan Type: Multiple reaction monitoring (MRM), Scheduled MRM: Yes, Polarity: Negative, Scan Mode: N/A, Ion Source: Turbo Spray, MRM detection window: 120 sec, Target Scan Time: 0.3000 sec, Resolution Q1: Unit, Resolution Q3: Unit, Intensity Threshold: 0.00 cps, Settling Time: 5<0.0010 msec, MR Pause: 2.0000 msec, multichannel analyser (MCA): No, Step Size: 0.00 dalton (Da) and negative modes (Settings: Scan Type: MRM, Scheduled MRM: Yes, Polarity: Negative, Scan Mode: N/A, Ion Source: Turbo Spray, MRM detection window: 120 sec, Target Scan Time: 0.4000 sec, Resolution Q1: Unit, Resolution Q3: Unit, Intensity Threshold: 0.00 cps, Settling Time: 5<0.0010 msec, MR Pause: 2.0000 m sec, MCA: No, Step Size: 0.00 Da), which was coupled to the liquid chromatograph (Kuda et al., 2016).

Deuterated internal standards were used to orientate the resulting UPLC-MS outputs and identify correct FA metabolite peaks using Analyst software (version 1.6.2 2013, AB SCIEX, Massachusetts, United States) (Table 4.2). The limit of detection was $\geq$ 0.1 pg for all metabolites; FA metabolite signals were determined to be genuine above this level and if 30% greater compared to background signals. An example of 3 deuterated internal standards matching experimental FA metabolite peaks is shown in Figure 4.2. FA metabolites displaying significant differences in peak area adjusted for tissue mass and/or relative concentration were quantified using calibration curve data. Not all lipids could be quantified due to lack of available calibration curve data or values falling below detection levels (i.e. the lowest curve concentration) but 23 FA metabolites were successfully quantified. Calibration curve slope and intercept values of mediators successfully quantified are reported in Table 4.1.

	Slope (absorbance/(ng/ml))	Intercept (absorbance)
5-HETE	106287.54	-282541.79
8-HETE	119356.05	-189434.72
12-HETE	167003.44	2316.01
17,18-DiHETE	26291.16	1655.63
5-oxo-ETE	90438.10	-932.40
PGE2	228519.39	4438.25
PGF2α	25256.51	477.08
6-keto-PGF1a	124109.59	-4482.28
TXB2	202087.85	-28088.87
11-dh-TXB2	175131.64	-3847.35
LXB4	46009.87	-2730.72
20-4-EA	86652.98	10829.27
2-AG	495.98	54.53
20-5-EA	79819.27	5388.10
4-HDoHE	100106.03	-1019.88
8-HDoHE	45865.37	-995.59
11-HDoHE	78715.85	-26.12
14-HDoHE	52580.74	2660.79
17-HDoHE	23304.65	3809.63
RvD1	243396.62	-17597.72
RvD2	243407.16	-17677.26
22-6-EA	60874.84	-150.98
18-1-EA	23201.16	36293.96

Table 4.1 Calibration curve slope and intercept values for fatty acid metabolites quantified for expression in ng/g scWAT.

Concentration range to obtain curve data was 0.005 ng/mL to 10 ng/mL.

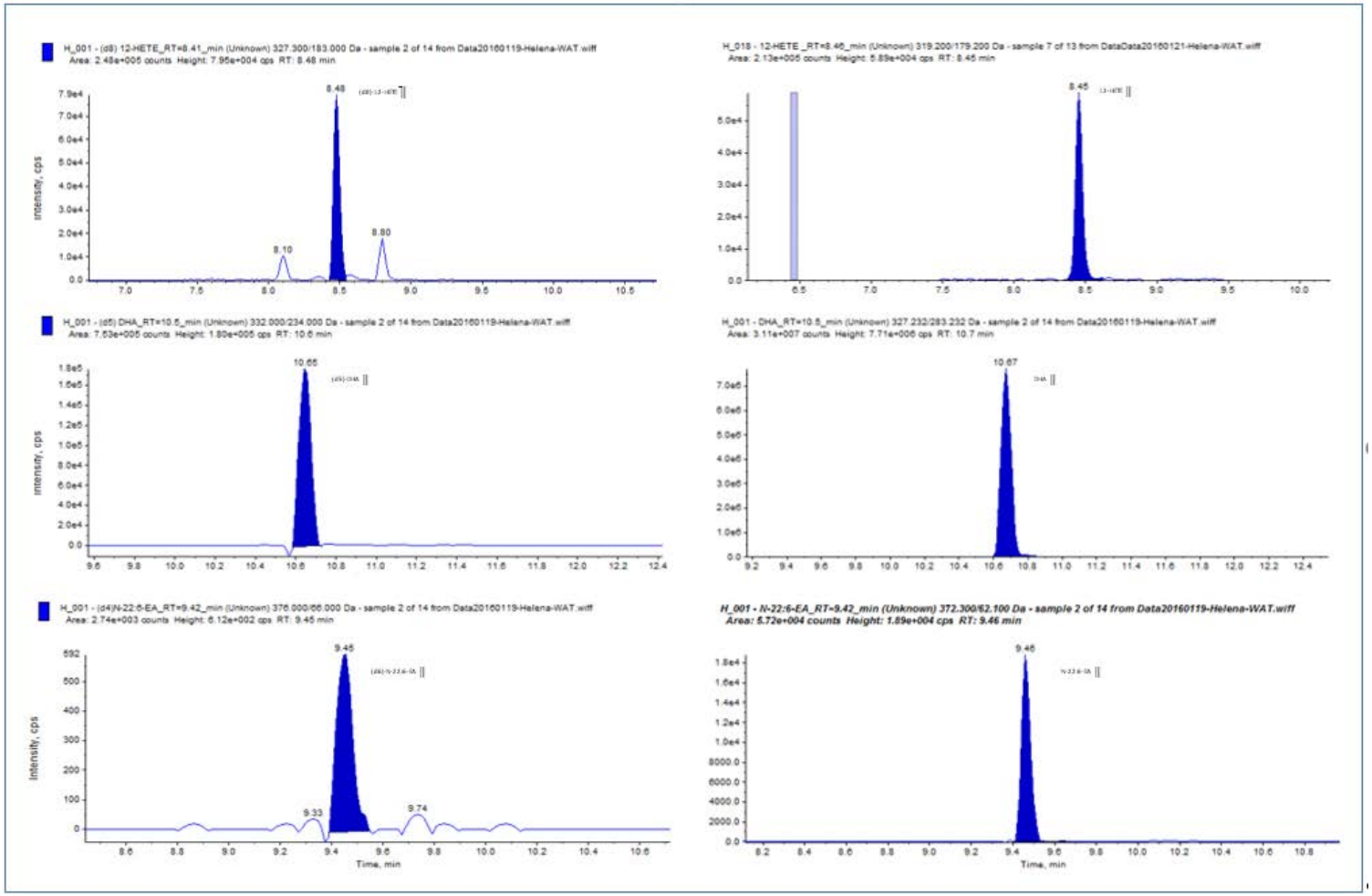


Figure 4.2 UPLC/MS-MS output of deuterated 12-HETE, DHA and N-22-6-EA internal standards added to an experimental sample (left hand images), and the corresponding non-deuterated peaks in the experimental sample (right hand images).

4.3.4 Statistical analyses

All statistical analyses were conducted using SPSS software (version 21; SPSS Inc.). The distribution of the data was assessed using the Shapiro-Wilk test and by plotting distributions of residuals obtained from general linear model (GLM) analysis of the data. Data did not conform to the normal distribution and remained not normally distributed following $\log_{10}$ transformation. Therefore, the Mann-Whitney U test was used to identify differences in FA metabolite composition of MHO individuals in comparison to normal weight individuals, and the Wilcoxon test was used on data split by BMI, selected by treatment, to compare paired post intervention data (week-12) with study entry (week-0). All data are reported as median $\pm$ interquartile range.

MHO individuals are referred to as 'obese' in all figures and tables herein.

4.4 Results

FA metabolites were extracted from scWAT biopsies collected from 36 normal weight and 37 MHO individuals at study entry and following 12-week intervention with FO or corn oil (CO) via SPE and were assessed by UPLC/MS-MS.

FA metabolite data are expressed as proportion (amount of specific FA metabolite expressed as a percentage (%) relative to total FA metabolites detected) for all metabolites, and as absolute concentration (ng/g scWAT) for mediators for which calibration curve data could be obtained. A list of lipids and mediators successfully measured in human scWAT using UPLC/MS-MS, and the instrument settings used to measure each mediator are detailed in Appendix D.

FA metabolites successfully isolated and reported in human scWAT in this analysis alongside their precursor FA are detailed in Table 4.2.

18:3n-3 (ALA)	9-HOTrE, 13-HOTrE,
18:2n-6 (LA)	9-HODE, 13-HODE, 9-oxo-ODE, 9-HpODE, 12,13-DiHOME, 18:2-EA, 2_18_2-glycerol
20:3n-6	15-HETrE, 11,12,15-TriHETrE, TXB_1, 6-keto-$PGF_{1\alpha}$, 2-3-dinor-6-keto-$PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_1, PGD_1
20:4n-6 (AA)	20-COOH-AA 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 16-HETE, 14-15-diHETE, 17-18-diHETE, 12-HHT LXA_4, LXB_4, LTB_4, LTC_4, LTD_4, LTE_4, $uLTC_4$, $uLTD_4$, 20-OH-LTB_4, 20-COOH-LTB_4, HXA_3 TXB_2, 11-dh-TXB_2 5-oxo-ETE, 9-oxo-ETE, 13-oxo-ETE, 15-oxo-ETE, 11-12-EET, 14-15-EET, 5-6-DHET, 11-12-DHET, 14-15-DHET PGB_2, PGD_2, $PGF_{2\alpha}$, PGJ_2 dhk-PGD_2, dhk-PGE_2, dhk-$PGF_{2\alpha}$, delta12-PGJ_2 8-iso-$PGF_{2\alpha}$ 20:4-EA, 1-20:4-glycerol, 2-20:4-glycerol
20:5n-3 (EPA)	5-HEPE, 12-HEPE, 15-HEPE, RvE1, 20:5-EA, 2-20:5-glycerol, LTB_5, TXB_3, $PGF_{3\alpha}$, PGD_3, PGE_3, LXA_5,
22:5n-3 (DPA)	19-20-DiHDPA, 22:5-EA, 2-22:5-glycerol
22:6n-3 (DHA)	4-HDoHE, 7-HDoHE, 8-HDoHE, 11-HDoHE, 13-HDoHE, 14-HDoHE, 16-HDoHE, 17-HDoHE 20-HDoHE 9-10-EpOME, 12-13-EpOME, RvD1, RvD2, 22:6-EA, 2-22:6-glycerol
14:0	14:0-EA
16:0	16:0-EA, 2-16:0-glycerol
16:1n-7	16:1-EA, 2-16:1-glycerol
18:0	18:0-EA
18:1n-9	18:1-EA, 2-18:1-glycerol

Table 4.2 Fatty acid metabolites and their precursor fatty acid reported in human scWAT.

4.4.1 Study entry results

4.4.1.1 Metabolically healthy obesity is associated with an altered scWAT fatty acid metabolite profile with particular dysregulation of DHA metabolites and the endocannabinoid system

At study entry, obesity was associated with an altered composition of FA metabolites in scWAT with statistically significant differences observed in metabolites derived from both omega-6 (n-6) and omega-3 (n-3) polyunsaturated FAs (PUFA). Lower proportions of the 18:3n-3 (ALA)-containing mediator 9-hydroxy-octadecatrienoic acid (9-HOTrE) ($P \leq 0.001$), the 18:2n-6 (LA)-containing mediators 9-hydroperoxy-octadecadienoic acid (9-HpODE), 9-oxo-octadecadienoic acid (9-oxo-ODE), 13-oxo-octadecadienoic acid (13-oxo-ODE), and 12,13-dihydroxy-octadecenoic acid (12,13-DiHOME) ($P \leq 0.05$), the FA ethanolamide of 14:0, 14:0-EA, and the glycerol ester of 16:1n-7, 16:1-glycerol ($P \leq 0.001$) (Figure 4.3) were observed in scWAT from MHO individuals.

scWAT from MHO individuals also had lower concentrations of the AA-containing mediator lipoxin B_4 (LXB_4) ($P \leq 0.05$, Figure 4.4) and lower proportions of 20-COOH-arachidonic acid (20-COOH-AA), 11,12-dihydroxy-eisoatrienoic acid (11,12-DHET), u-leukotriene-D_4 ($uLTD_4$), and hepoxilin-A_3 (HXA_3) ($P \leq 0.05$, Figure 4.4).

There were also higher concentrations of the AA (20:4n-6) derived mediators prostaglandin-$F_2\alpha$ ($PGF_2\alpha$) and arachidonoyl-ethanolamide (20_4_EA, anandamide, AEA) ($P \leq 0.001$, Figure 4.4) observed in scWAT from MHO individuals.

The concentrations of EPA (20:5n-3) and DHA (22:6n-3) derived metabolites also differed in scWAT from MHO individuals. There was a higher concentration of eicosapentaenoyl-ethanolamide (20:5_EA, EPEA) ($P < 0.05$, Figure 4.5), and lower concentrations of the hydroxy-DHA metabolites 4-hydroxy-DHA (4-HDHA) and 11-HDHA ($P < 0.05$, Figure 4.5). There was also lower proportions of 8-HDHA, 14-HDHA, 15-HDHA, 17-HDHA, 20-HDHA, and resolvin-D_2 (RvD_2) ($P < 0.05$, Figure 4.5).

A summary of mediators and their proportion and concentration in scWAT from MHO individuals in comparison to normal weight is reported in Figure 4.6 and Figure 4.7.

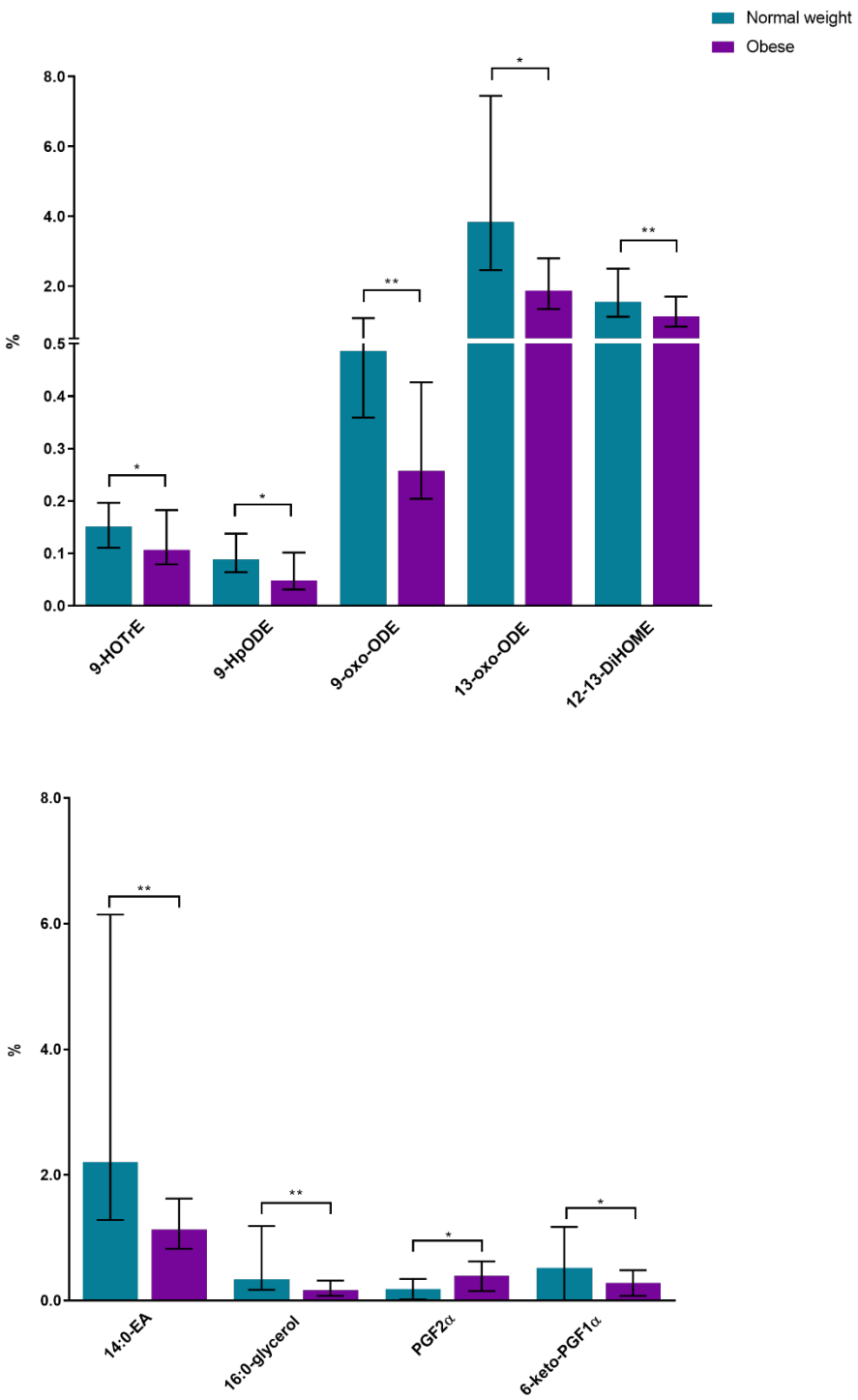


Figure 4.3 ALA (18:3n-3), LA (18:2n-6), 14.0, 16.0, and DGAL (20:3n-6) derived metabolites in scWAT of normal weight (n= 36) and metabolically healthy obese (n= 37) individuals at study entry (week-0).

Median (± interquartile range) scWAT from metabolically healthy obese individuals compared to scWAT from normal weight individuals. *P* obtained from Mann-Whitney U test. * = $P \leq 0.05$, ** = $P \leq 0.001$.

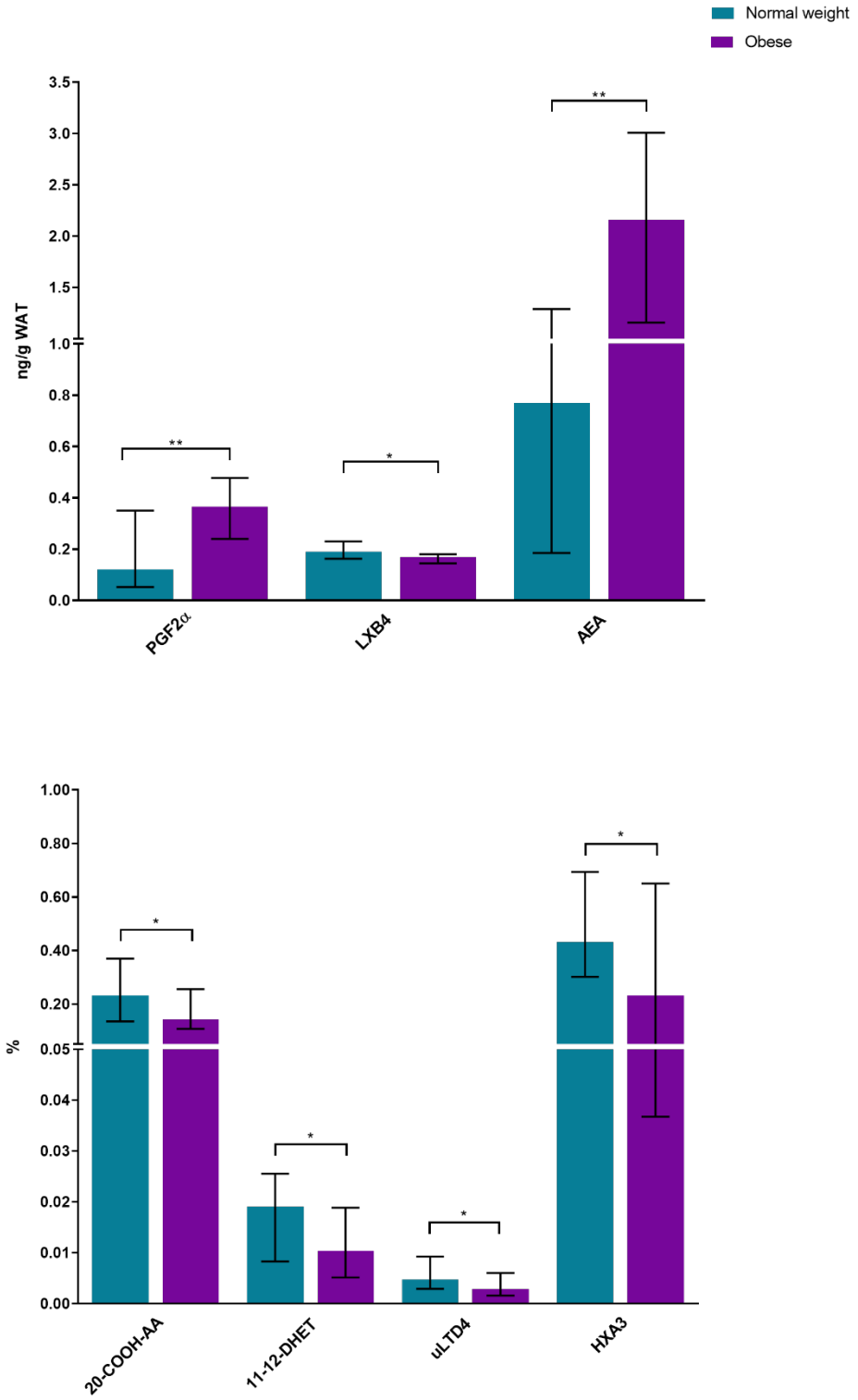


Figure 4.4 AA (20:4n-6) derived metabolites in scWAT of normal weight (n= 36)and metabolically healthy obese (n= 37) individuals at study entry (week-0).

Median (± interquartile range) scWAT from metabolically healthy obese individuals compared to scWAT from normal weight individuals. *P* obtained from Mann-Whitney U test. * = $P \leq 0.05$, ** = $P \leq 0.001$.

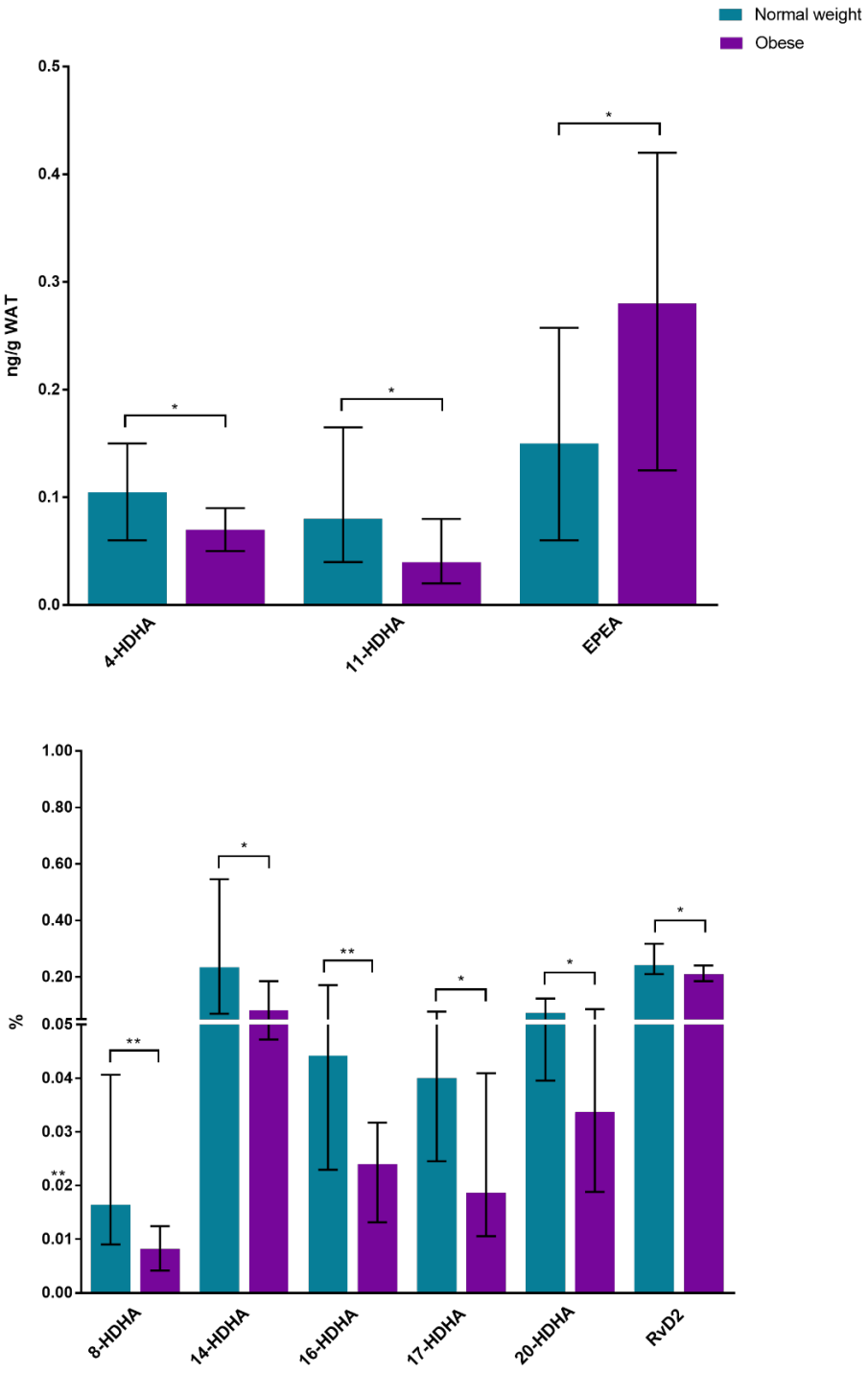


Figure 4.5 EPA (20:5n-3) and DHA (22:6n-3) derived metabolites in scWAT of normal weight (n= 36) and metabolically healthy obese (n = 37) individuals at study entry (week-0).

Median (± interquartile range) scWAT from metabolically healthy obese individuals compared to scWAT from normal weight individuals. *P* obtained from Mann-Whitney U test. * = $P \leq 0.05$, ** = $P \leq 0.001$.

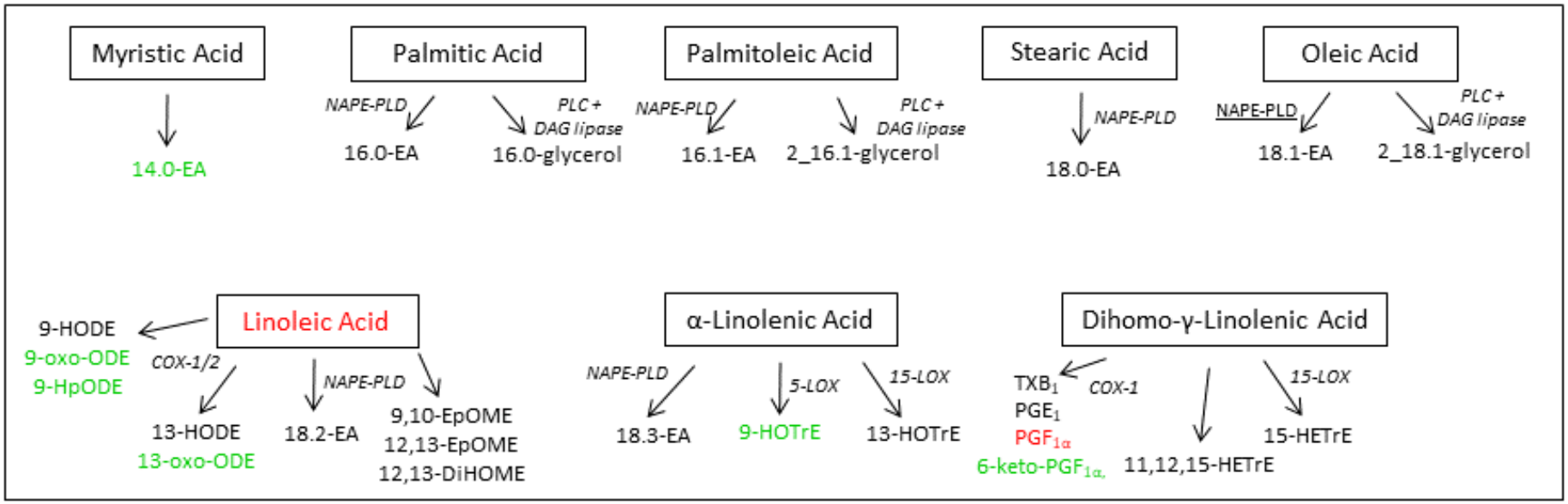


Figure 4.6 Differences in saturated fatty acids, monounsaturated fatty acids and shorter chain PUFA and their respective metabolites in scWAT from metabolically healthy obese (n= 37) in comparison to normal weight (n= 36) individuals at study entry (week-0).

Green = decreased ($P < 0.05$ all); Red = increased ($P < 0.05$ all); Black = no difference in concentration between normal weight and metabolically healthy obese individuals.

COX, cyclooxygenase; EA, ethanolamide; EpOME, epoxy-octadecaenoic acid; HETrE, hydroxyl-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxyl-octadecatrienoic acid; HpODE, hydroperoxy-octadecadienoic acid; LOX, lipoxygenase; NAPE-PLD, N-acyl phosphatidyl ethanolamide phospholipase-D; oxo-ODE, oxo-octadecadienoic acid; PLC, phospholipase C.

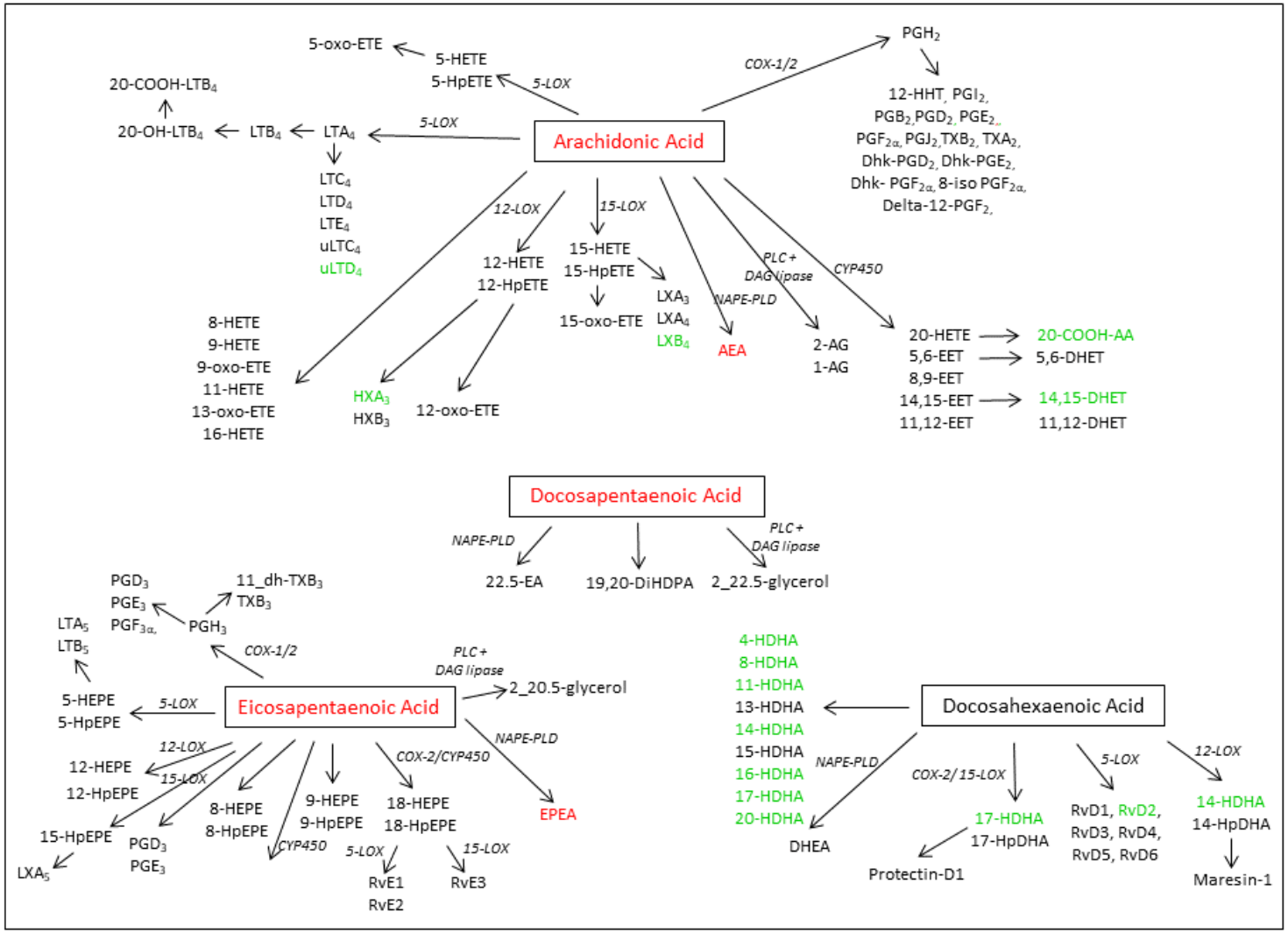

Arachidonic Acid
5-oxo-ETE
5-HETE
5-HpETE
5-LOX
20-COOH-LTB4
20-OH-LTB4
LTB4
LTA4
5-LOX
LTC4
LTD4
LTE4
uLTC4
uLTD4
COX-1/2
PGH2
12-HHT, PGI2,
PGB2, PGD2, PGE2,
PGF2α, PGJ2, TXB2, TXA2,
Dhk-PGD2, Dhk-PGE2,
Dhk- PGF2α, 8-iso PGF2α,
Delta-12-PGF2,
12-LOX
15-LOX
12-HETE
12-HpETE
15-HETE
15-HpETE
15-oxo-ETE
LXA3
LXA4
LXB4
NAPE-PLD
AEA
PLC + DAG lipase
2-AG
1-AG
CYP450
20-HETE
5,6-EET
8,9-EET
14,15-EET
11,12-EET
20-COOH-AA
5,6-DHET
14,15-DHET
11,12-DHET
8-HETE
9-HETE
9-oxo-ETE
11-HETE
13-oxo-ETE
16-HETE
HXA3
HXB3
12-oxo-ETE
Docosapentaenoic Acid
NAPE-PLD
PLC + DAG lipase
22.5-EA
19,20-DiHDPA
2_22.5-glycerol
PGD3
PGE3
PGF3α,
11_dh-TXB3
TXB3
PGH3
LTA5
LTB5
COX-1/2
PLC + DAG lipase
2_20.5-glycerol
5-HEPE
5-HpEPE
5-LOX
Eicosapentaenoic Acid
NAPE-PLD
EPEA
12-LOX
15-LOX
12-HEPE
12-HpEPE
COX-2/CYP450
8-HEPE
8-HpEPE
9-HEPE
9-HpEPE
18-HEPE
18-HpEPE
CYP450
15-HpEPE
LXA5
PGD3
PGE3
5-LOX
15-LOX
RvE1
RvE2
RvE3
4-HDHA
8-HDHA
11-HDHA
13-HDHA
14-HDHA
15-HDHA
16-HDHA
17-HDHA
20-HDHA
Docosahexaenoic Acid
NAPE-PLD
DHEA
COX-2/15-LOX
17-HDHA
17-HpDHA
Protectin-D1
5-LOX
RvD1, RvD2,
RvD3, RvD4,
RvD5, RvD6
12-LOX
14-HDHA
14-HpDHA
Maresin-1

Figure 4.7 Differences in long chain PUFA and their respective fatty acid metabolites in scWAT from metabolically healthy (n= 37) in comparison to normal weight (n= 36) individuals at study entry (week-0).

Green = decreased ($P < 0.05$ all); Red = increased ($P < 0.05$ all); Black = no difference in concentration between normal weight and metabolically healthy individuals.

1-AG, 1-arachidonoyl-glycerol; 2-AG, 2-arachidonoyl-glycerol; AEA, N-arachidonoyl-ethanolamide (anandamide, 20-4-EA); COX, cyclooxygenase; CYP450, cytochrome-P450; DAG, diacyglycerol; DHEA, docosahexaenoyl-ethanolamide; dh-TXB, dihydro-thromboxane-B; Dhk-PGD, dihydro-keto-prostaglandin-D; DHET, dihydroxy-eicosatrienoic acid; DiHDPA, dihydroxy-docosapentaenoic acid; DiHETE, di-hydroxy-eicosatetraenoic acid; EET, epoxy-eicosatrienoic acid; EPEA, eicosapentaenoyl-ethanolamide; HDHA, hydroxy-docosahexaenoic acid (HDoHE); HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHT, hydroxy-heptadecatrenoic acid; HpEPE, hydroperoxy-eicosapentaenoic acid; HpHDHA, hydroperoxy-docosahexaenoic acid; HXA, hepoxilin-A; HXB, hepoxilin-B; LOX, lipoxygenase; LTA, leukotriene-A; LTB, leukotriene-B; LTC, leukotriene-C; LTE, leukotriene-E; LXA, lipoxins-A, LXB, lipoxins-B; NAPE-PLD, N-acyl phosphatidyl ethanolamide phospholipase-D; oxo-ETE, oxo-eicosatetraenoic acid; PGB, prostaglandin-B; PGD, prostaglandin-D; PGE, prostaglandin-E; PGF, prostaglandin-F; PGH, prostaglandin-H; PGI, prostaglandin-I; PGJ, prostaglandin-J; PLC, phospholipase-C; RvD, resolvin-D; RvE, resolvin-E; TXB, thromboxane-B; TXA, thromboxane-A..

In addition, a number of FA metabolites were associated with the metabolic status of the scWAT. Of particular interest was the positive correlation of AEA with adipose-IR and the negative correlation of a range of hydroxy-DHA metabolites and RvD2 with adipose-IR ($P \leq 0.050$, Table 4.3). This suggests that the metabolic health of the tissue is associated with EC signalling and reduced resolution of inflammation. The LA derived metabolites 9-HpODE and 13-oxo-ODE, and a number of AA derived metabolites were negatively correlated with adipose-IR ($P \leq 0.050$, Table 4.3) highlighting the complex regulation of these FA metabolites.

		Correlation with Adipose-IR[1]	
Precursor FA	FA metabolite	Coefficient[2]	*P*[2]
LA	**9-HpODE**	-0.238	0.050
LA	**13-oxo-ODE**	-0.307	0.011
AA	**20-COOH-AA**	-0.235	0.054
AA	**PGFα2**	0.280	0.021
AA	**11, 12-DHET**	-0.323	0.007
AA	**14, 15-DHET**	-0.295	0.014
AA	**17, 18-DiHETE**	-0.265	0.029
AA	**HXA3**	-0.367	0.002
AA	**AEA**	0.389	0.002
DHA	**11-HDHA**	-0.241	0.048
DHA	**13-HDHA**	-0.247	0.043
DHA	**14-HDHA**	-0.332	0.016
DHA	**20-HDHA**	-0.248	0.042
DHA	**RvD2**	-0.275	0.023

Table 4.3 Correlation between scWAT FA metabolites and scWAT adipose-IR scores for the whole cohort at study entry (week-0).

[1]Adipose-IR = (NEFAs µmol/L) x (insulin µIU/L)

[2]Coefficient and *P* value obtained using Spearman's correlation, significance is deemed $P \leq 0.05$.

4.4.2 Post intervention results

4.4.2.1 12-week fish oil intervention significantly modulated omega-3 and omega-6 fatty acid metabolites in scWAT from normal weight individuals

The change in proportion (%) of all FA metabolites in response to 12 weeks of FO intervention in normal weight individuals is presented in Appendix G, and the change in ng/g scWAT of FA

metabolites for which calibration curve data allowed quantification in Appendix H. Significant changes in % and ng/g scWAT of FA metabolites are presented in Table 4.4

In response to 12-week FO intervention in normal weight individuals, the proportion of the 18:3n-3 (ALA) metabolite 9-HOTrE, and the 18:2n-6 (LA) metabolites, 9,10-EpOME, 9-HODE, and 9-HpODE, significantly decreased in the scWAT ($P \leq 0.006$, Table 4.4). The proportion of the 20:4n-6 (AA) metabolites 20-COOH-AA, lipoxin-A4 (LXA_4), leukotriene-B4 (LTB_4), LTD_4, 11,12-dihydroxy-eicosatrienoic acid (11-12-DHET), 14-15-DHET, 14,15-dihydroxy eicosatetraenoic acid (14,15-DiHETE), and 17-18-DIHETE were significantly decreased ($P \leq 0.048$, Table 4.3), and the AA metabolite dhk-PGE_2 significantly increased in the scWAT of normal weight individuals ($P = 0.035$, Table 4.4).

FO intervention in normal weight individuals also significantly altered the concentration of n-3 PUFA along with many EPA and DHA derived metabolites. The proportion of the EPA metabolite 15-hydroxy eicosapentaenoic acid (15-HEPE) ($P = 0.035$, Table 4.4) and the absolute concentration of EPA and DHA containing endocannabinoids, the FA ethanolamides EPEA and DHEA, were significantly increased in the scWAT of normal weight individuals in response to 12-week FO intervention ($P \leq 0.039$, Table 4.3, Figure 4.8). Despite significant associations with adipose-IR score and the EC system at study entry, neither the response of EPA and DHA or AA containing ECs were significantly associated with the metabolic health of the tissue (as indicated by adipose-IR score, $P \geq 0.370$).

Many hydroxy-DHA metabolites were altered in response to 12-FO intervention; the proportion of 4-HDHA, 13-HDHA, 17-HDHA, and 20-HDHA significantly decreased, and the proportion of 8-HDHA, 11-HDHA, and 16-HDHA significantly increased in the scWAT of normal weight individuals ($P \leq 0.030$, Table 4.4). The absolute concentration of 14-HDHA significantly increased in response to 12-week FO intervention in the scWAT of normal weight individuals ($P = 0.017$, Table 4.4).

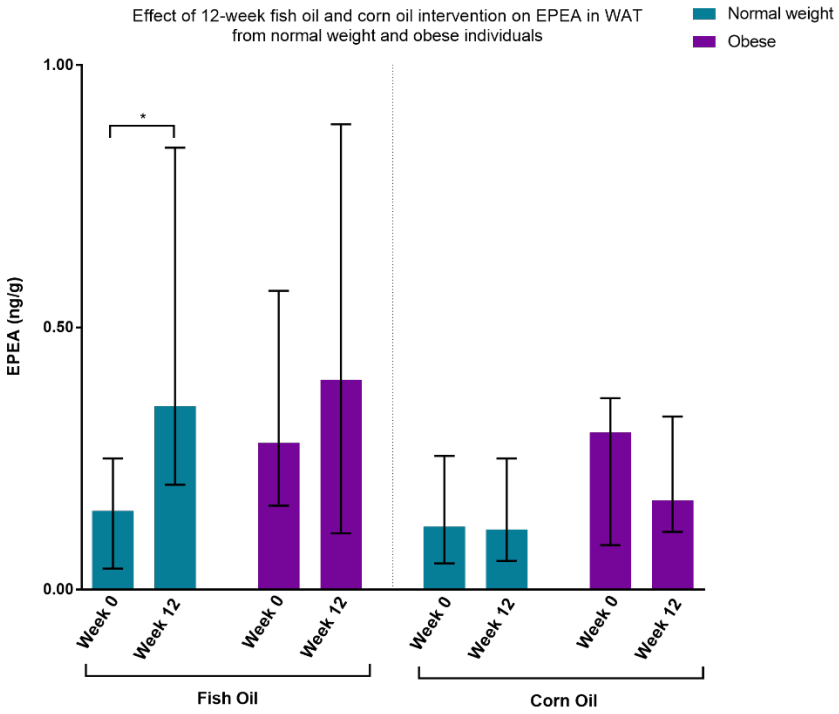
Normal Weight				
Increased				
Precursor fatty acid	ng/g	Study entry[1]	Post fish oil[1]	*P*
20:5n-3	EPEA	0.17 (0.04, 0.26)	0.35 (0.21, 0.82)	0.006
22:6n-3	14-HDHA	0.36 (0.20, 0.66)	1.00 (0.59, 2.44)	0.017
22:6n-3	DHEA	0.77 (0.42, 0.82)	0.95 (0.53, 1.57)	0.039

Decreased				
Precursor fatty acid	%	Study entry[1]	Post fish oil[1]	*P*
18:3n-3	9-HOTrE	0.0271 (0.0223, 0.0339)	0.0248 (0.0203, 0.0280)	0.011
18:2n-6	9-10-EpOME	0.3044 (0.2401, 0.3788)	0.2683 (0.2229, 0.3381)	0.003
18:2n-6	9-HODE	1.0193 (0.7542, 1.1406)	0.8100 (0.7000, 1.1035)	0.005
18:2n-6	9-HpODE	0.0178 (0.0150, 0.0212)	0.0120 (0.0090, 0.0169)	0.006
18:2n-6	12-13-diHOME	0.3299 (0.2280, 0.4856)	0.2743 (0.2392, 0.3751)	0.022
20:4n-6	20-COOH-AA	0.0648 (0.0420, 0.0850)	0.0388 (0.0304, 0.0611)	0.002
20:4n-6	LXA_4	0.0019 (0.0013, 0.0030)	0.0018 (0.0013, 0.0022)	0.016
20:4n-6	LTB_4	0.0221 (0.0175, 0.0506)	0.0213 (0.0133, 0.0288)	0.048
20:4n-6	LTD_4	0.0079 (0.0037, 0.0189)	0.0068 (0.0034, 0.0084)	0.041
20:4n-6	11-12-DHET	0.0039 (0.0029, 0.0048)	0.0025 (0.0018, 0.0030)	0.002
20:4n-6	14-15-DHET	0.0055 (0.0036, 0.0077)	0.0030 (0.0024, 0.0040)	0.004
20:4n-6	14-15-DiHETE	0.0066 (0.0035, 0.0104)	0.0056 (0.0044, 0.0067)	0.011
20:4n-6	17-18-DiHETE	0.0197 (0.0134, 0.0233)	0.0186 (0.0139, 0.0215)	0.005
20:5n-3	LTB_5	0.0015 (<0.0018, 0.0035)	0.0015 (<0.0018, 0.0023)	0.008
20:5n-3	RvE_2	0.0474 (0.0289, 0.0622)	0.0433 (0.0213, 0.0868)	0.048
22:6n-3	4-HDoHE	0.0105 (0.0047, 0.0174)	0.0078 (0.0069, 0.0110)	0.013
22:6n-3	13-HDoHE	0.0017 (0.0010, 0.0030)	0.0015 (<0.0018, 0.0030)	0.019
22:6n-3	17-HDoHE	0.0063 (0.0027, 0.0131)	0.0036 (0.0021, 0.0087)	0.005
22:6n-3	20-HDoHE	0.0130 (0.0049, 0.0210)	0.0074 (0.0058, 0.0185)	0.002
14:0	14:0-EA	0.3778 (0.2424, 0.9788)	0.2931 (0.1908, 0.4043)	0.048

Increased				
Precursor fatty acid	%	Study entry[1]	Post fish oil[1]	*P*
20:4n-6	dhk-PGE_2	0.0020 (0.0013, 0.0032)	0.0023 (<0.0018, 0.0045)	0.035
20:5n-3	15-HEPE	0.0054 (0.0028, 0.0125)	0.0058 (<0.0013, 0.0109)	0.035
22:6n-3	8-HDoHE	0.0021 (0.0010, 0.0071)	0.0023 (0.0011, 0.0034)	0.019
22:6n-3	11-HDoHE	0.0073 (0.0053, 0.0128)	0.0075 (0.0038, 0.0106)	0.022
22:6n-3	16-HDoHE	0.0062 (0.0035, 0.0127)	0.0078 (0.0037, 0.0099)	0.030

Table 4.4 Significant effects of 12-week fish oil intervention on the fatty acid metabolite composition (% and ng/g) of human scWAT from normal weight individuals.

[1] Median (± interquartile range), *P* obtained from Wilcoxon test comparing week-12 to week-0 data, Significance is deemed $P \leq 0.05$.



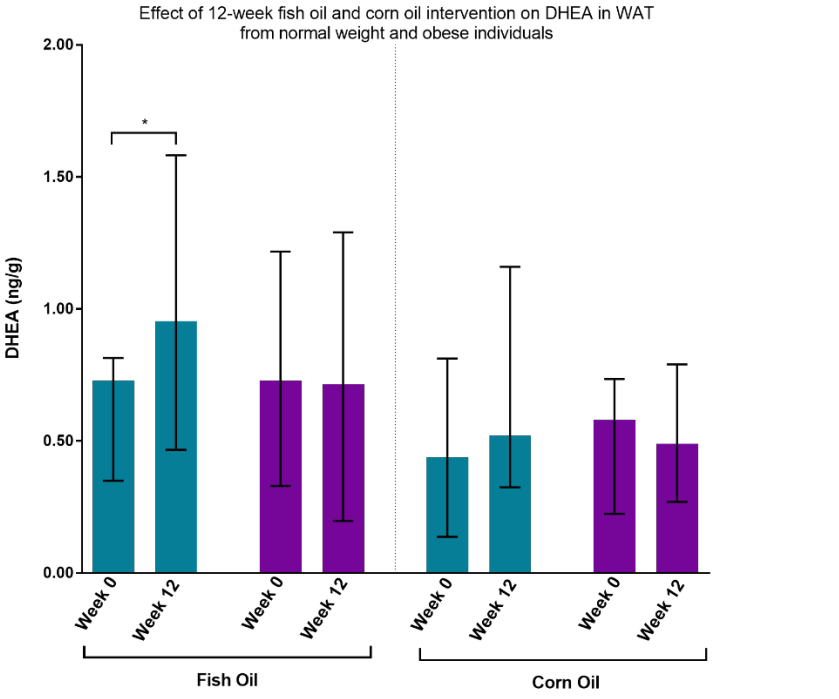


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Figure 4.8 Effect of 12-week fish oil and corn oil intervention on EPA and DHA derived endocannabinoids in normal weight and metabolically healthy obese individuals. Median (± interquartile range), *P* obtained from Wilcoxon test comparing week-12 to week-0 data, * = $P \leq 0.039$.

4.4.2.2 12-week fish oil intervention modulated omega-6 fatty acid metabolites in scWAT of MHO individuals only

12-week FO intervention in MHO individuals significantly increased the proportion of the 20:3n-6 metabolite, 15-hydroxyeicosatrienoic acid (15-HETrE), and the EPA (20:5n-e) metabolite LTB_5 ($P \leq 0.036$, Table 4.5), and decreased the proportion and absolute concentrations of the AA metabolite, 12-hydroxyeicosatetraenoic acid (12-HETE) ($P \leq 0.047$, Table 4.5) and the AA containing endocannabinoid, the glycerol ester, 2-arachidonoyl-glycerol (2-AG) $P = 0.006$, Table 4.5, Figure 4.9) in scWAT from MHO individuals. Although it was not deemed to be statistically significant, FO intervention also decreased the concentration of AEA by 55% (P 0.088, Table 4.5).

Obese				
Precursor fatty acid	ng/g	Study entry[1]	Post fish oil[1]	*P*
20:4n-6	12-HETE	3.68 (0.48, 8.02)	1.01 (0.25, 2.17)	0.028
20:4n-6	2-AG	1364.32 (721.95, 1933.46)	620.78 (265.36, 1128.07)	0.006
20:4n-6	AEA	2.21 (1.60, 2.98)	0.99 (0.31, 2.62)	0.088

Precursor fatty acid	%	Study entry[1]	Post fish oil[1]	*P*
20:3n-6	15-HETrE	0.0194 (0.0139, 0.0346)	0.0216 (0.0109, 0.0317)	0.036
20:4n-6	12-HETE	0.4831 (0.2703, 1.7955)	0.2146 (0.0975, 0.1081)	0.047
20:5n-3	LTB_5	0.0013 (<0.0016, 0.0038)	0.0015 (<0.0019. 0.0048)	0.012

Table 4.5 Significant effects of 12-week fish oil intervention on the fatty acid metabolite composition (% and ng/g) of human scWAT from metabolically healthy obese individuals.

[1] Median (± interquartile range), *P* obtained from Wilcoxon test comparing week-12 to week-0 data. Significance is deemed $P \leq 0.05$.

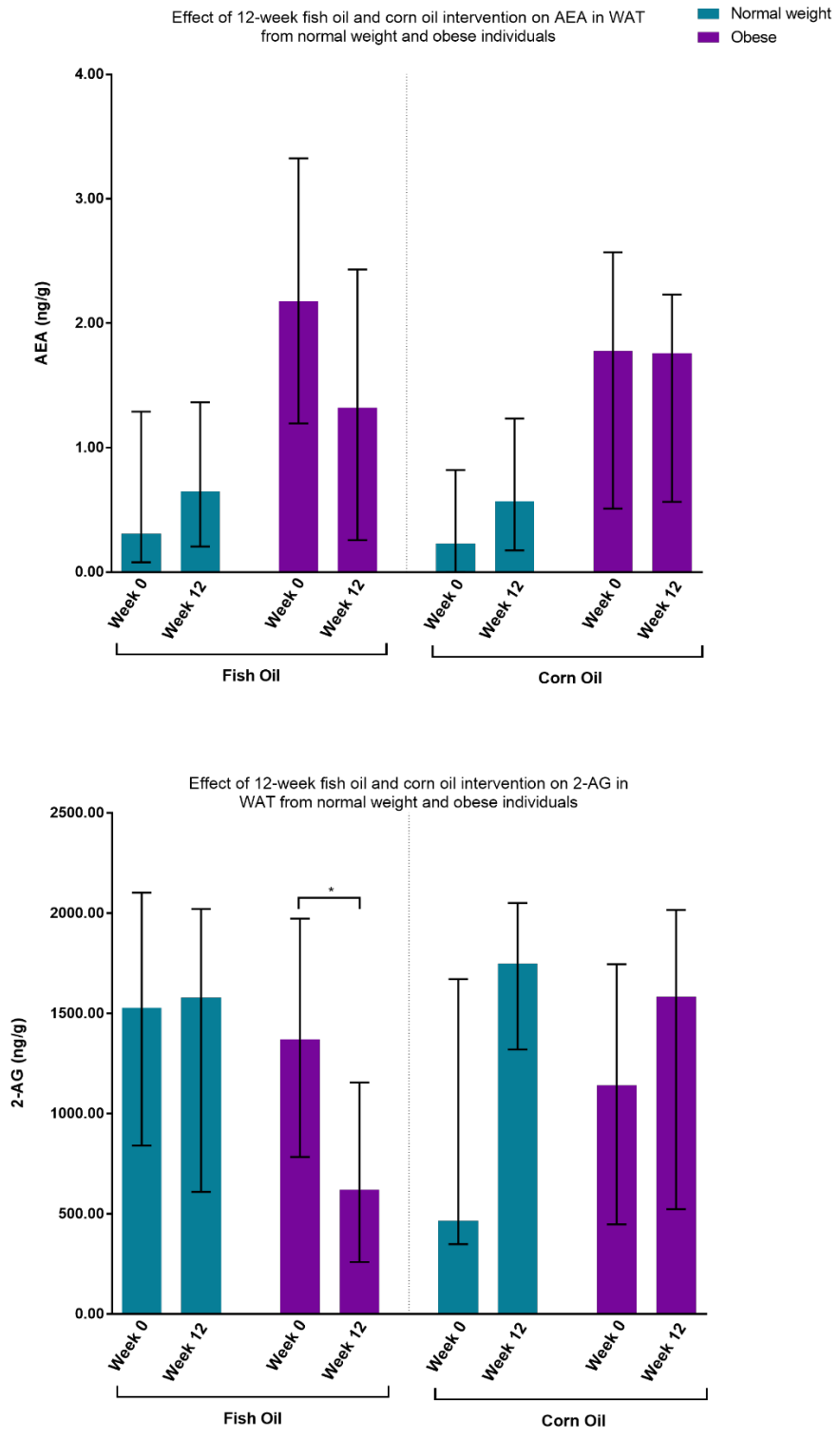


Figure 4.9 Effect of 12-week fish oil and corn oil intervention on AA derived endocannabinoids in normal weight (n= 36) and metabolically healthy obese individuals (n = 37).

Median (± interquartile range), *P* obtained from Wilcoxon test comparing week-12 to week-0 data, * = *P* = 0.006.

4.4.2.3 12-week corn oil intervention has minimal effects on scWAT fatty acid metabolite composition in normal weight and MHO individuals

Dietary supplementation with CO was used as a comparator oil for FO. In brief, CO is high in 18:2n-6 (LA) and contains no EPA + DHA; the full composition is reported in Chapter 2, Table 2.1. The change in the % and ng/g scWAT of all FA metabolites in response to 12 week CO intervention is presented in Appendix G and Appendix H respectively, with those changes that were significant displayed in Table 4.6.

Intervention with CO for 12 weeks resulted in a significant decrease in the proportion of the AA metabolite, 8-iso-$PGF_{2\alpha}$ in normal weight individuals, and of the EPA metabolite, 14,15-DiHETE in MHO individuals ($P \leq 0.048$, Table 4.6). 12-week CO intervention significantly increased the proportion of the AA metabolite, dihydro-keto-PGE_2 (dhk-PGE_2) ($P = 0.050$, Table 4.6).

There was no significant effect of CO intervention on the absolute concentrations of any FA metabolite in scWAT of normal weight individuals (Appendix H).

Normal Weight				
Precursor fatty acid	%	Study entry[1]	Post corn oil[1]	P^2
20:4n-6	8-iso-$PGF2_a$	0.0978 (0.0239, 0.1737)	0.0860 (0.1139, 1.0917)	0.008
20:4n-6	dhk-PGE_2	0.011 (0.0048, 0.0194)	0.0194 (0.0106, 0.0361)	0.050

Obese				
Precursor fatty acid	%	Study entry[1]	Post corn oil[1]	P^2
20:4n-6	14,15-DiHETE	0.0198 (0.0152, 0.0270)	0.0112 (0.0058, 0.0233)	0.048

Table 4.6 Significant effects of 12-week corn oil intervention on the fatty acid metabolite composition of human scWAT from normal weight (n= 36) and metabolically healthy obese individuals (n= 37).

[1] Median (25th, 75th percentile).

P value obtained from Wilcoxon test comparing week-12 to week-0 data.

Significance is deemed $P \leq 0.05$.

4.5 Discussion and conclusion

The current study successfully identified 111 and quantified 23 FA metabolites including those derived from LA (18:2n-6), ALA (18:3n-3), dihomo-gamma linoleic acid (DGLA) (20:3n-6), AA (20:4n-6), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3) in human scWAT (Table 4.2). Data from the current study indicates i) a significantly differing FA metabolite profile in MHO individuals in comparison to normal weight individuals; ii) significant effects of FO intervention on the levels of many of these metabolites, particularly those derived from AA, EPA and DHA; and iii) scWAT from MHO individuals is less sensitive to the effects of FO than that from normal weight individuals.

Overall, at study entry, scWAT from MHO individuals had decreased concentrations of specialised pro-resolving mediators (SPMs) and increased concentrations of ECs. The FA metabolites dysregulated in obesity are therefore involved in both pro- and anti-inflammatory signalling and the pattern seen is consistent with inflammatory events observed during enhanced WAT expansion but may also be indicative of dysregulated self-resolution of inflammation within the tissue (Turcotte et al., 2015, Scher and Pillinger, 2009). The use of CO as a comparative oil is justified by the minimal effects 12-week intervention posed on AA and EPA derived metabolites in both normal weight and MHO individuals, with no changes to LA derived metabolites.

4.5.1 FA metabolites associated with adipose expansion in obesity

The altered regulation of FA metabolite signalling observed in scWAT from MHO individuals is consistent with enhanced scWAT expansion which is part of 'normal' adipose function; however, expansion beyond the 'normal' capacity results in inflammation, indicated by the presence of pro-inflammatory stress signals including FA metabolites (Figure 4.10). Both classically described 'pro'- and 'anti'- inflammatory FA metabolite signals are likely to be required for proper scWAT function and maintenance, and energy homeostasis. In a state of homeostasis (as seen in the 'normal weight' state) there is equilibrium between these 'pro' and 'anti'-inflammatory signals.

During the onset of obesity, the tissue responds to nutritional surplus and lipid accumulates. To accommodate the increased lipid mass, adipocytes undergo hypertrophy, increasing in size, and hyperplasia, in which new adipocytes differentiate, and the surrounding microenvironment undergoes remodelling (Drolet et al., 2008, Jo et al., 2009). The ECs, AEA (20:4-EA) and EPEA (20:5-EA) are capable of promoting adipocyte differentiation and lipid accumulation (Balvers et al., 2010) and higher concentrations of these were observed in MHO individuals which would be consistent with increased adipose expansion.

There are few reports of dysregulated EC concentrations in the scWAT specifically in humans, with previous studies reporting predominantly increased plasma AA-containing ECs in obese individuals (Engeli et al., 2005, Côté et al., 2007, Bluher et al., 2006). However, Annuzzi *et al.* do report increased AEA in WAT of obese diabetic humans along with increased concentrations of the ethanolamides of 18:1n-9 and 16:1n-7 (Annuzzi et al., 2010). The current study reports similar findings of increased concentrations of AEA in scWAT in obesity but to my knowledge, there are no reports of increased EPEA in scWAT from obese subjects regardless of their metabolic health status. Higher levels of this mediator may be due to increased EPA availability as substrate for synthesis of EPEA in scWAT in obesity (see Chapter 3, section 3.4.2).

The ECs AEA and EPEA also have a role in anti-inflammatory signalling and suppress TNFα, IFNγ, MCP-1 and IL-6 secretion *in vivo* (Balvers et al., 2010). This may also be indicative of 'normal adipose expansion' in these MHO individuals; however, If obesogenic conditions persist and there is sustained demand to store surplus lipids, this demand may exceed the storage capacity leading to a state of severe obesity accompanied by WAT dysfunction which is known to be accompanied by inflammation (Figure 4.10). In this state, the WAT is no longer sensitive to FAs, glucose or insulin; the control of adipogenic and lipogenic signalling is lost, and inflammatory signalling is dysregulated resulting in loss of energy homeostasis and 'normal' WAT function (Figure 4.10).

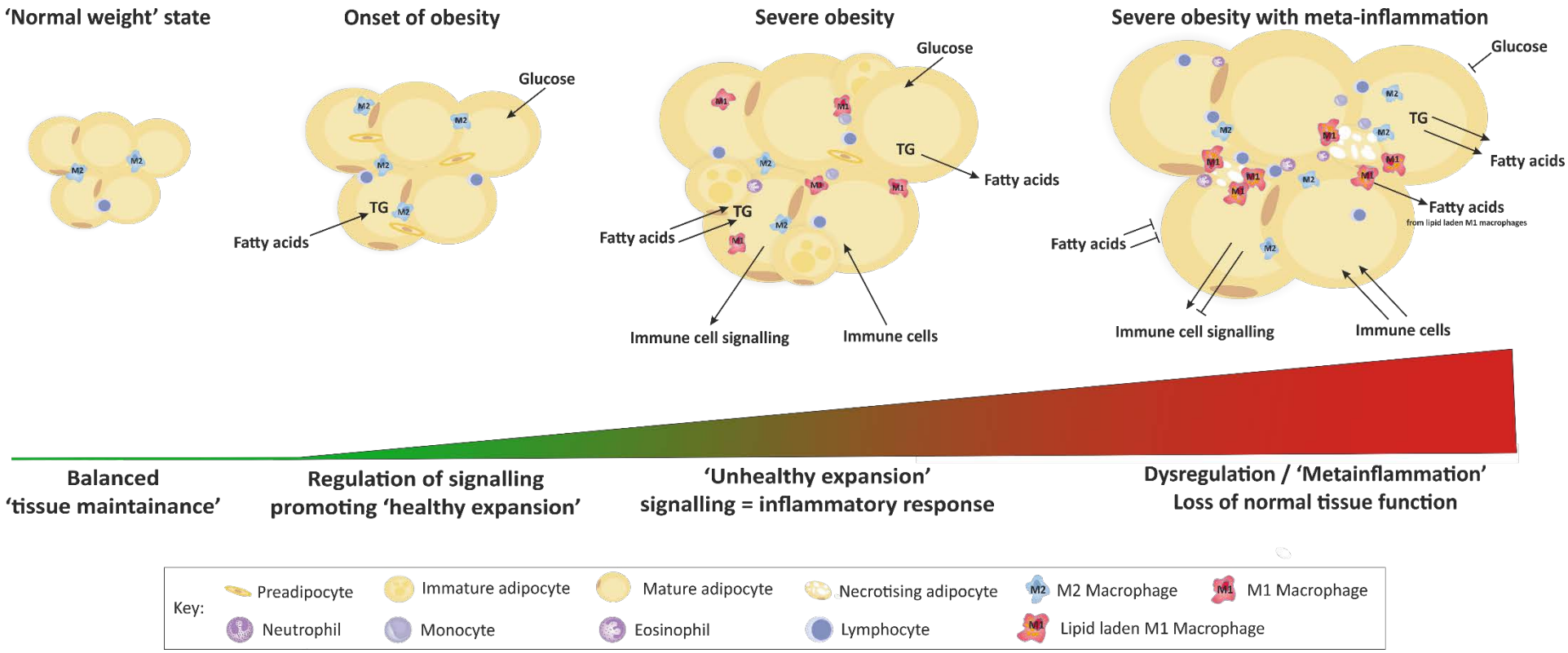


Figure 4.10 Regulation of fatty acid metabolite signalling during adipose tissue expansion and obesity related adipose tissue inflammation.

4.5.2 FA metabolites associated with the resolution of inflammation in obesity

Resolvins (Rvs) have anti-inflammatory actions including inhibition of immune cell infiltration, pro-inflammatory cytokine secretion and IL-1 induced NF-κB activation (Schwab et al., 2007, Croasdell et al., 2016, Claria et al., 2012, Serhan, 2014). The current study reports decreased concentrations of RvD_2 along with a range of hydroxy-DHA metabolites which are precursors to Rvs, protectins (PDs) and maresins (MaRs) (Serhan et al., 2015) in the WAT of MHO individuals.

HDHAs have been reported to have anti-angiogenic actions, to inhibit platelet aggregation, to inhibit PMN infiltration, and to promote phagocytosis of dying cells by macrophages (Sapieha et al., 2011, Yeung et al., 2017, Serhan et al., 2009). 17-HDHA is of particular interest as the precursor to RvD_1 and has been reported to be decreased in WAT in murine obesity (Neuhofer et al., 2013, Crouch et al., 2018). However, the specific effects of the other HDHAs reported in the current study, along with their concentrations in human obesity are under investigated and their physiological impact not understood. Therefore, reports of decreased concentrations of these mediators in scWAT in human obesity in the current study are, to my knowledge, novel.

Decreased proportions of these FA metabolites may be consistent with enhanced adipose expansion occuring in obesity; down regulation of such metabolites that inhibit signalling promoting remodelling and angiogenesis would be consistent with this scenario. A decrease in the proportion of DHA derived metabolites may also suggest decreased availability of DHA in the tissue as substrate; however, FA data from GC analysis reported in Chapter 3, section 3.4.1, indicates MHO individuals have a higher proportion of EPA and no change in DHA proportion suggesting the metabolic pathway of EPA and DHA for the synthesis of FA metabolites may be altered in obesity.

There are limited reports of SPMs in human adipose (Claria et al., 2013, Titos et al., 2016). A single study using human visceral WAT (vWAT) reported increased RvD1, RvD6, RvE2, MaR1, and PD1, and decreased RvD2 and RvD3 in obese individuals with normal blood triglycerides and total cholesterol, with glucose levels just above the normal range (114mg/dL) and no diagnosis of inflammatory bowel conditions or cancer; however, no information on insulin was available to suggest whether insulin resistance had an influence on these data (Titos et al., 2016). The current study provides evidence of decreased RvD2 in scWAT of MHO individuals but no increased levels of SPMs and may suggest the association of obesity and adipose SPMs are depot specific.

Greater concentrations of 'pro'-inflammatory FA metabolites and lower concentrations of SPMs would result in inability to resolve obesity-related inflammation. This would result in the

dysregulation of adipose and immune cell signalling, adipose composition and structure, and ultimately adipose function (Figure 4.11) (Serhan, 2014). This is concordant with the FA metabolite profile reported for scWAT in metabolically healthy obesity in the current study which suggests enhanced scWAT expansion with onset of associated inflammation and reduced self-resolution of scWAT inflammation.

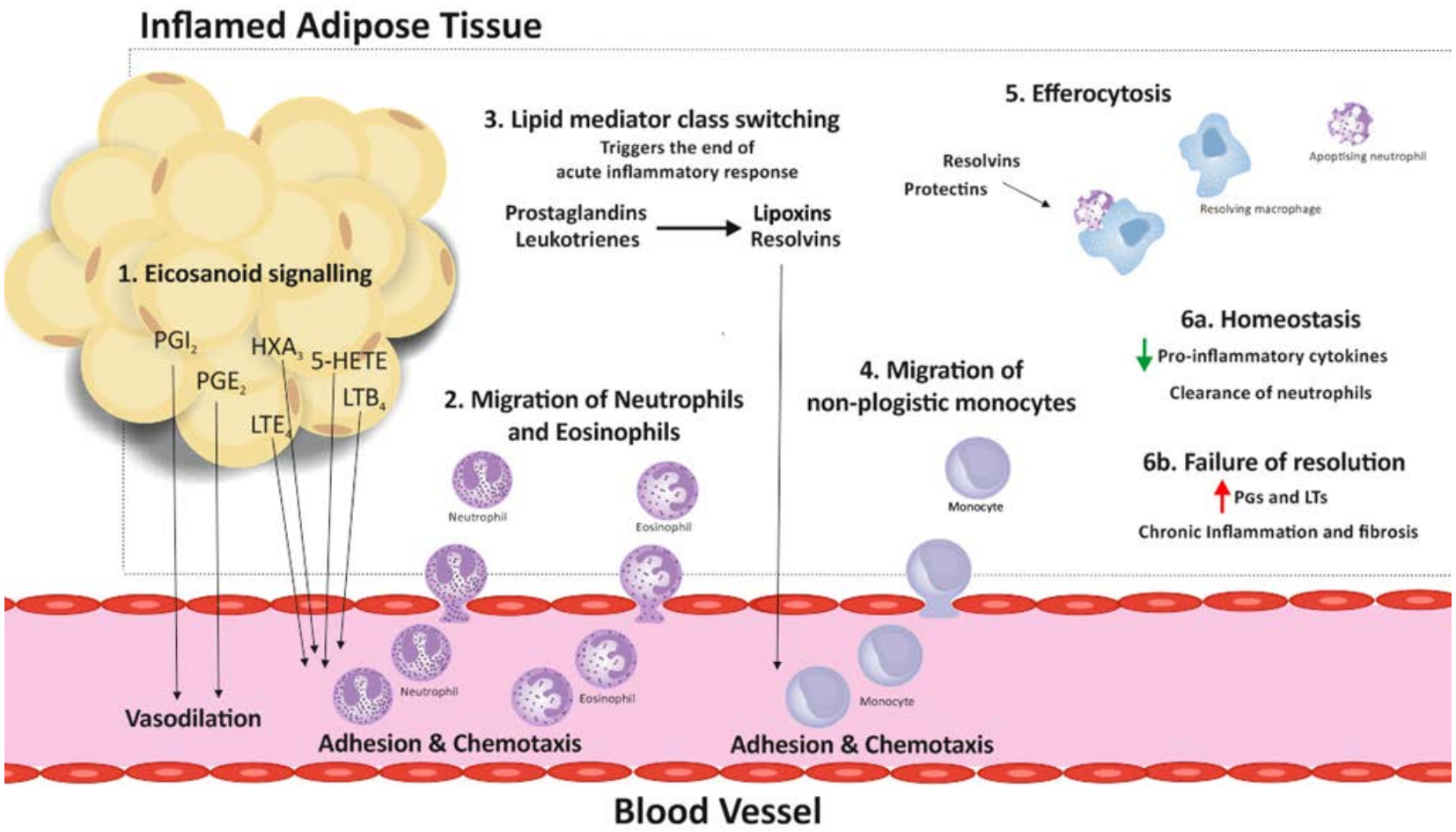


Figure 4.11 Inflammatory signalling and self-resolution of inflammation in adipose tissue.

4.5.3 Effect of 12-week fish oil intervention

Dietary supplementation with FO over 12 weeks results in significant changes in not only the FA composition of scWAT in both normal weight and MHO individuals (Chapter 3, section 3.4.2), but also in resultant FA metabolites. Daily supplementation with FO provided 1.1 g EPA (20:5n-3) and 0.8 g DHA (22:6n-3); as a result of increasing the intake of these FAs, concentrations of FA metabolites derived from and containing EPA and DHA, such as the FA ethanolamides 20:5-EA and 22:6-EA, and hydroxy-DHA metabolites, were significantly increased in scWAT from normal weight individuals.

Whole scWAT FA composition was measured in Chapter 3. Much of the lipid present would be in the form of triglycerides. Incorporation of EPA and DHA from fish oil into scWAT could be into stored triglycerides, cell membranes (mainly phospholipids) of adipocytes or other cells present in the tissue (e.g. macrophages) or both. The increased incorporation of EPA and DHA into adipose resident cell membranes, and therefore increased availability as substrate for the synthesis of pro-resolving mediators, is likely to be partly at the expense of AA (20:4n-6) and as such, FO intervention was also associated with changes in the concentration of many FA metabolites derived from and containing AA including FA ethanolamides and glycerol esters, LTs and DHETs. However, scWAT from MHO individuals appears to be less sensitive to the effects of FO on EPA and DHA derived fatty acid metabolite regulation in that these metabolites were not altered following the intervention period. Despite this, there was significant modulation of the EC system in both groups in response to FO intervention.

4.5.3.1 Effect of 12-week fish oil intervention – Modulation of the endocannabinoid system

Increased concentrations of 20:5-EA and 22:6-EA may indicate FO has a role in promoting adipose expansion and the resolution of inflammation (Balvers et al., 2010). Murine models report significantly altered ratios of n-3/n-6 derived FA metabolites in response to EPA + DHA intervention; murine WAT had increased EPA and a range of n-3 derived FA metabolites including 20:5-EA, 22:6-EA, HEPEs, 3-series PGs and TXBs, as well as increased 17-HDHA despite no significant increase in DHA, and a decrease in a range of AA derived mediators including 20:4-EA, 2-AG, EETs, and HETEs (Balvers et al., 2012).

Some other studies report specific effects on the EC system including increased 20:5-EA and 22:6-EA, and decreased 20:4-EA, and 2-AG in response to EPA + DHA intervention in murine/rat WAT (Batetta et al., 2009, Kim et al., 2015) or in human plasma (Banni et al., 2011) but reports in human scWAT are limited. To my knowledge, confirmed in a recent review of by Saleh-Ghadimi (Saleh-Ghadimi et

al., 2020), only one study reports modulation of the EC system in human scWAT by EPA + DHA. Rossmeisl *et al.* reported no change in AA-containing ECs but an increase in 20:5-EA and 22:6-EA with positive correlations to the n-3 index in the serum and scWAT of 16 type 2 diabetic individuals in response to FO intervention (receiving metformin alongside 2.8 g EPA + DHA daily for 24 weeks) (Rossmeisl et al., 2018). In contrast this, the current study provides novel evidence for significant increase in 20:5-EA and 22:6-EA in normal weight individuals only and for decreased 2-AG (with a similar effect seen for AEA) in response to FO intervention in non-diabetic obese individuals. There was an increase in EPEA observed in MHO individuals which was not to the significant extent reported in normal weight individuals but there was no change in DHEA at all in MHO individuals. The composition of the fish oil used in the current study differs from that used by Rossemeisl *et al*. in that it is richer in EPA than DHA. It may be that an oil with a higher proportion of DHA than EPA is required in obese individuals to raise the concentrations of DHEA. Data from the current study suggests MHO individuals are less sensitive to the effects of FO intervention on synthesis of ECs containing EPA and DHA, yet responsive to modulation of AA-containing ECs which to my knowledge is previously unreported and is therefore of great interest.

The proportional increase in EPA and DHA was similar in normal weight and MHO individuals; however proportions of AA were not changed following intervention, indicating MHO individuals still had higher proportions of AA following intervention in comparison to normal weight individuals (Chapter 3). Therefore, it may also be that MHO individuals require higher doses of EPA + DHA to alter the n-3/n-6 ratio of adipose resident cell membranes. Rossmeisl *et al.* report significant increases in EPEA and DHEA in MHO diabetic males following a higher dose of EPA + DHA, which a greater ratio of DHA: EPA for a longer period (2.8 g EPA + DHA daily for 24 weeks) which may contribute to the significant effects seen. Further to this, the effects of metformin on lipid handling and EC synthesis would be important to consider when comparing the data between studies.

4.5.3.2 Effect of 12-week fish oil intervention – Modulation of fatty acid metabolite pathway enzymes

The mechanisms behind differences in response to FO in obesity may involve differential expression or activity of enzymes involved in the synthesis or breakdown of these metabolites, or of proteins involved in transport and activation of FAs required for EC synthesis. Differences in these at study entry in obesity may have an impact on response to FO intervention, and differential modulation of these by FO intervention itself may also contribute to differences in response. Coinciding with this, differences in cell heterogeneity of scWAT between normal weight and MHO individuals may also

contribute to the differential metabolism of supplemental FAs and subsequent synthesis of FA metabolites.

Previous studies report significant differences in the expression of genes encoding COX and LOX in obesity (Garcia-Alonso et al., 2016, Chan et al., 2016) and type 2 diabetes (Heemskerk et al., 2015, Lieb et al., 2014), as well as differences in the concentrations of resultant mediators such as PGs, HETEs, LTs, and HEPEs (Wang et al., 2017, Pickens et al., 2017, Pickens et al., 2014). There is also evidence for the dysregulation of the expression of enzymes involved in the synthesis and hydrolysis of ECs as well as the EC receptors in obesity. Increased expression of genes encoding N-acyl phosphatidyl ethanolamide phospholipase D (NAPE-PLD) and MGLL, and decreased expression of genes encoding cannabinoid receptor 1 (CB1, CNR1) and FA amide hydrolase (FAAH) have been reported in obesity (Bluher et al., 2006, Engeli et al., 2005). The expression of CB1 and FAAH was further reported to negatively correlate to circulating concentrations of ECs (Bluher et al., 2006, Engeli et al., 2005), and the expression of cannabinoid receptor 2, NAPE-PLD, and FAAH mRNA is reported to be downregulated by EPA in MC3T3-E1 osteoblast-like cells, and FAAH by EPA+DHA intervention in murine WAT (Hutchins et al., 2011, Rossmeisl et al., 2018).

The expression of such genes will be examined by RNA-Sequencing and qRT-PCR as reported in Chapter 5 and Chapter 6 and will be key in further understanding the mechanisms by which the differences in FA metabolite profile currently reported in obesity, and the modulatory effects of FO intervention, may be occurring.

4.5.4 Conclusion

In conclusion, the current study reports both confirmatory evidence for the dysregulation of classic FA metabolites in scWAT, and novel evidence for the dysregulation of ECs and DHA metabolites in scWAT in human obesity. This dysregulation suggests enhanced adipose expansion with associated onset of inflammation in scWAT in metabolically healthy obesity but also reduced capacity for self-resolution of inflammation. Current data provides evidence for the modulation of n-3 LC PUFA and AA derived mediators by FO, and suggest there may be effects on the regulation of adipose expansion and remodelling, the EC system, and infiltration of immune cells and resulting inflammation as depicted in Figure 4.12. Further analyses are to be reported in Chapter 8 in which the morphology, state of fibrosis, and infiltration of macrophages in the scWAT will be investigated with the aim to provide more conclusive evidence for this.

The current study further reports differential effects of FO intervention on scWAT between normal weight and MHO individuals and that scWAT of MHO individuals is less sensitive to modulation of n-

3 LC PUFA metabolites by FO. This evidence in human scWAT is novel to my knowledge, and overall data from the current study indicate not only a differing FA metabolite profile in scWAT in obesity, but also dysregulation in the metabolism of EPA + DHA from FO for FA metabolite synthesis in scWAT in metabolically healthy obesity.

The mechanisms of action and physiological relevance of the results report herein are discussed theoretically but would be worthy of future investigation. Whole transcriptome profiling of scWAT from normal weight and obese individuals will provide insight into differences in metabolically healthy obesity and in response to FO intervention at a transcriptional level. In addition to this, further analysis of gene expression by qRT-PCR will provide an insight into the regulation of key genes transcribing enzymes involved in lipid metabolism and the synthesis of FA metabolites, receptors targeted by such signalling molecules, and proteins involved in tissue remodelling. These investigations are reported in Chapter 6 and Chapter 7.

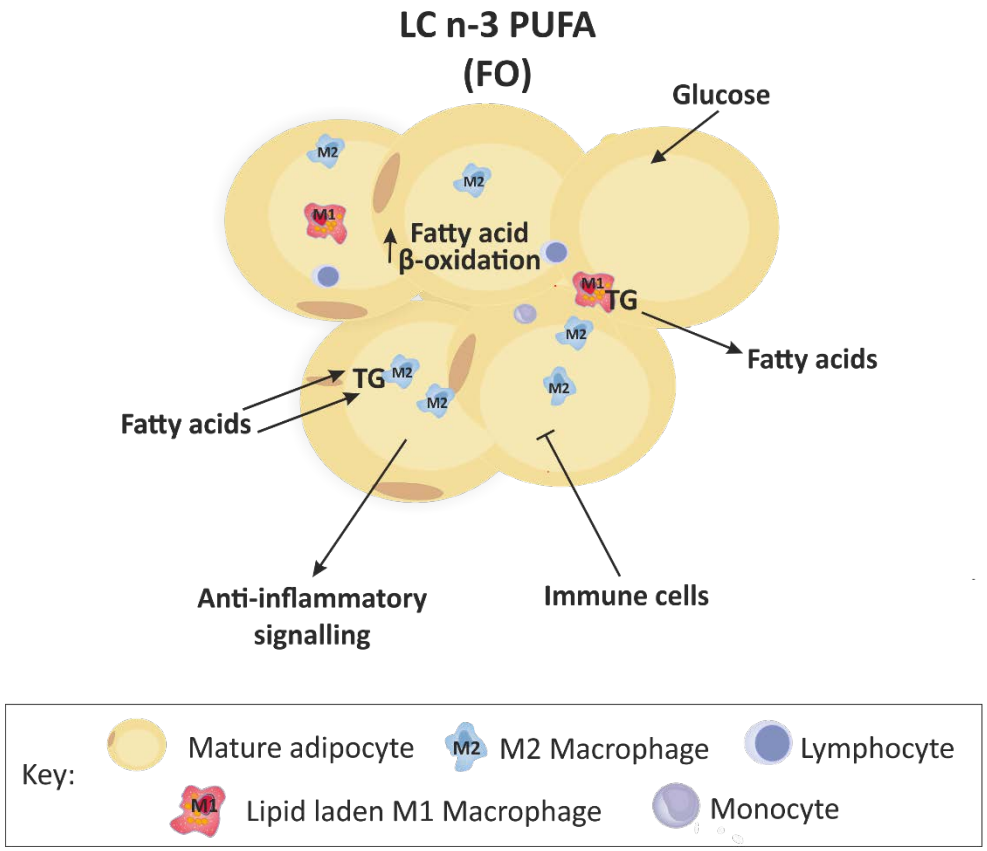


Figure 4.12 Effects of fish oil intervention on the regulation of fatty acid metabolite signalling and lipid metabolism in adipose tissue.

Chapter 5 Expression and activity of cyclooxygenase-2 and fatty acid amide hydrolase in human subcutaneous white adipose tissue and in response to 12-week fish oil intervention in normal weight and metabolically healthy obese individuals

5.1 Introduction

Fatty acid (FA) metabolites (also termed lipid mediators) have a role in regulating adipose homeostasis and inflammation. These include several functions including tissue expansion (Fain et al., 2001, Balvers et al., 2010, Hallenborg et al., 2010, Yamada et al., 2014, Chan et al., 2019), immune cell recruitment (Bittleman and Casale, 1995, Wright et al., 2010, Mothe-Satney et al., 2012, Chan et al., 2019), lipolysis (Fain et al., 2001), and lipid accumulation (Balvers et al., 2010, Chan et al., 2019) in the tissue.

A number of FA metabolites that were observed to be altered in metabolically healthy obese (MHO) individuals at study entry (chapter 4), are metabolised from their parent FA by cyclooxygenase (COX). This enzyme is involved in the synthesis of hydroxyoctadecadienoic acids (HODEs), prostaglandins (PGs), hydroxyeicosapentaenoic acids (HEPEs) which can be further metabolised to resolvins, and hydroxydocosahexaenoic acids (HDHAs) which can be further metabolised to resolvins and protectins (Masoodi et al., 2014, Powell and Rokach, 2015, Calder, 2017). Metabolism of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into their respective metabolites has been described in detail in chapter 1 and figure 1.8.

These metabolites are implicated in both the progression and resolution of inflammation systemically and within the white adipose tissue (WAT). An increase in concentrations of these metabolites in obesity may occur due to increased availability of the precursor FA, altered expression of COX or altered regulation of COX activity. The current study reports a higher proportion of AA in the scWAT of metabolically healthy obese (MHO) individuals (Chapter 3), and an increase in the expression of COX-2 alongside increased concentration of COX-2 derived metabolites such as PGE_2 has been reported in the AT obese humans (Garcia-Alonso et al., 2016, Chan et al., 2016). In the current study scWAT from MHO individuals had altered concentrations of several COX

metabolites including lower concentrations of 9-oxo-ODE, 9-HpODE, 13-oxo-ODE, and 17-HDHA, and increased $PGF_{1\alpha}$ (Chapter 4).

In addition, the concentrations of the FA ethanolamides arachidonoyl ethanolamide (AEA) and eicosapentaenoyl ethanolamide (EPEA) were higher in MHO individuals at study entry (Chapter 4). These metabolites are synthesised by N-acyl phosphatidyl ethanolamine phospholipase-D (NAPE-PLD) and are degraded by fatty acid amide hydrolase (FAAH). Lower scWAT and VWAT expression of FAAH is reported in obese male and females (Bluher et al., 2006). Increased concentrations of AA and EPEA available as substrates for AEA and EPEA, and reduced degradation of these may result in the increased concentrations observed in MHO individuals. The activity of FAAH may also influence the concentrations of AEA and EPEA in the scWAT and contribute to the differences observed in HMO individuals; however, evidence for this is limited and inconclusive. FAAH activity is observed to be increased in adipocytes from obese rats (Cable et al., 2014), and to be positively correlated with BMI in healthy normal weight, overweight and obese individuals (considered as a whole cohort) (Cable et al., 2011). However it has also been reported not to correlate with BMI in obese humans or to be significantly different between healthy obese individuals and those with either MetS or type-2 diabetes (T2DM) (Cable et al., 2014). Investigation of activity in the current study cohort as a whole and split by BMI group in addition to correlation with scWAT endocannabinoids (ECs) will add new data to this under-investigated area,

In addition to differences in FA metabolites at study entry, how EPA and DHA from chronic fish oil (FO) intervention were handled to form these also differed between normal weight and MHO individuals. In particular, the increased concentration of EPA and DHA ethanolamides, particularly DHEA, was only observed in normal weight individuals in the current study despite a similar increase in precursor FAs in both subgroups (chapter 4). EPEA and DHEA have been shown to have anti-inflammatory actions in macrophages and adipocytes *in vitro*, reducing concentrations of LPS induced monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (Balvers et al., 2010). These actions would be beneficial in obese individuals where higher concentrations of IL-6 and increased immune cell infiltration into the scWAT are often observed (Lafontan and Langin, 2009).

Lack of a significant increase in EPEA and DHEA concentrations following FO intervention I MHO individuals may be due to the retention of a higher concentration of AA vs EPA+DHA following intervention and competition between these substrates for enzymes, or to differences in the expression of genes/proteins involved in metabolism of n-3 PUFA or the metabolites themselves.

Greater expression of FAAH mRNA has been previously reported in AT from obese humans (Pagano et al., 2007, Murdolo et al., 2007, Cable et al., 2011). This may suggest that the differences in the concentration of FA ethanolamides in response to FO intervention between the two body weight groups may be due to the greater expression of FAAH and more rapid degradation of these metabolites in those with MHO. In addition to the expression of FAAH, the activity of this enzyme may also be altered in response to FO intervention, further contributing to the increased breakdown of these FA ethanolamides.

Current evidence for changes in the regulation of lipid metabolites in obesity and response to FO intervention is limited for human scWAT, and the mechanisms behind any alterations are underexplored. Therefore, the following aims were created in order to assess the contribution of expression and activity of two key enzymes involved in the synthesis and degradation of lipid metabolites observed to be altered in Chapter 4.

5.2 Aims

The aim of the research described in this chapter was to:

- Investigate whether adipose tissue from MHO individuals had greater expression and activity of COX-2 and FAAH in comparison to that from normal weight individuals.
- Investigate whether the expression and activity of these enzymes correlated to concentrations of resultant lipid metabolites
- Investigate whether FO intervention alters the expression and activity of COX-2 and FAAH in scWAT
- Investigate whether the response of scWAT to FO intervention is altered in obese individuals.

5.3 Methods

5.3.1 Experimental reagents and materials

Reagents and materials used for experiments are described in **Appendix C**.

5.3.2 Sample selection

Samples were selected for enzyme activity assessment in pre- and post- intervention pairs and were matched as best as possible for sex, age, and treatment between normal weight and MHO individuals. A total of 8 complete paired samples (4 FO and 4 CO) were available from normal weight

and MHO individuals, totalling 16 paired samples (16 week-0 samples, 8 post 12-week FO samples, and 8 post 12-week CO samples), as detailed in Table 5.1.

Normal weight				Metabolically healthy obese			
Paired sample	Sex	Age	Treatment	Paired sample	Sex	Age	Treatment
1	Female	57	FO	1	Female	57	FO
2	Female	29	FO	2	Female	28	FO
3	Female	55	FO	3	Male	61	FO
4	Male	23	FO	4	Male	31	FO
5	Male	21	CO	5	Male	36	CO
6	Female	28	CO	6	Female	31	CO
7	Female	57	CO	7	Male	57	CO
8	Female	19	CO		Female	56	CO

Table 5.1 Paired week-0 and week -12 samples selected for enzyme activity assays

5 of the 8 normal weight and 5 of the 8 MHO obese paired samples were available for Western blot analysis, totalling 10 paired samples (10 week-0 samples, 4 post 12-week FO samples, and 6 post 12-week CO samples).

5.3.3 COX-2 activity assay

~ 50 mg scWAT was washed in 3 x 500 µL PBS and then homogenised on ice in 300 µL of lysis buffer (1% NP-40 in PBS + protease inhibitor cocktail (1:200)). Homogenates were centrifuged at 12,000 x g for 4 minutes (min) at 4°C and supernatant collected. This step was repeated to ensure minimal lipid contamination. Protein concentrations were checked via the bicinchoninic acid (BCA) assay read at 500 nm using the Pierce™ BCA Protein Assay Kit (Fisher Scientific, Loughborough, UK) following the manufacturer's instructions. Total COX, COX-1 and COX-2 activity were measured using the COX Activity Assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions and read in loop mode at Ex/Em 520/ 580-640nm for 30 min. Delta RFU (30 minute data – 0 minute data) data were calculated against a standard curve (the standard was provided with the kit) and adjusted to mg protein used to give activity in µU/mg.

5.3.4 FAAH activity assay

~ 10 mg scWAT was homogenised on ice in 100 µL of FAAH lysis buffer. Homogenates were centrifuged at 12,000 x g for 5 min at 4°C and supernatant collected. Protein concentrations were

checked via the bicinchoninic acid assay read at 500 nm using the Pierce™ BCA Protein Assay Kit (Fisher Scientific, Loughborough, UK) following the manufacturer's instructions. FAAH activity was measured using the FAAH Activity Assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions read in loop mode at Ex/Em 365/ 415-445nm for 60 mins. Delta RFU (20 minute data – 0 minute data) data were calculated against the standard curve (the standard was provided with the kit) and adjusted to mg protein used to give activity in µU/mg.

5.3.5 Western blotting of proteins

Protein was extracted from ~50 mg of tissue using NP-40 as described in section 5.3.2. The concentration of protein was analysed using the BCA assay as described in section 5.3.2. 15 µg of protein was diluted with Western loading buffer and adjusted to a total 35 µl volume with H_2O. The protein was denatured at 95°C for 5 min. 2.5 µL of ladder and 35 µL of diluted samples were loaded into appropriate gel wells. The gel was submerged in running buffer and an 80 V current run through until the samples and ladder reached the stacking layer of the gel. The voltage was then increased to 180 V and run for 1.5 hr at room temperature (RT). The protein was then transferred to a pre-treated membrane (in methanol and transfer buffer) via a 100 V current for 1 hr. Proteins were stained for by subsequent incubations with blocking buffer (milk powder and PBS + tween (0.05%)) for an hour at RT, primary antibody on a roller overnight at 4°C followed by 3 x washes with PBS + tween (0.05%), and incubation with secondary antibody diluted in blocking buffer for 1 hour at RT on a roller. Primary antibodies used were: anti FAAH and anti COX-2 (Abcam, Cambridge, UK), which were normalised to the housekeeping protein antibody anti-FAA (Abcam, Cambridge, UK). Secondary antibody, goat-anti-rabbit-HRP (abcam, Cambridge, UK) was washed from the membrane with PBS + tween (0.05%) and supersignal (1:1) was added for visualisation of proteins on GeneGnome XRQ (Syngene, Bangalore, India).

5.4 Statistical analysis

COX and FAAH expression and activity data were normally distributed and therefore, an independent sample t-test was performed to assess differences with BMI group as the factor. Paired sample t-tests were performed to assess the effect of 12-week FO and CO intervention on normal weight and obese individuals' data separately. These data are reported as mean $\pm$ SEM.

MHO individuals are referred to as 'obese' in all figures and tables herein.

5.5 Results

5.5.1 Study entry results

5.5.1.1 Selection of appropriate loading control (reference) protein

Both FAAH and COX-2 protein were expressed in the scWAT of both normal weight and MHO individuals. However, an appropriate loading control or 'house-keeping' protein which should exhibit adequate specific binding and stable expression across all conditions (obesity, FO and CO treatments) could not be found meaning the accurate quantification of these proteins could not be determined. Four reference proteins were trialled based upon those previously reported in the literature and by selection of housekeeping genes for qRT-PCR. GAPDH, β-Actin, hypoxanthine phosphoribosyltransferase (HPRT), and fumarylacetoacetate hydrolase (FAA) were trialled. HPRT exhibited poor binding which was inconsistent between samples and could not be resolved by increasing concentration of antibody used (Figure 5.1). β-Actin and GAPDH produced visible binding but expression was not consistent amongst all conditions (Figure 5.1). FAA (N-terminal recognition) exhibited non-specific binding and inconsistent expression amongst conditions; therefore FAA with C-terminal recognition was trialled which exhibited very poor binding (Figure 5.1).

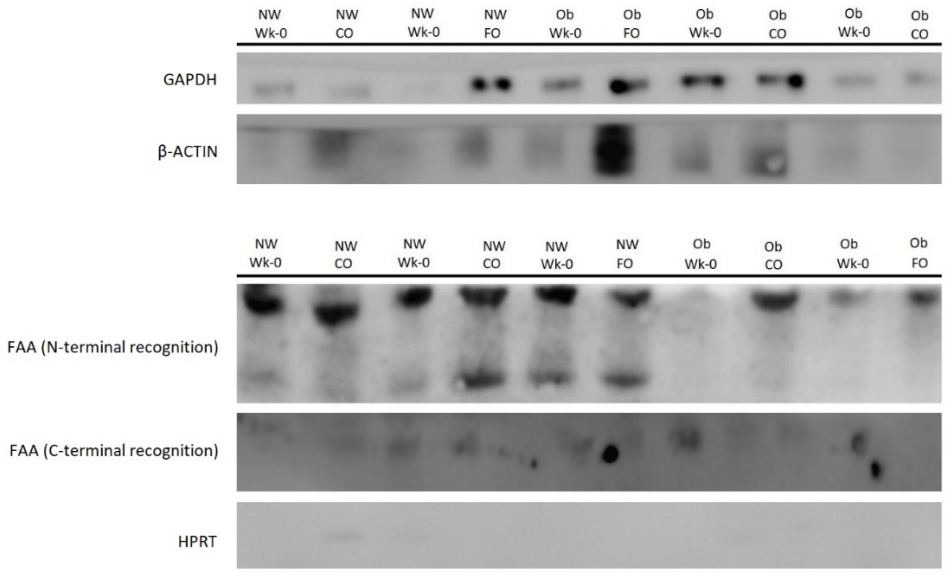


Figure 5.1 Trial of loading control proteins in human scWAT from normal weight and MHO individuals at study entry (week-0) and following fish oil or corn oil intervention (12-week)

NW, normal weight; Wk-0, week-0; CO, following 12-week corn oil intervention; FO, following 12-week FO intervention; Ob, metabolically healthy obese.

Due to lack of further potential reference proteins and limited availability of sample, no further investigation of suitable reference protein was possible. Therefore, for the purposes of this thesis, the Western blot images were quantified to give net values (artificial units, AU) representative of 10 µg of protein per sample but not controlled for by loading control. Usually, the value of the loading control is used to give a relative result that considers differences in the loading of the 10 µg of protein and stages of the immunoblotting process such as transferring, blocking, and staining in which variation could occur. This is why it is important for the loading control to exhibit good specific binding and consistent expression across samples from different conditions. Failure to validate published reference proteins in human scWAT in MHO and normal weight individuals poses the question of reproducibility between studies and appropriate use of specific reference proteins in human scWAT in obesity. This will be discussed later in this chapter.

5.5.1.1.1 COX-2 but not FAAH protein expression (net) is altered in scWAT in metabolically healthy obesity

Net expression of COX-2 was significantly greater in scWAT from MHO individuals; however there were no differences in the net expression of FAAH between normal weight and MHO individuals at study entry (week-0) (P = 0.024 and 0.495 respectively, Figure 5.2).

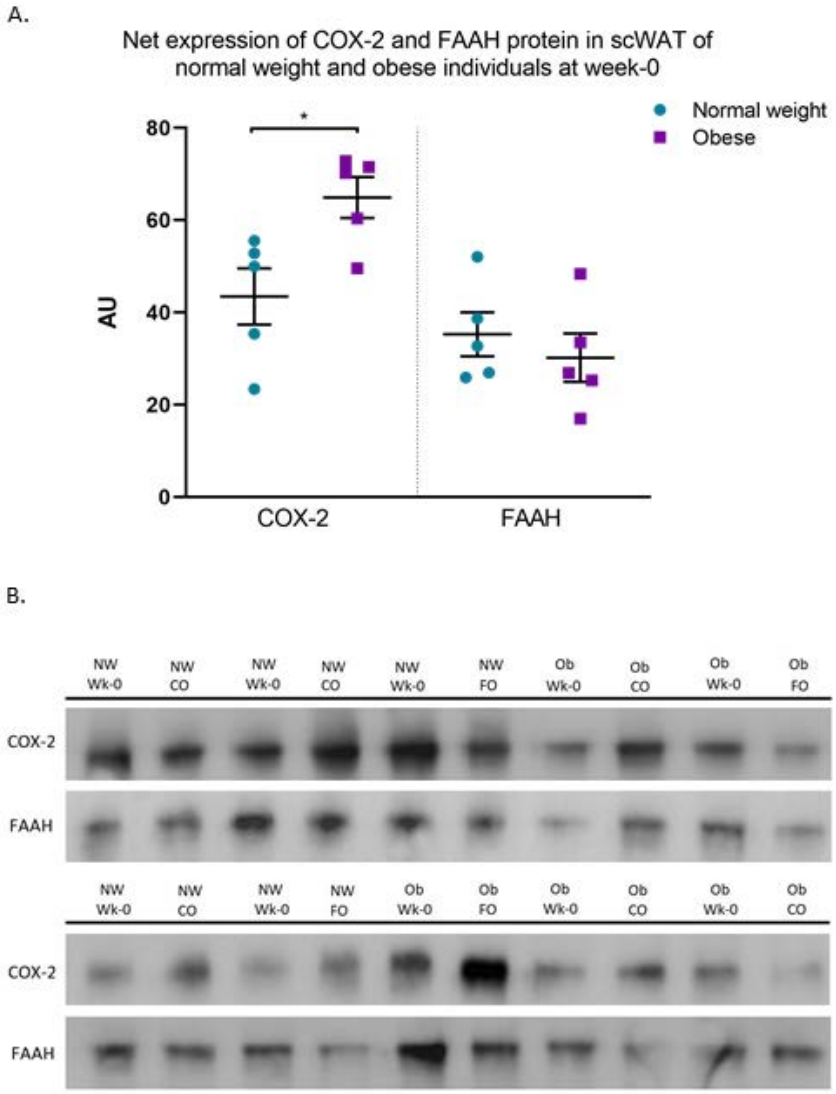


Figure 5.2 A. Net expression of COX-2 and FAAH in scWAT of normal weight (n=5) and metabolically healthy obese individuals (n-5) at study entry (week-0).

B. Western blot images.

Mean ± SEM. * *P* = 0.024.COX-2, cyclooxygenase-2; FAAH, fatty acid amide hydrolase; NW, normal weight, Wk-0, week-0; CO, following 12-week corn oil intervention; FO, following 12-week fish oil intervention; Ob, metabolically healthy obese.

5.5.1.1.2 The net expression of COX-2 and FAAH is significantly associated with scWAT lipid metabolite concentrations

A number of scWAT FA metabolites were significantly correlated with net COX-2 and net FAAH expression (P <0.05,Table 5.2). Relative concentrations of scWAT FA metabolites were more strongly associated with net FAAH expression than with net COX-2 expression and this was more evident in normal weight individuals (Table 5.2). In contrast, a number of AA derived FA metabolites were significantly associated with net expression of COX-2 but only in MHO individuals ($P \leq 0.05$, Table 5.2). This may be reflective of increased net COX-2 expression in MHO individuals and indicates dysregulation of FA metabolism with regards to FAAH associated FA metabolites in MHO individuals. Net expression of FAAH was positively correlated with the relative concentrations of EPA and DHA containing FA metabolites including RvE1 and -D2 in normal weight individuals only (P 0.020 and 0.007 respectively, Table 5.2). Significant associations between net expression of FAAH and non-EC FA metabolites was not expected as this enzyme is not known to be involved in other FA metabolite metabolism.

The absolute concentration of EPEA and 14-HDHA was positively associated with the net expression of FAAH in scWAT in MHO individuals (P = 0.037 both, Table 5.2). The absolute concentrations of AEA and DHEA were negatively correlated with net COX-2 expression (P = 0.037 both, Table 5.2).

Relative concentrations of FA metabolites correlated with:

Precursor FA	Metabolite	net COX-2 expression				net FAAH expression			
		Normal weight		Obese		Normal weight		Obese	
		[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*
AA	[2]20-COOH-AA			0.956	0.011				
	PGJ_2					0.956	0.011		
	dhk_PGE_2					0.884	0.047		
	8-HETE					0.956	0.011		
	15-HETE			-0.932	0.021				
	11,12-ETE			-0.955	0.011				
	5-oxo-ETE	0.900	0.037						
	15-oxo-ETE					0.980	0.003		
	14, 15-diHETE			0.921	0.026				
	15-HETrE					0.912	0.031		
	[2]u_LTD_4			0.0943	0.016				
	LXA_4					0.893	0.042		
	5,6-EET					0.987	0.011		
	14,15-EET					0.903	0.036		
	14, 15-DHET			0.876	0.051				
EPA	12-HEPE					0.941	0.017		
	15-HEPE					0.899	0.038		
	RvE1					0.935	0.020		
	PGD_3					0.882	0.048		
	11-dh-TXB_3					0.936	0.008		
DHA	RvD1					0.968	0.007		
	[2]14-HDHA					0.993	0.001		
	[2]16-HDHA			0.968	0.007				
	[2]17-HDHA			-0.090	0.038	0.948	0.015		

		Absolute concentrations of FA metabolites correlated with:							
		net COX-2 expression				net FAAH expression			
Precursor FA	Metabolite	Normal weight		Obese		Normal weight		Obese	
		[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*	[1]Coefficient	[1]*P*
AA	11-dh-TXB_2	-0.900	0.037						
	2-AG					-0.900	0.037		
EPA	[2]AEA			-0.009	0.037				
	[2]EPEA							0.900	0.037
DHA	DHEA			-0.900	0.037				
	14-HDHA					0.900	0.037		

Table 5.2 Association of scWAT FA metabolites and net expression of COX-2 and FAAH in normal weight and MHO individuals

[1]*P* values and correlation coefficients obtained from Spearman's correlation split by BMI group; significance defined as ≤ 0.05. [2] scWAT FA metabolite concentration significantly altered in obesity at study entry (Chapter 4). A, fatty acid; COX2, cyclooxygenase-2; FAAH, fatty acid amide hydrolase; 20-COOH-AA, 20-carboxy-arachidonic acid; PGD_3, prostaglandin-D_3; PGJ_3, prostaglandin-J_2; dhk_PGE_2, dehydro-keto-prostaglandin-E_2; 11_dh_TXB_3, 11-dehydro-thromboxane-B_3; 8-HETE, 8-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 11,12-ETE, 11,12-eicosatetraenoic acid; 5-oxo-ETE, 5-oxo- eicosatetraenoic acid; 15-oxo-ETE, 15-oxo- eicosatetraenoic acid; 14, 15-diHETE, 14,15-dihydroxyeicosatetraenoic acid; 15-HETrE, 15-Hydroxyeicosatrienoic acid; u_LTD_4, u_leukotriene-D_4; LXA_4, lipoxin-A_4; 5,6-EET, 5,6-eicosatrienoic acid; 14,15-EET, 14,15-eicosatrienoic acid; 14,15-DHET, dihydroxyeicosatrienoic acid; 12-HEPE, 12-hydroxyeicosapentaenoic acid; 15-HEPE, 15-hydroxyeicosapentaenoic acid; RvE1, resolvin-E1; RvD1, resolvin-D1; 14-HDHA, 14-hydroxydocosahexaenoic acid; 16-HDHA, 16-hydroxydocosahexaenoic acid; 17-HDHA, 17-hydroxydocosahexaenoic acid; 11-dh-TXB_2, 11-dehydro-thromboxane-B_2; a-AG, 2-arachidonoylglycerol; AEA, arachidonoyl ethanolamide; EPEA, eicosapentaenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide.

5.5.1.2 Metabolically healthy obesity is not associated with changes to COX-2 enzyme activity in scWAT

Background FAAH activity was detected via fluorometric assay but changes in activity (20 minute data – 0 minute data) could not be detected in either normal weight or MHO individuals (Figure 5.3). Background FAAH activity was significantly higher in MHO individuals in comparison to normal weight ($P = 0.010$) and was considerably lower than COX-2 activity (Figure 5.3 and Figure 5.4). Expression of FAAH protein was detectable by Western blotting so the protein is present. These assays provide substrate to the enzyme and measure the product of the enzymatic reaction. It may be that FAAH action occurs rapidly and the substrate was used up within the first minute of the incubation as the assay did detect study entry activity for both normal weight and MHO individuals. A way to test this would be to lower the concentration of FAAH protein used in the assay. Unfortunately, due to sample availability and the enzyme assay kit requiring the full volume of protein extracted, there was not enough tissue to re-extract protein from to trial at a lower concentration (which would involve using less tissue to buffer ratio).

COX-2 activity was successfully detected via fluorometric assay. There was no effect of sex or age on COX-2 activity ($P \geq 0.311$, data not shown) and therefore these factors were not controlled for in the analyses. There was no significant difference in the activity of COX-2 in scWAT in MHO individuals when compared to normal weight individuals ($P = 0.600$, Figure 5.4).

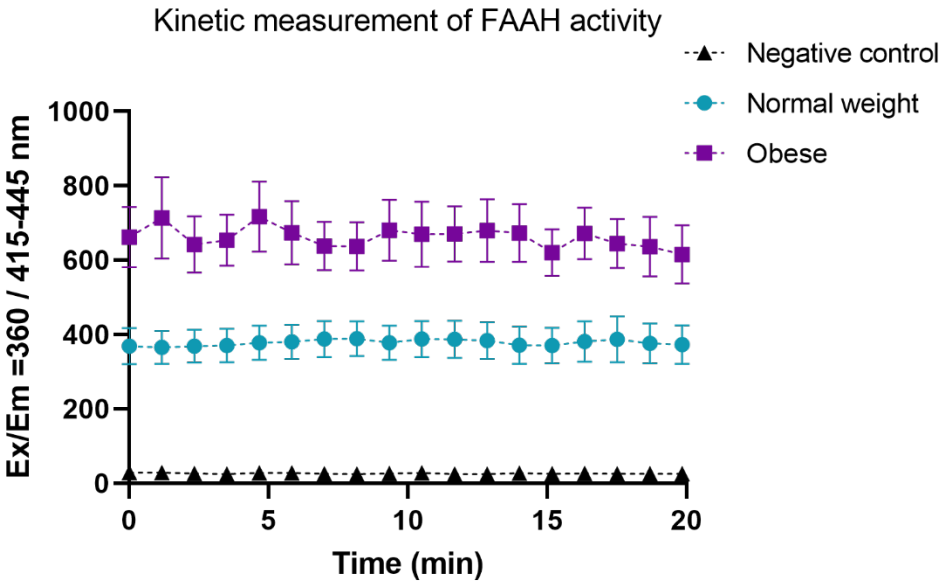


Figure 5.3 Activity of fatty acid amide hydrolase in normal weight (n-8) and metabolically healthy obese individuals (n-8) at study entry (week-0).

Mean ± SEM. FAAH, fatty acid amide hydrolase.

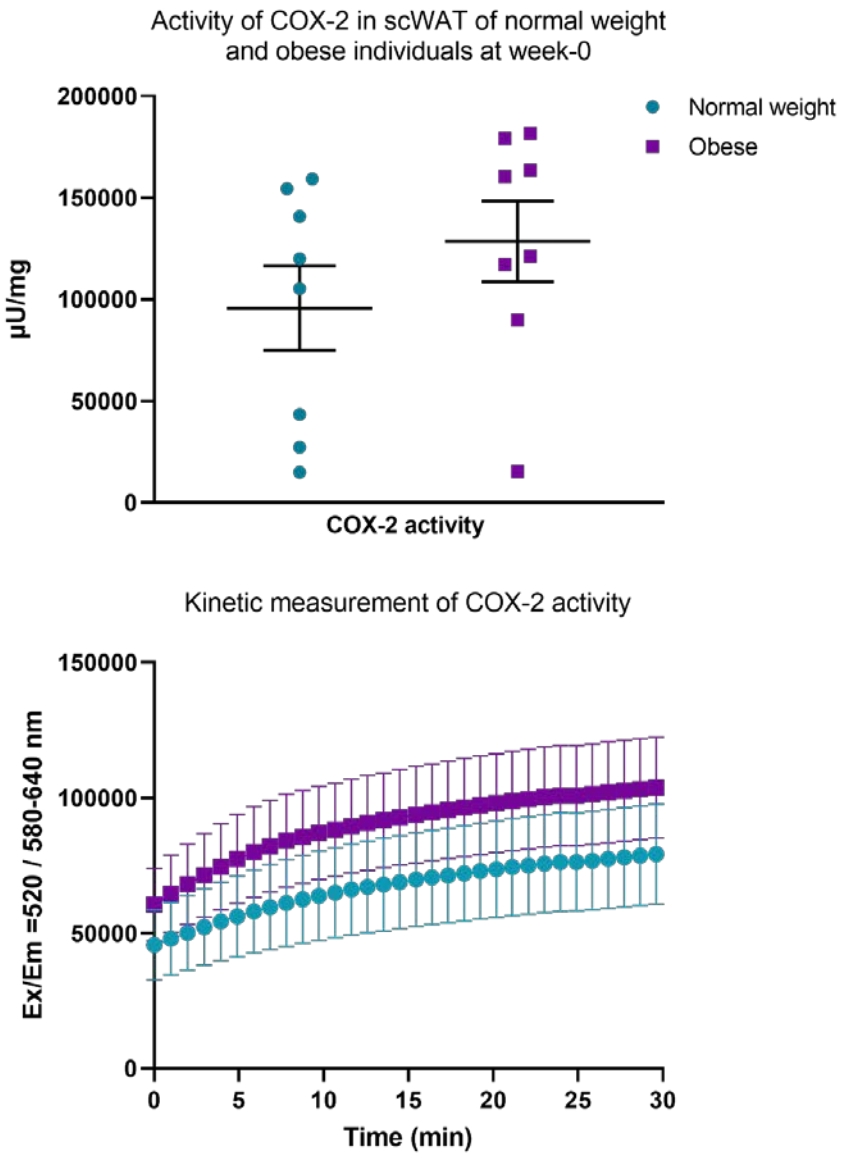


Figure 5.4 Activity of cyclooxygenase-2 in scWAT of normal weight (n=8) and metabolically healthy obese individuals (n=8) at study entry (week-0).

Mean ± SEM. COX-2, cyclooxygenase-2.

5.5.1.2.1 scWAT COX-2 activity is significantly correlated with a range of n-6 and n-3 fatty acid metabolites

COX-2 activity was not significantly correlated with COX-2 protein expression (P = 0.791, data not shown).

COX-2 activity was significantly correlated with a range of bioactive lipid metabolites synthesised from n-3 and n-6 FAs. COX-2 activity was negatively correlated with the relative concentration of COX mediated AA metabolite dihydro-keto-prostaglandin F2-alpha (dhk-PGF2α), the DHA metabolite, resolvin-D2 (RvD2), and the LOX generated DHA metabolites 16-HDHA and 20-HDHA ($P \leq 0.05$, Table 5.3). However, when the group was split by BMI, COX-2 activity was positively correlated with the COX mediated AA metabolite 8-iso-PGF2α, the LOX mediated alpha-linolenic acid (ALA) metabolite 13-HOTrE and the LOX mediated linoleic acid (LA) metabolite 9-HODE in normal weight individuals only ($P \leq 0.05$, Table 5.3). There were no significant correlations between COX-2 activity and any FA metabolite in scWAT from MHO individuals.

		All		Normal weight	
Precursor FA	Metabolite	Coefficient	*P*	Coefficient	*P*
AA	8-iso-PGF2α			0.886	0.019
	dhk-PGF2α	-0.540	0.046		
LA	9-HODE			0.829	0.042
ALA	13-HOTrE			0.886	0.019
DHA	16-HDHA	-0.535	0.049		
	20-HDHA	-0.563	0.036		
	RvD2	-0.543	0.045		

Table 5.3 Correlations between COX-2 activity and fatty acid metabolites in scWAT at study entry (week-0).

P obtained using Spearman's correlation between COX-2 activity and % fatty acid metabolite data at week-0.

5.5.2 Post intervention results

5.5.2.1.1 12-week fish oil intervention significantly downregulated net expression of FAAH in MHO individuals

The net expression of FAAH significantly decreased in scWAT from MHO individuals in response to 12-week FO intervention ($P = 0.042$, Figure 5.5). A similar directional change was observed in normal weight individuals, but this was not statistically significant ($P = 0.186$). Figure 5.5 depicts a visual increase in the net expression of COX-2 in both normal weight and MHO individuals in response to 12-week FO intervention; however this was not statistically significant ($P = 0.166$ and 0.244 respectively).

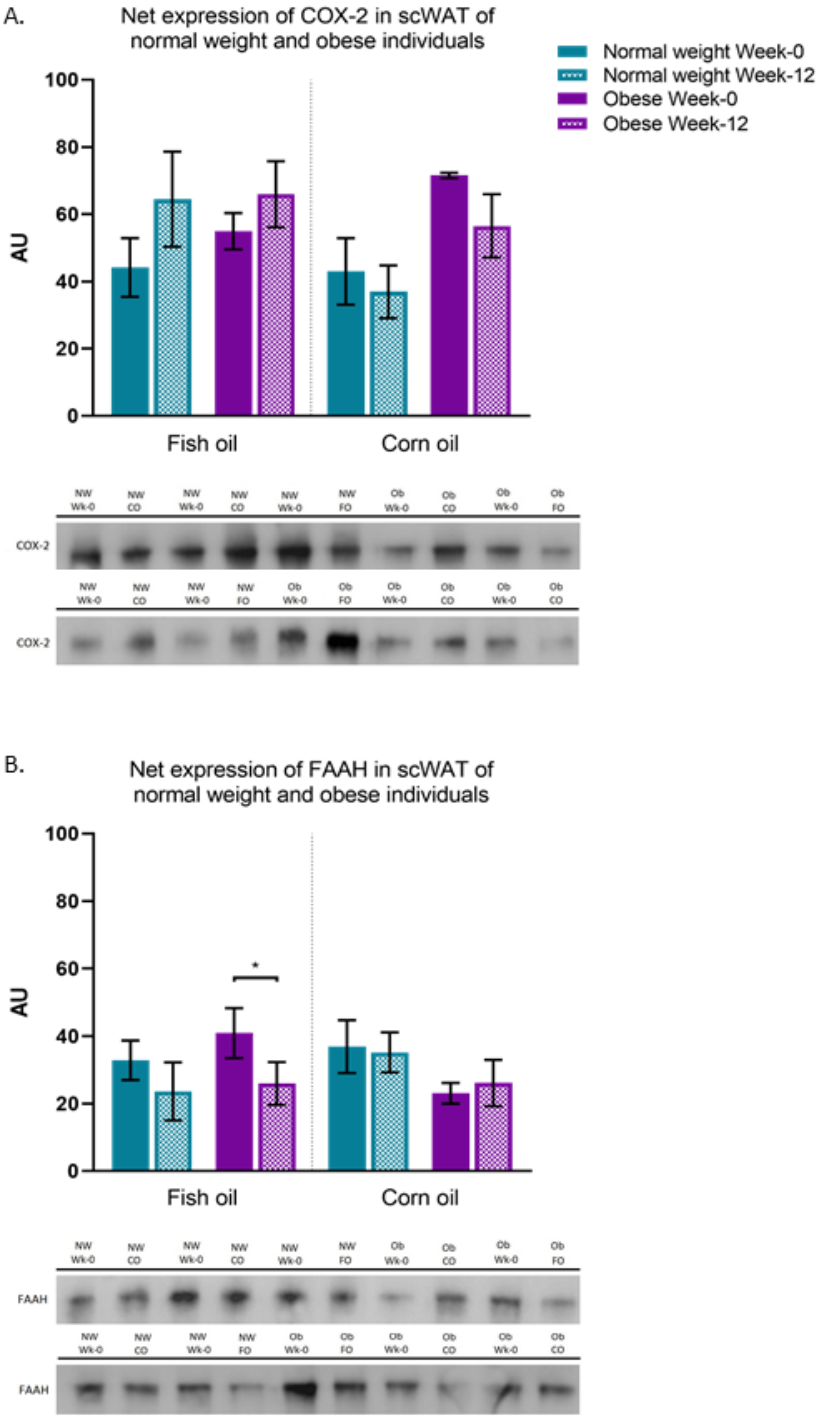


Figure 5.5 Effect of 12-week fish oil and corn oil treatment on A) net expression of COX-2 in scWAT of normal weight (n= 4 FO, 3 CO pairs) and metabolically healthy individuals (n= 4 FO, 3 CO pairs) and B) net expression of FAAH in scWAT of normal weight (n= 4 FO, 3 CO pairs) and metabolically healthy obese individuals (n= 4 FO, 3 CO pairs).

Mean ± SEM. * *P* = 0.042. COX-2, cyclooxygenase-2; FAAH, fatty acid amide hydrolase; NW, normal weight, Wk-0, week-0; CO, following 12-week corn oil intervention; FO, following 12-week fish oil intervention; Ob, metabolically healthy obese.

5.5.2.2 scWAT COX-2 activity was not significantly altered by 12-week FO or CO intervention in either normal weight or MHO individuals

There were no significant changes in COX-2 activity in the scWAT of either normal weight or MHO individuals in response to either 12-week FO or CO intervention ($P > 0.05$, Figure 5.6).

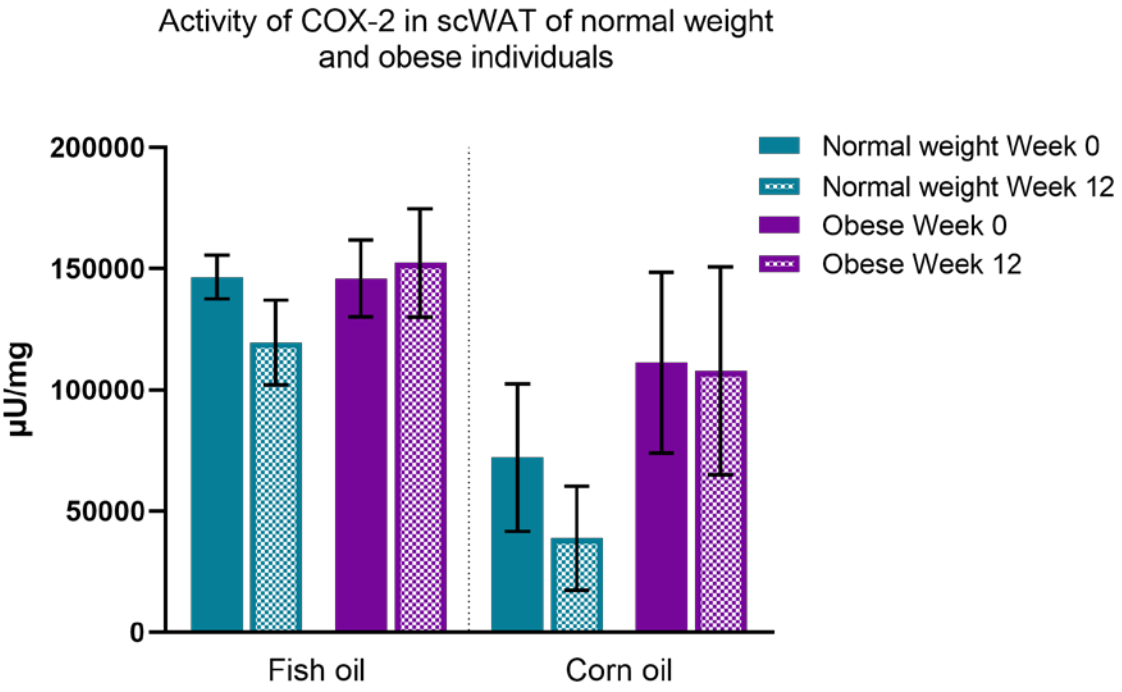


Figure 5.6 Effect of 12-week fish oil and corn oil intervention on scWAT COX-2 activity in normal weight (n= 3 FO, 2 CO pairs) and metabolically healthy individuals (n= 3 FO, 2 CO pairs)

Mean ± SEM. COX-2, cyclooxygenase-2.

5.6 Discussion

Chapter 4 details significant differences in scWAT lipid mediator profiles in MHO compared with normal weight individuals and in the lipid mediator profile in MHO individuals, and the EC system in normal weight individuals in response to 12-week FO intervention. It was hypothesized that these differences and changes may be due to altered expression and activity of enzymes involved in the metabolic pathways of these signalling molecules. COX-2 is responsible for the synthesis of many FA metabolites including HODEs and PGs which were altered in MHO individuals, and FAAH

is responsible for the breakdown of ECs, of which greater concentrations were observed in MHO individuals at week-0. Therefore, it was hypothesised that there may be altered expression and activity of scWAT COX-2 and FAAH in MHO individuals in comparison to normal weight individuals. It was further hypothesised that the expression and activity of these enzymes may also be altered by FO intervention as a number of metabolites influenced by these enzymes were altered in response to 12-week FO intervention.

FAAH protein was detected in the scWAT of both normal weight and MHO individuals and the net expression was similar between these two BMI subgroups. COX-2 protein was also detected in scWAT of both normal weight and MHO individuals with a greater net expression in MHO in comparison to normal weight individuals. A limitation of this work is the inability to accurately quantify Western blot data due to lack of appropriate loading control (reference protein). Four reference proteins were trialled with none exhibiting consistent expression across all conditions (obesity, FO, and CO intervention). This questions the use of these as reference proteins in publications and the reproducibility of studies in which these have been used as a reference to calculate expression of experimental proteins against.

Selection of reference proteins for the use in scWAT and visceral WAT (vWAT) in obesity has been reviewed by Pérez-Pérez *et al.* who concluded that FAA, ENOA and PARK7 were consistent when comparing obese and non-obese tissue, and that FAA expression was most consistent when comparing scWAT and vWAT depots (Pérez-Pérez et al., 2012). Examination of reference proteins (Pérez-Pérez et al., 2012) also showed βactin expression to be stable amongst normal weight and obese vWAT and as such its use as a reference protein is often reported (Gómez-Serrano et al., 2016, Grzegorczyk. et al., 2018) (Table 5.4). However, one study examining scWAT in addition to vWAT, published an image of just two samples for β-Actin immunoblotting, one lean and one obese sample, to represent consistency among their cohort. This may be misleading as there may be two samples that appear more consistent than others and scWAT may show greater variation in expression than vWAT. In the current study, βactin expression was not consistent between scWAT collected from normal weight and MHO individuals and was therefore not suitable for use as a reference protein.

Author	Tissue	Reference Protein	Notes
Ahn 2019	scWAT - human but BMI not defined	GAPDH	GAPDH consistent across multiple tissues in comparison with scWAT
(Grzegorczyk. et al., 2018)	vWAT and scWAT from morbidly obese and lean controls	β-ACTIN	Only published immunoblotting of 2 samples to represent consistency
(Gómez-Serrano et al., 2016)	vWAT from obese humans without diabetes and obese diabetics taking oral antidiabetic drugs or insulin	β-ACTIN	Consistent expression diabetic vs non-diabetic
(Pérez-Pérez et al., 2012)	vWAT and scWAT from non-obese and obese humans without diabetes	Examined use of: β-ACTIN, CALX, ENOA, GAPDH, FAA, PARK7, and TBB5.	β-ACTIN, ENOA, FAA, and PARK7, consistent comparing obese and non-obese vWAT FAA most consistent when comparing vWAT and scWAT
(Fisher et al., 2002)	vWAT and scWAT from obese humans	ACTIN	Consistent expression between depots
(Fisher et al., 2001)	vWAT and scWAT from lean and obese humans	ACTIN	Consistent expression between depots and BMI

Table 5.4 Reference proteins used for western blotting of samples from human adipose tissue.

Net expression of COX-2 was significantly greater in MHO individuals and was significantly associated with a number of scWAT FA metabolites including AEA, u_LTD_4, 20-COOH-AA, 16-HDHA and 17-HDHA which exhibited altered concentrations in obesity in Chapter 4. The consequences of this are not clear as COX-2 expression was positively associated with 16-HDHA which would suggest the greater the expression of COX-2, the greater the concentration of 16-HDHA produced from DHA by COX-2; however, the concentration of 16-HDHA was observed to be less in MHO individuals in comparison to normal weight (Chapter 4). In addition, COX-2 expression was negatively associated with 17-HDHA concentration suggesting the greater the expression of COX-2, the less 17-HDHA is produced from DHA. This does not align with the expected metabolic process but this is concordant with metabolite data in which the concentration of 17-HDHA was lower in MHO individuals in comparison to normal weight individuals. Therefore, it appears that expression does not necessarily directly dictate the amount of metabolite; the activity of the enzyme may be more important than the amount. Net expression of FAAH was not different between normal weight and MHO individuals but was significantly associated with scWAT concentrations of EPEA, 16-HDHA, and 17-HDHA in normal weight individuals only. The concentrations of these metabolites were significantly higher in MHO individuals as reported in Chapter 4. These changes being limited to one or the other BMI group may further highlight dysregulation in lipid metabolite signalling in obesity.

How FAAH may affect the concentrations of these metabolites is not certain as this enzyme is primarily involved in the degradation of FA ethanolamides including AEA, EPEA, and docosahexaenoyl ethanolamide (DHEA). Indeed FAAH was significantly associated with EPEA in normal weight individuals, but this association was positive suggesting the greater the net expression of FAAH, the higher the concentration of scWAT EPEA which would not reflect greater hydrolysis of EPEA that may be expected with an increase in expression of this protein. Unfortunately, investigation of whether the concentration of scWAT endocannabinoids was associated with the activity of FAAH was not possible as change in activity could not be detected for this enzyme.

Calculation of enzyme activity considers the background activity of the enzyme (T0); the background activity of FAAH is visually higher in MHO individuals in comparison to normal weight individuals (P = 0.010) but is very low in both BMI groups when compared to COX-2 activity (Figure 5.3 and Figure 5.4). Alteration to FAAH mRNA expression has been previously reported in obese humans but with conflicting results as both increased (Pagano et al., 2007, Murdolo et al., 2007, Cable et al., 2011) and decreased expression (Engeli et al., 2005, Bluher et al., 2006, Lofgren et al., 2007) are reported in comparison to normal weight individuals. In addition, the influence of hyperinsulinemia which often develops in obesity, has been investigated and observed to increase

FAAH mRNA expression (Murdolo et al., 2007). Therefore, consideration of the metabolic health of the obese individuals may be of importance when assessing the expression and activity of these enzymes. Indeed, the expression of FAAH protein is negatively correlated with HOMA2-IR scores (-0.648, *P* = 0.043, data not shown) suggesting net expression of FAAH decreases with increasing insulin resistance. However, the background activity of FAAH is positively correlated with HOMA2-IR (-607, *P* = 0.016, data not shown) suggesting study entry activity of the enzyme increases with increasing insulin resistance. Therefore, the expression and activity of this enzyme, and alteration of these in obesity and MetS is complex.

A further limitation of this work is that a small number of samples were available for Western blot analysis. Despite this, results from these data were statistically significant for COX-2 at study entry, and FAAH following the intervention period suggesting an appropriate sample size to detect such changes.

A strength of the current study is that it provides information on the net protein expression, background activity, and mRNA expression (to be investigation in chapters 5 and 6). The inclusion of absolute activity data (not just background activity) for FAAH would improve this and may be obtained by trialling an alternative method of assessing enzyme activity. The majority of studies describing FAAH expression in scWAT do not reference the mRNA expression to the total protein level or the activity of the enzyme. A single study that did investigate FAAH activity in metabolically healthy humans, reports a positive correlation between FAAH activity in isolated scWAT adipocytes and BMI (Cable et al., 2011). Another limitation of the current study is the inability to detect change in FAAH activity. Cable *et al.* measured FAAH activity by incubation of the particulate fraction of isolated adipocyte homogenate with radiolabelled AEA ([^{3}H]-AEA). The radioactive label in the reaction product was then measured by scintillation counting to determine enzyme activity (Cable et al., 2011, Boldrup et al., 2004). It may be that this method is superior at detecting activity in comparison to the fluorometric assay used in the current study and would be worth considering when planning future studies to ensure appropriate tissue weights are collected to isolate adipocytes and perform such analyses.

There was no significant difference in the activity of COX-2 between normal weight and MHO individuals but COX-2 activity was correlated with two COX pathway metabolites of AA, neither of which were altered at study entry in MHO individuals, and one COX -2 pathway metabolite of LA, 9-HODE, which was significantly lower in MHO individuals at study entry. However, this was only observed in normal weight individuals. COX-2 activity was negatively correlated with several LOX pathway metabolites of DHA including hydroxy-DHA metabolites and RvD2 in normal weight individuals. This may suggest COX-2 activity and preferential metabolism of FAs by COX-2 may

alter the metabolism of FAs by other pathways, perhaps through diversion of substrate. These metabolites have anti-inflammatory or 'pro-resolving' actions in the tissue and significantly lower proportions of these were observed in MHO individuals at study entry (Chapter 4). The restriction of the significant correlation between COX-2 activity, 9-HODE and DHA metabolites to only one BMI group, supports similar findings for net protein expression and may indicate dysregulation in the handling and metabolism of FA in MHO individuals.

To conclude, net COX-2 protein expression was significantly higher in MHO individuals but COX-2 activity was not altered; neither net COX-2 protein expression nor activity were altered by 12-week FO intervention. Net COX-2 protein expression and COX-2 activity were significantly associated with concentrations of scWAT LA, AA, EPA, and DHA metabolites. These correlations were predominantly observed in normal weight individuals with regards to COX-2 activity, and in MHO individuals with regards to net COX-2 protein expression. This may suggest that changes in scWAT lipid metabolites may be due to altered net protein expression of COX-2; however it does not provide evidence for COX-2 activity contributing to the altered regulation of LA and AA COX-2 mediated pathway metabolites which were observed to be altered in MHO individuals at study entry. There was no difference in the net expression of FAAH in MHO individuals compared to normal weight, but this was negatively correlated with HOMA2-IR suggesting net expression decreases with increasing insulin resistance. Net FAAH expression was significantly positively associated with EPEA, the concentration of which was significantly higher in MHO individuals at study entry (Chapter 4). Furthermore, background FAAH activity was significantly greater in MHO individuals. FAAH is responsible for the degradation of EPEA so this data may suggest dysregulation of normal FAAH action in obesity, specifically with declining metabolic health. Furthermore, these data being limited to particular BMI subgroups may suggest further dysregulation of FA metabolite metabolism in obesity.

12-week FO intervention did not alter the net expression or activity of COX-2, but did significantly lower the net expression of FAAH. In Chapter 4, an increase in the concentration of scWAT EPEA and DHEA in response to 12-week FO intervention was observed in normal weight individuals only. A decrease in FAAH net expression may result in a reduction in the degradation of AEA, EPEA, and DHEA. However, study entry data indicates neither net FAAH expression nor background activity corresponds to lower concentrations of ECs and may suggest the normal action of FAAH is altered in obesity. Furthermore, the action of FAAH may be minimal in comparison to enzymes responsible for the synthesis of ECs such as N-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD). Use of radiolabelled FA ethanolamides to measure FAAH activity in addition to NAPE-PLD, and diacylglycerol lipase-α, which were not

available as a fluorometric assay, as well as monoacylglycerol lipase, would be worthy of future investigation to dissect changes in EC metabolism further.

Overall, these data suggest the greater proportions of LA, AA, and EPE derived FA metabolites observed in HMO individuals at study entry in Chapter 4, may more likely be due to the greater proportion of the precursor FA available as substrate rather than to direct changes in the enzymes involved in their metabolism. The reasons for the differences in the EC system in response to 12 week FO intervention require further investigation.

Chapter 6 The transcriptomic profile of human subcutaneous white adipose tissue is altered in metabolically healthy obese subjects and in response to 12-week fish oil intervention

6.1 Introduction

Chapter 3 and Chapter 4 discuss the role of the subcutaneous white adipose tissue (scWAT) as a storage and endocrine organ detailing its capacity to incorporate and store dietary fatty acids (FAs) and to utilise these to produce a range of signalling molecules involved in autocrine and paracrine processes; these include: immune and inflammatory responses, adipose structure and maintenance, glucose homeostasis and therefore homeostasis of whole body energy. The processes of lipid accumulation, synthesis of lipid metabolites and their resultant actions such as tissue expansion and remodelling, are tightly regulated by transcriptional expression of genes encoding the proteins involved in these pathways. Many of these processes are observed to be altered in human obesity and by lipid intervention (Kalupahana et al., 2020), and it was demonstrated in Chapter 5 that proteins involved in these pathways are altered in both these scenarios. Therefore, the expression of the genes encoding these proteins as well as those regulating the wider biological processes may also be altered by obesity and by dietary FAs.

The transcriptome contains information about all RNA transcribed (i.e. both coding and non-coding) within a specific tissue or cell at a particular stage of development and under certain physiological or pathological conditions such as obesity (Jacquier, 2009, Casamassimi et al., 2017). Investigation of the whole transcriptome in such conditions not only provides understanding of the human genome at a transcriptional level but also provides novel information on the regulation of gene expression and alteration of biological processes to provide an overview that is unique to that subpopulation aiding understanding of the underlying mechanisms in addition to identification of specific markers of the condition that could be used for diagnosis and therapeutic intervention (Jacquier, 2009, Casamassimi et al., 2017). In the current study, the investigation of the whole scWAT transcriptome in metabolically healthy obese (MHO) individuals in comparison to normal weight individuals could provide information on the regulation of tissue specific processes such as expansion and remodelling, immune response, inflammation, and metabolism. Identification of a novel profile unique to these individuals that is indicative of obesity associated changes to the scWAT may provide insight into the progression of obesity and identify novel

transcripts indicative of early onset metabolic complications which may be suitable for targeting with therapeutic intervention such as long chain omega-3 polyunsaturated FAs (LC n-3 PUFA).

Changes in scWAT morphology are commonly observed in obesity in which there is expansion and remodelling of the tissue. Tissue expansion, termed hyperplasia, is regulated by genes involved in the maturation of adipocytes and the differentiation of preadipocytes into mature lipid accumulating adipocytes (Jo et al., 2009, Drolet et al., 2008). ScWAT expansion is a mechanism required for maintaining energy homeostasis by regulating the balance between storage and liberation of lipids from the scWAT. Therefore, dysregulation in the expression of lipogenic and lipolytic genes has been described in human obesity and in metabolically dysregulated WAT in which there is greater expression of genes associated with lipolysis (Vidal-Puig et al., 1997, Grzegorczyk. et al., 2018, Kern et al., 2004), and lower expression of genes involved in the uptake of lipids into the adipocytes (Kolehmainen et al., 2001, Sewter et al., 2002, Queipo-Ortuno et al., 2012)

Limitations in scWAT expandability and an increasing hypoxic state seen accompanying obesity influence a range of other adipose processes including inflammatory and immune signalling, and tissue and whole body energy metabolism and homeostasis (Halberg et al., 2009, Pasarica et al., 2009b, Lee et al., 2010, Queipo-Ortuno et al., 2012). This is an example of how gene networks interact between specific biological and cellular processes that can be explored in more detail by techniques such as whole transcriptome analysis.

scWAT plays an important role in secreting hormones and other signalling molecules to maintain whole body homeostasis, including insulin sensitivity and lipid metabolism, and to regulate immune and inflammatory processes (Kershaw and Flier, 2004, Jensen, 2006, Masoodi et al., 2014). Under hypoxic conditions and a state of chronic low-grade inflammation accompanying obesity, the synthesis and secretion of such molecules and subsequent signalling pathways become altered. Dysregulation of such pathways in obesity includes the activation of the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF-κB) (Delves et al., 2006). and the NOD like receptor 3 (NLRP3) inflammasome (Dewhurst-Trigg et al., 2020). The activation of NF-κB regulates the expression of many genes encoding inflammatory signalling molecules including enzymes involved in lipid metabolism, cytokines and adhesion molecules (Baldwin, 1996, Barnes and Karin, 1997, Rossi et al., 2000, Grabbe et al., 2011) as well as genes involved in regulating adipose metabolism and glucose homeostasis (Straczkowski et al., 2002, Bruun et al., 2004, Trujillo and Scherer, 2006, Hotamisligil, 2006, Jager et al., 2007, Cawthorn and Sethi, 2008, Titos and Claria, 2013, Park and Ahima, 2014, Bing, 2015).

Activation of the NLRP3 inflammasome which can occur via toll like receptor (TLR) -4 mediated NF-κB, activation by free fatty acids, damage associated molecular pattens (DAMPs) from dying adipocytes, and LPS, results in secretion of mature 'active' IL-1β (Dewhurst-Trigg et al., 2020, Charles-Messance et al., 2020, Kalupahana et al., 2020). Increased IL-1β is observed in obesity and is associated with insulin and glucose signalling in humans (Bing, 2015, Charles-Messance et al., 2020). IL-1β has been shown to decrease protein expression of insulin receptor substrate-1 (IRS-1) in human adipocytes, which is essential to activate phosphoinositide 3-kinase (PI3-k) in response to insulin and results in subsequent protein kinase B (Akt) phosphorylation and upregulation of glucose transport protein -4 (GLUT-4) translocation (Bing, 2015). IL-1β has been observed to decrease PI3-k, phosphorylated Akt, and GLUT4 proteins in addition to IRS-1 in human adipocytes (Bing, 2015). Therefore, the regulation of these genes, their upstream regulator such as TLR-4 and NF-κB, and cross network interactions with other inflammatory signals such as cytokines and inflammatory lipid metabolites may be altered in obesity.

Chapter 4 details altered lipid metabolite profiles in MHO individuals which may occur due to differences in the expression of genes encoding cyclooxygenase (COX), lipoxygenase (LOX), and endocannabinoid (EC) pathway enzymes (Engeli et al., 2005, Bluher et al., 2006, Lieb et al., 2014 , Heemskerk et al., 2015, Chan et al., 2016, Garcia-Alonso et al., 2016). The current study provides evidence for greater net expression of COX-2 protein in MHO individuals and the association of this, in addition to protein activity, with some FA metabolite concentrations (Chapter 5). In addition, alterations in immune response and immune cell types present in the WAT can influence cytokine and FA metabolite secretion. An increase in M1 macrophages, $CD4^+$ Th1 cells, dendritic cells (DCs), B cells, natural killer cells, $CD8^+$ cells, and decreased T-reg cells and $CD4^+$ Th2 cells, is reported in WAT in obesity (Nishimura et al., 2009, Bertola et al., 2012, Fabbrini et al., 2013, Travers et al., 2015, Liddle et al., 2017).

An upregulation of many transcriptional regulators such as the class II transactivator gene (CIITA) and interferon inducible protein 30 play a role in major histocompatibility complex class II (MHCII) expression on DCs, B cells, and macrophages, and therefore the upregulation of immune response priming is reported in obesity (Deng et al., 2013). IFN-γ signalling results in Th1 cell activation and the secretion of Th1 cell derived pro inflammatory cytokines (Deng et al., 2013). These pro-inflammatory cytokines can elicit further activation of pro-inflammatory cascades; for example, TNF can regulate the transcription of NRLP3 inflammasome components (McGeough et al., 2017). Therefore, crosstalk between cells, and regulation across gene networks is evident and could be explored in greater detail via whole transcriptomic analysis.

The whole transcriptome can also be influenced by dietary lipids; LC n-3 PUFA have been reported to affect gene expression and consequently a range of adipose parameters including modulating lipid metabolism and energy regulation through hypolipidemic and anti-obesity effects as described via the suppression of lipogenesis and increase in FA oxidation (Couet et al., 1997, Mori et al., 1999, Kunešová et al., 2006, Kopecky et al., 2009, Flachs et al., 2009, Kuda et al., 2018, Kuda et al., 2009). These FAs have also been observed to prevent body fat accumulation described through inhibition of adipocyte differentiation and induction of apoptosis (Mori et al., 1999, Ruzickova et al., 2004, Kim et al., 2006, Kuda et al., 2009). Evidence in humans, suggests a role for LC n-3 PUFA in weight maintenance and it is seen that a higher n-6:n-3 ratio during pregnancy is associated with increased adiposity in childhood (Vidakovic et al., 2016). The switch towards catabolism and suppressed lipogenesis may be dependent on LC n-3 PUFA mediated alterations to the transcriptional control of genes involved in these processes such as peroxisome proliferator activated receptors (PPARs) and adenosine monophosphate-activated protein kinase (AMPK) activity (Luquet et al., 2005, Madsen et al., 2005, Carling, 2004, Mejia-Barradas et al., 2014).

In addition to eliciting their effects via direct interaction with receptors, changes to inflammatory state by LC n-3 PUFA may reflect changes in cellularity and expression of genes associated with lipid metabolism and inflammatory molecule signalling such as NF-kB, PPARs, GPR120 , and the NLRP3 inflammasome (Nisoli et al., 2000, Oh et al., 2010, Mejia-Barradas et al., 2014, Kalupahana et al., 2020). LC n-3 PUFA are incorporated into cell membranes and disrupt lipid raft formation required for TLR4 signalling (Schoeniger et al., 2016, Calder, 2018, Hellwing et al., 2018, Kalupahana et al., 2020) therefore modulating inflammatory signalling (Hutchins et al., 2011, Calder, 2013, Naughton et al., 2013, Kalupahana et al., 2020). LC n-3 PUFA have been observed to inhibit NLRP3 inflammasome activation in humans likely mediated by interaction with GPR120 and GPR40 (Yiqing Yan et al., 2013).

Gut derived lipopolysaccharide (LPS) can potentiate an inflammatory response in the scWAT via TLR4 and subsequent NF-κB activation (Kalupahana et al., 2020); EPA and DHA have been reported to decrease the phosphorylation of inhibitor of nuclear factor kappa B (IκB) in human monocytes, decrease LPS-induced activation of NF-κB, and the expression and secretion of IL-1β, IL-6, TNF-α, and NF-κB in LPS stimulated macrophages derived from humans (Weldon et al., 2007), and decrease NF-κB activation in cultured DCs and stimulated macrophages (Lee et al., 2001, Novak et al., 2003, Kong et al., 2010). In a murine model, EPA and DHA were observed to inhibit LPS induced secretion of IL-1β from macrophages and hepatocytes in LDLR deficient mice by reducing cleavage of caspase-1 and NLRP3 activation (Charles-Messance et al., 2020). Therefore, LC n-3 PUFA may attenuate obesity related adipose inflammation via NLRP3 inflammasome and related pathways to elicit regulatory actions on glucose homeostasis as well as on inflammatory

status; however, evidence for this has not been observed in humans (Charles-Messance et al., 2020). In addition, DHA intervention may improve metabolic function and is reported to modulate adipogenesis by down regulating PPAR-γ1, PPAR-γ2, CEBP-α, and GLUT4 (Valli et al., 2018).

Current data from transcriptomic studies in human obesity are limited and are predominantly in the area of weight reduction and exercise intervention (Cancello et al., 2005, Capel et al., 2009, Marquez-Quinones et al., 2010, Mutch et al., 2011, Armenise. C et al., 2017); furthermore, consideration of the metabolic health of obese individuals is often overlooked or there is focus on obesity with a metabolic complication such as type-2 diabetes (Stenvers et al., 2019, Rodriguez-Ayala et al., 2020, Kerr et al., 2020). Only one investigation to my knowledge examines the effects of EPA on human WAT transcriptome but this is alongside calorie restriction in healthy obese women (Huerta et al., 2017). Therefore, understanding of how obesity, in the stages where metabolic complication has not developed, affects the transcriptome is of great interest and may improve understanding of the progression of inflammation and related metabolic consequences, and reveal novel transcripts or pathways as markers of this disease state that can be utilised as targets for intervention to prevent development of metabolic complications.

With a growing evidence base from *in vitro*, animal, and limited human interventions (predominantly circulating parameters), LC n-3 PUFA appear to have anti-adiposity and insulin sensitising effects, in addition to positive regulation of inflammatory signalling. Therefore, these lipids may pose benefit in the resolution of WAT inflammation and are worthy of investigation. How these lipids elicit these actions and their effects at the transcriptome level in human WAT is not reported for metabolically healthy obese individuals, a population which may be the best target for therapeutic intervention to prevent metabolic complications. Therefore, the research detailed herein aims to explore the whole scWAT transcriptome in conditions of metabolically healthy obesity and following 12-week FO intervention to provide much needed novel, evidence in this area.

6.2 Aims:

The aim of the research detailed in this chapter was to obtain scWAT whole transcriptome information to:

- Provide a comprehensive overview of the whole human scWAT transcriptome
- Investigate the differential transcriptome in metabolically healthy obese individuals in comparison to normal weight individuals and hypothesise the associated biological processes

- Investigate the effect of 12-week FO vs CO intervention on the transcriptome and hypothesise associated biological processes in scWAT from normal weight and metabolically obese individuals

6.3 Methods:

Please refer to 'Chapter 2– Study design and participant characteristics.

6.3.1 Experimental reagents and materials

Reagents and materials used for experiments are described in Appendix C.

6.3.2 Isolation of RNA from white adipose tissue

Ribonucleic acid (RNA) was isolated from the scWAT collected at week-0 and at week-12 stored in RNA*later*® (see Chapter 2, section 2.2.4 for sample processing), using the RNeasy lipid tissue mini kit™ (QIAGEN, Hilden, Germany). The following protocol was carried out twice to yield RNA from 150 mg of scWAT in total from each participant yielding an average of 2.47 µg of total RNA with an average 260 nm:280 nm ratio of 2.0, 260 nm:230 nm ratio of 1.0, and RNA integrity (RIN) score of >6.5. Extraction was carried out in two batches conducted on the same day; resultant RNA was stored at -80°C.

Tissue (75 mg) was homogenised with 1 ml QIAzol lysis reagent and stored at RT (15-20°C) for 5 minutes to promote the dissociation of nucleoprotein complexes. 200 µl of chloroform was added and the homogenate shaken vigorously for 15 seconds and then stored at RT for 2-3 minutes. The upper phase containing RNA was extracted from the homogenate following centrifugation at 12,000 x g for 15 minutes at 4°C. 600 µl of 70% C_2H_6O (ethanol) was added to the upper phase and vortex mixed. 700 µl of the resulting sample was transferred to an RNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 8000 x g for 15 seconds at RT. The flow through was discarded and the remaining sample was transferred to the mini spin column and centrifuged at 8000 x g for 15 seconds at RT. The flow through was again discarded and the mini spin column membrane was washed by the addition of 700 µl of buffer RW1 and centrifugation at 8000 x g for 15 seconds at RT, the addition of 500 µl of buffer RPE and centrifugation at 8000 x g for 15 seconds at RT, and by a further addition 500 µl of buffer RPE and centrifugation at 8000 x g for 2 minutes at RT. The flow through from each centrifugation was discarded. The mini spin column was then placed into a new collection tube and 30 µl of RNA grade, sterile, deoxyribonuclease (DNase), ribonuclease (RNase) and protease free, diethylpyrocarbonate (DEPC) treated H_2O was

added and left to sit on the column for 5 minutes at RT. RNA was eluted from the mini spin column by centrifuging at 8000 x g for 1 minute at RT.

20 samples were selected for RNA-Sequencing (RNA-Seq) analysis based upon the RNA quality and integrity. Samples with a RIN score of $\geq$ 6.5 were deemed of sufficient integrity for RNA-Seq analysis according to the requirements written by BGI Tech Solutions, Hong Kong. 10 samples were selected from normal weight participants and 10 from obese participants; these were matched for intervention group and as closely for age and sex as possible as detailed in Table 6.1.

Pairs	Sex	Treatment	Age (y)	BMI (kg/m^2)	Waist (cm)	Body fat (%)
Pair 1	Female	Fish Oil	21	22.7	74.1	31.6
	Female	Fish Oil	39	32.8	88.7	42.8
Pair 2	Female	Fish Oil	29	19.4	62.9	19
	Female	Fish Oil	41	32.4	102.6	40.7
Pair 3	Female	Fish Oil	55	22.7	75.8	28.3
	Female	Fish Oil	23	35.2	112.2	47.8
Pair 4	Male	Fish Oil	26	24.1	85.1	19
	Female	Fish Oil	46	32.4	96.8	44.9
Pair 5	Male	Fish Oil	29	24.1	76.7	11.7
	Male	Fish Oil	31	30.2	106.8	23.9
Pair 6	Female	Corn Oil	20	19.6	66.6	20.7
	Female	Corn Oil	23	38	107.6	48.1
Pair 7	Female	Corn Oil	27	22.7	75.1	21
	Female	Corn Oil	31	33.9	101.2	43.3
Pair 8	Female	Corn Oil	19	22.1	66.8	22.9
	Female	Corn Oil	35	34.9	89.4	45
Pair 9	Female	Corn Oil	47	24.2	82.9	27.8
	Male	Corn Oil	36	38.1	133.2	-
Pair 10	Male	Corn Oil	21	25	84.6	11.8
	Male	Corn Oil	35	33.7	114.4	32.8

Table 6.1 Participant characteristics of normal weight (> 18.5 to <25.0 kg/m^2) and MHO (30 - 40kg/m^2) pairwise samples selected for RNA-sequencing.

6.3.3 Whole transcriptome RNA-Sequencing

6.3.3.1 Principle of RNA-Sequencing

RNA-Seq is a high throughput deep sequencing technique used for whole transcriptome investigation. It can be used to quantify changes in transcript expression during particular conditions and although not performed in this study, it can be used to provide information about transcriptional structure including start sites, 5' and 3' ends and post translation modifications

such as splicing patterns. RNA extracted from scWAT is fragmented and converted to complementary DNA (cDNA). These cDNA fragments are then sequenced using high-throughput sequencing technology to provide short sequence reads. These reads are aligned to a reference human genome which allows identification of novel transcripts and estimation of the abundance of transcripts.

Read count tables can be generated from RNA-sequencing information which represents the total number of reads aligning to each gene. These are generally proportional to the length and the expression of the transcript. These counts can be affected by the depth of the sequencing, which refers to the number of times the nucleotide is read during the sequencing process, and the composition of the RNA. This can be overcome by using software, such as EdgeR (open source, Bioconductor org), that adjusts the results for the length and sequencing depth of transcripts as well as normalising for RNA composition. Use of such programmes allows selection of genes based on specific inclusion criteria such as minimum number of counts in a minimum number of samples.

6.3.3.2 RNA-Sequencing procedure

Total RNA was extracted and quantified from 20 scWAT samples (detailed in Table 6.1). This was sent to BGI Tech Solutions, Hong Kong for RNA seq. Sequencing was performed on a Hiseq2000 platform with 5 samples per lane sequenced in a total of 8 lanes (SE50) with a total of 20 million reads. The RNA was sequenced and consequent raw reads filtered for contamination and low-quality reads. In this process, messenger RNA (mRNA) was separated from non-coding RNA in the total RNA sample by detecting mRNA polyadenylated tails, and was then fragmented for cDNA synthesis. Single strand cDNA was synthesized from the mRNA by reverse transcription and was used to create a library. Double stranded cDNA (ds-cDNA) was then synthesized using the single strand cDNA template by a DNA polymerase (Figure 6.1). The ds-cDNA underwent end repair and 3’ and 5’ adapters were ligated to the appropriate ends; it was then amplified and sequenced, and the known orientation of adapters was used as a reference to obtain RNA strand information (Figure 6.1).

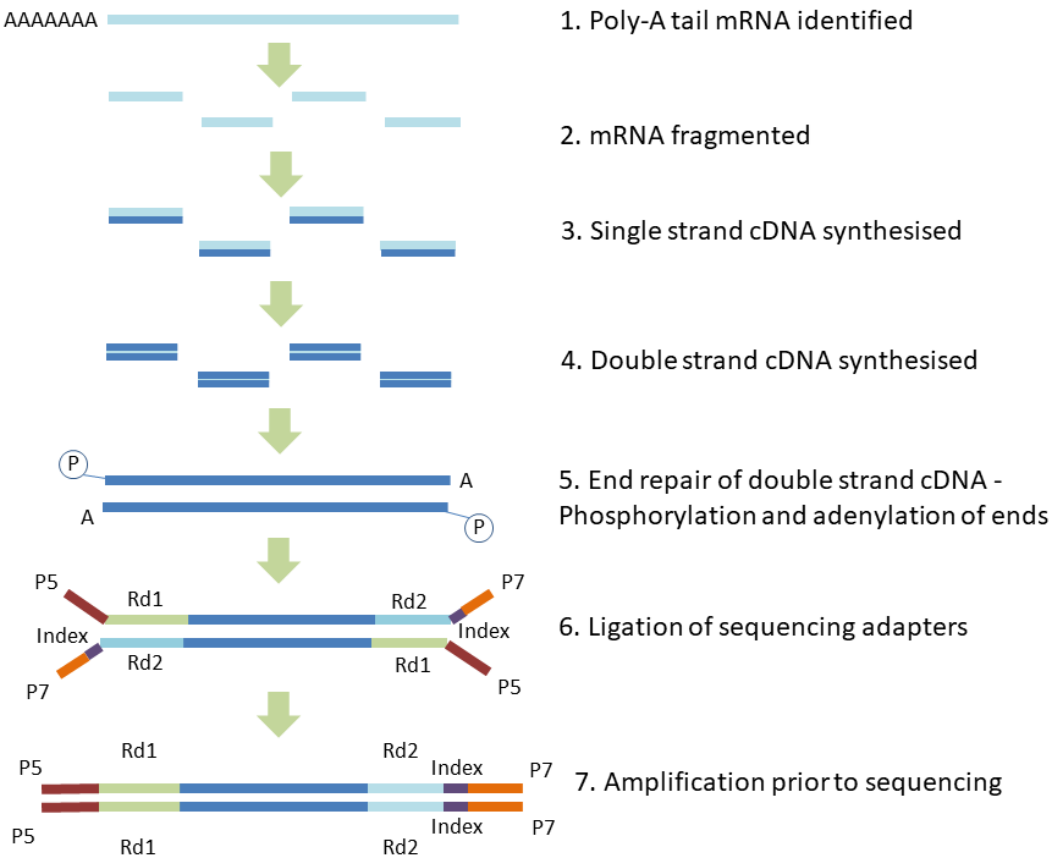


Figure 6.1 Overview of cDNA synthesis and library building for RNA-Sequencing.

6.3.3.3 RNA-Sequencing data analysis

Raw reads were analysed by Elie Antoun, a PhD student from the Developmental Epigenetics lab at the University of Southampton, using the institution's high performance computer - Iridis (BMI, New York, Unites States and OCF Plc, Sheffield, United Kingdom). The RNA-Seq reads were aligned to the hg38.0 reference genome using TopHat (open source, John Hopkins University, Center for Computational Biology, Baltimore, United States) (Trapnell et al., 2009), and a read count table detailing the number of reads mapping to each gene was produced using HTSeq (open source, Huber group, Heidelberg, Germany) (Anders et al., 2015). The read counts were then analysed in EdgeR (open source, Bioconductor.org) (Robinson et al., 2010) in which genes with greater than 5 counts in a minimum of 10 samples were selected for. Any un-mapped genes with large read count values were removed to avoid skewed statistics and the library size re-calculated for these deletions. The data were subsequently normalised by a generated normalisation factor based upon the depth of sequencing which refers to the number of times a nucleotide is read during the sequencing process. Data were further normalised based upon the biological coefficient of

variation which describes the variation in the unknown abundance of a gene between biological replicates, and finally normalised by the estimated tagwise dispersion.

A total of nine different pair-wise comparisons were made using EdgeR. The differential expression of genes between each BMI group (normal weight and MHO) and experimental conditions (CO or FO) was analysed as detailed in Table 6.2

	BMI group	Week	Treatment	VS	BMI group	Week	Treatment	n
1	Obese	0	-	/	Normal weight	0	-	5/5
2	Normal weight	12	Fish Oil	/	Normal weight	0	-	5/5
3	Normal weight	12	Corn Oil	/	Normal weight	0	-	5/5
4	Normal weight	12	Corn Oil	/	Normal weight	12	Fish Oil	5/5
5	Obese	12	Fish Oil	/	Obese	0	-	5/5
6	Obese	12	Corn Oil	/	Obese	0	-	5/5
7	Obese	12	Corn Oil	/	Obese	12	Fish Oil	5/5
8	Obese	12	Fish Oil	/	Normal weight	12	Fish Oil	5/5
9	Obese	12	Corn Oil	/	Normal weight	12	Corn Oil	5/5

Table 6.2 RNA-Seq comparisons for differential expression analysis.

Comparisons between 'normal weight' and MHO participant samples were conducted in a pair wise fashion as detailed in Table 6.2.

False discovery rate (FDR) adjusted *P* values describing the likelihood that a gene was differentially expressed compared to not, and Log2Ratio values describing the log fold change (FC) for each gene were obtained from EdgeR analysis. Genes were deemed to be differentially expressed if meeting criteria of $P \leq 0.05$ and FDR ≤ 0.05. (FDR ≤ 0.1 for post FO intervention due to the small number of genes meeting FDR ≤ 0.05).

6.4 Results

6.4.1 Study entry results

Twenty scWAT samples were selected in a pairwise fashion to ensure an equal number of samples in the FO and CO intervention groups from normal weight and MHO individuals at study entry and

post intervention (study entry and post intervention samples of the same individual were used for pairwise comparisons). These were matched for age (P = 0.119 and 0.132 age in normal weight vs MHO individuals and between intervention treatment groups respectively, Table 6.3). MHO individuals had a higher average BMI as well as a higher percentage and mass (kg) of body fat (P <0.001, Table 6.3).

MHO individuals are referred to as 'obese' in all figures and tables herein.

6.4.1.1 Principal component analysis

Principal component analysis (PCA) was undertaken in collaboration with Prof Joanna Holbrook to allow visual assessment of sample variation and clustering. PCA revealed clear clustering of normal weight individuals and clustering of MHO individuals at study entry as well as clustering of samples from the same individual (study entry and post intervention visits) (Figure 6.3). This suggests normal weight and MHO sample data should be analysed individually and that pairwise comparison would be most appropriate for this sample set (Figure 6.3). There was no clustering of data following intervention treatments (not shown).

	Male	Female	Treatment		Age	BMI	Body Fat (%)	Body Fat Mass (kg)	Total RNA Week 0	Total RNA Week 12
	(n)	(n)	Fish Oil (n)	Corn Oil (n)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	µg	µg
Normal weight	3	7	5	5	29.37 ± 12.08	22.39 ± 1.86	21.38 ± 6.61	13.12 ± 3.67	2.11 ± 1.09	2.93 ± 1.09
Obese	3	7	5	5	36.99 ± 8.42	34.34 ± 2.42	41.03 ± 7.87	39.52 ± 6.68	2.31 ± 0.72	2.53 ± 0.59

Table 6.3 scWAT samples selected for RNA extraction. BMI, Body Fat (%) and Body Fat Mass (kg) were significantly different between normal weight (n=10) and metabolically healthy obese individuals (n=10) $P < 0.001$ but there were no significant differences in age $P > 0.100$.

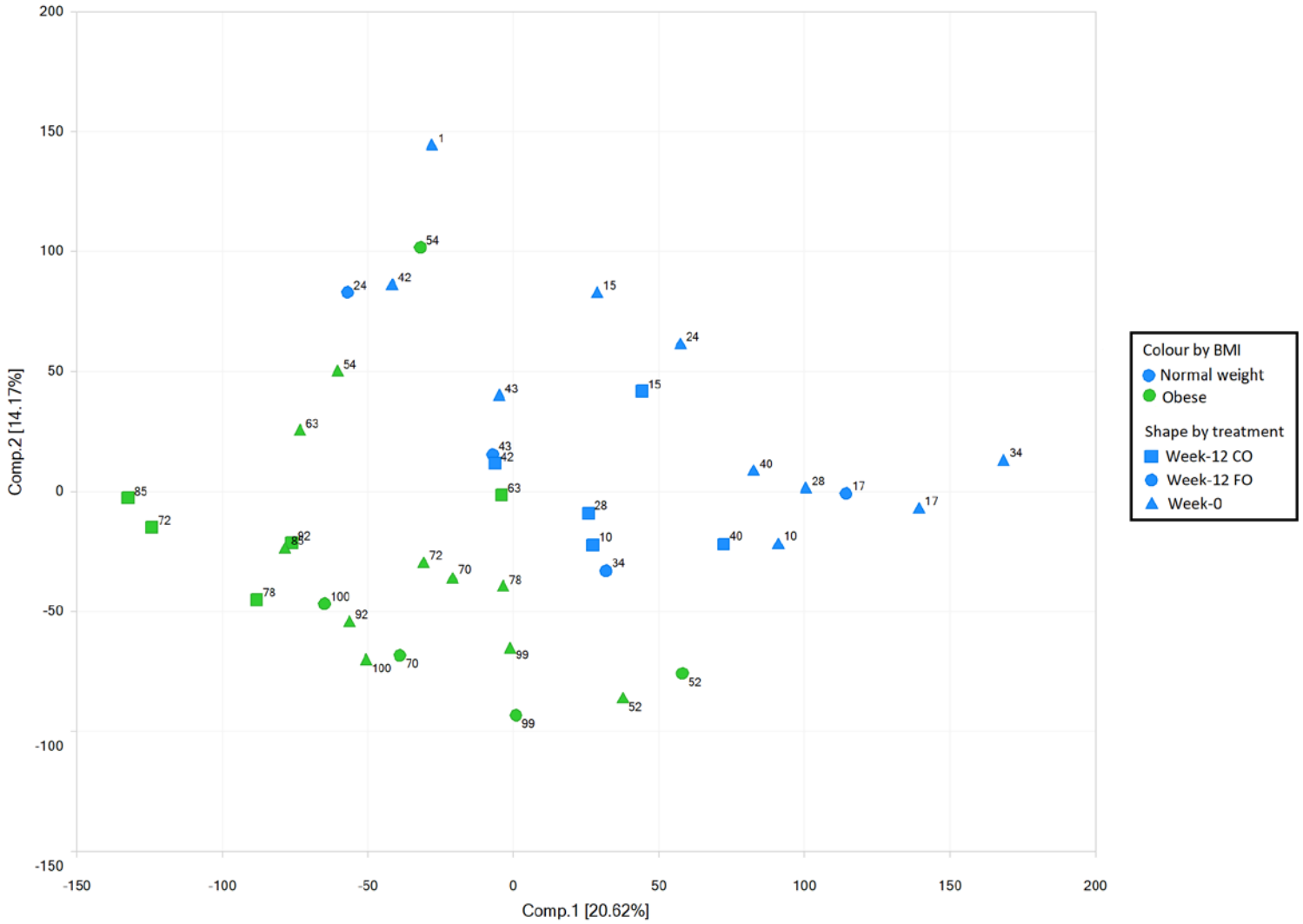


Figure 6.2 Principal component analysis of study entry (week-0) samples used for RNA-Seq.

6.4.1.2 Metabolically healthy obesity is associated with a significantly altered scWAT transcriptome with upregulation of immune and inflammatory responses and adipose specific processes

Sequencing of RNA extracted from human scWAT revealed 4461 genes (meeting significance and FDR criteria of ≤ 0.05) to be differentially expressed in MHO individuals compared to normal weight individuals. Of these, 789 genes had at least a two-fold change i.e. 2 x FC (positive 2 x FC signified by a $\log_2$-FC of at least 1 and negative 2 x FC by a $\log_2$-FC of at least -1) in expression in metabolically healthy obesity. Of these, 623 were up regulated and 175 down regulated (Appendix I). The top 20 up regulated (selected from the 623 up regulated) and top 20 down regulated (selected from the 175 down regulated) genes selected by fold change, in scWAT from MHO individuals are displayed in Table 6.4. *P* values were obtained by comparison of MHO vs normal weight data in a GLM likelihood ratio test in Edge R software.

Up-Regulated				
Gene Symbol	Gene Name	[1]log_2Fold Change	[1]*P* Value	FDR
EGFL6	Epidermal growth factor like domain multiple-6	5.44	≤0.001	≤0.001
MMP7	Matrix metalloproteinase-7	5.41	≤0.001	≤0.001
CCL22	C-C motif chemokine 22 (macrophage derived chemokine)	4.71	≤0.001	≤0.001
MMP9	Matrix metalloproteinase-9	4.03	≤0.001	≤0.001
DCSTAMP	Dendrocyte expressed seven transmembrane protein	3.91	≤0.001	≤0.001
URAD	Ureidoimidazoline	3.9	≤0.001	≤0.001
LINC01010	Long intergenic non-protein coding RNA 1010	3.88	≤0.001	≤0.001
AADACL3	Arylacetamide deacetylase like-3	3.61	≤0.001	≤0.001
CHIT1	Chitinase-1	3.6	≤0.001	≤0.001
SPP1	Secreted phosphoprotein-1	3.57	≤0.001	≤0.001
PLA2G7	Phospholipase A2 group VII	3.54	≤0.001	≤0.001
STMN2	Stathmin-2	3.45	≤0.001	≤0.001
TREM2	Triggering receptor expressed on myeloid cells-2	3.4	≤0.001	≤0.001
SDS	Serine dehydratase	3.37	≤0.001	≤0.001
KRT16	Keratin-16	3.34	≤0.001	≤0.001
UNC13C	Protein unc-13 homolog C	3.33	≤0.001	≤0.001
CHI3L1	Chitinase-3-like protein 1	3.3	≤0.001	≤0.001
TM4SF19	Transmembrane 4 L six family member 19	3.2	≤0.001	≤0.001
COL11A1	Collagen alpha-1(XI) chain	3.07	≤0.001	≤0.001
IGHV5-51	Immunoglobulin heavy variable 5-51	3.04	≤0.001	≤0.001

Down-Regulated				
Gene Symbol	Gene Name	[1]log_2Fold Change	[1]*P* Value	FDR
SLC27A2	Very long-chain acyl-CoA synthetase	-3.42	≤0.001	≤0.001
RORB	Nuclear receptor ROR-beta	-3.3	≤0.001	≤0.001
SPX	Spexin hormone	-3.26	≤0.001	≤0.001
CA3	Carbonic anhydrase-3	-3.25	≤0.001	≤0.001
CECR2	Cat eye syndrome critical region protein-2	-2.71	≤0.001	≤0.001
WDR86-AS1	WDR86 Antisense RNA	-2.56	≤0.001	≤0.001
KCNU1	Potassium channel subfamily U member-1	-2.48	≤0.001	≤0.001
ASPG	Asparaginase	-2.47	0.002	≤0.001
BMP3	Bone morphogenetic protein-3	-2.45	≤0.001	≤0.001
RASSF6	Ras-association domain family protein-6	-2.42	≤0.001	≤0.001
TTC36	Tetratricopeptide repeat protein-36	-2.41	≤0.001	≤0.001
GJC3	Gap junction gamma-3 protein	-2.31	≤0.001	≤0.001
KCTD8	BTB/POZ domain-containing protein KCTD8	-2.28	≤0.001	≤0.001
RPS28	40S ribosomal protein-S28	-2.19	≤0.001	≤0.001
SCEL	Sciellin	-2.17	≤0.001	0.004
TSHR	Thyrotropin receptor	-2.17	≤0.001	≤0.001
GFRA3	GDNF family receptor alpha-3	-2.07	≤0.001	≤0.001
NDRG4	Protein NDRG4	-2.02	≤0.001	≤0.001
RASL10B	Ras-like protein family member-10B	-2	≤0.001	≤0.001
AZGP1	Zinc-alpha-2-glycoprotein	-1.99	≤0.001	≤0.001

Table 6.4 Top 20 up regulated and down regulated genes in MHO individuals at week-0.

[1]Log2 Fold change and *P* values were obtained by comparison of MHO vs normal weight data in a general linear model likelihood ratio test in Edge R software.

GeneGO software (GeneGo Inc.) was used to analyse gene ontology of all differentially expressed genes in metabolically healthy obesity to categorize them into specific groups including cellular localisation, component organisation and biological processes (Table 6.5). These data show a range of associated processes with a predominant theme of cellular organisation and localisation, transport, and immune and inflammatory responses (Table 6.5).

Ingenuity® Pathway Analysis (IPA®) (QIAGEN, Hilden, Germany) revealed associations between the 789 genes differentially expressed in obesity with a FC of $\geq$ 2 with predicted activation and inhibition of a range of canonical signalling pathways (Figure 6.3). The top significantly predicted pathway 'activated' (up-regulated) in scWAT from MHO individuals was related to the maturation of DCs (Figure 6.3); 28 specific transcripts that are involved in DC maturation and were upregulated in scWAT from MHO individuals are highlighted in Figure 6.4. Other predicted pathways show a predominant theme of inflammatory signalling, including the predicted activation of a number of inflammatory cytokine signalling pathways, reactive oxygen species (ROS) production in macrophages and inflammatory lipid signalling (e.g. ceramide signalling), tissue response to nutritional stimulus including Wnt/β-catenin signalling, and regulation of lipid signalling including predicted activation of lipid metabolism pathway enzymes, and ceramide signalling (Figure 6.3). Type-2 diabetes mellitus (T2DM) is accompanied by predicted inhibition of LXR/RXR signalling which may have implications for fatty acid synthesis, lipid and cholesterol transport, and insulin sensitivity (Figure 6.3).

Processes	*P*	FDR	Genes
Localization	2.293E-86	2.640E-82	1583
Cellular component organization	1.533E-82	8.824E-79	1707
Cellular component organization or biogenesis	4.782E-80	1.835E-76	1730
Single-organism cellular process	4.055E-69	1.167E-65	3062
Positive regulation of biological process	6.621E-68	1.525E-64	1691
Establishment of localization	2.236E-67	4.290E-64	1312
Single-organism process	1.895E-64	3.116E-61	3285
Transport	2.550E-63	3.671E-60	1267
Regulation of response to stimulus	7.538E-60	9.644E-57	1233
Positive regulation of cellular process	1.982E-59	2.282E-56	1485
Immune system process	4.876E-59	5.104E-56	873
Cellular localization	1.507E-54	1.446E-51	830
Locomotion	3.288E-54	2.913E-51	582
Single-organism localization	1.579E-53	1.298E-50	1119
Movement of cell or subcellular component	2.364E-52	1.814E-49	616
Response to stress	1.553E-50	1.117E-47	1221
Establishment of localization in cell	2.025E-50	1.371E-47	712
Single-organism transport	2.581E-50	1.651E-47	1057
Single-multicellular organism process	2.982E-50	1.807E-47	1713
Single-organism metabolic process	3.717E-50	2.140E-47	1455
Cellular process	2.452E-49	1.344E-46	3493
Positive regulation of response to stimulus	2.648E-49	1.386E-46	756
Regulation of immune system process	3.170E-48	1.587E-45	632
Regulation of multicellular organismal process	4.423E-47	2.122E-44	931
System development	9.712E-47	4.473E-44	1366
Protein complex subunit organization	1.273E-46	5.638E-44	616
Regulation of biological quality	3.795E-46	1.618E-43	1182
Regulation of localization	8.931E-46	3.672E-43	860
Multicellular organismal development	3.675E-43	1.459E-40	1497
Anatomical structure development	6.507E-43	2.497E-40	1515
Regulation of signalling	1.043E-42	3.875E-40	991
Regulation of cell communication	1.866E-42	6.713E-40	994

Response to organic substance	6.137E-42	2.141E-39	1034
Single-organism developmental process	6.467E-42	2.190E-39	1643
Macromolecular complex subunit organization	1.616E-41	5.316E-39	791
Developmental process	2.772E-41	8.867E-39	1657
Translational termination	3.468E-41	1.079E-38	114
Cell surface receptor signalling pathway	4.607E-41	1.396E-38	811
Regulation of molecular function	1.073E-40	3.168E-38	947
Cellular component disassembly	2.121E-40	6.105E-38	297
Positive regulation of immune system process	2.591E-40	7.277E-38	421
Small molecule metabolic process	4.201E-40	1.152E-37	797
Translational initiation	5.119E-40	1.371E-37	136
Regulation of signal transduction	3.599E-39	9.417E-37	880
Response to external stimulus	8.351E-39	2.137E-36	831
Cellular response to chemical stimulus	8.902E-39	2.228E-36	933
Regulation of cellular component organization	1.865E-38	4.568E-36	772
Negative regulation of biological process	2.075E-38	4.977E-36	1390
Intracellular transport	3.203E-38	7.527E-36	561
Regulation of intracellular signal transduction	3.297E-38	7.591E-36	607

Table 6.5 Gene GO™ gene ontology (GO) of differentially expressed transcripts in MHO individuals at week 0.

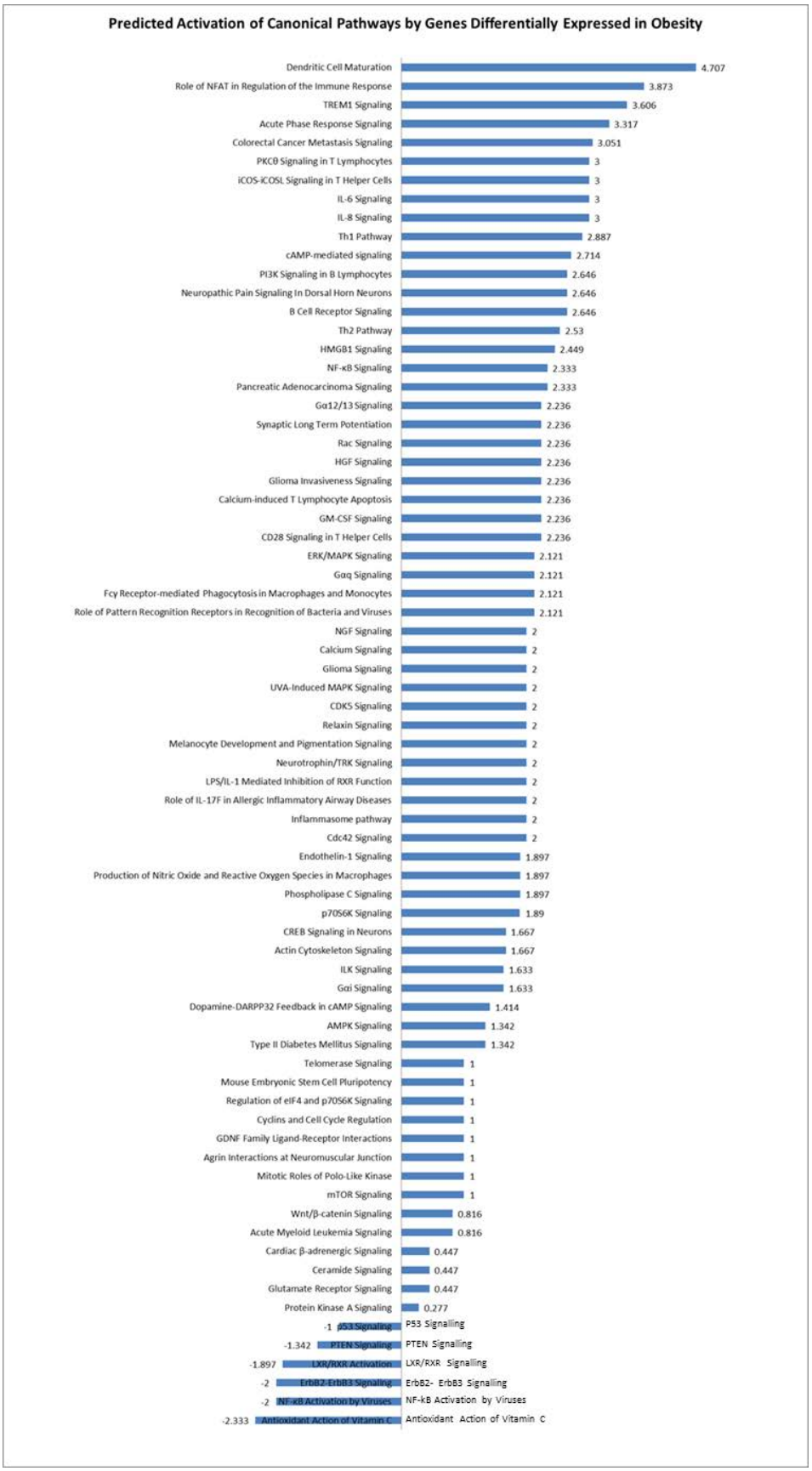


Figure 6.3 Predicted activation and inhibition of canonical pathways by genes differentially expressed in metabolically healthy obese individuals at study entry (week-0). Values are Z scores indicating relationship between the expected relationship direction and the observed gene expression.

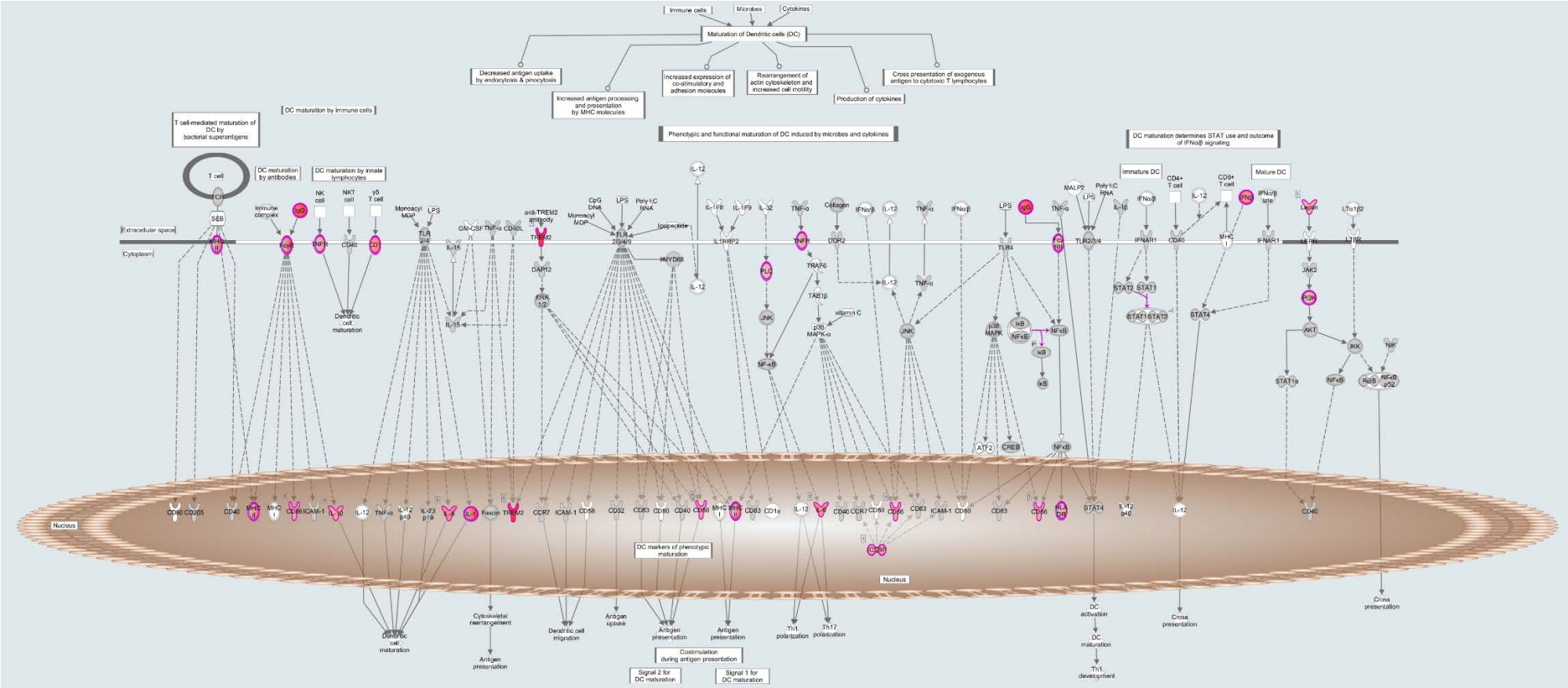


Figure 6.4 Genes differentially expressed in metabolically healthy obese individuals at study entry (week-0) involved in the maturation of dendritic cells. Red/pink highlight indicates upregulation.

A further method of interrogating differential gene expression is assessment of biologically functional pathways. These pathways were sub-divided into the categories: 'Disease and Bio Function', 'Molecular and Cellular Function', and 'Physiological System Development and Function' as displayed in Table 6.6.

Further interrogation of these pathways revealed differentially expressed genes in metabolically healthy obesity at week-0 were associated with parameters of scWAT expansion and remodelling including the up-regulation of cell proliferation and death, cell-cell signalling, migration and chemotaxis of immune cells including macrophages, and upregulation of haematopoiesis. The differential expression of genes involved in many of these processes overlapped with resulting inflammation of organ (Figure 6.5).

'Disease and Bio Function' pathways
1. Organismal Injury and Abnormality (631 genes – 80%)
2. Cancer (629 genes – 80%)
3. Dermatological diseases and conditions (406 genes – 51%)
4. Immunological disease (265 genes- 34%)
5. Inflammatory disease (154 genes -20%)
'Molecular and Cellular Function' pathways
1. Cellular growth and proliferation (164 genes – 21%)
2. Cell to cell signalling (132 genes – 17%)
3. Cell death and survival (128 genes – 16%)
4. Cellular development (94 genes – 12%)
5. Cellular movement (96 genes – 12%)
Physiological System Development and Function' pathways
1. Haematological system development and function (107 genes – 14%)
2. Immune cell trafficking (79 genes – 10%)
3. Lymphoid tissue structure and development (60 genes – 8%)
4. Haematopoiesis (31 genes – 4%)
5. Cell mediated immune response (22 genes – 3%)

Table 6.6 Biologically functional pathways affected with differentially expressed genes in metabolically healthy obese individuals at study entry (week-0).

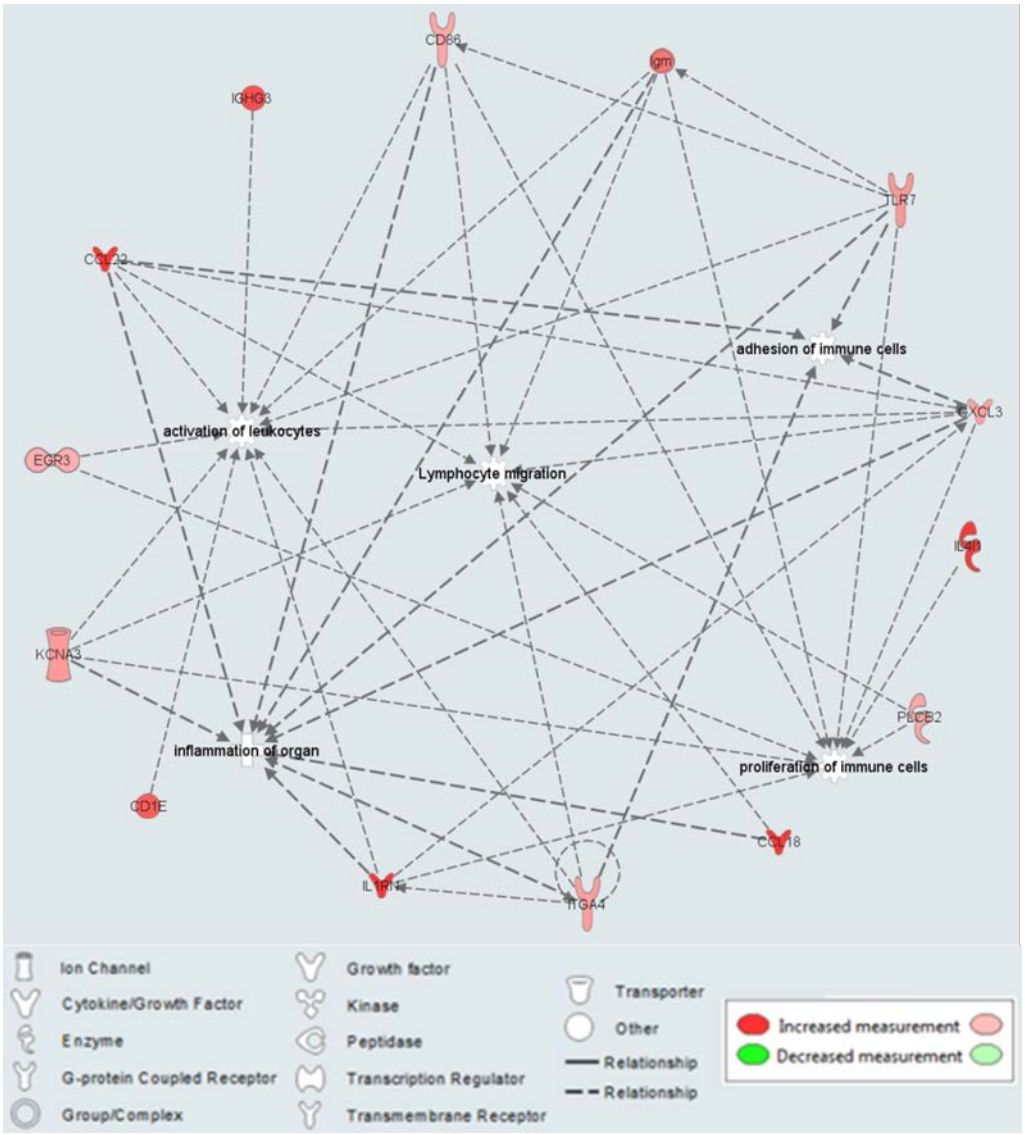


Figure 6.5 Network of genes differentially expressed in scWAT from metabolically healthy obese individuals in comparisons to normal weight at study entry (week-0) featured in 'Molecular and Cellular Function' pathways with roles in proliferation, movement, adhesion and activation of immune cells and resulting inflammation of organ.

Differentially expressed genes in obesity were further significantly associated with inflammation and immune response attributed to by the predicted upregulation of immune cell proliferation and activation, differentiation of blood cells and immune cell trafficking. These associations result in significant prediction for the upregulation in the development of cancer, inflammation of organ and diabetes (Figure 6.6).

Further interrogation of specific transcripts involved in adipose expansion, remodelling, and inflammation revealed the differential expression of a range of cytokines and chemokines in obesity, which were associated with the predicted regulation of a range of upstream regulators.

Upstream analysis revealed predicted activation of *NF-κB, STAT, IFN-γ, TNF-α, JUN, JNK, IL-4, IL-32, IL-27, IL-21, IL-1β, IL-17A, TLR2, TLR5, TLR7* and *TLR9, CD3, CD40*, and triggering receptor expressed on myeloid cells 1 (*TREM1*) (Figure 6.7). Activation of these upstream regulators not only results in the regulation of scWAT gene expression, but also further activation and regulation of other local upstream regulators. *C-JUN* itself is regulated by *TNF-α* and *IL-1β*; *JNK* by IL-1β, *TNF-α* and *IL-1 β; NF-κB* by *TLR2, IL-1β, TNF-α* and *CCL19*; *IL-1β* by *IL-10, Fcer1, TLR2* and *TREM1*; and *TNF-α* by *TREM1* and *TLR7* (8). An enhanced view of *NF-κB* regulation is detailed in Figure 6.8.

When a less stringent filter (i.e. FC of $\geq$1.0) was applied to the data, a further 157 transcripts of interest were identified to be differentially expressed in scWAT from MHO individuals which are displayed in Appendix J. This includes differential expression of genes encoding proteins also involved in:

- Lipid metabolism: including beta-oxidation of FAs, lipoprotein transport, lipid metabolism and elongation of FAs into longer chain FAs
- Immune responses: including JAK/STAT and TLR signalling, chemotaxis and adhesion of immune cells, and Ag responses
- Tissue remodelling and angiogenesis
- Inflammatory response: including inflammasome activation, cytokine and lipid mediator synthesis and secretion
- Adipogenesis

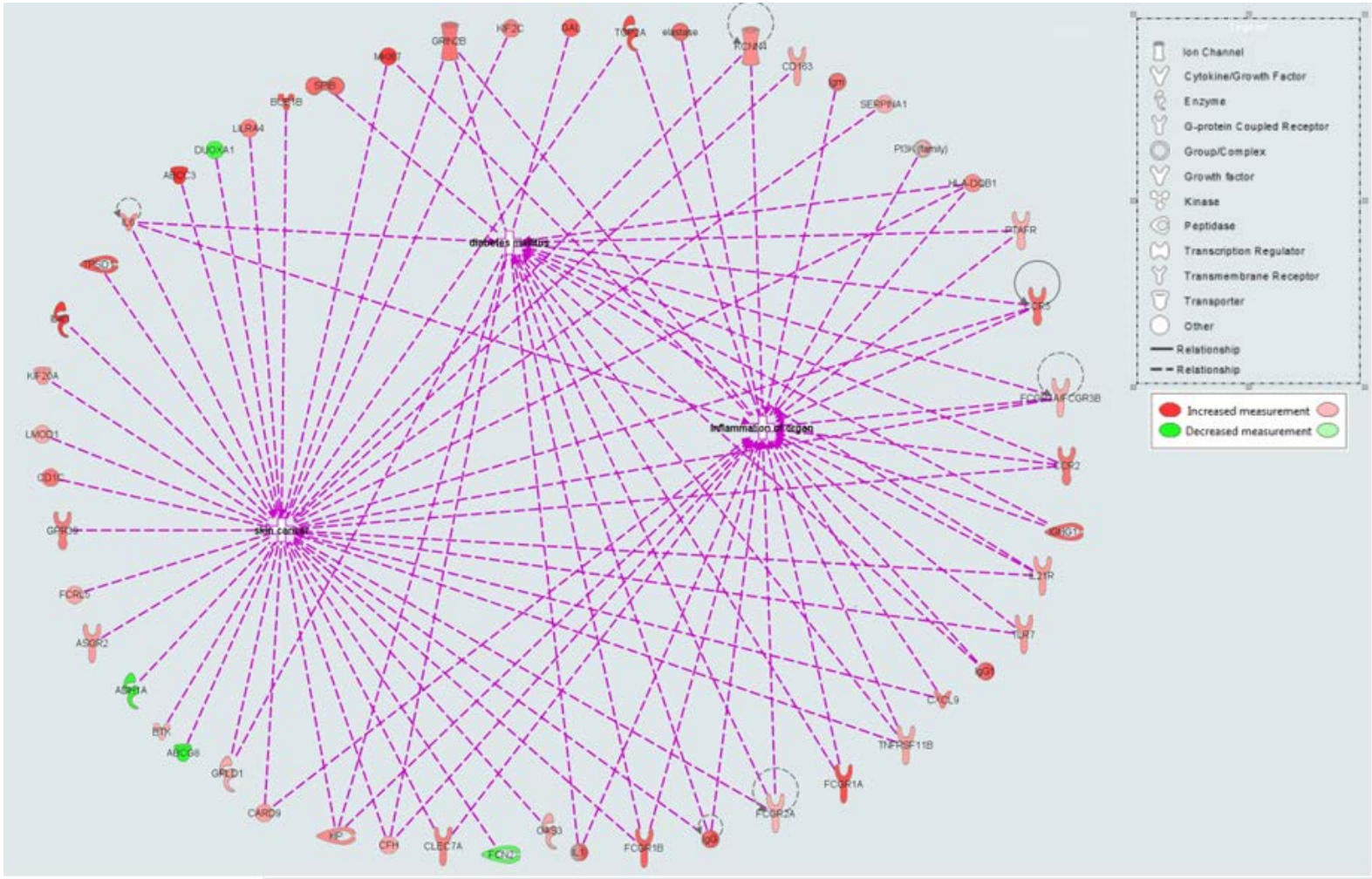
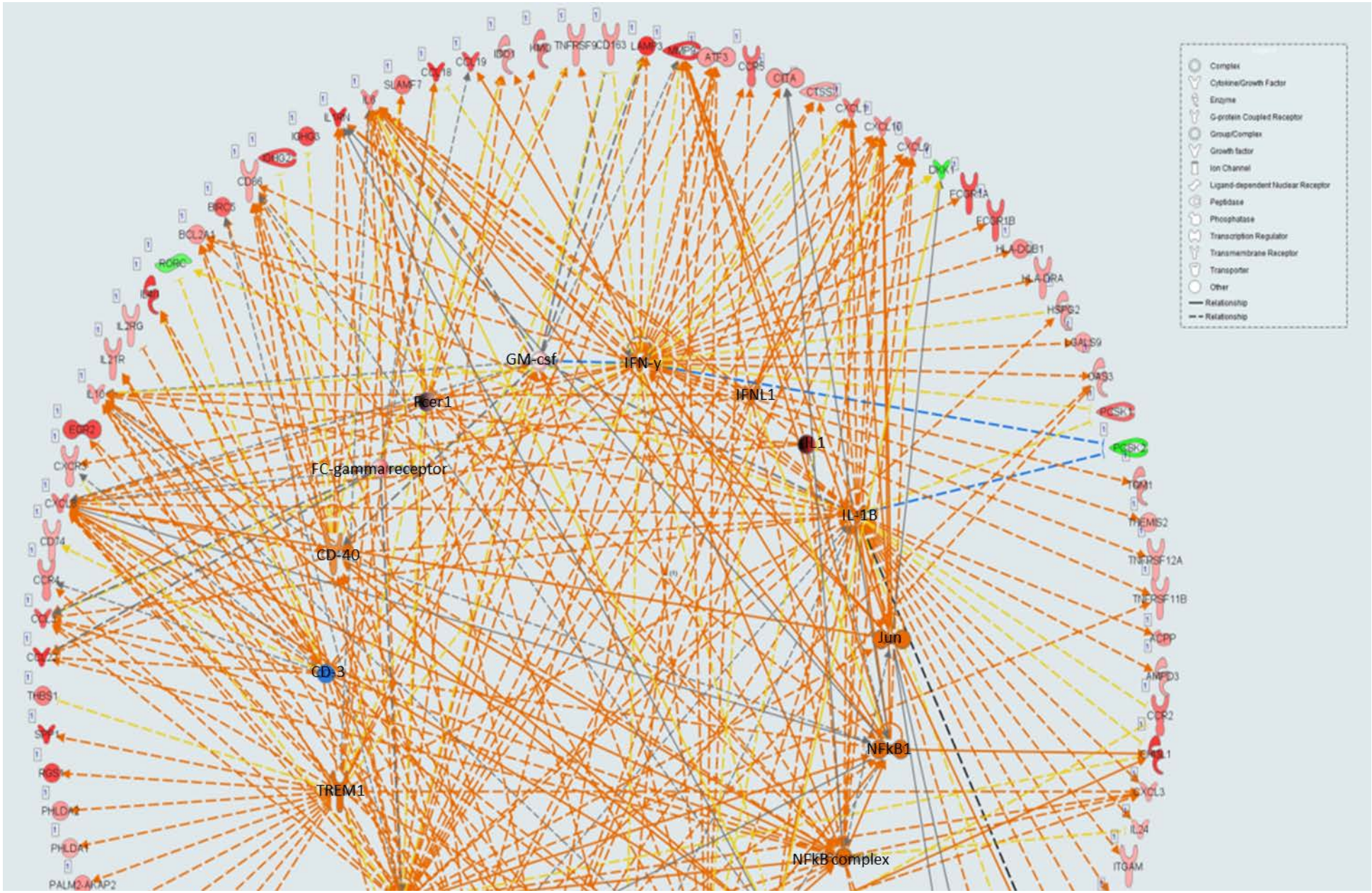


Figure 6.6 Network of genes differentially expressed in scWAT from metabolically healthy obese individuals in comparison to normal weight at study entry (week 0) featured in 'Disease and Bio Function' pathways with roles in skin cancer, diabetes and inflammation of organ.


GM-csf
IFN-γ
IFNL1
Rcer1
IL1
FC-gamma receptor
IL-1B
CD-40
Jun
CD-3
NFkB1
TREM1
NFkB complex
Complex
Cytokine/Growth Factor
Enzyme
G-protein Coupled Receptor
Group/Complex
Growth factor
Ion Channel
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter
Other
Relationship
Relationship

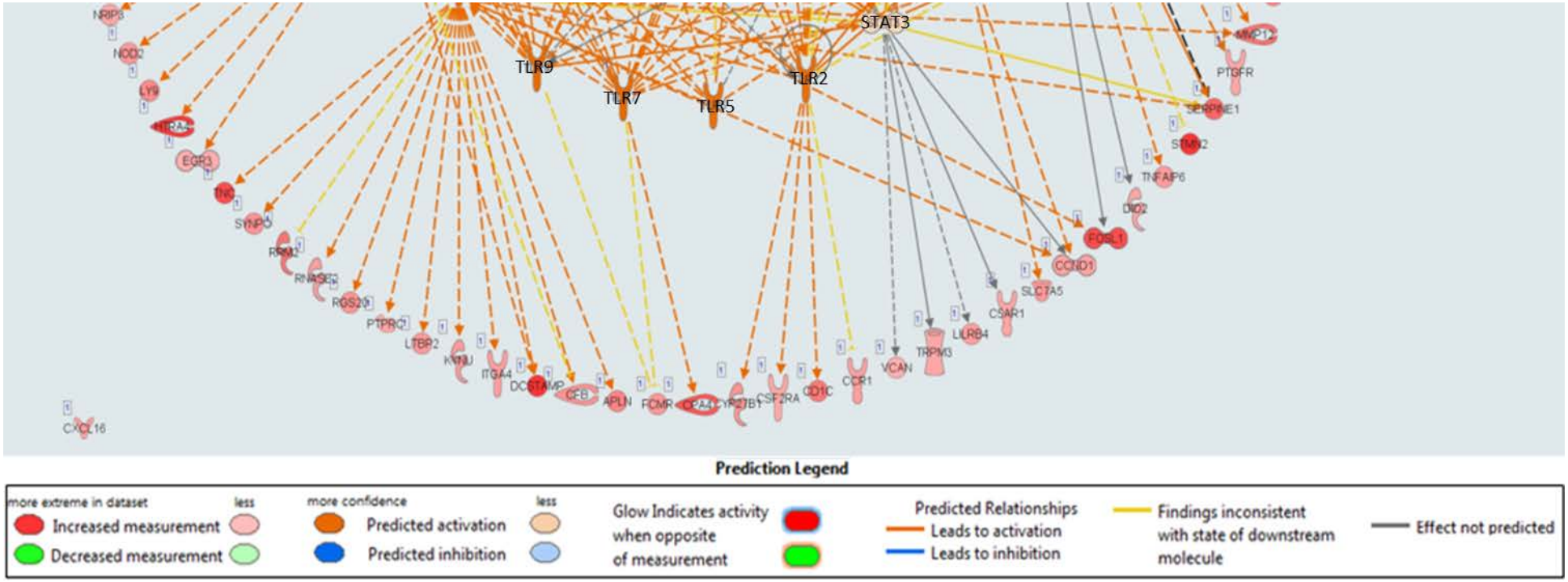


Figure 6.7 Upstream regulator analysis: Network of selected upstream regulators and their predicted activation and association with differentially expressed genes in scWAT from metabolically healthy obese individuals in comparison to normal weight at study entry (week 0).

Red = up-regulated, green = down-regulated.

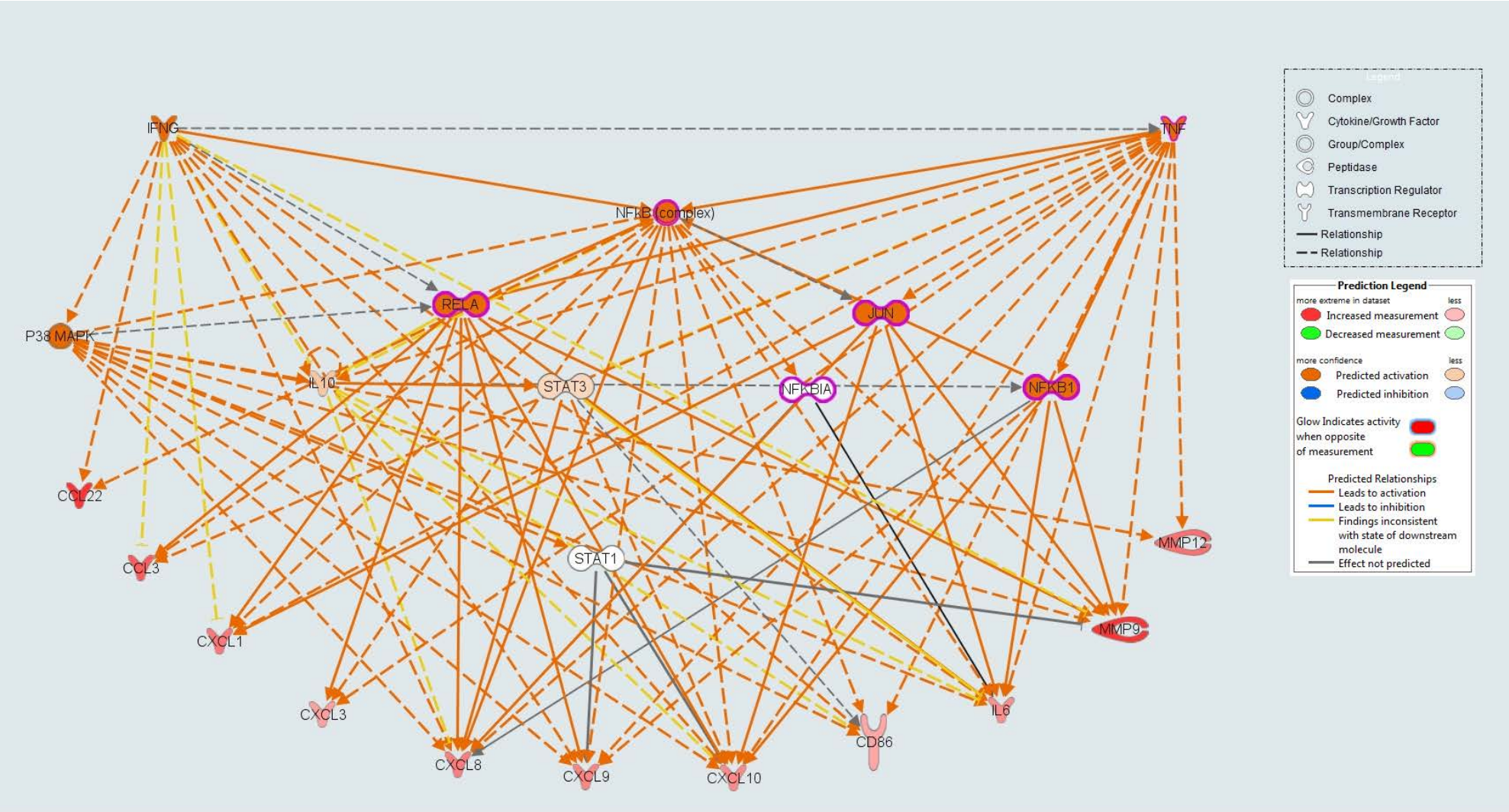


Figure 6.8 Enhanced view of upstream regulation of NF-κB by IFN-γ and TNF in metabolically healthy obese individuals at study entry (week 0)

6.4.2 Post intervention results

6.4.2.1 12-week fish oil intervention significantly modulated the expression of scWAT genes involved in inflammatory and immune response and tissue remodelling in normal weight individuals

Due to the limited number of genes (n= 7) meeting FDR criteria of $\leq$ 0.05 in MHO individuals following the intervention period, the FDR threshold was decreased to $\leq$ 0.1. To keep the analyses consistent, this revised FDR was also applied to data obtained from normal weight individuals. Sequencing of RNA extracted from the scWAT from normal weight individuals revealed the differential expression of 51 genes following 12-week FO intervention (meeting significance criteria of $\leq$ 0.05 and FDR $\leq$ 0.1). Of these, 26 genes had at least a 2 x FC in expression (14 up regulated and 12 down regulated) (Table 6.7). A full list of genes differentially expressed in the scWAT from MHO individuals following FO intervention outside the cut off criteria for IPA analysis is reported in Appendix L.

Investigation of networks and upstream analysis was not appropriate due to the limited number of genes differentially expressed in response to FO intervention in MHO individuals. Instead GO was investigated and genes upregulated in normal weight individuals in response to 12-week FO intervention were associated with: cell-cell signalling, cell adhesion, cell differentiation, clearance of apoptotic cells, blood vessel remodelling, amino acid and potassium transport, and glucose homeostasis (GO biological processes, Table 6.6). Genes downregulated in normal weight individuals in response to 12-week FO intervention were associated with: inflammatory response, immunoglobulin production, complement activation and immune response, and regulation of circadian rhythm. These results may suggest FO mediated promotion of tissue remodelling in the resolution of inflammation including immune cell signalling and autophagy, and improvement of glucose tolerance, as well as dampening of over-active inflammatory and immune responses in normal weight scWAT (GO biological processes, Table 6.7).

Gene ID	Full name	[1]log_2-Fold Change	[1]*P* Value	FDR	GO: Biological Processes
KCNH2	Potassium voltage gated channel subfamily H member 2	2.67	≤0.001	0.054	Potassium ion transport
FAM101A	Refilin-A	1.74	≤0.001	0.058	Regulation of chondrocyte development Actin filament binding
GCGR	Glucagon receptor	1.74	≤0.001	0.05	Glucose homeostasis
FOXC2	Forkhead box-C2	1.71	≤0.001	0.044	Blood vessel remodelling Cell differentiation
POF1B	Actin binding protein	1.69	≤0.001	0.019	Actin cytoskeleton organisation Epithelial cell morphogenesis
KIAA1644	Shisha like-1	1.56	≤0.001	0.015	Integral component of membrane
FBXO40	F box protein-40	1.45	≤0.001	0.089	Muscle cell differentiation Post translational protein modification
CSN1S1	Casein alpha-S1	1.43	≤0.001	≤0.001	Response to 11-deoxycorticosterone, estradiol and progesterone
MMRN1	Multimerin-1	1.41	≤0.001	0.101	Blood coagulation and cell adhesion
SLC36A2	Solute carrier family 36 member-2	1.26	≤0.001	0.101	Amino acid transport
PCDHAC2	Protocadherin alpha subfamily-C2	1.12	≤0.001	0.058	Cell adhesion and cell-cell signalling
TGM2	Transglutamase-2	1.10	≤0.001	0.054	Apoptotic cell clearance Blood vessel remodelling
ERICH4	Glutamine rich-4	1.01	≤0.001	0.05	Uncharacterised

PCDHGB7	Protocadherin gamma subfamily-B7	1.00	≤0.001	0.101	Cell adhesion and cell-cell signalling
PROK2	Prokineticin-2	-1.87	≤0.001	0.024	Angiogenesis Inflammatory response Circadian rhythm
IGLV1-44	Immunoglobulin lambda variable 1-44	-1.84	≤0.001	0.014	Complement activation Immunoglobulin production Immune response
IGLV1-51	Immunoglobulin lambda variable 1-51	-1.68	≤0.001	0.014	Complement activation Immunoglobulin production Immune response
IGHG4	Immunoglobulin heavy constant gamma-4	-1.58	≤0.001	0.094	B cell receptor signalling Complement activation Cytokine mediated signalling
S100A8	S100 calcium binding protein-A8	-1.53	≤0.001	0.024	Acute and chronic inflammatory response
RPS27P23	Ribosomal protein S27 pseudogene 23	-1.42	≤0.001	0.101	Uncharacterised
IGLC3	Immunoglobulin lamdba constant-3	-1.33	≤0.001	0.014	B cell receptor signalling Complement activation Cytokine mediated signalling
TREM1	Triggering receptor expressed on myeloid cells-1	-1.33	≤0.001	0.014	Humoral and innate immune response
LINC01260	Long intergenic non-protein coding RNA-1260	-1.19	≤0.001	0.014	Uncharacterised
AADACL2	Arylacetamide deacetylase	-1.14	≤0.001	0.024	Catabolic process

IGHA2	Immunoglobulin heavy constant alpha-2	-1.1	≤0.001	0.041	B cell receptor signalling Complement activation Cytokine mediated signalling
MTRNR2L1	MT-RNR2 like-1	-1.07	≤0.001	0.014	Associated with Smith-Magenis syndrome

Table 6.7 Differentially expressed genes meeting significance $P \leq 0.05$ and FDR < 0.1 in response to FO intervention in normal weight individuals.

[1]Log_2 Fold change and *P* values were obtained by comparison of study entry week-0) and post fish oil intervention (week-12) data of scWAT from normal weight in a general linear model likelihood ratio test in Edge R software.

6.4.2.2 12-week fish oil intervention modulates the expression of scWAT genes involved in immune response and signalling in MHO individuals but to a lesser extent than in normal weight individuals

An FDR threshold ≤ 0.1 was also applied to post intervention data from MHO individuals. Sequencing of RNA extracted from scWAT from MHO individuals following 12-week FO intervention revealed the differential expression of 21 genes meeting significance $P \leq 0.05$, with 8 of these displaying at least a 2 x FC in expression (3 up regulated and 5 down regulated) (Table 6.7). A full list of genes differentially expressed in the scWAT from MHO weight individuals following FO intervention outside the cut off criteria for IPA analysis is reported in Appendix K.

Investigation of networks and upstream analysis was not appropriate due to the number of genes differentially expressed in response to FO intervention in MHO individuals. Instead, GO was investigated and genes upregulated in MHO individuals in response to 12-week FO intervention were associated with cytokine mediated signalling, negative regulation of JAK/STAT cascade, and negative regulation of cell proliferation (GO biological processes, Table 5.8). Genes downregulated in MHO individuals in response to 12-week FO intervention were associated with: immunoglobulin production and complement activation in immune response, cell differentiation and negative regulation of adhesion, positive regulation of chronic inflammatory response, and regulation of Wnt signalling in MHO scWAT (GO biological processes, Table 6.8).

Gene ID	Full name	[1]log_2-Fold Change	[1]*P* Value	FDR	GO: Biological Processes
FGF12-AS2	FGF12 Antisense RNA-12	1.46	≤0.001	0.073	Uncharacterised
LRRTM4	Leucine rich repeat transmembrane neuronal-4	1.09	≤0.001	0.099	Cytokine mediated signalling pathway Negative regulation of JAK/STAT cascade
MAB21L1	MAB-21 like-1	1.06	≤0.001	0.002	Anatomical structure morphogenesis Negative regulation of cell proliferation
IGLV1-47	Immunoglobulin lambda variable 1-47	-2.56	≤0.001	0.073	Complement activation Immunoglobulin production immune response
IGLV1-44	Immunoglobulin lambda variable 1-44	-1.99	≤0.001	0.092	Complement activation Immunoglobulin production immune response
TDRD12	Tudor domain containing-12	-1.99	≤0.001	0.043	Cell differentiation DNA methylation in gamete generation
DACT2	Dishevelled binding antagonist of beta catenin-2	-1.25	≤0.001	0.08	Negative regulation of cell adhesion Regulation of Wnt signalling
IDO1	Indoleamine 2, 3-dioxygenase-1	-1.08	≤0.001	0.077	Cytokine production in immune response Negative regulation of chronic inflammatory response

Table 6.8 Differentially expressed genes meeting significance $P \leq 0.05$ and FDR < 0.1 in response to FO intervention in metabolically healthy obese individuals.

[1]Log_2 Fold change and *P* values were obtained by comparison of study entry (week-0) and post fish oil intervention (week-12) data of scWAT from metabolically healthy obese individuals in a general linear model likelihood ratio test in Edge R software.

6.4.2.3 Non-significant effects of 12-week fish oil intervention

Despite FO intervention significantly regulating only a limited number of genes, there were trends for the regulation of many transcripts that were differentially expressed in scWAT from MHO individuals in comparison to normal weight at study entry (week-0) including *MMP7, MMP9, IL-10, IL-1β, CCL3 (*MIP1α*)* and *CCL18* (Figure 6.12). There were no direct significant effects of FO intervention on these in either normal weight or MHO individuals but when comparing the FC difference of genes differentially expressed in obesity at study entry and post 12-week FO intervention, large differences were observed. At study entry *MMP7* and *MMP9* were significantly upregulated in MHO individuals in comparison to normal weight by a FC of 42.5 and 16.3 respectively; following FO intervention, this upregulation of *MMP7* and *MMP9* decreased to only 10.7 FC (a change of 31.8 fold) and 8.2 (a change of 8.1 fold) in MHO individuals in comparison to normal weight individuals (Figure 6.11).

The regulation of key inflammatory genes also exhibited changes following 12-week FO intervention; *IL-1β, NF-κB, IKBKB (IKK), IKBKE* and *TNF* were no longer differentially expressed in scWAT from MHO individuals compared to scWAT from normal weight individuals despite no direct significant effects of FO intervention on these genes in either MHO or normal weight individuals (Figure 6.11). *IKBKE* encodes the inhibitor of NF-κB epsilon and is predominantly expressed in macrophages (Patel et al., 2015); increased expression in obesity may indicate an increase in the presence of these cells in the scWAT in comparison to normal weight individuals. *IKBKE* expression is reported to positively correlate to BMI in humans and knock-out murine models have provided evidence for its involvement in NLRP3 inflammasome priming, adipose energy balance, and obesity associated dysregulation of metabolism (Patel et al., 2015). The upregulation of NF-κB and IKK, which phosphorylates IKB subsequently activating NF-κB, in obesity is likely to result in NF-κB mediated inflammatory responses including inflammasome priming.

The loss of differential expression of *IL-1β, NF-κB, IKBKB (IKK), IKBKE* and *TNF* following 12-week FO intervention may suggest negative regulation of pro-inflammatory cascades including NF-κB signalling and inflammasome priming, and possible changes in scWAT immune cell populations. Changes in the differential expression of *CCL3* and *CCL18* following FO intervention may provide further evidence for potential effects of FO intervention on modulating scWAT immune cell populations, in particularly macrophages; and changes in MMP expression following FO intervention may suggest a role of FO in the regulation of scWAT remodelling.

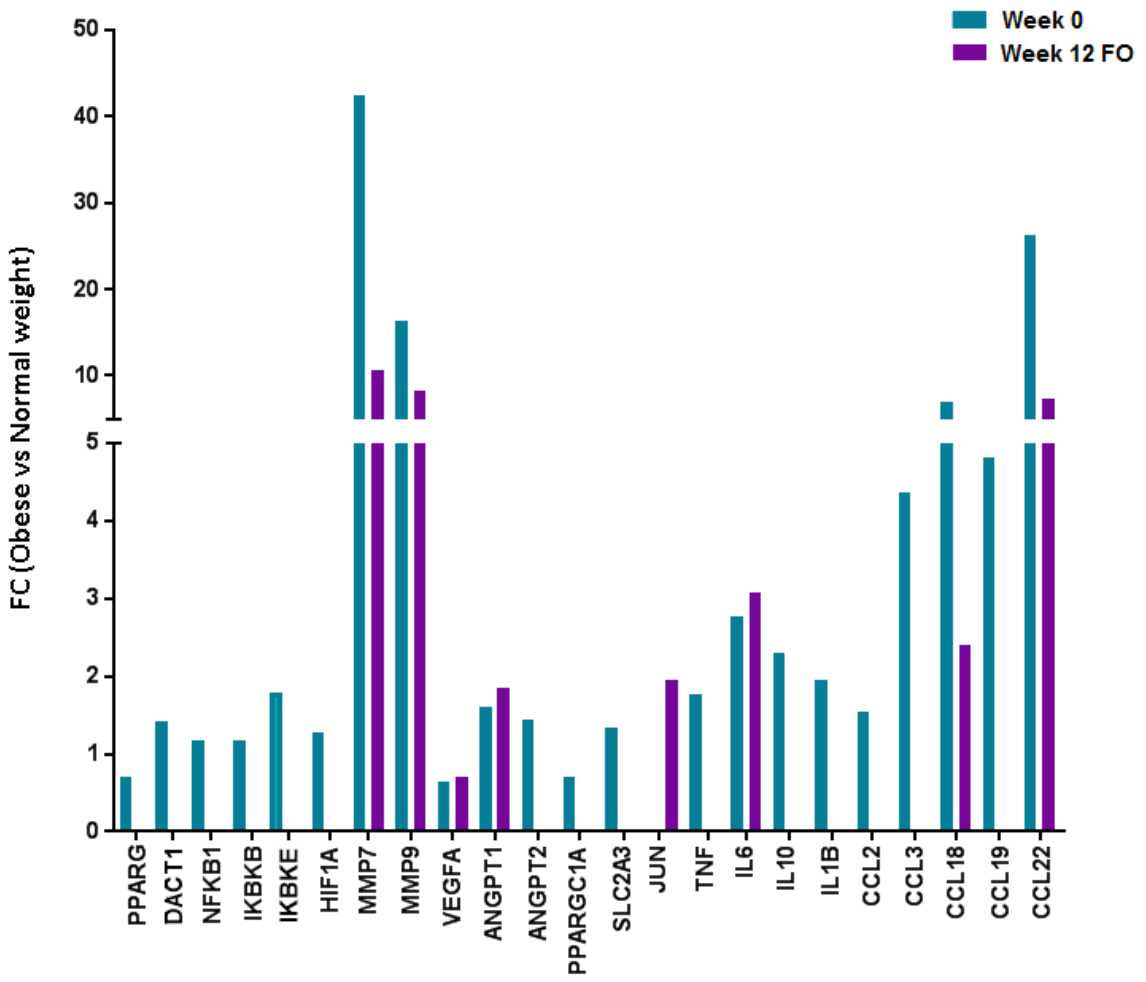


Figure 6.9 Fold change (FC) in expression of scWAT genes which display noticeable changes in FC expression between metabolically heathy obese and normal weight individuals following 12-week FO intervention in comparison to the fold change between metabolically healthy obese and normal weight at study entry (week-0), despite no significant effect of FO intervention.

6.4.2.4 Differential gene expression in scWAT from normal weight individuals following 12-week corn oil intervention

Sequencing of RNA extracted from the scWAT of normal weight individuals revealed 17 genes differentially expressed following 12-week CO intervention (meeting significance and FDR criteria of ≤ 0.05). Of these, 7 genes had FC ≥ 2 in expression (all up regulated) (Table 6.9).

Investigation of networks and upstream analysis was not appropriate due to the number of genes differentially expressed. Instead, GO was investigated which indicated genes upregulated in normal weight individuals in response to CO intervention were associated with cell differentiation,

adhesion and apoptotic processes, regulation of blood vessel size, nervous system development, oxidation reduction, and inactivation of MAPK activity (GO biological processes, Table 6.9). No genes were significantly downregulated in response to CO intervention in normal weight individuals.

These data suggest minor effects of CO intervention in normal weight individuals which may result in changes to cell activity including differentiation, survival and adhesion, and nervous system development. However, the significance of the contribution these genes have to such pathways at a biologically relevant level is not understood. Supplementation with CO would likely increase dietary intake of LA by 10% (compared to the average UK daily intake of 10 g/d $\pm$ 4.7 g/day) (Pot et al., 2012) and therefore effects of CO were not the primary focus of the intervention.

Gene ID	Full name	[1]log_2-Fold Change	[1]*P* Value	FDR	GO: Biological Processes
NTS	Neurotensin	5.76	≤0.001	0.035	G protein coupled receptor signalling pathway Regulation of blood vessel size
ANLN	Anillin actin binding protein	1.63	≤0.001	0.044	Cortical cytoskeleton organisation Hematopoietic progenitor cell differentiation
CNTN6	Contactin-6	1.61	≤0.001	≤0.001	Cell adhesion Central nervous system development
FMO6P	Flavin containing monooxygenase-6 pseudogene	1.55	≤0.001	0.016	Oxidation reduction process
TOP2A	DNA topoisomerase II-alpha	1.45	≤0.001	0.013	Cellular response to DNA damage stimulus
PAX3	Paired box-3	1.39	≤0.001	0.004	Apoptotic process Nervous system and muscular organ development
DUSP8	Dual specificity phosphatase-8	1.01	≤0.001	≤0.001	De-phosphorylation Inactivation of MAPK activity

Table 6.9 Differentially expressed genes meeting significance and FDR < 0.05 in response to CO intervention in normal weight individuals.

[1]Log_2 Fold change and *P* values were obtained by comparison of study entry (week-0) and post corn oil intervention (week-12) data of scWAT from normal weight individuals in a general linear model likelihood ratio test in Edge R software.

6.4.2.5 Differential gene expression in scWAT in MHO individuals following corn oil intervention

Sequencing of RNA extracted from the scWAT from MHO individuals revealed 22 genes differentially expressed following 12-week CO intervention (meeting significance and FDR criteria of ≤ 0.05). Of these, 13 genes had at least a 2 x FC in expression (7 up regulated and 6 down regulated) (Table 6.10).

Investigation of networks and upstream analysis was not appropriate due to the number of genes differentially expressed. Instead, GO was investigated which indicated that genes upregulated in MHO individuals in response to 12-week CO intervention were associated with retinol metabolism, brain development, proteolysis, cell adhesion and negative regulation of Wnt signalling pathways (GO biological processes, Table 6.10). Genes that were downregulated in MHO individuals in response to 12-week CO intervention were associated with cellular protein metabolism, glycogen biosynthesis, ageing, and epidermis development (GO biological processes, Table 6.10).

These data suggest minor effects of CO intervention in MHO individuals which may result in potential changes to regulation of central nervous system development and function, skin development and down regulation of glycogen biosynthesis. The significance of the contribution these genes have to such pathways at a biologically relevant level are not understood, and the effects of CO were not the primary focus of the intervention.

Gene ID	Full name	[1]log_2-Fold Change	[1]*P* Value	FDR	GO: Biological Processes
STRA6	Stimulated by retinoic acid-6	1.82	≤0.001	0.005	Retinol metabolism
GRIN2A	Glutamate ionotropic membrane exocytosis-4	1.61	≤0.001	0.009	Brain development
RIMS4	Regulating synaptic membrane exocytosis-4	1.42	≤0.001	0.007	Regulation of synaptic vesicle exocytosis and synaptic transmission
WFDC2	WAP four disulfide core domain-2	1.99	≤0.001	0.034	Negative regulation of peptidase activity Proteolysis
TPSG1	Tryptase gamma-1	1.57	≤0.001	0.031	Proteolysis
HAPLN2	Hyaluronan and proteoglycan link protein-2	1.39	≤0.001	0.034	Cell adhesion Central nervous system development
NKD1	Naked cuticle homolog-1	1.28	≤0.001	0.027	Negative regulation of Wnt signalling pathway
BPIFB2	BPI fold containing family B member-2	-1.67	≤0.001	0.001	Antimicrobial humoral response Cellular protein metabolic process
GYS2	Glycogen synthase-2	-1.47	≤0.001	≤0.001	Glycogen biosynthetic process
LUZP2	Leucine zipper protein-2	-1.03	≤0.001	≤0.001	Uncharacterised
KRT16	Keratin-16	-1.77	≤0.001	0.013	Aging Epidermis development
KRT14	Keratin-14	-1.64	≤0.001	0.028	Aging Epidermis development
RPL7P28	Ribosomal protein L7 pseudogene-28	-1.48	≤0.001	0.029	Uncharacterised

Table 6.10 Differentially expressed genes meeting significance and FDR < 0.05 in response to CO intervention in metabolically healthy obese individuals.

[1]Log_2 Fold change and *P* values were obtained by comparison of study entry (week-0) and post corn oil intervention(week-12) data of scWAT from metabolically healthy obese individuals in a general linear model likelihood ratio test in Edge R software.

6.5 Discussion and conclusion

The aim of the research described in this chapter was to obtain whole transcriptome information for human scWAT to compare expression profiles between normal weight and MHO individuals, and to identify effects of 12-week FO intervention in order to understand more about the biological processes that may be affected by obesity and LC n-3 PUFA. The current study reports altered regulation of many genes in scWAT from MHO individuals in comparison to those of normal weight consistent with enhanced scWAT inflammation, remodelling and progression to metabolic dysfunction. The current study further reports significant effects of FO intervention on inflammatory gene expression in scWAT; however, MHO individuals appear less sensitive to the effects of FO, potentially providing evidence for decline in scWAT function in metabolically healthy obesity.

6.5.1 Dysregulation of scWAT transcriptome in obesity

scWAT from MHO individuals displayed a dysregulated transcriptomic profile in comparison with that from normal weight individuals. To simplify the information, regulatory data and pathway analysis information in section 6.4.1.2 were combined and categorised into 7 main biological processes. Figure 6.10 details the percentage of genes differentially expressed by at least a 2 FC in obesity that are associated with these 7 main processes. The current study can conclude there was an upregulation of inflammatory and immune response, particularly in the context of tissue expansion and remodelling, as well as changes to lipid and glucose signalling and metabolism in scWAT from MHO individuals (Figure 6.10). Figure 6.11 depicts the actions of this signalling in the scWAT in MHO individuals.

At a cellular level, obesity was associated with the differential regulation of genes that promote cellular development and death, cell proliferation, cell-cell interaction, and cell movement. This may reflect increases in adipocyte generation and expansion to accommodate increased TG storage (Drolet et al., 2008, Wang et al., 2013), as well as adipocyte necrosis, in part due to increased hypoxia in the expanding tissue (Yin et al., 2009), the differentiation of immune cells, and communication between cells involved in the recruitment of immune cells to the site of necrosing tissue (Cinti et al., 2005, Grant and Dixit, 2015) (Figure 6.12). Figure 6.12 depicts a summary of the signalling pathways with the associated genes that were differentially expressed in scWAT from MHO individuals in the current study.

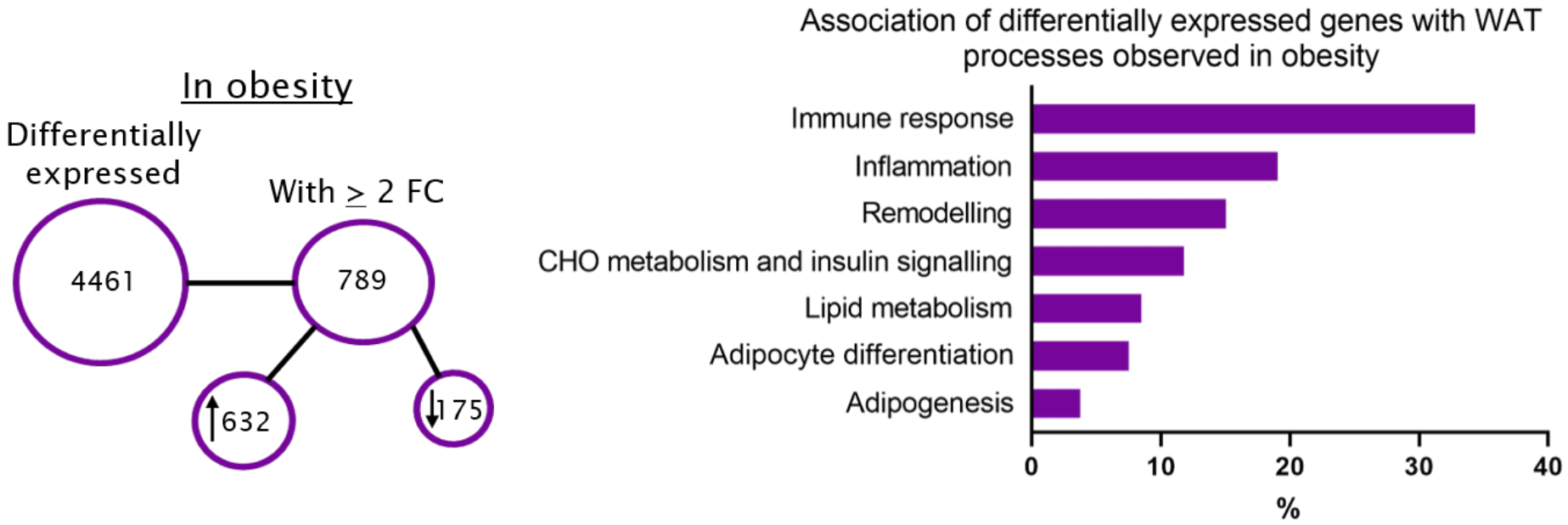


Figure 6.10 Regulation of gene expression and percentage of differentially expressed genes associated with scWAT processes in metabolically healthy obese individuals at study entry (week-0).

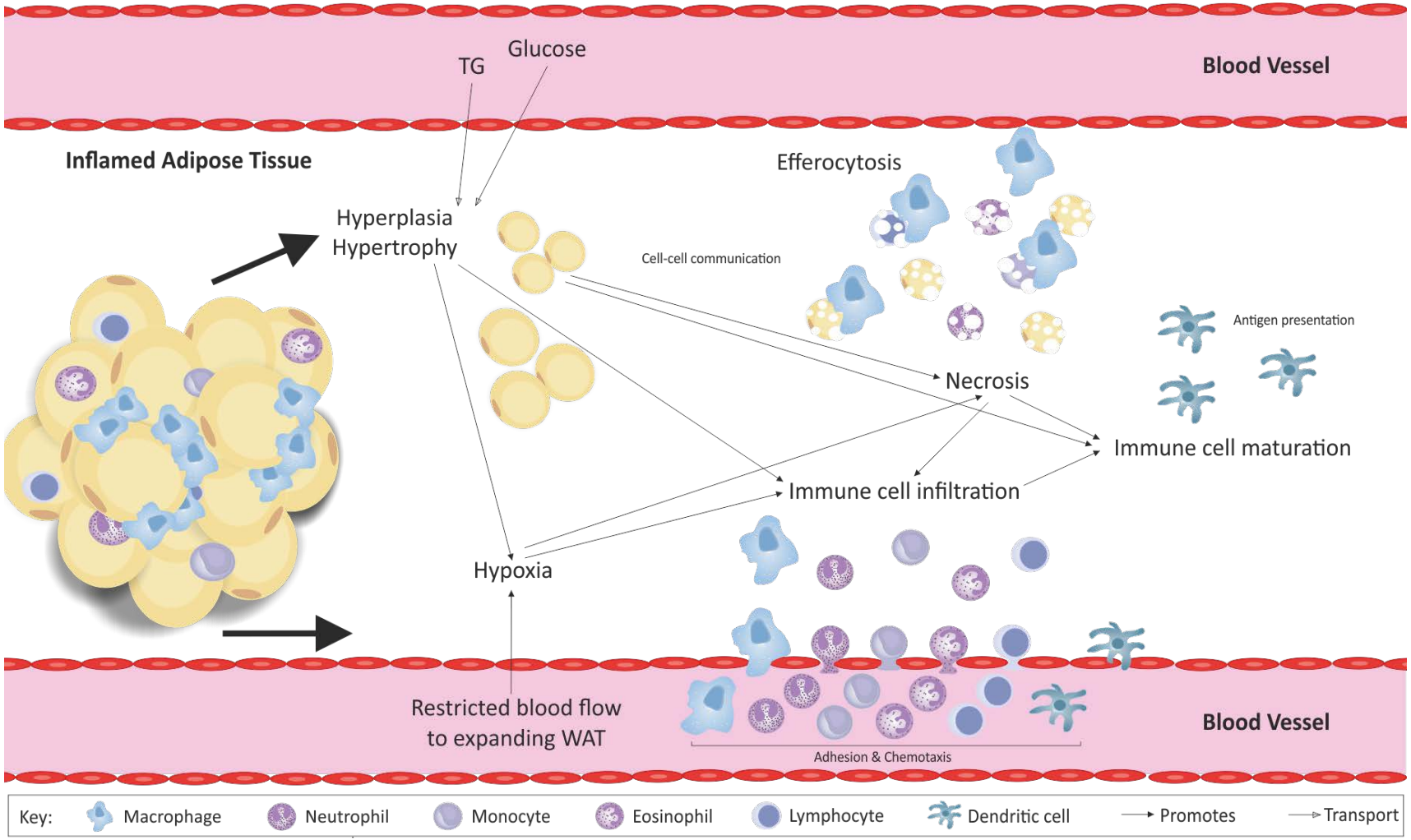


Figure 6.11 Upregulation of inflammatory and immune cell signalling in scWAT from metabolically healthy obese individuals at study entry (week-0).

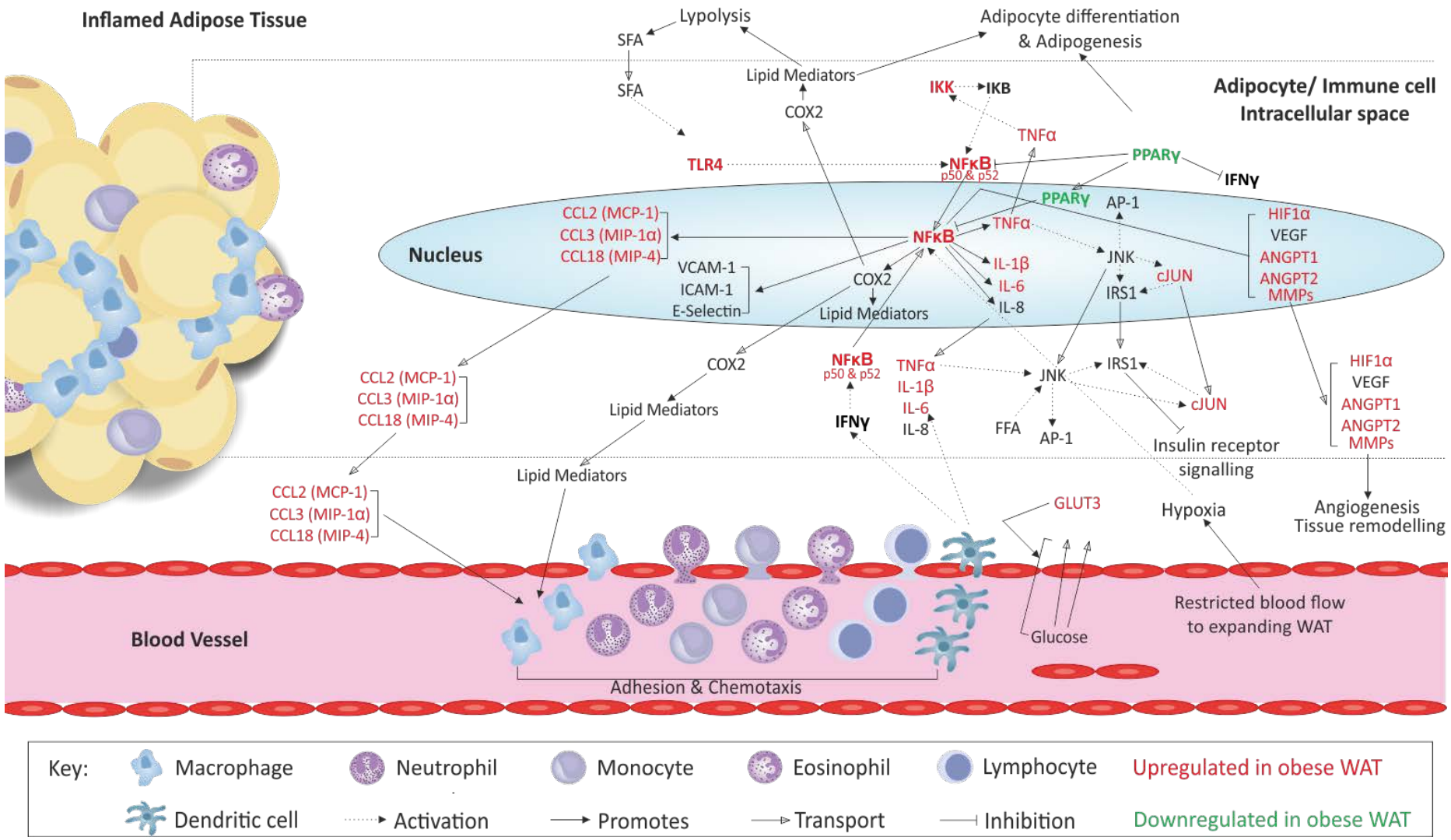


Figure 6.12 Inflammatory signalling by genes dysregulated in metabolically healthy obese individuals at study entry (week-0) and their upstream regulators.

6.5.1.1 Dysregulation of scWAT transcriptome in obesity: Adipose expansion and remodelling

scWAT hyperplasia is regulated by a range of transcription factors including CEBP-α, -β, and -δ, SREBP1C and PPAR-γ-1, and -2 (Tontonoz et al., 1994). Collectively, these factors act to induce and maintain adipocyte differentiation as well as to promote the expression of lipogenic genes, such as GLUT4, FAS, and acetyl-coA carboxylase, to promote the formation of mature lipid accumulating adipocytes (Tontonoz et al., 1994, Gregoire et al., 1998, Tamori et al., 2002, Choe et al., 2016). The current study reports a 2.38 fold decrease in *CEBP-AS1* (P <0.001, Appendix I) which encodes CEBP antisense RNA; antisense RNA binds with mRNA and blocks its's translation into an active protein. This may suggest an increase in expression of CEBP in scWAT from MHO individuals due to increased opportunity for mRNA translation to protein.

The current study further reports a 1.4 fold decrease in the expression of *PPAR-γ* in the scWAT from MHO individuals in comparison to scWAT from normal weight individuals (P <0.001, Appendix J). As well as regulating adipogenesis, PPAR-γ has been reported to interfere with NF-κB signalling to decrease inflammation in activated macrophages and T cells (Ricote et al., 1998, Castrillo et al., 2000 , Straus et al., 2000, Yang et al., 2000, Chawla et al., 2001). There was significant 1.17 fold increase in the expression of *NFKB1* (p50/105 subunit), a 1.42 fold increase in *NFKB2* (p52/100 subunit) expression, a 1.60 increase in *REL* expression, a 1.76 fold increase in *RLB* expression, and a 1.18 fold increase in the expression of the upstream activating kinase, *IKK-β* in obesity at study entry, as well as significant increases in the expression of the downstream NF-κB target genes *IKBKB, IKBKE, IL-1β* and *TNF* (P <0.05, Appendix I and Appendix J.).

As previously discussed, activation of IKK-β leads to the phosphorylation-dependent degradation of Inhibitor of kappaB (IkB) resulting in the nuclear translocation and activation of the transcription factor NF-κB. Free NF-κB can migrate to the nucleus where it acts as a transcription factor regulating the expression of many genes encoding cytokines, adhesion molecules, lipid mediators and hormones such as leptin to further promote inflammation and scWAT dysfunction in obesity (Baldwin, 1996, Barnes and Karin, 1997, Rossi et al., 2000, Baker et al., 2011, Grabbe et al., 2011) (Figure 6.13). IKK-β has also been shown to play a role in adipocyte differentiation; inhibition of IKK prevented adipocyte differentiation in human stem cells *in vitro* and in mice *in vivo*, and protected against high fat diet induced obesity in mice (Helsley et al., 2016). IKK has been further implicated in inflammation associated with obesity in response to nutrient excess, in which it mediates inflammatory metabolite and immune cell activation, and is implicated in lipid induced insulin resistance (Figure 6.14).

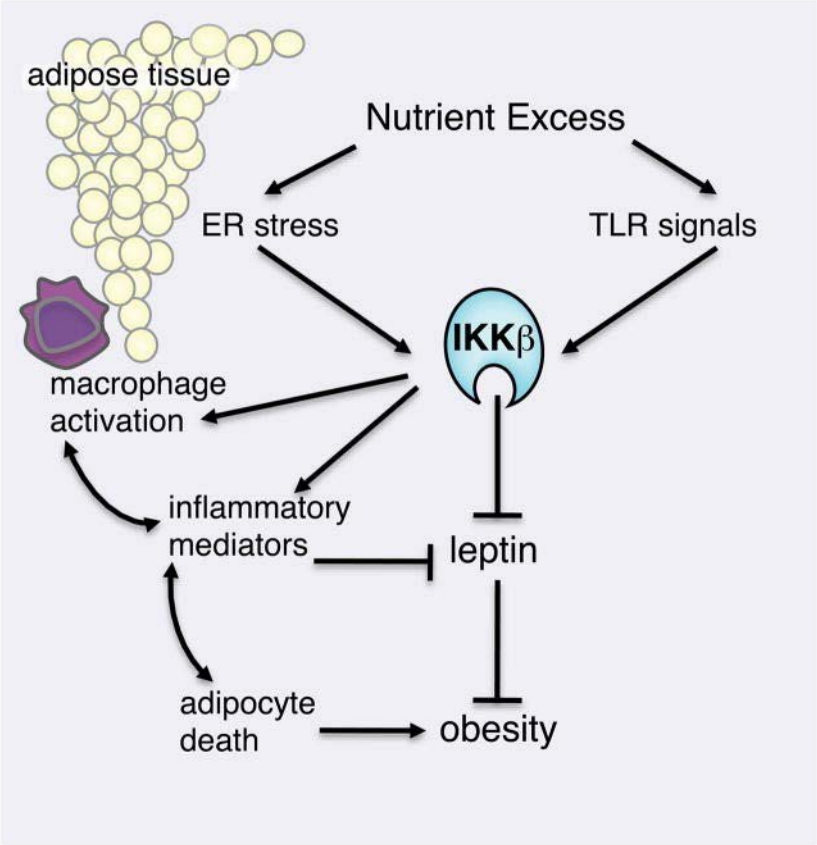


Figure 6.13 Effect of nutrient excess (obesity) on IKK-beta signalling in scWAT (Baker et al., 2011).

As discussed above, when scWAT expands, blood flow is reduced and oxygen supply is compromised resulting in hypoxic conditions (Halberg et al., 2009, Pasarica et al., 2009b, Lee et al., 2010). An increase in HIF1-α is often seen in these conditions which stimulates the expression of genes involved in angiogenesis and erythropoiesis, promoting blood vessel formation and ECM remodelling in the expanding tissue (Hosogai et al., 2007, Wang et al., 2007, Halberg et al., 2009, Pasarica et al., 2009a, Sun et al., 2013, Tahergorabi and Khazaei, 2013). In murine models, *HIF1-α* KO has been shown to improve glucose tolerance and insulin sensitivity, as well as to decrease infiltration of inflammatory macrophages, tissue fibrosis, and the concentration of inflammatory cytokines including TNF-α, MCP-1, RANTES, IL-6, and MIP-1α (Kihira et al., 2014). In concordance with this, the activation of HIF1-α has been observed to induce tissue fibrosis that was associated with the upregulation of a number of ECM molecules and their regulators. Without ECM remodelling, adipocytes become restricted, lose function and necrose, promoting a pro-inflammatory cascade of immune cell infiltration and signalling (Sun et al., 2013).

Degradation of existing ECM and formation of new vasculature is promoted by the expression of MMPs and pro-angiogenic factors such as HIF1-α, VEGF-α, and Ang-1 and-2 (Arany et al., 2008, Adair and Montani, 2011, Corvera and Gealekman, 2014). There is evidence for an increase in these pro-angiogenic factors in human obesity and positive correlations with BMI (Chavey et al.,

2003, Silha et al., 2005); data from the current study are in agreement with these reports and MHO was associated with a profile indicative of hypoxic conditions. *HIF1-α, ANGPT1, ANGPT2, PPARGC1α* and a range of MMPs, most notability *MMP7* and *MMP9*, were upregulated in scWAT from MHO individuals in comparison to normal weight at study entry ($P < 0.001$, Appendix J) indicative of hypoxia and the promotion of angiogenesis and remodelling in an attempt to re-vascularise the tissue (Figure 6.13).

6.5.1.2 Dysregulation of scWAT transcriptome in obesity: Lipid and carbohydrate metabolism and signalling

A number of genes involved in lipid metabolism were differentially expressed in obesity (Figure 6.11) of particular interest and relevance to the differences observed in lipid metabolite profiles described in Chapter 4, may be the regulation of phospholipase A_2 (PLA_2) isoenzymes. Several genes encoding PLA_2 isoenzymes were upregulated in MHO individuals; of note, the expression of *PLA2G4A* by 1.3-fold, *PLA2G2D* by 2.5-fold, and *PLA2G7* by 11.6-fold ($P < 0.01$, FDR < 0.05). Upregulation of PLA2G4A has been reported with high fat feeding in rats (Iyer et al., 2012), and the upregulation of a number of PLA_2 isoenzymes has been reported in human non-diabetic scWAT with *PLA2G7* expression observed to correlate with circulating lipids, indicating association with an unfavourable lipid profile, and to have importance for the oxidation of LDL in adipose macrophages (Jackisch et al., 2018).

These PLA_2 isoenzymes have a role in the liberation of FAs, which is required for the synthesis of fatty acid metabolites as described in Chapter 4. A recent study by Sato *et al.* describes the importance of PLA2G2D in the mobilisation of membrane bound PUFA in the WAT (Sato et al., 2020). They have previously reported down regulation of *PLA2G2D* in high fat fed mice (Sato et al., 2014), and now further report exclusive expression in WAT macrophages, most enriched in M2 macrophages, and a pivotal role in the mobilisation of WAT PUFA, particularly LC n-3 PUFA, which are observed to be lower in *PLA2G2D* knock out animals (Sato et al., 2020). This isoenzyme is however upregulated in the scWAT of MHO individuals in the current study. Unfortunately, total FAs are reported in the current study so associations between *PLA2G2D* and levels of "free" PUFA cannot be examined. Furthermore, Sato *et al.* report that despite these changes in *PLA2G2D* and PUFA, no changes to metabolites of these FAs were observed suggesting that WAT PUFA are hardly converted to lipid metabolites (Sato et al., 2020). The current study observes *PLA2G2D* to be negatively correlated with a number of AA, EPA, and DHA derived metabolites in addition to measures of metabolic health of the tissue including insulin resistance, number of macrophages and crown like structures ($P \leq 0.050$, Table 6.11). This may suggest association between *PLA2G2D* and the metabolic health of WAT and further evidence that the mobilised FAs are not readily

converted into lipid metabolites as it would seem that as *PLA2G2D* expression increases, which as Sato *et al.* report is associated with enhanced mobilisation of membrane bound PUFA (Sato et al., 2020), the levels of many LC n-3 PUFA, particularly DHA derived metabolites actually decrease.

	Coefficient	*P*
BMI	0.711	< 0.001
BF (%)	0.467	0.044
BF (kg)	0.631	0.004
Insulin	0.501	0.025
HOMA2-IR	0.507	0.023
Adipocyte diameter	0.589	0.006
Macrophage number	0.612	0.004
CLS number	0.627	0.003
HDL	-0.432	0.057
RvE3	-0.451	0.053
PGF1a	-0.400	0.090
14-15-DiHETE	-0.469	0.043
17-18-DiHETE	-0.470	0.043
11-HETE	-0.456	0.036
8-HDHA	-0.450	0.053
11-HDHA	-0.515	0.024
13-HDHA	-0.482	0.036

Table 6.11 Correlation of *PLA2G2D* with metabolic parameters and WAT fatty acid metabolites in the whole cohort (normal weight and metabolically healthy obese individuals) at study entry (week-0). (Adipocyte diameter, macrophage number, and CLS number are reported in Chapter 9).

It is important to note that *PLA2G2D* expression is positively correlated with BMI, body fat % and kg; an altered FA metabolite profile in obesity is described in Chapter 4 which includes a lower proportion of hydroxy-DHA metabolites. Whether this is due to a significantly greater proportion of n-6 PUFA in WAT cell membranes resulting in preferential utilisation over LC n-3 PUFA, or more complex pathways involving the dysregulation of many genes involved in lipid metabolism including *PLA2G2D* requires further investigation and dissection.

In addition to lipid metabolism, ~12% of differentially expressed genes at study entry in MHO individuals were associated with carbohydrate metabolism and signalling (Figure 6.11). Several of these genes were observed to be involved in metabolic disease pathways including T2DM. One physiological state that is reported to occur in obesity, that affects glucose metabolism in the scWAT is hypoxia. The transcriptome profile suggests enhanced tissue expansion in MHO

individuals in the current study and alteration of genes associated with hypoxia is reported in section 6.5.1.1. There is evidence for upregulation of genes encoding glucose transporters and proteins in mice and 3T3-L1 adipocytes under these conditions (Wood et al., 2007, Yin et al., 2009), and dysregulation of such genes is reported to be accompanied by decreased FFA uptake, increased lipolysis, and decreased insulin stimulated glucose uptake in hypoxic scWAT (Wood et al., 2007, Yin et al., 2009).

In the current study, an upregulation of the GLUT3 gene *SLC2A3*, which is expressed in circulating white blood cells, was observed in scWAT from MHO individuals (1.34 FC $P < 0.001$, Appendix J) with no dysregulation of *GLUT1* or *GLUT4* expression, the predominant glucose transporters in adipocytes, at study entry or in response to FO intervention. Further to dysregulation in GLUT3 gene expression, an upregulation of the *LEP* gene which encodes leptin was observed in the scWAT of MHO individuals (P <0.001, Appendix I); this is concordant with reports of increased circulating leptin in this cohort (Chávez, 2014). Further to this, a positive correlation was reported between circulating leptin concentrations and HOMA-IR scores (Chávez, 2014).

6.5.1.3 Dysregulation of scWAT transcriptome in obesity: Inflammatory signalling and immune response

The dysregulated transcriptomic profile observed in MHO individuals was also indicative of enhanced inflammatory signalling which may occur through the synthesis of FA metabolites. Two key genes encoding enzymes regulating the synthesis of inflammatory FA metabolites, discussed in Chapter 4, were observed to be upregulated in the scWAT from MHO individuals. *ALOX5*, encoding 5-LOX, and *PTGS1*, encoding COX-1 had a 1.75 and 1.29 FC in expression respectively (P <0.001, Appendix J) in the scWAT from MHO individuals in comparison to normal weight. This is likely to result in increased synthesis of many inflammatory fatty acid metabolites, and the dysregulation of pro- and anti-inflammatory metabolites in the scWAT which the current study provides evidence for in Chapter 4 (Appendix H). In addition, altered regulation of ECs in obesity and in response to FO intervention is further reported Chapter 4.

The expression of genes involved in EC biosynthetic and degradation pathways was observed to be dysregulated in obesity; genes involved in the synthesis of AA containing ECs and their interaction with EC receptors are depicted in Figure 6.14. No information was available for the expression of *NAPE-PLD*, the enzyme responsible for the synthesis of 20:4-EA which is reported to be increased in obesity in Chapter 4, or for *MGLL*. Further assessment of expression of genes involved in these pathways by qRT-PCR is reported in Chapter 7.

The current study also reports a -1.34 FC downregulation in expression of the CB1 gene *CNR1* in scWAT from MHO individuals (*P* = 0.01, Appendix J) which is concordant with reports by Bennetzen *et al.* and Sarzani *et al.* of a decrease in *CNR1* expression in scWAT from MHO humans in comparison to normal weight (Sarzani et al., 2009, Bennetzen et al., 2010). ECs interact with cannabinoid receptors to elicit a range of actions including stimulating adipogenesis and glucose transport, improving insulin sensitivity, and lowering the secretion of pro-inflammatory mediators such as IL-6 and MCP-1 (Bouaboula et al., 2005, Motaghedi and McGraw, 2008, Karaliota et al., 2009, Balvers et al., 2010). Downregulation of *CNR1* in obesity is likely to result in altered EC signalling despite increased concentrations of ECs reported in obesity in Chapter 4 as EC-cannabinoid receptor interaction is compromised, providing evidence for dysfunctional EC signalling in obesity. Further investigation of *CNR1* by qRT-PCR is reported in Chapter 7.

Upregulation of inflammatory signalling in obesity may also occur through increased cytokine secretion, which is activated through NF-κB, TNF-α, and IFN-γ mediated signalling (Figure 6.8 and Figure 6.12). Genes encoding IL-6, IL-10, IL1-β, TNF, and a range of chemokines including CCL2 (MCP-1), CCL3 (macrophage inflammatory protein 1-α (MIP1-α)), CCL5 (RANTES), CCL18 (macrophage inflammatory protein 4 (MIP-4)), CCL7, CCL19 and CCL22 were upregulated in scWAT from MHO individuals (*P* <0.001, Appendix I and Appendix J). These signalling molecules play a role in a range of functions including immune and inflammatory processes such as cell activation and chemotaxis, glucose metabolism (insulin sensitivity), and the modulation of cytokine secretion (Appay and L.Rowland-Jones, 2001, Trujillo and Scherer, 2006, Coppack, 2007, Deshmane et al., 2009, Finucane et al., 2012, Park and Ahima, 2014).

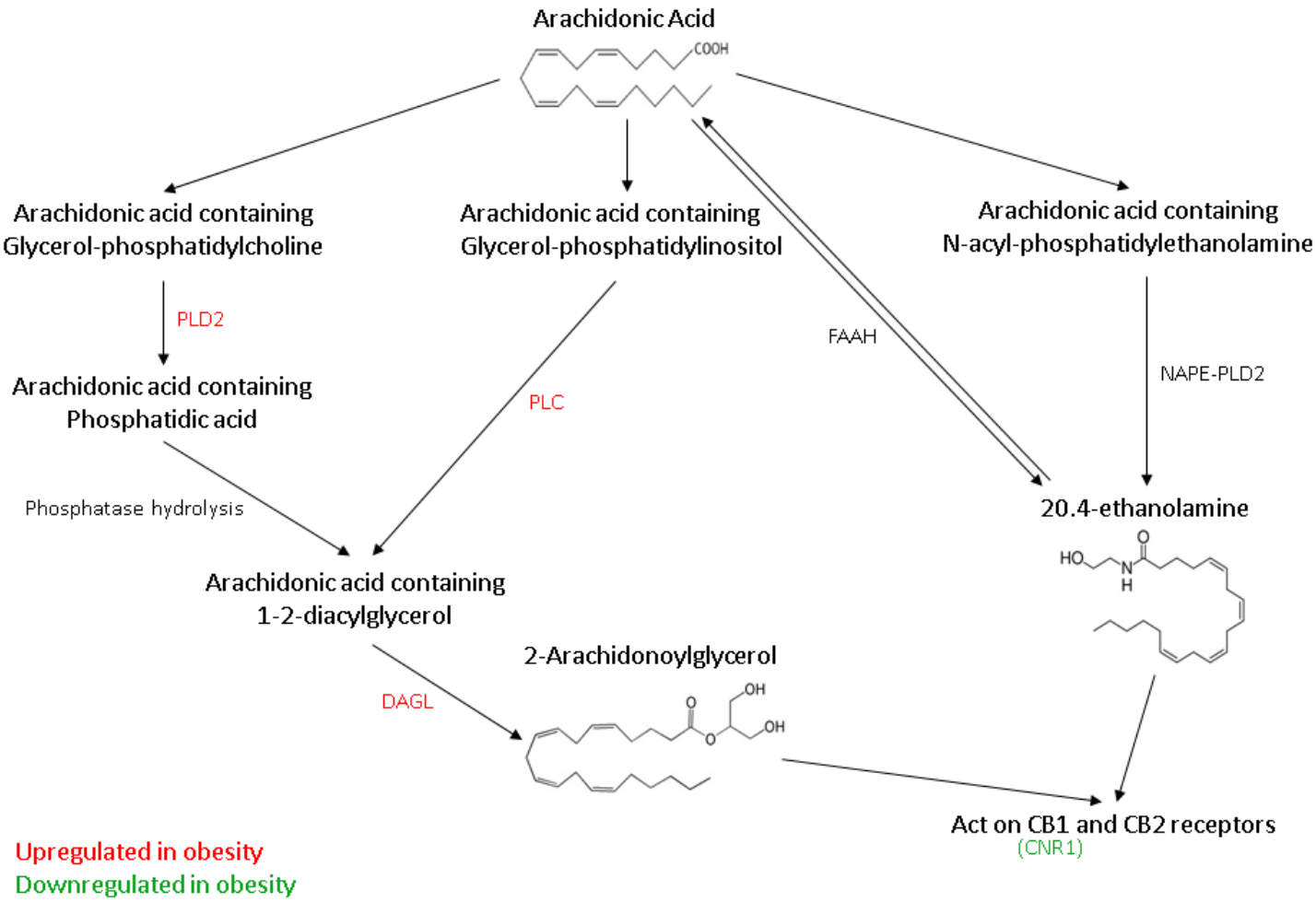


Figure 6.14 Biosynthetic pathways of 20:4-glycerol (2-arachidonoyl glycerol) and 20:4-ethanolamide. Adapted from (Turcotte et al., 2015)

Infiltration of immune cells into inflamed scWAT and alteration of scWAT immune cell populations are widely reported in obesity (Nishimura et al., 2009, Yang et al., 2010, Bertola et al., 2012, Fabbrini et al., 2013, Travers et al., 2015). The current study reports upregulation of genes encoding proteins secreted by immune cells as well as cell surface markers related to immune cells and resident endothelial cells. Immune cell infiltration, maturation and differentiation, activation, and communication is suggested to occur in scWAT from MHO individuals based upon the dysregulation of genes involved in these pathways, is detailed in Figure 6.11 and Figure 6.15. There is also evidence for increased expression of adhesion molecules in obesity as demonstrated in both murine and human models reporting dysregulation of circulating and adipose adhesion molecules with significant depot specific differences (Brake et al., 2006, Nishimura et al., 2008, Vachharajani and Granger, 2009, Bošanská et al., 2010). The current study reports a significant increase in genes encoding VCAM-1 (1.89 FC), ICAM-1 (1.93 FC), E-selectin (1.88FC), and P-selectin (FC 1.42) (P <0.001, Appendix J) which are involved in the adhesion and migration of leukocytes to the site of inflammation (Golias et al., 2007). The current study further reports upregulation of a range of immune cell markers including: *CD1c* (3.36), a marker of DCs; *FOXP3* (1.93 FC), a marker of T-reg cells; *CD4* (1.88 FC), a marker of T-helper cells; *CD14* (1.53 FC), a marker of macrophages, and *CD163* (2.43), a marker of alternative macrophage activation (P <0.001, Appendix I and Appendix J). These data suggest increased presence of T-reg cells, Th cells, and classically and alternatively activated macrophages in scWAT from MHO individuals.

The top predicted canonical pathway activated by obesity was the maturation of DCs (Figure 6.4 and Figure 5.8). Upon antigenic stimulation, $CD4^+$ cells are capable of polarising into Th-1, Th-2, Th-17, or T-reg cells (Bertola et al., 2012). The current study reports an upregulation of *IgG* in scWAT from MHO individuals which results in the upregulation of FCγR. FCγR directly promotes the expression of *MHCII*, *CD86* and *IL-10*, all of which are seen to be upregulated in obesity in the current study. MHCII and CD86 promote antigen presentation, which is a major function of mature DCs (Cella et al., 1997, Lim et al., 2012). MHC molecules translocate to the surface of DCs during maturation, aiding their transformation into fully functional antigen presenting cells (Cella et al., 1997, Lim et al., 2012) and promote Th1 cell activation, the secretion of Th1 cell derived pro inflammatory cytokines, and the suppression of Th2 cell differentiation (Deng et al., 2013) (Figure 6.15). The current study further reports the upregulation of a direct transcriptional regulator of MHCII, CIITA which is previously reported to be increased in obesity (Deng et al., 2013) (P <0.001, Appendix I). The activation of $CD4^+$ T cells and subsequent synthesis of pro-inflammatory cytokines requires the recognition of MHCII on antigen presenting cells such as macrophages and DCs (Deng et al., 2013).

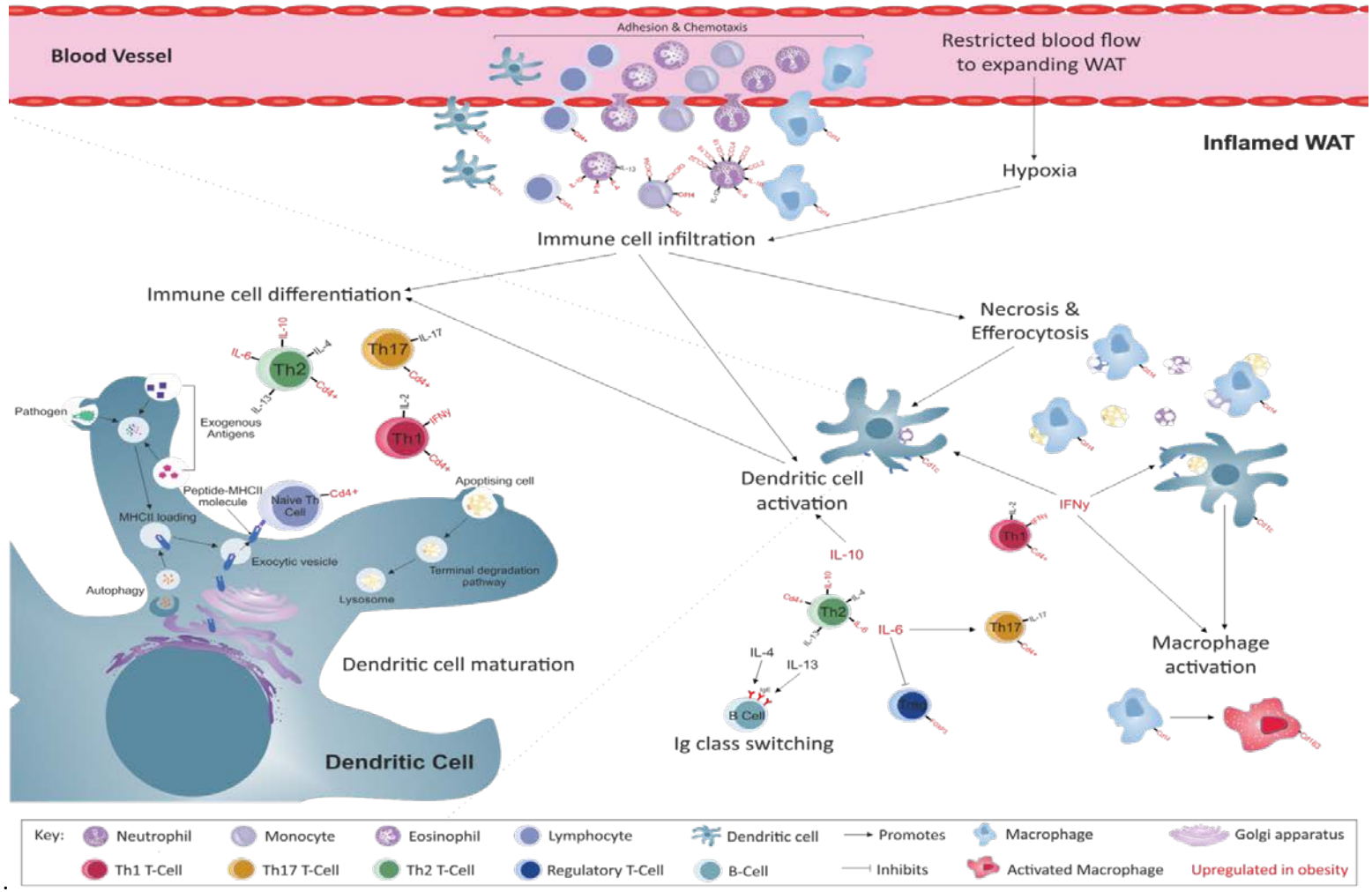


Figure 6.15 Regulation of genes involved in immune cell infiltration, maturation & differentiation, activation, and communication in inflamed scWAT.

Therefore, the dysregulated scWAT transcriptome in obesity may be reflective of enhanced pro-inflammatory mediator signalling, but also of changes in immune cell population and phenotype, and therefore, heterogeneity of the tissue.

6.5.2 Modulation of scWAT transcriptome by FO intervention

FO intervention had a greater effect on gene expression in the scWAT of normal weight individuals modulating 26 genes in comparison to 8 genes in scWAT of MHO individuals (P <0.001, FDR $\leq$ 1.0). Despite this, FO intervention in general significantly down regulated genes involved in inflammatory and immune responses in both BMI subgroups. Many of the genes affected in both BMI subgroups were unrelated and consequently, regulation of few pathways could be attributed to these genes. However, GO assessment allowed for attribution of the genes to specific processes in the scWAT. The proportion of differentially expressed genes associated with such processes is depicted for both normal weight and MHO individuals in Figure 6.16. The profile in normal weight individuals also suggests upregulation of glucose homeostasis and positive regulation of tissue expansion in response to FO intervention.

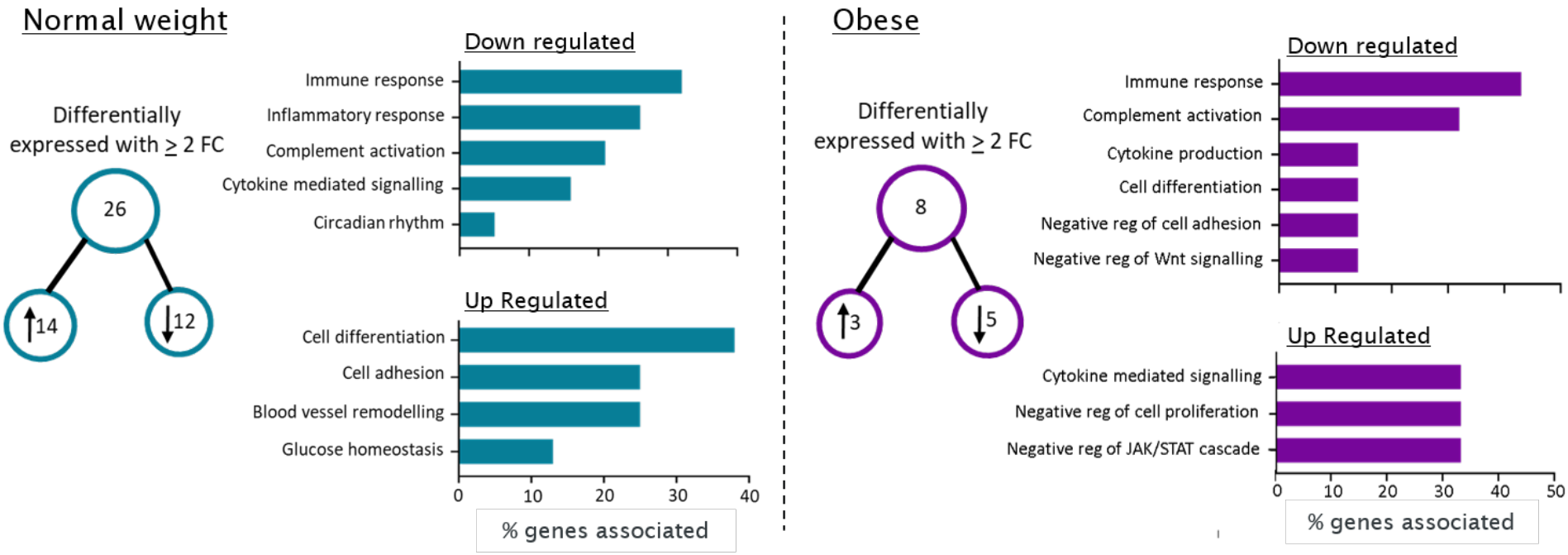


Figure 6.16 Effect of 12-week FO intervention on scWAT transcriptome in normal weight and metabolically healthy obese individuals and proportion of differentially expressed genes associated with biological processes.

6.5.2.1 Modulation of scWAT transcriptome by FO intervention: Adipogenesis and carbohydrate metabolism and signalling

FO intervention significantly modulated genes involved in adipogenesis (*DACT2, TGM2* and *FOXC2*); however, the biological effects of this are uncertain as there may be an increase in adipogenesis via enhanced Wnt signalling due to the downregulation of its inhibitor *DACT2* with FO intervention (Appendix I and Table 6.8) (Davis et al., 2004, Sethi and Vidal-Puig, 2010, Myneni et al., 2015, Suryawanshi et al., 2016).

DACT scaffold proteins, particularly DACT1, have been shown to influence the activity of Wnt signalling which is involved in regulating adipogenesis and AT expansion. Evaluation by GO biological processes suggests the association of DACT2 in the regulation of Wnt signalling but this is yet to be defined in human AT (Table 6.8). In physiological conditions, Wnt signalling is responsive to nutritional cues but this can be hijacked by pro-inflammatory cytokine signalling, particularly TNF-α, which is increased in obesity (Christodoulides et al., 2006, Christodoulides et al., 2009, Sethi and Vidal-Puig, 2010). Therefore, the actions downregulation of *DACT2* may confer on the Wnt signalling system and regulation of adipogenesis and expansion cannot be described in these individuals without further dissection and investigation of the signalling pathway.

The current study reports a significant decrease in expression of both Wnt-10b and -3a ligands in the scWAT of MHO individuals at study entry (-1.89 and -2.81 FC respectively Appendix I), confirming previous reports of downregulation of fat expansion in obesity. To my knowledge, there are no reports of Wnt signalling via regulation of DACTs by FO intervention and therefore, the current study provides novel evidence for the effects of FO intervention on DACT expression in human scWAT. However, the consequences of this on adipogenesis and scWAT expansion require further investigation. The expression of Wnt and DACT genes is further examined in Chapter 7.

Further to regulating adipogenesis, Wnt/β-catenin signalling has been shown to be sensitive to glucose and lipid concentrations in physiological conditions, and becomes inhibited with overfeeding/weight gain (Sethi and Vidal-Puig, 2010, Alligier et al., 2012, Suryawanshi et al., 2016). FO intervention significantly modulated genes involved in glucose homeostasis in normal weight individuals. Of note, the current study reports upregulation of glucagon receptor2 (*GCGR*) and *TGM2* following FO intervention which, in addition to being associated with decreased adipogenesis, may result in improved glucose tolerance and insulin sensitivity, as well as accelerated clearance of apoptotic cells in areas of inflammation, which are key steps in the resolution of scWAT dysfunction. Evidence from murine models reports glucose intolerance in *TGM2* knock out mice as well as impaired insulin secretion (Bernassola F et al., 2002), and there

are mutations in *TGM2* in humans with early onset diabetes development (Porzio et al., 2007), suggesting a role of *TGM2* in glucose homeostasis. However, there is also evidence for glucose homeostasis to be independent of *TGM2* expression, so the role of *TGM2* in this area is still undefined (Iismaa et al., 2013). *TGM2* is also implicated in inflammation as is it highly expressed in monocytes and macrophages, and is involved in the phagocytosis of apoptotic cells (Murtaugh, 1984).

The effects of FO intervention on CHO metabolism would be beneficial in scWAT in obesity in which there is an increase in adipose mass, inflammation, and the risk of developing insulin resistance; however, the current study reports increased expression of genes positively regulating glucose homeostasis in normal weight individuals only. The lack of effects of FO intervention on scWAT gene regulation in MHO individuals may indicate scWAT dysfunction occurring in these individuals. This may relate to lipid and glucose sensitivity and handling, as well as inflammatory signalling, which may contribute to differential effects of FO intervention on gene expression.

6.5.2.2 Modulation of scWAT transcriptome by FO intervention: Inflammation and immune response

FO intervention significantly modulated the scWAT transcriptome profile negatively regulating inflammatory and immune responses (Table 6.7, Table 6.8 and Figure 6.16). This included the down regulation of many genes encoding Immunoglobulin light and heavy chains in both normal weight and MHO individuals, which may result in a decrease in antigen recognition and binding, and decreased immune response (Table 6.7 and Table 6.8). This is of particular benefit to MHO individuals in which components of the immune response are reported to be over active (Table 6.6, and Figure 6.12).

Of note, the expression of *S100A8*, *IGHA2*, and *TREM1* was decreased in scWAT from normal weight individuals in response to FO (Table 6.7). TREM1 is expressed predominantly on monocytes and macrophages and its signalling pathways result in Ca^{2+} mobilisation, subsequent activation of transcription factors including NFκB, and therefore the transcription of genes encoding pro-inflammatory cytokines and chemokines (Yuan et al., 2014, Subramanian et al., 2017, Tammaro et al., 2017). TREM1 can also promote cell survival by inhibiting pro-apoptotic factors, further contributing to inflammatory cascades (Yuan et al., 2014). An increase in TREM1 expression has been previously observed in human obesity and related T2DM, and its expression is positively correlated with markers of classically activated M1 pro-inflammatory macrophages (Subramanian et al., 2017). There is also evidence for the modulation of TREM signalling by EPA and DHA in which many genes involved in TREM signalling pathways were downregulated by EPA and both up-and downregulated by DHA when incubated at 10 uM and 75 uM respectively with

LPS stimulated Thp-1 macrophages (Allam-Ndoul et al., 2017). Downregulation of *TREM1* reported in scWAT from normal weight individuals in the current study may indicate a decrease in inflammatory TREM1 expressing cell populations and may result in downregulation of transcription factors such as NF-κB and subsequent pro-inflammatory mediator secretion.

S100A8 which encodes 'S100 calcium binding protein A8' has also been shown to induce inflammation in obesity; increased expression results in the activation of the TLR4-MyD88 pathway in activated adipose macrophages and subsequent inflammasome activation and IL-1β secretion (Nagareddy et al., 2014). There is evidence for decreased expression of *S100A8* in human LPS stimulated Thp-1 monocytes treated with EPA and DHA, decreased expression in non-stimulated adipocytes treated with EPA and DHA, and increased expression in LPS stimulated adipocytes treated with EPA and DHA, highlighting alteration of effects of EPA and DHA during the state of inflammation (Shah et al., 2017). Decreased expression of *S100A8* in scWAT may result from decreased secretion of pro-inflammatory cytokines mediated by downregulation of TLR4 activation. Parás Chávez (2014) previously reported an increase in $CD14^+$ $TLR4^+$ monocytes and a decrease in $CD14^+$ $TLR2^+$ monocytes in whole blood from the cohort of individuals included in the current scWAT analysis (Chávez, 2014). Parás Chávez (2014) further reported down regulation of TLR4 expression on blood monocytes from normal weight individuals following the 12-week FO intervention period, but no effects of FO in MHO individuals or on the expression of TLR2 (Chávez, 2014).

Of further note, there was a significant decrease in the expression of *IDO1* in the scWAT from MHO individuals following FO intervention. *IDO1* encodes the enzyme indoleamine 2, 3-dioxygenase-1, which degrades tryptophan to produce metabolites that suppress effector T cell function and promote differentiation of T-reg cells (Prendergast et al., 2011). Decreased levels of circulating tryptophan, indicating increased IDO1 activity, and increased expression of IDO1, have been reported in human scWAT and VAT, accompanied by increased T-reg cell infiltration (Wolowczuk et al., 2012). Onodera *et al.* report a decrease in IDO1 and TGF-β in adipose macrophages following EPA intervention but still report enhanced murine adipose derived macrophage mediated T-reg cell induction (Onodera et al., 2017). They attribute this to EPA mediated inhibition of ALOX5 and subsequent inhibition of 5-HEPEproduction and hypothesise that IDO1 does not have involvement in the T-reg cell enhancement observed with EPA intervention (Onodera et al., 2017). Therefore, whether a decrease in IDO1 is significantly associated with a decrease in scWAT T-reg cell population is undetermined. Evaluation into immune cell population of the scWAT would be required to address this further.

6.5.2.3 Modulation of scWAT transcriptome by CO intervention: use of CO as comparative oil

CO intervention also had significant effects on the expression of a small number of genes in both normal weight and MHO individuals. These effects differed to those associated with FO intervention in that CO intervention significantly up regulated a very small number of genes involved in central nervous system and brain development in both normal weight and MHO individuals, and down regulated a very small number of genes involved in epidermis development and ageing in MHO individuals only. The amount of LA administered in CO is around +10% of the average UK daily intake, as reported in Chapter 3, this was not enough to significantly increase the proportion of LA in the scWAT and there were no differences in the proportion of LA at week-0 or week-12 between normal weight and MHO individuals. Therefore, with the addition of evidence for the minimal effect on gene expression reported within the current chapter, CO is confirmed to have been a suitable control oil for this study.

6.5.3 Conclusion

WAT is a sensitive organ and can respond to stimuli to maintain energy homeostasis; in obesity, the function scWAT may become altered as demonstrated by a dysregulated scWAT transcriptomic profile associated with adipose tissue expansion, immune and inflammatory processes, CHO and lipid metabolism, and diseases including metabolic diseases and cancer. In summary, genes upregulated in obesity indicate a state of hypoxia, increased immune cell differentiation and presence, increased pro-inflammatory signalling, dysregulation of glucose homeostasis and lipid signalling, and changes to the scWAT structure and vasculature through angiogenesis and remodelling.

12-week FO intervention modulated the scWAT transcriptomic profile with changes in the expression of genes associated with immune and inflammatory processes including immunoglobulin development, immune cell differentiation, TREM and possibly TLR4 pathway signalling. These results may indicate a change in cellular heterogeneity in scWAT and down regulation of pro-inflammatory signalling. 12-week FO intervention further modulated the expression of genes involved in glucose homeostasis and adipogenesis suggesting improvement in glucose tolerance, insulin sensitivity, and the negative regulation of scWAT mass and subsequent dysfunction. However, the effects of FO intervention were greater in normal weight individuals with MHO individuals exhibiting some resistance to the effects of FO intervention on gene expression in scWAT from MHO individuals.

The data reported herein provide further evidence for the dysregulation of scWAT in metabolically healthy obesity in agreement with data detailed in chapters 4 and 5, and indicate differential handling of FO by MHO individuals at the transcriptome level which may be attributed to altered lipid and glucose sensitivity, transport and metabolism, as well as altered heterogeneity and subsequent inflammatory signalling in obesity.

Transcriptomic studies in human scWAT have started to become of greater interest but the few studies to date lack consideration for the metabolic health of the individuals included (Stenvers et al., 2019, Rodriguez-Ayala et al., 2020, Kerr et al., 2020). Therefore, the data reported herein, specifically defining MHO individuals is novel and of importance. Further to this, transcriptomic studies investigating interventions in obesity are predominantly focussed on calorie restriction and weight reduction (Cancello et al., 2005, Capel et al., 2009, Marquez-Quinones et al., 2010, Mutch et al., 2011, Armenise. C et al., 2017) with a few investigating the effects of FO intervention on the whole transcriptome of human blood (Polus et al., 2016) or human scWAT (in addition to a calorie restriction) (Huerta et al., 2017). Therefore, the results reported herein provide novel evidence for the dysregulation of the adipose transcriptome in metabolically healthy obesity, identify altered biological pathways that may lead to metabolic complications if the condition persists, and identify targets for the therapeutic use of FO intervention to successfully modulate adipose transcriptome, particularly pathways involved in inflammation and whole body energy regulation. Furthermore, the current study highlights dysregulation in response to FO intervention at the transcriptome level in MHO individuals which may identify an underlying mechanism for the ineffectiveness of FO often reported on wider body measures of inflammation and metabolism in obesity such as whole body glucose sensitivity and blood cytokine concentrations.

The 20 samples of scWAT from humans that were selected in a pairwise fashion from a cohort of 100 individuals (50 normal weight, 50 MHO) for this analysis will undergo validation by qRT-PCR in addition to further investigation of expression of key scWAT genes reported within this chapter in the full cohort in Chapter 7.

Chapter 7 Expression of metabolic and inflammatory genes in subcutaneous white adipose tissue is altered in metabolically healthy obese subjects and in response to 12-week fish oil intervention – Validation of RNA-Seq data by qRT-PCR

7.1 Introduction:

Chapter 6 describes the principles and full procedure involved in RNA-Sequencing (RNA-Seq) analysis of human subcutaneous white adipose tissue (scWAT). In brief, RNA-Seq is a high throughput sequencing technique that allows whole transcriptome analysis. This allows a vast number of genes to be identified and changes in their expression quantified using a small amount of sample. However, the cost of this technique remains high and it is often not feasible to analyse large cohorts; therefore, analysis of a subset of samples from those available is often performed. This subset should be carefully selected to represent the full cohort, and in the context of the current study, samples were selected based upon condition, being metabolically healthy obese (MHO) or normal weight, and treatment, consuming either fish oil (FO) or corn oil (CO) for 12-weeks. These samples were paired for study entry and post intervention and were best matched for sex and age between the two BMI groups as detailed in Chapter 6, Table 6.1. The RNA-seq analysis in Chapter 6 highlights a significantly altered transcriptome in 10 MHO individuals in comparison to 10 normal weight, and significant modulation of the scWAT transcriptome with FO intervention in this subset of individuals. Validation of these data by a second expression quantifying technique should be performed to not only validate the data obtained by RNA-seq in this subset, but also for its generalisation with regards to the whole cohort.

Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) is a technique that quantifies gene expression through the measurement of complementary DNA (cDNA). This technique is performed in a candidate approach in that it is used to measure genes of particular interest as opposed to obtaining a large profile of all transcript changes within a particular condition, such as obtained by RNA-Seq analysis. qRT-PCR is deemed to be the gold standard test to validate RNA-Seq data and adequate concordance in seen between qRT-PCR and RNA-Seq (Fang and Cui, 2011, Trost et al., 2015, Casamassimi et al., 2017). Therefore, investigation of the top differentially expressed genes in each condition should be performed by qRT-PCR analysis on

the full study cohort and is reported herein. Some transcripts of interest were not reported by RNA-Seq; these would be candidates for further investigation by qRT-PCR and as such, are investigated within the current chapter.

RNA-Seq highlighted the upregulation of genes involved in tissue expansion and remodelling in MHO individuals as well as modulation of these processes in response to FO intervention (Chapter 6). As previously discussed, where there is nutrient excess beyond the body's needs, the scWAT expands to allow continued storage of excess lipid in the form of triglyceride (TG) in adipocytes. This occurs through expansion of pre-existing adipocytes, termed hypertrophy, as well as through the formation of new adipocytes (adipogenesis), termed hyperplasia (Drolet et al., 2008, Jo et al., 2009). One group of proteins controlling adipogenesis are encoded by Wnt genes; Wnt signalling proteins are anti-adipogenic and are inhibited by DACT proteins so when there is high expression of DACTs, Wnt signalling is inhibited and adipogenesis can occur. RNA-Seq highlighted the altered regulation of Wnt signalling pathways in MHO individuals and in response to FO intervention (Figure 6.3 and Table 6.8).

During WAT expansion, the expression of Wnt3A and Wnt10B is decreased, often seen in conjunction with increased expression of DACT genes, to allow adipogenesis to occur (Sethi and Vidal-Puig, 2010, Bennett et al., 2002). Therefore, differential expression of both Wnt and DACT genes may be observed in obesity. Furthermore, the Wnt signalling pathway can be hijacked by inflammatory cytokines such as TNF-α and IL-6 (Cawthorn et al., 2007), both of which are increased in obesity (Park and Ahima, 2014, Popko et al., 2010, Straczkowski et al., 2002). There are reports of mutations in Wnt10B in human obesity but investigation into the expression of other Wnt and DACT genes, and their regulation by lipids, particularly LC n-3 PUFA, is under reported and is therefore of great interest (Christodoulides et al., 2006).

Gene expression can be modulated by lipids; transcription factors including NF-κB as well as other proteins effecting gene transcription such as PPAR-γ and the Wnt signalling proteins, are sensitive to changes in dietary lipids (Calder, 2015a, Sethi and Vidal-Puig, 2010). Not only can dietary lipids stored within the scWAT regulate the expression of genes themselves, they can be used to synthesise metabolites which are involved in pro- and anti-inflammatory signalling which can further regulate the expression of genes within the tissue (Masoodi et al., 2014). The synthesis and degradation of fatty acid (FA) metabolites is dependent on the availability of FA substrate as well as the activity of the enzymes involved in the conversion of FAs to the resultant metabolites (Masoodi et al., 2014, Calder, 2017, Powell and Rokach, 2015, Lopategi et al., 2016). Therefore, if there are changes in the expression of the genes encoding these enzymes, there may be changes in the amount and type of lipid metabolites in the WAT.

Free FAs can undergo oxygenation by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to form a range of lipid metabolites involved in both pro- and anti-inflammatory signalling. There are 3 well described LOX enzymes, 5-LOX, 12-LOX and 15-LOX, encoded by *ALOX5*, *ALOX12*, and *ALOX15* respectively, and 2 COX enzymes, COX-1 and COX-2, encoded by *PTGS1* and *PTGS2* respectively (Lopategi et al., 2016, Masoodi et al., 2014) (a full description of lipid metabolites produced from COX and LOX oxygenation is provided in Chapter 4). WAT lipids can also undergo hydrolysis by N-acyl phosphatidylethanolamine-D (NAPE-PLD) and diacylglycerol lipase-alpha (DAGLα) to form endocannabinoids (ECs) including FA ethanolamides and FA glycerol esters respectively (Masoodi et al., 2014). The degradation of these metabolites is also regulated by expression of genes and proteins involved in further oxygenation and hydrolysis such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGLL) (Masoodi et al., 2014).

Differences in scWAT EC concentrations in MHO individuals in comparison to normal weight individuals are reported in Chapter 4, and previous studies report increased expression of *NAPE-PLD* and *MGLL*, and decreased expression of *FAAH* in AT in obesity (Bluher et al., 2006, Engeli et al., 2005). Further to the concentrations of these metabolites, their actions may also be altered if there are changes in the expression of the receptors they interact with. ECs are known to act through a range of receptors including cannabinoid receptor-1 (CB1), the expression of which is reported to be decreased in obesity (Bennetzen et al., 2010, Engeli et al., 2005). Metabolite-receptor interactions can trigger a cascade of events including the regulation of adipogenesis, lipid accumulation, inflammation, and the expansion and remodelling of AT (Masoodi et al., 2014, Calder, 2015a). Therefore, evaluation of the expression of CB1 (*CNR1*) by qRT-PCR will provide greater insight into EC signalling in metabolically healthy obesity.

Investigation of COX-2 and FAAH protein expression and enzyme activity in Chapter 5 highlighted greater expression of COX-2 in scWAT from MHO individuals, and down regulation of FAAH in response to 12-week FO intervention in scWAT from normal weight individuals. RNA-Seq data indicated increased expression of *PTGS1* (Chapter 6) but did not detail expression of *PTGS2* or *FAAH*. Therefore, qRT-PCR analysis of these, in addition to other genes involved in FA metabolite pathways not identified by RNA-Seq, will provide greater insight into the regulation of FA metabolite synthesis and degradation at a transcriptional level.

As described in detail in Chapter 6.1, long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) can influence gene expression directly via interaction with receptors and indirectly through alteration of lipid raft structure (Calder, 2018, Hellwing et al., 2018, Kalupahana et al., 2020). As such, modulation of the whole scWAT transcriptome is reported in Chapter 6 with effects on immune and inflammatory response and tissue expansion. Therefore, investigation of candidate

gene expression following 12-week FO intervention will provide greater insight into the regulation of genes not assessed by RNA-seq to provide data that can be used alongside pathway analysis reported in Chapter 6 to provide a more comprehensive overview of the mechanisms behind LC n-3 PUFA modulation of scWAT processes.

7.2 Aims:

The aims of the research described in this chapter were to:

1. Validate results obtained from RNA-Seq by:
   - Investigating the expression of the top differentially expressed genes in scWAT in metabolically healthy obesity identified by RNA-Seq.
   - Investigating the expression of the top differentially expressed genes in scWAT in normal weight individuals in response to FO and CO intervention identified by RNA-Seq.
   - Investigating the expression of the top differentially expressed genes in scWAT in metabolically healthy obese individuals in response to FO and CO intervention identified by RNA-Seq.
2. Investigate the expression of genes of interest involved in significantly enriched pathways identified by RNA-Seq in metabolically healthy obesity.
3. Investigate the expression of genes of interest involved in significantly enriched pathways identified by RNA-Seq in response to FO and CO interventions.

7.3 Methods:

Please refer to 'Chapter 2-Study design and population.

7.3.1 Experimental reagents and materials

Reagents and materials used for experiments are described in Appendix C and full primer sequence information is described in Appendix M.

7.3.2 Isolation of RNA from white adipose tissue

RNA was isolated from 132 scWAT samples (27 paired normal weight, 39 paired MHO) according to the protocol described in Chapter 6, section 6.3.2.

The protocol was carried out once to yield RNA from an average of 85 mg of scWAT in total from each participant yielding an average of 1.96 µg of total RNA with an average 260/280 ratio of 1.95, 260/230 ratio of 1.14, and RIN score of >6.5.

7.3.3 Investigation of gene expression by qRT-PCR

7.3.3.1 Principles of qRT-PCR

qRT-PCR is a technique used to investigate gene expression to provide data in 'real-time'. qRT-PCR using Taqman style probes was performed in the current study; these are DNA probes which have a fluorescent reporter dye (fluorophore) at the 5' end and a quencher at the 3' end. When the primer is intact the quencher blocks fluorescence of the reporter dye. During the PCR process, the quencher and reporter are separated allowing fluorescence of the reporter dye which is measured in real time (per cycle) (Bonetta, 2005).

cDNA is first synthesised from isolated RNA (as described in Chapter 6, section 6.3.2) and the resultant cDNA is subjected to a high temperature to denature the double strand structure (Figure 7.1). As the temperature is lowered, the Taqman probe anneals to the 5' end; the amplification primers then anneal and extension occurs. As Taq polymerase extends the amplification primers, it digests the probe with each PCR cycle, which separates the quencher from the reporter dye allowing fluorescence to occur. This is repeated for 40 cycles, with further fluorescence emitted each time the probe is cleaved (each cycle) (Figure 7.1). Transcripts with high abundance fluoresce faster than transcripts of low abundance; this is representative of the expression of that transcript in real time reported as a cycle threshold (Ct) value. The lower the Ct, the higher the expression of the gene (Bonetta, 2005).

7.3.3.2 Synthesis of cDNA by reverse transcription

GoScript Reverse Transcription System (Promega, Southampton, UK) was used to synthesise cDNA from total RNA isolated from scWAT. 1 µg of purified RNA was mixed with 1 ul of random primer and DEPC water to make an 11 µl total volume. This was incubated at 75°C for 5 min using a thermal reactor then immediately placed on ice for 5 min. 9 µl of a reverse transcription mix consisting of 4 µl GoScript 5 x reaction buffer, 2 µl magnesium chloride (MgCl), 1 µl PCR nucleotide mix, 20 units recombinant RNasin ribonuclease inhibitor and 1 µl GoScript reverse transcriptase, was added to the sample to make a total volume of 20 µl. The sample was mixed well and re-loaded into the thermal reactor to complete the following temperature protocol: Anneal = 25°C, 5 min; Extend = 42°C, 1 hour; and Inactivate = 70°C, 15 min.

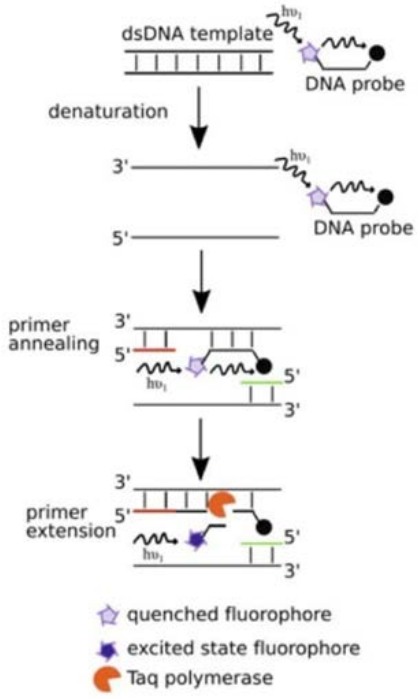


Figure 7.1 RT-PCR procedure (Nair. U, 2018).

7.3.3.3 qRT-PCR protocol

cDNA obtained from reverse transcription (as described in section 7.3.3.2) was diluted 1:10 with DEPC H_2O for use with the QuantiNova Probe PCR kit (GIAGEN, Hilden, Germany).

Kit components were thawed according to the manufacturer's guidelines and all reactions were carried out on ice. For genes assessed individually, 16 µl of PCR reaction mix consisting of: 10 µl of 2 x QuantiNova Probe PCR Master Mix, 0.1 µl ROX reference dye (for Low ROX qRT-PCR machines), 1 µl of the primer + probe of interest and 4.9 µl of DEPC H_2O, was aliquoted into a 96 well PCR plate. 4 µl of 1:10 diluted cDNA was then added. Non-template controls in which only 16 µl of the PCR reaction mix was added to a well were used in each analysis. For genes assessed in a duplex manner (two genes analysed per aliquot of cDNA), 16 µl of PCR reaction mix consisting of: 10 µl of 2 x QuantiNova Probe PCR Master Mix, 0.1 µl ROX reference dye (for Low ROX qRT-PCR machines), 1 µl of each primer + probe of interest (totalling 2 µl) and 3.9 µl of DEPC H_2O, was aliquoted into a 96 well PCR plate. 4 µl of 1:10 diluted cDNA was then added. Non-template controls in which only 16 µl of the PCR reaction mix was added to a well were used in each analysis. The PCR plate was centrifuged to ensure all liquid remained in the bottom of the wells and was sealed with a plate cover. The plate was loaded into an Applied Biosystems 7500 qRT-PCR machine to complete the following qRT-PCR protocol: PCR initial activation = 95°C, 2 min;

Denaturation = 95°C, 10 min; Combined annealing and extension = 60°C, 1 min for a number of 40 cycles.

7.3.4 Housekeeping gene selection

Due to limited sample availability, a subset of samples representative of the full sample set was selected to investigate candidate housekeeping (reference) genes. This subset was composed of 8 normal weight and 8 MHO scWAT samples from week 0 (study entry), 4 normal weight and MHO scWAT samples at week 12 following FO intervention (paired with week 0 samples), and 4 normal weight and MHO scWAT samples at week 12 following CO (paired with week 0 samples), totalling 16 samples (Table 7.1). These were run in duplicate alongside non-template controls.

Samples were investigated for housekeeping genes commonly reported for humans: 18s, CYCP1, GAPDH, RPL13A, SDHA, YWHAZ (Primer Design, Eastleigh, UK), ACTB and B2M (BD, New Jersey, USA).

Due to poor stability across conditions, further housekeeping genes reported to be specifically expressed and stable in human WAT across conditions including metabolic syndrome (Mets) and obesity were selected with the aid of RNA-Seq data. RNA-Seq data was consulted to check the expression of potential new housekeeping genes across all experimental conditions; this resulted in the selection of GUSB, ACTG1, IPO8, GABARAP and the novel gene VPS36.

Ct values from housekeeping gene analysis were analysed using qbase+ GeNorm software (Biogazelle, Gent, Belgium). 18s, CYCP1, GAPDH, RPL13A, SDHA, ACTB, B2M, GUSB, ACTG1 and IPO8 showed high variation between the experimental groups and were deemed not to be appropriate for use as reference genes. Analysis revealed some genes that would be most appropriate to use as housekeeping genes; GABARAP, YWHAZ and VPS36 were the top three most stable genes and were therefore selected as housekeeping genes for the full cohort analysis

Sex	Age	Treatment group	BMI (kg/m^2)	Body fat (%)	Body fat mass (kg)
Male	28.8	Fish Oil	21.7	12.2	8.9
Male	27.31	Fish Oil	23.4	4.5	3.2
Female	22.4	Corn oil	25.1	26.3	18.0
Male	20.4	Fish Oil	23.9	9.0	6.4
Female	25.1	Fish Oil	21.6	23.3	12.6
Female	27.6	Corn oil	20.2	23.6	11.9
Female	27.1	Corn oil	22.9	22.2	14.3
Female	47.4	Corn oil	23.4	27.5	17.7
Female	38.5	Fish Oil	32.2	43.0	34.5
Male	31	Fish Oil	27.1	20.9	20.9
Female	60.9	Corn oil	37.3	45.9	44.4
Female	62.1	Fish Oil	38.3	44.9	44.6
Female	34.2	Corn oil	32.9	38.6	32.1
Female	48	Fish Oil	34.0	43.5	36.0
Female	23.2	Corn oil	39.1	48.6	50.5
Male	22.5	Corn oil	30.3	26.8	26.0

Table 7.1 Characteristics of individuals whose scWAT samples were selected to analyse housekeeping gene stability.

7.3.4.1 Experimental gene selection

Genes reported to be significantly differentially expressed from RNA-Seq analysis (Chapter 6) were selected for validation by qRT-PCR based on their significance. The top genes differentially expressed in obesity and in response to FO intervention in normal weight and MHO individuals selected for qRT-PCR are listed in Table 7.2. A selection of other genes of interest that were differentially expressed in obesity with FC $\geq$ 1 were also selected for qRT-PCR.

Gene	Condition	Log FC	P	FDR	CPM
ALOX5	Obesity	0.809	<0.001	<0.001	17.755
CNR1	Obesity	-0.418	0.011	0.05	10.84
DACT1	Obesity	0.504	0.013	0.056	8.767
DACT2	Obesity	-1.177	0.01	0.046	6.23
DAGLA	Obesity	0.76	<0.001	0.004	6.851
EGFL6	Obesity	5.44	<0.001	<0.001	136.58
IKBKE	Obesity	0.83	<0.001	<0.001	4.761
MMP9	Obesity	4.027	<0.001	<0.001	45.522
SLC27A2	Obesity	-3.42	<0.001	<0.001	3.06
WNT3	Obesity	-1.492	<0.001	<0.001	3.768
WNT10B	Obesity	-0.917	0.034	0.11	3.501
KCNH2	FO intervention (Normal weight)	2.67	<0.001	0.054	1.18
MAB21L1	FO intervention (Normal weight)	1.06	<0.001	0.002	10.09
PROK2	FO intervention (Normal weight)	-1.87	<0.001	0.024	1.00

Table 7.2 Genes selected for qRT-PCR validation of RNA-Seq data
Obesity, metabolically healthy obesity.

These included *ALOX12*, *ALOX15*, *FAAH*, FATP1 (*SLC27A1*), *MGLL*, *NAPE-PLD*, and *PTGS2*, which are of particular interest due to their involvement in lipid mediator synthesis and signalling pathways, but for which fold change (FC) data were not reported following RNA-seq analysis. Significant differences in lipid mediator concentrations in obesity and in response to FO intervention are reported in Chapter 4

7.3.5 Statistical analysis

qRT-PCR data were analysed using the comparative Ct approach. The Ct values of the genes of interest were adjusted for the average Ct value for the three housekeeping genes to calculate the ΔCt value. ΔΔCt and FC values were subsequently calculated. At study entry, normal weight values were taken as the 'control' with FC data for obese individuals calculated in comparison to this; and following the interventions, the study entry data for each individuals were taken as the 'control' with post intervention FC calculated in a pairwise fashion in comparison to each individuals study entry result. The resultant FC data were analysed using SPSS software (version

21; SPSS Inc.). The distribution of the data was assessed using the Shapiro-Wilk test and by plotting distributions of residuals obtained from GLM analysis of the data. Data not conforming to the normal distribution were log transformed and the distribution re-assessed. Log_{10} transformation was unable to normalise the distribution of data and therefore non-parametric tests were used for all analyses. The Mann Whitney-U test was used to assess the significance of FC of genes differentially expressed in obesity at study entry, and the Wilcoxon test was used to assess the significance of FC of genes differentially expressed in response to the interventions. As FC is calculated as a mean value, figures are presented as mean ± SEM to reflect these values

7.4 Results

7.4.1 Study entry results

7.4.1.1 The expression of scWAT genes is significantly altered in MHO individuals and correlates with body fat parameters

At study entry, there was significantly greater expression of *EGFL6*, *ALOX5* and *MMP9*, and lower expression of *ALOX15*, *SLC27A2*, *DACT1*, *PTGS2*, *WNT10B*, *PROK2*, *WNT3*, *MAB21L1*, *ALOX12*, *CNR1*, *FAAH*, *DACT2*, *KCNH2, SLC27A1, MGLL, DAGLA, NAPE-PLD, NF-κB*, and *IKBKE* in the scWAT of MHO individuals in comparison to normal weight individuals ($P \leq 0.05$, Figure 7.2, Table 7.3). There was only a significant ≥ 1 FC in expression of *EGFL6*, *ALOX5* and *MMP9* but this equated to an increase in expression of 963%, 171% and 43% respectively ($P \leq 0.05$, Figure 7.2, Table 7.3). *SLC27A2* had the greatest decrease in expression in obesity at -92%; all other significantly differentially expressed genes in obesity were altered by -26 to -44% of the expression seen in normal weight individuals (Figure 7.2, Table7.3).

There were significant correlations between the expression of genes in the scWAT and body fat mass (kg) and body fat % including for genes not significantly affected by BMI (Table 7.4). The expression of *FAAH* was negatively correlated with both body fat % and body fat mass ($P = 0.03$ and 0.05 respectively), and the expression of *ALOX12* and *DAGLA* was negatively correlated with body fat mass ($P = 0.03$ and 0.01 respectively), with a trend for *DAGLA* to be negatively correlated with body fat % also ($P = 0.06$) (Table 7.4). *PTGS2* expression was not correlated with net COX-2 protein expression or activity (reported in Chapter 5) but *FAAH* expression was positively correlated with net protein expression in normal weight individuals only ($r = 0.900$, $P = 0037$, Table 7.4). *FAAH* expression was also negatively correlated with scWAT concentrations of the ECs, 2-AG and DHEA, with a trend for negative correlation with EPEA also ($P= 0.02$, 0.005, and 0.079 respectively, Table 7.4). As discussed previously, FAAH is responsible for the degradation of these

metabolites so the negative correlation with its expression would be in agreement with its function. However, in MHO individuals, not only was *FAAH* expression not correlated with FAAH protein but it was not significantly correlated with any EC; there were trends for correlation with AEA and EPEA but the direction of these was positive suggesting higher concentrations of ECs regardless of higher expression of FAAH which further highlights dysregulation of the EC system in MHO individuals (Table 7.3). There was no significant correlation between concentrations of ECs and *NAPE-PLD* ($P \geq 0.556$ and ≥ 0.439, normal weight and obese respectively) or *MGLL* ($P \geq 0.721$ and ≥ 0.104, normal weight and obese respectively).

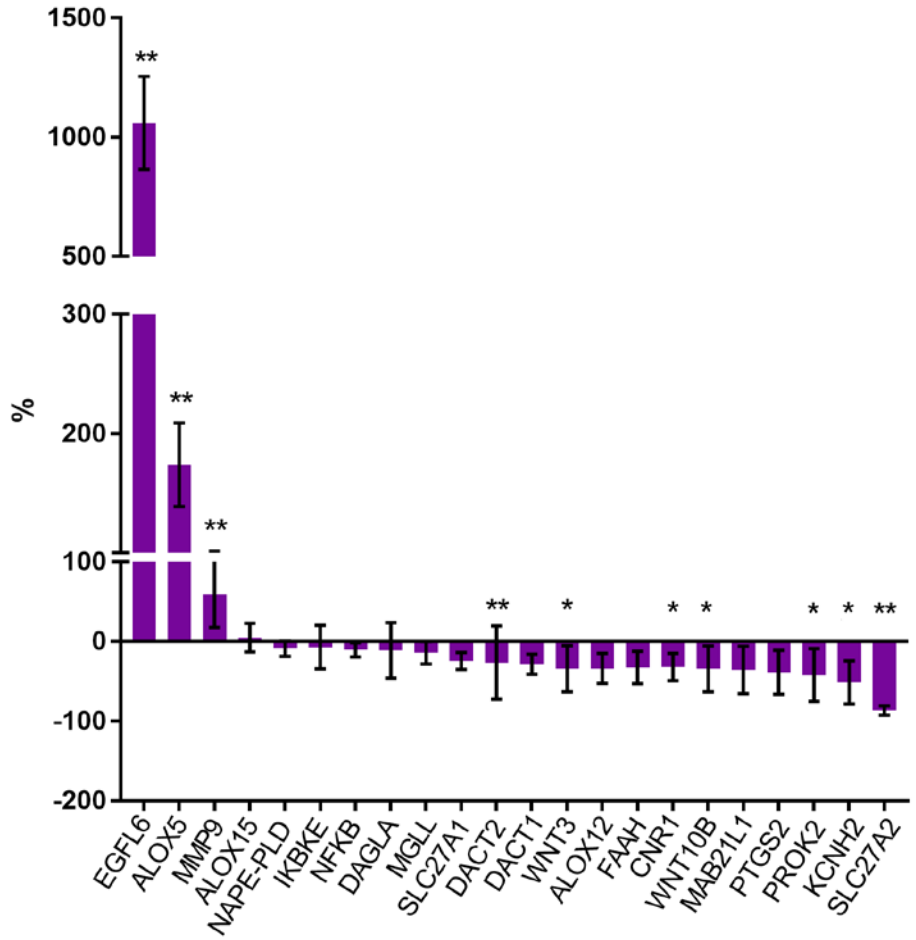


Figure 7.2 % difference in gene expression in scWAT from metabolically healthy obese (n=39) compared with normal weight individuals (n=27) at study entry (week-0).

Mean (± SEM) scWAT from metabolically healthy obese individuals compared with scWAT from normal weight individuals at study entry (week 0). * $P \leq 0.05$ ** $P \leq 0.001$.

Obese in comparison to Normal weight individuals at study entry							
Gene	Log FC[1]	Change (%)	BMI (kg/m^2)	Body fat mass (kg)		Body fat (%)	
			(*P*)[2]	(*P*)[3]	Coefficient[3]	(*P*)[3]	Coefficient[3]
EGFL6	**3.41**	**963**	**≤ 0.001**	**≤ 0.001**	**0.761**	**≤ 0.001**	**0.825**
ALOX5	**1.44**	**171**	**≤ 0.001**	**≤ 0.001**	**0.457**	**0.001**	**0.423**
MMP9	**0.52**	**43**	**0.042**	0.179	0.236	0.273	0.193
ALOX15	0.06	4	0.780	0.544	-0.077	0.561	-0.074
SLC27A2	**-3.69**	**-92**	**≤ 0.001**	**≤ 0.001**	**-0.635**	**≤ 0.001**	**-0.619**
DACT1	-1.04	-27	0.884	0.370	-0.114	0.332	-0.123
PTGS2	-0.90	-46	0.135	0.121	-0.196	0.121	-0.196
WNT10B	**-0.83**	**-44**	**0.048**	**0.042**	**-0.256**	**0.015**	**-0.302**
PROK2	**-0.78**	**-42**	**0.033**	**0.013**	**-0.31**	**0.011**	**-0.315**
WNT3	**-0.71**	**-39**	**0.003**	**≤ 0.001**	**-0.432**	**≤ 0.001**	**-0.474**
MAB21L1	-0.66	**-37**	**0.013**	**≤ 0.001**	**-0.44**	**≤ 0.001**	**-0.483**
ALOX12	-0.62	-35	0.121	0.155	-0.180	**0.036**	**-0.263**
CNR1	**-0.60**	**-34**	**0.047**	**0.023**	**-0.284**	**0.010**	**-0.319**
FAAH	-0.57	-33	0.121	**0.034**	-0.265	**0.051**	**-0.245**
DACT2	**-0.44**	**-27**	**0.025**	0.022	-0.287	**0.006**	**-0.343**
KCNH2	**-0.43**	**-26**	**0.005**	**0.001**	**-0.4**	**0.001**	**-0.392**
SLC27A1	-0.40	-24	0.197	0.174	-0.173	0.096	-0.212
MGLL	-0.27	-17	0.376	0.668	-0.056	0.930	0.011
DAGLA	-0.24	-16	0.215	0.061	-0.235	**0.014**	**-0.305**
NAPE-PLD	-0.19	-12	0.648	0.186	-0.169	0.242	-0.150
NFκB	-0.16	-10	0.703	0.742	-0.042	0.845	-0.025
IKBKE	-0.14	-9	0.670	0.302	-0.131	0.156	-0.179

[1]Log FC calculated as Log_2 mean (2-ΔΔCt divided by mean 2-ΔΔCt of normal weight data)

[2]Effect of BMI analysed by Mann Whitney-U test

[3]Correlations between body fat mass and body fat % analysed by spearman's rank correlation coefficient

Table 7.3 Association of metabolically healthy obesity and the expression of selected genes in human scWAT.

	Normal weight		Obese	
	FAAH protein		FAAH protein	
	Coefficient	*P*	Coefficient	*P*
FAAH	0.900	0.037	-0.300	0.624
AEA ng/g	-0.234	0.231	0.311	0.054
2-AG ng/g	-0.460	0.027	0.157	0.341
EPEA ng/g	-0.338	0.079	0.289	0.075
DHEA ng/g	-0.569	0.005	0.265	0.103

Table 7.4 Correlation between scWAT FAAH gene expression, net protein expression and endocannabinoids (ng/g) at study entry (week-0) in normal weight and metabolically healthy obese individuals

The qRT-PCR data support that obtained from RNA-Seq analysis (Chapter 6) which revealed greater expression of *EGFL6* and *MMP9* as well as the lower expression of *DACT2*, *WNT3* and *WNT10B* in scWAT from MHO individuals. In general, data generated from whole cohort qRT-PCR analysis are concordant with that obtained from RNA-Seq analysis of a subset of the cohort. Therefore, these data validate the RNA-Seq data and suggest subset analysis is representative of the whole cohort at study entry.

7.4.2 Post intervention results

7.4.2.1 12-week fish oil intervention significantly downregulates the expression of scWAT COX-2 in MHO individuals

In normal weight individuals, there was a non-significant ≥ 1 FC increase in the expression of *ALOX15, SLC27A2, MAB21L1, KCNH2, FAAH, MGLL, NFKB, SLC27A1* and *DACT1*, and a non-significant ≥ 1 FC down-regulation in the expression of *MMP9* with FO supplementation (Table 7.5). *ALOX15, SLC27A2, MAB21L1* and *KCNH2* showed the greatest change in which a non-significant 258%, 38%, 31% and 30% increase in expression was observed following FO intervention. *MMP9* expression decreased by 803% following FO intervention; however, variation in the expression of this gene in normal weight individuals resulted in large error margins which may have resulted in the non-significant result.

In MHO individuals, there was a significant ≥2 FC (167%) increase in expression of *PTGS2* in response to FO intervention ($P = 0.05$). Despite this, there were no significant changes in COX-2 protein as reported in Chapter 5, section 5.5.2.1.1. Furthermore, there were non-significant ≥ 1 FC increases in the expression of *ALOX15, SLC27A2, MAB21L1, KCNH2, FAAH, MGLL, SLC27A1, DACT1, CNR1, ALOX5*, *DACT2, WNT10B, EGFL6, IKBKE, ALOX12* and *NAPE-PLD*, as well as a non-significant ≥ 1 FC decrease in the expression of *PROK2* (Table 7.5). *SLC27A2, MAB21L1* and *KCNH2* showed the greatest, albeit non-significant, change in which a 349%, 94% and 93% increase in expression was observed in response to FO intervention (Table 7.5).

In general, FO intervention decreased the expression of many genes in normal weight individuals but increased the expression of these in MHO individuals. Genes that had an increase in expression in both BMI groups showed greater up-regulation in MHO individuals apart from *ALOX15*, which was more greatly increased in normal weight individuals (Table 7.3).

Despite the up-regulation of *MAB21L1* and *KCNH2* being non-significant, the magnitude of the FC is comparable to that reported in Chapter 6 from RNA-Seq analysis of a subset of the cohort (Table 7.5). RNA-Seq data further reports *PROK2* to be significantly down regulated by FO intervention in normal weight individuals; the current qRT-PCR analysis reports a non-significant 0.93 FC decrease in *PROK2* expression in response to FO intervention so is concordant with the overall trend of the data obtained from RNA-Seq. Therefore, whole cohort qRT-PCR analysis is concordant with, and validates results obtained from, RNA-Seq in which analysis of a subset of the cohort was performed.

Gene	Fish oil					
	Normal weight			Obese		
	Log FC[1]	Change (%)	(*P*)[2]	Log FC[1]	Change (%)	(*P*)[2]
ALOX15	1.84	58	0.600	0.44	36	0.157
SLC27A2	0.46	38	0.807	2.17	349	0.527
MAB21L1	0.39	31	0.552	0.96	94	0.856
KCNH2	0.38	30	0.861	0.95	93	0.133
FAAH	0.10	7	0.249	0.47	38	0.314
MGLL	0.09	7	0.780	0.15	11	0.556
NFkB	0.06	4	0.422	-0.01	-1	0.968
SLC27A1	0.05	4	0.778	0.24	18	0.732
DACT1	0.04	3	0.650	0.39	31	0.841
NAPE-PLD	-0.09	-6	0.388	0.30	23	0.958
PROK2	-0.10	-7	0.917	0.88	84	0.629
CNR1	-0.11	-7	0.594	0.86	82	0.432
ALOX12	-0.19	-12	0.807	0.45	36	0.984
IKBKE	-0.26	-17	0.221	0.59	50	0.777
EGFL6	-0.28	-17	0.937	0.15	11	0.240
DAGLA	-0.29	-18	0.701	0.58	50	0.456
WNT10B	-0.35	-22	0.972	0.57	49	0.845
PTGS2	-0.41	-25	0.972	**1.42**	**167**	**0.050**
DACT2	-0.96	-48	0.133	0.79	73	0.952
WNT3	-0.98	-49	0.861	0.80	74	0.556
ALOX5	-1.09	-53	0.975	0.38	30	0.904
MMP9	3.17	-803	0.650	-0.52	-30	0.674

[1]Log FC calculated as Log_2 mean (2-ΔΔCt divided by mean 2-ΔΔCt of normal weight data)

[2]Effect of fish oil analysed by Wilcoxon test

Table 7.5 Effect of fish oil on the expression of selected genes in scWAT form normal weight (n=27) and metabolically healthy obese individuals (n=39).

7.4.2.2 The effect of 12-week corn oil intervention on gene expression in scWAT from normal weight and MHO individuals.

12-week CO intervention significantly up-regulated *MAB21L1* by 379% in normal weight individuals and up-regulated *MGLL, SLC27A1* and *WNT10B* by 105%, 146% and 2% respectively in MHO individuals in comparison to study entry (week-0) expression levels ($P \leq 0.05$, Table 7.6). 12-week CO intervention also significantly down-regulated *ALOX5* and *EGFL6* in MHO individuals by 25% and 14% respectively ($P \leq 0.05$, Table 7.6).

Many genes in normal weight individuals were non-significantly up-regulated in response to CO intervention with the greatest changes in expression observed for *ALOX15* (626%), *SCL27A2* (471%), *KCNH2* (373%), *FAAH* (138%), *NFκB* (108%) and *SLC27A1* (100%) (Table 7.6). In obesity however, a less potent effect of CO was seen on the up-regulation of these genes, and a down-regulation of *SLC27A2* (-7%) and *KCNH2* (-44%) was observed in response to CO intervention in these individuals. The expression of *DACT1, ALOX5, WNT3, DACT2* and *IKBKE* was also decreased in response to CO intervention in MHO individuals, but increased in normal weight individuals (non-significant, Table 7.6). Therefore, there appears to be an association between obesity and the changes in adipose gene expression in response to CO.

CO was used as a comparator oil in this study; 12-week intervention with this oil only significantly increased the expression of one gene in normal weight individuals suggesting this oil was an appropriate choice. However, there appears to be an interaction in MHO individuals as greater effects of CO were observed in these individuals. This may be due to differences in scWAT FA composition at study entry, in which MHO individuals had significantly higher proportions of linoleic acid (LA) and arachidonic acid (AA) (Chapter 3, section 3.4.1), or due to potentially altered lipid metabolism in obesity. CO is predominantly comprised of LA (for full intervention oil composition see Chapter 2, Table 2.1) but there was no increase in the proportion of either this FA, or its longer chain metabolite AA, in scWAT in response to CO intervention in either normal weight or MHO individuals (Chapter 3, section 3.4.2). Further to this, there were also no significant changes in saturated FAs, monounsaturated FAs or omega-3 polyunsaturated FAs in scWAT in response to CO intervention (Chapter 3, section 3.4.2). Therefore, the effects of intervention on gene expression cannot significantly be attributed to an increase in the proportion of LA or other CO derived FAs in scWAT.

Gene	Corn oil					
	Normal weight			Obese		
	Log FC[1]	Change (%)	(*P*)[2]	Fold Change[1]	Change (%)	(*P*)[2]
ALOX15	1.49	626	0.499	0.26	13	0.158
SLC27A2	0.34	471	0.612	-0.11	-7	0.152
MAB21L1	**0.52**	**379**	**0.041**	0.43	11	0.177
KCNH2	2.24	373	0.866	1.30	-44	0.221
FAAH	2.51	180	0.499	-0.45	22	0.300
MGLL	0.66	138	0.138	**0.23**	**105**	**0.043**
NFkB	1.05	108	0.398	-0.03	9	0.470
SLC27A1	0.11	101	0.735	**0.26**	**146**	**0.022**
DACT1	0.85	80	0.310	-0.41	-27	0.507
CNR1	0.55	78	0.398	0.03	35	0.540
ALOX5	0.68	60	0.499	**-0.19**	**-25**	**0.041**
WNT3	0.65	58	0.327	0.29	-8	0.944
DACT2	0.27	57	0.612	-0.84	-36	0.096
PTGS2	2.26	55	0.237	0.16	17	0.551
WNT10B	0.23	46	0.735	**0.18**	**2**	**0.041**
DAGLA	1.01	43	0.499	-0.65	20	0.780
EGFL6	0.14	38	0.866	**0.37**	**-14**	**0.016**
IKBKE	0.83	26	0.735	0.13	-9	0.311
ALOX12	2.86	20	0.612	-0.13	20	0.594
PROK2	0.46	17	0.866	-0.10	-2	0.221
NAPE-PLD	1.25	11	0.779	-0.21	30	0.345
MMP9	0.64	8	0.398	1.04	-12	0.756

[1]Log FC calculated as Log_2 mean (2-ΔΔCt divided by mean 2-ΔΔCt of normal weight data)

[2]Effect of corn oil analysed by Wilcoxon test

Table 7.6 Effect of corn oil on the expression of selected genes in scWAT from normal weight (n=27) and metabolically healthy obese individuals (n=39).

7.5 Discussion

The data described herein provides evidence for the appropriate use of a subset of the study cohort for RNA-Seq, as well as validating data obtained by RNA-Seq.

Direction and magnitude of changes in expression with MHO and FO intervention were highly concordant between qRT-PCR and RNA-Seq. Testing the difference between the FC values obtained from the two techniques would not be appropriate as they do not employ the same principle for adjustment/correction of expression data. Expression determined by qRT-PCR is adjusted for the expression of housekeeping (reference) genes. The selection of housekeeping genes in the current study proved to be difficult due to the large variation in expression between BMI subgroups and treatment conditions. It was determined that three housekeeping genes would be most appropriate and the three most stable genes (GABARAP, YWHAZ and VPS36) were selected. Difficulty in determining stable housekeeping genes was an indication of the variation amongst the sample cohort, which was further observed in experimental gene expression between BMI and treatment groups. This was not surprising given the inability to select an appropriate reference protein in Chapter 5. RNA-Seq on the other hand does not adjust expression values against housekeeping gene expression and therefore data generated from these two techniques will differ and cannot be directly compared. Expression values indicated by Ct values were, overall, particularly low. This was not due to lack of cDNA or poor quality RNA (average 260/280 of 1.95, 260/230 of 1.14, and RIN score of $\geq$6.5), so suggests the expression of these genes in the samples was biologically low. This low expression may also contribute to error seen in some of the reported genes and contribute to loss of significance for many large changes in expression.

However, evaluating the overall pattern of data, genes that were selected as being the top differentially expressed quantified by RNA-Seq analysis, were also the top differentially expressed genes when analysed by qRT-PCR. Expression direction and pattern is concordant between the two techniques and therefore, qRT-PCR analysis of the full cohort successfully validated data obtained from RNA-Seq performed on a subset of the sample cohort and suggests use of a subset is appropriate for analyses in which the full cohort cannot be used due to cost or sample availability.

Data from the current study further indicate dysregulation of scWAT gene expression in obesity, particularly of genes involved in tissue growth, remodelling, and inflammatory signalling, as well dysregulation of changes in gene expression modulated by FO intervention.

As discussed previously, Wnt proteins are involved in the regulation of adipogenesis; findings from the current study indicate a significant 39% decrease in *Wnt3* expression and 44% decrease in *Wnt10B* expression in the scWAT of MHO individuals in comparison to normal weight individuals, which may result in the positive regulation of adipogenesis ($P \leq 0.05$, Table 7.4). However, the current study also reports a significant 27% decrease in *DACT2* expression and non-significant 27% decrease in *DACT1* expression in these individuals; *DACT1* and *DACT2* encode scaffold proteins known to influence Wnt signalling (Table 7.3). *DACT1* expression has been shown to inhibit Wnt signalling, but the actions of *DACT2* remain undefined in human WAT (Christodoulides et al., 2009). In conditions of high pro-inflammatory stimulus such as increased concentrations of TNF-α in obesity, Wnt signalling is hijacked and no longer responds to nutritional cues (Sethi and Vidal-Puig, 2010). Data from the current study may suggest such dysregulation of Wnt/DACT signalling and is consistent with reports that *DACT* expression increases only to the point where the expansion limit of the scWAT is reached. Obesity beyond this point results in loss of adipose function and in this scenario, loss of *DACT* expression in conjunction with loss of Wnt signalling is observed (Sethi and Vidal-Puig, 2010).

Proteins encoded by *EGFL6* and *KCNH2* are also involved in cell differentiation and therefore adipogenesis (Oberauer et al., 2010, Vasconcelos et al., 2016, Hu et al., 2009), and the expression of these was increased in the scWAT of MHO individuals in the current study. Greater expression of epidermal growth factor like domain multiple-6 (EGFL6) has been previously observed in obesity, with a decrease in expression resulting from weight loss (Oberauer et al., 2010). Expression of EGFL6 has been seen to increase with differentiation of adipocytes indicating mature adipocytes to be the main source of EGFL6 expression in scWAT, with lower expression seen in SVCs. However, EGFL6 elicits effects on SVCs (enhanced proliferation) and has been shown to mediate cell adhesion (Oberauer et al., 2010). KCNH2 is also implicated in adipogenesis; it encodes potassium voltage-gated channel subfamily H member-2a, a protein involved in formation of potassium ion channels. Potassium transduction has involvement in the development of obesity as adipogenesis is dependent on ion channel function (Vasconcelos et al., 2016) and expression of KCNH2 has been shown to promote the proliferation of adipocytes (Hu et al., 2009).

Obesity was also associated with the expression of genes involved in lipid transport and accumulation. *SLC27A2* encodes FATP2, also referred to as very long-chain acyl co-A synthetase (ACSVL1), which has a role in activating LCFAs (Watkins, 2008). Once these FAs have been activated to their Co-A thioesters, they can undergo a range of reactions including incorporation into phospholipids or triglycerides, FA elongation and unsaturation, and β-oxidation (Watkins, 1997, Watkins, 2008). A decrease in the expression of *SLC27A2* was observed in MHO individuals

in comparison to normal weight (Table 7.3) which may suggest altered VLCFA activation and subsequent transport, incorporation and metabolism in obesity.

Not only does the differential expression of genes suggest enhanced scWAT expansion and altered lipid handling in obesity, it further suggests remodelling of the surrounding environment.

In agreement with this, there is a greater expression of *MMP9* in obesity; the protein encoded by this degrades type IV and V collagens as well as other ECM proteins to allow changes to the surrounding environment during scWAT expansion (Yabluchanskiy et al., 2013). Therefore, expression of *MMP9* has been reported to be significantly increased during adipocyte differentiation (Bouloumie et al., 2001). MMP9 has also been reported to be highly expressed in activated adipose resident macrophages, which have a role in clearing cells undergoing apoptosis and ECM debris (Bourlier et al., 2008). Therefore, significantly greater expression of such genes in obesity is consistent with enhanced adipose expansion and remodelling and may suggest a greater number of activated adipose resident macrophages in obesity.

Differential expression of genes encoding proteins involved in inflammatory signalling in obesity may further suggest differential adipose immune cell populations in obesity. There was a significantly greater expression of *ALOX5* in the scWAT of MHO individuals in comparison to normal weight, which may suggest an increase in capacity to synthesise lipid metabolites via the 5-LOX pathway. As such, it is reported in Chapter 4 that MHO individuals had a significantly higher proportion (%) and absolute concentration (ng/g scWAT) of the 5-LOX oxygenation product, 5-HETE, derived from AA. Increased expression of 5-HETE may result in enhanced neutrophil activation and migration into the AT in obesity (Bittleman and Casale, 1995).

There were no significant effects of FO intervention on the expression of genes in either normal weight or MHO individuals. However, there were many large non-significant changes and the overall pattern of the data is consistent with that obtained from RNA-Seq (Chapter 6) with an increase of 78% in *MAB21L1* expression, 101% in *KCNH2* expression, and 471% in *PROK2* expression, which were the top up-regulated genes in response to FO intervention in normal weight individuals (Table 7.5). FO intervention non-significantly increased the expression of genes involved in adipogenesis in normal weight individuals such as *WNT10B*, *WNT3* and *KCNH2*, and further up-regulated genes encoding enzymes responsible for synthesising lipid metabolites in normal weight individuals.

There was a 57% increase in *DAGLA* expression, 43% increase in *ALOX15* expression, 38% increase in *NAPE-PLD* expression and 46% increase in *CNR1* expression, as well as an 80% increase in the expression of *FAAH*. This may result in an increase in the synthesis of 15-LOX pathway metabolites

including 15-HETE and the resultant pro-resolving lipoxins synthesized from this, as well as altering the synthesis and degradation of ECs containing EPA, DHA and AA. This may result in anti-inflammatory actions within the tissue including reduction of pro-inflammatory cytokine signalling and modulation of adipogenesis (Balvers et al., 2010). Obese individuals appear to be less sensitive to these effects of FO intervention in that there was a much lower increase in expression of Wnt genes ($\leq$ 22%), only a 2% increase in expression of *CNR1*, and a 14% decrease in the expression of *NAPE-PLD* (Table 7.5).

Effects of CO intervention on the regulation of adipose gene expression were observed predominantly in MHO individuals only. The main FA contributing to the composition of CO is LA (Chapter 2); MHO individuals had a significantly greater proportion of LA in their scWAT than normal weight individuals at study entry (Chapter 3). The amount of LA given per day during the 12-week intervention period was around 14-15% above the normal daily intake (Baker et al., 2016); however, adipose FA composition following the intervention period indicated no significant changes in the proportion of LA in either normal weight or MHO individuals (Chapter 3). This would suggest that MHO individuals would continue to have significantly greater amounts of LA in their scWAT following the intervention period, which may contribute to the majority of these effects on adipose gene expression observed in the MHO individuals only. Actions of the genes differentially expressed in MHO individuals in response to CO intervention may involve greater transport and metabolism of very long chain PUFA (Watkins, 2008), changes to lipid metabolite signalling through enhanced degradation of ECs and decreased synthesis of 5-LOX pathway metabolites, as well as decreasing scWAT expansion (Oberauer et al., 2010).

In conclusion, the current study reports evidence for the altered regulation of gene expression in human scWAT in obesity, and the modulation of gene expression by FO and CO in addition to the validation of data obtained by RNA-Seq. Differential expression of genes determined by qRT-PCR is consistent with differential expression of genes determined by RNA-Seq and may suggest enhanced tissue expansion and remodelling, enhanced transport and metabolism of VLCFAs, and an increase in pro-inflammatory signalling and lipid metabolite synthesis in obesity. This chapter provides evidence for the significant modulation of *PTGS2* expression, as well as the non-significant modulation of *MMP9* and *SLC27A2* expression by FO, which suggests effects of FO on scWAT FA handling, including FA metabolite synthesis, and tissue remodelling. The current study further reports altered regulation in the response of scWAT gene expression in response to FO and CO intervention between normal weight and MHO individuals. Many large, non-significant findings were reported which were attributed to by large variation between individuals within both normal weight and MHO subgroups. With this considered, full cohort data reported herein,

is concordant with data (significant and trends) obtained from RNA-Seq of a subset of individuals therefore validating the use of these data as well as the use of a subset for future analyses.

Chapter 8 Adipocyte size and presence of macrophages in crown like structures is altered in metabolically healthy obese individuals but not in response to fish oil intervention

8.1 Introduction:

It is discussed in the previous chapters that the subcutaneous white adipose tissue (scWAT) of obese individuals without metabolic complications (metabolically healthy obese; MHO) is associated with a fatty acid (FA) metabolite, transcriptome, and gene expression profile consistent with enhanced scWAT inflammation, expansion and remodelling.

When there is an excess of energy beyond the required needs, the adipose tissue (AT) can respond by increasing uptake of excess lipid in the form of triglyceride (TG) and storing this within existing adipocytes expanding them in size by tissue hypertrophy (Rutkowski et al., 2015, Walther and Farese, 2012, Thiam et al., 2013). Enlarged adipocytes have been observed in human obesity and in obesity with accompanied insulin resistance (Salans et al., 1973, Drolet et al., 2008, Verboven et al., 2018, Belligoli et al., 2019). As adipocytes enlarge, the local tissue environment needs to adapt to accommodate the larger cells and provide adequate oxygen and nutrient supply to the expanding tissue. If cells become trapped within a restricted extracellular matrix (ECM), they release inflammatory stress signals and may undergo necrosis (Sun et al., 2013).

Tissue remodelling involves the regeneration of ECM components as part of the normal repair process in response to inflammation. However, if tissue damage persists, the production of fibrillar ECM components continues and these accumulate resulting in a characteristic fibrotic appearance (Sun et al., 2013). Fibrosis is not just the accumulation of ECM components, but the imbalance between production of these components, such as collagen I, II, and VI, and the impairment of degradation of these by proteins such as the matrix metalloproteinases (MMPs)(Stamenkovic, 2003, Sun et al., 2011).

Hypoxic conditions accompanying scWAT expansion (Lee et al., 2010, Halberg et al., 2009, Pasarica et al., 2009b) have been shown to upregulate the expression of many ECM components such as collagens (Sun et al., 2013, Pasarica et al., 2009a) which is observed with increasing BMI (Pasarica et

al., 2009a). The first reports of fibrosis in human WAT came from examining transcriptional profiles of WAT from obese and normal weight individuals which revealed dysregulation of many ECM components in the WAT of obese individuals (Henegar et al., 2008, Mutch et al., 2009). Data from the current study detailed in Chapters 5 and 6 are concordant with such observations and report altered expression of many ECM components in obese individuals. Furthermore, overfeeding experiments show the fibrotic program to be induced shortly after high fat diet (HFD) challenge in mice, and suggest early changes to ECM genes occur with moderate weight gain with trends towards collagen deposition in the scWAT in humans (Halberg et al., 2009, Alligier et al., 2012).

Immunohistochemistry techniques have also been employed to confirm fibrosis and collagen accumulation which is considered to be a hallmark of WAT in obese humans with increased positive staining for ECM observed in both scWAT and visceral AT (VAT) of morbidly obese individuals (Divoux et al., 2012). Further to this, relationships between fibrosis and obesity related metabolic complications such as insulin resistance have been reported. Positive correlations in humans between collagen VI and impaired glucose metabolism, and higher presence of ECM components in the scWAT of obese insulin resistant individuals, suggest the involvement of WAT fibrosis in the development of metabolic dysregulation (Pasarica et al., 2009a, Spencer et al., 2011). In addition to this, collagen VI knock out mice have impaired ECM stability and reduced fibrosis, and in response to HFD challenge or being genetically ob/ob, exhibit improved local and systemic metabolic parameters despite increase in adipocyte size (Khan et al., 2009). This may further suggest fibrosis, in particularly an accumulation of collagen VI, has an important role in the development of metabolic dysregulation.

Positive relationships between ECM status, adipocyte survival, and inflammation, including M1 phenotype macrophage infiltration and polarisation, have also been reported (Pasarica et al., 2009a). Adipocytes within a restrictive fibrotic ECM lose function and undergo necrosis triggering infiltration of lymphocytes, neutrophils and macrophages, creating a pro-inflammatory environment (Sun et al., 2013). Macrophages accumulating around dying adipocytes form crown like structures (CLS) consisting predominantly of M1 phenotype macrophages which produce pro-inflammatory signals (Lumeng et al., 2007a, Lumeng et al., 2007b, Boutens and Stienstra, 2016, Engin et al., 2019). CD68 staining allows identification of macrophages by their localisation, i.e. stained macrophages observed clustered in CLS are likely to be predominantly of the M1 phenotype (Lumeng et al., 2007a, Lumeng et al., 2007b, Boutens and Stienstra, 2016, Engin et al., 2019).

Reports of the influence of long chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA) on WAT remodelling and macrophage infiltration, particularly in the context of metabolically healthy obesity

are limited. A single report in human scWAT of insulin resistant obese individuals observed a reduction of $CD68^+$ stained macrophages following 12-week intervention with 4 g/day omega-3 ethyl esters (Spencer et al., 2013). Further to this, a report in non-diabetic obese individuals observed no change in the number of macrophages but a decrease in the expression of M1 phenotype macrophage marker CD40 following 8-week intervention with 3.36 g EPA + DHA/day (Itariu et al., 2012). Therefore, limitation in data on the effects of FO derived EPA and DHA on macrophage populations has limited understanding the possible benefits of LC n-3 PUFA in obesity, , but the existing data suggests it might be important to distinguish between insulin resistant and 'metabolically healthy' obese individuals.

As reviewed by Kuda *et al.*, reports of LC n-3 PUFA effects on tissue remodelling are also limited and are predominantly obtained from HFD-based animal models (Kuda et al., 2018). LC n-3 PUFA supplemented diets have been observed to decrease the number of epididymal WAT cells and inhibit HFD induced upregulation of a range of remodelling proteins including MMPs, procollagens and fibrillin in obese and obese diabetic mice (Huber et al., 2007, Adamcova et al., 2018). Furthermore, LC n-3 PUFA were observed to decrease the amount of ECM in gonadal WAT of obese diabetic mice (Huber et al., 2007). However, these observations have not been translated to human WAT or to human obesity.

Aggregation of available information in obesity lead to the hypotheses that a destabilised ECM results in reduced mechanical stress to the rapidly expanding adipocytes observed in obesity, and that inflammation, including macrophage infiltration and re-polarisation, only persists at the later stages of adipose dysfunction in response to an increasingly fibrotic environment. Further to this, intervention with LC n-3 PUFA may attenuate obesity-induced changes to WAT remodelling, including hypertrophy and ECM formation/degradation, and inflammation.

However, such observations and comprehensive data in human scWAT in obesity, particularly in the context of metabolically healthy obesity, and in response to LC n-3 PUFA to support or refute such hypotheses are lacking. Such data are required to give biologically relevant evidence to the observations described in the previous chapters of this thesis. The need for additional investigations led to the creation of research aims outlined below in section 8.2.

8.2 Aims:

The aims of the research described in this chapter were to:

- Investigate whether adipose tissue from MHO individuals had

i) adipocytes of larger diameter
ii) a greater degree of fibrosis
iii) a higher number of macrophages and more pro-inflammatory type macrophages identified as crown like structures (CLS)

in comparison to adipose tissue from normal weight individuals.

- Investigate whether FO intervention decreases fibrosis and the number of macrophages and CLS in WAT of normal weight and obese individuals
- Investigate whether this response to FO intervention is altered in MHO individuals.

8.3 Methods:

8.3.1 Experimental reagents and materials

Reagents and materials used for experiments are described in Appendix C.

8.3.2 Histochemical analysis of WAT

Human WAT was fixed in 4 mL of 10% neutral buffered formalin and stored at room temperature and embedded in paraffin wax. 4 µM sections were cut using a Leica RM2125 RTS microtome (Leica Biosystems, Wetzlar, Germany) and dried onto glass slides at 37°C overnight.

A sub-set of 40 samples were selected for H&E staining, Picro-sirius red staining, and CD68 staining. These samples were selected based on the sub-set analysed for RNA-Sequencing, which was validated by qPCR and shown to be representative of the whole cohort.

The characteristics of the subjects from whom samples were selected are reported in Table 8.1.

Pairs	Sex	Treatment	Age (y)	BMI (kg/m^2)	Waist (cm)	Body fat (%)
1	Male	Fish Oil	29	21.4	76.7	11.7
2	Female	Corn Oil	19	22.1	66.8	22.9
3	Female	Corn Oil	27	22.7	75.1	21.0
4	Female	Fish Oil	29	19.4	68.9	19.0
5	Male	Fish Oil	26	24.1	85.1	19.0
6	Female	Corn Oil	47	24.2	82.9	27.8
7	Female	Fish Oil	54	22.7	75.8	28.3
8	Female	Corn Oil	20	19.6	66.6	20.7
9	Male	Corn Oil	21	25.0	84.6	11.8
10	Female	Fish Oil	21	22.7	74.1	31.6
11	Female	Fish Oil	38	32.8	88.7	42.8
12	Male	Fish Oil	31	30.2	106.8	23.9
13	Female	Fish Oil	23	38.0	107.6	48.1
14	Female	Fish Oil	46	34.2	96.8	44.9
15	Male	Corn Oil	35	33.7	114.4	32.8
16	Female	Corn Oil	35	34.9	89.4	45.0
17	Male	Corn Oil	36	38.1	133.2	
18	Female	Corn Oil	31	33.9	101.2	43.3
19	Female	Fish Oil	41	32.4	102.6	40.7
20	Female	Fish Oil	53	35.2	112.2	47.8

Table 8.1 Characteristics of subjects from whom scWAT samples were selected for histochemical analysis

Pairs' is defined as both stud entry (week-0) and post intervention (week-12) samples of each individual, totalling 40 samples.

Body fat % data not available for participant 17.

8.3.2.1 H&E staining

Sections were dewaxed in tissue-clear (Sakura Finetek, Thatcham, UK) and rehydrated sequentially through graded alcohol, absolute alcohol, and 70% alcohol. Sections were stained with Mayer's Haematoxylin for 5 min then "blued" under running water. Sections were then stained with eosin for 5 min and briefly rinsed under running water. Sections were then dehydrated through graded alcohols and cleared 3 times in tissue-clear (Sakura Finetek, Thatcham, UK). Coverslips were mounted using Expert XTF mounting medium (Cell Path, Newtown, Wales).

8.3.2.2 Picro-sirius red staining

Sections were dewaxed in tissue-clear (Sakura Finetek, Thatcham, UK) and rehydrated through graded alcohols up to 70% alcohol. Sections were washed in distilled water twice for 5 min then treated with 0.2% phosphomolybdic acid for 5 min. Sections were then stained with Picro-sirius red for 2 hours then rinsed briefly in 0.01% HCl then distilled water. Sections were then stained with Mayer's haematoxylin for 2 min and blued under running water. Sections were then dehydrated through graded alcohols and cleared 3 times in tissue-clear (Sakura Finetek, Thatcham, UK). Coverslips were mounted using Expert XTF mounting medium (Cell Path, Newtown, Wales).

8.3.2.3 CD68 immunochemical staining

Sections were dewaxed in tissue-clear (Sakura Finetek, Thatcham, UK) and rehydrated through graded alcohols up to 70% alcohol. Endogenous peroxidase was inhibited with 0.5% hydrogen peroxide in methanol for 10 min. Sections were washed with tris buffered saline (TBS) 3 times for 2 min and then microwaved in citrate buffer (pH 6) at 80% power for 25 min to retrieve antigens. Sections were gradually cooled under running water before blocking avidin was applied for 20 min. Sections were washed with TBS 3 times for 2 min, drained, and blocking biotin applied for 20 min. Sections were washed with TBS 3 times for 2 min and blocking medium applied for 20 min. Sections were drained before primary CD68 PGM antibody (Dako, Denmark) (1:100 in TBS) was applied overnight, incubated at 4°C. Sections were washed with TBS 3 times for 5 min, drained, and biotinylated secondary antibody (goat, anti-mouse 1:800 in TBS (Vector Laboratories, Peterborough UK)) was applied for 30 min. Sections were washed 3 times for 5 min, drained, and avidin (1:800 in TBS) biotin (1:1000 TBS)-peroxidase complex applied for 30 min. Sections were washed with TBS 3 times for 5 min and drained before DAB substrate was applied for 5 min. Sections were rinsed in TBS and washed in running water for 5 min before counterstaining with Mayer's haematoxylin for 30 seconds. Sections were "blued" under running water, dehydrated through graded alcohols, cleared

in tissue-clear (Sakura Finetek, Thatcham, UK) and coverslips mounted with Expert XTF mounting medium (Cell Path, Newtown, Wales).

8.3.3 Image analysis

8.3.3.1 H&E imaging - Determination of adipocyte size

Sections were imaged using an Olympus VS110 scanner microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at 10 x magnification under normal light. Whole sample images were obtained and cropped into individual regions of interest (ROI), creating several smaller images totalling the whole image. Each individual ROI was then analysed in Fiji (open source, https://fiji.sc/) to identify adipocytes and quantify their size.

Three adipocyte analysis tools were compared; these were the Adiposoft plugin (open source, https://imagej.net/Adiposoft CIMA, University of Navarra), the Adipocyte Tools macro (open source, https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Adipocytes-Tools), and the Adipocount program (open source, http://www.csbio.sjtu.edu.cn/bioinf/AdipoCount/ CSBIO). Out of the 3 tools, Adiposoft had the best accuracy at identifying adipocyte membranes, particularly in poor quality samples such as that depicted in Figure 8.1 and was easiest to remove incorrectly selected cells.

Therefore, adipocytes were identified and counted using the Adiposoft plugin as depicted in Figure 8.2. Once identified, the counted adipocytes were assigned a number as shown in Figure 8.2B. Following counting, the identified adipocyte areas and diameters were automatically calculated based upon the pixel and unit information provided by the users (0.692 pixels per µm for all H&E images) and recorded in an excel file. Parameters for minimum and maximum diameters were set as follows: minimum = 40, maximum = 250. These were selected to exclude areas outside the range of the adipocytes in the ROI to avoid identification of non-adipocyte structures. Data from >100 adipocytes were averaged to give a single average diameter measurement per sample.

The success rate of the plugin was high despite some under/overzealous membrane selection of some cells, particularly those with faint or broken membranes as shown in Figure 8.2.

To rectify this, the image shown in Figure 8.2C was matched to the excel sheet containing the data labelled by adipocyte number and any unacceptable adipocyte selections were removed from the data sheet.

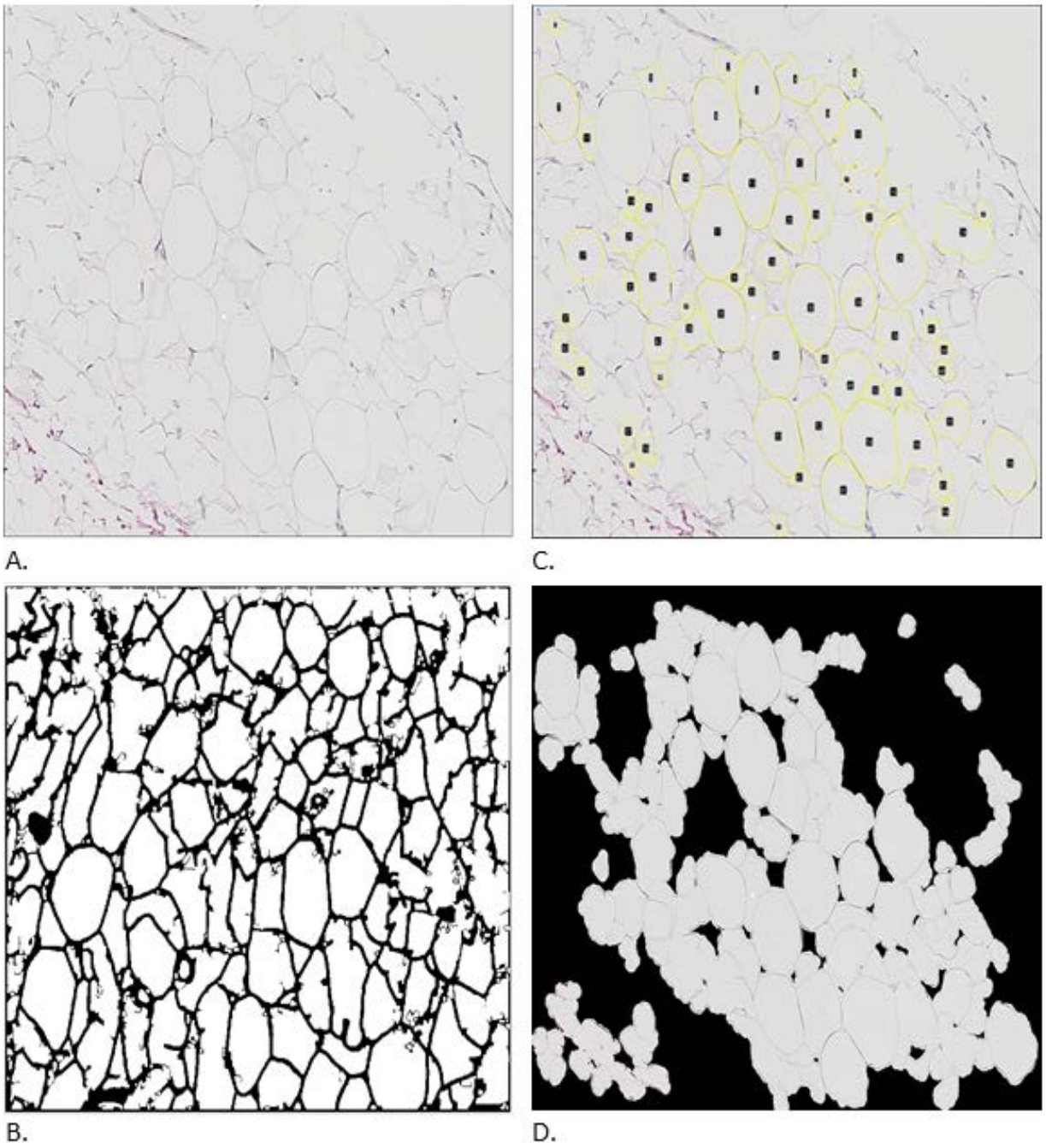


Figure 8.1 Comparison of adipocyte area quantification tools.

A. Original WAT ROI image; B. Adipocytes identified by Adiposoft plugin; C Adipocytes identified by Adipocount program; D. Adipocytes identified by Adipocyte Tools macro.

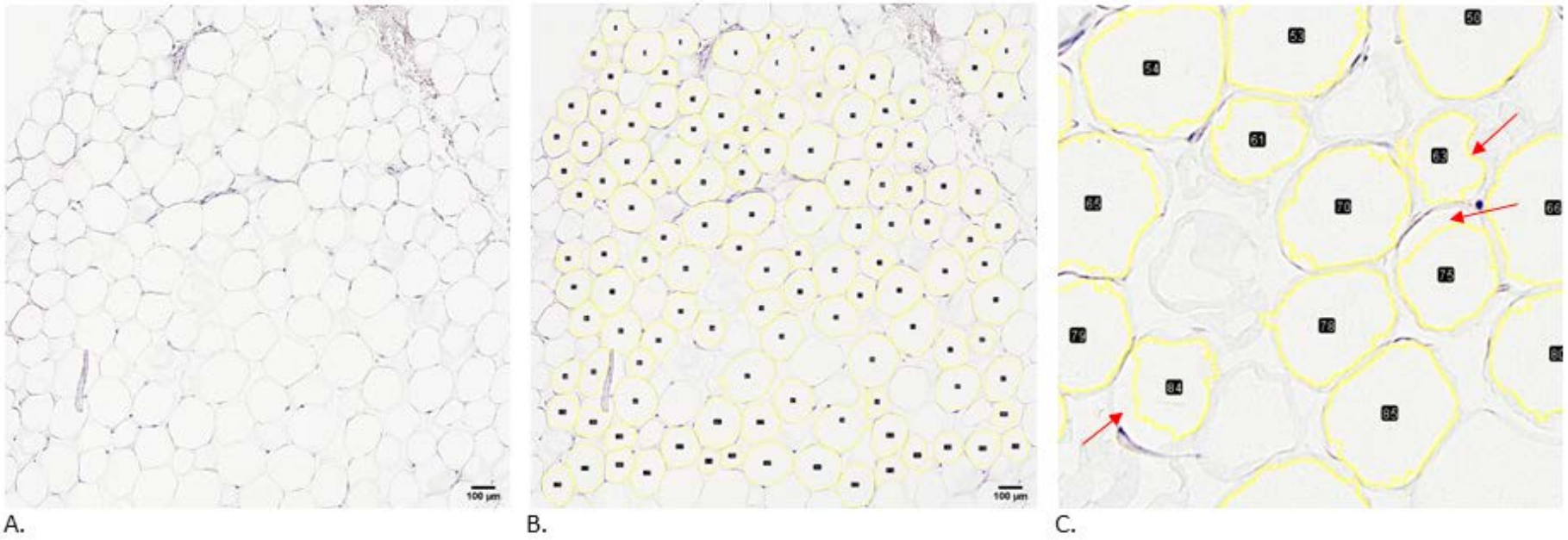


Figure 8.2 Adipocyte membrane recognition by the Adiposoft plugin

A. Original WAT ROI image; B. Adipocytes identified and counted by Adiposoft plugin; C. Incorrect selection of adipocytes by Adiposoft plugin.

Picro-sirius red imaging - Determination and quantification of total fibrosis

Sections were imaged using an Olympus VS110 scanner microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at 10 x magnification under normal light. Whole sample images were obtained and cropped into individual ROIs totalling the whole image. Each individual ROI was then analysed in Fiji (open source, https://fiji.sc/) using the MRI Fibrosis Tool (open source, http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Fibrosis_Tool), which employs a colour deconvolution macro created by Gabriel Landini.

This macro separates multiple colours of the staining into single images by splitting the RGB colour channels; it then employs a colour threshold to measure picro-sirius red staining. A selection of the image was set to define the total image area, ensuring no empty background was selected. The macro was then run on the selection, and picro-sirius staining in relation to non-stained tissue within the area selected was measured to give a relative area of fibrosis (picro-sirius staining) as depicted in Figure 8.3.

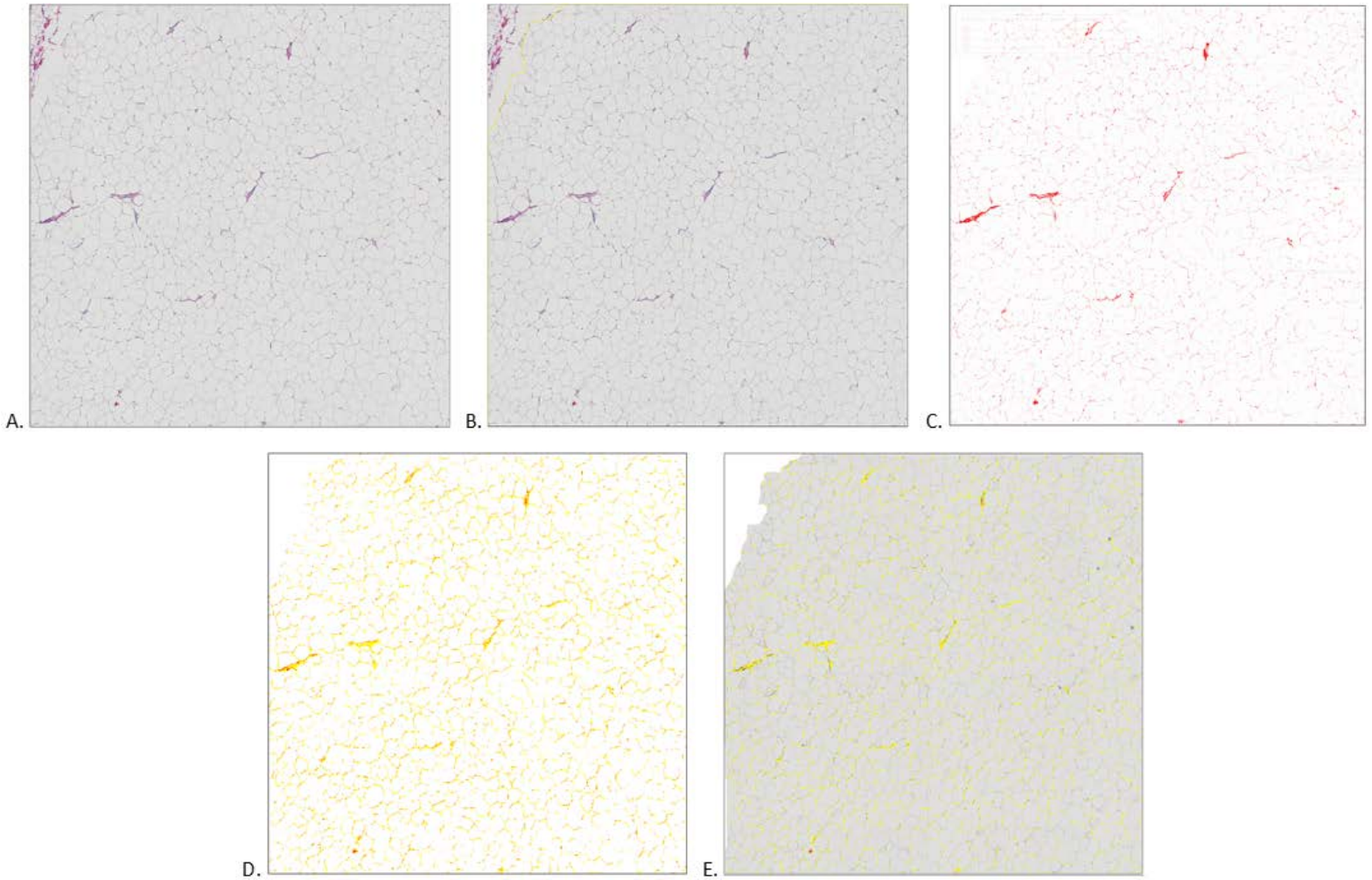


Figure 8.3 Workflow of MRI fibrosis tools in Fiji

A. Original WAT ROI image; B. WAT ROI selection for fibrosis measurement; C. Colour deconvolution - red; D. Colour deconvolution, red and yellow; E. WAT ROI post colour deconvolution with picro-sirius staining only detected.

8.3.3.2 Picro-sirius red imaging - Determination and quantification of pericellular fibrosis

Sections were analysed as described in 8.3.3.2 using the MRI Fibrosis Tool (open source, http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Fibrosis_Tool) in Fiji (open source, https://fiji.sc/). However, instead of selecting the entire tissue area, blood vessels and bundles of fibres were excluded from the selection to be analysed to ensure only fibrosis between cells, 'pericellular fibrosis', would be measured. An example of such selection is depicted in Figure 8.4.

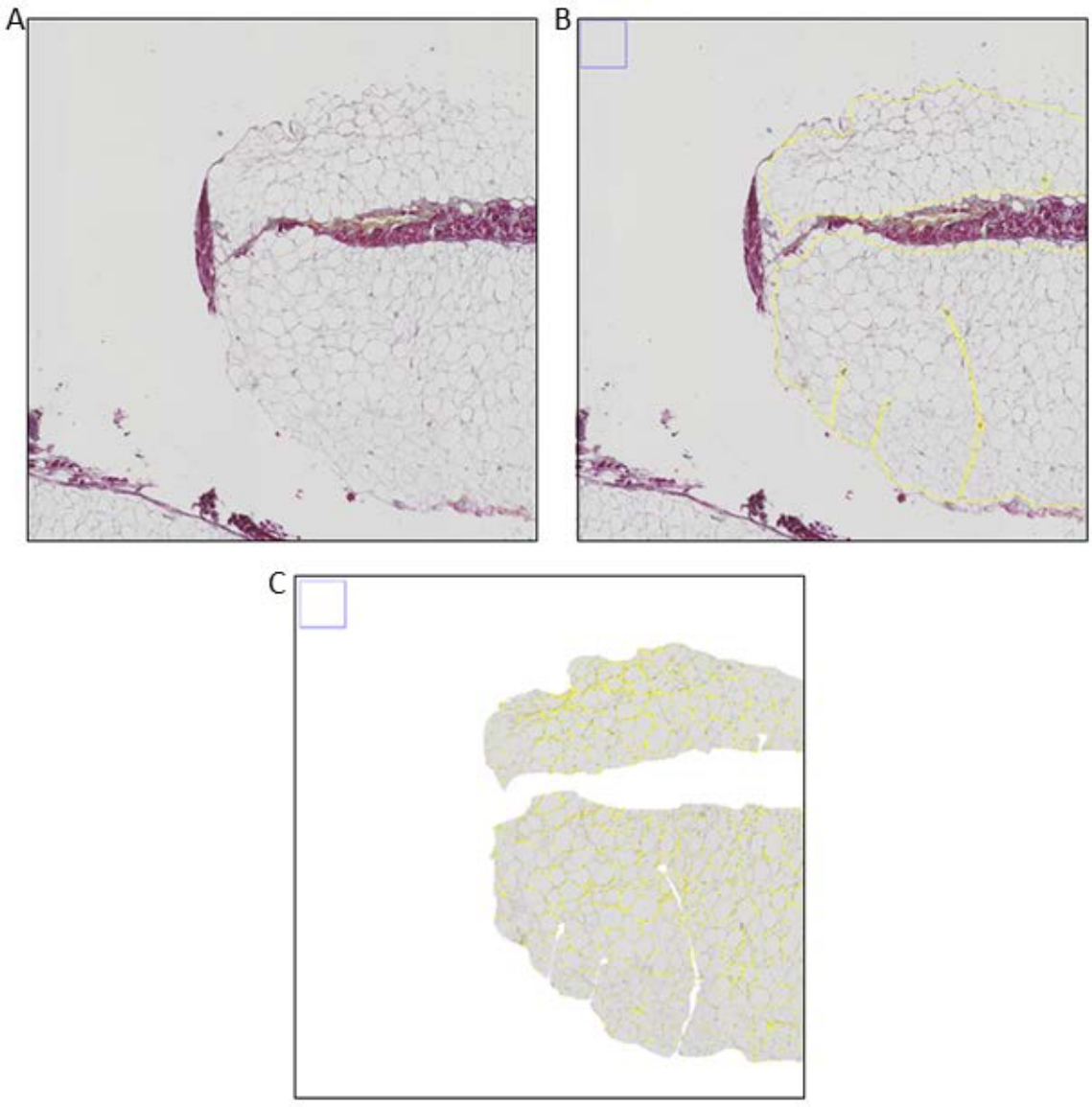


Figure 8.4 Workflow of pericellular staining selection using MRI fibrosis tools in Fiji.

A. Original WAT ROI image; B. WAT ROI selection for fibrosis measurement; C. Colour deconvolution - red; D. Colour deconvolution, red and yellow; D. WAT ROI post colour deconvolution with picro-sirius staining only detected.

8.3.3.3 CD68 imaging - Determination and quantification of macrophages

Sections were imaged using an Olympus VS110 scanner microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at 20 x magnification under normal light. Whole sample images were obtained and cropped into individual ROIs totalling the whole image. Each individual ROI was then analysed with number of macrophages quantified by eye due to incompatibility of automated macro detection due to faint staining.

A CLS was defined as $\geq$ 3 macrophages clustered around an adipocyte (Figure 8.5). The number of macrophages and CLS are presented in relation to the area (cm^2) of scWAT occupied by adipocytes. The area of tissue occupied by adipocytes was analysed using the using the MRI Fibrosis Tool (open source, http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Fibrosis_Tool) in Fiji (open source, https://fiji.sc/) as described for pericellular fibrosis quantification in 8.3.3.3 allowing measurement of area in μm^2 which was transposed to cm^2. The median area of the tissue samples analysed was 3894.64 (2096.68, 7046.70) cm^2 in normal weight individuals and 4090.64 (3641.43, 6239.63) cm^2 in obese individuals.

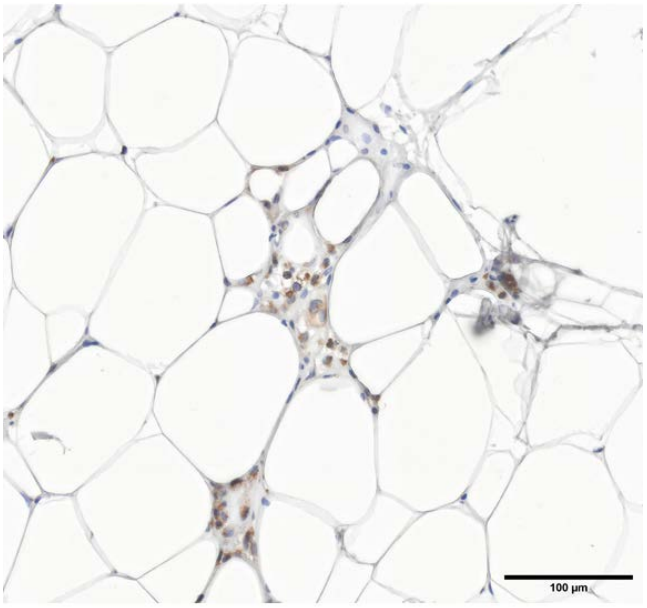


Figure 8.5 Identification of crown like structures (CLS) following CD68 staining in scWAT from metabolically healthy obese individual.

8.3.4 Measurement of plasma cytokines and adipokines

Fasted blood was processed to obtain plasma as described by Dr Carolina Paras-Chavez in her doctoral thesis (Chávez, 2014). Fasted plasma was analysed by Luminex Performance Assay multiplex kits from R&D to measure concentrations of cytokines and chemokines. I performed a number of these analyses and report data for interleukin (IL)-6, IL-10, tumour necrosis factor-alpha (TNF-α), vascular cell adhesion molecule-1 (VCAM-1), C-reactive protein (CRP), adiponectin and leptin in correlation with measures of adipose morphology herein.

Luminex assays employ the use of antibody coated colour-coded microparticles specific to the analytes of interest. Immobilised antibodies bind to the analyte of interest and a biotinylated antibody cocktail specific to the individual analytes is added to bind the complex. A streptavidin-phycoerythrin conjugate is then added which binds to the biotinylated detection antibody and is detected using a Luminex platform. The magnitude of the signal detected by Luminex is directly proportional to the amount of analyte present.

All reagents, standards, and samples were prepared as recommended by the manufacturer. A filter-bottomed microplate was pre-wet with 100 µL of wash buffer. The liquid was removed via vacuum and 100 µL of microparticle cocktail and 100 µL of sample or standards were added to the plate and incubated for 2-3 hr at room temperature (RT) on a microplate shaker at 200 rpm. After incubation, the plate was washed with 3 x 100 µL of wash buffer removed via vacuum. 50 µL of biotin antibody cocktail was added to each well and incubated for 1hour at RT on a microplate shaker at 200rpm. The plate was washed after incubation with 3 x 100ul wash buffer removed via vacuum and 50 µL of streptavidin-PE was added to the wells and incubated for 30 min at RT on a microplate shaker at 200 rpm. The microplate was washed 3 x with 100 µL wash buffer removed via vacuum and then 75 µL of wash buffer was added. The microplate was incubated for 2 min at RT on the microplate shaker at 500 pm and analysed immediately on the Biorad Luminex 200 using Bio-Plex Manager 6.1 software.

8.3.5 Statistical analysis

Adipocyte size data were normally distributed and therefore, independent sample t-tests were performed to assess differences in adipocyte area and diameter, and number of macrophages and CLS with BMI group as the factor. Paired sample t-tests were performed to assess the effect of 12-week FO and CO intervention on normal weight and obese individuals' data separately. These data are reported as mean $\pm$ SEM. Fibrosis and macrophage count data were not normally distributed and remained so following $\log_{10}$ transformation; therefore, non-parametric test

equivalents of the above analyses were performed using the original data. These data are reported as median (interquartile range).

MHO individuals are referred to as 'obese' in all figures and tables herein.

8.4 Results

8.4.1 Study entry results:

There was no effect of age or sex on adipocyte diameter, frequency, fibrosis or macrophage infiltration ($P \geq 0.301$; data not shown).

8.4.1.1 Metabolically healthy obesity is associated with a larger average size and higher frequency of enlarged scWAT adipocytes

The average area and diameter of adipocytes in the scWAT from MHO individuals were significantly greater in comparison to those in the scWAT of normal weight individuals ($P \leq 0.001$, Figure 8.6 and Figure 8.7). On average, adipocytes in the scWAT of MHO individuals were 84.98 ± 1.88 µm in diameter with an area of 5912.80 ± 253.04 µm^2, in comparison to those in the scWAT of normal weight individuals, which were on average 68.50 ± 1.51 µm^2 in diameter with an area of 3832.2 ± 175.35 µm^2.

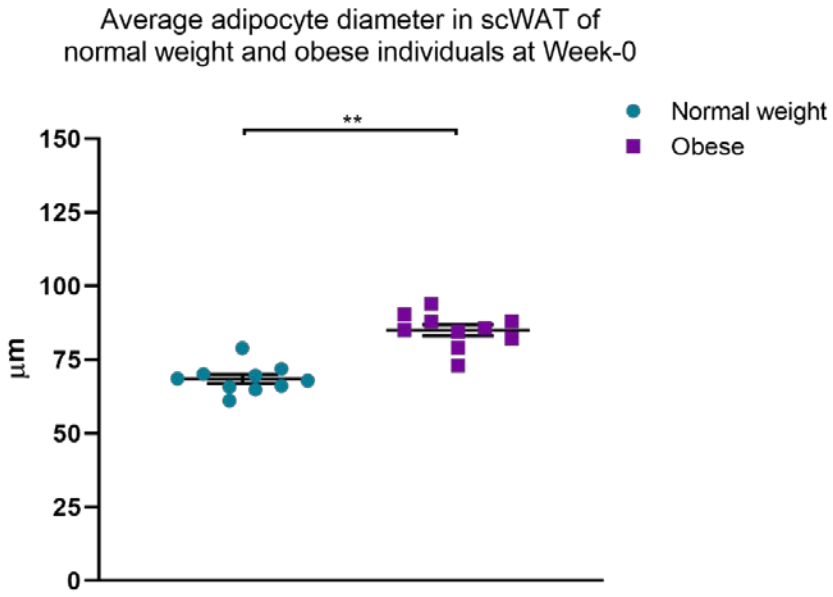


Figure 8.6 Adipocyte diameter in scWAT of normal weight (n=10) and metabolically healthy obese individuals (n=10) at study entry (week-0).

Mean (± SEM), *P* obtained using an Independent t-test comparing scWAT data from normal weight and metabolically healthy obese individuals at study entry (week-0), ** $P < 0.001$.

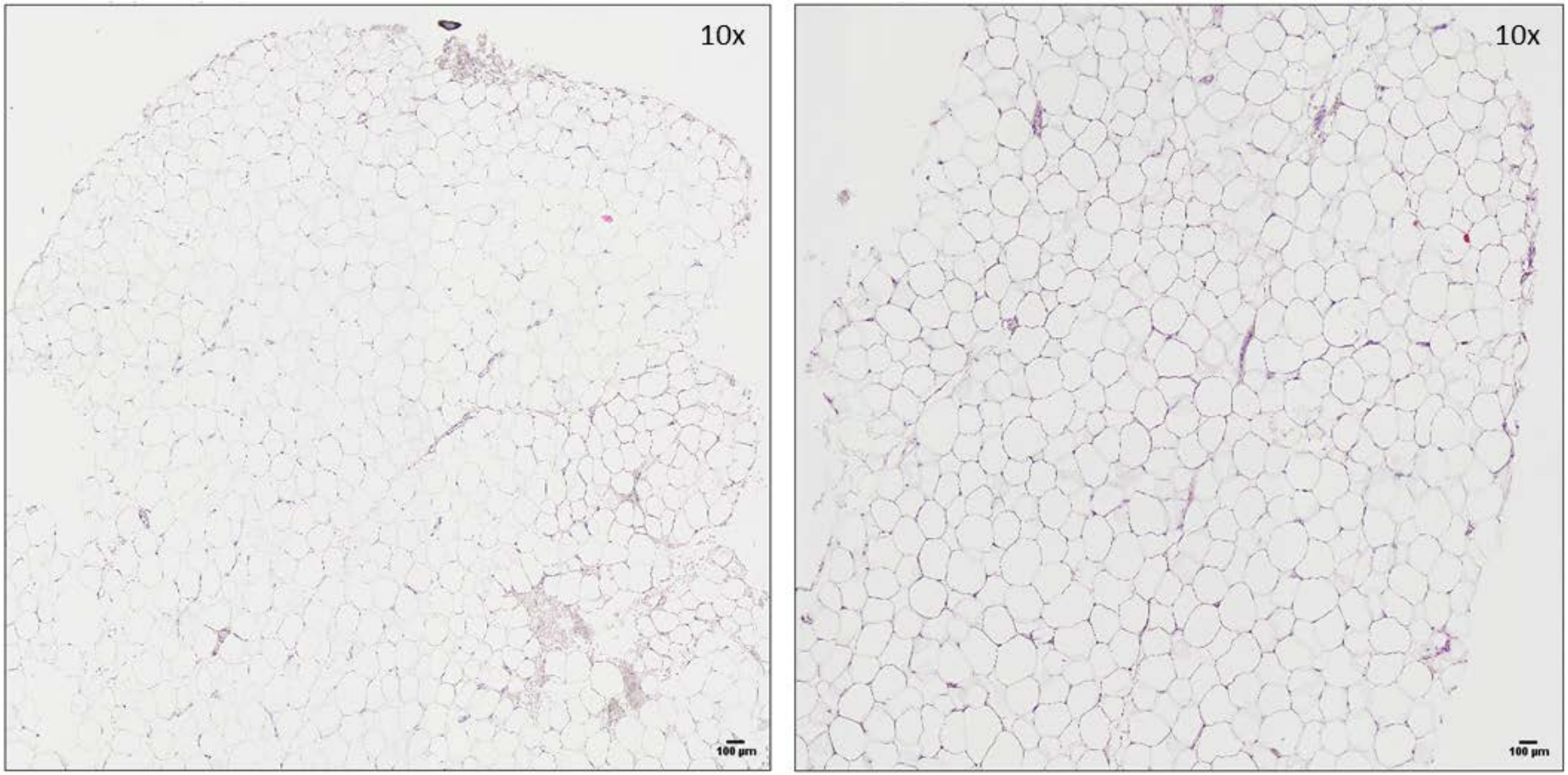


Figure 8.7 H&E histology of scWAT from normal weight and metabolically healthy obese individuals at study entry (week-0).

A. WAT from a normal weight individual collected at week-0; B. WAT from a metabolically healthy obese individual collected at study entry (week-0). Scale bar depicts 100 µm.

The distribution of scWAT adipocyte size was also investigated. Normal weight individuals had significantly higher frequency of small (40-60 µm) and medium (61-80 µm) sized adipocytes than MHO individuals ($P \leq 0.001$ and $P \leq 0.05$, Figure 8.8), and lower frequency of large (81-100 µm), very-large (101-120 µm) and extra-large (121-14 0µm) sized adipocytes ($P \leq 0.001$ and $P \leq 0.05$, Figure 8.8). A small number of extra, extra-large adipocytes (>141 µm) were observed in some MHO individuals only (Figure 8.8).

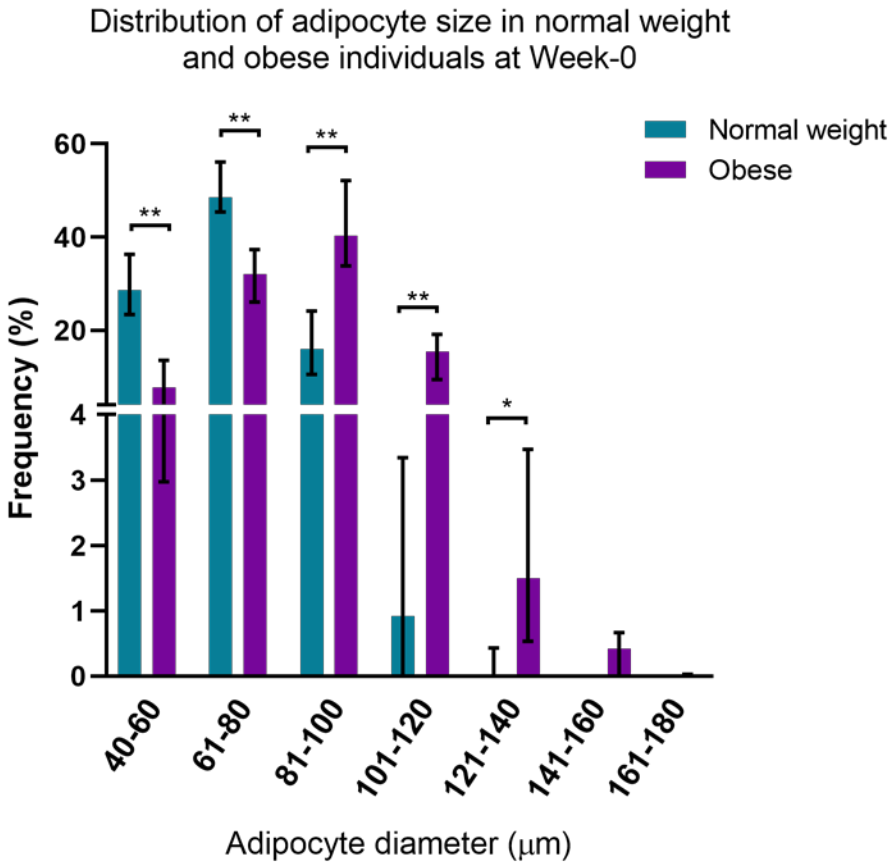


Figure 8.8 Distribution of scWAT adipocyte size (diameter) in normal weight (n=10) and metabolically healthy obese individuals (n=10) at study entry (week-0).

Median (± interquartile range), *P* obtained using a Mann-Whitney U model comparing scWAT data from normal weight and metabolically healthy obese individuals at study entry (week-0), ** $P \leq 0.001$, * $P < 0.05$.

Adipocyte diameter was significantly correlated with BMI, kg body fat and HOMA2-IR scores ($P \leq 0.001$, Figure 8.9), as well as % body fat ($P < 0.005$, Figure 8.9)**.**

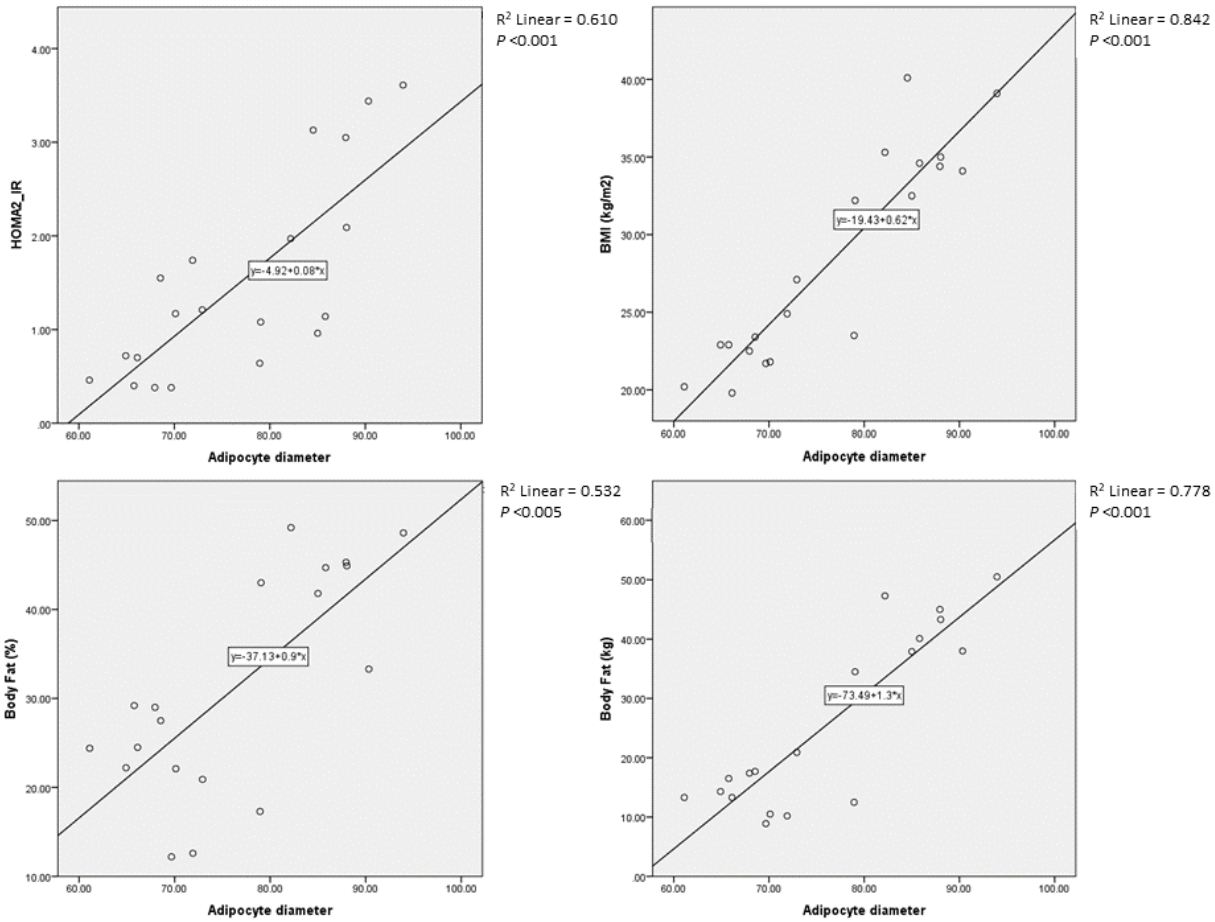


Figure 8.9 Correlation of adipocyte size (diameter) with BMI, body fat and insulin resistance in whole cohort.

Adipocyte diameter (µm) correlated with HOMA2 IR, BMI (kg/m^2), and Body Fat % and Body fat kg.

P obtained using a Spearman's rank correlation coefficient.

8.4.1.2 The relative area of total and pericellular fibrosis in scWAT is not altered in metabolically healthy obesity

Overall, the relative area of total fibrosis in WAT was 3.06 (2.14, 7.20) % in normal weight individuals and 3.51 (2.16, 4.21) % in MHO individuals at study entry (week-0) with no significant difference between the two groups (P = 0.616, Figure 8.10 and Figure 8.11). The relative area of pericellular fibrosis in WAT was 0.62 (0.34, 0.81) % in normal weight individuals, and 0.86 (0.55, 1.03) % in MHO individuals at study entry (week-0) with no significant difference between the two groups (P = 0.280) (Figure 8.10 and Figure 8.11).

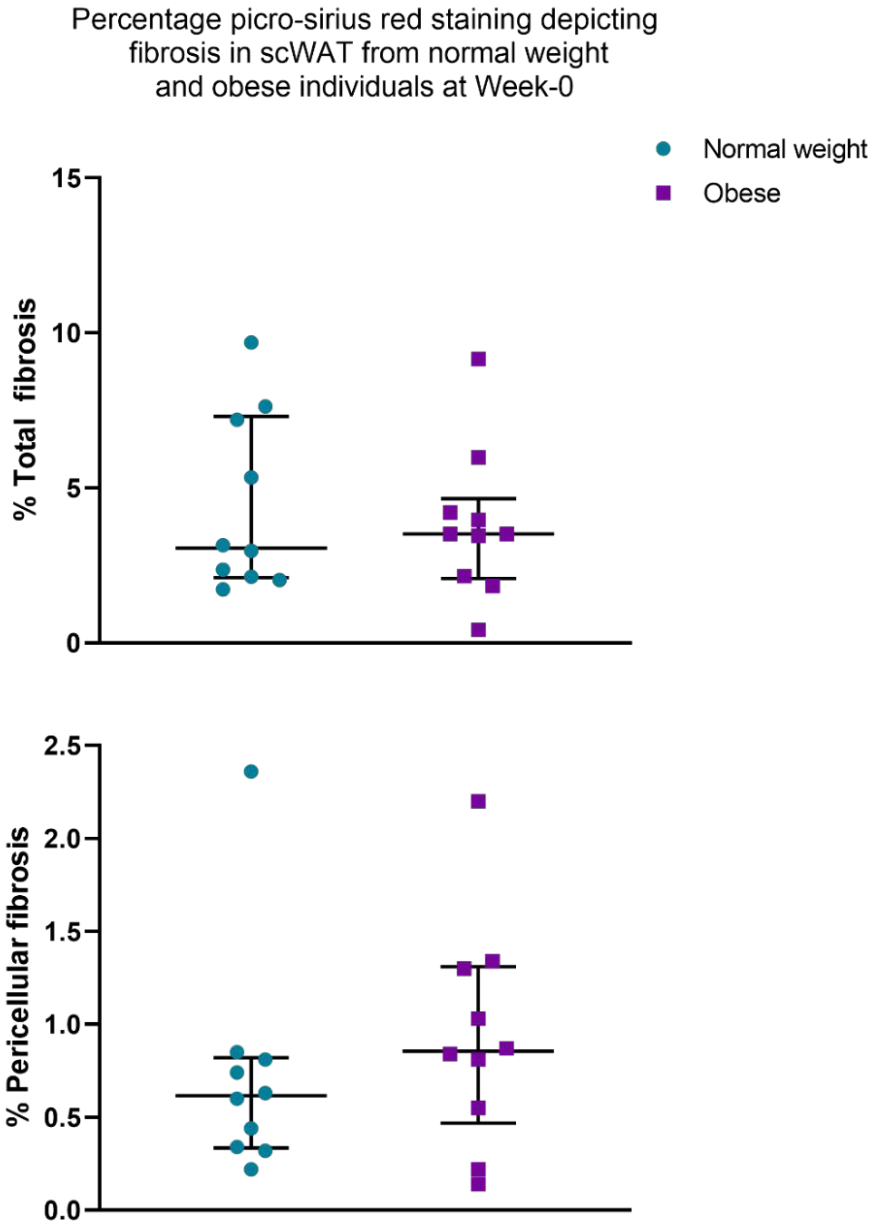


Figure 8.10 scWAT fibrosis in normal weight (n=10) and metabolically healthy obese individuals (n=10) at study entry (week-0).

Median (25^{th}, 75^{th} percentile), % area of total fibrosis defined as: (area of positive staining for picro-sirius red/total tissue surface)*100, % area of pericellular fibrosis defined as ((area of positive staining for pirco-sirius red excluding positively stained blood vessels and fibre bundles) / (tissue surface area excluding area of blood vessels and fibre bundles)) *100.

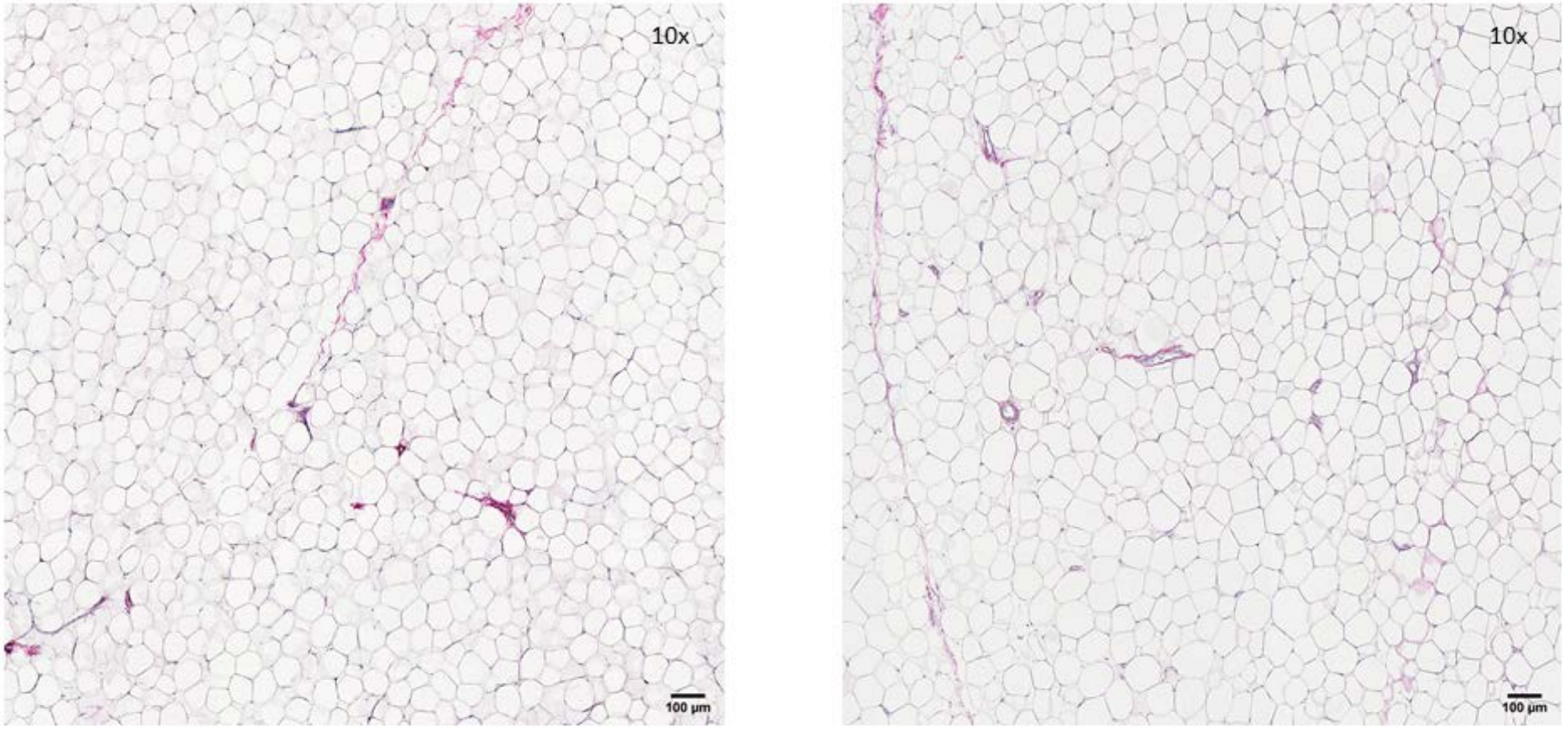


Figure 8.11 Picro-sirius stained sections of scWAT for total fibrosis in normal weight and metabolically healthy obese individuals at study entry (week-0).

A. WAT from a normal weight individual collected at week-0; B. WAT from a metabolically healthy individual collected at week-0.

The relative amount of total fibrosis was positively correlated with study entry (week-0) plasma cholesterol levels (*P* = 0.024) and exhibited a trend for positive correlation with study entry plasma NEFA levels in the cohort as a whole (*P* = 0.058, Table 8.2). The relative amount of pericellular fibrosis was positively correlated with study entry plasma insulin and HOMA2-IR in the cohort as a whole (*P* 0.027 both, Table 8.2).

When normal weight and MHO individuals' data were examined separately, relative total fibrosis was positively correlated with study entry plasma glucose (*P* = 0.023) and exhibited a trend for positive correlation with plasma NEFA in normal weight individuals only (*P*= 0.060, Table 8.2). Relative pericellular fibrosis was positively correlated with study entry plasma NEFA in normal weight individuals only (*P* = 0.043) and BMI (*P* = 0.048) and body fat (kg) (*P* = 0.050) in MHO individuals (Table 8.2).

Pericellular fibrosis was positively correlated with total fibrosis in both normal weight and MHO individuals (*P* = 0.022, and *P* = 0.037, Table 8.2).

% Total Fibrosis

	Whole cohort		Normal weight (n=10)		Obese (n=10)	
	P	Coefficient	*P*	Coefficient	*P*	Coefficient
BMI (kg/m^2)	-	-	-	-	-	-
Body fat (kg)		-	-	-	-	-
Cholesterol	0.024	0.503	-	-	-	-
NEFA	0.058	0.431	0.060	0.612	-	-
Glucose	-	-	0.023	0.703	-	-
Insulin	-	-	-	-	-	-
HOMA2-IR	-	-	-	-	-	-
% Pericellular Fibrosis	0.017	0.528	0.022	0.709	0.037	0.663

% Pericellular Fibrosis

	Whole cohort		Normal weight (n=10)		Obese (n=10)	
	P	Coefficient	*P*	Coefficient	*P*	Coefficient
BMI (kg/m^2)	-	-	-	-	0.048	0.829
Body fat (kg)	-	-	-	-	0.050	0.667
Cholesterol	-	-	-	-	-	-
NEFA	-	-	0.043	0.648	-	-
Glucose	-	-	-	-	-	-
Insulin	0.027	0.494	-	-	-	-
HOMA2-IR	0.027	0.493	-	-	-	-
% Total Fibrosis	0.017	0.528	-	-	-	-

Table 8.2 Correlations between BMI, body fat, and blood metabolic markers and WAT total and pericellular fibrosis.

P obtained using a Spearman's rank correlation coefficient.

8.4.1.3 Metabolically healthy obesity is associated with a greater number of macrophages arranged in crown like structures

Overall, the relative number of macrophages and CLS per 100 cm^2 of scWAT occupied by adipocytes was 0.19 (0.06, 0.32) and 0.02 (0.00, 0.03) respectively in normal weight individuals and 0.53 (0.20, 1.76) and 0.08 (0.02, 0.25) respectively in MHO individuals at study entry (week-0). The number of CLS was significantly higher in MHO individuals (P = 0.023) and there was a trend for significantly higher numbers of macrophages overall (P = 0.063, Figure 8.12 and Figure 8.13). The higher number of CLS present in scWAT of MHO individuals may reflect a higher proportion of pro-inflammatory M1 macrophages.

Number of CLS per 100 cm^2 of scWAT was positively correlated with BMI and body fat (kg) (P = 0.023 and 0.029 respectively) and number of macrophages per 100 cm^2 of scWAT was also positively correlated with body fat (kg) (P = 0.041, Figure 8.14). However, despite being significant, these correlations are not particularly strong and do not indicate BMI or body fat (kg) to be a clear predictor of macrophage or CLS numbers. There were no significant correlations with insulin or HOMA2-IR (data not shown). There is variation in CLS number in MHO which is not explained by variation in blood lipid or metabolic parameters but may be due to variation in adipokines and cytokines.

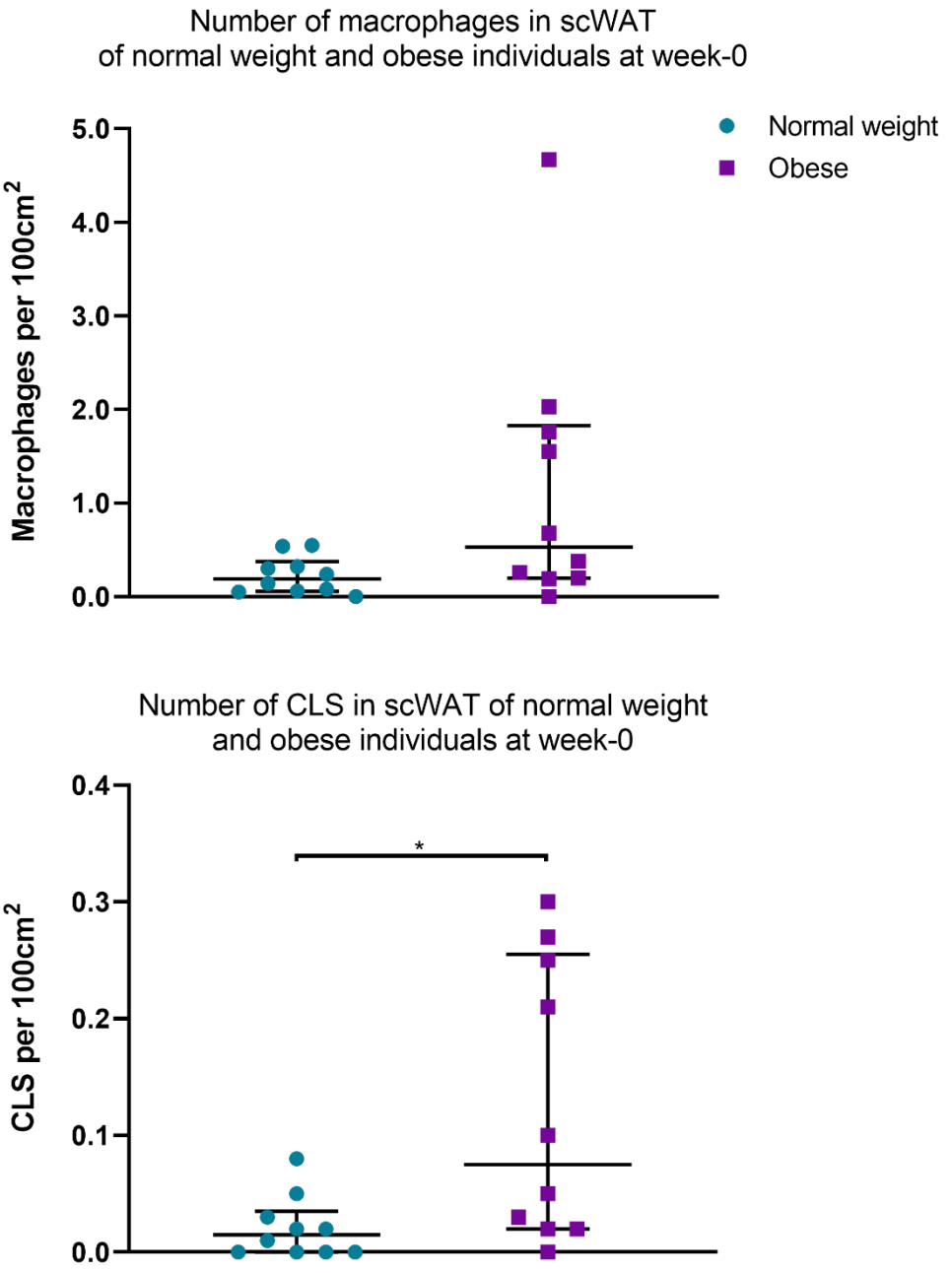


Figure 8.12 Macrophages and CLS present in scWAT from normal weight (n=10) and metabolically healthy obese individuals (n=10) at study entry (week-0).

Median (25^{th}, 75^{th} percentile), macrophages and CLS counted per $100cm^2$ of scWAT occupied by adipocytes. *P* obtained using a Mann-Whitney U model comparing scWAT data from normal weight and metabolically healthy obese individuals at study entry (week 0). * *P* = 0.023.

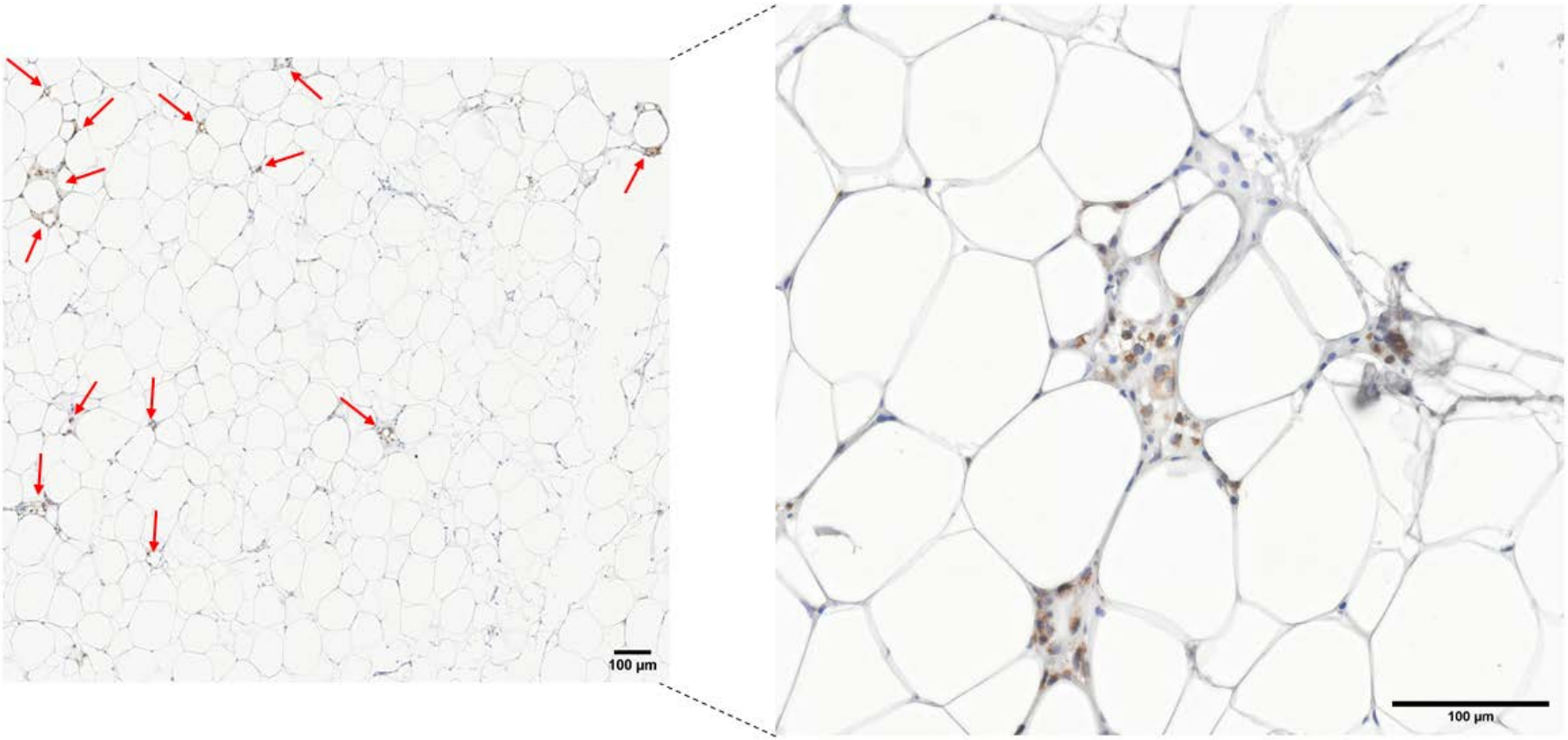


Figure 8.13 CLS as identified by CD68 staining in a section of scWAT from a metabolically healthy obese individual collected at study entry (week-0), and an enhanced view.

WAT from a metabolically healthy obese individual at week-0 showing presence of multiple crown-like structures (CLS) and an enhanced view of the number of macrophages within the CLS.

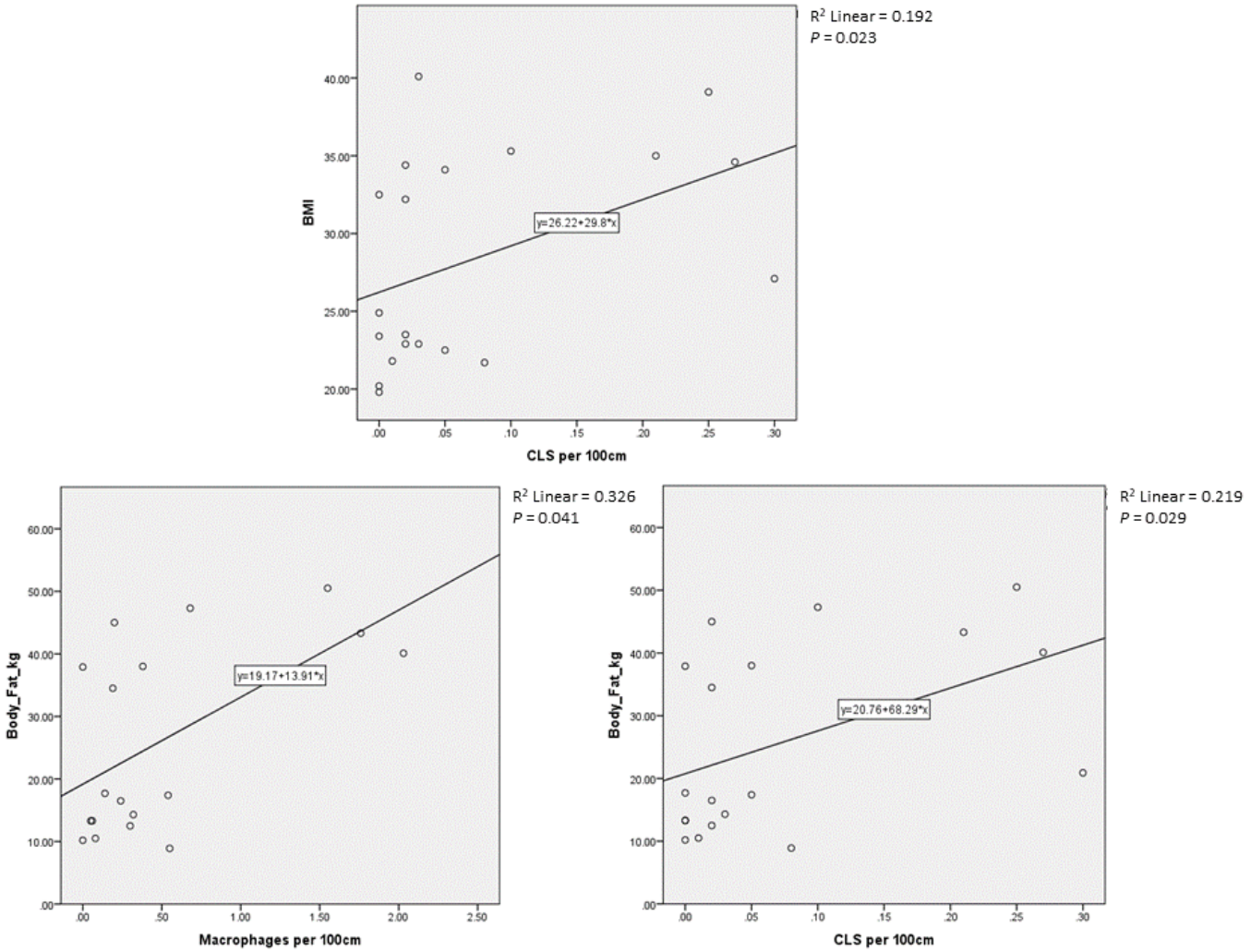


Figure 8.14 Correlation between number of scWAT macrophages and CLS, and BMI (kg/m^2), and body fat (kg) in whole cohort.

P obtained using a Spearman's rank correlation coefficient.

8.4.1.4 scWAT morphology is associated with blood cytokines and adipokines

Measures of adipose morphology were significantly correlated with study entry cytokines and adipokines. scWAT adipocyte size was positively correlated with plasma IL-6, CRP, and leptin, and negatively correlated with plasma VCAM-1 and adiponectin concentrations ($P \leq 0.05$, Table 8.3). There was a trend for a negative correlation between total fibrosis and adiponectin concentrations (P 0.068) and there were significant negative correlations between pericellular fibrosis and plasma TNF-α, IL-10, VCAM-1 and adiponectin concentrations ($P \leq 0.05$, Table 8.3). The number of macrophages was positively correlated with plasma IL-6, and number of CLS positively correlated with IL-6 and negatively correlated with adiponectin.

	Adipocyte size		Total Fibrosis		Pericellular Fibrosis		# Macrophages		# CLS	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
IL-6	0.499	0.025	-	-	-	-	0.472	0.035	0.491	0.028
IL-10	-	-	-	-	-0.555	0.011	-	-	-	-
TNF-a	-	-	-	-	-0.438	0.062	-	-	-	-
VCAM-1	-0.749	< 0.001	-	-	-0.536	0.015	-	-	-	-
CRP	0.731	< 0.001	-	-	-	-	-	-	-	-
Adiponectin	-0.597	0.005	-0.417	0.068	-0.617	0.004	-	-	-0.477	0.033
Leptin	0.683	0.001	-	-	-	-	-	-	-	-

Table 8.3 Association of adipocyte size, tissue and pericellular fibrosis and macrophage presence with plasma cytokines in the whole cohort.

P value and correlation coefficient obtained from Spearman's rank correlation.

8.4.2 Post intervention results:

8.4.2.1 12-week fish oil intervention does not alter adipocyte size in scWAT

There were no significant effects of either 12-week FO (P = 0.263) or CO (P = 0.880) intervention on the average size of scWAT adipocytes in normal weight individuals. In MHO individuals, there was a trend for an increase in adipocyte size with FO intervention (P = 0.071), and there was no significant change in size following CO intervention (P = 0.507) (Figure 8.15).

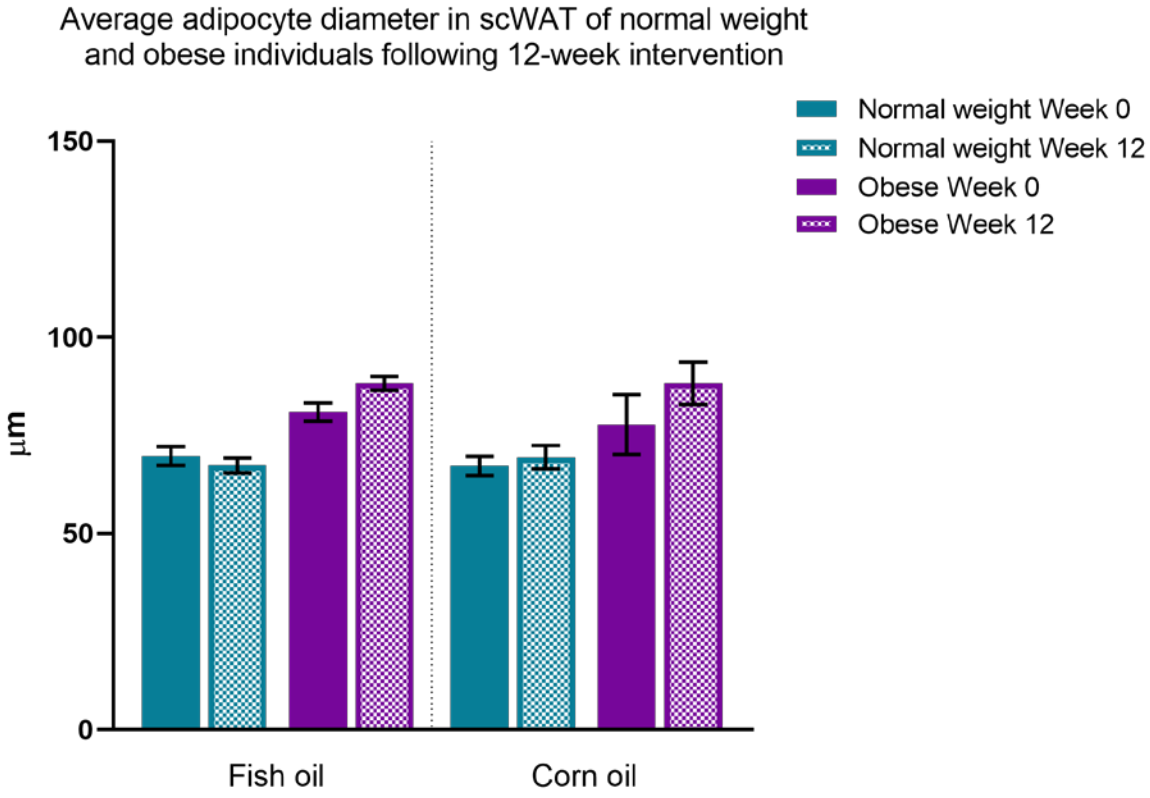


Figure 8.15 Adipocyte size (diameter) following 12-week fish oil and corn oil intervention. Mean (± SEM), Normal weight (n=10), metabolically healthy obese (n=10).

8.4.2.2 12-week fish oil intervention does not alter relative total or pericellular fibrosis in scWAT

Despite a slightly higher percentage of total fibrosis observed in the scWAT of obese individuals following 12-week CO intervention, there were no significant effects of either 12-week FO (P = 0.500 both) or CO (P = 0.500 normal weight, P = 0.686 MHO) intervention on the relative area of total fibrosis or the relative area of pericellular fibrosis (FO: P = 0.345 both, CO: P = 0.686 normal weight, P = 0.500 MHO) (Figure 8.16).

Figure 8.17 depicts typical tissue histology of WAT collected from two normal weight individuals at week-0 and week-12 following CO intervention (Figure 8.17A and B paired samples) and FO intervention (Figure 8.17C and D paired samples) stained with picro-sirius red.

Figure 8.18 depicts typical tissue histology of WAT collected from two MHO individuals at week-0 and week-12 following CO intervention (Figure 8.18A and B paired samples) and FO intervention (Figure 8.18C and D paired samples) stained with picro-sirius red.

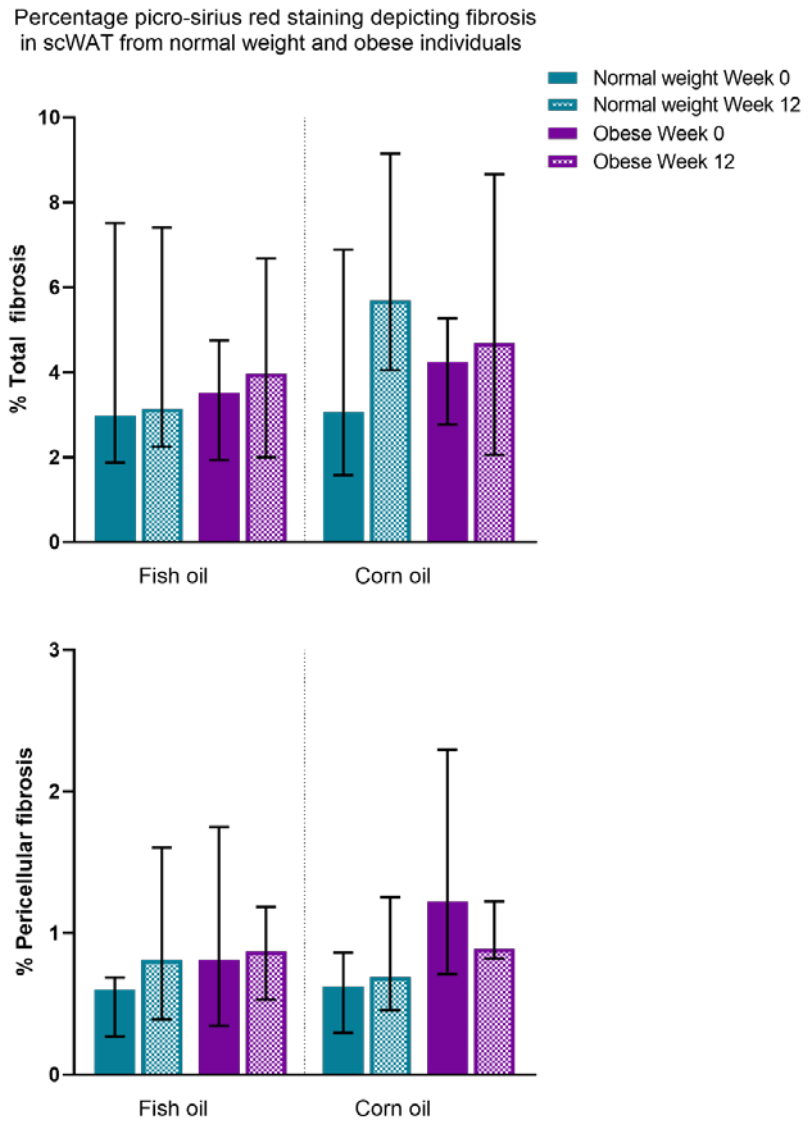


Figure 8.16 scWAT fibrosis following 12 week fish oil and corn oil intervention.

Median (25^{th}, 75^{th} percentile), Normal weight (n=10), metabolically healthy obese (n=10), % area of total fibrosis defined as: (area of positive staining for picro-sirius red/total tissue surface)*100, % area of pericellular fibrosis defined as ((area of positive staining for pirco-sirius red - positively stained blood vessels and fibre bundles) / (tissue surface area – area of blood vessels and fibre bundles)) *100.

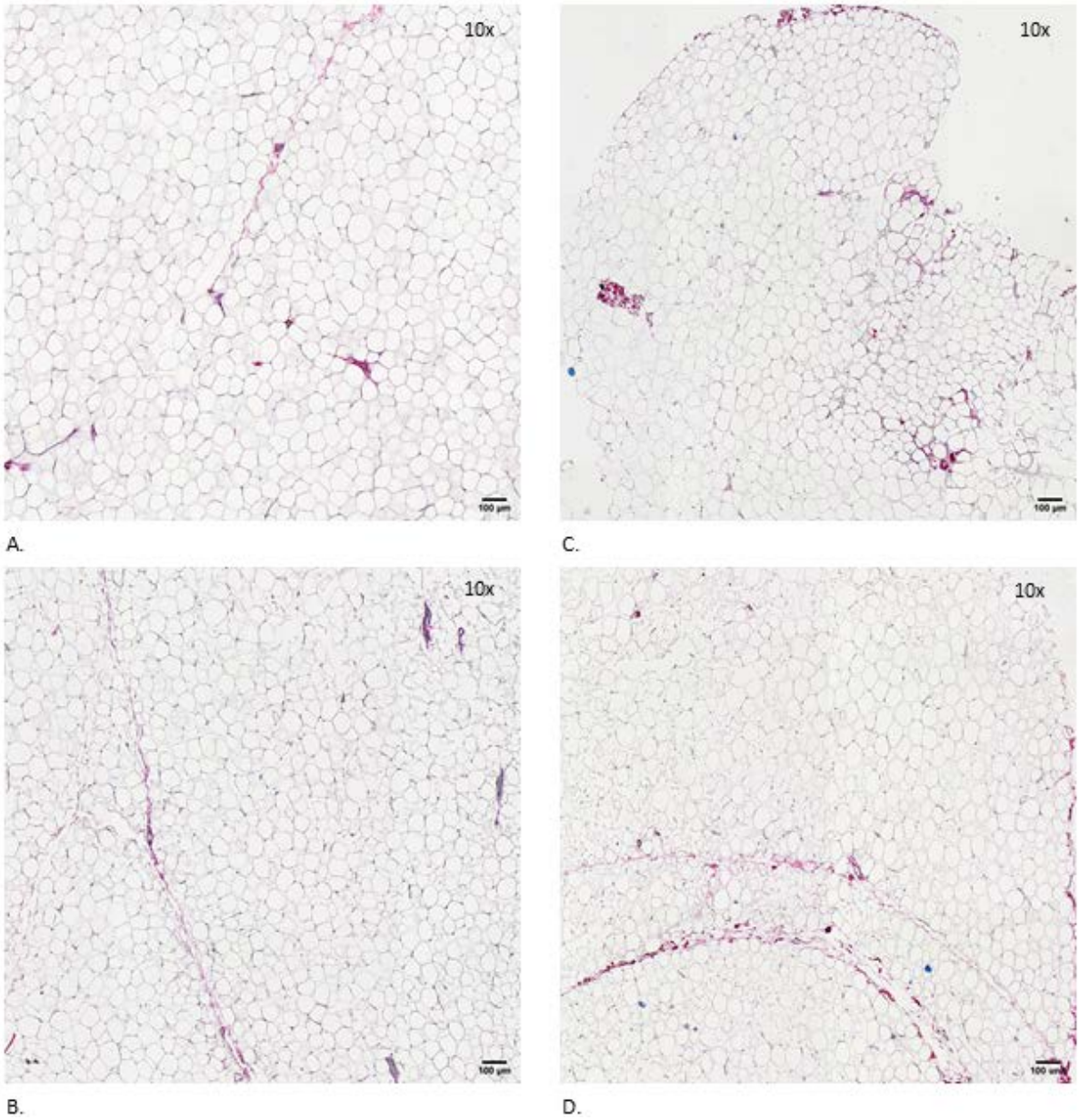


Figure 8.17 Picro-sirius stained WAT from normal weight individuals at study entry (week-0) and following CO and FO interventions (week-12).

A, WAT from normal weight individual at week-0; B, paired WAT from normal weight individual at week-12 following CO intervention; C, WAT from normal weight individual at week-0; D, paired WAT from normal weight individual at week-12 following FO intervention.

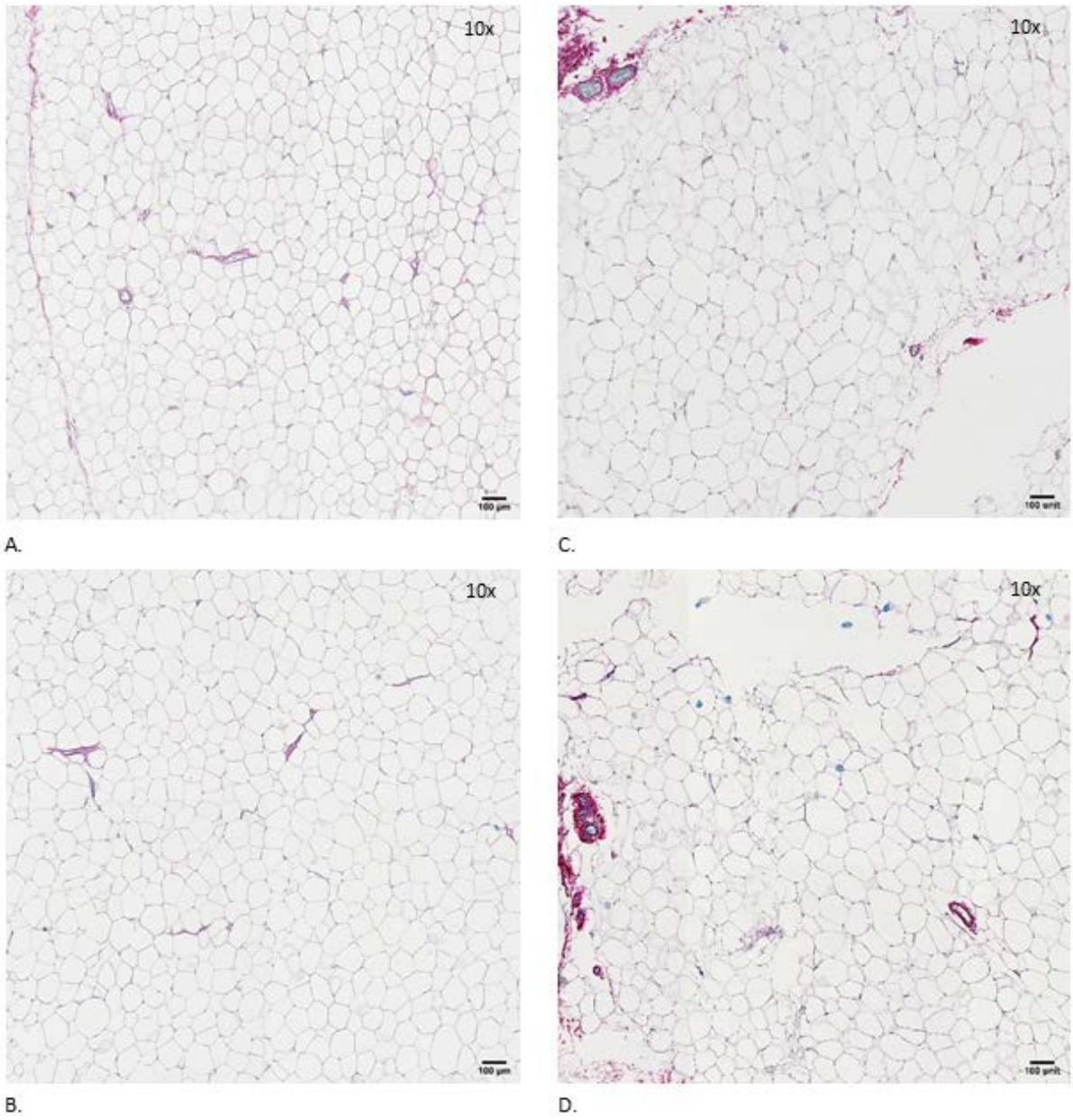


Figure 8.18 Picro-sirius red stained WAT from metabolically healthy obese individuals at study entry (week-0) and following CO and FO interventions (week-12).

A, WAT from a metabolically healthy obese (MHO) individual at week-0; B, paired WAT from a MHO individual at week-12 following CO intervention; C, WAT from a MHO individual at week-0; D, paired WAT from a MHO individual at week-12 following FO interventions.

8.4.2.3 12-week fish oil intervention does not alter macrophage or CLS numbers in scWAT

12-week intervention with FO did not significantly alter the number of macrophages or CLS in scWAT from either normal weight or MHO individuals ($P > 0.05$). CO intervention did not alter macrophage or CLS numbers either, validating its use as a comparator oil.

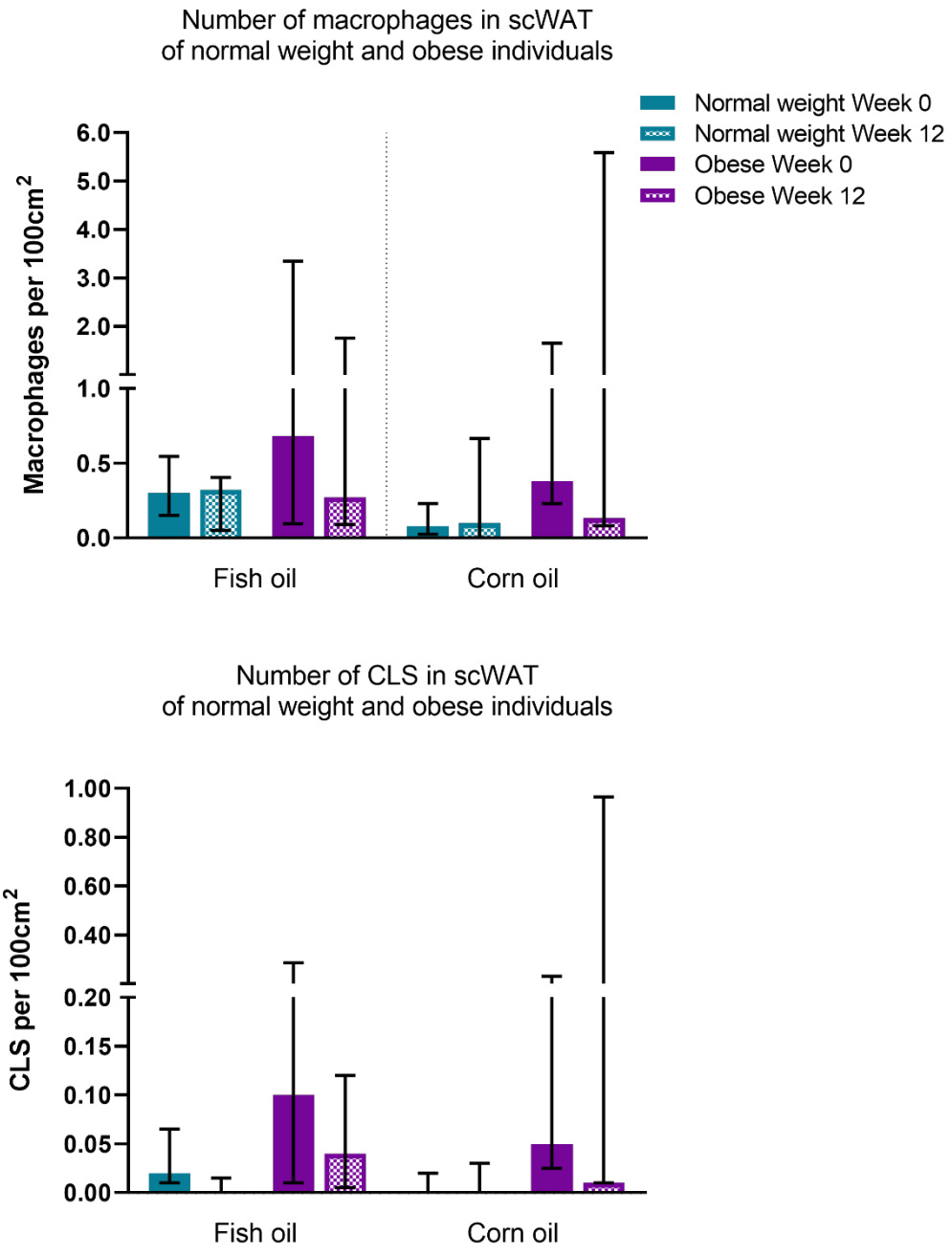


Figure 8.19 Macrophages and CLS in scWAT following fish oil and corn oil intervention.

Median (25th, 75th percentile), normal weight (n=10), metabolically healthy obese (n=10), macrophages and CLS counted per 100cm^2 of scWAT occupied by adipocytes.

8.5 Discussion

This study demonstrates that the scWAT of MHO individuals had a significantly enlarged adipocyte area, higher frequency of larger adipocytes, and lower frequency of smaller adipocytes in addition to a higher presence of CLS compared to scWAT from normal weight individuals. Tissue fibrosis did not differ in MHO individuals in comparison to normal weight individuals but was positively correlated with insulin and HOMA2-IR scores indicating significant association with metabolic health. Furthermore, it was demonstrated that 12-week FO intervention had no significant effects on adipose morphology, described as size of adipocytes, degree of tissue fibrosis, or macrophage infiltration and CLS formation.

Adipocytes in the scWAT from MHO individuals were significantly larger than in that from normal weight individuals and adipocyte diameter was positively correlated with BMI, body fat % and kg, and HOMA2-IR indicating a significant association with body fatness and metabolic health. Despite this, previous reports investigating adipocyte size in MHO and metabolically unhealthy obese individuals (with accompanying type-2 diabetes) do not report a difference in adipocyte size between these groups (McLaughlin et al., 2007, O'Connell et al., 2010, Smith et al., 2019). The observation of enlarged adipocytes in obese individuals is largely confirmatory and supports evidence for the role of adipocytes in lipid storage and the manifestation of obesity being an accumulation of excess lipid.

The MHO individuals recruited to the current study had fasting blood triglycerides (TG) within the healthy range (Chapter 2). This may suggest that the scWAT of these individuals retains some normal function in that it is able to respond to and store excess dietary lipid, which is further demonstrated by the incorporation of LC n-3 PUFA following 12-week intervention. However; adipocyte size was not correlated with fasting blood TG (P = 0.329, data not shown). Despite fasting blood TG and HOMA2-IR scores being within the normal range, they were still elevated in MHO individuals (by $\geq$ 54% and $\geq$ 43% respectively) in comparison to normal weight individuals. This may be indicative of progression towards declining adipose function and capability to store continued excess lipid in the MHO individuals.

The phenotype and function of adipocytes is related to their size; insulin resistant hypertrophic adipocytes exhibit increased levels of basal lipolysis, which is positively correlated with fasting blood TG (Tchoukalova et al., 2008, Ryden and Arner, 2017, Haczeyni et al., 2018). Rates of lipolysis, and lipogenesis also differ between small, medium, and larger adipocytes with small insulin sensitive adipocytes exhibiting a higher ratio of lipogenesis: lipolysis (Roberts et al., 2009, Haczeyni et al., 2018). In addition, hypertrophic adipocytes influence paracrine signalling and have

been observed to secrete more pro-inflammatory cytokines and less adiponectin (Meyer et al., 2013). This has implications for insulin signalling and the development of systemic insulin resistance (Meyer et al., 2013).

The current study provides further evidence for altered paracrine signalling associated with adipose hypertrophy; adipocyte size was negatively correlated with plasma adiponectin concentration and positively correlated with plasma IL-6, CRP and leptin concentrations. Furthermore, pericellular fibrosis was also negatively correlated with plasma adiponectin and IL-10 concentrations, which may have further implications on insulin signalling and sensitivity in the tissue and whole body (Meyer et al., 2013). Indeed, the current study reports positive correlations between pericellular fibrosis and both insulin concentrations and HOMA2-IR scores.

Fibrosis is the imbalance of ECM degradation and the deposition of new ECM components; this remodelling occurs as an adaptive response to adipocyte hypertrophy with the aim to provide additional space and nutrient supply to support the expanding tissue. Existing ECM is degraded by MMPs and new supporting ECM is formed by secretion of collagens. When there is excessive deposition of ECM components and the tissue exceeds expansion capability, the restrictive matrix results in adipocyte necrosis and release of stress signals resulting in immune cell infiltration and activation of macrophages. This is commonly seen in obesity with accompanied insulin resistance in which a fibrotic adipose environment is a key feature of the pathology. The interaction between immune cell infiltration and fibrotic state of the environment is not clear but the progression of inflammation in the tissue in obesity is thought to be due to a cascade of these events.

The current study demonstrates that the degree of both total and pericellular scWAT fibrosis was comparable between MHO and normal weight individuals and was not significantly correlated with macrophage infiltration or CLS formation. This is in contrast to existing reports of positive correlations between the two, and positive association with inhibited insulin signalling and insulin resistance (Spencer, 2010). The current study reports similar percentage of pericellular fibrosis for normal weight individuals but a lower percentage in MHO in comparison to unhealthy obese individuals currently described in literature (Divoux et al., 2010). Furthermore, the current study demonstrates that neither macrophage number nor CLS formation was associated with HOMA2-IR despite greater CLS formation and higher HOMA2-IR scores observed in MHO individuals. However, both number of macrophages and formation of CLS were positively correlated with IL-6, and formation of CLS was negatively correlated with adiponectin which plays a role in insulin sensitivity. This highlights the complexity of the relationship between fibrosis, immune cell recruitment, and insulin sensitivity.

Adipose dysfunction may manifest as a higher frequency of hypertrophic adipocytes in association with stress signals, adipocytes undergoing necrosis, deposition of ECM components, and immune cell recruitment attributed to the pathological enlargement of adipocytes. Destabilisation of the ECM may lead to a reduction in mechanical stress on the expanding adipocytes and environment, and that inflammation including macrophage infiltration only persists at the later stages of adipose dysfunction in response to an increasingly fibrotic ECM. Data from the current study is conflicting in that increased expression of genes encoding MMPs and collagens is reported in chapters 5 and 6 suggesting enhanced remodelling, but no physical differences in adipose fibrosis, despite a greater number of CLS in MHO individuals, are reported in the current chapter. This may suggest that a balance between ECM breakdown and deposition is maintained in metabolically healthy obesity. However, the current study further observes associations between number of CLS and pro-inflammatory cytokines, adiponectin and HOMA2-IR scores suggesting a more pro-inflammatory environment and association with insulin signalling even in the absence of enhanced fibrosis.

LC n-3 PUFA have been widely studied for their anti-inflammatory actions and it was therefore hypothesized that EPA + DHA may decrease parameters of inflammation in scWAT. Previous studies report that EPA + DHA intervention decreases AT expression of genes related to fatty acid uptake and storage, and increases expression of those related to beta-oxidation of fatty acids (Camargo et al., 2014, Todorcevic and Hodson, 2016). This may suggest a reduction in adipose lipid uptake and potentially adipocyte size. Indeed, reduced adipocyte diameter was seen in females with type-2 diabetes consuming 1.1 g of EPA and 0.7 g DHA daily for 8 weeks (Kabir et al., 2007) in addition to reduction of body fat determined by dual energy x-ray absorptiometry (DEXA) scanning; however, subsequent CT scans did not observe a significant reduction in either trunk scWAT or VAT following intervention with LC n-3 PUFA (Kabir et al., 2007). This is the only study to my knowledge in humans to report a reduction in adipocyte size following LC n-3 PUFA intervention. Hames *et al.* report no significant effects of 6 month 3.9 g EPA + DHA/day on adipocyte size, fat mass or % in insulin resistant adults (Hames et al., 2017) and the current study provides further evidence for no significant effect of EPA + DHA intervention on scWAT adipocyte size or on body fat. However, the duration of the FO intervention in the current study was only 3 months and the dose of EPA + DHA used (1.1g EPA + 0.8g DHA) was modest. This is reflected in the small, though significant, enrichment of scWAT in EPA and DHA reported in Chapter 3. Nevertheless, a higher intake of EPA+DHA for a much longer period of time may influence the outcomes assessed in the current chapter.

There is currently limited and unclear information on the effects of LC n-3 PUFA on adipose tissue macrophage infiltration and polarisation in humans. A negative correlation between the number

of pro-inflammatory macrophages and plasma membrane n-3 PUFA, particularly EPA, has been observed in VAT of healthy individuals (Poledne et al., 2019), and a decreased number of macrophages and CLS with FO intervention has been observed in scWAT of non-diabetic individuals with impaired glucose tolerance (Spencer et al., 2013). In contrast, 6-month intervention with 4.2 g EPA+DHA/day resulted in no change to macrophage or CLS numbers or polarisation of macrophages in insulin resistant overweight and obese individuals (Hames et al., 2017). Data from the current study further reports no significant correlations between either study entry or Δ LC n-3 PUFA and macrophage or CLS numbers following 12-week intervention. In addition to this, no significant change in the number of macrophages or CLS in scWAT of either normal weight or MHO individuals was observed following 12-week FO intervention.

Currently, there is no information on the effects of LC n-3 PUFA on fibrosis in human scWAT. LC n-3 PUFA have however been reported to decrease hepatic fibrosis accompanying MetS, as observed through a decrease in collagen associated genes and presence of collagen fibres in the tissue (Zhang et al., 2016, Tanaka et al., 2008). In addition, EPA has been observed to impair collagen reorganisation in wound healing in mice, which was attributed to elevation of IL-10 levels (Burger et al., 2019). Therefore, it could be hypothesized that collagen organisation and fibrosis observed in adipose tissue in obesity and the development of MetS may be reduced with LC n-3 PUFA intervention. However, to date there are no reports to confirm or refute this and therefore, the current study provides novel evidence for not only no effect of FO on the expression of genes associated with collagen degradation (Chapter 5), but also no effect of FO on collagen deposition (fibrosis) as determined by histochemical staining of scWAT from healthy normal weight or MHO individuals in the current chapter.

8.6 Conclusion

It can be concluded that metabolically healthy obesity is associated with greater adipocyte size and frequency of larger cells, and a higher number of CLS in the scWAT, but no differences in tissue fibrosis compared with heathy weight. In addition to data from Chapter 3 which reports a significant incorporation of EPA and DHA in response 12-week FO intervention, this may suggest some normal function of the adipose tissue is maintained as it can respond to changes in dietary lipid and remodelling processes are balanced. However; both adipocyte size and fibrosis were significantly correlated with HOMA2-IR suggesting metabolic health is implicated and that some individuals may be nearing the tissues' expansion and remodelling capacity which could result in the development of insulin resistance and further metabolic complications. In addition, the correlation between plasma adipokines, fibrosis, and macrophage presence in the tissue further

reflects the variation in the obese individuals and suggests a risk for the development of unhealthy obesity in a proportion of these.

12-week intervention with FO was unable to alter the level of fibrosis or macrophage numbers in scWAT from either normal weight or MHO individuals. It may be that the effects of LC n-3 PUFA on scWAT are preventative rather than therapeutic; indeed, LC n-3 PUFA interventions in animal models have shown prevention of increased lipid accumulation associated with feeding a high fat diet. Investigation into the effect of sustained high fat/calorie diet (observed in obesity) in addition to EPA+DHA supplementation on expansion of scWAT, adipocyte size, and accumulation of macrophages and collagen deposition in humans would be controversial (from an ethical perspective) but would provide greater insight.

Chapter 9 Discussion and conclusion

9.1 Rationale

Inflammation is a protective response which involves cellular activation and the secretion of chemical mediators in the context of immunity and tissue repair (Bessesen et al., 2015). In normal circumstances, inflammation is self-limiting; however, failure to resolve inflammation results in a sustained increase in the concentrations of chemical mediators and inflammatory cells at the site of inflammation as well as in the circulation (Serhan and Savill, 2005, Johnson et al., 2012). This is described as a state of chronic inflammation and is observed at the onset of, and during, obesity (Johnson et al., 2012, Chen et al., 2014, Masoodi et al., 2014, Bessesen et al., 2015). This chronic inflammation associated with obesity increases the risk of developing further metabolic disease such as insulin resistance and type-2 diabetes (T2DM) (Masoodi et al., 2014, Choe et al., 2016, Gerlini et al., 2018). Dietary fats can modulate inflammation and saturated fatty acids (SFA) are ligands for toll-like receptors (TLR) -2 and TLR-4 (Hwang et al., 2016) resulting in activation of pro-inflammatory pathways including NF-κB and the inflammasome (Roche, 2019) with subsequent increased concentrations of pro-inflammatory cytokines (Lee et al., 2001, Finucane et al., 2015, Dewhurst-Trigg et al., 2020). On the other hand, long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) have been shown to regulate the expression of transcription factors key to inflammatory signalling such as NF-κB and PPARγ, and to resolve a pro-inflammatory environment through reducing expression and secretion of inflammatory cytokines, and modulating expression of inflammasome related genes (Titos and Claria, 2013, Mejia-Barradas et al., 2014, Serhan et al., 2015, Martinez-Fernandez et al., 2015, Polus et al., 2016, Calder, 2017, Roberts Lee et al., 2019).

Therefore, intervention with these LC n-3 PUFA may have beneficial anti-inflammatory actions in scWAT and help to resolve obesity-associated inflammation. Previous investigations of inflammation in obese subjects are limited in humans, predominantly to investigations of circulating cells and mediators and/or in the context of metabolically unhealthy obesity (MUO) in which insulin resistance occurs, or to investigations which do not distinguish between metabolically healthy obese (MHO) and MUO individuals (Perreault et al., 2014, Pickens et al., 2014, Fjeldborg et al., 2014, Pickens et al., 2017). Few studies have comprehensively investigated the effects of obesity on a number of inflammatory parameters in human scWAT in the context of metabolically healthy obesity. Further to this, the effects of LC n-3 PUFA on obesity-associated inflammation are predominantly limited in humans to reports in the circulation, and again, do not consider the metabolic health of the individuals or assess the effects of these FAs on multiple

inflammatory markers in human adipose tissue (Rossmeisl et al., 2018, Polus et al., 2016, Martinez-Fernandez et al., 2015).

With the aim to provide a more comprehensive overview of inflammation in scWAT of MHO subjects, the current study investigated multiple parameters in these individuals including scWAT FA and lipid metabolite composition, expression and activity of proteins involved in lipid metabolite pathways, the transcriptomic profile of scWAT, the expression of scWAT genes involved in lipid metabolism and inflammation, adipose morphology (adipocyte size and fibrosis) and presence of macrophages in the scWAT. Further to this, with the aim to provide comprehensive evidence for the effect of LC n-3 PUFA on obesity associated scWAT inflammation, the current study examined the parameters detailed above, prior to and following 12-week intervention with a fish oil (FO) or corn oil (CO) supplement.

9.2 Summary of findings in scWAT of MHO individuals at study entry

9.2.1 Fatty acid and fatty acid metabolite composition of scWAT in metabolically healthy obesity

In general, the FA composition of scWAT obtained from normal weight individuals was concordant with previous reports (Bouéa et al., 2000, Baylin et al., 2002, Hodson et al., 2008, Browning et al., 2012, Walker et al., 2014, Hames et al., 2017). Reports of FA composition of scWAT in obesity are limited, but the composition detailed in the current study is consistent with that reported by (Kunešová et al., 2012) at study entry prior to a weight loss intervention. Comparison of scWAT FA composition between normal weight and MHO individuals is yet to be reported and the data in this thesis provides novel evidence for significantly altered FA composition in these individuals. scWAT from MHO individuals had higher proportions of several monounsaturated FA (MUFA) and omega-6 (n-6) PUFA including arachidonic acid (AA), and the LC n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as lower proportions of SFA and alpha-linolenic acid (ALA).

Dietary information at study entry was not obtained from the participants; therefore, differences in scWAT FA composition could not be correlated with dietary fat intake. However, there is great variability in strength and significance of correlations between dietary FA intake and concentration of FAs in adipose tissue (Hodson et al., 2008, Arab, 2003) , particularly with regard to SFA and MUFA which can be synthesised and elongated endogenously suggesting differences in de novo synthesis of FAs contributing to adipose tissue FA composition independent of dietary intake (Connor et al., 1996, Summers et al., 2000). Therefore, carbohydrate (providing glucose for

de novo lipogenesis) intake in the diet may be of as much, if not greater, importance when evaluating SFA and MUFA content of adipose tissue and would be of benefit to assess in future studies. With regard to LC n-3 PUFA, differences in scWAT content are likely to reflect dietary intake, altered synthesis and/or mobilisation of these FAs (Reynisdottir et al., 1995, Tsujita et al., 1995, Ryden and Arner, 2007, McQuaid et al., 2011, Arner and Langin, 2014, Morigny et al., 2016). In addition to highlighting compositional differences in scWAT in MHO individuals, data from the current study provides novel evidence for association between FAs in RBC and scWAT.

Examination of ratios of the parent FA to its corresponding elongated/desaturated FA derivative representing the activity of enzymes involved in the desaturation and elongation of FAs, indicates significantly greater activity of delta-6-desaturase (D6D), stearoyl-CoA-desaturase (SCD) n-7 and SCDn-9 in MHO individuals. This is concordant with previous reports of greater D6D and SCDn-7 activity in obesity, determined from ratios amongst circulating FAs (Pickens et al., 2014). These data suggest FA metabolism is significantly altered in metabolically healthy obesity and provide novel evidence for an altered scWAT FA profile in MHO individuals.

Complex lipids such as phospholipids and individual FAs within the scWAT can be metabolised to form metabolites that have potent regulatory actions on inflammatory and metabolic processes. The current study successfully identified 111 such lipid metabolites in human scWAT, of which 23 were quantifiable, to provide a lipid mediator profile for scWAT from normal weight and MHO individuals. Reports of altered FA metabolites including ECs associated with obesity are under reported in human scWAT (Annuzzi et al., 2010); however, there are a number of reports detailing dysregulation of FA metabolites in human plasma including HETEs, dihydroxy-HETEs (DiHETEs), epoxyoctadecaenoic acid (EpOMEs), dihydroxy-octadeacenoic acids (diHOMEs), and plasma LC n-3 PUFA derived mediators such as hydroxy-docosapentaenoic acids (HDPAs) (Pickens et al., 2017, Wang et al., 2017). The lipid metabolite profile of scWAT obtained from MHO individuals displayed a large degree of altered regulation with over 30 lipid metabolites differentially expressed with particular dysregulation observed for the endocannabinoid system (ECS). This altered regulation in lipid signalling metabolites suggests enhanced adipose expansion and remodelling in obesity, along with enhanced infiltration of immune cells and an overall greater pro-inflammatory state of the tissue environment.

The lipid metabolite profile may be reflective of both the FA composition of the scWAT and the availability of these FAs to be used as substrate to synthesize such metabolites, but also the activity of enzymes involved in the synthetic and degradation pathways. Indeed, there were altered concentrations of AA derived mediators, including prostaglandins (PGs) and hydroxyeicosatetraenoic acids (HETEs) and of the AA containing endocannabinoids (ECs) 2-

arachidonoyl glycerol (2-AG) and arachidonoyl ethanolamide (anandamide, AEA) in scWAT from MHO individuals. Increased concentrations of AA containing ECs is consistent with a greater proportion of AA observed in the tissue of these individuals, however there was lower concentrations of a number of AA derived metabolites. Investigation of protein expression, enzyme activity, as well as expression of the genes encoding the enzymes involved in both lipid transport and the synthesis and degradation of lipid metabolites was performed to provide greater insight into the mechanisms behind these differences in lipid mediators metabolically healthy obesity.

Data from these analyses reported significantly greater expression of *ALOX5* (*5*-LOX) and *PTGS1* (COX-1) in obesity, but no differences in the expression of *ALOX12* (12-LOX*), ALOX15 (*15-LOX*), PTGS2* (COX-2)*, NAPE-PLD, DAGLα, MGLL* or *FAAH* was observed. There was significantly greater expression of COX-2 protein but no difference in COX-2 activity or the expression of FAAH in scWAT from MHO individuals in comparison with normal weight individuals. Greater COX-2 expression in MHO individuals may suggest greater concentrations of COX-2 derived metabolites in the MHO individual. This is consistent with the higher concentrations of PGF1α.

The expression and activity of COX-2 was significantly negatively associated with concentrations of scWAT HETEs, DHA metabolites including hydroxy-DHAs and resolvin D2 (RvD2), and the FA ethanolamides AEA and docosahexaenoylethanolamide (DHEA). These are synthesised via lipoxygenase (LOX) and N-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD) pathways, respectively; therefore, it may be suggested that greater COX-2 expression and activity results in less favourable metabolism by alternative pathways. Due to a lack of positive associations between COX-2 expression, activity, and COX-2 derived FA metabolites, the data suggest differences in FA metabolites synthesized by these pathways are unlikely to be due to significantly increased expression of genes or activity of enzymes and may instead be due to increased availability of AA as substrate for metabolism by these pathways. This may also hold for FAAH, where expression did not negatively correlate with FA ethanolamide concentrations.

9.2.2 Expression of scWAT genes and the whole transcriptome in metabolically healthy obesity

The few transcriptomic studies conducted in human scWAT have failed to consider the metabolic health of the individuals included (Stenvers et al., 2019, Rodriguez-Ayala et al., 2020, Kerr et al., 2020). Defining individuals by their metabolic status has been shown to be of importance in the data reported in Chapters 4 and 5 which details associations of FA metabolites and enzymes involved in their metabolism with measures of insulin resistance (HOMA2-IR, glucose, and insulin

concentrations). Furthermore, transcriptomic studies in obese individuals to date have been predominantly focussed on calorie restriction and weight reduction with no comparison between normal weight and obese individuals (Cancello et al., 2005, Capel et al., 2009, Marquez-Quinones et al., 2010, Mutch et al., 2011, Armenise. C et al., 2017). Therefore, the data reported herein, specifically defining MHO individuals are novel and of importance.

RNA-sequencing and quantitative real-time polymerase chain reaction (qRT-PCR) analyses revealed significant changes in the expression of genes involved in FA transport and activation in scWAT in obesity. Of note, FATP1, which has a role in the transport of LC PUFA, and FATP2 which has a role in the activation of very LC PUFA, were significantly down regulated in obesity, and FATP4, which has a role in the transport of LC FA, was significantly upregulated in obesity. These data provide evidence that obesity is associated with a) altered FA activation and therefore incorporation into phospholipid (PL) and triglyceride (TG) and shunting towards β-oxidation; b) altered FA transport (Watkins, 1997, Watkins, 2008). The effects on FA transport may depend upon FA chain length and it is possible that there is an increased influx of some FAs into the scWAT in obesity but decreased transport (and activation) of LC PUFA. Reduced activation of LC n-3 PUFA may affect their conversion into metabolites and this could explain the differences in concentration of DHA metabolites between MHO and normal weight despite similar concentrations of DHA being present in the scWAT.

Significant differences in the scWAT transcriptome in MHO individuals were identified by RNA-sequencing and further validated by qRT-PCR, with additional genes of interest being examined using the latter technique. There was a significant dysregulation in the expression of genes involved in tissue expansion and remodelling in MHO individuals indicating enhanced adipogenesis, tissue remodelling, and overall tissue expansion. In addition, there was altered expression of genes involved in CHO metabolism and insulin signalling indicating enhanced risk of insulin resistance in these MHO individuals. Further to this, there was altered regulation in the expression of genes involved in inflammation and immune responses where there was crossover with CHO metabolism. There was a significant upregulation of genes involved in pro-inflammatory lipid metabolite synthesis, glucose and insulin regulation, cell activation, adhesion, and cytokine secretion. These data are indicative of enhanced pro-inflammatory signalling, immune cell infiltration and immune response, and are likely to suggest a greater pro-inflammatory state of the scWAT and changes to scWAT heterogeneity in obesity.

9.2.3 Evidence of tissue expansion and enhanced inflammation is observed in adipose tissue morphology

Changes in scWAT morphology support data indicative of enhanced tissue expansion and inflammation in MHO individuals. Adipocytes of MHO individuals were significantly larger than those of normal weight individuals and they had a higher proportion of large, very large and extra-large adipocytes in addition to a lower proportion of small and medium adipocytes. There were however no significant changes in either whole tissue or pericellular fibrosis, but this was positively associated with study entry HOMA2-IR and insulin measurements suggesting association with metabolic health and that fibrosis may persist once metabolic complications such as insulin resistance are established. Adipocyte size was also positively associated with HOMA2-IR as well as BMI and body fat % also suggesting an influence of metabolic health in addition to obesity on fat cell expansion and accumulation of TG. The greater number of macrophages accumulating in crown like structures (CLS) supports suggestions of enhanced inflammation and immune response in the scWAT of MHO individuals. Furthermore, adipocyte size, tissue fibrosis, and presence of macrophages and CLS were associated with a number of circulating cytokines which are indicative of systemic inflammation. Adipocyte size, and macrophage and CLS number were positively associated with IL-6 and, in addition to tissue fibrosis, were negatively associated with adiponectin. Adipocyte size was further associated positively with CRP and leptin. This suggests an influence of scWAT inflammation and function on whole body inflammation.

An overview detailing the dysregulation of scWAT from MHO individuals is depicted in Figure 9.1

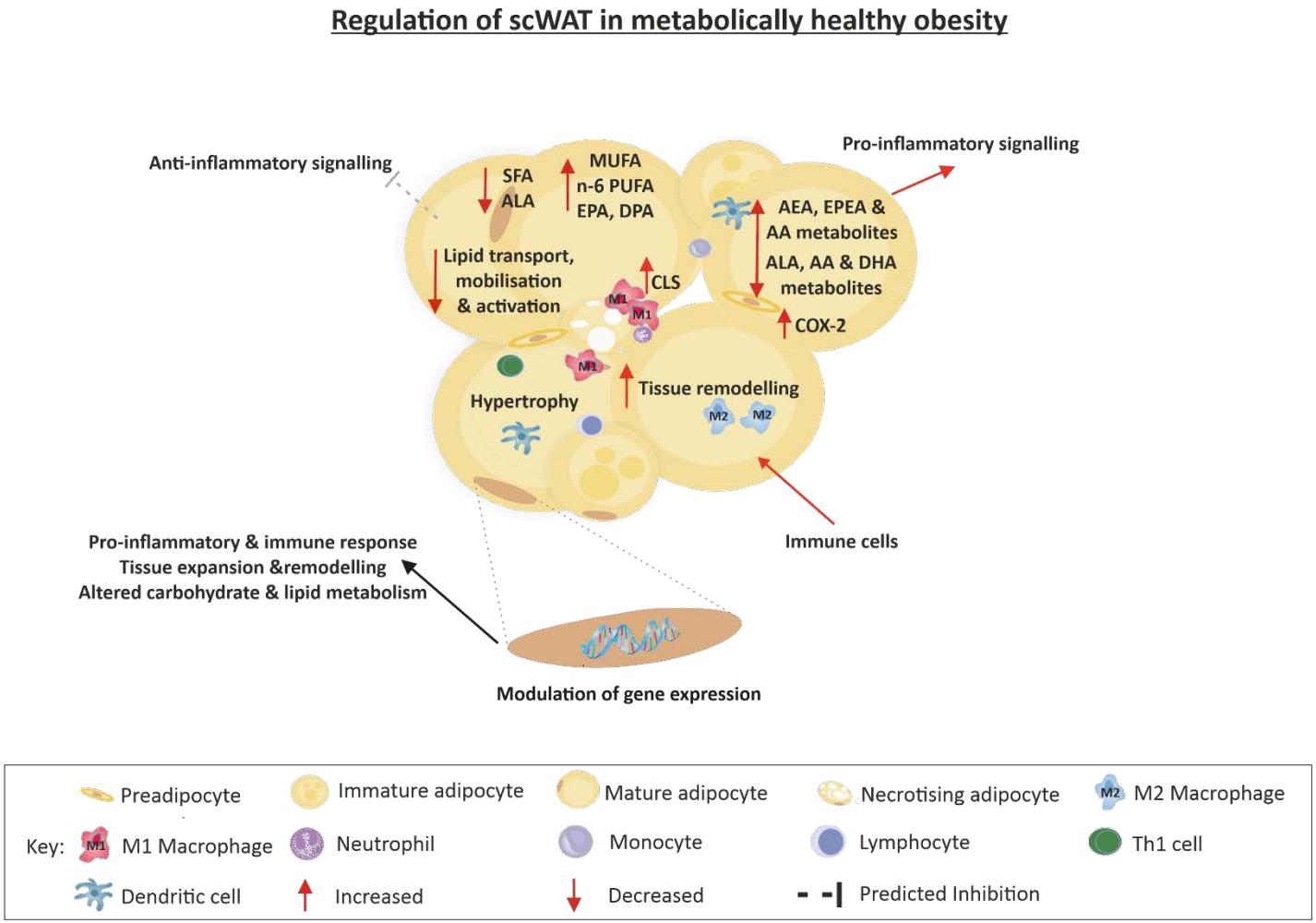


Figure 9.1 Dysregulation of scWAT in MHO individuals in comparison to normal weight individuals

9.3 Summary of findings: Effect of 12-week intervention with fish oil on scWAT from normal weight and MHO individuals

Omega-3 FAs have been widely examined for their anti-inflammatory effects; however, studies investigating their incorporation into adipose tissue in human obesity and the subsequent effects on scWAT biology and inflammation are limited. There are studies of n-3 LC PUFA and inflammatory markers in the blood of obese humans (Browning et al., 2007, Kabir et al., 2007, Mas et al., 2010, Banni et al., 2011) and studies using adipose tissue in animal models (Batetta et al., 2009, Balvers et al., 2012, Kim et al., 2015). Therefore, the current study aimed to provide evidence for the effects of LC n-3 PUFA on scWAT in obesity compared to in normal weight individuals.

12-week FO intervention significantly increased the proportion of EPA, DPA and DHA in scWAT in normal weight individuals, but only significantly increased the proportion of EPA in scWAT in MHO individuals, despite a similar incorporation of DHA observed in MHO individuals. These data may suggest altered FA transport and or storage in obesity, which in part may be attributed to the decreased expression of the LC PUFA transporter FATP1 in obese individuals as discussed above. There are no reports to my knowledge reporting incorporation of EPA and DHA in scWAT in metabolically healthy obesity; therefore, reports of similar incorporation in normal weight and MHO individuals is novel.

Further to this, 12-week FO intervention significantly modulated scWAT lipid metabolites with profound effects on the ECS. FO significantly increased EPA and DHA containing ECs (EPEA, DHEA, and the glycerol ester of EPA) in the scWAT of normal weight individuals, and significantly decreased AA containing ECs predominantly in MHO individuals (Figure 9.2). In general, FO significantly increased anti-inflammatory lipid metabolites in scWAT in normal weight individuals and decreased pro-inflammatory metabolites in MHO individuals, with the greatest effects predominantly seen in normal weight individuals (Figure 9.2). These data suggest FO may be capable of promoting tissue repair, increasing β-oxidation of FAs, and inhibiting immune cell infiltration, alternative polarisation of macrophages, pro-inflammatory cytokine secretion and subsequent inflammation (Figure 9.2). The current study is the first to report effects of obesity and FO intervention on EPA, DHA and AA containing ECs and further reports that scWAT of MHO individuals may be less sensitive to the effects of FO on lipid metabolite regulation.

The increase in synthesis of EPA and DHA derived metabolites may result from the increased contribution of these FAs to total scWAT lipids following intervention, their effects on enzymes

involved in the synthesis and degradation of such metabolites, or both. The proportion of scWAT AA was unchanged following FO intervention; therefore, changes in AA derived mediators may not be attributed to a decrease in AA, but to effects of n-3 LC PUFA on the enzymes involved in lipid metabolite pathways, or an increase in membrane LC n-3 PUFA and altered preferential use of substrate for lipid mediator synthesis. There was no effect of FO intervention of the expression of *NAPE-PLD*, *DAGLa*, *MGLL*, *FAAH*, COX or LOX genes in normal weight individuals, but a significant increased expression of *PTGS2* (COX-2) in MHO individuals. However, there was no change to the protein expression or activity of COX-2.

COX-2 is responsible for the metabolism of EPA into 18-HEPE, the precursor to the E-series Rvs, and for the metabolism of DHA into 17-HDHA, the precursor to protectin-D1; however, no significant changes in the concentrations of these lipid metabolites were observed with FO intervention. This may be explained by increased expression of the gene not resulting in greater COX-2 protein expression or any there being any changes in COX-2 enzyme activity in response to FO intervention. However, it may also be that the balance between EPA and AA may not have been altered enough to change the overall proportion of EPA derived metabolites in MHO individuals, but was significant enough to decrease the proportion of AA derived metabolites.

From wider gene expression analysis, the current study reports that FO significantly modulated the transcriptome of scWAT in both normal weight and MHO individuals. FO significantly down-regulated genes involved in overall immune and inflammatory response in both normal weight and MHO individuals; further to this, it significantly down-regulated *DACT2*, the action of which is uncharacterised in human AT, but similarly to DACT1 may play a role in Wnt signalling and adipogenesis, in MHO individuals. FO further significantly up-regulated genes involved in the negative regulation of cell proliferation and cytokine mediated signalling suggesting inhibition of adipose expansion and inflammatory signalling in normal weight individuals only (Figure 9.2). These effects were opposed in MHO individuals in which FO significantly up-regulated genes that promote cell differentiation, blood vessel remodelling, amino acid and potassium transport, and the regulation of glucose transport, suggesting promotion of adipose remodelling and expansion. This may indicate an attempt to resolve excess lipid and inflammatory processes occurring by a restricted ECM in scWAT of MHO individuals.

To date there are very few studies investigating the effects of FO intervention on the whole transcriptome at all, with one study investigating effects in human blood (Polus et al., 2016) and one in human scWAT (in addition to a calorie restriction) (Huerta et al., 2017). Therefore, observations from the current study providing evidence for the modulation of inflammatory,

immune, and remodelling pathways by LC n-3 PUFA, and reduced sensitivity to these actions in MHO individuals are novel.

An overview of the effect of 12-week FO intervention on scWAT processes in normal weight and MHO individuals is depicted in Figure 9.2

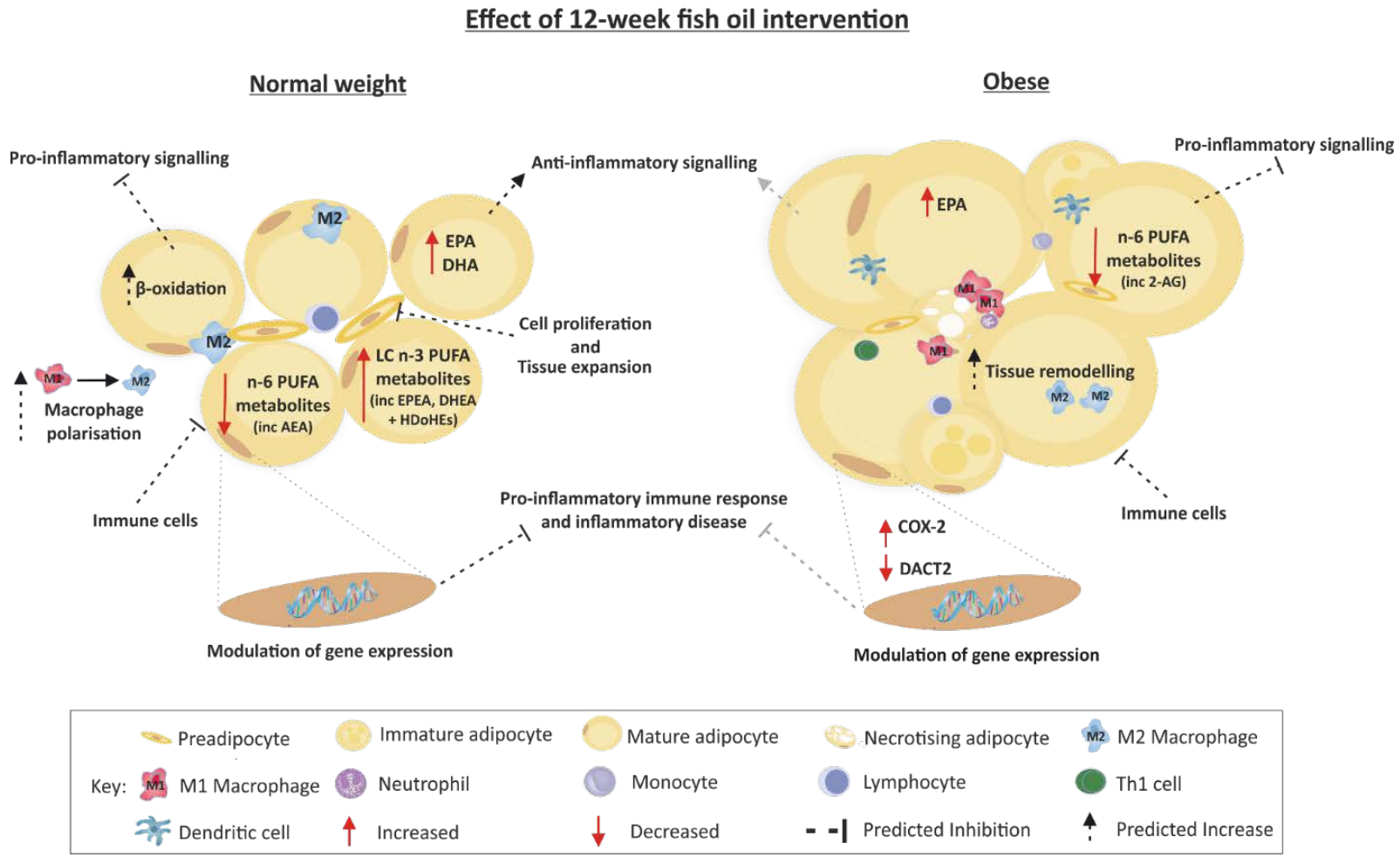


Figure 9.2 Summary of the effects of 12-week fish oil intervention on scWAT processes in normal weight and MHO individuals.

9.4 Conclusion

To conclude, the current study provides novel evidence for the altered regulation of scWAT FA and lipid metabolite composition, particularly ECs, in MHO as well as an altered scWAT transcriptome and the expression of many genes involved in lipid metabolism, scWAT remodelling, and inflammation, and further provides evidence of enhanced expansion and inflammation in the tissue itself. Taken together the evidence indicates enhanced inflammation in the scWAT of MHO individuals in the context of tissue expansion and remodelling, dysregulated lipid and CHO metabolism, increased pro-inflammatory signalling and immune response. In addition, associations of a number of inflammatory parameters with genes and proteins involved in dysregulated pathways, suggest the metabolic status of obese individuals is of importance and the relationship between inflammation, tissue dysfunction and metabolic complication is complex.

Further to this, the current study provides novel evidence for altered metabolism of EPA and DHA in metabolically healthy obesity, the modulation of scWAT EPA, DHA and AA derived ECs and enzymes involved in their metabolism with FO intervention, as well as regulation of the expression of genes involved in inflammation, immune response, glucose homeostasis, and tissue expansion and remodelling. These effects of FO suggest amelioration of scWAT inflammation in both subgroups but to a lesser extent in MHO individuals and data within this thesis indicate scWAT from MHO individuals was less sensitive /responsive to FO intervention. Mechanisms behind the differences in response to FO intervention may be explained by a number of altered processes seen at study entry in MHO individuals including mobilisation and metabolism of EPA and DHA and greater concentrations of n-6 FAs despite similar incorporation of EPA and DHA following the 12-week period. The concentrations of n-6 FAs were higher in MHO individuals at study entry and were unchanged following 12-week FO intervention. It has been discussed that the ratio of n-6:n-3 FA is important and it may be that a higher dose of EPA and DHA is required to change this ratio in favour of n-3 FA metabolism and subsequent anti-inflammatory signalling, or that an additional dietary intervention reducing n-6 intake alongside increasing n-3 intake may be beneficial.

9.5 Limitation and strengths

A limitation of this study is that FA internal standards were not added to scWAT when analysing the FA composition. This would have provided information on the absolute concentrations (ng/g) of FAs present in the tissue at study entry and following the intervention period. The proportion

of a FA may differ between subgroups such as normal weight and MHO individuals, whilst the absolute concentration may not; conversely, the absolute concentration of a FA may differ but the proportion may not. Therefore, there may be significant changes in the absolute concentrations of FAs, in particular, AA, following FO intervention, which are not reflected in the proportional composition. However, the proportion a FA contributes to total FAs is still of importance as FAs often compete for metabolism, as discussed for lipid metabolite synthesis, and therefore the relative concentrations (proportion) of these will be informative in this scenario.

A further limitation is the lack of data collected on the habitual diets of the normal weight and MHO individuals. This information would have allowed attribution of diet to differences in scWAT FA at study entry. However, as discussed, there is great variability in the strength and significance of these correlations as many other biological processes can influence the concentrations of scWAT FAs. Dietary data would have been most beneficial for the evaluation of the essential fatty acids ALA and LA which cannot be synthesized in the body, and of the LC n-3 PUFA of which a small proportion is made in the body from the elongation and desaturation of ALA.

A further limitation may be the use of a subset for some of the analyses reported herein. RNA-seq analysis was performed on a much smaller subset; ideally, profiles from all individuals recruited would have been obtained but due to cost and sample availability, this was not possible. However, qRT-PCR data performed on the full cohort investigating the top up- and down-regulated genes highlighted from RNA-seq analysis was concordant with data obtained from the RNA-seq subset analysis therefore, validating the RNA-seq data and providing evidence for the appropriate use of a cohort subset. In light of these data, the use of a subset for other analysis including protein expression was deemed to be appropriate.

On the other hand the study has many strengths. The techniques used in the current study are robust and cutting edge and this is the first study of its kind to utilise these to provide a comprehensive overview of human scWAT inflammatory status in metabolically healthy obesity and in response to FO intervention. Evidence obtained from FA composition profiles, lipid mediator profiles, protein expression, enzyme activity, and whole transcriptome information, in addition to physical evidence of changes in the tissue, covers multiple aspects of tissue status and not only allows for large scale detection of differences in obesity, but also to explore the underlying mechanisms contributing to these changes. Further to this, the current study has a large cohort population, which is often lacking in human studies, and treatment compliance was high (greater than 90%).

9.6 Future work

A main feature of scWAT from MHO individuals was a lower proportion of SFA and a higher proportion of MUFA. It was discussed that, in addition to dietary intake of these FAs and carbohydrate as a source for de-novo synthesis of these FAs, the activity of enzymes involved in the metabolism of SFA to MUFA (with the exception of LA and ALA which cannot be synthesized by humans and are therefore essential) and MUFA to PUFA may be altered in obesity. Suggestions of increased D6D and SCDn-7 activity and of decreased D5D activity in obese individuals have been made previously using plasma FAs and the ratio of product to substrate of these enzymes (Pickens et al., 2014). Data from the current study based on ratios of SFA:MUFA in adipose tissue suggest significantly increased SCDn-7 and SCDn-9 activity observed in MHO individuals, which is supported by increased proportions of 16:1n-7, 18:1n-7, and 18:1n-9, and a decreased proportion of 18:0 observed in adipose tissue from MHO individuals. Although FA ratios are often used to determine enzyme activity they can only be estimates because of other factors that might influence FA proportions, and so future work should include incubation of scWAT homogenates with labelled FAs to follow the fate of 16:0, 18:0, 18:2n-6 and 20:3n-6 to more accurately assess SCD and desaturase activities.

Similarly, assays used to determine the activity of enzymes involved in FA metabolite pathways were limited and these assays did not perform well for all enzymes; assays utilising labelled substrates should be performed to provide further information about these pathways. The FA metabolite profile of scWAT from MHO individuals was altered, including COX and LOX metabolites, and ECs. Investigation of the expression of genes encoding these enzymes suggested significantly greater *PTGS2* (COX-2) expression which may contribute to greater concentrations of COX-2 metabolites, significantly greater *ALOX5* expression which may contribute to greater concentrations of 5-LOX metabolites, and non-significant but notably lower expression of *FAAH* and *ALOX-12*. The activity of COX-2 and FAAH was assessed using fluorometric assays; this was suitable and gave reliable results for COX-2 activity but was not able to detect changes in FAAH activity. The assay was able to detect study entry FAAH activity so it may be that the enzymatic reaction occurs very quickly, and substrate was used up at the beginning of the reaction, or that it was not sensitive enough to detect small changes in activity. An alternative method would be to incubate scWAT homogenates with radio-labelled AEA and to measure the subsequent radio-labelled AA by scintillation counting (Boldrup 2004, Cable 2011).

In addition, there was greater expression of phospholipase A_2 isoenzymes in MHO individuals which may influence the availability of substrates for EC synthesis and contribute to greater concentrations of FA ethanolamides at study entry. However, in contrast, there was 92% lower

expression of SLC27A2 in scWAT of MHO individuals which encodes FATP2 (ACSVL1), which has a role in activating LCFAs (Watkins, 2008). If there is lower activation of DHA to its Co-A ester which is required for synthesis of DHA derived metabolites, there may be lower concentrations of hydroxy-DHA metabolites which was observed in the scWAT of MHO individuals at study entry and well as lower DHEA concentrations in response to FO intervention in these individuals (Watkins, 2008). Assessment of PLA_2 and acyl-CoA synthetase activity should be made using the fluorometric assay available from Abcam (Cambridge, UK). This work was not carried out as part of the analyses included in this thesis due to unavailability of a sufficient number of samples. An option may to pool the remaining scWAT samples within their BMI and treatment subgroups to provide representative samples for this analysis. Fluorometric assays are commercially available for LOX activity (Abcam, Cambridge, UK) and these could also be performed using remaining pooled representative samples.

There is further data mining that could be outsourced from the RNA-seq data to uncover further information such as splice variants. One particular pathway highlighted from the current analysis of this data that would be worthy of further exploration is Wnt signalling in MHO and its regulation by LC n-3 PUFA. Wnt signalling is responsive to nutritional cues and has a role in the regulation of adipogenesis and hyperplasia in the scWAT (Sethi and Vidal-Puig, 2010). Wnt proteins are in part modulated by DACT proteins and when DACT and Wnt expression is high, adipogenesis is inhibited (Sethi and Vidal-Puig, 2010). Wnt and DACT1 ligands are downregulated in response to nutritional surplus; however *WNT10B* and *WNT3* were upregulated in MHO individuals suggesting dysregulation of Wnt signalling pathways. In response to 12-week FO intervention, there was a 74% decrease in *WNT3* and 73% decrease in *DACT2* expression which may suggest EPA and DHA have a role in upregulating adipogenesis and tissue expansion via Wnt signalling; however, there was also a 49% increase in *WNT10B* and 31% increase in *DACT1* which is conflicting. The role of DACT2 has not been characterised in Wnt signalling and adipogenesis in human WAT (Christodoulides et al., 2009). and therefore further dissection of these pathways would be of great interest. Cell culture experiments measuring the differentiation of 3T3-L1 pre adipocytes into mature adipocytes would be one possible way to explore the role of the Wnt and DACT proteins in adipogenesis. The use of siRNA to knock out Wnt and DACT proteins in subsequent experiments followed by the measurement of adipogenesis by neutral lipid staining of adipocytes and expression of adipogenic genes (PPARγ and CEBPα) and markers of mature adipocytes (FABP4 and GLUT4) via qRT-PCR could be a way to investigate the role of DACT 2 in adipogenesis. Furthermore, to assess the potential effects of FO intervention on these pathways, these experiments could be repeated with the incubation with EPA and DHA.

Lastly, a number of the inflammatory and adipose function parameters reported herein were association with HOMA2-IR and/or insulin and glucose concentrations at study entry. The data provides a comprehensive overview of a more inflamed scWAT in MHO individuals with alteration to carbohydrate and lipid signalling suggesting complications occur long before manifestation of insulin resistance and diagnosis of MetS. Therefore, it is important to further investigate the influence of insulin signalling in the progression of obesity, not just when resistance has occurred, and its role in adipose function and dysregulation.

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Appendix A**Participant information sheet**

You are invited to take part in a research study. Before you decide whether or not you wish to take part it is important that you understand what the research will involve. Please read this information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Please take as much time as you would like.

What is the purpose of this study?

Fish oil type supplements contain omega-3 fats. These are believed to be good for health. One effect of omega-3s is to dampen inflammation, which can harm the body and lead to disease. Researchers have discovered that inflammation within adipose (fat) tissue plays a role in obesity. We do not know if omega-3s have the same anti-inflammatory effects in normal weight and in obese people. Nor do we know if omega-3s can influence inflammation within fat tissue. We want to find out about the effects of omega-3s in normal weight and obese people. This is important in identifying whether dietary supplements will be equally effective in people with different body fatness. We plan to compare the effects of an omega-3 supplement in normal weight and obese individuals. A dummy called a placebo will be used in half of the people enrolled in the study in order to make a comparison. The dummy will contain vegetable oil. The fish oil supplement to be used is available in pharmacies, supermarkets, health food shops etc. Since the supplement contains fish oil and has a gelatin coating it is not suitable for

vegetarians and vegans. In the study we will measure the amounts of inflammatory chemicals in the bloodstream and in adipose tissue.

Why have I been chosen?

You have been contacted because you have expressed an interest in our research and you appear to meet the inclusion criteria. We need people aged 18 to 65 years. We will be recruiting 100 subjects in total.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time, and without giving a reason. If you do withdraw, you will be asked whether you want us to destroy any samples you have given as part of the study.

What will happen to me if I take part?

If you decide to take part you will asked to attend 4 study appointments at the Clinical Research Facility at Southampton General Hospital. The first of these appointments will last about an hour and will be at any time of the day depending upon your availability. The other three study appointments will begin in the morning, before your breakfast, and each of these will last about seven hours.

At the first visit the amount of blood taken will be about 9 ml (two teaspoons full). We will use this blood to look at your DNA to identify whether there are any

features that relate to body weight, to the way you handle fat from your diet or to inflammation.

On study visits two, three and four you will be asked to fast (no food or drink apart from water) from 9 pm the night before. When you arrive for these visits a cannula will be placed into one of your arm veins and a small blood sample (20 ml, equivalent to just over a tablespoonful) will be taken. On visit four an additional 9 ml of blood will be taken, again for analysis of your DNA. After the initial blood sample is taken you will be given a breakfast and asked to take some omega-3 ("fish oil") or dummy capsules while you eat your breakfast. You will remain in the clinic for a further six hours and a series of blood samples will be taken over this time. Each sample will be 5 ml, so that over the entire visit you will provide 45 ml (about 3 tablespoons) of blood (54 ml at visit four). After the last blood sample is taken you will be provided with some food and a drink.

At the end of the second and fourth visits a small fat sample will be taken from your lower abdomen. Prior to this you will get a small injection to make your skin numb. Then a small cut up to an inch long will be made in the skin and a small piece of fat (about a teaspoon full) will be taken from under the skin. Then the cut will be stitched (usually two stitches will be required). You will need to come back to the Clinical Research Facility at Southampton General Hospital about a week later to have the stitches removed; this visit will be short lasting less than 15 minutes and will be at your convenience.

You will be given a supply of fish oil or dummy capsules with instructions on how to take these. You will take the capsules daily for 3 months between your third and fourth visits.

All samples are treated as a gift from you, and we will respect your wishes when using them. They will be stored for five years before being destroyed, and with your consent we may use them for future studies.

What do I have to do?

As described in the previous section, you will attend the Clinical Research Facility at SGH for 4 study visits. The first visit will last about one hour and visits two, three and four will each last about seven hours. All the visits will take place over four to five months. The second and third visits will be two or three weeks apart. The fourth visit will be about 3 months after the third visit.

For the period between the third and fourth visits, you will be asked to take some omega-3 ("fish oil") or dummy capsules each day.

What are the possible benefits of taking part?

You will not receive any direct benefit from taking part in the study. Knowledge gained from this study will help our research and will ultimately be of use to other researchers and to regulators, consumers and the supplements industry.

What are the possible disadvantages and risks of taking part?

There is a very small chance of bruising at the site of insertion of the needle for collection of blood samples. There is a very small chance of infection and a chance of bleeding and bruising at the site of the cut made in your skin for collecting the fat tissue. These risks will be minimised by using sterile techniques and trained members of staff.

What will happen if anything goes wrong?

If something goes wrong during an investigation day any procedures will be stopped and you will be seen by a doctor. Your involvement in the rest of the study may be stopped.

If you have a concern about any aspect of the study you should speak to the researcher involved who will do their best to answer your questions (telephone 023 8079 5252; email: p.noakes@soton.ac.uk). If you remain unhappy and wish to complain formally you should contact the Welcome Trust Clinical Research Facility in the first instance (telephone 023 8079 4989; email: wtcrf@suht.swest.nhs.uk). Any

complaint about the way that you have been dealt with during the study or any possible harm you might suffer will be addressed.

Will my taking part in this study be kept confidential?

Your GP will be notified that you are participating in this study, but any information that is collected about you during the course of the research will be kept strictly confidential.

What will happen to the study results?

We will inform you of the overall study results. As the samples from the study will not be processed until the end of the study, your results will not be available for several months. The overall results may be presented at scientific meetings or published in a scientific journal. You will not be identified in any of these presentations or publications. We will be happy to discuss the results with you when the study is completed, and will let you know where you can obtain a copy of the published results.

Will I be reimbursed for my time?

In recognition of your time commitment, you will be paid an honorarium of £150. Reasonable travel expenses will also be paid.

Who is organising and funding the study?

This study is being organised by The Institute of Human Nutrition at The University of Southampton. The European commission is funding the research. The legal sponsor of the study is the Southampton University Hospitals NHS Trust.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the NRES Committee South Central – Berkshire.

Contact for further information

If you have any further questions then please contact Paul Noakes at The Institute of Human Nutrition on 023 8979 5252 or e-mail p.noakes@soton.ac.uk

And finally...

Thank you for having taken the time to read this information sheet and your interest in the study. If you do decide to take part in the study, you will be given a copy of this information sheet and a signed consent form for you to keep.

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton

Omega-3 fatty acids and inflammation in normal weight and obese subjects

Information Sheet for Study Participants

Ethical Approval number 11/SC/038
Chief Investigator: Prof P C Calder Mailpoint 887
Southampton General Hospital
Southampton SO16 6YD
Researchers: Dr Paul Noakes
Dr James Hopkins
Miss Annette West
Miss Carolina ParasChavez
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Version 4a, 27th June 2012

Appendix B **Participant consent form**

CONSENT FORM

Southampton NHS
University Hospitals NHS Trust

Omega-3 fatty acids and inflammation in normal weight and obese subjects

LREC Reference Number: 11/SC/0384

Name of Chief Investigator: Professor Philip Calder

Please initial box

I confirm that I have read and understand the information sheet (version .... dated .....) for the above study and have had the opportunity to ask questions ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected ☐

I consent to my general practitioner being notified of my participation in this research ☐

I consent to give blood samples according to the schedule described in the information sheet ☐

I consent to giving adipose tissue biopsies according to the schedule described in the information sheet ☐

I consent to my DNA being used for the purposes described in the information sheet ☐

I agree that samples taken as part of the protocol of this study may be stored for 5 years and used in further research studies during that period. Samples will only be used in studies that have been approved by the appropriate Ethics Committee ☐

I understand that I can withdraw this consent at any time by notifying the study team ☐

I agree to take part in the above study ☐

Volunteer: Print_____Date_____
Signature_____

Member of research team taking consent: Print_____Date_____
Signature_____

3 copies required: 1 for researcher, 1 for volunteer, Original to be kept with investigator site file

Version 2: 1st August 2011

Appendix C **Reagents and materials**

Analysis	Reagent/Material	Supplier	Cat. No.
Fatty acid analysis:	NaCl	Sigma- Aldrich	S9888
	Chloroform	Fisher Scientific	C/4966/17
	Methanol	Fisher Scientific	M/4056/17
	BHT	Sigma- Aldrich	W218405
	Nitrogen (oxygen free) cylinder	BOC	44-w
	Toluene	Fisher Scientific	T/2300/15
	Sulfuric acid	Sigma- Aldrich	339741
	Potassium carbonate	Sigma- Aldrich	209619
	Potassium bicarbonate	Sigma- Aldrich	237205
	Hexane	Fisher Scientific	H/0406/17
	Molecular sieves	Sigma- Aldrich	334324
	SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 µm)	SGE Analytical Science	054612
	Gas chromatograph (HP- 6890)	Agilent	8012-30-1019
Lipid mediator analysis:	AA-d8	Cayman Chemical	390010
	DHA-d5	Cayman Chemical	10005057
	EPA-d5	Cayman Chemical	10005056
	PGD2-d4	Cayman Chemical	312010
	15 (s) -HETE-d8	Cayman Chemical	334720
	12 (s)-HETE-d8	Cayman Chemical	334570
	5 (s)-HETE-d8	Cayman Chemical	334230
	6k-PGF1α-d4	Cayman Chemical	31520
	8-iso-PFG2α-d4	Cayman Chemical	316350

	PGF2-d9	Cayman Chemical	10008643
	15d-PGJ-d4	Cayman Chemical	318570
	8,9-DHET-d11	Cayman Chemical	1000998
	Methanol	Fisher Scientific	M/4056/17
	Hexane	Fisher Scientific	H/0406/17
	Ethyl acetate	Sigma- Aldrich	1.00789
	Hydrochloric acid	Sigma- Aldrich	H1758
	Glycerol	Sigma- Aldrich	G5516
	Methyl formate	Sigma- Aldrich	291056
	Acetonitrile	Sigma- Aldrich	1.00016
	Acetic acid	Sigma- Aldrich	695084
	Polyvinylidene Fluoride (PVDF) micro-filter	Merck	UFC30VV25
	Vacuum pump	Sigma- Aldrich	2656-194GB-1EA
	Solid phase extraction (SPE) tank - VacElut 20 Manifold	Agilent	NA
	Strata-X Polymeric reversed phase 500mg 6ml SPE cartridges	Phenomenex	8B-S043-HCH
	Thermo Dionex Ultimate 3000 RSLC Liquid Chromatograph	Thermo Fisher Scientific	NA
	Kinetex UPLC column (1.7um diameter, C18, 100A pore size, 150x2.1mm)	Phenomenex	00F-4475-AN
	AC SCIEX SelexIon QTRAP 5500 Mass Spectrometer	AB SCIEX	NA
RNA Sequencing:	QIAGEN RNeasy lipid tissue mini RNA extraction kit (Qiazol reagent, RW1 buffer, RPE buffer)	QIAGEN	74804
	Ethanol	Sigma- Aldrich	2483
	Chloroform	Fisher Scientific	C/4966/17
qRT-PCR:	QIAGEN RNeasy lipid tissue mini RNA extraction kit (Qiazol reagent, RW1 buffer, RPE buffer)	QIAGEN	74804

	Ethanol	Sigma- Aldrich	2483
	Chloroform	Fisher Scientific	C/4966/17
	GoScript™ Reverse Transcriptase	Promega	A5003
	QuantiNova Probe PCR kit	QIAGEN	208256
	Double-dye (TaqMan style) gene expression primers: GUSB (NM_000181), ACTG1 (NM_00119995), IPO8 (NM_006390.3), GABARAP (NM_007278.1), VPS36 (NM_016075.3), MGLL (NM_007283), FAAH (NM_001441), CNR1 (NM_016083), WNT3 (NM_030753.4), WNT10B (NM_003394.3), DACT2 (NM_214462.4), DACT1 (NM_016651.5), ALOX5 (NM_000698), IKBKE (NM_014002), PTGS2 (NM_000963), ALOX15 (NM_001140), aLOX12 (NM_000697.2), MMP9 (NM_004994), PROK2 (NM_00112612), KCNH2 (NM_000238), MAB21L1 (NM_ 005584.4), SLC27A2 (NM_003645), EGFL6 (NM_015507), DAGLA (NM_006133), NAPE-PLD (NM_00112283), FATP1 (NM_198580), NFkB (NM_003990	Primer Design	DD-hu-300
Enzyme activity assays:	Cyclooxygenase (COX) activity assay kit (fluorometric)	Abcam	Ab204699
	Phosphate buffered saline	Fisher Scientific	10388739
	Nonidet P-40 substitute	Sigma- Aldrich	11754599001
	Protease inhibitor cocktail	Abcam	Ab201111
	Dimethyl sulfoxide	Fisher Scientific	10103483
	Fatty acid amid hydrolase activity assay kit (fluorometric)	Abcam	Ab252895
	Pierce™ BCA Protein Assay Kit	Fisher Scientific	23225
	Optiblot SDS-Page Gel 16% (10 x 10 cm) - 12 Well	Abcam	ab139592
	Anti-COX-2 / Cyclooxygenase 2 antibody [EP1978Y	Abcam	ab62331
	Anti-FAAH1 / Fatty acid amide hydrolase antibody [EPR7549]	Abcam	ab128917

Western blotting:	Anti-HPRT / Hypoxanthine Phosphoribosyl transferase 1 antibody [EPR5298] (	Abcam	ab133242
	Anti-GAPDH / Glyceraldehyde 3-phosphate dehydrogenase antibody [EPR6256]	Abcam	ab128915
	Anti-Beta Actin antibody [EPR21241]	Abcam	ab213262
	Anti-FAA / Fumarylacetoacetate hydrolase antibody	Abcam	ab151998
	Tris base	Fisher Scientific	10103203
	Glycine	Fisher Scientific	10101620
	Sodium Dodecyl Sulfate	Fisher Scientific	15865168
	Methanol	Fisher Scientific	M/4056/17
	Phosphate buffered saline	Fisher Scientific	10388739
	Laemmeli SDS Sample Buffer (4x)	Fisher Scientific	15492859
	Tween™ 20	Fisher Scientific	15825398
	Oxoid™ Skim Milk Powder	Fisher Scientific	10651135
	Protein ladder	Abcam	ab116028
	10% Neutral buffered formalin	Fisher Scientific	12673026
	Paraffin wax	Fisher Scientific	10244250
	Glass microscope slides	Cell path	MAG-1000-03P
	Glass cover slips	Cell path	SAJ-2250-03A
	Tissue clear	Sakura Finetek	T/2300/15
	Ethanol -absolute	Fisher Scientific	10680993
	Mayers' Haematoxylin	Sigma- Aldrich	MHS1-100ML
	Eosin	Sigma- Aldrich	E4009
	Expert XTF mounting medium	Cell path	SEA-1904-00A
	0.2% phosphomolybdic acid	Fisher Scientific	10660175
	Picro Sirius red	Sigma- Aldrich	365548
	HCl	Fisher Scientific	10467640
	Methanol	Fisher Scientific	M/4056/17
	Tris buffered saline	Fisher Scientific	10467583

Immunohistochemistry:	Citric Acid	Fisher Scientific	10599180
	Hydrogen Peroxide	Fisher Scientific	10386643
	Blocking Avidin and blocking biotin kit	Vector	SP-2001
	Blocking medium/ buffer	Fisher Scientific	10076713
	CD68 PGM primary antibody	Dako	GA61361-2
	Goat anti-mouse secondary antibody	Vector	BA-9200-1.5
	Avidin-biotin complex	Vector	PK-4002
	DAB chromogen	Vector	SK-4105
	Leica RM2125 RTS microtome	Leica	RM2125 RTS
	Olympus VS110 scanning microscope	Olympus	

Appendix D **Effect of 12-week fish oil intervention on the proportion (%) of scWAT fatty acids.**

Fatty Acid	Normal weight Fish oil (%)			Metabolically healthy obese Fish oil (%)		
	Week 0[1]	Week 12[1]	*P*	Week 0[1]	Week 12[1]	*P*
14:0	2.88 (2.70, 3.30)	2.93 (2.70, 3.25)	.607	2.28 (2.14, 2.71)	2.38 (2.18, 2.69)	.123
16:0	23.01 (22.33, 24.05)	22.94 (22.40, 23.32)	.101	22.31 (21.19, 23.91)	22.85 (22.10, 23.60)	.946
16:1n-7	4.43 (4.01, 4.97)	4.40 (3.76, 4.87)	.368	5.10 (4.69, 6.78)	5.35 (4.54, 6.44)	.446
18:0	4.77 (4.36, 5.36)	4.78 (4.57, 5.34)	.692	3.13 (2.23, 3.40)	2.95 (2.57, 3.40)	.573
18:1n-9	46.62 (46.44, 48.41)	46.94 (46.25, 47.78)	.800	49.11 (48.00, 50.12)	48.54 (47.42, 50.03)	.169
18:1n-7	2.22 (2.07, 2.46)	2.26 (1.94, 2.42)	.727	2.93 (2.55, 3.17)	2.96 (2.87, 3.10)	.304
18:2n-6	11.90 (11.34, 12.70)	11.98 (11.25, 12.79)	.227	11.19 (10.24, 12.34)	11.24 (10.25, 11.70)	.794
18:3n-6	0.09 (0.08, 0.10)	0.09 (0.08, 0.10)	.831	0.09 (0.08, 0.10)	0.09 (0.08, 0.11)	1.000
18:3n-3	0.82 (0.65, 0.95)	0.79 (0.64, 0.99)	.208	0.63 (0.58, 0.77)	0.67 (0.61, 0.81)	.219
20:0	0.24 (0.22, 0.27)	0.26 (0.23, 0.29)	.084	0.13 (0.09, 0.29)	0.12 (0.10, 0.27)	.823
20:1n-9	0.76 (0.72, 0.83)	0.77 (0.70, 0.89)	.388	0.72 (0.68, 0.80)	0.73 (0.64, 0.79)	.291
20:2n-6	0.18 (0.17, 0.21)	0.21 90.18, 0.23)	.056	0.20 (0.18, 0.23)	0.21 (0.20, 0.25)	.202
20:3n-6	0.18 (0.15, 0.25)	0.17 (0.15, 0.21)	.814	0.35 (0.26, 0.42)	0.32 (0.26, 0.38)	.281

20:4n-6	0.28 (0.25, 0.36)	0.28 (0.25, 0.31)	.407	0.49 (0.42, 0.55)	0.49 (0.46, 0.53)	.411
20:4n-3	0.09 (0.07, 0.12)	0.09 (0.07, 0.13)	.276	0.06 (0.05, 0.09)	0.07 (0.05, 0.09)	.847
20:5n-3	0.08 (0.07, 0.14)	0.14 (0.12, 0.17)	.006	0.09 (0.08, 0.11)	0.16 (0.14, 0.22)	<0.001
22:5n-3	0.17 (0.15, 0.22)	0.21 (0.20, 0.27)	<0.001	0.28 (0.24, 0.36)	0.33 (0.26, 0.39)	.090
22:6n-3	0.14 (0.12, 0.21)	0.22 (0.19, 0.29)	<0.001	0.18 (0.16, 0.25)	0.25 (0.18, 0.32)	.091

[1]Median (25th, 75th percentile).

P value obtained using the Wilcoxon model comparing week-12 vs week-0 data split by BMI group and treatment.

Significance is deemed $P \leq 0.05$.

Appendix E **Effect of 12-week corn oil intervention on the proportion (%) of scWAT fatty acids.**

Fatty Acid	Normal weight Corn oil (%)			Metabolically healthy obese Corn oil (%)		
	Week 0[1]	Week 12[1]	*P*	Week 0[1]	Week 12[1]	*P*
14:0	3.29 (2.65, 3.51)	3.25 (2.66, 3.49)	.222	2.37 (2.04, 2.65)	2.31 (2.13, 2.78)	.496
16:0	23.07 (21.50, 24.31)	23.66 (21.00, 24.76)	.359	22.57 (21.80, 23.57)	23.57 (21.37, 24.11)	.445
16:1n-7	4.21 (3.67, 4.63)	4.51 (3.36, 5.13)	.390	5.36 (4.66, 5.83)	5.35 (4.56, 5.82)	.745
18:0	4.86 (3.86, 5.48)	4.68 (3.72, 5.45)	.575	2.60 (2.33, 3.07)	2.65 (2.45, 3.28)	.437
18:1n-9	48.01 (47.57, 49.05)	48.01 (45.95, 48.73)	.334	49.46 (46.70, 50.47)	48.85 (46.60, 50.70)	.313
18:1n-7	2.26 (2.16, 2.44)	2.32 (2.12, 2.43)	.209	2.94 (2.68, 3.49)	2.73 (2.46, 3.29)	.379
18:2n-6	11.07 (10.49, 11.53)	11.13 (10.50, 12.08)	.060	11.33 (10.29, 11.71)	11.30 (10.49, 12.26)	.777
18:3n-6	0.08 (0.07, 0.11)	0.09 (0.08, 0.10)	1.000	0.10 (0.08, 0.11)	0.10 (0.08, 0.12)	.396
18:3n-3	0.83 (0.61, 0.93)	0.82 (0.68, 0.93)	.684	0.72 (0.63, 0.76)	0.71 (0.60, 0.83)	.516
20:0	0.27 (0.22, 0.28)	0.23 (0.20, 0.27)	.814	0.13 (0.10, 0.27)	0.16 (0.09, 0.30)	.983
20:1n-9	0.76 (0.71, 0.91)	0.75 (0.72, 0.80)	.920	0.69 (0.63, 0.73)	0.67 (0.59, 0.73)	.518
20:2n-6	0.17 (0.15, 0.20)	0.16 (0.15, 0.20)	.359	0.19 (0.16, 0.23)	0.19 (0.16, 0.21)	.828
20:3n-6	0.16 (0.12, 0.18)	0.15 (0.14, 0.17)	.443	0.32 (0.24, 0.43)	0.28 (0.22, 0.37)	.260

20:4n-6	0.25 (0.24, 0.29)	0.27 (0.24, 0.34)	.239	0.49 (0.43, 0.58)	0.45 (0.38, 0.54)	.616
20:4n-3	0.11 (0.08, 0.12)	0.09 (0.07, 0.10)	.362	0.07 (0.06, 0.09)	0.06 (0.05, 0.08)	.123
20:5n-3	0.09 (0.07, 0.12)	0.10 (0.07, 0.12)	1.000	0.11 (0.09, 0.17)	0.10 (0.08, 0.12)	.306
22:5n-3	0.19 (0.13, 0.28)	0.21 (0.14, 0.23)	.800	0.25 (0.18, 0.30)	0.22 (0.17, 0.25)	.198
22:6n-3	0.16 (0.13, 0.24)	0.17 (0.13, 0.18)	.733	0.15 (0.12, 0.22)	0.13 (0.10, 0.17)	.124

[1]Median (25th, 75th percentile).

P value obtained using the Wilcoxon model comparing week-12 vs week-0 data split by BMI group and treatment.

Significance is deemed $P \leq 0.05$.

Appendix F **UPLC-MS/MS instrument settings**

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Scheduled MRM:	Yes
Polarity:	Negative
Scan Mode:	N/A
Ion Source:	Turbo Spray
MRM detection window:	120 sec
Target Scan Time:	0.4000 sec
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Threshold.:	0.00 cps
Settling Time:	5<0.0010 msec
MR Pause:	2.0000 msec
MCA:	No
Step Size:	0.00 Da

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Parameter	Start	Stop
LA	279.232	261.232	10.9	DP	-140	-140
				CE	-26	-26
				CXP	-15	-15

9-HODE	295.2	171.1	8	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
13-HODE	295.2	195.2	7.89	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
9-oxo-ODE	293	185	8.16	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
13-oxo-ODE	293	113	8.06	DP	-100	-100
				CE	-30	-30
				CXP	-15	-15
9-HpODE	311.2	185.2	8.06	DP	-70	-70
				CE	-20	-20
				CXP	-15	-15
13-HpODE	311.2	179.1	7.96	DP	-70	-70
				CE	-20	-20
				CXP	-15	-15
9-HOTrE	293	171	6.83	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
13-HOTrE	293	195	6.92	DP	-110	-110
				CE	-25	-25
				CXP	-15	-15
DGLA	277.217	233.217	10.1	DP	-140	-140
				CE	-20	-20
				CXP	-15	-15
15-HETrE	321	221	8.6	DP	-90	-90

				CE	-21	-21
				CXP	-15	-15
11,12,15-TriHETrE	353.3	167.1	3.91	DP	-100	-100
				CE	-30	-30
				CXP	-15	-15
AA	303.232	259.232	10.8	DP	-140	-140
				CE	-17	-17
				CXP	-15	-15
20-COOH-AA	333.1	289.1	6.84	DP	-125	-125
				CE	-22	-22
				CXP	-15	-15
5-HETE	319.2	115.1	8.75	DP	-80	-80
				CE	-20	-20
				CXP	-22	-22
8-HETE	319.2	155.1	8.51	DP	-80	-80
				CE	-20	-20
				CXP	-15	-15
9-HETE	319	151	8.59	DP	-80	-80
				CE	-20	-20
				CXP	-15	-15
11-HETE	319.2	167.2	8.35	DP	-80	-80
				CE	-20	-20
				CXP	-15	-15
12-HETE	319.2	179.2	8.46	DP	-80	-80
				CE	-20	-20
				CXP	-15	-15
15-HETE	319.2	219.2	8.11	DP	-70	-70
				CE	-15	-15

				CXP	-18	-18
16-HETE	319.2	189	7.65	DP	-100	-100
				CE	-25	-25
				CXP	-15	-15
17-HETE	319.2	247	7.54	DP	-100	-100
				CE	-25	-25
				CXP	-15	-15
18-HETE	319.2	261	7.43	DP	-100	-100
				CE	-25	-25
				CXP	-15	-15
20-HETE	319.2	289.2	7.3	DP	-95	-95
				CE	-25	-25
				CXP	-15	-15
5,6-diHETE	335	163	7.17	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
5,15-diHETE	335.2	115.2	5.3	DP	-110	-110
				CE	-22	-22
				CXP	-17	-17
8,15-diHETE	335	127	5.11	DP	-80	-80
				CE	-25	-25
				CXP	-15	-15
14,15-diHETE	335.3	207.15	5.58	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
17,18-diHETE	335.3	247.2	5.33	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15

12-HHT	279	163	6.36	DP	-60	-60
				CE	-30	-30
				CXP	-15	-15
LXA4	351.2	235.1	4.06	DP	-95	-95
				CE	-22	-22
				CXP	-11	-11
LXA5	349	115	3.29	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
LXB4	351.2	221.1	4.01	DP	-90	-90
				CE	-30	-30
				CXP	-21	-21
LTB4	335.2	195.1	5.37	DP	-120	-120
				CE	-22	-22
				CXP	-17	-17
LTB5	333	195	4.52	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
20-OH-LTB4	351.3	195.2	2.96	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
20-COOH-LTB4	365.3	347.2	3.4	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
HXA3	335.2	171.1	6.79	DP	-110	-110
				CE	-20	-20
				CXP	-15	-15
HXB3	335	183	6.45	DP	-70	-70

				CE	-20	-20
				CXP	-15	-15
5-oxo-ETE	317	203	8.98	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
12-oxo-ETE	317	153	8.51	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
15-oxo-ETE	317	113	8.21	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
5-HpETE	335.2	155.1	8.72	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
12-HpETE	335.2	153.1	8.34	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
15-HpETE	335.2	113.1	8.15	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
14,15-EpETE	317.2	247.2	8.15	DP	-90	-90
				CE	-15	-15
				CXP	-15	-15
17,18-EpETE	317.2	259.2	7.96	DP	-90	-90
				CE	-15	-15
				CXP	-15	-15
5,6-EET	319	191	9.72	DP	-60	-60
				CE	-20	-20

				CXP	-15	-15
8,9-EET	319	155	9.17	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
11,12-EET	319	167	8.35	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
14,15-EET	319	219	8.11	DP	-80	-80
				CE	-15	-15
				CXP	-15	-15
5,6-DHET	337	145	7.5	DP	-95	-95
				CE	-25	-25
				CXP	-15	-15
8,9-DHET	337	127	7.03	DP	-90	-90
				CE	-30	-30
				CXP	-15	-15
11,12-DHET	337	167	6.71	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
14,15-DHET	337	207	6.33	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
PGA2	333.2	235.3	4.81	DP	-50	-50
				CE	-20	-20
				CXP	-15	-15
PGB2	333.2	271.2	4.77	DP	-60	-60
				CE	-20	-20
				CXP	-15	-15

PGD1	353.3	273	4.02	DP	-80	-80
				CE	-25	-25
				CXP	-15	-15
PGD2	351.2	189	3.66	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
PGD3	349.2	189	3.33	DP	-75	-75
				CE	-25	-25
				CXP	-15	-15
PGE1	353.3	317.2	3.86	DP	-80	-80
				CE	-25	-25
				CXP	-15	-15
PGE2	351.2	271.2	3.58	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
PGE3	349.2	269	3.21	DP	-75	-75
				CE	-25	-25
				CXP	-15	-15
PGF1a	355	293	3.63	DP	-95	-95
				CE	-30	-30
				CXP	-15	-15
PGF2a	353.2	193.2	3.37	DP	-80	-80
				CE	-30	-30
				CXP	-15	-15
PGF3a	351	193	2.92	DP	-105	-105
				CE	-30	-30
				CXP	-15	-15
PGJ2	333.2	233.1	4.62	DP	-50	-50

				CE	-20	-20
				CXP	-15	-15
dhk-PGD2	351	207	4.19	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
dhk-PGE2	351	235	4	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
dhk-PGF2a	353	291	4.01	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
8-iso-PGF2a	353.2	309.3	3.12	DP	-80	-80
				CE	-30	-30
				CXP	-15	-15
2,3-dinor-6-keto-PGF1a	341	135	2.77	DP	-90	-90
				CE	-30	-30
				CXP	-15	-15
6-keto-PGF1a	369.3	163.2	2.62	DP	-85	-85
				CE	-35	-35
				CXP	-15	-15
delta12-PGJ2	333.2	271	4.77	DP	-110	-110
				CE	-15	-15
				CXP	-15	-15
15d-PGD2	333.2	271.2	5.5	DP	-50	-50
				CE	-20	-20
				CXP	-15	-15
15d-PGJ2	315.2	271.3	6.86	DP	-80	-80
				CE	-15	-15

				CXP	-15	-15
TxB1	371	171	2.83	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
TXB2	369.2	169.1	3.21	DP	-75	-75
				CE	-25	-25
				CXP	-15	-15
TXB3	367.2	169.1	2.76	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
11-dh-TXB2	367.1	305.1	3.48	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
11-dh-TXB3	367.2	169.1	2.93	DP	-70	-70
				CE	-25	-25
				CXP	-15	-15
EPA	301.217	257.217	10	DP	-80	-80
				CE	-15	-15
				CXP	-15	-15
5-HEPE	317	115	7.8	DP	-60	-60
				CE	-22	-22
				CXP	-11	-11
8-HEPE	317	127	7.45	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
11-HEPE	317	121	7.33	DP	-90	-90
				CE	-24	-24
				CXP	-15	-15

12-HEPE	317	179	7.47	DP	-70	-70
				CE	-21	-21
				CXP	-15	-15
15-HEPE	317	219	7.23	DP	-65	-65
				CE	-19	-19
				CXP	-8	-8
18-HEPE	317	259	6.91	DP	-65	-65
				CE	-18	-18
				CXP	-23	-23
5-HpEPE	333.2	155	7.58	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
12-HpEPE	333.2	151	7.3	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
15-HpEPE	333.2	111	7.11	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
5,15-diHEPE	333	115	4.96	DP	-110	-110
				CE	-22	-22
				CXP	-17	-17
RvE1	349.2	195.1	2.8	DP	-80	-80
				CE	-24	-24
				CXP	-16	-16
RvE2	333.3	253.2	3.63	DP	-40	-40
				CE	-22	-22
				CXP	-17	-17
RvE3	333.3	201.1	3.09	DP	-40	-40

				CE	-22	-22
				CXP	-17	-17
DPA	329.248	285.248	11	DP	-80	-80
				CE	-15	-15
				CXP	-15	-15
10,17-DiHDPA	359	153	5.14	DP	-70	-70
				CE	-20	-20
				CXP	-15	-15
19,20-DiHDPA	361	229	6.23	DP	-90	-90
				CE	-25	-25
				CXP	-15	-15
16,17-EpDPE	343.5	273.5	8.76	DP	-80	-80
				CE	-15	-15
				CXP	-15	-15
19,20-EpDPE	343.5	281.2	8.52	DP	-80	-80
				CE	-20	-20
				CXP	-15	-15
DHA	327.232	283.232	10.5	DP	-140	-140
				CE	-15	-15
				CXP	-15	-15
4-HDoHE	343	101	8.88	DP	-40	-40
				CE	-18	-18
				CXP	-13	-13
7-HDoHE	343	141	8.52	DP	-40	-40
				CE	-18	-18
				CXP	-13	-13
8-HDoHE	343	109	8.56	DP	-100	-100
				CE	-20	-20

				CXP	-15	-15
10-HDoHE	343	181	5.31	DP	-80	-80
				CE	-17	-17
				CXP	-15	-15
11-HDoHE	343	149	8.43	DP	-90	-90
				CE	-19	-19
				CXP	-15	-15
13-HDoHE	343	221	8.24	DP	-90	-90
				CE	-17	-17
				CXP	-15	-15
14-HDoHE	343	205	8.31	DP	-70	-70
				CE	-21	-21
				CXP	-15	-15
16-HDoHE	343	233	8.1	DP	-105	-105
				CE	-19	-19
				CXP	-15	-15
17-HDoHE	343	245	8.14	DP	-80	-80
				CE	-20	-20
				CXP	-18	-18
20-HDoHE	343	241	7.87	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
9,10-EpOME	295.2	171.2	8	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15
12,13-EpOME	295.2	195.2	8	DP	-90	-90
				CE	-20	-20
				CXP	-15	-15

9,10-diHOME	313.2	201.1	8.72	DP	-90	-90
				CE	-30	-30
				CXP	-15	-15
12,13-diHOME	313.2	183.1	5.88	DP	-90	-90
				CE	-30	-30
				CXP	-15	-15
RvD1	375.2	215.2	3.81	DP	-90	-90
				CE	-22	-22
				CXP	-13	-13
RvD2	375.2	141.2	3.6	DP	-90	-90
				CE	-24	-24
				CXP	-13	-13
Maresin1	359.2	250.1	5.09	DP	-40	-40
				CE	-22	-22
				CXP	-17	-17
FA 16:1	253.217	71	10.6	DP	-140	-140
				CE	-25	-25
				CXP	-15	-15
FA 18:1	281.248	263.248	11.8	DP	-140	-140
				CE	-25	-25
				CXP	-15	-15

Window (sec) = 120, Primary/Secondary = 1, Dwell Weight = 1000000, Group = 1, for all mediators

Period 1 Experiment 2:

Scan Type: MRM (MRM)

Scheduled MRM: Yes

Appendix F

Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
MRM detection window:	120 sec
Target Scan Time:	0.3000 sec
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Threshold.:	0.00 cps
Settling Time:	5<0.0010 msec
MR Pause:	2.0000 msec
MCA:	No
Step Size:	0.00 Da

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Parameter	Start	Stop
N-14:0-EA	272.26	62.1	8.9	DP	100	100
				CE	30	30
				CXP	16	16
N-16:0-EA	300.23	62.1	10.23	DP	100	100
				CE	30	30
				CXP	16	16
N-16:1-EA	298.27	62.1	9.2	DP	100	100
				CE	30	30
				CXP	16	16
N-18:0-EA	328.2	62.1	11.55	DP	110	110
				CE	35	35

				CXP	16	16
N-18:1-EA	326.2	62.1	10.52	DP	100	100
				CE	30	30
				CXP	16	16
N-18:2-EA	324.2	62.1	9.62	DP	100	100
				CE	30	30
				CXP	16	16
N-18:3a-EA	322.3	62.1	8.83	DP	100	100
				CE	30	30
				CXP	16	16
N-20:4-EA	348.3	62.1	9.55	DP	90	90
				CE	30	30
				CXP	16	16
N-20:5-EA	346.3	62.1	8.85	DP	90	90
				CE	30	30
				CXP	16	16
N-22:4-EA	376.3	62.1	9.7	DP	90	90
				CE	35	35
				CXP	16	16
N-22:5-EA	374.3	62.1	9.6	DP	90	90
				CE	35	35
				CXP	16	16
N-22:6-EA	372.3	62.1	9.42	DP	90	90
				CE	35	35
				CXP	16	16
2-16:0-glycerol	331.3	239.3	10.88	DP	90	90
				CE	20	20
				CXP	16	16

2-16:1-glycerol	329.3	237.3	9.6	DP	90	90
				CE	20	20
				CXP	16	16
2-18:1-glycerol	357.3	265.2	10.83	DP	90	90
				CE	20	20
				CXP	16	16
2-18:2-glycerol	355.3	263.2	10.04	DP	90	90
				CE	20	20
				CXP	16	16
1-20:4-glycerol	379.3	287.2	10.06	DP	90	90
				CE	20	20
				CXP	16	16
2-20:4-glycerol	379.4	287.2	9.94	DP	90	90
				CE	20	20
				CXP	16	16
2-20:5-glycerol	377.3	285.3	9	DP	90	90
				CE	20	20
				CXP	16	16
2-22:5-glycerol	405.3	313.3	10.15	DP	90	90
				CE	20	20
				CXP	16	16
2-22:6-glycerol	403.3	311.3	9.77	DP	90	90
				CE	20	20
				CXP	16	16
LTC4	626	308	4.74	DP	90	90
				CE	20	20
				CXP	15	15
LTD4	497	189	4.6	DP	90	90

LTE4	440.3	189	4.9	CE	20	20
				CXP	15	15
				DP	100	100
				CE	20	20
				CXP	16	16

Window (sec) = 120, Primary/Secondary = 1, Dwell Weight = 1000000, Group = 1, for all mediators

Appendix G Effect of 12-week fish oil intervention on relative fatty acid metabolite concentration (%) of human scWAT from normal weight and metabolically healthy obese individuals

	Normal Weight (%)			Obese (%)		
Fatty acid metabolite	Study event[1]	Post Fish oil[1]	*P*[2]	Study event[1]	Post Fish oil[1]	*P*[2]
9-10-EpOME	0.3044 (0.2401, 0.3788)	0.2683 (0.2229, 0.3381)	0.003	0.2894 (0.1830, 0.4345)	0.2539 (0.1836, 0.7518)	0.733
12-13-EpOME	0.9129 (0.7426, 2.4941)	0.7055 (0.4537, 1.8743)	0.397	0.6626 (0.3955 (1.7699)	0.7383 (0.5012, 4.0015)	0.427
9-HODE	1.0193 (0.7542, 1.1406)	0.8100 (0.7000, 1.1035)	0.005	0.8379 (0.5470, 1.5706)	0.7785 (0.6342, 2.2039)	0.691
13-HODE	2.1540 (1.5372, 2.7986)	1.9809 (1.3132, 3.0111)	0.056	1.6250 (0.8264, 12.5255)	1.6803 (1.0043, 15.1074)	0.112
9-oxo-ODE	0.1063 (0.0657, 0.6463)	0.0631 (0.0502, 1.1359)	0.397	0.0594 (0.0441, 0.1039)	0.0843 (0.0372, 0.1397)	0.955
13-oxo-ODE	0.0866 (0.03549, 8.7215)	0.6751 (0.2804, 14.6427)	0.433	0.3667 (0.1743, 1.4096)	0.7365 (0.1947, 2.0476)	0.156
9-HpODE	0.0178 (0.0150, 0.0212)	0.0120 (0.0090, 0.0169)	0.006	0.0085 (0.0052, 0.0161)	0.0080 (0.0044, 0.0126)	0.078
9-HOTrE	0.0271 (0.0223, 0.0339)	0.0248 (0.0203, 0.0280)	0.011	0.0207 (0.0147, 0.0254)	0.0184 (0.0117, 0.0266)	0.112
13-HOTrE	0.0227 (0.0169, 0.0348)	0.0193 (0.0126, 0.0260)	0.009	0.0200 (0.0130, 0.0357)	0.0276 (0.0151, 0.0360)	0.650
15-HETrE	0.0163 (0.0096, 0.0289)	0.0185 (0.0102, 0.0264)	0.221	0.0194 (0.0139, 0.0346)	0.0216 (0.0109, 0.0317)	0.036
11-12-15-TriHETrE	0.0063 (0.0042, 0.0116)	0.0084 (0.0044, 0.0126)	0.331	0.0055 (0.0032, 0.0153)	0.0067 (0.0023, 0.0144)	0.733
18:2-EA	0.2874 (0.2400, 0.6011)	0.2904 (0.2399, 0.3426)	0.245	0.3516 (0.2054, 0.4337)	0.2700 (0.1511, 0.3798)	0.307
2-18:2-glycerol	0.1994 (0.1302, 0.3540)	0.2460 (0.1732, 0.3595)	0.778	0.1611 (0.1009, 0.2598)	0.1182 (0.0621, 0.1961)	0.112
20-COOH-AA	0.0648 (0.0420, 0.0850)	0.0388 (0.0304, 0.0611)	0.002	0.0243 (0.0148, 0.0449)	0.0309 (0.0079, 0.0519)	0.427
5-HETE	0.0193 (0.0157, 0.0258)	0.0179 (0.0152, 0.0244)	0.363	0.0198 (0.0167, 0.0325)	0.0156 (0.0079, 0.0240)	0.156
8-HETE	0.0618 (0.0301, 0.0717)	0.0512 (0.0309, 0.0767)	0.730	0.0440 (0.0288, 0.0940)	0.0697 (0.0308, 0.1146)	0.460
9-HETE	0.0021 (0.0017, 0.0035)	0.0027 (0.0015, 0.0039)	0.638	0.0023 (0.0013, 0.0037)	0.0015 (<0.0017, 0.0025)	0.100
11-HETE	0.0292 (0.0235, 0.0558)	0.0259 (0.0155, 0.0447)	0.875	0.0364 (0.0230, 0.0591)	0.0200 (0.0104, 0.0446)	0.088
12-HETE	0.6172 (0.1463, 1.3299)	0.6533 (0.1837, 0.9917)	0.730	0.4831 (0.2703, 1.7955)	0.2146 (0.0975, 0.10806)	0.047
15-HETE	0.0110 (0.0055, 0.0202)	0.0104 (0.0062, 0.0157)	0.470	0.0125 (0.0052, 0.0273)	0.0069 (0.0039, 0.0233)	0.281

16-HETE	0.0245 (0.0142, 0.0358)	0.0160 (0.0112, 0.0181)	0.778	0.0121 (0.0063, 0.0185)	0.0098 (0.0049, 0.0197)	0.256
14-15-diHETE	0.0066 (0.0035, 0.0104)	0.0056 (0.0044, 0.0067)	0.011	0.0033, 0.0021, 0.0050)	0.0048 (0.0017, 0.0069)	0.532
17-18-DiHETE	0.0197 (0.0134, 0.0233)	0.0186 (0.0139, 0.0215)	0.005	0.0106 (0.0070, 0.0169)	0.0093 (0.0048, 0.0181)	0.363
12-HHT	0.0117 (0.0029, 0.0170)	0.0088 (0.0042, 0.0137)	0.470	0.0108 (0.0061, 0.0241)	0.0091 (0.0036, 0.0186)	0.363
LXA_4	0.0019 (0.0013, 0.0030)	0.0018 (0.0013, 0.0022)	0.016	0.0017 (<0.0019, 0.0029)	0.0019 (<0.0018, 0.0044)	0.733
LXA_5	<0.0016 (<0.0015, <0.0019)	<0.0016 (<0.0013, 0.0016)	0.177	<0.0015 (<0.0012, 0.0010)	<0.0014 (<0.0012, 0.0013)	0.776
LXB_4	0.0042 (0.0022, 0.0088)	0.0028 (0.0022, 0.0061)	0.177	0.0025 (<0.0019, 0.0070)	0.0022 (0.0015, 0.0043)	0.363
LTB_4	0.0221 (0.0175, 0.0506)	0.0213 (0.0133, 0.0288)	0.048	0.0186 (0.0143, 0.0259)	0.0204 (0.0064, 0.0333)	0.256
LTB_5	0.0015 (<0.0018, 0.0035)	0.0015 (<0.0018, 0.0023)	0.008	0.0013 (<0.0016, 0.0038)	0.0015 (<0.0019. 0.0048)	0.012
LTC_4	<0.0018 (<0.0015, 0.0022)	<0.0019 (<0.0014, 0.0019)	0.124	<0.0019 (<0.0014, 0.0021)	<0.0019 (<0.0013, 0.0013)	0.650
LTD_4	0.0079 (0.0037, 0.0189)	0.0068 (0.0034, 0.0084)	0.041	0.0062 (0.0030, 0.0110)	0.0042 (0.0022, 0.0082)	0.363
LTE_4	0.0083 (0.0067, 0.0215)	0.0113 (0.0052, 0.0137)	0.551	0.0080 (0.0049, 0.0124)	0.0049 (0.0022, 0.0081)	0.125
u-LTC_4	<0.0016 (<0.0015, 0.0010)	<0.0017 (<0.0013, 0.0011)	0.090	<0.0014 (<0.0012, 0.0015)	<0.0014 (<0.0012, 0.0014)	0.158
u-LTD_4	0.0011 (<0.0015, 0.0020)	0.0011 (<0.0015, 0.0022)	0.140	<0.0015 (<0.0013, 0.0012)	<0.0019 (<0.0013, 0.0016)	0.691
20-OH-LTB_4	0.0021 (<0.0018, 0.0031)	0.0020 (<0.0019, 0.0026)	0.245	<0.0019 (<0.0016, 0.0017)	<0.0018 (<0.0015, 0.0016)	0.100
20-COOH-LTB_4	0.0056 (0.0045, 0.0104)	0.0042 (0.0037, 0.0069)	0.140	0.0038 (0.0018, 0.0060)	0.0036 (0.0013, 0.0074)	0.733
HXA_3	0.0931 (0.0624, 0.1426)	0.0574 (0.0376, 0.0712)	0.109	0.0585 (0.0381, 0.0946)	0.0306 (0.0088, 0.0680)	0.061
5-oxo-ETE	0.0053 (0.0043, 0.0072)	0.0048 (0.0034, 0.0060)	0.074	0.0051 (0.0038, 0.0081)	0.0034 (0.0030, 0.0058)	0.650
15-oxo-ETE	0.0087 (0.0060, 0.0111)	0.0067 (0.0036, 0.0100)	0.096	0.0087 (0.0068, 0.0150)	0.0081 (0.0051, 0.0114)	0.609
11-12-EET	0.0291 (0.0168, 0.0390)	0.0180 (0.0109, 0.0345)	0.826	0.0197 (0.0135, 0.0358)	0.0164 (0.0087, 0.0296)	0.460
14-15-EET	0.0076 (0.0046, 0.0120)	0.0121 (0.0047, 0.0174)	0.551	0.0082 (0.0058, 0.0264)	0.0096 (0.0037, 0.0184)	0.394
5-6-DHET	0.0030 (0.0022, 0.0037)	0.0024 (0.0013, 0.0038)	0.198	0.0020 (<0.0019, 0.0052)	0.0020 (<0.0019, 0.0044)	0.865
11-12-DHET	0.0039 (0.0029, 0.0048)	0.0025 (0.0018, 0.0030)	0.002	0.0021 (0.0011, 0.0038)	0.0016 (<0.0017, 0.0031)	0.532
14-15-DHET	0.0055 (0.0036, 0.0077)	0.0030 (0.0024, 0.0040)	0.004	0.0035 (0.0023, 0.0043)	0.0027 (0.0015, 0.0050)	0.061
PGB_2	0.0028 (0.0013, 0.0042)	0.0021 (0.0017, 0.0024)	0.109	0.0015 (<0.0018, 0.0030)	0.0022 (<0.0019, 0.0036)	0.173
PGD_1	0.0025 (0.0019, 0.0062)	0.0026 (<0.0019, 0.0036)	0.177	0.0017 (<0.0018, 0.0055)	0.0019 (0.0011, 0.0046)	0.363
PGD_2	0.0018 (0.0014, 0.0047)	0.0020 (0.0013, 0.0035)	0.510	0.0025 (0.0010, 0.0055)	0.0018 (<0.0019, 0.0041)	0.125
PGD_3	0.0012 (<0.0017, 0.0017)	<0.0018 (<0.0016, 0.0019)	0.683	<0.0018 (<0.0016, 0.0017)	<0.0018 (<0.0012, 0.0015)	0.570

PGE1	0.0032 (0.0017, 0.0048)	0.0020 (0.0012, 0.0040)	0.470	0.0016 (0.0011, 0.0041)	0.0020 (0.0011, 0.0041)	0.496
PGE2	0.0081 (0.0062, 0.0132)	0.0091 (0.0023, 0.0121)	0.975	0.0112 (0.0040, 0.0236)	0.0076 (0.0020, 0.0163)	0.211
PGE3	0.0014 (0.0010, 0.0017)	0.0016 (0.0013, 0.0029)	0.140	0.0010 (<0.0017, 0.0017)	0.0011 (<0.0015, 0.0023)	0.570
PGF1a	0.0030 (0.264, 0.0497)	0.0283 (0.0230, 0.0343)	0.510	0.0183 (0.0103, 0.0246)	0.0177 (0.0063, 0.0253)	0.088
PGF2a	0.0058 (0.0043, 0.0143)	0.0093 (0.0041, 0.0121)	0.778	0.0136 (0.0078, 0.0236)	0.0129 (0.0077, 0.0277)	0.078
PGF3a	0.0087 (0.0057, 0.0151)	0.0093 (0.0051, 0.0202)	0.064	0.0074 (0.0041, 0.0132)	0.0096 (0.0036, 0.0326)	0.307
PGJ_2	0.0023 (0.0018, 0.0048)	0.0022 (0.0013, 0.0040)	0.331	0.0014 (<0.0014, 0.0043)	0.0020 (0.0010, 0.0045)	0.394
dhk-PGD_2	0.0013 (<0.0019, 0.0022)	0.0014 (<0.0019, 0.0034)	0.140	0.0015 (<0.0017, 0.0018)	<0.0017 (<0.0013, 0.0017)	0.570
dhk-PGE_2	0.0020 (0.0013, 0.0032)	0.0023 (<0.0018, 0.0045)	0.035	0.0018 (0.0011, 0.0050)	0.0023 (0.0011, 0.0058)	0.820
dhk-PGF_{2a}	0.0032 (0.0021, 0.0056)	0.0029 (0.0017, 0.0050)	0.124	0.0021 (0.0013, 0.0033)	0.0014 (0.0011, 0.0034)	0.955
8-i-PGF_{2a}	0.0105 (0.0070, 0.0299)	0.1446 (0.0767, 0.2316)	0.056	0.0133 (0.0057, 0.0297)	0.0472 (0.0122, 0.1561	0.173
2-3-d6k-PGF_{1a}	0.0018 (<0.0019, 0.0029)	0.0018 (0.0015, 0.0041)	0.158	0.0018 (0.0011, 0.0033)	0.0018 (0.0011, 0.0050)	0.609
6-k-PGF_{1a}	0.0603 (0.0455, 0.1369)	0.0431 (0.0256, 0.0625)	0.124	0.0348 (0.0185, 0.0539)	0.0266 (0.0078, 0.0516)	0.140
delta12-PGJ_2	0.0011 (<0.0017, 0.0014)	<0.0019 (<0.0016, 0.0018)	0.245	<0.0019 (<0.0013, 0.0021)	<0.0018 (<0.0015, 0.0015)	0.820
TxB_1	<0.0017 (<0.0015, 0.0010)	<0.0017 (<0.0013, 0.0015)	0.084	<0.0016 (<0.0013, 0.0013)	<0.0018 (<0.0014, 0.0021)	0.496
TXB_2	0.0387 (0.0118, 0.2011)	0.0483 (0.0125, 0.1485)	0.470	0.0601 (0.0262,0.3071)	0.0411 (0.0114, 0.1253)	0.140
TXB_3	<0.0016 (<0.0014, 0.0017)	0.0014 (<0.0014, 0.0031)	0.925	<0.0019 (<0.0014, 0.0019)	0.0011 (<0.0013, 0.0021)	0.460
11-dh-TXB_2	0.0029 (0.0017, 0.0051)	0.0031 (0.0013, 0.0074)	0.875	0.0056 (0.0016, 0.0164)	0.0024 (0.0010, 0.0070)	0.910
11-dh-TXB_3	0.0013 (<0.0016, 0.0016)	0.0013 (<0.0018, 0.0024)	0.530	<0.0019 (<0.0015, 0.0022)	0.0010 (<0.0017, 0.0014)	0.691
20:4-EA	0.0649 (0.0310, 0.1101)	0.0414 (0.0214, 0.0677)	0.397	0.1228 (0.0661, 0.1484)	0.0893 (0.0608, 0.1159)	0.156
1-20:4-glycerol	0.4218 (0.2971, 0.6533)	0.2554 (0.1769, 0.4903)	0.470	0.2732 (0.1505, 0.3685)	0.1798 (0.1118, 0.2554)	0.650
2-20:4-glycerol	1.2426 (0.7013, 1.9927)	0.9796 (0.7034, 1.2810)	0.397	0.8539 (0.4097, 1.1644)	0.5755 (0.3568, 0.7162)	0.125
5-HEPE	0.0041 (0.0011, 0.0109)	0.0067 (0.0034, 0.0112)	0.109	0.0047 (0.0027. 0.0080)	0.0055 (0.0015, 0.0128)	1.000
12-HEPE	0.0536 (0.0120, 0.0927)	0.0893 (0.0168, 0.2394)	0.221	0.0489 (0.0282, 0.1442)	0.0310 (0.0130, 0.1546)	0.776
15-HEPE	0.0054 (0.0028, 0.0125)	0.0058 (<0.0013, 0.0109)	0.035	0.0057 (0.0028, 0.0133)	0.0070 (0.0026, 0.0126)	0.955
RvE_1	<0.0018 (.0005, 0.0011)	<0.0018 (<0.0015, 0.0012)	0.363	<0.0014 (<0.0012, 0.0016)	<0.0015 (<0.0012, <0.0018)	0.820
RvE_2	0.0474 (0.0289, 0.0622)	0.0433 (0.0213, 0.0868)	0.048	0.0365 (0.0215, 0.0555)	0.0363 (0.0191, 0.1212)	0.532
RvE_3	0.0157 (0.0052, 0.0339)	0.0143, 0.0081, 0.0309)	0.730	0.0078 (0.0030, 0.0150)	0.0057 (0.0014, 0.0135)	0.053

20:5-EA	0.0091 (0.0056, 0.0138)	0.0145 (0.0054, 0.0212)	0.875	0.0097 (0.0055, 0.0134)	0.0146 (0.0084, 0.0291)	0.865
2-20:5-glycerol	0.0356 (0.0270, 0.0500)	0.0695 (0.0499, 0.0977)	0.826	0.0263 (0.0218, 0.0429)	0.0357 (0.0237, 0.0445)	0.363
19-20-DiHDPA	0.0026 (0.0018, 0.0050)	0.0033 (0.0027, 0.0049)	0.272	0.0021 (0.0017, 0.0030)	0.0022 (0.0011, 0.0044)	0.820
22:5-EA	0.0158 (0.0063, 0.0192)	0.0139 (0.0090, 0.0187)	0.064	0.0131 (0.0102, 0.0168)	0.0083 (0.0050, 0.0192)	0.691
2-22:5-glycerol	0.0353 (0.0289, 0.0407)	0.0455 (0.0361, 0.0585)	0.470	0.0373 (0.0220, 0.0532)	0.0331 (0.0177, 0.0443)	0.334
4-HDoHE	0.0105 (0.0047, 0.0174)	0.0078 (0.0069, 0.0110)	0.013	0.0060 (0.0045, 0.0122)	0.0107 (0.0022, 0.0192)	0.496
7-HDoHE	0.0067 (0.0017, 0.0116)	0.0069 (0.0034, 0.0195)	0.140	0.0058 (0.0027, 0.0097)	0.0056 (0.0028, 0.0190)	0.307
8-HDoHE	0.0021 (0.0010, 0.0071)	0.0023 (0.0011, 0.0034)	0.019	0.0011 (<0.0014, 0.0024)	0.0014 (<0.0012, 0.0036)	0.955
11-HDoHE	0.0073 (0.0053, 0.0128)	0.0075 (0.0038, 0.0106)	0.022	0.0070 (0.0029, 0.0120)	0.0044 (0.0028, 0.0169)	0.394
13-HDoHE	0.0017 (0.0010, 0.0030)	0.0015 (<0.0018, 0.0030)	0.019	0.0014 (0.0012, 0.0034)	0.0023 (<0.0013, 0.0039)	0.334
14-HDoHE	0.0274 (0.0125, 0.0749)	0.0303 (0.0058, 0.0502)	0.300	0.0236 (0.0106, 0.0404)	0.0123 (0.0070, 0.0632)	0.191
16-HDoHE	0.0062 (0.0035, 0.0127)	0.0078 (0.0037, 0.0099)	0.030	0.0030 (0.0018, 0.0065)	0.0033 (0.0024, 0.0133)	0.256
17-HDoHE	0.0063 (0.0027, 0.0131)	0.0036 (0.0021, 0.0087)	0.005	0.0039 (0.0020, 0.0120)	0.0054 (0.0010, 0.0095)	0.776
20-HDoHE	0.0130 (0.0049, 0.0210)	0.0074 (0.0058, 0.0185)	0.002	0.0060 (0.0038, 0.0193)	0.0077 (0.0023, 0.0167)	0.650
12-13-diHOME	0.3299 (0.2280, 0.4856)	0.2743 (0.2392, 0.3751)	0.022	0.2087 (0.1480, 0.2415)	0.1927 (0.1213, 0.3065)	0.256
RvD_1	0.0021 (0.0010, 0.0032)	0.0022 (0.0011, 0.0029)	0.331	0.0019 (<0.0019, 0.0027)	0.0014 (<0.0013, 0.0030)	0.173
RvD_2	0.0464 (0.0285, 0.0735)	0.0279 (0.0185, 0.0657)	0.158	0.0187 (0.0090, 0.0429)	0.0162 (0.0065, 0.0642)	0.112
22-6-EA	0.0513 (0.0408, 0.0735)	0.0736 (0.0576, 0.1159)	0.826	0.0608 (0.0418, 0.0716)	0.0659 (0.0376, 0.1030)	0.609
2-22:6-glycerol	0.1210 (0.0638, 0.1693)	0.1658 (0.1117, 0.2035)	0.925	0.1096 (0.0857, 0.1360)	0.0840 (0.0481, 0.1747)	0.532
14:0-EA	0.3778 (0.2424, 0.9788)	0.2931 (0.1908, 0.4043)	0.048	0.1894 (0.1212, 0.2869)	0.1772 (0.1146, 0.2403)	0.910
16:0-EA	1.9921 (1.6119, 3.0000)	1.9995 (0.9202, 2.4019)	0.245	0.17991 (1.3474, 2.3517)	1.5939 (1.2270, 2.4118)	0.460
16:1-EA	0.3639 (0.2608, 0.5878)	0.2697 (0.1673, 0.4978)	0.683	0.3722 (0.2081, 0.4549)	0.2296 (0.1004, 0.4972)	0.078
18:0-EA	0.2977 (0.1945, 0.4426)	0.2738 (0.2047, 0.3641)	0.272	0.3683 (0.2780, 0.4542)	0.2956 (0.2140, 0.4660)	0.281
18:1-EA	1.3836 (0.8579, 1.7911)	0.9583 (0.6654, 1.1041)	0.096	1.1311 (0.7925, 1.4349)	0.9831 (0.6543, 1.5732)	0.496
18:3a-EA	0.0184 (0.0157, 0.0260)	0.0196 (0.0133, 0.0236)	0.177	0.0163 (0.0128, 0.0187)	0.0143 (0.0080, 0.0220)	0.427
2-16:0-glycerol	0.1205 (0.0467, 0.3463)	0.0810 (0.0385, 0.2612)	0.433	0.0342 (0.0232, 0.0640)	0.0253 (0.0131, 0.0460)	0.125
2-16:1-glycerol	0.0779 (0.0425, 0.1362)	0.0901 (0.0488, 0.1314)	0.177	0.0745 (0.0483, 0.1189)	0.0594 (0.0333, 0.1081)	0.570
2-18:1-glycerol	0.5465 (0.3183, 0.8354)	0.6086 (0.4812, 0.7560)	0.331	0.5712 (0.3423, 0.8140)	0.4095 (0.1818, 0.6664)	0.100

Appendix G

[1] Median (25^{th}, 75^{th} percentile).

[2] *P* value obtained using the Wilcoxon signed ranks test.

Significance deemed $P \leq 0.05$.

Appendix H Effect of 12-week fish oil intervention on absolute fatty acid metabolite concentration (ng/g) of human scWAT from normal weight and metabolically healthy obese individuals

	Normal Weight (ng/g)			Obese (ng/g)		
Fatty acid metabolite	Study entry[1]	Post Fish oil[1]	*P*[2]	Study entry[1]	Post Fish oil[1]	*P*[2]
5-HETE	5.78 (2.56, 6.17)	5.83 (5.51, 6.19)	0.861	6.09 (5.65, 6.53)	6.12 (5.60, 6.35)	0.627
8-HETE	3.55 (3.39, 3.84)	3.71 (3.40, 3.89)	0.807	3.79 (3.57, 4.12)	3.87 (3.55, 4.06)	0.823
12-HETE	3.03 (0.25, 5.87)	3.29 (0.32, 6.24)	0.311	3.68 (0.48, 8.02)	1.01 (0.25, 2.17)	0.028
17-18-DiHETE	0.47 (0.15, 0.59)	0.32 (0.25, 0.47	0.530	0.17 (0.12, 0.26)	0.09 (0.01, 0.22)	0.074
5-oxo-ETE	0.06 (0.05, 0.08)	0.06 (0.04, 0.07)	0.834	0.07 (0.05, 0.08)	0.04 (0.03, 0.07)	0.086
PGE_2	0.02 (0.01, 0.04)	0.08 (0.03, 0.11)	0.285	0.09 (0.02, 0.22)	0.06 (0.02, 0.07)	0.285
PGF_{2a}	0.16 (0.07, 0.35)	0.19 (0.06, 0.27)	0.347	0.34 (0.24, 0.48)	0.25 (0.22, 0.45)	0.486
6-keto-PGF_{1a}	0.38 (0.28, 1.09)	0.28 (0.17, 0.51)	0.279	0.29 (0.15, 0.39)	0.19 (0.15, 0.33)	0.305
TXB_2	0.46 (0.33, 0.93)	0.41 (0.32, 1.43)	0.249	0.60 (0.38, 1.67)	0.45 (0.34, 0.56)	0.232
11-dh-TXB_2	0.06 (0.05, 0.09)	0.07 (0.06, 0.29)	0.834	0.06 (0.05, 0.15)	0.06 (0.05, 0.08)	0.145
LXB_4	0.19 (0.17, 0.24)	0.18 (0.17, 0.22)	0.552	0.17 (0.15, 0.19)	0.17 (0.15, 0.19)	0.681
AEA	0.31 (0.07, 1.32)	0.65 (0.24, 1.35)	0.861	2.16 (1.19, 3.44)	1.32 (0.31, 2.37)	0.094
2-AG	1527.80 (883.37, 2104.65)	1579.81 (635.81, 1919.35)	0.600	1364.32 (721.95, 1933.46)	620.78 (265.36, 1128070)	0.006
EPEA	0.17 (0.04, 0.26)	0.35 (0.21, 0.82)	0.006	0.28 (0.16, 0.60)	0.40 (0.12, 0.88)	0.433
4-HDoHE	0.11 (0.05, 0.15)	0.09 (0.05, 0.13)	0.463	0.07 (0.05, 0.10)	0.06 (0.05, 0.10)	0.370
8-HDoHE	0.08 (0.06, 0.11)	0.08 (0.06, 0.12)	0.576	0.07 (0.06, 0.09)	0.06 (0.05, 0.08)	0.324
11-HDoHE	0.05 (0.04, 0.11)	0.06 (0.03, 0.12)	0.235	0.06 (0.02, 0.13)	0.03 (0.02, 0.05)	0.073
14-HDoHE	0.36 (0.20, 0.66)	1.00 (0.59, 2.44)	0.017	0.44 (0.10, 0.72)	0.16 (0.05, 1.20)	0.241
17-HDoHE	0.07 (0.02, 0.12)	0.03 (0.01, 0.04)	0.465	0.07 (0.01, 0.14)	0.03 (0.01, 0.05)	0.686
18:1-EA	39.64 (8.72, 59.02)	23.30 (7.79, 46.84)	0.917	38.93 (15.07, 55.45)	27.84 (4.64, 42.28)	0.108

RvD_1	0.16 (0.14, 0.17)	0.16 (0.15, 0.17)	0.861	0.16 (0.15, 0.18)	0.16 (0.16, 0.17)	0.888
RvD_2	0.26 (0.21, 0.34)	0.24 (0.21, 0.29)	0.753	0.24 (0.20, 0.26)	0.20 (0.19, 0.23)	0.073
DHEA	0.77 (0.42, 0.82)	0.95 (0.53, 1.57)	0.039	0.69 (0.29, 1.26)	0.72 (0.21, 1.29)	0.737

[1] Median (25^{th}, 75^{th} percentile).

[2] *P* value obtained using the Wilcoxon signed ranks test.

Significance deemed $P \leq 0.05$.

Appendix I Effect of 12-week corn oil intervention on relative concentration (%) of fatty acid metabolites in scWAT of normal weight and metabolically healthy obese individuals

	Normal Weight (%)			Obese (%)		
Fatty acid metabolite	Study entry[1]	Post Corn oil[1]	*P*[2]	Study entry[1]	Post Corn oil[1]	*P*[2]
9-10-EpOME	1.5400 (0.9822, 1.7584)	1.3947 (1.2879, 1.7949)	1.000	1.6659 (1.3837, 1.8097)	1.7349 (1.4647, 1.9425)	0.733
12-13-EpOME	5.8448 (3.9639, 7.1862)	4.7148 (3.7575, 5.0430)	0.158	4.4627 (2.8954, 5.2791)	4.8605 (3.8524, 5.2659)	0.809
9-HODE	4.6653 (3.2634, 5.6386)	5.2182 (3.5441, 5.7980)	0.814	5.2668 (4.4889, 5.8769)	5.5177 (4.8333, 6.6817)	0.778
13-HODE	11.1479 (8.7645, 14.7507)	9.4024 (8.0776, 11.8581)	0.433	46.8432 (8.432, 60.2910)	48.9743 (8.8717, 64.4809)	0.601
9-oxo-ODE	0.4871 (0.4155, 2.3124)	0.5208 (0.4134, 1.0690)	0.583	0.2365 (0.1998, 0.3094)	0.2593 (0.1843, 0.3637)	0.872
13-oxo-ODE	4.0932 (3.2335, 34.6122)	4.4298 (2.3262, 11.6820)	0.695	1.7921 (1.3786, 2.4444)	2.1480 (1.6325, 6.0036)	0.212
9-HpODE	0.0902 (0.0408, 0.1162)	0.0954 (0.0723, 0.1120)	0.239	0.0452 (0.0215, 0.0538)	0.0313 (0.0222, 0.0796)	0.546
9-HOTrE	0.1312 (0.1073, 0.1768)	0.1272 (0.0981, 0.1650)	0.814	0.0947 (0.0796, 0.1522)	0.0704 (0.0440, 0.1571)	0.260
13-HOTrE	0.1139 (0.0619, 0.1661)	0.1108 (0.0842, 0.2063)	0.937	0.0935 (0.0705, 0.1284)	0.0641 (0.0492, 0.1386)	0.147
15-HETrE	0.0946 (0.0375, 0.1340)	0.1506 (0.1058, 0.2188)	0.060	0.0603 (0.0388, 0.1389)	0.0583 (0.0221, 0.1421)	0.260
11-12-15-TriHETrE	0.0612 (0.0167, 0.0907)	0.0444 (0.0299, 0.0849)	0.875	0.0230 (0.0153, 0.0333)	0.0191 (0.0083, 0.0520)	0.936
18:2-EA	1.6148 (0.7582, 2.2184)	1.7222 (1.2640, 1.9361)	0.754	1.5485 (0.9855, 2.3358)	1.1033 (0.5454, 2.6248)	0.494
2-18-2-glycerol	0.8660 (0.2956, 1.5054)	1.4598 (1.0419, 2.0804)	0.182	0.7212 (0.4748, 1.0588)	0.7975 (0.3649, 1.6530)	0.334
20-COOH-AA	0.2183 (0.1149, 0.3149)	0.2817 (0.1899, 0.3532)	0.695	0.1342 (0.1132, 0.1676)	0.1035 (0.0687, 0.2430)	0.601
5-HETE	0.1020 (0.0707, 0.1532)	0.1170 (0.0964, 0.1873)	0.158	0.0958 (0.0765, 0.1510)	0.0667 (0.0293, 0.1498)	0.469
8-HETE	0.2963 (0.1196, 0.5545)	0.3599 (0.2897, 0.6564)	0.158	0.1809 (0.0976, 0.3408)	0.1251 (0.0569, 0.4663)	0.136
9-HETE	0.0123 (0.0072, 0.0208)	0.0145 (0.0109, 0.0208)	0.347	0.0113 (0.0046, 0.0160)	0.0080 (0.0020, 0.0128)	0.277
11-HETE	0.1530 (0.1126, 0.2494)	0.2096 (0.1572, 0.3865)	0.084	0.1632 (0.0991, 0.2514)	0.1300 (0.0424, 0.2430)	0.717

12-HETE	4.0312 (0.9840, 6.6703)	8.2509 (0.8895, 19.4987)	0.084	1.8655 (0.5348, 3.1966)	1.5131 (0.2218, 4.4708)	0.629
15-HETE	0.0488 (0.0288, 0.0695)	0.0708 (0.0347, 0.1033)	0.583	0.0413 (0.0271, 0.1321)	0.0321 (0.0267, 0.0827)	0.376
16-HETE	0.0673 (0.0411, 0.1353)	0.1149 (0.0707, 0.1501)	0.136	0.0799 (0.0562, 0.1182)	0.0554 (0.0197, 0.1027)	0.227
14-15-diHETE	0.0229 (0.0135, 0.0431)	0.0283 (0.0246, 0.0395)	0.388	0.0198 (0.0152, 0.0270)	0.0112 (0.0058, 0.0233)	0.005
17-18-DiHETE	0.0836 (0.0236, 0.1539)	0.0851 (0.0589, 0.1159)	0.638	0.0477 (0.0314, 0.0780)	0.0374 (0.0256, 0.0607)	0.295
12-HHT	0.0441 (0.0232, 0.0705)	0.0674 (0.0212, 0.1602)	0.209	0.0429 (0.0154, 0.0673)	0.0257 (0.0138, 0.0568)	0.469
LXA_4	0.0093 (0.0049, 0.0145)	0.0144 (0.0080, 0.0177)	0.754	0.0084 (0.0041, 0.0110)	0.0059 (0.0020, 0.0123)	0.778
LXA_5	0.0024 (0.0015, 0.0056)	0.0036 (0.0018, 0.0059)	0.388	0.0022 (<0.0017, 0.0041)	0.0014 (<0.0015, 0.041)	0.778
LXB_4	0.0186 (0.0085, 0.0239)	0.0268 (0.0210, 0.0387)	0.084	0.0091 (00037, 0.0125)	0.0086 (0.0026, 0.0218)	0.904
LTB_4	0.1129 (0.0513, 0.2070)	0.1739 (0.0715, 0.2131)	0.638	0.0535 (0.0364, 0.0965)	0.0666 (0.0281, 0.1347)	0.546
LTB_5	0.0090 (0.0026, 0.0157)	0.0098 (0.0056, 0.0185)	0.480	0.0048 (0.0025, 0.0085)	0.0056 (0.0017, 0.0164)	0.778
LTC_4	0.0024 (0.0014, 0.0074)	0.0059 (0.0035, 0.0122)	0.610	0.0045 (0.0013, 0.0059)	0.0026 (0.0013, 0.0069)	0.748
LTD_4	0.0322 (0.0155, 0.0994)	0.0362 (0.0254, 0.0790)	0.480	0.0261 (0.0126, 0.0529)	0.0169 (0.0114, 0.0377)	0.601
LTE_4	0.0508 (0.0162, 0.0773)	0.0564 (0.0408, 0.0804)	0.638	0.0312 (0.0227, 0.0661)	0.0370 (0.0144, 0.0549)	0.520
u-LTC_4	0.0029 (0.0019, 0.0057)	0.0034 (0.0022, 0.0059)	0.937	0.0010 (<0.0015, 0.0029)	<0.0019 (<0.0013, 0.0026)	0.879
u-LTD_4	0.0047 (0.0023, 0.0059)	0.0069 (0.0047, 0.0149)	0.099	0.0021 (0.0016, 0.0036)	0.0027 (0.0012, 0.0066)	0.546
20-OH-LTB_4	0.0055 (0.0021, 0.0126)	0.0119 (0.0091, 0.0132)	0.136	0.0049 (0.0015, 0.0072)	0.0031 (0.0010, 0.0047)	0.421
20-COOH-LTB_4	0.0281 (0.0187, 0.0400)	0.0290 (0.0250, 0.0419)	0.272	0.0152 (0.0070, 0.0341)	0.0228 (0.0073, 0.0297)	0.778
HXA_3	0.3172 (0.1153, 0.4759)	0.3828 (0.1437, 0.7031)	0.583	0.0774 (0.0305, 0.4059)	0.0666 (0.0374, 0.2248)	0.629
5-oxo-ETE	0.0266 (0.0196, 0.0371)	0.0308 (0.0212, 0.0412)	0.480	0.0269 (0.0161, 0.0455)	0.0122 (0.0064, 0.0398)	0.107
15-oxo-ETE	0.0525 (0.0241, 0.0678)	0.0393 (0.0289, 0.0795)	0.638	0.0501 (0.0256, 0.0721)	0.0393 (0.0175, 0.0798)	0.658
11-12-EET	0.1272 (0.1114, 0.1695)	0.1379 (0.0834, 0.2655)	0.136	0.1262 (0.0883, 0.1880)	0.0785 (0.0290, 0.2042)	0.147
14-15-EET	0.0430 (0.0300, 0.0858)	0.0698 (0.0360, 0.1111)	0.480	0.0335 (0.0150, 0.0774)	0.0370 (0.0103, 0.0728)	0.936
5-6-DHET	0.0110 (0.0084, 0.0225)	0.0099 (0.0062, 0.0173)	0.158	0.0081 (0.0059, 0.0130)	0.0092 (0.0044, 0.0156)	0.968
11-12-DHET	0.0119 (0.0062, 0.0187)	0.0183 (0.0114, 0.0331)	0.136	0.0060 (0.0049, 0.0122)	0.0070 (0.0019, 0.0114)	0.711
14-15-DHET	0.0262 (0.0093, 0.0355)	0.0188 (0.0101, 0.0348)	0.388	0.0155 (0.0089, 0.0244)	0.0117 (0.0030, 0.0257)	0.494
PGB_2	0.0114 (0.0056, 0.0146)	0.0140 (0.0091, 0.0184)	1.000	0.0084 (0.0042, 0.0111)	0.0076 (0.0027, 0.0154)	0.469
PGD_1	0.0147 (0.0080, 0.0275)	0.0178 (0.0147, 0.0241)	0.583	0.0100 (0.0044, 0.0167)	0.0076 (0.0026, 0.0213)	0.445

PGD_2	0.0100 (0.0047, 0.0197)	0.0157 (0.0073, 0.0206)	0.158	0.0118 (0.0040, 0.0193)	0.0046 (0.0018, 0.0100)	0.184
PGD_3	0.0063 (0.0044, 0.0102)	0.0070 (0.0035, 0.0097)	0.388	0.0034 (0.0016, 0.0072)	0.0033 (0.0018, 0.0075)	0.469
PGE_1	0.0115 (0.0083, 0.0153)	0.0153 (0.0080, 0.0177)	0.875	0.0091 (0.0045, 0.0175)	0.0061 (0.0040, 0.0142)	0.936
PGE_2	0.0348 (0.0269, 0.0632)	0.0531 (0.0245, 0.1518)	0.071	0.0669 (0.0168, 0.1133)	0.0155 (0.0025, 0.0735)	0.243
PGE_3	0.0077 (0.0044, 0.0106)	0.0094 (0.0060, 0.0147)	0.158	0.0041 (0.0024, 0.0072)	0.0038 (0.0013, 0.0103)	0.445
PGF_{1a}	0.1530 (0.0629, 0.2268)	0.1820 (0.1280, 0.2819)	0.158	0.0891 (0.0211, 0.1178)	0.0760 (0.0171, 0.1607)	0.546
PGF_{2a}	0.0280 (0.0110, 0.0773)	0.0367 (0.0234, 0.0657)	0.875	0.0492 (0.0233, 0.0840)	0.0458 (0.0216, 0.0921)	0.968
PGF_{3a}	0.0386 (0.0214, 0.0703)	0.0529 (0.0490, 0.0930)	0.388	0.0350 (0.0243, 0.4091)	0.0316 (0.0113, 0.1043)	0.717
PGJ_2	0.0111 (0.0052, 0.0216)	0.0133 (0.0075, 0.0272)	0.875	0.0056 (0.0022, 0.0073)	0.0047 (0.0019, 0.0141)	0.825
dhk-PGD_2	0.0078 (0.0031, 0.0129)	0.0094 (0.0052, 0.0115)	0.638	0.0048 (0.0024, 0.0085)	0.0029 (0.0012, 0.0084)	0.717
dhk-PGE_2	0.0106 (0.0048, 0.0194)	0.0194 (0.0106, 0.0361)	0.050	<0.0016 (0.0041, 0.0119)	0.0064 (0.0015, 0.0185)	0.748
dhk-PGF_{2a}	0.0173 (0.0072, 0.0215)	0.0173 (0.0122, 0.0267)	0.754	0.0102 (0.0069, 0.0125)	0.0098 (0.0030, 0.0123)	0.421
8-i-PGF_{2a}	0.0978 (0.0239, 0.1737)	0.8595 (0.1139, 1.0917)	0.008	0.0376 (0.0172, 0.0897)	0.0254 (0.0168, 0.1612)	0.546
2-3-d6k-PGF_{1a}	0.0102 (0.0029, 0.0189)	0.0142 (0.0064, 0.0181)	0.638	0.0057 (0.0015, 0.0107)	0.0081 (0.0020, 0.0171)	0.444
6-k-PGF_{1a}	0.2098 (0.1603, 0.4251)	0.3426 (0.2201, 0.4096)	0.272	0.1340 (0.0359, 0.1833)	0.0615 (0.0181, 0.1409)	0.243
delta12-PGJ_2	0.0042 (0.0030, 0.0070)	0.0090 (0.0048, 0.0125)	0.099	0.0031 (0.0020, 0.0061)	0.0030 (<0.0015, 0.0060)	0.314
TxB_1	0.0028 (0.0013, 0.0059)	0.0034 (0.0024, 0.0102)	0.388	0.0022 (<0.0018, 0.0035)	0.0018 (<0.0019, 0.0107)	0.763
TXB_2	0.2741 (0.0921, 0.6998)	0.5027 (0.2243, 2.5349)	0.099	0.1914 (0.0847, 0.7305)	0.1409 (0.0397, 0.4682)	0.520
TXB_3	0.0025 (0.0020, 0.0045)	0.0089 (0.0036, 0.0125)	0.084	0.0041 (0.0021, 0.0090)	0.0036 (0.0011, 0.0067)	0.445
11-dh-TXB_2	0.0174 (0.0091, 0.0242)	0.0246 (0.0101, 0.0584)	0.583	0.0231 (0.0069, 0.0517)	0.0094 (0.0050, 0.0377)	0.494
11-dh-TXB_3	0.0066 (0.0046, 0.0079)	0.0080 (0.0057, 0.0124)	0.239	0.0034 (0.0015, 0.0060)	0.0033 (0.0014, 0.0061)	0.968
20:4-EA	0.2436 (0.1115, 0.3094)	0.2266 (0.1512, 0.4379)	0.480	0.4816 (0.1892, 0.7718)	0.3890 (0.2342, 0.7455)	0.398
1-20:4-glycerol	1.8475 (0.4981, 2.9603)	2.0352 (1.0088, 2.9093)	0.388	1.2297 (0.7029, 1.7755)	0.9613 (0.3779, 1.7139)	0.601
2-20:4-glycerol	4.8049 (1.4355, 9.9953)	6.6573 (3.3716, 9.5528)	1.000	4.2739 (1.3175, 5.6107)	2.6433 (1.4625, 5.3830)	0.520
5-HEPE	0.0217 (0.0084, 0.0537)	0.0268 (0.0179, 0.0617)	0.308	0.0177 (0.0124, 0.0437)	0.0173 (0.0114, 0.0371)	0.872
12-HEPE	0.3767 (0.0817, 0.6987)	0.9233 (0.1032, 2.3415)	0.347	0.1807 (0.0566, 0.3779)	0.1447 (0.0365, 0.3470)	0.936
15-HEPE	0.0342 (0.0112, 0.0601)	0.0515 (0.0237, 0.0758)	0.099	0.0118 (0.0096, 0.0404)	0.0171 (0.0050, 0.0414)	0.968
RvE_1	0.0044 (0.0021, 0.0076)	0.0043 (0.0025, 0.0068)	0.638	0.0019 (<0.0018, 0.0035)	0.0022 (<0.0016, 0.0055)	0.459

RvE_2	0.2399 (0.0705, 0.3529)	0.3483 (0.1664, 0.4269)	0.754	0.1195 (0.0914, 0.0035)	0.1380 (0.0693, 0.0055)	0.717
RvE_3	0.0693 (0.0254, 0.1178)	0.0770 (0.0272, 0.1978)	0.530	0.0221 (0.0074, 0.0596)	0.0227 (0.0063, 0.0527)	0.494
20:5-EA	0.0318 (0.0137, 0.0520)	0.0824 (0.0319, 0.1045)	0.060	0.0370 (0.0214, 0.0684)	0.0344 (0.0191, 0.0708)	0.601
2-20:5-glycerol	0.1384 (0.1084, 0.2917)	0.3048 (0.2102, 0.5179)	0.084	0.1065 (0.0627, 0.1663)	0.0818 (0.0420, 0.1666)	0.376
19-20-DiHDPA	0.0132 (0.0064, 0.0225)	0.0149 (0.0085, 0.0237)	0.695	0.0094 (0.0058, 0.0144)	0.0063 (0.0034, 0.0122)	0.117
22-5-EA	0.0455 (0.0205, 0.0901)	0.0724 (0.0518, 0.1352)	0.272	0.0623 (0.0291, 0.0918)	0.0374 (0.0187, 0.0831)	0.629
2-22:5-glycerol	0.1728 (0.0698, 0.2617)	0.3067 (0.1949, 0.3765)	0.117	0.1300 (0.0930, 0.2202)	0.1560 (0.0695, 0.2568)	0.687
4-HDoHE	0.0574 (0.0258, 0.0832)	0.0690 (0.0407, 0.0965)	0.239	0.0279 (0.0158, 0.0393)	0.0202 (0.0078, 0.0776)	0.968
7-HDoHE	0.0386 (0.0257, 0.0889)	0.0452 (0.0277, 0.1460)	0.638	0.0217 (0.0109, 0.0285)	0.0219 (0.0050, 0.0693)	0.717
8-HDoHE	0.0183 (0.0063, 0.0437)	0.0184 (0.0083, 0.0289)	0.695	0.0082 (0.0040, 0.0126)	0.0059 (0.021, 0.0156)	0.658
11-HDoHE	0.0428 (0.0261, 0.0957)	0.0744 (0.0401, 0.1003)	0.638	0.0174 (0.0103, 0.0484)	0.0235 (0.0040, 0.0263)	0.601
13-HDoHE	0.0096 (0.0037, 0.0236)	0.0145 (0.0083, 0.0307)	0.638	0.0082 (0.0027, 0.0115)	0.0071 (0.0023, 0.0106)	0.717
14-HDoHE	0.1636 (0.0881, 0.3301)	0.4337 (0.0803, 0.6612)	0.272	0.0785 (0.0343, 0.1495)	0.0623 (0.0104, 0.1376)	0.546
16-HDoHE	0.0303 (0.0195, 0.1383)	0.0764 (0.0455, 0.1355)	0.583	0.0266 (0.0115, 0.0321)	0.0205 (0.0080, 0.0300)	0.117
17-HDoHE	0.0369 (0.0148, 0.0584)	0.0392 (0.0219, 0.0754)	0.754	0.0118 (0.0099, 0.0280)	0.0151 (0.0042, 0.0205)	0.968
20-HDoHE	0.0636 (0.0242, 0.0876)	0.0585 (0.0462, 0.0734)	1.000	0.0296 (0.0169, 0.0444)	0.0257 (0.0134, 0.0541)	0.469
12-13-diHOME	1.4099 (0.6284, 2.4354)	1.7981 (1.3803, 2.7901)	0.308	1.0937 (0.9215, 1.4815)	0.8065 (0.2918, 1.7152)	0.334
RvD_1	0.0109 (0.0034, 0.0173)	0.0130 (0.0052, 0.0188)	0.480	0.0070 (0.0020, 0.0110)	0.0027 (0.0013, 0.0104)	0.778
RvD_2	0.1193 (0.0846, 0.3872)	0.2510 (0.1140, 0.6390)	0.099	0.0597 (0.0206, 0.0800)	0.0361 (0.0160, 0.1057)	0.658
22:6-EA	0.2049 (0.0895, 0.3018)	0.4089 (0.2955, 0.5804)	0.239	0.1813 (0.1194, 0.3703)	0.1941 (0.0850, 0.4281)	0.872
2-22:6-glycerol	0.5245 (0.1738, 1.1751)	0.9371 (0.5712, 1.4828)	0.239	0.3466 (0.1909, 0.5593)	0.2704 (0.0944, 0.5136)	0.643
14:0-EA	1.6634 (1.2802, 7.2252)	1.2618 (0.9603, 2.4326)	0.117	0.9533 (0.6968, 1.3002)	0.8891 (0.3126, 1.4195)	0.212
16:0-EA	9.0451 (5.1380, 12.7021)	8.1503 (5.6006, 14.2092)	0.695	8.5930 (5.1296, 14.0019)	7.1142 (2.8532, 14.6969)	0.314
16:1-EA	2.1235 (0.6725, 2.6774)	1.0445 (0.8627, 1.5585)	0.182	1.5780 (0.8039, 2.6871)	1.1327 (0.4707, 2.3156)	0.084
18:0-EA	1.4173 (0.9545, 2.1391)	1.7665 (1.2675, 2.2492)	0.695	1.5962 (0.6403, 2.7573)	1.4964 (0.6083, 2.6454)	0.936
18:1-EA	4.4791 (2.3446, 6.7843)	4.3754 (2.9462, 7.0181)	0.754	4.4286 (2.9738, 8.0824)	3.8172 (2.1504, 9.7444)	0.355
18:3a-EA	0.0904 (0.0455, 0.1254)	0.0978 (0.0677, 0.1308)	0.754	0.0981 (0.0454, 0.1348)	0.0511 (0.0321, 0.1386)	0.355
2-16:0-glycerol	0.3388 (0.1760, 1.1089)	0.6772 (0.2736, 2.5190)	0.272	0.1186 (0.0727, 0.1684)	0.1559 (0.0480, 0.2116)	0.904

2-16:1-glycerol	0.3839 (0.0832, 0.6054)	0.6174 (0.4125, 1.1211)	0.272	0.3393 (0.2614, 0.5136)	0.4941 (0.1816, 0.6401)	0.658
2-18:1-glycerol	2.5426 (0.4872, 4.4029)	4.0765 (2.2316, 5.8345)	0.695	1.6495 (1.0042, 4.3391)	3.0008 (0.8103, 4.1943)	0.968

[1] Median (25^{th}, 75^{th} percentile).

[2] *P* value obtained using the Wilcoxon signed ranks test.

Significance deemed $P \leq 0.05$.

Appendix J Effect of 12-week corn oil intervention on the absolute concentration (ng/g) of fatty acid metabolites in scWAT of normal weight and metabolically healthy obese individuals

	Normal Weight (ng/g)			Obese (ng/g)		
Fatty acid metabolite	Study entry[1]	Post Corn oil[1]	*P*[2]	Study entry[1]	Post Corn oil[1]	*P*[2]
5-HETE	6.258 (5.5410, 6.2580)	6.136 (5.5980, 6.1360)	0.441	5.61 (5.4420, 6.3670)	6.02 (5.6140, 6.6260)	0.184
8-HETE	3.837 (3.4620, 3.8370)	3.942 (3.5190, 3.9420)	0.767	3.52 (3.3620, 3.9110)	3.724 (3.4890, 4.1050)	0.147
12-HETE	10.762 (2.5170, 10.7620)	68.236 (3.5310, 68.2360)	0.214	1.264 (0.3230, 3.2310)	1.153 (0.1310, 3.4010)	0.778
17-18-DiHETE	0.641 (0.0940, 0.6410)	0.281 (0.0640, 0.2810)	0.917	0.209 (0.1210, 0.4870)	0.1885 (0.1290, 0.2310)	0.182
5-oxo-ETE	0.078 (0.0410, 0.0782)	0.102 (0.0460, 0.1020)	0.236	0.059 (0.0450, 0.0820)	0.055 (0.0400, 0.0880)	0.528
PGE_2	0.127 (0.0360, 0.1270)	0.204 (0.0130, 0.2040)	0.655	0.03 (0.0260, 0.0960)	0.033 (0.0310, 0.0360)	1.000
PGF_{2a}	0.137 (0.0280, 0.1370)	0.531 (0.0200, 0.5310)	0.575	0.373 (0.2430, 0.4710)	0.245 (0.1800, 0.3930)	0.184
6-keto-PGF_{1a}	0.396 (0.1680, 0.3960)	0.521 (0.1550, 0.5210)	0.173	0.204 (0.1220, 0.3480)	0.202 (0.1220, 0.3410)	0.744
TXB2	1.37 (0.5000, 1.3700)	12.366 (0.9940, 12.3660)	0.097	0.419 (0.3460, 0.6070)	0.394 (0.3520, 0.6190)	0.702
11-dh-TXB_2	0.092 (0.0530, 0.0920)	0.289 (0.0550, 0.2890)	0.594	0.054 (0.0490, 0.0900)	0.058 (0.0540, 0.0770)	1.000
LXB_4	0.209 (0.1600, 0.2090)	0.191 (0.1580, 0.1910)	0.407	0.157 (0.1400, 0.1730)	0.171 (0.1490, 0.1780)	0.384
AEA	0.773 (0.0020, 0.7730)	1.165 (0.2300, 1.1650)	0.214	1.78 (0.5170, 2.4300)	1.7565 (0.5870, 2.2020)	0.711
2-AG	465.82 (348.6530, 1671.6430)	1748.34 (1374.0250, 1959.1110)	0.066	1141.409 (456.6160, 1631.3290)	1584.73 (523.8450, 2015.4510)	0.421
EPEA	0.253 (0.0620, 0.2530)	0.201 (0.0600, 0.2010)	0.878	0.295 (0.0980, 0.3530)	0.172 (0.1060, 0.3260)	0.334
4-HDoHE	0.154 (0.0580, 0.1540)	0.13 (0.0590, 0.1300)	0.889	0.07 (0.0520, 0.0900)	0.066 (0.0520, 0.0820)	0.277
8-HDoHE	0.164 (0.0760, 0.1640)	0.161 (0.0630, 0.1610)	0.594	0.074 (0.0570, 0.1000)	0.073 (0.0600, 0.0930)	0.760
11-HDoHE	0.197 (0.0460, 0.1970)	0.301 (0.0590, 0.3010)	0.859	0.037 (0.0230, 0.0540)	0.033 (0.0200, 0.0490)	1.000
14-HDoHE	1.658 (0.4550, 1.6580)	4.876 (0.3480, 4.8760)	0.575	0.192 (0.0940, 0.6410)	0.226 (0.0465, 0.4375)	0.674
17-HDHA	0.0523 (0.0135, 0.0523)	0.6568 (0.0234, 0.6568)	0.500	0.0299 (0.0101, 0.1561)	0.0380 (0.0160, 0.0745)	0.655

RvD_1	0.165 (0.1500, 0.1650)	0.163 (0.1520, 0.1630)	0.262	0.156 (0.1460, 0.1730)	0.165 (0.1530, 0.1830)	0.093
RvD_2	0.297 (0.2040, 0.2970)	0.357 (0.2590, 0.3570)	0.406	0.21 (0.1760, 0.2170)	0.198 (0.1760, 0.2310)	0.601
DHEA	0.711 (0.1280, 0.7110)	0.741 (0.3040, 0.7410)	0.441	0.584 (0.2350, 0.7310)	0.488 (0.2690, 0.7900)	0.809
18:1-EA	34.066 (6.0010, 34.0660)	29.188 (6.1900, 29.1880)	0.314	30.779 (11.0230, 44.0420)	34.086 (10.7160, 40.0020)	0.717

[1] Median (25^{th}, 75^{th} percentile).

[2] *P* value obtained using the Wilcoxon signed ranks test.

Significance deemed $P \leq 0.05$.[2]

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Appendix K **Full list of genes differentially expressed in scWAT of metabolically healthy obese individuals in comparison to normal weight at study entry (meeting Log2 FC ≥ 2, *P* ≤ 0.05, and FDR ≤0.05)**

Up-regulated				
hgnc_symbol	**Log2 FC**	**FC**	***P***	**FDR**
EGFL6	5.44	43.41	<0.001	<0.001
MMP7	5.41	42.52	<0.001	<0.001
CCL22	4.71	26.17	<0.001	<0.001
MMP9	4.03	16.34	<0.001	<0.001
DCSTAMP	3.91	15.03	<0.001	<0.001
URAD	3.9	14.93	<0.001	<0.001
LINC01010	3.88	14.72	<0.001	<0.001
AADACL3	3.61	12.21	<0.001	<0.001
CHIT1	3.6	12.13	<0.001	<0.001
SPP1	3.57	11.88	<0.001	<0.001
PLA2G7	3.54	11.63	<0.001	<0.001
STMN2	3.45	10.93	<0.001	<0.001
TREM2	3.4	10.56	<0.001	<0.001
SDS	3.37	10.34	<0.001	<0.001
KRT16	3.34	10.13	<0.001	<0.001
UNC13C	3.33	10.06	<0.001	<0.001
CHI3L1	3.3	9.85	<0.001	<0.001

Down-regulated				
hgnc_symbol	**Log2 FC**	**FC**	***P***	**FDR**
SLC27A2	-3.42	-10.70	<0.001	<0.001
RORB	-3.30	-9.85	<0.001	<0.001
SPX	-3.26	-9.58	<0.001	<0.001
CA3	-3.25	-9.51	<0.001	<0.001
CECR2	-2.71	-6.54	<0.001	<0.001
WDR86-AS1	-2.56	-5.90	<0.001	<0.001
KCNU1	-2.48	-5.58	<0.001	<0.001
ASPG	-2.47	-5.54	0.002	0.013
BMP3	-2.45	-5.46	<0.001	<0.001
RASSF6	-2.42	-5.35	<0.001	<0.001
TTC36	-2.41	-5.31	<0.001	<0.001
GJC3	-2.31	-4.96	<0.001	<0.001
KCTD8	-2.28	-4.86	<0.001	<0.001
RPS28	-2.19	-4.56	<0.001	<0.001
SCEL	-2.17	-4.50	<0.001	0.004
TSHR	-2.17	-4.50	<0.001	<0.001
GFRA3	-2.07	-4.20	<0.001	<0.001

TM4SF19	3.2	9.19	<0.001	<0.001
COL11A1	3.07	8.40	<0.001	<0.001
IGHV5-51	3.04	8.22	<0.001	<0.001
IGLV3-21	3.03	8.17	<0.001	<0.001
SLC28A3	3	8.00	<0.001	<0.001
IL1RN	2.97	7.84	<0.001	<0.001
LAMP3	2.92	7.57	<0.001	<0.001
SULT1C2	2.8	6.96	<0.001	0.003
CCL18	2.8	6.96	<0.001	<0.001
UBE2QL1	2.79	6.92	<0.001	<0.001
IGHV2-5	2.74	6.68	<0.001	0.001
IGLV2-11	2.72	6.59	<0.001	0.007
KIF18B	2.72	6.59	<0.001	<0.001
ABCC3	2.7	6.50	<0.001	<0.001
DNASE2B	2.69	6.45	<0.001	<0.001
FCGBP	2.66	6.32	<0.001	<0.001
TIFAB	2.63	6.19	<0.001	<0.001
HTRA4	2.58	5.98	<0.001	<0.001
IL4I1	2.58	5.98	<0.001	<0.001
GDA	2.54	5.82	<0.001	0.004
HS3ST2	2.52	5.74	<0.001	<0.001
MKI67	2.51	5.70	<0.001	<0.001
IGHG2	2.46	5.50	<0.001	<0.001
TCF23	2.45	5.46	<0.001	<0.001
IGLV1-40	2.41	5.31	<0.001	0.004
CLEC5A	2.41	5.31	<0.001	<0.001
SLC22A12	2.4	5.28	<0.001	0.005

NDRG4	-2.02	-4.06	<0.001	<0.001
RASL10B	-2.00	-4.00	<0.001	<0.001
AZGP1	-1.99	-3.97	<0.001	<0.001
CSDC2	-1.94	-3.84	<0.001	<0.001
LINC01612	-1.93	-3.81	<0.001	<0.001
COL6A6	-1.86	-3.63	<0.001	<0.001
PCSK2	-1.83	-3.56	<0.001	<0.001
MAB21L1	-1.82	-3.53	<0.001	<0.001
CIDEA	-1.82	-3.53	<0.001	<0.001
CHODL	-1.81	-3.51	<0.001	<0.001
C6	-1.80	-3.48	<0.001	<0.001
LINC01230	-1.77	-3.41	<0.001	<0.001
LEFTY2	-1.76	-3.39	<0.001	<0.001
CERS3-AS1	-1.71	-3.27	<0.001	0.001
CPNE4	-1.71	-3.27	<0.001	<0.001
DNER	-1.69	-3.23	<0.001	<0.001
LINC00284	-1.67	-3.18	0.001	0.005
LDHC	-1.67	-3.18	0.003	0.018
USP32P1	-1.63	-3.10	0.012	0.053
WDR86	-1.62	-3.07	<0.001	<0.001
NRXN1	-1.61	-3.05	<0.001	<0.001
SPTB	-1.60	-3.03	<0.001	<0.001
ABCG8	-1.59	-3.01	<0.001	0.004
DLGAP2	-1.58	-2.99	<0.001	<0.001
NCAM2	-1.58	-2.99	<0.001	<0.001
GPAT3	-1.56	-2.95	<0.001	<0.001
BMP7	-1.55	-2.93	0.002	0.013

COMP	2.39	5.24	<0.001	0.027
P2RX6	2.38	5.21	<0.001	<0.001
TOP2A	2.38	5.21	<0.001	<0.001
RGS1	2.37	5.17	<0.001	<0.001
ANLN	2.37	5.17	<0.001	<0.001
TMEM155	2.35	5.10	<0.001	<0.001
EGR2	2.35	5.10	<0.001	<0.001
FOSL1	2.33	5.03	<0.001	<0.001
TNC	2.32	4.99	<0.001	<0.001
IGHG3	2.31	4.96	<0.001	0.001
CDC20	2.29	4.89	<0.001	<0.001
FCGR1A	2.28	4.86	<0.001	<0.001
CCL19	2.27	4.82	<0.001	<0.001
IGHV3-21	2.26	4.79	<0.001	<0.001
LINC00184	2.25	4.76	<0.001	<0.001
TEX26	2.25	4.76	<0.001	<0.001
ASPM	2.25	4.76	<0.001	<0.001
CDCP1	2.24	4.72	<0.001	<0.001
MYRFL	2.21	4.63	<0.001	<0.001
CHI3L2	2.21	4.63	<0.001	<0.001
BUB1B	2.2	4.59	<0.001	<0.001
IGHV3-15	2.2	4.59	<0.001	<0.001
TPSD1	2.17	4.50	<0.001	0.020
LINC00601	2.17	4.50	<0.001	<0.001
COL4A2-AS2	2.17	4.50	<0.001	<0.001
IGLV2-8	2.15	4.44	<0.001	0.001
C1orf168	2.14	4.41	<0.001	0.003

AADACL2	-1.55	-2.93	0.009	0.042
FOXN4	-1.55	-2.93	<0.001	<0.001
ALDH1L1-AS2	-1.55	-2.93	<0.001	<0.001
SYT17	-1.55	-2.93	<0.001	<0.001
DKK1	-1.53	-2.89	0.009	0.041
MOV10L1	-1.53	-2.89	<0.001	<0.001
ADH1A	-1.52	-2.87	<0.001	<0.001
XKR4	-1.52	-2.87	<0.001	<0.001
WNT3	-1.49	-2.81	<0.001	<0.001
EYA1	-1.48	-2.79	<0.001	0.002
PGM5-AS1	-1.48	-2.79	<0.001	<0.001
TRIM55	-1.47	-2.77	0.002	0.014
MAL	-1.46	-2.75	<0.001	<0.001
DUOX2	-1.45	-2.73	<0.001	0.003
TMEM52	-1.45	-2.73	<0.001	<0.001
GABRD	-1.44	-2.71	<0.001	<0.001
AMN	-1.43	-2.69	<0.001	<0.001
RPL27AP	-1.42	-2.68	0.001	0.006
SGCA	-1.42	-2.68	0.005	0.027
ADH1B	-1.41	-2.66	<0.001	<0.001
MKX	-1.40	-2.64	<0.001	0.002
ALPK3	-1.40	-2.64	<0.001	<0.001
DUOXA1	-1.39	-2.62	0.002	0.012
C19orf33	-1.39	-2.62	<0.001	<0.001
FHOD3	-1.38	-2.60	<0.001	0.002
CSRP2	-1.38	-2.60	<0.001	<0.001
CYP4F29P	-1.37	-2.58	0.001	0.006

CD300E	2.14	4.41	<0.001	<0.001
GAL	2.13	4.38	<0.001	0.001
IGKV1-5	2.13	4.38	<0.001	0.002
SLAMF8	2.13	4.38	<0.001	<0.001
IGHA2	2.13	4.38	<0.001	<0.001
LAMC3	2.13	4.38	<0.001	<0.001
PLEK2	2.13	4.38	<0.001	<0.001
CD1E	2.13	4.38	<0.001	<0.001
IGLV3-1	2.12	4.35	<0.001	0.014
CCL3	2.12	4.35	<0.001	<0.001
LIPG	2.12	4.35	<0.001	<0.001
IGHV4-39	2.11	4.32	<0.001	0.022
AOC1	2.11	4.32	<0.001	<0.001
PRND	2.1	4.29	<0.001	<0.001
IGHV3-23	2.09	4.26	<0.001	<0.001
IGLC3	2.08	4.23	<0.001	0.005
KCNK13	2.07	4.20	<0.001	<0.001
SIGLEC15	2.07	4.20	<0.001	<0.001
IGHG1	2.06	4.17	<0.001	0.005
MXRA5Y	2.06	4.17	<0.001	0.018
FAM84A	2.06	4.17	<0.001	<0.001
FCGR1B	2.04	4.11	<0.001	<0.001
AKR1B15	2.03	4.08	<0.001	<0.001
RASGRF1	2.03	4.08	<0.001	<0.001
SLC6A12	2.02	4.06	<0.001	<0.001
FAM111B	2.02	4.06	<0.001	<0.001
MATK	2.02	4.06	<0.001	<0.001

CASQ2	-1.37	-2.58	<0.001	0.003
C8orf89	-1.37	-2.58	<0.001	0.003
CNTD2	-1.37	-2.58	<0.001	<0.001
MYOC	-1.36	-2.57	<0.001	<0.001
ALK	-1.34	-2.53	<0.001	0.001
STOX1	-1.34	-2.53	<0.001	<0.001
MIR181A2HG	-1.34	-2.53	<0.001	<0.001
IZUMO4	-1.34	-2.53	<0.001	<0.001
DMRT2	-1.33	-2.51	<0.001	<0.001
ARHGEF16	-1.32	-2.50	<0.001	<0.001
ZBED9	-1.31	-2.48	<0.001	0.001
PGM5P4	-1.30	-2.46	<0.001	<0.001
C14orf39	-1.30	-2.46	<0.001	<0.001
CSPG5	-1.29	-2.45	<0.001	<0.001
CKB	-1.29	-2.45	<0.001	<0.001
TMEM27	-1.28	-2.43	<0.001	<0.001
IGSF11	-1.27	-2.41	0.011	0.047
FCN2	-1.27	-2.41	<0.001	<0.001
CEBPA-AS1	-1.25	-2.38	0.036	0.116
SOX9-AS1	-1.25	-2.38	<0.001	0.002
CCDC144A	-1.24	-2.36	0.007	0.034
KRT222	-1.23	-2.35	<0.001	0.002
ARF4-AS1	-1.21	-2.31	<0.001	0.001
LINC00853	-1.21	-2.31	<0.001	0.004
CYB5A	-1.21	-2.31	<0.001	<0.001
PDE4C	-1.19	-2.28	<0.001	<0.001
TMEM25	-1.19	-2.28	<0.001	<0.001

SERPINE1	1.99	3.97	<0.001	<0.001
BIRC5	1.99	3.97	<0.001	<0.001
MXRA5	1.99	3.97	<0.001	<0.001
CD300LB	1.97	3.92	<0.001	<0.001
CKAP2L	1.96	3.89	<0.001	0.002
PCSK1	1.96	3.89	<0.001	<0.001
ST14	1.96	3.89	<0.001	<0.001
TREML1	1.94	3.84	<0.001	<0.001
CLEC12A	1.93	3.81	<0.001	<0.001
IFI30	1.93	3.81	<0.001	<0.001
DLGAP5	1.93	3.81	<0.001	<0.001
SPTA1	1.93	3.81	<0.001	<0.001
MYCL	1.92	3.78	<0.001	<0.001
CCR5	1.91	3.76	<0.001	<0.001
HLA-DQB1-AS1	1.9	3.73	<0.001	0.013
IGHA1	1.9	3.73	<0.001	<0.001
ITGAD	1.9	3.73	<0.001	<0.001
SPOCD1	1.9	3.73	<0.001	<0.001
SPIB	1.9	3.73	<0.001	<0.001
CEP55	1.88	3.68	<0.001	<0.001
SCIN	1.88	3.68	<0.001	<0.001
SPINK5	1.87	3.66	<0.001	0.002
IGHGP	1.87	3.66	<0.001	0.047
IGKV3-20	1.87	3.66	<0.001	<0.001
HJURP	1.87	3.66	<0.001	<0.001
ITGAX	1.87	3.66	<0.001	<0.001
KRT7	1.86	3.63	<0.001	<0.001

SULT4A1	-1.18	-2.27	0.002	0.011
FAM156A	-1.18	-2.27	0.003	0.016
DACT2	-1.18	-2.27	0.010	0.046
RPS27	-1.18	-2.27	<0.001	<0.001
EIF4EBP1	-1.18	-2.27	<0.001	<0.001
PPP1R16A	-1.18	-2.27	<0.001	<0.001
PEX5L	-1.17	-2.25	0.003	0.016
TBX4	-1.17	-2.25	0.004	0.025
PXDNL	-1.17	-2.25	0.009	0.043
HRCT1	-1.17	-2.25	<0.001	0.002
AACS	-1.17	-2.25	<0.001	<0.001
FADS1	-1.17	-2.25	<0.001	<0.001
RPL37P6	-1.17	-2.25	<0.001	<0.001
RORC	-1.17	-2.25	<0.001	<0.001
S100A1	-1.16	-2.23	0.012	0.052
ZNF334	-1.16	-2.23	<0.001	<0.001
SLC22A25	-1.16	-2.23	<0.001	<0.001
SCN7A	-1.16	-2.23	<0.001	<0.001
HACD1	-1.16	-2.23	<0.001	<0.001
RARB	-1.16	-2.23	<0.001	<0.001
RPL41P2	-1.15	-2.22	0.001	0.007
GRIK3	-1.15	-2.22	0.002	0.014
ARL17B	-1.15	-2.22	0.003	0.019
SCN2B	-1.15	-2.22	0.007	0.035
PLD6	-1.15	-2.22	<0.001	0.001
ADSSL1	-1.15	-2.22	<0.001	<0.001
TRDC	-1.15	-2.22	<0.001	<0.001

MMP12	1.85	3.61	<0.001	<0.001
LGALS2	1.85	3.61	<0.001	<0.001
IGKV4-1	1.84	3.58	<0.001	0.022
HLA-DQA1	1.84	3.58	<0.001	<0.001
TTK	1.84	3.58	<0.001	<0.001
ZNF804A	1.83	3.56	<0.001	<0.001
CYP2S1	1.82	3.53	<0.001	<0.001
CLEC12B	1.82	3.53	<0.001	<0.001
IGKC	1.81	3.51	<0.001	0.001
HLA-DQA2	1.81	3.51	<0.001	0.041
CCR2	1.81	3.51	<0.001	<0.001
IGHM	1.81	3.51	<0.001	<0.001
TCHH	1.8	3.48	<0.001	<0.001
RRM2	1.8	3.48	<0.001	<0.001
GRIN2B	1.8	3.48	<0.001	<0.001
KCNJ1	1.8	3.48	<0.001	<0.001
KIAA0125	1.8	3.48	<0.001	<0.001
LBP	1.79	3.46	<0.001	<0.001
LINC00968	1.79	3.46	<0.001	<0.001
JCHAIN	1.78	3.43	<0.001	0.001
BCL11A	1.78	3.43	<0.001	<0.001
CXCL1	1.77	3.41	<0.001	0.001
UBE2C	1.75	3.36	<0.001	0.001
CLIC6	1.75	3.36	<0.001	0.024
UCHL1	1.75	3.36	<0.001	<0.001
CECR1	1.75	3.36	<0.001	<0.001
CD1C	1.75	3.36	<0.001	<0.001

ALB	-1.14	-2.20	0.001	0.007
APOL4	-1.14	-2.20	0.002	0.015
PCK1	-1.13	-2.19	0.001	0.005
AK5	-1.13	-2.19	0.005	0.028
ZP1	-1.13	-2.19	<0.001	<0.001
ANKRD53	-1.13	-2.19	<0.001	<0.001
GLUL	-1.13	-2.19	<0.001	<0.001
NAALAD2	-1.13	-2.19	<0.001	<0.001
SLC25A21	-1.13	-2.19	<0.001	<0.001
SMIM1	-1.13	-2.19	<0.001	<0.001
NTRK3	-1.12	-2.17	0.001	0.010
PLIN5	-1.12	-2.17	<0.001	<0.001
ANXA3	-1.12	-2.17	<0.001	<0.001
PPP1R14A	-1.12	-2.17	<0.001	<0.001
OR7E13P	-1.11	-2.16	0.001	0.007
GRIK4	-1.11	-2.16	0.002	0.015
GCK	-1.11	-2.16	0.005	0.028
S100P	-1.10	-2.14	0.004	0.024
C11orf53	-1.10	-2.14	0.006	0.032
RPS27P23	-1.10	-2.14	<0.001	0.003
PXMP2	-1.10	-2.14	<0.001	<0.001
KRBOX1	-1.07	-2.10	0.002	0.013
GCHFR	-1.07	-2.10	<0.001	<0.001
KANSL1-AS1	-1.07	-2.10	<0.001	<0.001
C9orf170	-1.06	-2.08	0.009	0.041
ERBB3	-1.06	-2.08	<0.001	0.004
RBPMS-AS1	-1.06	-2.08	<0.001	<0.001

ASPHD1	1.74	3.34	<0.001	<0.001
SLAMF7	1.74	3.34	<0.001	<0.001
ANKRD30B	1.73	3.32	<0.001	0.001
COL4A4	1.72	3.29	<0.001	<0.001
FLT3	1.72	3.29	<0.001	<0.001
ALCAM	1.71	3.27	<0.001	<0.001
GPR39	1.71	3.27	<0.001	<0.001
MSR1	1.7	3.25	<0.001	<0.001
AATBC	1.7	3.25	<0.001	<0.001
AURKB	1.69	3.23	<0.001	0.004
CXCL8	1.69	3.23	<0.001	0.025
U2AF1	1.69	3.23	<0.001	0.034
CASC5	1.69	3.23	<0.001	<0.001
KMO	1.69	3.23	<0.001	<0.001
TRDN	1.68	3.20	<0.001	0.008
AOAH	1.68	3.20	<0.001	<0.001
NPR3	1.68	3.20	<0.001	<0.001
RPL7AP64	1.67	3.18	<0.001	0.001
SEZ6	1.67	3.18	<0.001	0.013
UCKL1-AS1	1.67	3.18	<0.001	<0.001
PRAM1	1.67	3.18	<0.001	<0.001
FCN1	1.66	3.16	<0.001	<0.001
LYZ	1.66	3.16	<0.001	<0.001
IGHG4	1.65	3.14	<0.001	0.009
HMMR	1.65	3.14	<0.001	<0.001
PAX3	1.64	3.12	<0.001	0.010
TUBB2B	1.64	3.12	<0.001	<0.001

DPH6-AS1	-1.05	-2.07	0.004	0.023
MT-ND3	-1.05	-2.07	<0.001	<0.001
PCBD1	-1.05	-2.07	<0.001	<0.001
SHOX2	-1.04	-2.06	0.001	0.005
NWD2	-1.04	-2.06	0.001	0.007
FAM46B	-1.04	-2.06	0.002	0.011
ARHGEF26-AS1	-1.04	-2.06	<0.001	0.003
GLYCTK	-1.04	-2.06	<0.001	<0.001
MPPED2	-1.04	-2.06	<0.001	<0.001
PLAG1	-1.04	-2.06	<0.001	<0.001
SLC16A11	-1.04	-2.06	<0.001	<0.001
ADAM20P1	-1.02	-2.03	<0.001	0.002
NIPSNAP3B	-1.02	-2.03	<0.001	<0.001
ASIC4	-1.01	-2.01	0.005	0.025
PKP2	-1.01	-2.01	0.006	0.031
RPL9P9	-1.01	-2.01	<0.001	0.002
PLPP2	-1.01	-2.01	<0.001	0.004
DMKN	-1.01	-2.01	<0.001	<0.001
IGFBP6	-1.01	-2.01	<0.001	<0.001
COL9A3	-1.00	-2.00	0.001	0.007
RPL31P61	-1.00	-2.00	0.001	0.009
CDH19	-1.00	-2.00	0.003	0.020
GPX3	-1.00	-2.00	<0.001	0.002
DCXR	-1.00	-2.00	<0.001	<0.001

IQGAP3	1.64	3.12	<0.001	<0.001
SAPCD2	1.63	3.10	<0.001	0.002
MZB1	1.63	3.10	<0.001	0.004
CLEC7A	1.63	3.10	<0.001	<0.001
KIF2C	1.63	3.10	<0.001	<0.001
NME8	1.63	3.10	<0.001	<0.001
SAA2	1.62	3.07	<0.001	0.040
LCP1	1.62	3.07	<0.001	<0.001
LILRA4	1.62	3.07	<0.001	<0.001
THBS1	1.62	3.07	<0.001	<0.001
GABRG1	1.62	3.07	<0.001	<0.001
TENM4	1.61	3.05	<0.001	<0.001
JAKMIP3	1.61	3.05	<0.001	<0.001
JPH2	1.6	3.03	<0.001	0.005
PDE6G	1.6	3.03	<0.001	<0.001
ADGRE4P	1.6	3.03	<0.001	<0.001
ACP5	1.6	3.03	<0.001	<0.001
APLN	1.6	3.03	<0.001	<0.001
CXCL9	1.59	3.01	<0.001	0.005
CLCA2	1.59	3.01	<0.001	<0.001
IGFN1	1.59	3.01	<0.001	<0.001
WDFY4	1.58	2.99	<0.001	<0.001
KCNC3	1.58	2.99	<0.001	<0.001
NUGGC	1.58	2.99	<0.001	<0.001
ADCYAP1	1.57	2.97	<0.001	0.017
DPP6	1.57	2.97	<0.001	0.022
RANBP3L	1.57	2.97	<0.001	<0.001

IGLV2-14	1.56	2.95	<0.001	0.005
CENPF	1.56	2.95	<0.001	<0.001
LINC01094	1.56	2.95	<0.001	<0.001
APOBR	1.56	2.95	<0.001	<0.001
KIF14	1.55	2.93	<0.001	0.002
C5orf64	1.55	2.93	<0.001	<0.001
GABRB2	1.55	2.93	<0.001	<0.001
CTSV	1.55	2.93	<0.001	<0.001
CLSPN	1.53	2.89	<0.001	0.001
TMC5	1.53	2.89	<0.001	0.028
PROSER2-AS1	1.53	2.89	<0.001	<0.001
FAM225A	1.53	2.89	<0.001	<0.001
CACNG8	1.53	2.89	<0.001	<0.001
MSC	1.53	2.89	<0.001	<0.001
IGKV3-11	1.52	2.87	<0.001	0.029
CXCL10	1.52	2.87	<0.001	0.045
SPINT1	1.52	2.87	<0.001	<0.001
ITGB2	1.52	2.87	<0.001	<0.001
HP	1.51	2.85	<0.001	0.004
IGLC2	1.51	2.85	<0.001	0.006
PLD4	1.51	2.85	<0.001	<0.001
TTYH3	1.51	2.85	<0.001	<0.001
NR4A3	1.51	2.85	<0.001	<0.001
DNAH10	1.51	2.85	<0.001	<0.001
WWC1	1.51	2.85	<0.001	<0.001
CD52	1.51	2.85	<0.001	<0.001
CNTN6	1.5	2.83	<0.001	0.002

MFSD2A	1.5	2.83	<0.001	<0.001
SPN	1.5	2.83	<0.001	<0.001
ARPP21	1.5	2.83	<0.001	<0.001
CLEC4D	1.49	2.81	<0.001	0.053
LY9	1.49	2.81	<0.001	<0.001
LINC01503	1.49	2.81	<0.001	<0.001
GSG2	1.49	2.81	<0.001	<0.001
LINC00877	1.49	2.81	<0.001	<0.001
FPR3	1.49	2.81	<0.001	<0.001
ESCO2	1.48	2.79	<0.001	0.002
HLA-DQB1	1.48	2.79	<0.001	0.020
KCNN4	1.48	2.79	<0.001	<0.001
SIGLEC9	1.48	2.79	<0.001	<0.001
XCR1	1.47	2.77	<0.001	0.001
MIR4767	1.47	2.77	<0.001	<0.001
OSCAR	1.47	2.77	<0.001	<0.001
IL6	1.46	2.75	<0.001	0.002
CP	1.46	2.75	<0.001	<0.001
LINC00942	1.45	2.73	<0.001	0.015
ANKRD30A	1.45	2.73	<0.001	<0.001
ACSM4	1.44	2.71	<0.001	0.001
HLA-DQB2	1.44	2.71	<0.001	0.008
ACPP	1.44	2.71	<0.001	<0.001
CCR4	1.44	2.71	<0.001	<0.001
TROAP	1.43	2.69	<0.001	0.002
SULT1A2	1.42	2.68	<0.001	0.001
CELSR1	1.42	2.68	<0.001	0.001

GPR141	1.42	2.68	<0.001	<0.001
BRCA2	1.42	2.68	<0.001	<0.001
PAQR5	1.42	2.68	<0.001	<0.001
CFB	1.42	2.68	<0.001	<0.001
PRG4	1.41	2.66	<0.001	0.010
C6orf183	1.41	2.66	<0.001	<0.001
NCAPH	1.41	2.66	<0.001	<0.001
SERINC2	1.41	2.66	<0.001	<0.001
GDF15	1.4	2.64	<0.001	0.001
P2RX6P	1.4	2.64	<0.001	0.004
CDHR5	1.4	2.64	<0.001	0.029
ADGRE2	1.4	2.64	<0.001	<0.001
LINC01272	1.4	2.64	<0.001	<0.001
MELK	1.4	2.64	<0.001	<0.001
KCNAB2	1.4	2.64	<0.001	<0.001
TPX2	1.4	2.64	<0.001	<0.001
MYH2	1.39	2.62	<0.001	0.001
RGS20	1.39	2.62	<0.001	<0.001
SYNPO	1.39	2.62	<0.001	<0.001
CYP19A1	1.39	2.62	<0.001	<0.001
LINC01347	1.39	2.62	<0.001	<0.001
HPD	1.39	2.62	<0.001	<0.001
CHST1	1.39	2.62	<0.001	<0.001
KCNQ3	1.39	2.62	<0.001	<0.001
DCHS2	1.38	2.60	<0.001	0.001
LINC01021	1.38	2.60	<0.001	0.023
DHRS9	1.37	2.58	<0.001	<0.001

FAT2	1.37	2.58	<0.001	<0.001
SLC4A3	1.37	2.58	<0.001	<0.001
B3GNT7	1.37	2.58	<0.001	<0.001
CIITA	1.37	2.58	<0.001	<0.001
CYP27B1	1.36	2.57	<0.001	0.002
SLC34A3	1.36	2.57	<0.001	0.015
TRPC4	1.36	2.57	<0.001	0.028
TLR8	1.36	2.57	<0.001	<0.001
NUF2	1.35	2.55	<0.001	0.005
CD300LF	1.35	2.55	<0.001	<0.001
POU2F2	1.35	2.55	<0.001	<0.001
TLR7	1.35	2.55	<0.001	<0.001
SIGLEC14	1.34	2.53	<0.001	0.002
CCNB2	1.34	2.53	<0.001	<0.001
SYNC	1.34	2.53	<0.001	<0.001
NGFR	1.34	2.53	<0.001	<0.001
PHLDA2	1.33	2.51	<0.001	0.003
USP2	1.33	2.51	<0.001	<0.001
MYBPC3	1.32	2.50	<0.001	0.001
CLECL1	1.32	2.50	<0.001	0.026
FCMR	1.32	2.50	<0.001	<0.001
CD300C	1.32	2.50	<0.001	<0.001
NOD2	1.32	2.50	<0.001	<0.001
LAMA5-AS1	1.32	2.50	<0.001	<0.001
CACNA2D1	1.31	2.48	<0.001	<0.001
HLA-DOA	1.31	2.48	<0.001	<0.001
SLC7A5	1.31	2.48	<0.001	<0.001

KCNA3	1.3	2.46	<0.001	0.001
ASGR2	1.3	2.46	<0.001	0.001
KIF20A	1.3	2.46	<0.001	0.008
JAKMIP2	1.3	2.46	<0.001	0.008
ADAMTS19	1.3	2.46	<0.001	0.047
SLC9A7	1.3	2.46	<0.001	<0.001
PALM2-AKAP2	1.3	2.46	<0.001	<0.001
SCIMP	1.3	2.46	<0.001	<0.001
TM4SF19-AS1	1.3	2.46	<0.001	<0.001
NCEH1	1.3	2.46	<0.001	<0.001
PANX2	1.3	2.46	<0.001	<0.001
NUP210	1.3	2.46	<0.001	<0.001
CENPE	1.3	2.46	<0.001	<0.001
LUCAT1	1.29	2.45	<0.001	0.002
CFAP221	1.29	2.45	<0.001	0.002
NFE4	1.29	2.45	<0.001	0.003
COL4A3	1.29	2.45	<0.001	0.028
PLA2G2D	1.29	2.45	<0.001	<0.001
TGM1	1.29	2.45	<0.001	<0.001
LTBP2	1.29	2.45	<0.001	<0.001
NETO2	1.29	2.45	<0.001	<0.001
NCR3LG1	1.29	2.45	<0.001	<0.001
HLA-DRA	1.29	2.45	<0.001	<0.001
SIGLEC7	1.29	2.45	<0.001	<0.001
DIAPH3	1.28	2.43	<0.001	0.001
IL21R	1.28	2.43	<0.001	0.001
TFCP2L1	1.28	2.43	<0.001	0.002

HTR7	1.28	2.43	<0.001	0.007
TRGV5	1.28	2.43	<0.001	0.029
DEPDC1	1.28	2.43	<0.001	0.030
CD163	1.28	2.43	<0.001	<0.001
SIRPB1	1.27	2.41	<0.001	0.001
KLHDC7B	1.27	2.41	<0.001	0.001
KRT5	1.27	2.41	<0.001	0.001
ZMYND15	1.27	2.41	<0.001	<0.001
ADAM28	1.27	2.41	<0.001	<0.001
KYNU	1.27	2.41	<0.001	<0.001
PIK3R5	1.27	2.41	<0.001	<0.001
TNFAIP6	1.27	2.41	<0.001	<0.001
PTCHD4	1.27	2.41	<0.001	<0.001
CHST11	1.27	2.41	<0.001	<0.001
ITGA4	1.27	2.41	<0.001	<0.001
HPSE	1.27	2.41	<0.001	<0.001
GM2AP1	1.26	2.39	<0.001	0.003
IDO1	1.26	2.39	<0.001	0.007
LINC00993	1.26	2.39	<0.001	0.046
CES1	1.26	2.39	<0.001	<0.001
DAPP1	1.26	2.39	<0.001	<0.001
RTN4R	1.26	2.39	<0.001	<0.001
KCNJ5	1.26	2.39	<0.001	<0.001
ATF3	1.26	2.39	<0.001	<0.001
SFRP4	1.25	2.38	<0.001	0.002
CDCA2	1.25	2.38	<0.001	0.007
NALCN	1.25	2.38	<0.001	0.032

SCX	1.25	2.38	<0.001	<0.001
LILRB4	1.25	2.38	<0.001	<0.001
CLDN18	1.25	2.38	<0.001	<0.001
CCDC162P	1.24	2.36	<0.001	0.004
TNFRSF9	1.24	2.36	<0.001	0.009
RNASE2	1.24	2.36	<0.001	0.036
BCL2A1	1.24	2.36	<0.001	<0.001
GALNT6	1.24	2.36	<0.001	<0.001
CXCL16	1.24	2.36	<0.001	<0.001
NOS1AP	1.24	2.36	<0.001	<0.001
NRP2	1.24	2.36	<0.001	<0.001
OTX1	1.23	2.35	<0.001	0.006
POPDC3	1.23	2.35	<0.001	0.036
SLC7A4	1.23	2.35	<0.001	<0.001
CCR1	1.23	2.35	<0.001	<0.001
ADCY7	1.23	2.35	<0.001	<0.001
NFAM1	1.23	2.35	<0.001	<0.001
DNM1P51	1.22	2.33	<0.001	0.001
SALL1	1.22	2.33	<0.001	0.014
GRIN2D	1.22	2.33	<0.001	0.014
TDRD9	1.22	2.33	<0.001	0.015
LCNL1	1.22	2.33	<0.001	0.018
BPIFB2	1.22	2.33	<0.001	0.020
CARD9	1.22	2.33	<0.001	<0.001
CXCL3	1.22	2.33	<0.001	<0.001
ALPL	1.21	2.31	<0.001	0.001
SLC4A10	1.21	2.31	<0.001	0.008

EPCAM	1.21	2.31	<0.001	0.009
ABHD11-AS1	1.21	2.31	<0.001	0.030
SMTNL1	1.21	2.31	<0.001	0.043
GPR183	1.21	2.31	<0.001	<0.001
CD84	1.21	2.31	<0.001	<0.001
TNFRSF11B	1.21	2.31	<0.001	<0.001
F2R	1.21	2.31	<0.001	<0.001
CD180	1.21	2.31	<0.001	<0.001
LINC01426	1.21	2.31	<0.001	<0.001
ZNF469	1.21	2.31	<0.001	<0.001
IL10	1.2	2.30	<0.001	0.001
CCDC144B	1.2	2.30	<0.001	0.002
UHRF1	1.2	2.30	<0.001	0.004
CILP2	1.2	2.30	<0.001	0.011
C9orf106	1.2	2.30	<0.001	0.012
HORMAD2	1.2	2.30	<0.001	0.027
HK3	1.2	2.30	<0.001	<0.001
PLEKHM3	1.2	2.30	<0.001	<0.001
RTKN2	1.2	2.30	<0.001	<0.001
KIF23	1.19	2.28	<0.001	0.001
CXCR3	1.19	2.28	<0.001	0.002
SHANK1	1.19	2.28	<0.001	0.006
SDC1	1.19	2.28	<0.001	0.006
RNF39	1.19	2.28	<0.001	0.010
KIF4A	1.19	2.28	<0.001	0.025
RAPH1	1.19	2.28	<0.001	<0.001
CCND1	1.19	2.28	<0.001	<0.001

FCHO1	1.19	2.28	<0.001	<0.001
NRXN3	1.19	2.28	<0.001	<0.001
PEG10	1.18	2.27	<0.001	0.001
ANGPTL6	1.18	2.27	<0.001	0.001
AQP9	1.18	2.27	<0.001	0.015
CUX2	1.18	2.27	<0.001	0.017
HSPG2	1.18	2.27	<0.001	<0.001
KCNB1	1.18	2.27	<0.001	<0.001
PARVG	1.18	2.27	<0.001	<0.001
ANKDD1A	1.18	2.27	<0.001	<0.001
TNRC18P1	1.18	2.27	<0.001	<0.001
FGF11	1.17	2.25	<0.001	0.002
E2F2	1.17	2.25	<0.001	0.005
CXorf65	1.17	2.25	<0.001	0.018
C5AR1	1.17	2.25	<0.001	<0.001
ASGR1	1.17	2.25	<0.001	<0.001
AMPD3	1.17	2.25	<0.001	<0.001
CD1D	1.17	2.25	<0.001	<0.001
CGNL1	1.17	2.25	<0.001	<0.001
DBNDD1	1.17	2.25	<0.001	<0.001
NLRC4	1.17	2.25	<0.001	<0.001
RAB3C	1.17	2.25	<0.001	<0.001
SEZ6L	1.16	2.23	<0.001	0.018
ECEL1	1.16	2.23	<0.001	0.026
CTSS	1.16	2.23	<0.001	<0.001
ZMIZ1-AS1	1.16	2.23	<0.001	<0.001
NCKAP1L	1.16	2.23	<0.001	<0.001

RAB39A	1.16	2.23	<0.001	<0.001
LGALS9	1.16	2.23	<0.001	<0.001
COL12A1	1.16	2.23	<0.001	<0.001
KIAA1522	1.16	2.23	<0.001	<0.001
GPR17	1.15	2.22	<0.001	0.001
DIO2	1.15	2.22	<0.001	0.003
DTHD1	1.15	2.22	<0.001	0.005
SIGLEC5	1.15	2.22	<0.001	0.029
LINC00677	1.15	2.22	<0.001	0.030
PPP2R2C	1.15	2.22	<0.001	0.033
PTGFR	1.15	2.22	<0.001	<0.001
KCNH3	1.15	2.22	<0.001	<0.001
AMICA1	1.15	2.22	<0.001	<0.001
LILRB1	1.15	2.22	<0.001	<0.001
AP1S3	1.15	2.22	<0.001	<0.001
STAC3	1.15	2.22	<0.001	<0.001
NRIP3	1.14	2.20	<0.001	0.002
OAS3	1.14	2.20	<0.001	0.003
PTPRJ	1.14	2.20	<0.001	<0.001
GCNT2	1.14	2.20	<0.001	<0.001
PTGIR	1.14	2.20	<0.001	<0.001
CD86	1.14	2.20	<0.001	<0.001
TFEC	1.14	2.20	<0.001	<0.001
GRIP1	1.13	2.19	<0.001	0.001
CSF2RA	1.13	2.19	<0.001	0.001
GAPT	1.13	2.19	<0.001	0.002
RASL11B	1.13	2.19	<0.001	0.032

FCRL5	1.13	2.19	<0.001	0.032
PIK3R6	1.13	2.19	<0.001	<0.001
TNFRSF12A	1.13	2.19	<0.001	<0.001
PTPRC	1.13	2.19	<0.001	<0.001
FYB	1.13	2.19	<0.001	<0.001
SLCO2B1	1.13	2.19	<0.001	<0.001
PLCB2	1.13	2.19	<0.001	<0.001
GPAT2	1.12	2.17	<0.001	0.001
TRPM3	1.12	2.17	<0.001	0.001
ZBED6	1.12	2.17	<0.001	0.007
SLC46A2	1.12	2.17	<0.001	0.016
CFH	1.12	2.17	<0.001	<0.001
ZDHHC20P4	1.12	2.17	<0.001	<0.001
HLA-DPA1	1.12	2.17	<0.001	<0.001
RCC2P7	1.11	2.16	<0.001	0.001
PCDHAC2	1.11	2.16	<0.001	0.001
DCC	1.11	2.16	<0.001	0.008
RPS4XP5	1.11	2.16	<0.001	0.018
PIK3AP1	1.11	2.16	<0.001	<0.001
NFIX	1.11	2.16	<0.001	<0.001
TPTEP1	1.11	2.16	<0.001	<0.001
SAMHD1	1.11	2.16	<0.001	<0.001
SLC37A2	1.11	2.16	<0.001	<0.001
BGN	1.11	2.16	<0.001	<0.001
CYP7B1	1.11	2.16	<0.001	<0.001
FMNL1	1.11	2.16	<0.001	<0.001
TNXB	1.1	2.14	<0.001	0.005

DPYSL4	1.1	2.14	<0.001	0.028
CSF2RB	1.1	2.14	<0.001	<0.001
ITIH5	1.1	2.14	<0.001	<0.001
FAM83H	1.1	2.14	<0.001	<0.001
RPS6KA1	1.1	2.14	<0.001	<0.001
DOCK3	1.09	2.13	<0.001	0.001
LMOD1	1.09	2.13	<0.001	0.001
FCER1A	1.09	2.13	<0.001	0.002
RPS24P6	1.09	2.13	<0.001	0.039
IQGAP2	1.09	2.13	<0.001	<0.001
C10orf90	1.09	2.13	<0.001	<0.001
HLA-DMB	1.09	2.13	<0.001	<0.001
VENTX	1.09	2.13	<0.001	<0.001
GPLD1	1.09	2.13	<0.001	<0.001
PTPN7	1.09	2.13	<0.001	<0.001
KCP	1.09	2.13	<0.001	<0.001
HLA-DRB6	1.08	2.11	<0.001	0.002
NOTCH3	1.08	2.11	<0.001	<0.001
C2	1.08	2.11	<0.001	<0.001
MMP24	1.08	2.11	<0.001	<0.001
PHLDA1	1.08	2.11	<0.001	<0.001
ITGAM	1.08	2.11	<0.001	<0.001
SEMA6B	1.08	2.11	<0.001	<0.001
BTK	1.08	2.11	<0.001	<0.001
THEMIS2	1.08	2.11	<0.001	<0.001
SERPINA1	1.07	2.10	<0.001	0.001
CPNE5	1.07	2.10	<0.001	0.001

LRRC43	1.07	2.10	<0.001	0.001
KCNK15	1.07	2.10	<0.001	0.002
NCAPG	1.07	2.10	<0.001	0.009
NPTX2	1.07	2.10	<0.001	0.022
KCNA2	1.07	2.10	<0.001	0.040
FCAR	1.07	2.10	<0.001	0.053
IRF8	1.07	2.10	<0.001	<0.001
POLR2A	1.07	2.10	<0.001	<0.001
CD74	1.07	2.10	<0.001	<0.001
GMIP	1.07	2.10	<0.001	<0.001
C1orf233	1.07	2.10	<0.001	<0.001
STC1	1.07	2.10	<0.001	<0.001
AGRN	1.07	2.10	<0.001	<0.001
IL2RG	1.07	2.10	<0.001	<0.001
EPHB1	1.06	2.08	<0.001	0.001
IL24	1.06	2.08	<0.001	0.006
OTOA	1.06	2.08	<0.001	0.011
ESPL1	1.06	2.08	<0.001	0.015
RELT	1.06	2.08	<0.001	<0.001
MS4A7	1.06	2.08	<0.001	<0.001
ID4	1.06	2.08	<0.001	<0.001
LILRB3	1.06	2.08	<0.001	<0.001
FERMT3	1.06	2.08	<0.001	<0.001
MYO1G	1.06	2.08	<0.001	<0.001
CARD11	1.06	2.08	<0.001	<0.001
NPY1R	1.06	2.08	<0.001	<0.001
VGLL3	1.06	2.08	<0.001	<0.001

C19orf35	1.05	2.07	<0.001	0.001
LINGO3	1.05	2.07	<0.001	0.001
PKP1	1.05	2.07	<0.001	0.006
SAMD5	1.05	2.07	<0.001	0.006
ARRDC5	1.05	2.07	<0.001	0.025
TLDC2	1.05	2.07	<0.001	<0.001
LEP	1.05	2.07	<0.001	<0.001
IGSF6	1.05	2.07	<0.001	<0.001
FCGR3A	1.05	2.07	<0.001	<0.001
SOWAHD	1.05	2.07	<0.001	<0.001
PALLD	1.05	2.07	<0.001	<0.001
HIST1H2BC	1.04	2.06	<0.001	0.001
VCAN	1.04	2.06	<0.001	0.001
NAPSB	1.04	2.06	<0.001	0.005
GPR68	1.04	2.06	<0.001	0.015
CCDC88B	1.04	2.06	<0.001	<0.001
SASH3	1.04	2.06	<0.001	<0.001
CD101	1.04	2.06	<0.001	<0.001
PTAFR	1.04	2.06	<0.001	<0.001
ARHGAP30	1.04	2.06	<0.001	<0.001
MSNP1	1.04	2.06	<0.001	<0.001
SLIT2	1.04	2.06	<0.001	<0.001
PLBD2	1.04	2.06	<0.001	<0.001
P2RX7	1.04	2.06	<0.001	<0.001
PEMT	1.04	2.06	<0.001	<0.001
LINC01106	1.03	2.04	<0.001	0.018
CSMD2	1.03	2.04	<0.001	0.030

MPO	1.03	2.04	<0.001	0.042
EGR3	1.03	2.04	<0.001	<0.001
HLA-DPB1	1.03	2.04	<0.001	<0.001
FCGR2A	1.03	2.04	<0.001	<0.001
VLDLR	1.03	2.04	<0.001	<0.001
DOK5	1.03	2.04	<0.001	<0.001
CXCR2P1	1.02	2.03	<0.001	0.010
CROCC2	1.02	2.03	<0.001	0.012
EPHX3	1.02	2.03	<0.001	0.022
TMEM139	1.02	2.03	<0.001	0.047
OSBPL3	1.02	2.03	<0.001	<0.001
MAP4K1	1.02	2.03	<0.001	<0.001
STXBP2	1.02	2.03	<0.001	<0.001
DOCK2	1.02	2.03	<0.001	<0.001
SYT12	1.02	2.03	<0.001	<0.001
CD248	1.02	2.03	<0.001	<0.001
FBXO41	1.02	2.03	<0.001	<0.001
GAS6-AS1	1.02	2.03	<0.001	<0.001
EOMES	1.01	2.01	<0.001	0.002
PMFBP1	1.01	2.01	<0.001	0.003
FBXL16	1.01	2.01	<0.001	0.004
DNAH2	1.01	2.01	<0.001	0.004
FAM225B	1.01	2.01	<0.001	0.006
SPAG17	1.01	2.01	<0.001	0.015
VSTM2L	1.01	2.01	<0.001	0.026
LINC00937	1.01	2.01	<0.001	0.032
IL6STP1	1.01	2.01	<0.001	<0.001

LACE1	1.01	2.01	<0.001	<0.001
LAPTM5	1.01	2.01	<0.001	<0.001
GLB1	1.01	2.01	<0.001	<0.001
GRM7	1.00	2.00	0.003	0.017
CHST13	1.00	2.00	0.010	0.055
MUC1	1.00	2.00	<0.001	0.006
TNS3	1.00	2.00	<0.001	<0.001
DLEC1	1.00	2.00	<0.001	<0.001
FGF1	1.00	2.00	<0.001	<0.001
TRIM14	1.00	2.00	<0.001	<0.001
IL18	1.00	2.00	<0.001	<0.001

FC, fold change; FDR, false discovery rate

Log2 FC and *P* values were obtained by comparison of data from obese individuals vs data from normal weight individuals in a general linear model likelihood ratio test in EdgeR software.

Appendix L **Genes differentially expressed in scWAT of metabolically healthy obese individuals in comparison to normal weight individuals <2 FC**

hgnc_symbol	Log FC	FC	*P*	FDR
IL2RB	0.99	1.99	<0.001	<0.001
IL1B	0.97	1.96	0.004	0.023
IL10RA	0.97	1.96	<0.001	<0.001
COL9A2	0.96	1.95	<0.001	0.002
IL1R2	0.95	1.94	<0.001	0.004
CD72	0.95	1.93	<0.001	0.001
ICAM1	0.95	1.93	<0.001	<0.001
FOXP3	0.95	1.93	0.002	0.012
CD48	0.94	1.92	<0.001	<0.001
CXCL2	0.94	1.91	0.002	0.014
IL7R	0.93	1.91	0.001	0.010
CD5	0.93	1.91	<0.001	<0.001
IL1RL1	0.93	1.90	0.001	0.008
CD28	0.92	1.89	<0.001	<0.001
VCAM1	0.92	1.89	<0.001	<0.001
CD4	0.91	1.88	<0.001	<0.001
HLA-DRB5	0.91	1.88	0.039	0.122
SELE	0.91	1.88	0.004	0.023
CD209	0.91	1.88	<0.001	<0.001
HLA-DRB1	0.91	1.87	0.003	0.017

hgnc_symbol	Log FC	FC	*P*	FDR
CEBPA-AS1	-1.25	-2.38	0.036	0.116
APOB	-0.93	-1.91	0.003	0.018
ELOVL6	-0.90	-1.87	0.052	0.151
APOE	-0.89	-1.85	0.002	0.011
KLF15	-0.86	-1.82	<0.001	0.001
FABP4	-0.85	-1.80	<0.001	<0.001
MMP27	-0.84	-1.79	0.014	0.059
FAAHP1	-0.83	-1.78	0.020	0.076
VEGFA	-0.64	-1.56	<0.001	0.002
APOD	-0.55	-1.46	<0.001	0.003
CCL14	-0.55	-1.46	0.003	0.020
ELOVL5	-0.53	-1.44	0.005	0.027
PPARGC1A	-0.49	-1.40	0.011	0.049
PPARG	-0.49	-1.40	0.001	0.005
CNR1	-0.42	-1.32	0.011	0.050
CIDEC	-0.37	-1.29	0.032	0.108
CD40	-0.34	-1.27	0.002	0.011
FOXP2	-0.33	-1.26	0.022	0.081
PPARA	-0.21	-1.16	0.050	0.146

CR1	0.90	1.86	<0.001	0.003
CD22	0.88	1.84	0.003	0.019
CCL8	0.87	1.83	0.005	0.027
CD37	0.87	1.82	<0.001	<0.001
CCL13	0.86	1.82	<0.001	0.003
IL12RB1	0.86	1.81	<0.001	<0.001
CD300A	0.85	1.81	<0.001	<0.001
PLCH2	0.84	1.79	0.001	0.010
CD53	0.83	1.78	<0.001	<0.001
PLIN3	0.82	1.77	<0.001	<0.001
TNF	0.82	1.77	0.001	0.005
COL1A1	0.82	1.76	0.007	0.036
COL8A2	0.81	1.76	0.002	0.014
RELB	0.81	1.76	<0.001	<0.001
ALOX5	0.81	1.75	<0.001	<0.001
CCL3L3	0.80	1.74	0.034	0.112
CCL4	0.79	1.73	0.026	0.091
JAK3	0.78	1.72	<0.001	0.001
CD244	0.78	1.71	0.005	0.027
DAGLA	0.76	1.69	<0.001	0.004
NFATC2	0.75	1.68	<0.001	<0.001
COL14A1	0.75	1.68	0.004	0.023
COL6A1	0.75	1.68	<0.001	0.002
CD3G	0.73	1.66	0.006	0.032
CD2	0.73	1.66	0.003	0.018
NLRP3	0.73	1.66	<0.001	0.003
SLC2A9	0.73	1.65	<0.001	0.001

IL5RA	0.73	1.65	0.028	0.098
PLA1A	0.72	1.65	0.058	0.163
CD163L1	0.72	1.65	<0.001	<0.001
COL6A2	0.72	1.64	0.001	0.007
NLRP12	0.69	1.62	0.006	0.032
COL5A2	0.69	1.62	<0.001	<0.001
TGFB1	0.68	1.60	<0.001	<0.001
ANGPT1	0.68	1.60	<0.001	<0.001
REL	0.67	1.60	<0.001	<0.001
KLF16	0.66	1.58	<0.001	0.003
STAT3	0.66	1.58	<0.001	<0.001
FAT1	0.65	1.57	<0.001	<0.001
IL17RE	0.65	1.57	<0.001	0.001
CD33	0.65	1.56	0.002	0.011
TLR1	0.64	1.56	<0.001	<0.001
COL18A1	0.64	1.56	<0.001	0.001
CD274	0.64	1.56	0.010	0.045
TLR6	0.63	1.55	<0.001	0.003
COL3A1	0.63	1.54	0.004	0.024
TGFA	0.62	1.54	0.009	0.041
TLR2	0.62	1.54	<0.001	0.002
CCL2	0.62	1.54	0.004	0.024
ACOX1	0.61	1.53	<0.001	<0.001
IL18BP	0.61	1.53	0.001	0.007
CD14	0.61	1.53	<0.001	0.003
COL16A1	0.61	1.52	0.001	0.010
STAT1	0.60	1.52	0.001	0.008

COL4A2	0.58	1.50	0.001	0.010
ELN	0.58	1.49	0.013	0.057
COL6A3	0.56	1.48	0.010	0.046
COL4A1	0.56	1.47	0.001	0.007
CD6	0.55	1.47	0.011	0.049
PLD3	0.55	1.46	<0.001	<0.001
CD226	0.55	1.46	0.016	0.065
KLF14	0.55	1.46	0.011	0.050
HIF1AP1	0.54	1.46	0.023	0.083
FOXP4	0.54	1.46	0.007	0.036
CD83	0.54	1.45	0.005	0.026
ANGPT2	0.53	1.45	0.008	0.039
CD109	0.53	1.44	0.001	0.005
COL8A1	0.53	1.44	0.020	0.077
CD200R1	0.52	1.44	0.009	0.043
COL5A1	0.52	1.43	0.007	0.033
PLA2G15	0.52	1.43	<0.001	<0.001
IL6R	0.51	1.43	<0.001	<0.001
NFKB2	0.51	1.42	<0.001	<0.001
SELP	0.51	1.42	0.011	0.049
TLR10	0.50	1.41	0.051	0.149
PLIN2	0.48	1.39	0.001	0.009
CD27	0.48	1.39	0.040	0.125
IL7	0.46	1.38	0.059	0.165
NFATC3	0.46	1.38	<0.001	0.004
CD3EAP	0.45	1.37	0.030	0.102
IL17RA	0.45	1.36	0.002	0.012

CD44	0.45	1.36	0.012	0.052
MMP19	0.44	1.36	0.001	0.007
CD93	0.44	1.36	0.001	0.005
CD9	0.43	1.35	0.025	0.088
STAT2	0.43	1.34	0.003	0.017
SLC2A3	0.42	1.34	0.002	0.013
COL15A1	0.41	1.33	0.007	0.035
TIMP1	0.41	1.33	0.038	0.120
MMP14	0.40	1.32	0.018	0.069
HLA-DMA	0.40	1.32	0.007	0.036
PLA2G4A	0.39	1.31	0.012	0.051
CXCL12	0.38	1.30	0.014	0.057
TLR5	0.37	1.30	0.021	0.078
IL12RB2	0.37	1.30	0.059	0.165
PTGS1	0.37	1.29	0.054	0.155
PLCG2	0.36	1.28	0.004	0.024
ACOX3	0.36	1.28	0.003	0.017
HIF1A	0.35	1.28	<0.001	0.003
PECAM1	0.35	1.27	0.013	0.055
STAT6	0.34	1.26	0.007	0.035
TIMP2	0.33	1.26	0.055	0.156
ELMSAN1	0.33	1.26	0.006	0.031
IL33	0.33	1.26	0.032	0.106
KLF7	0.33	1.26	0.008	0.039
GATAD2A	0.33	1.25	0.001	0.008
NFATC1	0.32	1.25	0.012	0.053
GATA2	0.29	1.23	0.027	0.094

FTO	0.29	1.22	0.005	0.027
JAK2	0.27	1.21	0.028	0.096
NFAT5	0.27	1.21	0.023	0.084
IL6ST	0.26	1.20	0.053	0.152
PLD2	0.25	1.19	0.046	0.138
FOXP1	0.25	1.19	0.021	0.078
NLRP1	0.25	1.19	0.035	0.113
TLR4	0.25	1.19	0.015	0.062
IFNAR1	0.24	1.18	0.021	0.079
IFNAR2	0.24	1.18	0.039	0.122
COL4A3BP	0.24	1.18	0.038	0.120
IKBKB	0.23	1.18	0.044	0.135
NFKB1	0.22	1.17	0.028	0.095
GATAD2B	0.22	1.16	0.047	0.141

FC, fold change; FDR, false discovery rate

Log2 FC and *P* values were obtained by comparison of data from obese individuals vs data from normal weight individuals in a general linear model likelihood ratio test in EdgeR software.

Appendix M Primer sequence (Primer design Ltd) information of genes investigated by qRT-PCR

Official Gene Symbol	Sequence Length	Sense Primer	Anti-sense Primer	Dye
ALOX15	2707	5' ATCTTCTGAGGGGACACTTGA	3' GTATCGCAGGTGGGGAATTATA	FAM
ALOX12	2358	5' CTCCTGGAACTGCCTSGAAGAC	3' CTGGTAGCTGAACAACTCATCATC	FAM
DAGLA	5761	5' CTGTGCTGGAGCGTGATGA	3' GCTGCGTGTCCTGTGAGT	FAM
EGFL6	2445	5' GGGTCTGTCACTSTGGAACTAAA	3' CCACGCACTCACCAAACTTA	FAM
FATP1	3562	5’ CGGCTGACGTGCTCTATGA	3' TTCTTGCGGAGGACGACTG	FAM
KCNH2	4307	5' TGCTGAAGGAGACGGAAGAA	3' GGTGGTGCGGAGTTGATG	FAM
MAB21L1	2907	5' ACACGATTTCCTATCTCCCTTGAA	3' GATGCACCTAAATCACCAGGATTG	FAM
MMP9	2387	5' CTTCCAGTACCGAGAGAAAGC	3' CAGGATGTCATAGGTCACGTAG	FAM
NAPE-PLD	5363	5' GGTCTCCTTGACTGGATGCAA	3' GGACACAATTCTCCTCCCACC	FAM
NFkB1	4104	5' GTAACTGCTGGACCCAAGGA	3' CCTCTGTCATTCGTGCTTCC	FAM
PROK2	1613	5'ACGGACTTCATTTAACCGATTTATTTG	3' ACAGGTAAGATGTGGCTATTCACA	FAM
PTGS2	4507	5' CAAATCATCAACACTGCCTCAAT	3' TCTGGATCTGGAACACTGAATG	FAM
SLC27A1	3562	5’ CGGCTGACGTGCTCTATGA	3’ TTCTTGCGGAGGACGACTG	FAM
SLC27A2	2408	5' TATGACCTGATTAAATATGATGTGGAG	3' CATTAAATGGTGTAAGTTGTGTGATT	FAM
ALOX5	2591	5' AAGCGATGGAGAACCTGTTCA	3' GTCTTCCTGCCAGTGATTCATG	VIC
CNR1	5486	5' TGGTGTATGATGTCTTTGGGAAG	3' CGTGTCGCAGGTCCTTACT	VIC
DACT1	3877	5' CTTCATGCTGTGGCTGTGC	3' GGACGGTAAGGAACTGTCTGT	VIC
DACT2	2992	5' GCCCCGGTCGGTTGATGAG	3' CCACGGCTGGCCTGCATC	VIC
FAAH	2105	5' GACCTCCATCCCTGCTCTG	3' GCTGTTTCTTGACTGTTAGTTGG	VIC
GABARAP	924	5' GCTCGGATAGGAGACCTGGA	3' GCTCGGAGATGAATTCGCTTC	VIC
IKBKE	3492	5' ATGATTCCAACACTAGGCTGAT	3' AGGAGGGAGGTAGGCTGAT	VIC
MGLL	4653	5' CTCATTTCGCCTCTGGTTCTTG	3' GAGAGAGCACGCTGGAGTC	VIC

VPS36	4473	5'GGAGGTAGAXGGXTXAGGAA	3' CCTGTCTGTGGCTTTGAGGTAA	VIC
WNT3	3385	5' ATGGGCAAGGTTCTGTTTCCA	3' GTGTGTCCTACTTCCTGACTCC	VIC
WNT10B	2370	5' GCCGGGCCATCTTCATTCATA	3' AGCTCTCCTGAGAGGCGAC	VIC

Appendix N Genes differentially expressed in scWAT of metabolically healthy obese individuals following 12-week fish oil intervention

hgnc_symbol	Log FC	FC	*P*	FDR
PTGFRN	-0.41	-1.33	<0.001	0.099
PLEKHO1	-0.44	-1.35	<0.001	0.105
WARS	-0.45	-1.36	<0.001	0.170
PLCB2	-0.45	-1.37	<0.001	0.128
OLMALINC	-0.48	-1.40	<0.001	0.128
NHSL1	-0.49	-1.41	<0.001	0.178
DGKE	0.54	-1.45	<0.001	0.160
PIK3R1	0.56	-1.47	<0.001	0.099
PI16	-0.57	-1.49	<0.001	0.167
SLCO2A1	0.65	-1.57	<0.001	0.167
LILRB4	-0.67	-1.59	<0.001	0.068
ABCB1	0.69	-1.61	<0.001	0.049
HECW2	0.71	-1.63	<0.001	0.096
SPN	-0.73	-1.66	<0.001	0.031
SMOC2	-0.74	-1.67	<0.001	<0.001
LYZ	-0.80	-1.74	<0.001	0.099
GREM1	-0.80	-1.74	<0.001	0.068
CETP	-0.81	-1.75	<0.001	0.178
SLAMF7	-0.82	-1.76	<0.001	0.086
PRAM1	-0.89	-1.85	<0.001	0.178
APOBR	-0.92	-1.89	<0.001	0.031
CHST8	-0.96	-1.94	<0.001	0.178
PDPN	-0.96	-1.94	<0.001	0.031
FREM2	-0.98	-1.97	<0.001	0.200
MCOLN2	-1.02	-2.03	<0.001	0.167
MAB21L1	1.06	-2.09	<0.001	0.002
IDO1	-1.08	-2.11	<0.001	0.077
LRRTM4	1.09	-2.13	<0.001	0.099
NA	-1.17	-2.24	<0.001	0.065
CCL22	-1.21	-2.31	<0.001	0.167
DACT2	-1.25	-2.38	<0.001	0.080
FGF12-AS2	1.46	-2.76	<0.001	0.073
TDRD12	-1.99	-3.96	<0.001	0.043
IGLV1-44	-1.99	-3.98	<0.001	0.092
IGLV2-8	-2.51	-5.71	<0.001	0.178
IGLV1-47	-2.56	-5.92	<0.001	0.073

FC, fold change; FDR, false discovery rate

Log2 FC and *P* values were obtained by comparison of data from obese individuals vs data from normal weight individuals in a general linear model likelihood ratio test in EdgeR software

Grey highlight indicates FDR >1.0

Appendix O Genes differentially expressed in scWAT of normal weight individuals following 12-week fish oil intervention

hgnc_symbol	Log FC	FC	*P*	FDR
KCNH2	2.67	6.35	<0.001	0.054
FAM101A	1.74	3.34	<0.001	0.058
GCGR	1.74	3.33	<0.001	0.054
MYH11	1.73	3.31	0.001	0.170
FOXC2	1.71	3.28	<0.001	0.044
POF1B	1.69	3.22	<0.001	0.019
NA	1.58	2.99	<0.001	0.101
KIAA1644	1.56	2.96	<0.001	0.015
KRT16	1.51	2.85	0.001	0.150
NA	1.45	2.74	<0.001	0.101
FBXO40	1.45	2.73	<0.001	0.089
CSN1S1	1.43	2.69	<0.001	<0.001
CPN2	1.42	2.68	0.001	0.176
MMRN1	1.41	2.66	<0.001	0.101
NA	1.38	2.60	<0.001	0.094
NA	1.35	2.55	0.001	0.173
NA	1.33	2.51	0.001	0.170
NA	1.32	2.51	0.001	0.138
SV2C	1.32	2.49	0.001	0.179
POTEKP	1.28	2.43	0.002	0.198
ST3GAL1P1	1.27	2.42	0.002	0.198
SLC36A2	1.26	2.39	<0.001	0.101
NPTX2	1.23	2.34	<0.001	0.105
SOSTDC1	1.22	2.32	0.001	0.138
NA	1.19	2.29	0.002	0.189
PCDHAC2	1.12	2.18	<0.001	0.058
NA	1.12	2.17	0.001	0.179
FGF12-AS2	1.11	2.16	0.002	0.187
DSG4	1.11	2.16	0.002	0.195
PNCK	1.10	2.14	<0.001	0.105
TGM2	1.10	2.14	<0.001	0.054
NA	1.08	2.12	0.001	0.170
NA	1.07	2.10	0.002	0.199
ASB2	1.06	2.08	0.002	0.198

NA	1.05	2.07	<0.001	0.044
TRPC4	1.04	2.06	0.002	0.179
FGF11	1.01	2.01	<0.001	0.115
ERICH4	1.01	2.01	<0.001	0.050
PCDHGB7	1.00	2.01	<0.001	0.101
ZBTB16	1.00	2.00	0.002	0.192
RAB26	0.98	1.97	0.001	0.139
FAM222A	0.98	1.97	0.001	0.179
NA	0.98	1.97	0.002	0.179
ACTA2	0.96	1.94	0.002	0.180
NA	0.95	1.94	<0.001	0.101
KCNK3	0.95	1.93	0.002	0.201
NA	0.94	1.92	<0.001	0.114
PRRG4	0.94	1.91	<0.001	0.101
NA	0.93	1.91	<0.001	0.101
GALNT4	0.93	1.91	0.002	0.179
NA	0.93	1.91	0.001	0.165
NA	0.93	1.91	0.002	0.197
PRUNE2	0.93	1.90	0.001	0.154
DIRAS1	0.93	1.90	0.001	0.138
NA	0.92	1.89	<0.001	0.115
NA	0.92	1.89	0.001	0.159
ACTA2-AS1	0.91	1.88	0.001	0.150
NA	0.91	1.87	0.001	0.158
NA	0.90	1.87	0.001	0.170
PLA1A	0.89	1.86	<0.001	0.101
GPR17	0.88	1.84	0.002	0.179
NA	0.88	1.84	0.001	0.169
PCDHGA9	0.88	1.84	<0.001	0.089
NOTCH3	0.87	1.83	0.002	0.201
NA	0.87	1.82	0.001	0.158
NA	0.86	1.81	0.001	0.179
TMPRSS9	0.85	1.80	<0.001	0.105
NA	0.85	1.80	<0.001	0.105
GIPC3	0.84	1.79	0.001	0.170
NFIX	0.83	1.78	0.002	0.194
PROB1	0.83	1.77	<0.001	0.094
COL23A1	0.82	1.77	0.001	0.168
UCKL1-AS1	0.81	1.76	0.002	0.201
CCNK	0.81	1.75	0.001	0.143
RAPH1	0.81	1.75	0.001	0.170
FAM155A	0.80	1.74	0.001	0.156

FASN	0.79	1.73	0.001	0.122
CNTFR-AS1	0.79	1.73	0.001	0.149
NA	0.79	1.73	0.002	0.198
NA	0.78	1.71	0.002	0.197
NA	0.78	1.71	0.002	0.199
POLR2A	0.77	1.71	0.002	0.187
WIPF3	0.77	1.71	0.001	0.150
MYH9	0.77	1.71	0.001	0.149
PCDHGA10	0.77	1.70	0.001	0.160
TRAPPC12-AS1	0.75	1.69	0.002	0.189
SPTBN2	0.74	1.67	0.002	0.194
JPH2	0.74	1.67	0.001	0.123
LMOD1	0.73	1.66	<0.001	0.115
ACLY	0.72	1.65	0.002	0.201
HOXC11	0.72	1.64	0.001	0.179
TPO	0.72	1.64	0.001	0.133
CELSR1	0.71	1.64	<0.001	0.115
ELFN1	0.70	1.62	<0.001	0.105
ITGA3	0.68	1.61	0.002	0.198
CP	0.68	1.60	0.002	0.179
MYLK	0.68	1.60	0.002	0.201
CLIC4P1	0.68	1.60	0.002	0.189
PER1	0.68	1.60	0.001	0.158
NA	0.68	1.60	0.001	0.158
CCND1	0.67	1.59	<0.001	0.014
SHROOM3	0.67	1.59	<0.001	0.071
KLC2	0.67	1.59	<0.001	0.101
ABCB1	0.67	1.59	0.002	0.179
MYH14	0.67	1.59	0.002	0.198
TSPAN18	0.66	1.58	<0.001	0.115
UNC5B	0.66	1.58	<0.001	0.115
FRMD3	0.66	1.58	<0.001	0.101
NA	0.66	1.58	0.002	0.184
ALDH1A2	0.65	1.57	<0.001	0.050
DSP	0.65	1.57	0.001	0.165
GAS6-AS1	0.64	1.56	0.001	0.174
COL18A1	0.63	1.55	0.002	0.198
KHSRPP1	0.62	1.54	0.002	0.198
NXPH4	0.62	1.54	0.002	0.182
COL7A1	0.61	1.53	<0.001	0.105
TFRC	0.61	1.52	0.002	0.197
TNRC18	0.60	1.52	0.002	0.194

SLC25A22	0.60	1.51	<0.001	0.105
ALPK3	0.60	1.51	0.001	0.123
ZFP41	0.59	1.51	0.002	0.201
NCOR2	0.59	1.51	0.002	0.187
MIR143HG	0.59	1.51	<0.001	0.107
ZNF469	0.59	1.51	0.002	0.179
CLIP2	0.59	1.50	0.002	0.187
HSP90AA2P	0.59	1.50	0.001	0.158
SLC4A3	0.59	1.50	0.002	0.184
LPCAT3	0.59	1.50	0.001	0.125
ERBB4	0.58	1.50	<0.001	0.114
RAPGEF3	0.58	1.50	<0.001	0.050
PDZD7	0.57	1.49	0.002	0.187
FAM129B	0.57	1.49	0.002	0.201
GBP4	0.57	1.49	0.002	0.197
SPTBN5	0.57	1.48	<0.001	0.103
Sep-09	0.57	1.48	<0.001	0.101
SCUBE1	0.55	1.47	0.001	0.140
PRRC2A	0.53	1.44	0.002	0.201
EPAS1	0.51	1.43	0.001	0.138
TMEM104	0.51	1.42	0.001	0.149
FN3K	0.51	1.42	0.002	0.189
NAV2	0.50	1.41	0.002	0.179
FBLIM1	0.50	1.41	<0.001	0.114
TTYH3	0.48	1.39	<0.001	0.101
MSR1	0.48	1.39	0.001	0.158
CADM4	0.46	1.38	0.002	0.189
HIF3A	0.45	1.36	<0.001	0.114
LIFR	0.44	1.35	0.002	0.179
SVIL	0.42	1.33	0.002	0.189
PROK2	-1.87	-0.27	<0.001	0.024
IGLV1-44	-1.84	-0.28	<0.001	0.014
IGLV1-51	-1.68	-0.31	<0.001	0.014
NA	-1.61	-0.33	<0.001	0.095
NA	-1.59	-0.33	<0.001	0.109
IGHG4	-1.58	-0.33	<0.001	0.094
S100A8	-1.53	-0.35	<0.001	0.024
RPS27P23	-1.42	-0.37	<0.001	0.101
S100P	-1.41	-0.38	<0.001	0.108
NA	-1.37	-0.39	0.001	0.171
S100A9	-1.34	-0.39	<0.001	0.105
TEX26	-1.33	-0.40	0.001	0.161

IGLC3	-1.33	-0.40	<0.001	0.014
TREM1	-1.33	-0.40	<0.001	0.014
NA	-1.32	-0.40	<0.001	0.101
CCL4L2	-1.32	-0.40	0.002	0.192
S100A14	-1.32	-0.40	0.001	0.179
NA	-1.31	-0.40	0.001	0.125
GLT1D1	-1.30	-0.41	0.001	0.133
PPBP	-1.25	-0.42	0.002	0.189
NA	-1.22	-0.43	0.002	0.197
LINC01260	-1.19	-0.44	<0.001	0.014
HBA2	-1.18	-0.44	0.001	0.158
AADACL2	-1.14	-0.45	<0.001	0.024
LINC00907	-1.11	-0.46	0.001	0.158
IGHA2	-1.10	-0.47	<0.001	0.041
MTRNR2L1	-1.07	-0.47	<0.001	0.014
NA	-1.06	-0.48	0.001	0.150
CCL26	-1.05	-0.48	0.001	0.149
NA	-1.04	-0.49	0.002	0.201
RPL37P6	-1.01	-0.50	0.002	0.187
ADAMTS18	-1.01	-0.50	0.002	0.189
SEMA3B-AS1	-1.00	-0.50	0.001	0.170
ATP5HP1	-1.00	-0.50	0.001	0.130
WBP1LP2	-0.96	-0.51	0.002	0.189
RPL41P2	-0.95	-0.52	0.002	0.189
LILRA5	-0.95	-0.52	<0.001	0.050
NA	-0.95	-0.52	0.002	0.194
NA	-0.93	-0.52	0.001	0.170
JCHAIN	-0.92	-0.53	<0.001	0.014
EEF1B2P2	-0.92	-0.53	0.001	0.133
ALX1	-0.92	-0.53	0.002	0.194
NA	-0.90	-0.54	0.001	0.165
SERPINA1	-0.89	-0.54	<0.001	0.101
NA	-0.89	-0.54	0.001	0.159
TRGC2	-0.88	-0.54	0.002	0.198
CDA	-0.86	-0.55	0.001	0.162
C4BPB	-0.85	-0.55	0.001	0.125
FGF16	-0.85	-0.56	<0.001	0.058
CD300LF	-0.84	-0.56	0.002	0.197
NA	-0.83	-0.56	<0.001	0.105
LINC00648	-0.82	-0.57	<0.001	0.094
RPS12P23	-0.81	-0.57	<0.001	0.032
TSPEAR-AS1	-0.80	-0.57	0.002	0.198

LSP1P1	-0.80	-0.57	<0.001	0.101
RPLP1P6	-0.79	-0.58	0.001	0.150
TNFAIP6	-0.79	-0.58	0.002	0.198
LINC01431	-0.78	-0.58	<0.001	0.105
MFAP5	-0.78	-0.58	<0.001	0.050
NA	-0.78	-0.58	<0.001	0.115
HSPB2	-0.76	-0.59	<0.001	0.035
NA	-0.76	-0.59	<0.001	0.015
CCL8	-0.76	-0.59	0.002	0.181
LINC01436	-0.76	-0.59	<0.001	0.115
NA	-0.75	-0.59	0.001	0.129
NA	-0.74	-0.60	<0.001	0.055
TPPP3	-0.74	-0.60	<0.001	0.115
NCF1	-0.74	-0.60	0.002	0.194
NA	-0.73	-0.60	0.002	0.194
PXDNL	-0.73	-0.60	0.001	0.150
FNDC5	-0.71	-0.61	0.002	0.194
NA	-0.71	-0.61	0.001	0.168
NA	-0.71	-0.61	0.001	0.158
CPB1	-0.71	-0.61	<0.001	0.075
TMEM71	-0.71	-0.61	0.002	0.189
NA	-0.70	-0.61	<0.001	0.108
CNFN	-0.70	-0.61	<0.001	0.115
SNHG9	-0.68	-0.63	0.001	0.138
NA	-0.66	-0.63	0.001	0.168
RPL30P14	-0.66	-0.63	<0.001	0.058
DNAJC27-AS1	-0.65	-0.64	<0.001	0.101
NA	-0.64	-0.64	0.001	0.138
PLAC9	-0.64	-0.64	0.001	0.170
NA	-0.63	-0.65	0.001	0.150
NA	-0.63	-0.65	0.002	0.197
C17orf58	-0.62	-0.65	<0.001	0.075
NA	-0.62	-0.65	0.002	0.194
NA	-0.62	-0.65	0.001	0.138
TNFAIP8L2	-0.61	-0.65	0.002	0.198
FCN1	-0.60	-0.66	0.002	0.197
B4GALT1-AS1	-0.60	-0.66	<0.001	0.115
RPL27AP	-0.60	-0.66	0.001	0.170
MMP27	-0.59	-0.66	0.001	0.162
RGCC	-0.59	-0.66	<0.001	0.115
NA	-0.58	-0.67	0.001	0.153
NA	-0.58	-0.67	0.002	0.201

LST1	-0.57	-0.67	0.002	0.181
MIR4458HG	-0.55	-0.68	0.001	0.179
FCER1G	-0.55	-0.68	0.002	0.179
NA	-0.54	-0.69	0.002	0.194
NA	-0.53	-0.69	0.001	0.159
MT2P1	-0.53	-0.69	0.001	0.159
HOXC-AS1	-0.52	-0.70	0.002	0.179
FMO3	-0.52	-0.70	0.001	0.150
MT1X	-0.52	-0.70	0.002	0.201
PIN4	-0.51	-0.70	0.002	0.191
ST3GAL5	-0.50	-0.71	0.001	0.158
UBL5P2	-0.49	-0.71	0.001	0.168
NA	-0.49	-0.71	0.002	0.194
ACKR3	-0.49	-0.71	0.001	0.163
EVI2A	-0.48	-0.72	0.002	0.189
NA	-0.45	-0.73	0.001	0.178
NA	-0.45	-0.73	0.002	0.194
CNRIP1	-0.42	-0.75	0.002	0.201
CEBPD	-0.41	-0.75	0.001	0.165
FSTL1	-0.40	-0.76	0.002	0.198
LOX	-0.40	-0.76	0.001	0.168

FC, fold change; FDR, false discovery rate

Log2 FC and *P* values were obtained by comparison of data from obese individuals vs data from normal weight individuals in a general linear model likelihood ratio test in EdgeR software

Grey highlight indicates FDR >1.0

Appendix P Gantt chart of activities undertaken for the duration of the project

Activities	Year 2 – 2015 - 2016											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												

Writing thesis												
	Year 3 – 2016 - 2017											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												
Writing thesis												

	Year 4 –2017 - 2018											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												
Writing thesis												

Appendix P

	Year 5 –2018 - 2019											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												
Writing thesis												

	Year 6 –2019 - 2020											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												
Writing thesis												

Appendix P

	Year 7 –2020- 2021											
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Reading												
Lab training												
Fatty acid data analysis												
Lipid mediator extraction												
UPLC-MS/MS Lipid mediator analysis												
Lipid mediator data analysis												
RNA-seq data analysis												
RNA extraction + cDNA synth												
qRT-PCR												
qRT-PCR data analysis												
Writing transfer thesis												
Conferences												
Immunohistochemistry lab work												
Immunohistochemistry data analysis												
Protein extraction												
Enzyme activity assay												
Western blot analysis												
Writing papers												
Writing thesis												

Appendix Q List of relevant publications

Publication of data reported within this thesis:

Fisk HL, Childs CE, Miles EA, Ayres R, Noakes PS, Paras-Chavez C, Kuda O, Kopecky J, Antoun E, Lillycrop K, Calder PC. Endocannabinoid concentrations in human subcutaneous adipose tissue in obesity: Modulation by omega-3 polyunsaturated fatty acids. Under revision for Clin Sci, Oct 2020.

Publication of methodology reported within this thesis:

Fisk HL, West AL, Childs CE, Burdge GC, Calder PC. The use of gas chromatography to analyse compositional changes of fatty acids in rat liver tissue during pregnancy. J Vis Exp. 13 (85), 2014, 10.3791/51445.

Publication of data not included in this thesis but using techniques reported herein:

1. **Fisk HL**, Kindberg GM, Hustvedt SO, Calder PC. A novel omega-3 glyceride mixture enhances enrichment of eicosapentaenoic acid and docosahexaenoic acid after single dosing in healthy older adults: results from a double-blind crossover trial. Br J Nutr. 2020 Oct 13:1-19Online ahead of print.
2. Kermack AJ, Wellstead SJ, **Fisk HL**, Cheong Y, Houghton FD, Macklon NS, Calder PC. The Fatty Acid Composition of Human Follicular Fluid Is Altered by a 6-Week Dietary Intervention That Includes Marine Omega-3 Fatty Acids. Lipids. 2020 Oct 12. Online ahead of print.
3. Kermack AJ, Lowen P, Wellstead SJ, **Fisk HL**, Montag M, Cheong Y, Osmond C, Houghton FD, Calder PC, Macklon NS. Effect of a 6-week "Mediterranean" dietary intervention on in vitro human embryo development: the Preconception Dietary Supplements in Assisted Reproduction double-blinded randomized controlled trial. Fertil Steril. 2020 Feb;113(2):260-269.
4. Crozier SR, Godfrey KM, Calder PC, Robinson SM, Inskip HM, Baird J, Gale CR, Cooper C, Sibbons CM, **Fisk HL**, Burdge GC. Vegetarian Diet during Pregnancy Is Not Associated with Poorer Cognitive Performance in Children at Age 6-7 Years. Nutrients. 2019 Dec 11;11(12):3029.
5. Candreva T, Kühl CMC, Burger B, Dos Anjos MBP, Torsoni MA, Consonni SR, Crisma AR, **Fisk HL**, Calder PC, de Mato FCP, Sernaglia EM, Vinolo MAR, Rodrigues HG. Docosahexaenoic acid slows inflammation resolution and impairs the quality of healed skin tissue. Clin Sci (Lond). 2019 Nov 29;133(22):2345-2360.
6. Russell K, Zhang HG, Gillanders LK, Bartlett AS, **Fisk HL**, Calder PC, Swan PJ, Plank LD. Preoperative immunonutrition in patients undergoing liver resection: A prospective randomized trial. World J Hepatol. 11(3), 2019, 305-317.

7. Burger B, Kühl CMC, Candreva T, Cardoso RDS, Silva JR, Castelucci BG, Consonni SR, **Fisk HL**, Calder PC, Vinolo MAR, Rodrigues HG. Oral administration of EPA-rich oil impairs collagen reorganization due to elevated production of IL-10 during skin wound healing in mice. Sci Rep. 2019, 9(1):9119.
8. Osowska S, Kunecki M, Sobocki J, Tokarczyk J, Majewska K, Omidi M, Radkowski M, **Fisk HL**, Calder PC. Effect of changing the lipid component of home parenteral nutrition in adults. Clin Nutr. 18, 2018, S0261-5614.
9. Crozier SR, Sibbons CM, **Fisk HL**, Godfrey KM, Calder PC, Gale CR, Robinson SM, Inskip HM, Baird J, Harvey NC, Cooper C, Burdge GC; Southampton Women's Survey (SWS) Study Group. Arachidonic acid and DHA status in pregnant women is not associated with cognitive performance of their children at 4 or 6-7 years. Br J Nutr. 119 (12), 2018, 1400-1407.
10. **Fisk HL**, Irvine M, Miles EA, Lietz G, Mathers JC, Packard CJ, Armah CK, Kofler BM, Curtis PJ, Minihane AM, Calder PC. Association of oily fish intake, sex, age, BMI and APOE genotype with plasma long-chain n-3 fatty acid composition. Br J Nutr. 120 (1), 2018 Jul, 120(1), 23-32.
11. de Pablo P, Romaguera D, **Fisk HL**, Calder PC, Quirke AM, Cartwright AJ, Panico S, Mattiello A, Gavrila D, Navarro C, Sacerdote C, Vineis P, Tumino R, Ollier WE, Michaud DS, Riboli E, Venables PJ, Fisher BA. High erythrocyte levels of the n-6 polyunsaturated fatty acid linoleic acid are associated with lower risk of subsequent rheumatoid arthritis in a southern European nested case-control study. Ann Rheum Dis. 77 (7), 2018 Jul, 981-987.
12. Fuller R, Moore MV, Lewith G, Stuart BL, Ormiston RV, **Fisk HL**, Noakes PS, Calder PC. Yeast-derived β-1,3/1,6 glucan, upper respiratory tract infection and innate immunity in older adults. Nutrition. 2017, 39-40:30-35.
13. Jeffery L, **Fisk HL**, Calder PC, Filer A, Raza K, Buckley CD, McInnes I, Taylor PC, Fisher BA. Plasma Levels of Eicosapentaenoic Acid Are Associated with Anti-TNF Responsiveness in Rheumatoid Arthritis and Inhibit the Etanercept-driven Rise in Th17 Cell Differentiation in Vitro. Rheumatol. 44 (6), 2017, 748-756.
14. Hall TC, Bilku DK, Neal CP, Cooke J, **Fisk HL**, Calder PC, Dennison AR. The impact of an omega-3 fatty acid rich lipid emulsion on fatty acid profiles in critically ill septic patients. Prostaglandins Leukot Essent Fatty Acids. 112, 2016, 1-11.
15. Eltweri AM, Thomas AL, **Fisk HL**, Arshad A, Calder PC, Dennison AR, Bowrey DJ. Plasma and erythrocyte uptake of omega-3 fatty acids from an intravenous fish oil based lipid emulsion in patients with advanced oesophagogastric cancer. Clin Nutr. 36 (3), 2017, 768-774.
16. A.M. Eltweri, A.L. Thomas, H.L. Fisk, P.C. Calder, A.R. Dennison, D.J. Bowrey. SUN-P071: Cellular and Plasma Uptake of Omega-3 Fatty Acids Following Omegaven® Infusion in Patients with Advanced Oesophago-Gastric Adenocarcinoma. Clin Nutr. 35 (s1), 2016, S70–S71.
17. Chong MF, Ong YL, Calder PC, Colega M, Wong JX, Tan CS, Lim AL, **Fisk HL**, Cai S, Pang WW, Broekman BF, Saw SM, Kwek K, Godfrey KM, Chong YS, Gluckman P, Meaney MJ, Chen H; GUSTO

Study Group. Long-chain polyunsaturated fatty acid status during pregnancy and maternal mental health in pregnancy and the postpartum period: results from the GUSTO study. J Clin Psychiatry. 76 (7), 2015 Jul; e848-56.

18. Olthof ED, Roelofs HM, **Fisk HL**, Calder PC, Wanten GJ. No Clinical or Biochemical Evidence for Essential Fatty Acid Deficiency in Home Patients Who Depend on Long-Term Mixed Olive Oil- and Soybean Oil-Based Parenteral Nutrition. JPEN J Parenter Enteral Nutr. 40 (7), 2016, 982-8.
19. Yu YM, Chan YH, Calder PC, Hardjojo A, Soh SE, Lim AL, **Fisk HL**, Teoh OH, Goh A, Saw SM, Kwek K, Gluckman PD, Godfrey KM, Chong YS, Shek LP, Pan A, Chong MF, van Bever HP; GUSTO study group. Maternal PUFA status and offspring allergic diseases up to the age of 18 months. Br J Nutr. 113 (6), 2015 Mar 28; 975-83.

Appendix R Conference presentations and awards received during student candidature

2020: SCI Young Lipid Scientist of the Year Award

Oral presentation: Adipose tissue inflammation in human obesity and response to chronic marine omega3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

Award: Winner.

2019: FENS 13th European Nutrition Conference

Oral poster presentation: Adipose fatty acid composition and gene expression in obesity, and response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

Oral poster presentation: Adipose tissue fatty acid and lipid mediator composition in obesity and response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

2018: 16th Euro Fed Lipid Congress Fats, Oils and Lipids: Science, Technology and Nutrition in a Changing World

Oral presentation: The effect of obesity on adipose tissue fatty acid composition and lipid mediators, and their response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

2018: 13th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL)

Oral presentation: The effect of obesity on adipose tissue fatty acid composition and lipid mediators, and their response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

Award: New investigator award.

2018: ISIN XI International Conference on Immunonutrition

Oral presentation: The effect of obesity on adipose tissue fatty acid composition and lipid mediators, and their response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

2017: SCI Young Lipid Scientist of the Year Award

Oral presentation: Adipose tissue fatty acid and lipid mediator composition in obesity and response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

Award: Finalist.

2017: Biochemical Society congress - Lipid Mediators in Ageing and Disease

Oral presentation: The effect of obesity on adipose tissue fatty acid composition and lipid mediators, and their response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

2016: 12th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL)

Poster presentation: The effect of obesity on adipose tissue fatty acid composition and lipid mediators, and their response to chronic marine omega-3 fatty acid supplementation: The BIOCLAIMS study at the University of Southampton.

Award: Young investigator award.

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