

Understanding the role of obesity-mediated white adipose tissue and liver crosstalk

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Abstract

Obesity drives white adipose tissue (WAT) changes which can lead to its dysregulation and insulin resistance. The obesity-mediated loss of WAT homeostasis can trigger liver steatosis through dysregulated lipid pathways such as those related to polyunsaturated fatty acid (PUFA)-derived oxylipins. Cross-tissue dynamics of the WAT and liver in progressive obesity remains elusive and the exact relationship between oxylipins, metabolic syndrome and metabolic dysfunction-associated liver disease (MASLD) is ill-defined. My aim was to understand the transition from adaptive to maladaptive WAT homeostasis in humans and its repercussion on liver homeostasis. Using oxylipins as potential messengers between WAT and the liver, my objective was to clarify their role in obesity-related T2DM/MASLD.

Methods: I phenotyped the omental WAT (oWAT) of N=88 patients with obesity undergoing bariatric surgery and n=9 control patients and correlated the obesity-related phenotype with MASLD progression in N=41 paired liver biopsy, plasma and oWAT samples. I integrated these findings with multi-tissue profiling (liver, oWAT and plasma) of PUFA-derived oxylipins during progression toward insulin resistance and MASH. Potential drug-effects were investigated through analysis of Metformin-treated fat explants *ex vivo*.

Findings: Obesity-mediated adipocyte hypertrophy is associated with down-regulation of PPAR γ and CEBP α independently of T2DM, increased macrophage infiltration and reduction in Collagen type I and VI. Obese oWAT explants treated with Metformin demonstrated increased adipogenic markers (PPARY, FABP4, CD36). Multi-tissue oxylipin profiling revealed a generalized down-regulation of cytochrome P450 (CYP)-derived diols during obesity conserved between the oWAT and plasma. Notably, epoxide:diol ratio, indicative of soluble epoxide hydrolase (sEH) activity, increased with hepatic steatosis and T2DM.

Interpretation: These results reveal a distinct oWAT phenotype occurring with obesity and metabolic syndrome and propose Metformin is adipogenic. The oxylipin signature in obesity suggests a possible role of fatty acid diols during metabolic syndrome and a dampened sEH activity in WAT and liver in T2DM/MASLD

Statement of Originality

I declare that all the work included in this thesis is my own. Where work from others has been included, this has been acknowledged within the text.

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Published work

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Abbreviations

A	AA	Arachidonic acid
	α -LA	Alpha-linoleic acid
	ACC	Acetyl-Co A carboxylase
	ACS	Acyl-CoA synthetase
	Akt	Protein Kinase B
	ALOX	Arachidonate lipoxygenase
	AMPK	AMMP-dependent protein kinase
	ANOVA	Analysis of Variance
	AUC	Area under the operator curve
	ATGL	Adipocyte triglyceride lipase
	ATM	Adipose tissue macrophages
B	BAT	Brown adipose tissue
	BMI	Body mass index
	BMP	Bone morphogenetic protein
	dsDNA	Double-stranded DNA
C	CAF	Cafeteria diet
	CCL	C-C motif chemokine ligands
	CD	Cluster of differentiation
	cDNA	Complementary DNA
	C/EBP	CCAAT enhancer-binding protein
	CLS	Crown-like-structures
	COX	Cyclooxygenase
	CPA	Collagen proportionate area
	CREB	CAMP-Responsive Element-Binding Protein
	CYP	Cytochrome-P450
D	DAG	Diacylglycerol
	DGAT	Diacylglycerol acyltransferase
	DHA	Docosahexaenoic acid

	DHET	Dihydroxyeicosatrienoic acid
	DiHOME	Dihydroxy-octadecenoic acid
	DiHDPE	Dihydroxy-docosapentaenoic acid
	DGLA	Dihomo- γ -linoleic acid
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	DNL	<i>de novo</i> lipogenesis
E	ECM	Extracellular matrix
	EET	Epoxyeicosatrienoic acid
	EPA	Eicosapentaenoic acid
	EPHX1	Epoxide hydrolase gene 1
	EpOME	Epoxyoctadecenoic acid
	ER	Endoplasmatic reticulum
	ERK	Extracellular signal-regulated kinases
F	FABP	Fatty acid binding protein
	FASN	Fatty acid synthase complex
	FATP	Fatty acid transport gene
	FDR	False discovery rate
	FFA	Free fatty acid
G	GLP-1	Glucagon like protein-1
	GSV	GLUT4-containing storage vesicles
	GWAS	Genome Wide Association Study
H	HbA1c:	Glycated haemoglobin
	HCC	Hepatocellular carcinoma
	HDL	High density lipoprotein
	HETE	Hydroxyeicosatetraenoic acid
	HFD	High fat diet
	HGP	Hepatic glucose production
	HODE	Hydroxyoctadecadienoic acid
	HOMA-IR	Homeostatic Model Assessment of Insulin Resistance

	HpETE	Hydroperoxyeicosatetraenoic acid
I	IHC	Immunohistochemistry
	IF	Immunofluorescence
	IR	Insulin resistance
	IRS	Insulin receptor substrate
K	KC	Kupffer cell
	KLF	Kruppel-Like factors
	KO	Knock out
L	LA	Linolenic acid
	LAMS	Lipid-associated macrophages
	LC-MS	Liquid chromatography-mass spectrometry
	LDL	Low density lipoprotein
	LEP	Leptin gene
	LOX	Lipoxygenase
	LPL	Lipoprotein lipase
	LPS	Lipopolysaccharide
M	MAGL	Monoacylglycerols
	MASH	Metabolic dysfunction-associated steatotic hepatitis
	MASLD	Metabolic dysfunction-associated steatotic liver disease
	MMe	Macrophage metabolically activated
	MMP	Matrix metalloproteinases
	mRNA	Messenger Ribonucleic acid
	MSC	Mesenchymal stem cells
	MUFA	Monounsaturated fatty acid
	MS	Metabolic Syndrome
N	NAS score	NAFLD Activity Score
	NICE	National Institute of Clinical Excellence
O	oWAT	Omental white adipose tissue
P	PCR	Polymerised chain reaction
	PI3k	Phosphoinositide 3-kinase

	PLA2	Phospholipase A2
	PLIN1	Perilipin1
	PPAR:	Peroxisome Proliferator-Activated Receptor
	PUFA	Polyunsaturated fatty acids
Q	qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
R	RNA	Ribonucleic acid
	ROS	Reactive oxygen species
S	SAT	Subcutaneous adipose tissue
	Sc-RNA-seq	Single cell RNA sequencing
	scWAT	Subcutaneous white adipose tissue
	sEH	Soluble epoxide hydrolase
	sEHi	Soluble epoxide hydrolase inhibitor
	SFA	Short chain fatty acids
	SLD	Steatotic liver disease
	SNP	Single Nucleotide Polymorphism
	SREBP1	Sterol response element binding protein
	STAT	Signal transducer and activator of transcription
	SVF	Stromal vascular fraction
T	TAG	Tryacylglyceride
	TCA	Tricarboxylic acid
	T2DM	Type 2 Diabetes Mellitus
	TG	Triglyceride
	TGF β	Transcription growth factor beta
	TLR	Toll-like receptor
	TNF	Tumour necrosis factor
	TREM	Triggering receptor expressed on myeloid cells protein
U	UPLC-MS/MS	Ultra-high-performance liquid-chromatography-mass spectrometry
V	VAT	Visceral adipose tissue
	VEGF	Vascular endothelial growth factor
	VLDL	Very low-density lipoprotein

W	WAT	White adipose tissue
	WB	Western blot
	WHO	World Health Organisation
	WT	Wild type

Chapter 1: Introduction

1.0 Obesity

The incidence of obesity is increasing worldwide, with the prevalence set to rise from 14% in 2020 to 24% by 2035 [1] and in the US, the prevalence is estimated to increase to 50% of the population by 2030 [2]. The global economic impact of obesity is predicted to rise to \$4 trillion by 2035[1] and the associated health burden is not limited to high-income countries; low- and middle-income countries are also suffering, adding to the burden of malnutrition they already experience [3]. The prevalence of obesity and overweight adolescents and children aged 5-19 is estimated to be 31% in boys and 28% in girls in 2023, up from just 4% in 1975 [4]. The World Health Organisation(WHO) set out a target for obesity: there would be '*no increase in 2010 levels by 2025*', however no country has reported a decline in obesity levels over the last decade [1] which demonstrates the serious health and financial impact globally that obesity will continue to have.

The definition as set by the World Health Organisation for obesity and being overweight is 'abnormal or excessive fat accumulation that may impair health' [5]. The most commonly used measure to classify people is based on their height-for-weight index i.e. Body Mass Index (BMI). A 'healthy' weight is described as a BMI of 18-25kg/m² while a Body Mass Index >25kg/m² is deemed overweight and a BMI > 30 kg/m² is considered obese.

Other anthropological measures including skeletal mass and waist circumference are also used and in some instances are considered more useful for predicting health complications that follow prolonged obesity. A waist circumference of 80-87.8cm in women and 94-101.9cm in men is considered moderate central fat accumulation and increases a person's risk of co-morbidities; a waist circumference of ≥102cm in men and ≥88cm in women is considered high central fat accumulation and leads to a high risk of developing co-morbidities [6].

1.1 Metabolic Syndrome

Obesity and being overweight are considered major risk factors for developing metabolic syndrome (MS) which is a cluster of abnormalities: visceral obesity, insulin resistance, impaired glucose tolerance, reduced high density lipoprotein (HDL) -cholesterol and elevated triglycerides (**Figure 1**). It was first described by Reaven in 1988 as 'syndrome X' or 'insulin resistance syndrome'. In 2001 the National Cholesterol Education Programme Adult Treatment Panel (NCEP ATP III) defined metabolic syndrome as three out of five of the following [7]

a) waist circumference >102cm for men or 88cm for women

b) Hypertriglyceridemia >150 mg/dl (1.695 mmol/L)

c) Low HDL-C < 40 mg/dL (1.04 mmol/dL) in men and <50 mg/dL (1.3mmol/dL) in women

d) Blood pressure $> 130/85$ mmHg

e) High fasting glucose > 100 mg/dL (6.1mmol/L)

This was updated by the International Diabetes Foundation (IDF) in 2005 [8]. Metabolic syndrome has been shown to increase the risk of developing type 2 diabetes (T2DM), cardiovascular disease, stroke, steatotic liver disease (SLD), polycystic ovarian syndrome and obstructive sleep apnoea. The association with MS and the increased risk of developing chronic diseases are the main concern for the growing obese epidemic. Diagnosis therefore poses an opportunity for individuals to take preventative measures and lifestyle choices to reduce the risks of further disease burden.

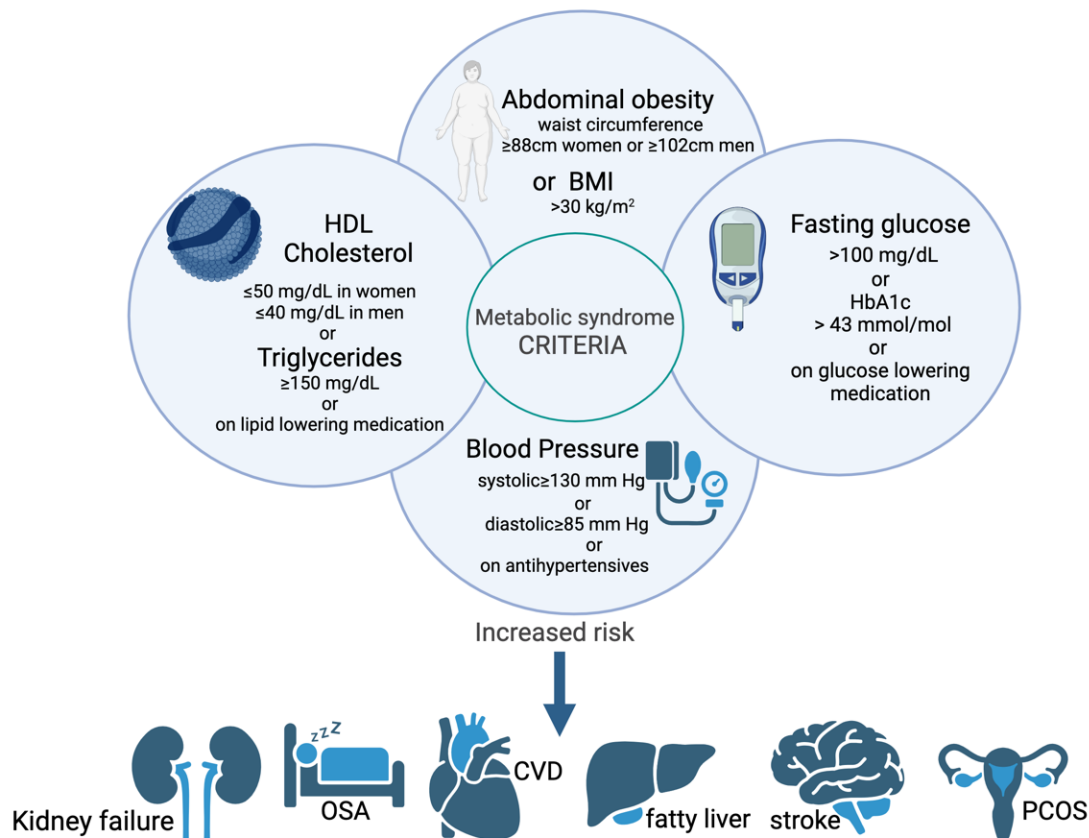


Figure 1 Metabolic syndrome: criteria for diagnosis and associated diseases. Metabolic syndrome is a cluster of abnormalities including abdominal obesity, raised fasting glucose, high blood pressure and high HDL cholesterol or triglycerides. Three out of five abnormalities are required for a diagnosis and predispose a person to increased risk of kidney failure, OSA, CVD, SLD, stroke and PCOS. BMI: body mass index; OSA: obstructive sleep apnoea; CVD: cardiovascular disease; PCOS: polycystic ovarian syndrome; SLD: steatotic liver disease. Figure created by Bio Render®

1.1.1 Aetiology of Obesity

The cause for increasing prevalence of obesity is multi-factorial. Easy access to cheap high energy foods rich in fat and sugars combined with reduced daily physical activity in modern life as a result of urbanization increases the chances of populations becoming overweight [5] and this situation has been coined the 'obesogenic' environment. It is important to note that not everyone who is obese or overweight develops metabolic syndrome nor the associated co-morbidities. One of the most significant factors for developing MS is in fact, childhood obesity with psychosocial stresses also having an important impact [9]. Furthermore, genetic and epigenetic factors also contribute to the variance seen. Twin studies and family adoption studies have demonstrated that obesity is inherited between 40% and 70% [10, 11].

In 2007 the first Genome Wide Associated study (GWAS) discovered the single nucleotide polymorphism (SNP) association of a gene with increased BMI now known as FTO (fat mass and obesity associated) which regulates energy expenditure and appetite [12]. Since then, hundreds of loci have been discovered but it is estimated only 6% of variation in BMI can be accounted for GWAS significant loci [9, 10], highlighting the polygenic nature of this disease.

There have been few single gene mutations associated with significant obesity and these are usually leptin-receptor defects (*LEPR* gene) or result in leptin deficiency due to genes involved in the melanocortin pathway (*MC4R*, *PCSK1* and *POMC*). Obesity related syndromes such as Prada-Willi (abnormality of chromosome 15) or Bardet-Biedl (autosomal recessive disease affecting up to 26 genes) are rare. They are usually the result of chromosomal defects and are present in childhood [9, 10].

1.1.2 Management of Obesity and Metabolic Syndrome

While lifestyle modifications and diet are the mainstay of weightloss and reversal of obesity, there are now several medications (**Table 1**) that have been commissioned by the National Institute of Clinical Excellence that can be used alongside lifestyle modifications to augment weightloss.

Orlistat and liraglutide were the main drugs available until 2021, when Semaglutide was also commissioned for weightloss therapy. Orlistat is an oral inhibitor of fat absorption by inhibiting gastric and pancreatic lipases. It has shown to reduce weight by 5% with dietary modification but side-effects (diarrhoea) are relatively common [13].

Liraglutide is a GLP-1 receptor agonist but is required to be taken daily as a subcutaneous injection. Semaglutide is the newest drug available and is another GLP-1 receptor agonist but is only required to be taken as an injection weekly [14]. NICE has recommended the use of Semaglutide in patients with 1 obesity associated co-morbidity and BMI >30 kg/m² for a

maximum of 2 years by a specialist weight management service. It is not without side-effects but has been shown to be more effective than orlistat and liraglutide and studies show patients lose between 9% and 16% of weight with combined dietary intervention compared to placebo [15, 16].

Other less commonly used drugs include Phentermine/topiramate, a combined oral therapy of amphetamine, an appetite suppressant, and topiramate, an anticonvulsant but should only be used as second line. Naltrexone/bupropion is an oral therapy containing naltrexone (opioid receptor antagonist) and bupropion (noradrenaline-dopamine reuptake inhibitor) but requires caution in patients with seizures, hepatic or renal impairment and previous drug abuse [13].

Stemalanotide (a melanocortin 4 receptor agonist) is used for rare genetic conditions that cause obesity including Bardet-Biedl Syndrome or genetically confirmed PCSK1 or LEPR deficiency [13].

If lifestyle changes and medical treatments do not enable significant weightloss and improve obesity-related co-morbidities, patients can undergo bariatric surgery which has increased 6-fold in the last 20 years. It is now an option for patients with BMI $>35\text{kg/m}^2$ plus one obesity related illness or patients with a BMI $>40\text{kg/m}^2$ [9, 17]. To be able to qualify for bariatric surgery, each patient must have had dietary and lifestyle support that has not led to significant weightloss and be assessed by a psychologist, ensuring they are fit enough to cope with the psychological impact of such life-altering surgery. The main types of surgery available are Roux-en-Y gastric bypass (17.9% of bariatric surgery undertaken), sleeve gastrectomy (59.4%) and revision procedures (16.7%) [18].

Surgery is now done laparoscopically, reducing the risks associated with intra-abdominal surgery. The major complication rate for this high-risk group of patients is 4% and risk of death is 0.1% [18]. Studies have demonstrated up to 70% maximum weightloss with median weightloss at 28% alongside improvement and even remission of T2DM and other obesity related diseases [19, 20]. Gastric bypass is the most successful option for weightloss followed by sleeve gastrectomy [21] but weight gain in an 8 year study was shown to be as high as 50% of patients which was associated with postoperative maladaptive eating habits [22]. Another study showed the failure rates at 55%, [19] with patients who underwent a sleeve gastrectomy requiring conversion to a bypass in 36% of cases due to gastroesophageal reflux or weight gain [20]. Therefore, this highlights the importance of postoperative dietary and lifestyle support. There have also been recent developments endoscopically, with the endoscopic sleeve gastroplasty now being utilised as a low risk (1-2% risk of adverse events) alternative or adjunct to surgery with weightloss of 10-14% of

initial body weight being reported. There is however, limited longitudinal data and only a handful of randomised-control trials [23].

Obesity is a complex, multifactorial disease and bears a significant health and economic burden for future generations despite emerging drugs and surgical options available. How obesity alters human pathophysiology and results in end organ damage still needs to be fully understood which may provide answers for much needed disease prevention. It is clear that insulin resistance and impaired glucose tolerance are closely linked with obesity, T2DM and steatotic liver disease and therefore likely an important factor in disease progression.

Table 1 Table of medications available for treating obesity. GLP-1: Glucagon-like peptide1; BMI: body mass index; CVD: cardiovascular disease.

Medication	Mechanism of action	Indications for use	Side-effects
Semaglutide	GLP-1 receptor analogue	First line: BMI >35kg/m ² , or <35kg/m ² plus non-diabetic hyperglycaemia, high risk of CVD	gastrointestinal, headache, fatigue, hypoglycaemia. Contraindicated in patients with multiple endocrine neoplasm syndrome type 2
Liraglutide	GLP-1 receptor analogue	First line: BMI >35kg/m ² , or <35kg/m ² plus non-diabetic hyperglycaemia, high risk of CVD	gastrointestinal, headache, fatigue, hypoglycaemia. Contraindicated in patients with multiple endocrine neoplasm syndrome type 2
Orlistat	gastric and pancreatic lipase inhibitor	BMI > 30 Kg/m ² b) consider combining with L-carnitine	gastrointestinal(diarrhoea) common
Setmelanotide	melanocortin 4 receptor agonist	rare genetic causes confirmed with genetic testing (POMC/PCSK1/LEPR deficiency)	gastrointestinal, headache, injection-site reactions, skin hyperpigmentation
Naltrexone/bupropion	opioid receptor antagonist plus noradrenaline-dopamine reuptake inhibitor	not NICE approved	avoid in patients with i) seizures ii) substance misuse iii) reduce in hepatic/renal impairment
Phentermine/Topiramate	appetite suppressant and anticonvulsant	not NICE approved	lower doses in hepatic and renal impairment. Topiramate associated with congenital malformations.

1.2 Insulin Resistance

Catabolism, the process in which the human body breaks down food sources for energy is key to cell survival. Metabolism is the sum of catabolic processes minus anabolic processes; where small molecules are transformed into larger molecules e.g. proteins for vital cell processes [24]. When catabolism outweighs anabolism, the body is required to store the excess energy in tissues, mainly the liver, skeletal muscle and adipose tissue. These metabolic processes and storage of excess energy are under the control of several key hormones, of which insulin is the master regulator.

Insulin is an endocrine peptide hormone, with 51 amino acids, an alpha and beta chain linked together by two disulphide bridges plus a further disulphide bridge in the alpha chain [25]. When normal metabolic processes occur, insulin is released by the pancreatic islet beta cells following food intake and increased circulating plasma glucose. Insulin is essential for systemic glucose and lipid metabolism (**Figure 2**). It acts by allowing the uptake of circulating glucose into cells and results in glycogen production. In the liver, insulin promotes glycogenesis (production of glycogen) and *de novo* lipogenesis (storage of fats) and prevents hepatic glucose production (HPG) through inhibiting gluconeogenesis (production of glucose) and glycolysis (glycogen breakdown). In the adipose tissue, insulin promotes lipogenesis (storage of fats through uptake of carbohydrates) and inhibits lipolysis (breakdown of lipids) [26, 27]. Insulin is recognised by the insulin signalling receptor on the cell surface which activates specific adapted proteins through phosphorylation of the insulin receptor substrate (IRS). The main signalling pathways are through activating phosphatidylinositol 3-Kinase (PI3K, a lipid kinase) and protein kinase B (AKT) which have several downstream pathways. AKT promotes GLUT4-containing storage vesicles (GSV) via PI3K to enable glucose uptake into the cell and promotes glycogen synthesis. It also phosphorylates factor forkhead box O1 (FOXO1) which inhibits gluconeogenesis and upregulates mTORC1 which induces protein synthesis and adipogenesis [25, 27]. *De novo* lipogenesis is promoted through dephosphorylation of ACC1/2, the product of upregulation of sterol regulatory element binding protein 1c (SREBP-1c) following mTORC1 upregulation and inhibition of AMPK-dependent protein kinase (AMPK), both downstream pathways to AKT.

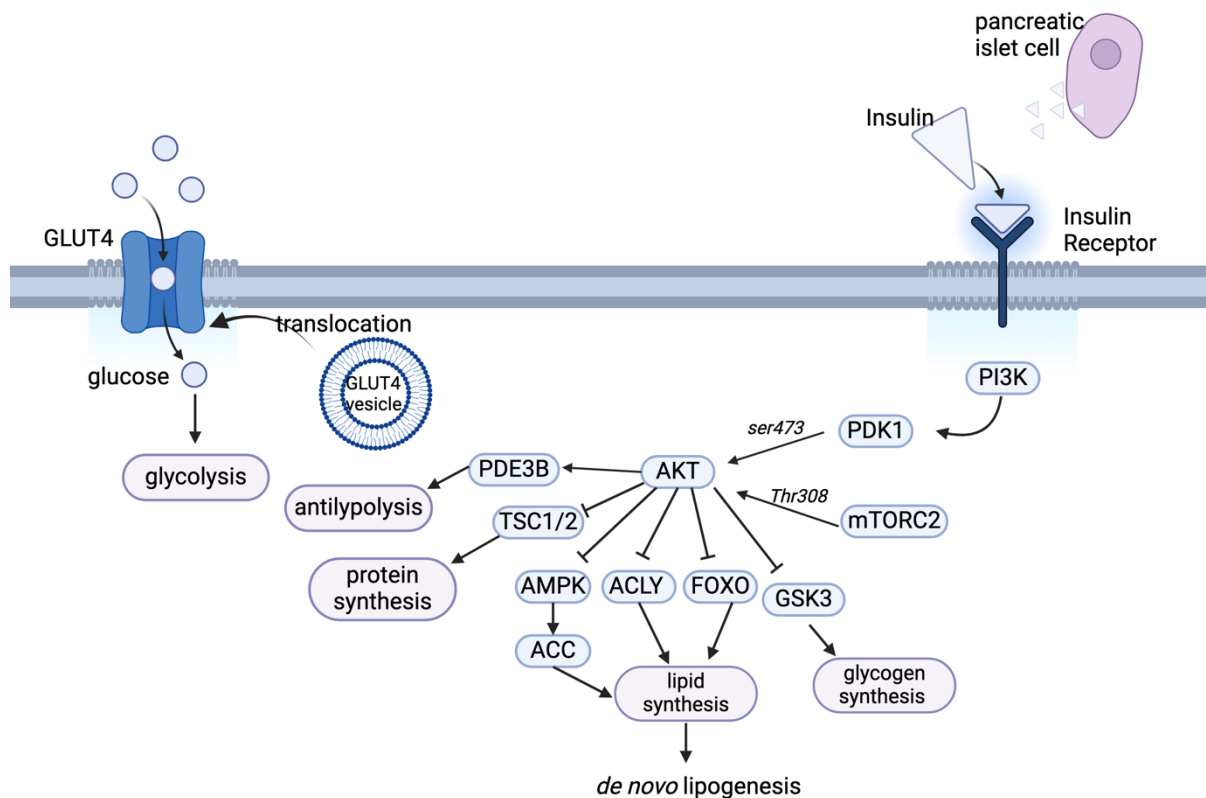


Figure 2 Insulin signalling pathways. Insulin is integral to glucose and lipid metabolism and is released from pancreatic islet cells following the increase of circulating glucose. It acts on the insulin receptor at the cell-surface which in turn activates specific adapted proteins through phosphorylation of the insulin receptor substrate (IRS). The main signalling pathways are through activating phosphatidylinositol 3-Kinase (PI3K, a lipid kinase) and protein kinase B (AKT) which modulate several downstream pathways to enable *de novo* lipogenesis, glycogen synthesis and protein synthesis. Importantly, AKT promotes GLUT4-containing storage vesicles (GSV) via PI3K to enable glucose uptake into the cell. PI3K: phosphatidylinositol 3-Kinase; AKT: protein kinase B; GLUT4: glucose transporter type 4; PDK1: 3-phosphoinositide-dependent kinase 1; mTORC2: Mammalian Target of Rapamycin Complex 2; GSK3: Glycogen synthase kinase 3; FOXO: Forkhead box O; ACLY: ATP citrate lyase; ACC: Acetyl-coenzyme A carboxylase; AMPK: AMP-activated protein kinase; TSC1/2: Tuberous sclerosis complex proteins 1 or 2. PDE3B: phosphodiesterase 3B. Image created using Bio Render®

Insulin resistance (IR), however, can develop when there is no longer effective homeostasis of glucose and lipid metabolism. It is defined as ineffective insulin-mediated glucose uptake into tissue with no or reduced suppression of glucose production or lipolysis. Systemic insulin resistance develops as a result of impaired insulin action in peripheral metabolically active organs including skeletal, adipose and hepatic tissue and is measured as a decrease in the ability to dispose of glucose into tissues [26, 28]. At a tissue level, hepatic IR is due to the failure of insulin to suppress gluconeogenesis with continuing fatty acid synthesis and in adipose tissue IR is due to impaired insulin-stimulated glucose transport and impaired inhibition of lipolysis [29]. This in turn leads to hyperglycaemia and thus increased insulin secretion from beta islet cells and hyperinsulinemia pursues. This theory for the development of systemic IR has been challenged in recent years; it has been proposed that

primary hyperinsulinemia in the context of overnutrition in fact precedes insulin resistance; and studies have shown hyperinsulinemia is a driver of insulin resistance itself and causes the metabolic dysregulation that is observed in obesity [26, 27].

What is clear is that IR has been shown to closely associate with obesity, T2DM and Metabolic dysfunction-associated steatotic liver disease (MASLD); the mechanisms of which are complex and multifactorial. Furthermore, it has been shown that IR is affected by sex [30, 31], ethnicity [9, 32-34], genetics [35] and hormones with gut microbiota also playing a part [36]. At a cellular level mitochondrial dysfunction has been proposed as potential mechanism [37] and the literature shows that insulin resistance can develop through disruptions in multiple downstream pathways involved in insulin signalling, with alterations in FOXO1, GLUT4, insulin receptor (INSR) and AKT pathways amongst others which ultimately prevents the suppression of HGP [38-40]. As insulin resistance progresses to T2DM I shall now discuss this disease and its association with obesity and MASLD.

1.3 Type 2 Diabetes Mellitus

Type 2 Diabetes is characterised by loss of glucose homeostasis through increased insulin resistance in skeletal, liver and adipose tissue driving hyperglycaemia and systemic IR and initially hyperinsulinemia. Eventually there is decreased production of insulin by beta pancreatic islet cells. The prevalence of T2DM worldwide is increasing with 1 in 10 people currently diagnosed as diabetic and over 643 million expected to be diabetic by 2030 [41]. 2.5million deaths in 2019 were attributed to diabetes and life expectancy is shown to be reduced by up to 10 years compared to people without T2DM [42]. The risk of developing coronary vascular disease (HR 2.0 95% CI 1.83-2.19), stroke (ischaemic: HR 1.56 CI 95% 1.19-2.05 haemorrhagic HR 1.84; CI 95% 1.59-2.13), and renal failure are all shown to increase two-fold with T2DM[43]. One third of patients with Type 2 diabetes will develop renal disease and 60% of people will develop retinopathy within 20 years [44]. Other complications from T2DM include diabetic neuropathy, diabetic foot disease, MASLD, sexual dysfunction and depression. This multi-organ disease is therefore a huge health burden and is thought to already cost 12 billion a year for the NHS [45].

1.3.1 Clinical Diagnosis of T2DM

The clinical diagnosis of T2DM as set by WHO in 2011 is:

Symptoms of diabetes (polyuria, polydipsia)

- i) a fasting glucose concentration of ≥ 7 mmol/l.
- ii) a random venous plasma glucose ≥ 11.1 mmol/l.

- iii) after administering 75g anhydrous glucose as oral glucose tolerance test (OGTT) a plasma glucose concentration of ≥ 11.1 mmol/l.
- iv) a haemoglobin A1c(HbA1c) of ≥ 48 mmol/mol (6.5%) [46]

HbA1c, also known as glycated haemoglobin, is a marker of the last 8-12 weeks' glucose control. Importantly, it is not affected by the individual's fasting status nor the time at which the blood sample was taken. Haemoglobin A in the erythrocyte bonds with monosaccharides in the plasma and once glycated, remains that way until the end of its life cycle which is on average 108(female) -117(male) days thus giving a measure of glucose exposure over this period. It has been shown to correlate with risk of developing both micro and macrovascular complications of diabetes [44]. However, if a person has a genetic haemoglobinopathy this can impact glycation and thus other diagnostic measures need to be used[47]. WHO sets the normal range of HbA1c as <42 mmol/mol, pre-diabetes (i.e. insulin resistance) 42-47mmol/mol and Diabetes >48 mmol/mol. It also used to guide treatment of T2DM [46] with the aim of reducing HbA1c to normal levels.

1.3.2 Treatment options for T2DM

The primary method of reversing insulin resistance and improved glucose homeostasis is weightloss through a healthy diet and exercise where loss of 3-10% of body weight can induce remission [48]. There are also multiple medication options available. NICE has comprehensive guidelines on initiating anti-glycaemic drugs(summarised in **Table 2**) [49]. The first line being Metformin; a drug that primarily reduces gluconeogenesis in the liver. It is the most widely used drug due to its minimal side-effect profile and does not cause hypoglycaemia. Sulfonylureas (e.g. Gliclazide) are used as a rescue therapy when a patient has symptomatic hyperglycaemia or if Metformin is contraindicated. Sulfonylureas directly stimulate the release of insulin from pancreatic beta islet cells. Sodium-glucose co-transporter (SGLT2) inhibitors ('flozins' e.g. Empagliflozin) are added if a patient has a high risk of cardiovascular disease. SGLT2 inhibitors prevent reabsorption of glucose in the proximal convoluted tubules of the kidney[25] thus reducing circulating glucose levels.

If patients are unable to tolerate Metformin or HbA1c is not within controlled range Dipeptidyl peptidase-4 (DPP-4) inhibitors ('gliptins' eg. Linagliptin), Sulfonylureas or Pioglitazone (PPAR γ agonist) can be used or added for dual therapy. DPP-4 inhibitors increase concentrations of incretin hormones GLP-1 and insulinotropic peptide GIP, inhibiting glucagon release and improving beta cell response [50]. Thiazolidinediones (Pioglitazone) is a PPAR γ agonist that improves insulin sensitivity. PPAR γ is a transcription factor mainly expressed in the adipose tissue and is the master regulator of adipocyte differentiation which in turn, allows increased storage of free fatty acids and glucose in white adipose tissue, thus

reducing circulating levels. It also induces the expression of GLUT4, adiponectin and decreases TNF- α , IL-6 and leptin [25] in adipose tissue, resulting in insulin sensitivity and improved glucose control, but paradoxically leads to weight gain and fluid retention which have limited its use in clinical practice [51].

If dual therapy with Metformin or 2 other drugs does not control HbA1C, insulin can be added to a regime. Triple oral therapy can also be used in combination with GLP-1 mimetic treatments if BMI > 30kg/m² or if insulin would have a significant impact on occupation [49]. The aim of treatment is to prevent complications of long term T2DM through maintaining a normal glycaemic level.

Table 2 Table of NICE approved medications for T2DM. SGLT2: Sodium-glucose co-transporter2; DDP-4: Dipeptidyl peptidase-4; GLP-1: glucagon-like-peptide-1; PPAR- γ : Peroxisome proliferator-activated receptor gamma; PCOS: Polycystic ovarian syndrome; T2DM: Type 2 Diabetes Mellitus.

Medication	Options	mechanism of action	Indications for use	Effect on weight	side-effects
Biguanide	Metformin	decreases gluconeogenesis	First line: T2DM or PCOS	none	gastrointestinal disorder, acute metabolic acidosis
Sulfonylureas	Gliclazide	increase insulin secretion from pancreatic β -cells by binding to β -cell sulfonyl urea receptor	First line: if Metformin contraindicated or Second line	gain	ketoacidosis, hypoglycaemia
Sodium-glucose co-transporter (SGLT2) inhibitors: 'flozins'	Empagliflozin Dapagliflozin	Sodium-glucose co-transporter (SGLT2) inhibitor, increasing renal sodium and glucose excretion	First line: T2DM plus cardiovascular disease of heart failure	loss	ketoacidosis
Dipeptidyl peptidase-4 (DPP-4) inhibitors: 'gliptins'	Linagliptin, Saxagliptin	Dipeptidyl peptidase-4 (DPP-4) inhibitor prevents degradation of incretins	First line: if Metformin contraindicated or Second line	none	ketoacidosis
Thiazolidinediones	Pioglitazone	PPAR- γ agonist	First line: if Metformin contraindicated or Second line	gain	bladder cancer, heart failure
GLP-1 receptor agonist	Liraglutide, Semaglutide	GLP-1 receptor analogue stimulates insulin secretion from pancreatic islets	Third line: If 2 drugs plus Metformin not tolerated/not effective/contraindicated	loss	ketoacidosis, gastrointestinal disorder,
Insulin	Long or short acting	Increases glucose uptake by directly binding to insulin receptor	Symptomatic hyperglycaemia or HbA1c not controlled	gain	hypoglycaemia

Bariatric surgery has also been shown to both reduce and, in some cases, completely reverse insulin resistance in patients with T2DM. Roux-en-Y gastric bypass seemed to improve glycaemic control the most (29% of patients) versus gastric bypass (23%)[52]. The mechanisms involved are not fully understood, reductions in visceral adipose tissue due to

weightloss, and the hormone changes that pursue, are thought to be the most likely cause [52, 53].

T2DM is characterised by insulin resistance and as previously described insulin homeostasis is tightly regulated by adipose tissue, skeletal muscle and the liver. Thus, the association between T2DM and metabolic associated steatotic liver disease (MASLD) is well characterised with metabolic syndrome a predisposing factor for both end-organ diseases. Next, I shall explore the relationship MASLD has with obesity, insulin resistance and T2DM.

1.4 Metabolic dysfunction associated steatotic liver disease

Metabolic dysfunction associated steatotic liver disease (MASLD) [54], previously known as non-alcoholic fatty liver disease (NAFLD) was first coined by Ludwig in the 1980s. A recent consortium has renamed the disease MASLD/MASH to prevent stigma associated with the previous nomenclature. Ludwig's seminal 20 patient case study of mainly women, described the salient histological features (discussed in detail below) of non-alcoholic steatohepatitis (NASH), now known as metabolic associated steatohepatitis (MASH) [55].

Several meta-analyses conducted in the last 5 years all demonstrate the rapid increase in prevalence of MASLD over the last 2 decades, in line with obesity and T2DM. There has been an overall increase from 25.5% of the world population pre 2005 with MASLD to 37.8% in 2019 [56] which equates to an overall increase of 0.7% per year [57, 58]. The prevalence varied from continent to continent, Western Europe and America had the highest rates and South America has had the most dramatic rise in cases. Li et al. estimated 58.2% (95% CI 52.5%-63.7%) of people classed as obese and 58.9% (95% CI 54.8%-63.0%) of people with T2DM had MASLD [58]. When assessing co-morbidities of the people diagnosed with MASLD, 40% were found to have metabolic syndrome, 14% T2DM and 37% hypertension [58].

1.4.1. Definition of MASLD

MASLD is described as fatty infiltration of the liver (>5% of hepatocytes) without a history of excessive consumption of alcohol (no more than 30g per day) and with no other secondary causes of hepatic fat accumulation (e.g. drugs) [59]. Steatotic liver disease is a spectrum; at one end simple steatosis, defined as fatty accumulation in the liver with no or minimal inflammation or hepatic injury (MASLD), and metabolic-associated steatohepatitis (MASH), cirrhosis and HCC at the other [60] (**Figure 3**).

Simple steatosis is classified as steatosis in >5% of hepatocytes either on histology or cross-sectional imaging. MASLD encompasses a) simple steatosis and b) steatosis with features of either lobular inflammation or ballooning, but not both features histologically [61]. Twenty-five

percent of people with steatosis will develop MASH [62] which is defined by lobular inflammation *and* hepatocyte cell ballooning alongside fatty infiltration; with the presence of all three features required for diagnosis. Mallory-Denk bodies and fibrotic changes may also be present but are not required for diagnosis. Ballooning is a histological feature used to describe the injured enlarged hepatocyte 1.5-2 times the normal size with the loss of its polygonal shape, various cytoplasmic vacuoles and often Mallory Denk bodies are present in the cytoplasm [63]. Once fibrosis progresses it can lead to cirrhosis in 25% of patients with MASH and end stage liver failure and the risk of hepatocellular carcinoma (HCC) increases [60, 64, 65].

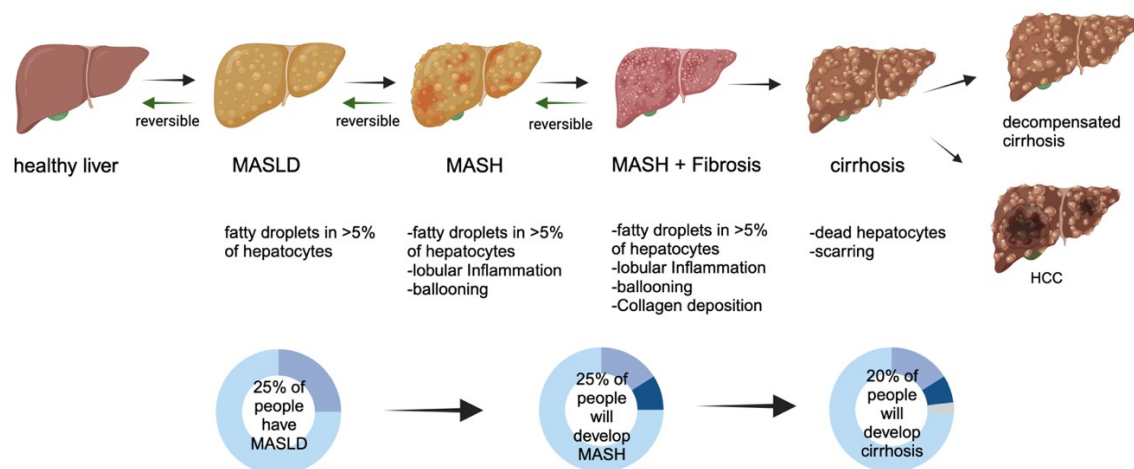


Figure 3 Spectrum of SLD progression from simple steatosis to cirrhosis and HCC. Diagnosis is based on histology from liver biopsies and determines the difference between MASLD (fatty droplets in >5% of hepatocytes), MASH (fatty droplets in >5% of hepatocytes, lobular inflammation and ballooning), MASH plus Fibrosis (MASH plus collagen deposition), cirrhosis (dead hepatocytes and scarring). Image created using BioRender® SLD: steatotic liver disease; MASLD: metabolic-associated steatotic liver disease; MASH: metabolic-associated steatohepatitis. Image produced by Bio Render®

1.4.2. Staging of MASLD: Histological score

The Pathology Committee of the NASH Clinical Research Network designed and validated a scoring system that encompasses the full spectrum of MASLD in 2005 and remains the most widely used scores to assess disease activity both clinically and in academia. Only two histochemistry stains are required to interpret the biopsies, haematoxylin and eosin (H&E) staining plus Masson's trichrome staining or Sirius red. The 'NAFLD Activity Score' (NAS) is split into three components: Steatosis (Score 0: <5% hepatocytes, Score 1: 5-33% hepatocyte, Score 2: >33%-66% hepatocytes, Score 3: >66%), lobular inflammation (Score 0 : no foci, Score 1 < 2 foci/200x, Score 2 : 2-4 foci/200x, Score 3: >4 foci/200x) and hepatocyte ballooning (Score 0: none, Score 1: few balloon cells, Score 2: many cells/ prominent ballooning). The NAS score has been validated to predict a diagnosis of

MASH/MASH with a total score out of 8. A total NAS score of 0-2 indicated no diagnosis of MASH, a score of 3-4 was considered borderline with some considered MASH and others non-MASH and a score of 5 or more were largely considered diagnostic of MASH [66]. However, in recent years, there has been concerns that ballooning is underrepresented in this scoring system.

Fibrosis grade is a separate scoring system and often called the Kleiner Fibrosis Score. Fibrosis is scored 0 to 4; no fibrosis :0 (F0), perisinusoidal or periportal :1 (1A: mild, zone 3, perisinusoidal, 1B: moderate, zone 3 perisinusoidal, 1C: portal/ periportal) (F1), perisinusoidal and portal/periportal :2 (F2), bridging fibrosis: 3 (F3) and cirrhosis: 4 (F4) [66].

1.4.3 Diagnosing MASLD/MASH and current limitations

The gold standard for diagnosis of MASLD/MASH is a liver biopsy, an invasive procedure not without risk of complications. Therefore, NICE, European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Disease (AASLD) all agree that it is not required in all patients to diagnose MASLD [59]. However, there is no standardised non-invasive single test that is reliably able to diagnose MASLD from MASH and cirrhosis [59]. Instead, if MASLD is suspected the first line investigation should be non-invasive imaging such as an abdominal ultrasound which can identify fatty infiltrates in the liver [59]. Routine liver function blood tests including alanine transaminases and aspartate aminotransferase can be raised or within normal range despite a person having MASLD related cirrhosis and therefore unreliable to use alone [67].

Scores are instead used to stratified patients into high or low risk of advanced disease. Fibrosis has been shown to be the leading predictor in progression of disease to cirrhosis and HCC and disease-related mortality [68]. Therefore Fibrosis-4 scoring (Fib-4) and NAFLD Fibrosis Score (NFS) use routine liver blood tests, anthropometric data and glycaemic status (NFS score only) to risk stratify patients based on risk of advanced fibrosis [67, 68]. Fib-4 has been shown in studies to have an AUROC of 0.7 (0.74-0.99) for progression of liver disease to advance fibrosis and NFS has an AUROC of 0.83 (0.74-0.93) [69]. Therefore, If FIB-4 or NFS scores suggest a patient has low risk of advanced fibrosis, then patients can be reassessed in 2 years' time with no requirement for further investigations.

Patients with intermediate risk have ultrasound elastography which uses ultrasonic waves to test the 'stiffness' of the liver (a surrogate marker for fibrosis) with 95% sensitivity and 77% specificity for advance fibrosis (F4) and was shown to have good accuracy with AUROC score 0.93 (95% CI: 0.89-0.96) for fibrosis score of 4 [70]. The reliability of identifying early fibrosis (score 2-3) however, is less robust. An alternative, which has been NICE approved,

is an enhanced liver fibrosis test (ELF™) which uses 3 biomarkers (type III procollagen peptide (PIIINP), hyaluronic acid (HA) and tissue inhibitor of metalloproteinase-1(TIMP1)) to stratify patients into low or high risk of advanced fibrosis [71]. The ELF test has a sensitivity of 90% for excluding advanced fibrosis but required high prevalence of MASLD (>50% population) to be able to diagnose advanced fibrosis accurately with specificity of 90% and sensitivity of 57% at threshold set at 10.18, the threshold recommended by NICE.

Patients that are high risk of having advanced fibrosis would likely have a liver biopsy to confirm the diagnosis and rule out any other causes of liver disease; in 5% of people there will be concurrent dual pathology, for example, viral hepatitis which would require specific treatment. This complex approach emphasises the unmet need of inexpensive non-invasive biomarkers that are both highly specific and sensitive to accurately diagnose a patient's stage of liver disease, particularly differentiating early fibrotic changes.

1.4.4. Treatment of MASLD/MASH and current limitations

Once a diagnosis of MASLD has been established, the primary treatment of dietary and lifestyle modifications is similar to that of obesity, IR and T2DM with studies showing relatively modest weightloss of 5% can reverse steatosis but 7%-10% is required to result in regression of MASH histologically [72]. Fructose has been associated with MASLD progression and therefore processed food should be avoided [61].

Thyroid hormone receptor beta is a transcription factor that is predominantly expressed in the liver, it can form heterodimers with PPAR α/β and liver X receptor and regulates free fatty acid uptake and lipolysis resulting in reduced circulating lipids and thus identified as a target for use in MASLD/MASH. The recent large phase 3 trial confirmed Resmetiron (thyroid hormone receptor beta agonist) prevents progression of fibrosis in 25.9% of patients on treatment versus 9.7% on placebo ($p<0.001$) and in March 2024 has been approved by the US Food and Drug administration (FDA) as the first direct treatment of MASH [73, 74], a huge step forward in the field. It currently remains under review by EU.

There are no other licenced drugs currently that successfully reverse MASH fibrosis and MASLD. NICE, AASLD and EASL only recommends the use of Pioglitazone and Vitamin E in patients with advanced fibrosis or cirrhosis due to weak evidence and associated risks of treatment. Vitamin E and Pioglitazone were evaluated in the landmark PIVENS randomized control trial[75]. Vitamin E is an antioxidant with free radical scavenging properties thought to improve MASH, while the PIVENS trial demonstrated it is well tolerated and improved MASH by 43% versus placebo 19% ($p=0.001$) there was no improvement in fibrosis scores ($p=0.24$). Other studies have linked vitamin E to increased all-cause mortality and small

increased risk of prostate cancer [76] therefore, use is suggested after a risk-benefit assessment.

Pioglitazone is thought to increase uptake of fatty acids in the adipose tissue and thus reduce FFA deposition and lipotoxicity in the liver. However, the PIVENS trial showed it was less beneficial than Vitamin E with no overall improvement in MASH (34% versus 19% placebo, $p=0.04$) and no improvement of fibrosis ($p=0.12$). On the other hand, steatosis ($p<0.001$) and lobular inflammation ($p=0.004$) were both shown to improve. Side effects include weight gain, bone fractures and rarely congestive heart failure therefore it is recommended only for selected patients with MASH on case-by-case basis [61, 77].

Metformin has not been shown to have a significant impact on disease progression and the current literature has conflicting results with some studies show that Metformin can cause hepatotoxicity[59]. Therefore, all advisory bodies suggest continuing with Metformin treatment, unless a patient has decompensated liver cirrhosis, for control of cholesterol and cardiovascular risk but it is not recommended actively for reversal of MASH [77].

There are several other classes of drugs that are undergoing clinical trials but there is currently insufficient evidence to commission their use in MASH/MASLD. Glucose metabolism modulators include GLP-1 mimetics, also licensed in T2DM and obesity with positive results, have been shown in several phase 2 trials, the LEAN trial for Liraglutide and a Semaglutide double-blinded trial, to result in MASH resolution but no significant improvement in fibrosis. Further longer term, larger trials are also underway [78, 79].

SGLT2 inhibitors have also been investigated with a small, randomised control trial showing Empagliflozin attenuated liver stiffness and steatosis, suggesting there was antifibrotic potential, the phase 3 DEAN trial is now underway to investigate this further [80, 81].

Bile acid metabolism has also been a target for drugs, the farnesoid X factor is a regulator of *de novo* lipogenesis in the liver through deactivation of the SREB-1c pathway. Therefore, obeticholic acid, an FXR agonist has been shown in the FLINT study to improve MASH activity ($p=0.0002$) but with a minimal anti-fibrotic effect [82]. Fibroblast growth factor (FGF) 19 and 21 are highly expressed in the liver on FXR and lead to PPAR α upregulation and inhibit lipolysis. Ongoing phase 3 trials aim to establish if these candidates are able to attenuate fibrosis [81].

Fibrogenesis inhibitors and anti-inflammatory therapies have also been studied with disappointing results, a selective ASK-1 inhibitor, a C-C chemokine receptor 2 and 5 dual antagonist and a monoclonal Lysyl oxidase like 2 antibody have all had negative results [83].

While new therapeutic options are currently being investigated, there are also surgical options. Bariatric surgery has been shown to improve MASH in 85% of patients at one year and improved fibrosis by 34% and therefore remains an option if patients reach the criteria set by NICE [84].

Liver transplantation remains the only definitive cure of MASH cirrhosis and therefore the race to find a drug to prevent progression of MASLD/MASH is crucial to stem the increasing burden of what is now the most common cause of chronic liver disease. Furthermore, the need for an accurate non-invasive biomarker which can predict the stage of MASLD is also key to disease prevention and treatment.

Therefore, I believe in order to bridge this gap in the current knowledge of obesity-driven MASLD and its link with insulin resistance, it is vital to further understand the crosstalk between the adipose tissue and the liver in disease progression. This may pave the way in identifying further treatment targets or potential biomarkers.

1.5 Adipose Tissue subtypes

There are 3 main subtypes of adipose tissue in the human body: white, brown and beige. White adipose tissue (WAT) is the predominant subtype with the primary function of storing lipids but its role as a master-regulator of whole-body metabolism is becoming more evident. WAT is uniloculated, with minimal mitochondria and low oxidative state; an excellent environment for the storage of triglycerides [85].

Brown adipose tissue (BAT) on the other hand was originally thought to be the main adipose type in newborn babies, which progressively decreases and is replaced by white adipose tissue in adolescence. The primary location of the BAT depots in neonates are the neck and subscapular region, however, positron emission tomography and computerised tomography (PET-CT) has now shown BAT dispersed throughout the adipose tissue deposits in adults, particularly from the anterior neck to thorax [86] challenging the hypothesis that BAT is only seen in childhood. Brown adipose tissue's main function, in contrast to white adipose tissue, is thermoregulation with a high number of mitochondria in multiloculated adipocytes and through mitochondria uncoupling protein 1 (UCP-1) allows the adipocytes to dissipate heat[87]. Young, lean women appear to have the largest volume of BAT but it is also determined by the local environment [86, 88].

The final main subtype is beige adipose tissue, which has been used to describe the pockets of 'browning' white adipose tissue that appear to have similar functions to brown adipocytes but with a different embryonic lineage [89]. WAT undergoes 'browning' on cold exposure and beta-3 adrenoceptor activation [90]. Beige, like brown adipocytes, are multiloculated with

high numbers of mitochondria expressing UCP-1 and are involved in thermogenic regulation but whether they have distinct functional purpose to BAT is still to be determined.

It has become increasingly apparent that mice with increased beige and brown fat are more resistant to obesity and as activated brown fat has high uptake of lipids it has also been shown to improve insulin sensitivity [89]. Human studies using FDA-approved B3-adrenergic receptor agonist, mirabegron, increased brown fat and improved insulin sensitivity [91].

While brown and beige adipocytes are evidently becoming potential targets in preventing obesity and IR and a plethora of research continues to investigate the beneficial properties of these depots in humans, I have focused my research on white adipose tissue firstly due to its prominent role in lipid storage and its link with metabolic syndrome and insulin resistance. Secondly, because it is the most abundant type of adipose tissue especially during prolonged obesity and thirdly due to the accessibility of white adipose tissue during bariatric surgery, as it would be technically challenging to take beige/brown adipose tissue samples in this cohort.

1.5.1 Anatomical location of white adipose tissue

White adipose tissue has evolved to store surplus energy as triglycerides and is found in multiple deposits throughout the human body which are heterogeneous with distinct tissue microenvironments and cellular compositions [92]. Adipose tissue can be subcutaneous (SAT: in between the muscle and dermis) or intra-abdominal. Subcutaneous depot locations are cranial, facial, abdominal, femoral or gluteal while intra-abdominal or visceral adipose tissue (VAT) can be pericardial (surround the heart), omental (layer around the stomach and intestine), retroperitoneal (layer behind the abdominal organs), mesenteric (layer attached between the intestines and abdominal wall) and perirenal (surrounding the kidneys) [85, 93]. There are also further depots peri-orbitally, intramuscularly and in the bone marrow. In chronic surplus nutrition, the adipose tissue is able to expand but whether the VAT depot or SAT is the predominant depot that increases is multi-factorial. Females are more likely to accumulate SAT while males are more likely to accumulate visceral fat and as humans age, there appears to be an accumulation of VAT as opposed to SAT [85].

There are distinct functional differences between SAT and intra-abdominal fat (VAT); SAT is more likely to have metabolic protective qualities while mounting evidence has shown that visceral adipose tissue accumulation is more closely linked to metabolic dysfunction and the complications of metabolic syndrome and insulin resistance [94-96]. The portal theory suggests this is due to the proximity of the omental WAT to the portal vein which delivers metabolites, cytokines and adipokines directly to the liver. In support of this theory, bariatric surgery has been shown to be more efficient in reducing visceral fat mass when compared

to subcutaneous fat mass, suggesting that its beneficial effects are largely due to the loss of visceral fat [97]. Mouse studies have elegantly shown that transplantation of SAT from lean mice to visceral compartments of obese mice led to beneficial effects on weight and metabolic profile. Furthermore, transplantation of the visceral fat to subcutaneous compartments had little effect on IR [98, 99]. However, mice do not share exactly the same hormone profile nor anatomical WAT depots as humans with the main difference being their large perigonadal (surrounding ovaries or testes) depot, and no equivalent omental depot thus cross-species comparisons must be done with caution. Furthermore, when obese individuals have had omental fat surgically removed, there was no improvement in IR [100] suggesting differences in VAT and SAT metabolism cannot be fully explained by location alone.

Other theories suggest it is due to the physical restriction of adipose tissue in the visceral compartment that prevents the adipocytes from fully expanding, unlike SAT that can expand unrestrained. The 'spill over' theory proposes excess free fatty acids (FFA) are released from the adipose tissue once the adipocytes are no longer able to expand or store excessive lipids [101, 102]. This leads to excess circulating levels of FFAs which are taken up by the surrounding organs such as the liver. As visceral adipose tissue expansion is thought to be constrained this process is therefore believed to occur more readily from VAT than SAT and leads to the metabolic consequences described [103].

In conclusion, while it appears VAT is associated with metabolic syndrome, T2DM and cardiovascular disease, the mechanisms for this are still incompletely understood [104]. I have chosen to focus on the role that visceral white adipose tissue has on T2DM and MASLD due to this association with metabolic syndrome characterised in the literature.

1.5.2 White adipose tissue development

The white adipose tissue originates embryonically from the mesoderm and mesenchymal stem cells (MSC) are thought to give rise to the early adipoblasts that form the precursor cells for white and brown adipocytes (pre-adipocytes) [105]. Adipocyte-derived stem cells (ADSC) are pluripotent MSCs found in the adipose tissue which are postnatal adult stem cells that have the capabilities of differentiating into adipocytes, osteoblasts, chondrocytes, connective tissue and myoblast in specific environments [105, 106].

Emerging studies seem to suggest that the lineage is more complex than originally anticipated, the number of intermediate steps from precursor to mature adipocyte is undefined and it remains to be determined if there are specific white preadipocytes for each WAT depot. Detailed lineage tracing is challenging in humans and thus most knowledge is from mouse studies or genetic lipodystrophies with rare mutations that produce different

WAT phenotypes. Individuals with the partial lipodystrophy, Baraquer-Simons syndrome have loss of facial deposits with sparing of lower extremities, suggesting different origins of WAT in upper versus lower extremity depots[93]. The facial SAT likely arises from the ectoderm, specifically the neural crest, with specific neural crest progenitor cells since isolated from this tissue. As the other WAT deposits arise from the mesoderm this would explain this varying fat distribution [105]. Furthermore, a true specific adipocyte precursor marker has not been identified, a mouse study showed that CD24⁺ precursor cells were able to proliferate and reconstitute a WAT depot in mice with A/Zip lipodystrophy [107] but this remains elusive in human WAT. Single-cell RNA sequencing of lean and obese individuals has also identified distinct progenitor cells with clear expression signatures dependent on the microenvironment and depot (SAT versus VAT) origin [108-110].

1.5.3 Cellular composition of white adipose tissue

The cellular heterogeneity of the WAT is an essential feature of its multifaceted function. Adipocytes are the predominant cell type, up to 40% of all cells in the tissue, and are of varying size from 25-200µm depending on lipid storage requirement. WAT also comprises of highly vascular cells and the stromal vascular fraction (SVF) which includes innate and adaptive immune cells (resident monocytes/macrophages, lymphocytes and leukocytes), fibroblasts, vascular smooth muscle cells, endothelial cells, erythrocytes, pericytes, plus adipocyte-derived stem cells and precursors cells (pre-adipocytes, and epithelial precursor cells, vascular precursor cells and haemopoietic progenitor cells) which are essential for healthy homeostasis [111, 112]. The SVF provides cells which enable regeneration of the adipose tissue when needed, through differentiation of precursor cells, the process involved in adipocyte turnover.

In recent years single cell-RNA sequencing has enabled further characterisation of adipose tissue composition and unveiled specific cell types present in lean and obese adipose tissue which have provided new insights in adipose tissue dynamics. These techniques have uncovered unique subsets of adipose-resident NK cells, innate lymphoid cell, macrophages and dendritic cells and highlights the vast heterogeneity of this tissue [113].

1.5.4 Adipogenesis and Adipocyte turn-over

¹⁴Carbon tracing experiments have demonstrated that lean adults have a constant number of adipocytes and this final number is determined by adolescence [114]. Spalding demonstrated around 10% of fat cells are regenerated yearly with the average turnover rate of an adipocyte occurring every 8.3 years [114]. SAT depots have been shown to develop as early as 14-24 weeks in utero with key depots being established by 28 weeks gestation [115], the number of adipocytes then remain the same until adolescence. On the other hand,

total VAT appears to remain small until adolescence when they expand through adipocyte proliferation and differentiation to form their total number by adulthood. While adipocyte numbers are stable in lean adults, in chronic obesity, adipocytes can respond to an increase in excess lipids in two ways, either through hypertrophy (i.e. the cell volume expanding) or through turnover of new cells (hyperplasia) by adipogenesis. [116, 117].

Adipogenesis is the process of adipocyte development from the commitment of mesenchymal stem cells (MSC) to resident tissue pre-adipocyte in the SVF which then undergo differentiation to terminal mature adipocyte formation. This process has been predominantly characterised using mouse models and an immortal mouse cell line 3T3-L1 *in vitro* [118]. Signalling pathways lead to proliferation of MSC in the SVF which are then committed to pre-adipocytes. The process of adipocyte differentiation has four key stages; the committed cells undergo proliferation until growth arrest, cells then undergo clonal expansion, early differentiation and finally terminal differentiation of mature adipocytes [119, 120] and this complex signalling pathway is regulated by multiple factors.

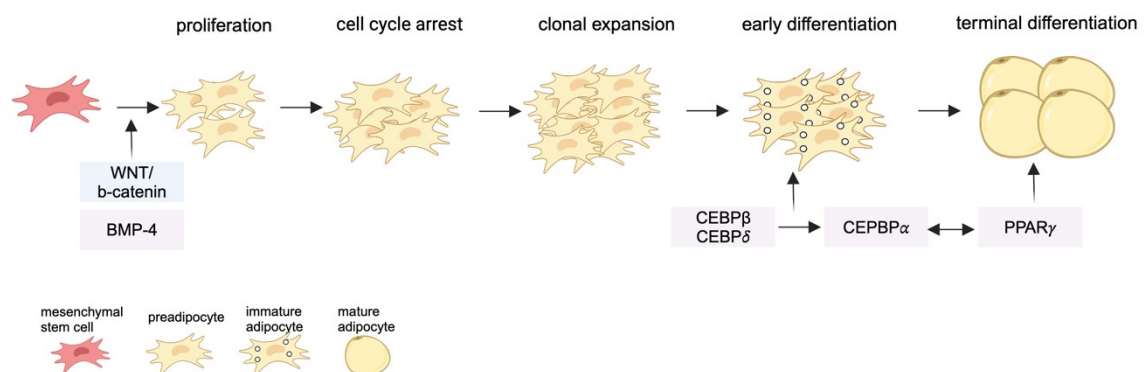


Figure 4 Adipogenesis pathway in adipose tissue. Mesenchymal stem cells are committed to preadipocytes through activation of WNT signalling pathway. BMP-4 activation in turn leads to the expression of transcription factors which regulate adipocyte differentiation. There are four key stages of adipocyte differentiation: proliferation, growth arrest, mitotic clonal expansion, and finally terminal differentiation. Pro- and anti-adipogenic factors regulate adipogenesis; PPAR γ has been identified as the master regulator and CEBPs are important for early differentiation. BMP-4: bone morphogenetic protein 4; CEBP β : CCAAT/enhancer-binding protein beta, CEBP δ : CCAAT/enhancer-binding protein delta, CEBP α : CCAAT/enhancer-binding protein alpha, PPAR γ : peroxisome proliferator-activated receptor gamma. Image created by Bio Render®

Mesenchymal stem cells (MSC) are first committed to a signalling cascade that leads to the formation of pre-adipocytes; WNT signalling through β -catenin activates BMP-4 which in turn commits the MSC to preadipocyte lineage. BMP-4 activates the Smad signalling pathway and leads to the expression of transcription factors for differentiation [121].

Studies have identified multiple pro-adipogenic factors that either act upon clonal expansion or differentiation [122]. PPAR γ nuclear transcription factor is considered the master regulator of adipogenesis [123, 124] and it has been clearly shown that without PPAR γ differentiation

does not occur. Furthermore adipogenesis is not terminal and PPAR γ is required to prevent dedifferentiation [118]. However, a specific ligand for PPAR γ remains elusive [120]. PPAR γ 3D structure has a ligand binding domain (LBD) which is 3 times bigger than the average nuclear transcription factor enabling it to bind with varying agonists. While fatty acids, eicosanoids and prostaglandins as well as CD36 (a scavenger receptor gene) and sterol response element binding protein 1c (SREBP1c) have all been shown to bind with PPAR γ they have a low affinity. cAMP dependent ligand activity has also been shown to be important for initiation of adipogenesis in 3T3-L1 cells but not for maintenance of PPAR γ expression [125]. Following activation PPAR γ translocates to the nucleus and heterodimerises with retinoid X factor (RXR) and other co-factors. The function of PPAR γ has been shown to be regulated by many co-factors either inhibiting or enhancing its expression [125, 126].

It has been well described that CEBPs (CCAAT/enhancer-binding protein) are the other key transcription factors with 5 members of the family involved in orchestrating early differentiation, CEBP β and CEBP δ work together in early differentiation to induce CEBP α and PPAR γ [122]. Knock out CEBP α mice have almost no WAT and are profoundly hypoglycaemic suggesting its role is also vital for insulin sensitivity [118].

Other pro-adipogenic co-factors including Kruppel-Like factors (KLFs) which are a large family of C2H2-zinc finger proteins that are important in regulating adipocyte differentiation, particularly KLF15 which acts through inducing expression of GLUT4 enabling uptake of glucose into the cell. KLF5 has been shown to be upregulated in early adipocyte differentiation and binds and activates *PPAR γ 2* promoter [127]. Signal Transducer and Activator of Transcription (STAT) family are also able to promote adipogenesis [122]. Further co-activators can act on transcription factor PPAR γ including HIC5 and TRAP220 which leads to FABP4 expression (fatty-acid binding protein 4) and BMPs have been shown to have varying effects on adipocyte differentiation, BMP4 commits MSC to adipocyte lineage while BMP2 appears to stimulate adipogenesis through nuclear localisation of Schnurri-2 (SHN2) [128].

There are also important anti-adipogenic factors particularly KLF2 and KLF7 and GATA2 which repress the *PPAR γ 2* promoter. The canonical Wnt/ β -catenin signalling pathway, while promoting commitment of MSCs, inhibits mature adipocyte formation through blocking PPAR γ and CEBP α expression [129]. Transcription growth factor (TGF β) also inhibits PPAR γ signalling through phosphorylation of SMAD3 pathway [119].

In summary, while PPAR γ and CEBP α are key regulators of adipogenesis, there are many other cofactors involved in the process with overlapping signalling molecules and

transcription factors that are also involved with lipid and glucose metabolism. As caloric excess occurs, WAT expands its mass through enlargement of adipocyte size (i.e. hypertrophy) and/or by recruitment of new adipocytes (hyperplasia) [130]. This enables the tissue to maintain a fine balance between lipogenic and lipid-scavenging roles. In chronic obesity and over nutrition adipocyte hypertrophy has been associated with dysfunctional adipose tissue and increased risk of developing insulin resistance and metabolic disorders while hyperplasia is more likely to result in metabolically healthy WAT [131, 132]. The mechanisms that determine how obesity disrupts adipogenesis homeostasis and leads to metabolic dysfunction are not fully elucidated. Certainly, the hypertrophied adipocyte has been shown to have structural changes which in turn alters lipogenic activity and downregulates DNL enabling the development of insulin resistance. In addition the obese microenvironment surrounding the MSC has been shown to lead to DNA destabilisation, senescence and telomere shortening, all of which effect cell survival and proliferation potential which result in restrained adipogenesis [133].

1.5.5 White adipose tissue lipid and glucose metabolism

WAT is an efficient fuel reservoir and is one of the key organs that control lipid and glucose metabolism. WAT tightly controls the synthesis and storage of triglycerides and glucose which are key for cellular pathways and whole-body energy homeostasis through the balance of lipogenesis, lipolysis and glycolysis. In surplus nutritional state lipogenesis stores excess circulating free fatty acids while at times of fasting lipolysis releases triglycerides as a critical energy source from the adipocytes (**Figure 5**).

Most of the fatty acids stored in the adipocytes, under normal daily feeding conditions, are from circulating triglycerides in the form of chylomicrons, produced by the small intestine or very low-density lipoprotein (VLDL), packaged and released by the liver which are taken up by the adipocyte and undergo lipogenesis. Postprandially, the adipocyte secretes lipoprotein lipase (LPL) which migrates to the surrounding vessel lumen and catalyses the hydrolysis of free fatty acids (FFAs) from the circulating TGs. CD36 the scavenger receptor transports the FFA into the adipocyte where diacylglycerol acyltransferase (DGAT) enables re-esterification of the free fatty acids to triacylglycerol (TAG) which are stored in a lipid droplet. Glucose is also taken up by the GLUT4 receptor which in turn is converted to glycerol and forms the backbone for re-esterification of FFA to TAG. This is the predominant pathway for lipids to be stored in the adipocyte.

The other process by which lipids are stored in the adipocyte is *De novo* lipogenesis. This is the process of synthesising TAG in the adipocyte themselves and can occur in both fasted and fed states [124], it accounts for only 2% of all lipogenesis processing in the body. It is

often used as a mechanism to store surplus carbohydrates in excess nutritional states. It utilises firstly the glycolysis of excess glucose which is taken up through the GLUT4 or GLUT1 receptor and phosphorylated by glucokinase (GK) to pyruvate which enter the tricarboxylic (TCA) cycle in the mitochondria to produce citrate. Citrate is then modified to acetyl-co enzyme A (acetyl-CoA) and malonyl-CoA. Acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN) convert the excess Acetyl-coA to palmitate (16:0) which undergoes elongation and desaturation to glycerol and other fatty acid species [134].

As described above, glucose uptake in the adipocyte is integral to lipogenesis and is governed by insulin and GLUT4 translocation and insulin-independent transporter GLUT1. While in other metabolic organs, glucose is primarily taken up and stored as glycogen, in the adipocyte this accounts for less than 5% of glucose uptake. Instead, glucose is utilised by the cell for glycolysis which contributes to a) TAG synthesis by providing a glycerol-phosphate backbone for the FFA esterification and b) *de novo* lipogenesis by providing acetyl-CoA. These two processes for storing glucose and lipids as TAGs however only accounts for 50% of glucose uptake and in fact a large proportion of glucose is metabolised to lactate and secreted from the cells as an energy source [135].

Lipolysis, on the other hand is the breakdown of TAG into FFA and is usually utilised at time of fasting. Three neutral lipases govern the breakdown of TAG. Adipose triglyceride lipase (ATGL encoded by PNPLA2) is the principal enzyme that hydrolyses TAG to DAG which is followed by hormone-sensitive lipase (HSL, encoded by LIPE) which hydrolyses DAG. Monoacylglycerol (MAGL) is then hydrolysed to glycerol by the catalysing enzyme monoglyceride lipase which releases the glycerol backbone. The free fatty acids fate can either be a) release into circulation for use by other tissues including the liver for gluconeogenesis or b) re-esterification in the adipocyte.

Perilipin, a lipid-droplet associated protein that is known as the gatekeeper of lipolysis has different roles in both HSL and ATGL hydrolysis of TAG and DAG mainly through perilipin 1 (PLIN1). cAMP dependent PKA-mediated phosphorylation of HSL triggers the translocation of HSL to the lipid droplet where the phosphorylated Plin1 resides and provides a docking point for HSL to bind with FABP4 which activates the hydrolysis of DAG to MAGL.

Phosphorylated ATGL is recruited to the lipid droplet and binds in a PLIN1 independent manner [122, 124, 136]. It is believed that Plin1 role is to modulate the co-activator ABMD5 which is released on phosphorylation from Plin1 and on binding with ATGL activates the hydrolysis of TAG to DAG. In insulin resistance the ability to inhibit this pathway is lost and results in the increased FFA levels in circulation which constantly exposes the liver to higher levels of FFA and the pathological effects of lipotoxicity [136, 137].

In prologued obesity, there is an overall downregulation of lipogenesis in the adipocyte in contrast to the liver where DNL increases. Lipogenic activity becomes dysregulated in the adipocyte and it has been shown that there is a decrease in several key transcription factors, SREBP1c, ChREBP- β , PPAR γ , ACLY, ACAC and FASN, that are required to maintain healthy WAT [138]. Down regulation of these key transcription factors potentially has a role in ectopic FFA accumulation and thus lipotoxicity within the liver and development of insulin resistance [132]

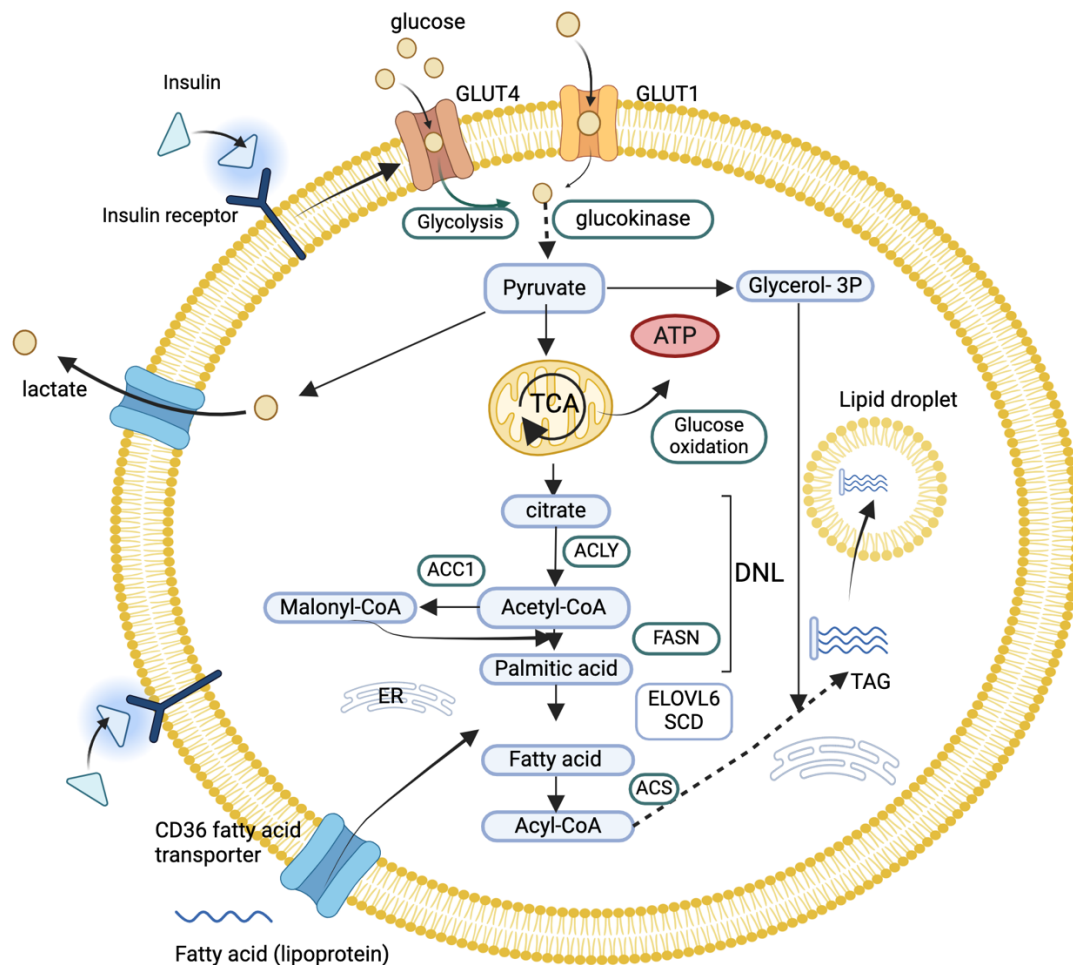


Figure 5 Lipid storage and glucose metabolism in the adipocyte. WAT is an important fuel reservoir and tightly regulates the production and storage of triglycerides and glucose through lipogenesis, lipolysis and glycolysis. There are two mechanisms of fatty acid production (lipogenesis) and storage. 1) In a fed state the major pathway is through the uptake of circulating cholesterol via CD36 fatty acid transporter which undergo re-esterification resulting in TAG formation. 2) while *de novo* lipogenesis (DNL) is the production of TAG through synthesis of fatty acids within the adipocyte itself utilising excess glucose taken up by the cell in states of nutritional excess. Glucose metabolism is integral to lipid production and storage either by providing a glycerol-phosphate backbone for the FFA esterification for TAG synthesis or *de novo* lipogenesis by providing acetyl-CoA. Most glucose is however broken down and excreted from the cell as lactate. GLUT4: Glucose transporter 4; TCA: tricarboxylic acid; ATP: Adenosine triphosphate; ACC1: acetyl-CoA carboxylase; ACLY: ATP Citrate Lyase; DNL: *de novo* lipogenesis; FASN: fatty acid synthase; TAG: triacyl glyceride; ELOVL6: Fatty Acid Elongase 6 SCD: Stearoyl-CoA desaturase; ACS: Acyl-CoA synthetase. Image produced by Bio Render®

1.5.6 The role of adipokines in whole-body homeostasis

In addition to lipid storage WAT is also an essential endocrine tissue [139]. Adipokines such as adiponectin and leptin are secreted from adipose tissue and heavily influence systemic insulin sensitivity and other endocrine functions including appetite [124, 140]. There have been multiple different hormones discovered that manipulate systemic functions with secretion of these bioactive species acting in both a paracrine and autocrine fashion (**Table 3**). Adiponectin was characterised as an adipocyte cytokine in 1995, it is a 28KDa protein secreted by the adipose tissue and is short acting, with a half-life of only 45-75 minutes. The serum level of adiponectin levels are relatively abundant in the plasma (0.01-0.05%) and are tenfold higher than insulin however they decrease with increasing obesity and insulin resistance [122, 141]. Adiponectin is insulin sensitising, increases fatty acid oxidation and reduces glucose production. There are two adiponectin receptors (AdipoR1 and AdipoR2) that are expressed by skeletal, liver and adipose tissue.

Adiponectin modulates fatty acid synthesis in the liver and adipose tissue via the AMPK pathway. Activation of AMPK then inhibits acetyl CoA carboxylase which is critical for DNL and thereby increasing long chain fatty acid oxidation[142]. Adiponectin has also been shown to reduce serum glucose levels predominantly through reducing hepatic glucose output both dependently and independently of the AMPK pathway and improves insulin sensitivity through increased phosphorylation of insulin receptor and other downstream molecules involved in the insulin pathway including AKT and glycogen synthase-3 β [143, 144].

Strikingly *ob/ob* mice that overexpress adiponectin have increased subcutaneous adipose tissue but overall adipocytes size is smaller with increased mRNA levels of PPAR γ suggesting that adiponectin plays a positive role in metabolic health and adipocyte turnover in an autocrine fashion [143].

Furthermore, adiponectin has a vital role in inflammatory pathways both locally through macrophages present in the adipose tissue and systemically. Adiponectin suppresses growth of granulocyte and macrophage progenitor cells from the bone-marrow and reduces TNF- α and IL-6 secretion from macrophages and increases IL-10 exerting an anti-inflammatory effect [141, 145].

Leptin is another key adipokine that has been studied over the last few decades. It is 16kDa and produced by the Ob gene. The adipose tissue is the main secretory tissue, and it is closely linked to satiety with decreased lipid and glucose uptake and increased energy expenditure and as patients develop chronic obesity the leptin levels increase.

Resistin is another hormone released by the adipocytes that interacts with toll-like receptor 4 (TLR4) and adenylyl cyclase-associated protein 1 and supports the production of TNF- α and Il-6 in contrast to adiponectin, two further adipokines. Other bioactive peptides include omentin, visfatin and chemerin which all exert affects both locally and systemically and have both positive and negative roles in obesity and T2DM summarised in **Table 3** below [146, 147]. Inflammation is a key feature of obesity and adipokines all exert effects on systemic and local inflammation, particularly TNF- α , which was the first cytokine described as an adipokine in 1980s. Therefore, it is important to understand the role of inflammation in white adipose tissue homeostasis and the effects obesity has on the inflammatory microenvironment which I will explore in the next section.

Table 3 Table of adipokines and their function. LCN-2: Lipocalin-2; TNF- α : Tumour necrosis factor alpha; IL-6: Interleukin 6.

Adipokine	Target tissue	receptors	Main metabolic actions	Inflammation	expression in obesity
Adiponectin	Liver, skeletal muscle, Adipose tissue,	AdipoR1, AdipoR2, T-cadherin	Insulin sensitizing, increase fatty acid oxidation and energy expenditure	anti-inflammatory	decreased
Leptin	Hepatocytes and B-cells	Leptin receptor	decrease food intake/increase energy expenditure	pro-inflammatory	increased
Resistin	adipose tissue, liver, endothelium, macrophages	TLR4 and CAP1	increase Insulin resistance /decrease adipogenesis/ decrease glucose uptake.	pro-inflammatory	increased
Omentin	adipose tissue, macrophages	Integrin $\alpha\beta3$ and $\alpha\beta5$	Insulin sensitising/ supresses fatty acid oxidation	anti-inflammatory	decreased
Visfatin	adipose tissue, liver tissue, macrophages			pro-inflammatory	increased
Chemerin	adipose tissue	CMKLR1	Insulin sensitising/ increase adipogenesis	pro-inflammatory /anti-inflammatory	increased
Vaspin	adipose tissue		insulin sensitising	anti-inflammatory	increased
LCN-2	immune cells, liver	SLC22A17 or 24p3R	transport of fatty acids and iron	pro-inflammatory	increased
TNF- α	adipose tissue, macrophages	TNFR1	decrease adipogenesis/ decrease lipid metabolism/ increase insulin resistance	pro-inflammatory	increased
IL-6	adipose tissue, macrophages	IL6R		pro-inflammatory	increased

1.5.7 Immune cells in the white adipose tissue

WAT has multiple resident immune cell types that play important roles in maintaining WAT homeostatic conditions through performing key housekeeping functions including apoptotic cell clearance, extracellular matrix remodelling, maintaining angiogenesis and metabolic functions. They are also integral to mounting an inflammatory response to infection and sterile inflammation through innate and adaptive pathways.

The WAT immune compartment comprises of myeloid (macrophages, mast cells, eosinophils, basophils, neutrophils and dendritic cells) and lymphoid cells (innate lymphoid, T-, B-cells and NK cells) and make up 10% of all cells in this tissue during homeostatic conditions [148]. The importance of immune cells for WAT homeostasis was first identified in 1993 when it was observed that TNF- α was secreted from adipose tissue in mouse studies which led to an increase in insulin resistance, lipolysis, hypertriglyceridemia and serum FFAs [149] and since then extensive work on understanding low-grade inflammation in obesity has been conducted. The largest population of immune cells that are thought to drive this through cytokine secretion are the macrophages which are discussed in detail below.

1.5.8 Macrophages and their role in WAT homeostasis

Extensive research into adipose tissue macrophages (ATMs) over the last 20 years has identified this group of cells as the most abundant myeloid cell within the adipose tissue making up 5% of cells in healthy tissue and up to 40% of the cells in obese humans [150].

With an increasing number of tools available to track macrophage ontogeny, this has allowed a deeper understanding of macrophage lineage in human adipose tissue. Initially it was thought that bone-marrow derived circulating monocytes were the sole precursors for all tissue-resident macrophages in adults [151]. However it is now understood that there are at least two distinct development origins of ATMs [152]; bone-marrow derived monocytes or yolk-sac erythro-myeloid progenitors (EMPs) which give rise to pre-macrophages or monocytes on embryonic day 8.25 that in turn can differentiate into long lived tissue-resident macrophages. This was observed by Felix et al. who demonstrated that Tim4⁺ macrophages, a marker of foetal-derived macrophages, were in fact, predominant in the adipose tissue [153].

In the prenatal period, the adipose tissue development relies on the EMP-derived macrophages, but after birth, this switches to include macrophages differentiated from bone-marrow derived monocytes [154]. Haematopoietic stem cells in the foetus undergo maturation and expansion in the developing foetal liver before they migrate to bone-marrow and generate bone-marrow derived stem cells giving rise to monocytes and monocyte-derived macrophages in the adipose tissue which are believed to be shorter lived than the tissue-resident macrophages [155].

A complex relationship, with crosstalk between macrophage subpopulations, develops to support adipose tissue function. Studies suggest however, in disease pathology, including obesity, it is the bone-marrow monocyte-derived macrophages that are the predominant cells involved in adaptive and maladaptive responses [156].

Independent of their origin, macrophage plasticity enables them to have multiple functions which govern WAT health in a homeostatic state including removal of apoptotic and necrotic adipocytes through phagocytosis and lysosomal activation, modulation of ECM and angiogenesis and differentiation of precursor adipocytes to mature adipocytes. Their functions are tightly controlled by the secretions of varying adipokines, miRNAs and FFA from adipocytes which trigger the infiltration of bone-marrow derived macrophages or the activation of tissue resident macrophages [157].

Macrophages are also important in the response to pathogens through activation of the innate immune response. Macrophages can express a wide range of pattern-recognition receptors that recognise 'danger signals' and instructs the macrophages to phagocytose the foreign particle or bacteria through an M1-phenotype which in turn activates the inflammasome. Traditionally it was described that the macrophages have two phenotypes, (M1 or M2) and are 'polarised' by the microenvironment which induces the adipocyte tissue macrophage (ATM) signalling cascade to determine their function[158]

The M1 or "classical" phenotype are considered pro-inflammatory and shown to express CD11b, CD11c macrophage markers and secrete TNF- α , IL-1 β , IL-6 and iNOs and this in turn has a role in metabolism by signalling glycolysis and oxidative phosphorylation of pyruvate and worsening metabolic health of the WAT [159].

While the M2 or anti-inflammatory "alternative" macrophage express CD11b, CD206 and CD301 markers and secrete IL-10 and are involved in wound repair through ECM regulation. These macrophages also have been shown to have a role in increasing fatty acid oxidation.

In 2003 Weisberg and Xu demonstrated in rodent models that WAT macrophages accumulated in obesity. Furthermore, adipocyte size correlated with increased macrophage numbers in the WAT and key macrophage markers were upregulated in obesity including CD68 and MCP-1 [160, 161]. Mouse models have shown that in an obese state there was a phenotypic switch from M2 protective anti-inflammatory phenotype to the classic inflammatory phenotype driven by adipokines [162, 163]. One of the most intensely studied chemokines is MCP1 and its receptor CCR2, both of which have important roles in macrophage recruitment and insulin sensitivity. Mouse models have shown MCP1 and CCR2 are expressed with increasing adiposity and led to macrophage recruitment but when MCP1 and CCR2 were deleted IR improved and attenuated hepatic steatosis [159, 164]. While pro-inflammatory macrophages have been shown to have deleterious effects on adipose tissue health, Sherer and colleagues importantly showed they are also vital for moderating WAT homeostasis. Using a mouse model – the 'adipochaser' they demonstrated that without the pro-inflammatory macrophage sensing there is loss of fat pad expansion and

inhibition of adipogenesis which leads to metabolic dysfunction [165] so dynamic moderating of macrophage function is essential for healthy WAT homeostasis.

Recent unbiased transcriptomic approaches and single cell RNA-seq analysis in both obese and lean mice and humans has demonstrated the M1/M2 macrophage phenotype is in fact an oversimplification and have identified several specific sub-sets of macrophages within the adipose tissue that have distinct roles which are modulated in obesity. Firstly, metabolically activated macrophages (MMe) have been described as a subset of macrophages with dual function. On one hand MMe are beneficial for adipocyte clearance through lysosomal exocytosis which in turn limits insulin resistance through maintaining adipocyte health and thus preventing ectopic lipid deposition in other metabolic organs. However, in early obesity they have also been shown to be pro-inflammatory and in this setting promote insulin resistance. This complex balance is regulated by TLR2, NOX2 and MYD88 and highlights the complex roles in a time-dependent manner that these macrophages have on adipocyte function [166].

Hall and colleagues also used transcriptomics to identify another unique subtype of macrophages with CD9⁺ markers that are lipid laden in lysosomal like-structures, produce IL-1 β and TNF- α [113] and are found localising in crown-like structures [167]. Due to the large size of adipocytes, individual macrophages are thought to be unable to phagocytose dying adipocytes and instead form a synapse with other macrophages to re-absorb the cells, this process causes a reorganisation of macrophages into crown-like structures (CLS) [168] described as multinucleated giant cells with enhanced ability of clearing dead adipocytes [169, 170] and their numbers have been shown to increase during obesity in mice and humans [113, 171]. A further subset of macrophages called lipid-associated macrophages (LAMs) have been shown to co-localise within these CLS and have been shown to express TREM2⁺ CD9⁺. LAMs have been suggested to play a vital role in preventing adipocyte hypertrophy, are insulin sensitising and prevent weight gain [167].

In contrast to ATM subsets forming CLS, MFe hi ATMs are localised to the intracellular space of adipose tissue and are a distinct population involved in iron handling in the adipose tissue, but their role in obesity is yet to be determined [172].

Macrophage biology and function in WAT has been the focus of interest for unravelling the chronic inflammatory phenotype seen in obesity and several drugs have been developed to target identified pathways with minimal success. However, there are also other important inflammatory cells that have vital roles in WAT homeostasis, including other myeloid cells.

1.5.9 Other Myeloid cells and their role in WAT homeostasis

Mast cells are the next most abundant myeloid cell after macrophages in the WAT. They are usually found in skin or at barrier sites and can rapidly degranulate to release inflammatory mediators. Liu et. al. showed in mast-cell deficient KitW-sh/KitW-sh mice fed a HFD there was almost no macrophage accumulation and improved insulin sensitivity and decreased body weight, it was therefore hypothesised that mast cells modulate IR indirectly through adipose tissue inflammation [173].

In lean individuals only around 1% of cells in the WAT are neutrophils, which in obesity rises 20-fold [174]. Their main role is to migrate to the site of infection and are the first cells to provide an immune response through degranulation and secretion of chemokines and cytokines (IFN- α , IFN- γ , TNF- α , IL-1 β and CCL3) and perform phagocytosis. In obesity they increase secretion of elastase and serine protease and maintain crosstalk with adipocytes through CD11b and ICAM-1 interaction. They are pro-inflammatory, and studies have shown elastase can decrease the number of proteins involved in insulin signalling [175].

Dendritic cells are the bridge between innate and adaptive immune responses and are both phagocytosing and important antigen presenting cell for T-cells. There are two types of dendritic cells: conventional dendritic cells (cDC) and plasmacytoid DCs (pDC). cDCs activate the Wnt/ β -catenin pathway and PPAR γ signalling which promotes an anti-inflammatory pro-adipogenic environment. In obese mouse models whereby these pathways are deleted there is inflammation and metabolic dysfunction [176]. Further studies also suggest there is promotion of a Th17 response, a pro-inflammatory response with release of IL-17 [177]. Plasmacytoid DCs on the other hand have been shown to accumulate in obesity and are recruited to the VAT by elevated levels of adipokines, this results in interferon (INF-1) signalling cascade and thus pro-inflammatory [178].

Eosinophils are best known for their inflammatory response to parasitic infections and provide a Th2 / anti-inflammatory response with release of anti-inflammatory cytokines such as IL-4, IL-10 and TGF- β [179]. The numbers of eosinophils in WAT are very low but despite this, some rodent studies have suggested that the M2 macrophage phenotype is driven by IL-4 secretion from eosinophils as M2 macrophages increase in line with eosinophils [180]. However, there is limited studies identifying the effects of eosinophils in obesity directly on WAT.

1.5.10 Lymphoid cells and WAT homeostasis

Lymphoid cells' role in WAT metabolic and inflammatory control have also been explored, this includes B-cells, T-cells, NK cells and NKT cells. T-cells (CD3⁺) are the next most abundant cell after macrophages found in WAT and can be split into 3 groups. CD4⁺ T-cells

secrete multiple cytokines which affects their function. They either have a T helper 1 (Th1) signature (IFN- γ release), Th2 signature (IL-4, IL-5 and IL-13) or Th17 (IL-17, IL-21 or IL-22) or are Foxp3⁺ T regulatory cells (Tregs) (IL-10 and TGF- β) [176]. CD4⁺ cells are shown to accumulate in obesity and polarise to an Th1 signature due to the increased IFN- γ excretion which affects insulin signalling, lipid storage and adipogenesis through JACK-STAT pathways [181]. In IFN- γ deficient mice models on HFD, insulin sensitivity improved and VAT inflammation reduced, suggesting it is the IFN- γ directly leading to Th1 polarisation [182, 183]. CD8⁺ T cells also accumulate in obesity and are shown to secrete IFN- γ and granzyme B which leads to IR.

Tregs on the other hand are found in high levels in lean WAT and are shown to be regulated by PPAR γ [184]. They are also involved in M2 macrophage polarisation. However, in obesity, studies have shown they are reduced and outnumbered by other T cells and their pro-inflammatory signatures [185].

B cells are unique cells involved in the humoral response and produce antibodies specific for pathogens but can also act as antigen presenting cells (APCs) for MHC class I and II [176]. Obesity increases the number of B-cells and most notably IgG2c antibodies that are preferentially found in CLS and drive macrophages to a pro-inflammatory phenotype and insulin resistance. Transferring IgG2c antibodies from obese to lean mice resulted in IR [186].

Therefore, it is evident that while macrophages are the most abundant immune cell type that drives inflammation and metabolic function in WAT, other immune cells also have roles directly or indirectly on macrophage insulin signalling and inflammatory pathways and a further understanding could provide further treatment targets.

1.5.11 The immune functions of the omentum

As described above, immune cells have key roles in all WAT depots but omental WAT has unique immune features due to its size and location in humans. While in mice the omentum is only a small strip of fat, in humans the omentum is a fibro-fatty layer extending from the greater curvature of the stomach spanning the abdominal cavity to the transverse colon and can vary in size depending on the individual [187, 188]. It is highly vascularised and has important physiological functions both in a steady state and in response to infection and inflammation by enabling tissue repair thus giving it the name the “policeman of the abdomen” [187].

One of the important features of the omentum is the presence of milky spots which are aggregates of leukocytes scattered between the adipocytes supported by fibroblastic stromal

cells. While similar structures are found in other fat depots, the number found in the omentum is the highest. They are composed of predominantly CD4⁺, CD8⁺ T cells and B cells but they also includes a unique set of CD4⁺ T regulatory cells [189]. High numbers of macrophages are also found in steady-state conditions and interestingly, there is a subset of macrophages derived from CCR2- expressing monocyte precursors that are differentiated by transcription factor IRF4 which requires specific microbiota for activation. The presence of this subset of macrophages demonstrate the important role the milky spots have in preserving gut and peritoneal cavity homeostasis [190]. The unique set of immune cells within the omental WAT enables them to react quickly to any free-floating cells, particulates or antigens that they are exposed to, from the fluid that is continuously drained from the peritoneal cavity [188].

The omentum also plays a key part in regulating tumour metastases, particularly those seen with ovarian cancer with specific tumour-associated macrophages being described as pro-angiogenic, acting as anti-tumour suppressors and correlating with disease progression [191].

Importantly, the omental immune cells also appear to have roles in obesity with increased expression of IL-6, TNF and adiponectin within the omentum and rapidly proliferating TNF-expressing macrophages have been identified as obesity and metabolic syndrome progresses [191, 192]. Due to the importance of the omental WAT in humans from an immunometabolism perspective, its predominance in obesity and link with metabolic syndrome clinically, I therefore chose to focus on this depot for my research.

1.5.12 Extracellular matrix remodelling in WAT homeostasis

The extracellular matrix (ECM) that embeds adipocytes is important in modulating systemic metabolism and WAT expansion. The most common ECM proteins are collagens and fibrinogen in the WAT with metalloproteinases (MMPs) regulating ECM degradation and tissue inhibitors of metalloproteinases (TIMPS) inhibiting MMP activity [157, 174, 193-195].

Collagens, specifically type I, III, IV and VI form the main components of ECM in mice and human adipose tissue [196, 197]. Both mouse and human studies have demonstrated links with dynamic collagen remodelling, inflammation and insulin resistance in the WAT [3, 198] and macrophages have been shown to have a direct role in the regulation of obesity-induced adipose tissue fibrosis [30].

While adipose tissue macrophages' role in adipocyte dysfunction is well established, the exact consequences of ECM remodelling during the maladaptive transformation of WAT are complex and depend on many factors, including site-specific influences (e.g. visceral vs.

subcutaneous depots), type of collagen involved, and their distribution patterns [196, 199]. In fact, each type of collagen is likely to be differentially influenced by the WAT microenvironment and while the term 'fibrosis' is used in the literature and is defined as 'thickening or increased deposition of ECM which has a deleterious effect on the tissue' total fibrosis may be a too broad term to describe ECM remodelling in obesity [200].

The literature landscape to date has contradicting evidence of the underlying changes that occur with the ECM in obesity. Some studies show that collagen abundance and fibrosis decrease with adipocyte hypertrophy, suggesting the collagen matrix is key to maintaining WAT health by preventing unconstrained expansion [196, 201, 202]. Muir et al. have shown that human subcutaneous and visceral WAT fibrosis is reduced (measured by Sirius red staining, collagen transcripts and immunohistochemistry) with increasing HbA1c levels [202]. On the other hand, work by Gugliemi reported increased total fibrosis positively correlated with insulin resistance in obese omental tissue but there was no significant difference in collagen transcripts or adipocyte size with increased insulin resistance [203]; others studies corroborated these findings [204-206].

Furthermore, there has been mounting interest in collagen type VI following a study by Khan et al. that showed improved metabolic phenotype and reduced inflammation in collagen type VI knockout mice [204]. Studies in humans have also demonstrated elevated collagen type VI deposition in WAT during obesity and insulin resistance [201, 205, 206] and macrophage depletion in WAT explants isolated from patients undergoing bariatric surgery led to reduced type VI collagen and improved insulin sensitivity [207]. Inazumi used an EP4 KO mouse model (the receptor for Prostaglandin E₂) to demonstrate that in obesity the PGE₂-EP4 axis is upregulated which results in increase of Col6A3 mRNA transcripts [208].

Type I collagen has also shown to be important for WAT homeostasis in a CYP2J2 knockout rat model used by the Behmoaras lab [209] and further studies in HFD-fed MMP14^{+/-} mice demonstrated adipocytes are unable to undergo expansion due to a lack of collagen type I [210]. Adapra et al. demonstrated the complexity of ECM modelling with both mice and human studies showing Collagen I increases at mRNA transcript level in HFD but protein measured by Western Blot in both SAT and VAT decreases; They postulate that this is due to MMP2 and MMP9 modifications [211].

While collagen composition is important in adipose tissue in obesity, there has been only one study that has established a functional role through measuring tensile strength which was decreased in Collagen VI KO mice, suggesting it is important for integrity of the cell and to enable expansion [212]. They also measure MMP2 and MMP9 which, contrary to previous

work that suggested MMP9 increases with obesity in VAT, were in fact decreased in this study, again highlighting the variability of studies to date in this field [212, 213].

Therefore, while inflammation has been extensively studied, it is clear that the role of ECM remodelling in WAT during chronic obesity is still relatively undefined and more studies are needed to further understand the importance of its role in maladaptive WAT homeostasis. It is well characterised in other organs such as the liver, that fibrosis is the end consequence of prolonged injury to the tissue often resulting in irreversible damage and scarring, however, whether this is the same in the WAT is yet to be confirmed. Through the work I performed in this thesis, I clarified the role of type I collagen and type 6 collagen in terms of adaptive and maladaptive ECM remodelling of the WAT.

1.5.13 Angiogenesis and its role in WAT homeostasis

Angiogenesis is the stimulation and growth of new blood vessels from existing vasculature and is an important aspect of tissue remodelling and adipocyte turnover within the adipose tissue in a steady state. As previously described, inflammation and ECM remodelling are central to the maladaptive changes seen in adipose tissue in prolonged obesity, however, it is still not fully understood what triggers these responses. One hypothesis is that in nutritional excess the rapid expansion of adipocytes through hypertrophy leads to hypoxia, specifically low partial pressure oxygen in the adipose tissue [214-216]. The hypoxia hypothesis was first demonstrated *in vivo* *ob/ob* mice and was shown to lead to inflammation, ECM remodelling and thus adipose tissue dysfunction. Hypoxia triggers hypoxia-inducible factors (HIFs) predominantly HIF-1 α and HIF-2 α subunits. HIF-1 α has classically been known to be pro-angiogenic and has been implicated in pro-fibrotic/proinflammatory pathways while HIF-2 α has been shown to attenuate inflammatory responses. HIF-1 α deletion mouse models have been shown to reverse obesity-mediated WAT inflammation and improve insulin sensitivity [217] [218]. HIF-2 α , on the other hand, has been shown to be important in UCP1 and VEGF expression in BAT, with increased expression improving AT vascularisation and preventing metabolic decline during adipose expansion [219]. While hypoxia is thought to be the precipitant of AT inflammation and metabolic decline another viewpoint is that macrophage infiltration in obesity in fact promotes angiogenesis through PDGF angiogenetic promoting factors [220].

While this loss in balance between oxygen demand and supply during the rapid expansion of adipocytes in obesity has been shown elegantly *in vivo*, there is relatively limited evidence to implicate these pathways in human adipose tissue. On one hand studies suggests partial oxygen pressure is reduced in obese individuals' subcutaneous upper arms [221], on the other, Goossens et al. reported the reverse and Hodson et al. did not find significant

evidence suggesting hypoxia drives metabolic signatures in human obesity [218, 222]. However, these are caveated by significant limitations in study design in humans and therefore the delicate balance of hypoxia, inflammation and ECM remodelling during the progression of obesity and adipocyte expansion in humans is still not fully elucidated. There are also conflicting viewpoints as to whether adipose tissue is a relatively lowly vascularised tissue or highly vascularised, which maybe in part due to the significant differences in brown thermogenic tissue versus white depots [221]. Further human studies are required to understand the role of HIF pathways in obesity, determine whether there are depot specific effects and identify if certain HIF subunits drive specific adaptive or maladaptive responses.

So far, I have discussed the importance of adipose-tissue specific adaptive and maladaptive responses to nutritional excess, however cross-talk between other organs such as the liver or gut are also important in understanding disease pathogenesis in obesity. The gut microbiota has gained interest due to its role in many disease pathologies and therefore I shall explore current literature below.

1.5.14 Microbiome-adipose tissue interactions and their role in obesity

The interactions between the microbiome and adipose tissue homeostasis have become a central focus in recent years. The gut microbiota encompasses bacteria, fungi, protozoa, viruses and archaea that are resident in the gastrointestinal tract with bacteria the most widely studied group of these organisms. The composition of the microbiota varies hugely from individual to individual and is dependent on diet, genetics, age and sex with other factors also influencing this dynamic ecosystem including sleep, drug use and smoking [223]. The gut microorganisms have an impact on metabolism through modulating appetite, producing nutrients, and influencing inflammatory pathways. They can produce a variety of metabolites including short chain fatty acids (SCFAs), bile acids, bioactive lipids including oxylipins, endocannabinoids and amino acid derivatives within the gut [223, 224].

The dynamic bidirectional relationship between the gut microbiome and adipose tissue was first shown to be important in metabolism homeostasis using animal models, when 'germ-free' rats and mice were shown to be protected from obesity, insulin resistance and metabolic decline [225]. In 2000 another group showed that changing the gut microbiota composition using prebiotics, reduced adipose mass, hepatic steatosis and glycaemia. Furthermore, in 2006 'obese mice' microbiota were transplanted into germ free mice which resulted in increased total body fat, supporting the idea that microbiota influence metabolism [226]. In humans, microbiota changes are observed pre and post Roux-en-Y gastric bypass and is thought to be an important reason for the significant weight changes observed following this treatment [227].

What links the microbiota to the modulations seen in adipose tissue metabolism is still unknown but certain mechanisms including bacterial translocation to the adipose tissue have been proposed. SCFAs, produced by the gut microbiota, have also been shown to have a role in modulating metabolism through stimulating excretion of gut peptides including glucagon-like-peptide (GLP1) and peptide YY (PYY). These pathways enable the gut-brain axis to activate free fatty acid receptors (FFARs) FFAR2 and FFAR3 which can in turn modulate intestinal transit time, inflammation and metabolism [224, 228]. Furthermore, these *in vivo* studies have been validated in humans with SCFAs found in higher levels in athletes compared to sedentary individuals [229].

Other products of the microbiota, including tryptophan metabolites, have also been implicated in modulating WAT metabolism. Microbiota have been shown to regulate other host responses including bile acid and endocannabinoid signalling. Bile acid- microbiota interactions, for example, have been implicated as a reason for rebound weight gain after dieting through bacterial metabolism altering the bioactivity and bioavailability of the bile acids [224]. Furthermore, as chronic low grade inflammation is the hallmark of obese WAT, it is likely that the gut microbiome has a key role in modulating the immune response; microbiota-associated molecular patterns (such as Toll-like receptors (TLR) or Nucleotide-binding oligomerization domain (NOD)-like receptors) are activated by specific changes in the gut microbiota during high fat diets resulting in immune cell activation [230].

While there are hundreds of gut organisms found to be associated with specific disease pathologies, there are a few organisms that are of increasing interest in obesity. Ley et al. compared lean and obese microbiota and identified people with obesity had lower *Bacteroidetes* and higher *Firmicutes* than lean people and interestingly, with dieting, this ratio could be partially restored. Other bacteria that play an important role in obesity have included *Lactobacillus paracaesei* and *Escherichia coli* [227]. A recent study shows *Dysosmobacter welbionis* has been found in lower levels in T2DM patients with obesity and *in vitro* studies suggest it is modulating bioactive lipid pathways by decreasing the oxylipin; prostaglandin j2, a PPAR γ agonist, and directly influencing lipid metabolism [231].

However, while there are multiple studies over the last two decades that have identified gut microbiota that are associated with development of obesity and metabolic decline, casual relationships remain relatively elusive and the complex relationship in humans between microbiota and adipose tissue still have unanswered questions.

Through the last few sections I have described how obesity can lead to perturbed WAT homeostasis through a multitude of cells and mechanisms resulting in IR and T2DM, I now

will review how obesity can affect liver function and the interplay between WAT and liver homeostasis.

1.6 The Liver during obesity and MASLD progression

Obesity has a deleterious impact on the liver and is closely related to the development of MASLD/MASH and therefore I am to explore how surplus nutrition leads to hepatic injury and the progression of liver disease.

1.6.1 Liver anatomy

The liver plays an important role in whole body homeostasis with involvement in metabolism, immunity, digestion, detoxification, vitamin storage, production of clotting factors and proteins and has been shown to account for 2-5% of total human body weight. It is made up of the left and right lobes which are split into eight segments delineated by their individual blood supply and is encased in a fibrous membrane known as the Glisson's capsule. The liver is unique as it has two major vessels – the hepatic artery providing 25% of blood supply and the portal vein providing 75%, that enable it to perform its vast functions[232].

The liver is formed from the foregut; it begins formation from the endodermal cells at around 4 weeks gestation developing from the hepatic diverticulum [233, 234].

1.6.2 Hepatic Cellular composition

At a cellular level the liver is made up of lobules that are hexagonal in morphology with the portal triad; a bile duct, hepatic arteriole and portal venule at each of the six corners. The portal venules all drain into a central vein via liver sinusoid capillaries and the bile ducts form canaliculi in a 3D lattice type structure [235].

Hepatocytes are the predominant cell type within a lobule which are split into three major zones: zone I hepatocytes are defined by their proximity to the portal triad and therefore closest to the oxygenated blood and nutrients which enables them to be the first to regenerate, these periportal hepatocytes are involved in gluconeogenesis, bile formation, cholesterol formation and amino acid catabolism. Zone II hepatocytes are further away from the portal triad and are known as pericentral region with Zone III hepatocytes located furthest away from the triad and thus have the lowest perfusion. Their function is rather different to zone I hepatocytes enabling glycolysis, lipogenesis, glycogen synthesis, glutamine formation, ketogenesis, detoxification and drug metabolism to occur. Hepatocytes make up 70% of the cell types in the liver with the others major cells types including liver sinusoidal endothelial cells(LSECs), Kupffer cells, stellate cells and cholangiocytes [232].

The sinusoidal endothelial cells are fenestrated and allow communication between blood in the specialised capillaries, known as sinusoids, and hepatocytes. The space of disse is the

perisinusoidal area between the endothelial cells and the basolateral membrane of the hepatocytes, which extend microvilli into the plasma filled lumen, and this allows bidirectional communication of the hepatocytes to the sinusoidal capillaries [235]. The space of disse contains collagens, proteoglycans and other proteins important for scaffolding the hepatocytes but is occupied by hepatic stellate cells (HSC) that in healthy state are quiescent and store vitamin A in lipid droplets. As hepatocytes become injured, the HSCs proliferate and lose vitamin A forming myofibroblasts and are integral to ECM deposition and fibrosis formation [235, 236]. Kupffer cells are resident monocyte/macrophages that patrol the space of disse, having both a scavenging role phagocytosing pathogens and immunological mediation through cytokine release. Further immune cells found in the liver are Pit cells which are specialist liver natural killer cells that have cytotoxic activity but are relatively rare. A further main cell type are cholangiocytes from bile ducts which are responsible for bile flow rate and pH regulation and secrete bicarbonate and water [233].

1.6.3 Hepatocytes' role in development of MASH and fibrosis

Hepatocytes are highly proliferative and key to liver homeostasis and regeneration. The hallmark of MASLD is the presence of fatty droplets within the hepatocytes termed steatosis with pericentral hepatocytes, which perform predominantly metabolic functions, more susceptible to fatty deposition compared to periportal. Due to this, MASLD characteristically develops firstly around the centrilobular vein and extends to the portal tracts as steatosis progresses [20]. The continued exposure of hepatocytes to 'stress' resulting in cellular damage through lipid accumulation is coined 'lipotoxicity' and histologically is represented by ballooning and Mallory-denk bodies which significantly correlate with disease progression and MASH. Ballooned hepatocytes are larger than the average hepatocytes, have irregular cytoplasm and loss of cytokeratin (CK) 8 and 18 and have been shown to be more susceptible to apoptosis [237, 238].

1.6.4 Hepatic Lipotoxicity

Lipotoxicity is driven mainly by free fatty acid (FFA) accumulation, the exact lipid metabolites that causes the hepatic injury have not been identified but are thought to be non-triglyceride species including ceramides, cholesterol, saturated fatty acids, lysophosphatidylcholine and diacylglycerol. The FFA accumulation within hepatocytes originates from increased adipose tissue lipolysis (59%), *de novo* lipogenesis (DNL) within the hepatocytes (26%) and increased lipid intake through diet (15%) [239]. Insulin resistance as a result of dietary, genetic and environmental factors plays an important role in MASH progression and lipotoxicity as it results in upregulation of proteins involved in *de novo* lipogenesis through transcription factors sterol regulatory element binding protein-1 (SREBP-1), carbohydrate response element-binding protein (ChREBP) and PPAR- γ and also leads to unrestrained

adipocyte lipolysis [240-242]. MASH has also been associated with increased phosphorylation of c-Jun N-terminal Kinase(JNK), a pro-apoptotic and pro-inflammatory mediator, preventing insulin receptor function and increased insulin resistance either directly through increased FFA or activation of nuclear factor kappa-beta kinase (NF- κ B) signalling cascade [243, 244].

Traditionally the 2-hit hypothesis for MASLD to MASH progression; by which lipotoxicity was the 'first hit' with further hepatic injury being the second, was proposed but this is now outdated and clearly too simplistic. Firstly, as MASLD to MASH progression is non-linear and secondly, because it is evident that the cascade of events that pursue lipid accumulation are complex, thus a multi-hit hypothesis has since been implicated [245].

Lipid toxicity has been shown to cause liver damage through three main mechanisms a) through impairing intracellular organelle such as mitochondria and the endoplasmic reticulum (ER) within the hepatocyte, b) altering signalling pathways within the hepatocyte c) interacting with proinflammatory cytokines on the cell surface and thus hepatocytes undergo oxidative stress, formation of reactive oxygen species(ROS) or trigger ER stress and injury [241].

1.6.5 Hepatic mitochondrial dysfunction

Hepatocytes have high numbers of mitochondria and when exposed to lipotoxicity become dysfunctional, no longer being able to efficiently synthesise ATP which results in the inhibition or depletion of antioxidants thioredoxin and glutathione and increased production of ROS [246].

Dysfunctional mitochondria are directly involved in MASH progression and several mechanisms have been identified. Morphologically mitochondria can become enlarged between 3-10um in MASH, coined megamitochondria, which is thought to be a result of impaired mitochondrial division and fusion and has been directly associated with hepatic cell death [238, 247]. ROS can induce cell death through NF- κ B signalling producing proinflammatory cytokines and encouraging apoptosis and necrosis of hepatocytes. Furthermore, mitochondria derived danger-associated molecular patterns(DAMPs) can directly affect hepatocyte cell death through NLRP3 inflammasome pathways or by being recognised by other pattern recognition receptors (PRRs) triggering the innate immune response [246]. Damaged mtDNA also has been shown in mice models to act through TLR9 on HSCs and Kupffer cells to activate the innate immune response and fibrosis formation [248].

1.6.5.1 Hepatic endoplasmic reticulum stress

The endoplasmic reticulum (ER) is extensive within hepatocytes and is the organelle in which DNL occurs, regulated by SREBP-mediated pathways. The ER is key to protein folding and functional development and is maintained by the unfolded protein response (UPR) which regulates the formation of proteins and ensures the degradation of misfolded proteins and thus enables cell survival [249]. Hepatic fat accumulation in MASLD causes impaired ER function. Injured hepatocytes produce increasing numbers of misfolded proteins overloading the ER and triggering the UPR through directly inducing RNA-dependent protein kinase-like ER eukaryotic initiation factor-2 α kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme-1 (IRE1) resulting in ER stress and DNL [240] and ultimately cell death. Misfolded proteins have been shown to activate IRE1 which can phosphorylate JNK and NF- κ B signalling promoting inflammatory response and insulin resistance [246] and animal models have shown silencing JNK decreases further hepatic steatosis. ER stress has also been shown to induce caspase 2 and reduce mitofusin 2 which contribute to MASH progression [250, 251].

1.6.6 Hepatic signalling pathways influencing MASLD progression

There are also several pathways that are involved in MASLD progression. TAZ is a part of the HIPPO-YAP/TAZ-TEAD pathway and has been shown to be upregulated in both mouse and human hepatocytes with MASH, but not simple steatosis, and targets Indian Hedgehog(IHH), a pro-fibrogenic molecule that acts on HSCs *in vitro* [252]. Notch signalling and the Hedgehog(Hg) pathways are also implicated in MASLD and are both considered profibrogenic[253].

In summary, lipotoxicity causing hepatic cell injury through ROS production, oxidative stress and ER stress can then either directly or indirectly activate an inflammatory response or hepatic stellate cell-mediated collagen deposition which are both integral to MASH progression and will be discussed in the next section.

1.6.7 The role of immune cells in MASLD to MASH transition

Macrophages are the most abundant immune cell within the liver and reside in the sinusoidal space however their lineage and functions are vast and increasingly complex with both homeostatic, immune and disease specific functions. Their abundance, plasticity and diverse range of functions makes them an important cell within the liver with their location, origin and current microenvironment all integral to the phenotype and function they poses.

The Kupffer cell (KC) are the tissue resident macrophages and are the predominant immune cell type under homeostatic conditions in humans that are maintained locally through proliferation in the sinusoidal blood and their role in immune tolerance means their location

has been shown to be mainly periportal[253]. They are able to quickly recognise and phagocytose bacteria passing through the large blood supply to the liver and act as antigen presenting cells(APCs) and thus important for the recruitment of other cells to the site of injury, including HSCs and adaptive responses[254]. While they are important for immunoregulation they also have key roles in tissue homeostasis, play a role in metabolism; specifically iron and cholesterol metabolism, respond to gut-derived pathogens and enable immunotolerance[255].

In humans monocytes and monocyte-derived macrophages from the bone marrow are recruited to the liver primarily as a response to an inflammatory insult [253] and following the initial response can mature into a pro-resolving phenotype enabling vessel, sinusoidal and extracellular matrix reconstruction[256]. Mounting evidence also shows these recruited monocyte-derived macrophages are also present at low levels in humans in a steady state, localising to the central vein with spatial transcriptomics demonstrating their ability to differentiate into KCs [257, 258].

Initially macrophages were thought to have two phenotypes – an M1 pro-inflammatory phenotype versus an M2- anti-inflammatory phenotype similar to that describe in the WAT (section 1.5.8) with polarisation dependent on the microenvironment, cytokine release and cross-talk from other cells [257, 259]. However, in recent years this view has been challenged by single cell transcriptomics which has identified several unique macrophage subsets including lipid-associated macrophages (TREM2⁺ Cd63⁺ Cd9⁺ Gpnmb⁺) and scar-associated macrophages (TREM2⁺CD9⁺), and thus enabling a more intricate understanding of macrophage and monocyte biology in healthy and diseased livers[260].

There is increasing evidence demonstrating the importance of KC in MASLD, both as inflammatory and metabolic mediators. Lipotoxicity occurs following excessive accumulation of lipids within hepatocytes resulting in CCL2 and CXCL10 release as a stress response triggering macrophage-mediated inflammation. Gut-derived endotoxins, free fatty acid (FFA) release from adipose tissue and high levels of circulating cholesterol have all been implicated in activating macrophage-mediated inflammation in the liver via pattern recognition receptors or scavenger receptors including toll-like receptor 4 (TLR4) and CD36[261]. Altered lipid products that directly lead to oxidative stress such as malondialdehyde have also been shown to lead to macrophage activation[262]. Once activated, Kupffer cells often release IL-1 β and induce further lipid accumulation in hepatocytes by PPAR α downregulation and reduced fatty acid oxidation[263].

Importantly, macrophage depletion in obese animal models demonstrated decreased steatosis and improved insulin sensitivity[259, 264, 265]. Both in humans and mouse models

Daemen et al. and Ramachandran et al. demonstrated Kupffer cells (TIM4⁺) are reduced with increased infiltration of bone marrow-derived monocytes (TIM4⁻) in MASLD and cirrhosis compared to healthy livers[260]. In an obese or MASH microenvironment i.e. steatotic zones, monocyte-derived macrophages in turn give rise to lipid-associated macrophages (LAMs) [253] which have repeatedly shown to be linked closely with TREM2 expression but there is conflicting evidence when deciphering TREM2's role; depletion in rodent models has been shown to protect against MASH development and is associated with reduced IL-1 β , IL-10 and TNF in mice fed a HFD[258] on one hand while accelerating fibrosis in mice fed a HFD [262] in another. Strikingly, these lipid-associated macrophages have been shown to form aggregates or hepatic crown-like structures similar to that observed in the WAT[109, 258] and are a feature that is observed in advanced MASH and fibrosis. The formation of these structures is thought to be due to the significant size of hepatocytes following excessive lipid uptake rendering them impossible to be phagocytosed by a single macrophage, so several lipid-associated macrophages form multinucleated aggregates and surround dying hepatocytes to engulf them through cell fusion[170]. Hepatic CLS seem to be a source of inflammation but there is some evidence to suggest that they are also protective against fibrosis formation[260, 266].

Scar-associated macrophages (SAMs) that have a distinct phenotype have now been described with CD9⁺ and TREM2⁺ markers and are associated with profibrotic genes (SPP1, LGALS3, CCL2, CXCL8, PDGFB and VEGFA) and have been found in pro-fibrotic niches in increased abundance in patients with advanced MASH versus early MASLD. Furthermore, *in vitro* cultures demonstrated SAMs conditioned media induce HSC proliferation [260].

While recent developments in advanced transcriptomic techniques have significantly changed the field of macrophage and monocyte biology and lineage and further highlighted the plasticity and importance of these cells in both immunometabolism homeostasis and disease pathogenesis, their function in distinct environmental niches and disease-types is still to be fully elucidated and moreover their cross-tissue and cell-cell relationship is still to be completely defined.

1.6.8 The role of fibrosis in MASLD to MASH transition

Liver fibrosis is the most important predictor of prognosis in MASH [68] and is primarily the result of the accumulation of extra cellular matrix (ECM) which in the liver comprises of collagens and non-collagenous glycoproteins (elastin, laminin, fibronectin), glycosaminoglycans (hyaluronan), and proteoglycans (fibromodulin, decorin, biglycan, glypicans and syndecans) [251]. In healthy livers collagen type I and collagen type III are

predominant in the space of Disse, capsule and stroma, with type IV mainly deposited in the basal lamina and type V within fibrils. Decreased levels of fibronectin, found in the capsule and stroma, has been associated with worsening cirrhosis and increased levels of collagen type I [267].

The main cell in the liver that leads to production of ECM are the myofibroblasts, but the origins of which cell types give rise to myofibroblasts are still debated. The resident hepatic stellate cells comprise of up to 80% of myofibroblasts in MASLD, while portal fibroblasts, bone marrow derived fibroblasts and mesenchymal progenitor cells can all also trans-differentiate into myofibroblasts in smaller numbers but are often dependent on location and disease pathology [250]. Quiescent HSCs in a healthy liver primarily store vitamin A. In liver injury, lipotoxicity initiates the trans-differentiation of quiescent HSCs by release of stored retinyl esters enabling them to become activated myofibroblasts that are able to proliferative, are contractile, inflammatory, increase ECM deposition and involved in chemotaxis[268]. Classically activated HSCs (aHSC) express the markers protein platelet-derived growth factor receptor-B(PDGFRB), the enzyme leichin retinol acyltransferase (LRAT), cytoskeletal proteins desmin and glial fibrillary acidic protein (GFAP) and transcription factor heart- and neural crest derivatives- expressed protein (HAND2) and release an abundance of pericellular matrix proteins including vimentin, alpha-smooth muscle antigen, non-muscle myosin and fibronectin [252, 269]. In a healthy liver they are important in targeting infection and the repair response and on resolution of these processes are inactivated or can undergo apoptosis however in pathogenic disease this balance is impaired which results in an accumulation of active myofibroblasts.

Multiple signalling pathways and small molecules activate HSCs including innate immune signalling from TLRs, LPs and DAMPs, hedghog signalling pathways, cytokines and adipokines, nuclear transcription factor receptors, epigenetic factors, cholesterol accumulation and often as a result of oxidative or ER stress [252].

Cholesterol accumulation in MASH has been associated with the hypertrophy of activated HSCs similar to that seen in hepatocytes. This is associated with increase collagen deposition and directly correlated with disease activity [268, 270]. Excess accumulation of FFA and cholesterol in hepatocytes activates HSCs through TLRs or indirectly through macrophage-mediated inflammatory response and secretion of TNF, IL-1 β and TGF- β [271].

TGF- β is the primary cytokine found to activate HSCs and TGFB mRNA and serum levels are increased in MASH patients thus appearing to be a significant driver of fibrosis [272]. It is produced by several cells including activated HSCs and in response to hepatic damage and ROS production and signals through SMAD2, SMAD3 and SMAD4 pathways in the aHSCs

leading to collagen I and III production. Rodent models that have inhibited TGF- β have shown reduced numbers of aHSCs [273, 274].

Furthermore, PDGF is another growth factor heavily involved in activating HSCs. Ramachandran et al. showed that scar associated macrophages increased proliferation of HSCs in specific hepatic niches through TNFSF12 and PDGFBB [260]. Overexpressing PDGF- β in mice caused liver fibrosis and patients with severe fibrosis versus early MASLD changes histologically had increased expression. Crosstalk between macrophages and HSCs is therefore heavily implicated in MASH-associated fibrosis formation [275, 276].

HSCs also play an important role in resolving fibrosis and can be 'inactivated' following liver injury repair. Nuclear receptors liver X receptor (LXR) and farnesoid X receptor (FXR) that control lipid and glucose metabolism can inhibit activation of HSCs. LXR ligands reduce markers of fibrosis, including TGF β , through regulating sterol regulatory element -binding protein (SREBPs) [277, 278]. Mice deficient in LXR or FXR have increased susceptibility to developing fibrosis [277] and therefore have become targets of interest for drug therapy [274]. PPAR γ has also been shown to induce quiescence and decrease α -SMA, Type I collagen and TGFB mRNA levels [279].

The role of HSCs in fibrosis formation is pivotal to preventing further liver damage and as discussed earlier in this chapter there are multiple treatment targets currently being trialled. However, while advances have been made in understanding myofibroblasts involvement in MASH, their role during different disease states and cell-cell crosstalk, it is still not clear at what stage fibrosis is irreversible. Moreover, as only 25-30% of patients with MASH will develop fibrosis and cirrhosis it remains to be elucidated exactly what triggers the ECM deposition and scar formation in certain individuals and not others.

1.7 Crosstalk between white adipose tissue and the liver

The consequences of prolonged obesity on WAT and liver tissue have been explored in the previous sections with key mechanisms, signalling pathways and cell-cell interactions identified as potential causes of hepatic injury and WAT dysfunction. The crosstalk between WAT and liver tissue in obesity and MASLD progression is yet to be fully understood however studies show a clear link between dysfunctional adipose tissue and progressive hepatic damage [280, 281]. The proportion of FFA from visceral AT rises to 50% in the portal circulation of obese adults from only 5-10% in lean adults and increased FFA release from omental WAT into the portal circulation is associated with progression of insulin resistance and MASLD [282]. Furthermore, AT lipolysis in a mouse model is shown to mediate hepatic

steatosis and IR through glycoprotein (gp)130, a transmembrane protein that forms a subunit of the type 1 cytokine and IL-6 receptor family [283].

1.7.1. Inflammation and liver-WAT crosstalk

There is also mounting evidence that adipose tissue inflammation is involved in insulin resistance and MASLD progression. Du Pleuiss et al. showed that histological progression of liver disease positively correlated with inflammatory genes in human biopsies of WAT [284]. Canello. et al. has also shown HAM⁺ omental macrophages increase in tandem with hepatic fibrosis and demonstrated that as adipose tissue insulin resistance progressed, MASH also progressed [285]. Several other human studies to date have also observed increasing WAT macrophage infiltration correlated with hepatic fibroinflammatory lesions in both obese adults and children [285-287]. The exact role and mechanisms involved are still to be elucidated and some studies have begun to try to understand this link. Pro-inflammatory AT macrophages (CD14⁺CD11c⁺) have been shown to increase as MASLD progressed while anti-inflammatory AT macrophages (CD14⁺CD136⁺) decreased in patients with worsening fibrosis [246]. A recent sc-RNA seq study suggested that the phenotype of ATMs change with the degree of MASH, which may be related to the VAT vascular barrier permeability, but the numbers are too small to make firm conclusions [288]. From a metabolic prospect, Jaitin and colleagues demonstrated that adipose tissue lipid-associated macrophages, specifically TREM2⁺ cells, are involved in moderating metabolic homeostasis [109].

Furthermore, cytokine release of inflammatory mediators from visceral adipose tissue have been studied in humans and IL-6, TNF-alpha, and MCP-1 has been associated with increasing severity of MASLD and systemic CCL2 corresponded with morphological changes of MASLD in obesity [283, 289-291].

Mouse studies have also explored this relationship; the first study that showed ATMs potentially modulate MASH progression was performed by transplanting VAT from an obese mouse to a lean mouse and observing the progression of steatohepatitis, this was prevented if ATMs were depleted prior to transplantation using clodronate liposomes [292]. Since this study, further mouse models have also demonstrated macrophage inflammation in the WAT is associated with hepatic steatosis. By surgically removing inflamed epididymal fat [293], reducing white adipose tissue inflammation by treating with rosiglitazone [294] and selectively depleting adipose tissue macrophages using dextran coupled with dexamethasone nanocarriers all ameliorated hepatic steatosis in obesity [295]. Insulin resistance has also been shown to improve by selectively knocking out adipose tissue macrophage FASN [296] and obese mice treated with lean ATM exosomes had increase

insulin sensitivity [297]. While these show that WAT macrophages are clearly linked to human MASLD the mechanisms are yet to be deciphered and remains a gap in current knowledge and highlights an interesting potential treatment target [298].

1.7.2. Adipokine/hormone signalling crosstalk

While the link between WAT inflammation and liver injury in recent years has become more evident, changes in the hormones released from WAT and SAT have also mounted interest and have been associated with insulin resistance, hepatic inflammation and fibrosis.

Leptin is a critical adipokine involved in metabolic and endocrine homeostasis and is secreted from white adipose tissue in proportion to the amount of white adipose tissue mass, thus reflecting a person's whole body energy stores [299]. There is clear evidence from a relatively recent meta-analysis that in obesity, systemic leptin is increased which is likely due to relative leptin resistance [300] and thus impaired leptin response. The hyperleptinemia also correlates with severity of liver injury [301] and hepatic IR [265]. There has been, therefore, interest in leptin's role in disease progression and experimental studies have suggested leptin has a dual functional response in MASLD; with leptin having a protective role in early hepatic steatosis but a profibrotic and proinflammatory effect as disease persists or progresses.

Leptin has been shown to have both direct and indirect effects on hepatic steatosis. On one hand animal models suggest leptin acts directly on hepatocytes and prevents hepatic glucose production and lipogenesis through activating the JACK2/STAT3 pathway [302, 303]. However, on the other hand a mouse model injected with intrathecal leptin suggested a central role of leptin on the parasympathetic autonomic response which resulted in reduced DNL and increased hepatic triglyceride export [304]. Furthermore, in later disease, leptin has been shown to act on Kupffer cells increasing TNF α production through JNK-MAPK pathways [305], TGF β [306] and pro-inflammatory cytokines in obesity as well as directly activating HSCs which release TGF β and Collagen I α and in turn leads to hepatic inflammation and fibrosis [307].

Adiponectin was first described by Berg et al. in 2001 as Acrp30 and was observed to be a potent insulin sensitiser that links adipose tissue and hepatic glucose control through primarily PPAR γ mediated reduction in gluconeogenesis and glycogenolysis [308]. Since then, research has demonstrated that it is integral to WAT and liver homeostasis with decreasing circulating levels correlating with increasing adipocyte diameter, insulin resistance and hepatic steatosis in obesity [285, 309]. The reverse has also been described with increased levels associated with decreased fatty liver and a shift from VAT to SAT fat distribution [310]. Postprandial adiponectin has been shown to predict liver dysfunction and

acts as a $\text{TNF}\alpha$ antagonist [311]. Adiponectin appears to reduce hepatic DNL, act through AMPK signalling pathways to stimulate fatty acid oxidation [312], and sensitises insulin signalling via IRS-2 and IL-6 dependent pathways [313].

A further secretory factor Neuregulin 4 (Nrg 4) has been suggested to have a protective role in MASLD with decreased serum levels in people with MASLD compared to non-MASLD, however no differences was observed between obese and lean individuals in this population [314].

In summary, crosstalk between WAT and the liver is increasingly important in understanding the complex multi-system effects of obesity and identifying potential mechanisms or soluble factors which could be utilised to prevent end-organ disease.

Previous research performed by the Behmoaras lab identified a link between WAT and liver tissue homeostasis through oxylipin signalling pathways (bioactive lipid mediators, discussed in detail, below) using a $\text{Cyp2j4}^{-/-}$ rat model fed a cafeteria (CAF) diet. Cyp2j4 is the rat orthologue of human CYP2J2, and unlike mice, the gene locus does not present allelic expansion. $\text{Cyp2j4}^{-/-}$ rat fed CAF demonstrated adipocyte dysfunction (decreased CEBPA and PPAR γ expression), raised WAT hydroxyproline levels, collagen VI deposition and CD68 $^{+}$ macrophage infiltration which correlated with lipid accumulation in the liver and therefore suggests an important role for bioactive lipids in obesity[209]. This study, which forms the basis of my project in humans, had some important conclusions which could be highlighted as

- (i) Oxylipins, specifically fatty acid epoxides, are important regulators of adipogenesis
- (ii) A disrupted adipogenesis process, accompanied by WAT inflammation/fibrosis is a pre-requisite for hepatic liver accumulation

However, this study did not investigate oxylipins in the liver and whether hepatic oxylipin metabolism contribute to MASLD/MASH was unclear. More importantly, there is no study comprehensively measuring oxylipin dynamics in WAT and liver during obesity and MASLD.

In the next section I discuss the role of PUFA-derived oxylipins in obesity and their involvement as potential mediators in crosstalk between WAT and the liver in the development of T2DM and MASLD. Firstly, I shall discuss how oxylipins are produced from their substrates polyunsaturated fatty acids (PUFAs), and their roles in cell signalling.

1.8 Polyunsaturated fatty acids

Oxylipins are oxygenated polyunsaturated fatty acids which are the products of fatty acids (FA). FAs are essential for cell biology as structural components of membranes, inflammatory mediators and an important source of energy. FAs are molecules that have a carbon chain of varying length, a methyl terminus and a carboxylic acid group and can be split into 3 groups: saturated (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) depending on the number of carbon chains and double bonds. SFA have the maximum number of hydrogen atoms with no double bonds and have 14-24 carbon chain length, while mono and polyunsaturated fatty acids are the products of the essential fatty acids, alpha-linoleic acid and linoleic acid, through desaturation and addition of a double bond. MUFA can have one double bond and chain length of 14-24 while PUFAs can contain 2-6 double bonds with chain length of 16-22.

Essential PUFAs are important for growth and development are predominantly required to be absorbed through dietary sources, the most common being alpha-linoleic acid (n-6 or omega-6; 18:3) and linolenic acid (n-3 or omega-3;18:2). In healthy homeostasis the levels of these free (non-esterified) PUFAs are low to ensure that their products, the oxylipins are tightly controlled due to their potency and short half-life [315]. They are sequestered to form phospholipids (PL) and stored in the membrane phospholipid bilayer (PLB) or as triglycerides (TAG) in the endoplasmic reticulum or lipid vesicles and can be released when required by the cell in a dynamic complex remodelling cycle [316].

1.8.1 Mechanisms of transport of PUFAs into cells

PUFAs are predominantly albumin bound and taken up by most cells in the human body including heart, adipose, brain, liver kidney, ovary, lung, spleen, pancreas, muscle and small intestine through protein mediated transport mechanisms. The cellular uptake of PUFAs can occur through three potential mechanisms utilising caveolin, fatty acid transport proteins (FATPs), fatty acid binding proteins (FABPs) and acetyl-coA binding proteins (ACBPs) which govern intracellular movement and are tissue specific. Firstly, FABPc can deliver the FA to the membrane where CD36 facilitates the PUFA across the membrane into the cell, bound to FABP. Secondly, FAs cross the membrane using passive diffusion, also described as the flip-flop mechanism. Finally, very long chain fatty acids (VLCFA) can be preferentially inserted into the cells by FATPs. On entering the cytol, FA co-A enters and FA are shuttled by one of 10 FABP and 3 ACBP or converted to FA-CoA by acyl-CoA synthetase (ACSL) [315, 317].

They are then metabolised by desaturases (FADS), elongases (Elovl) and peroxisomal beta-oxidation to the PUFAs; DHA, EPA and AA (**Figure 6**) and their derivatives and then

transported to different organelles by fatty acid transport proteins (FABP) and G-protein coupled receptors (GPCR) [318].

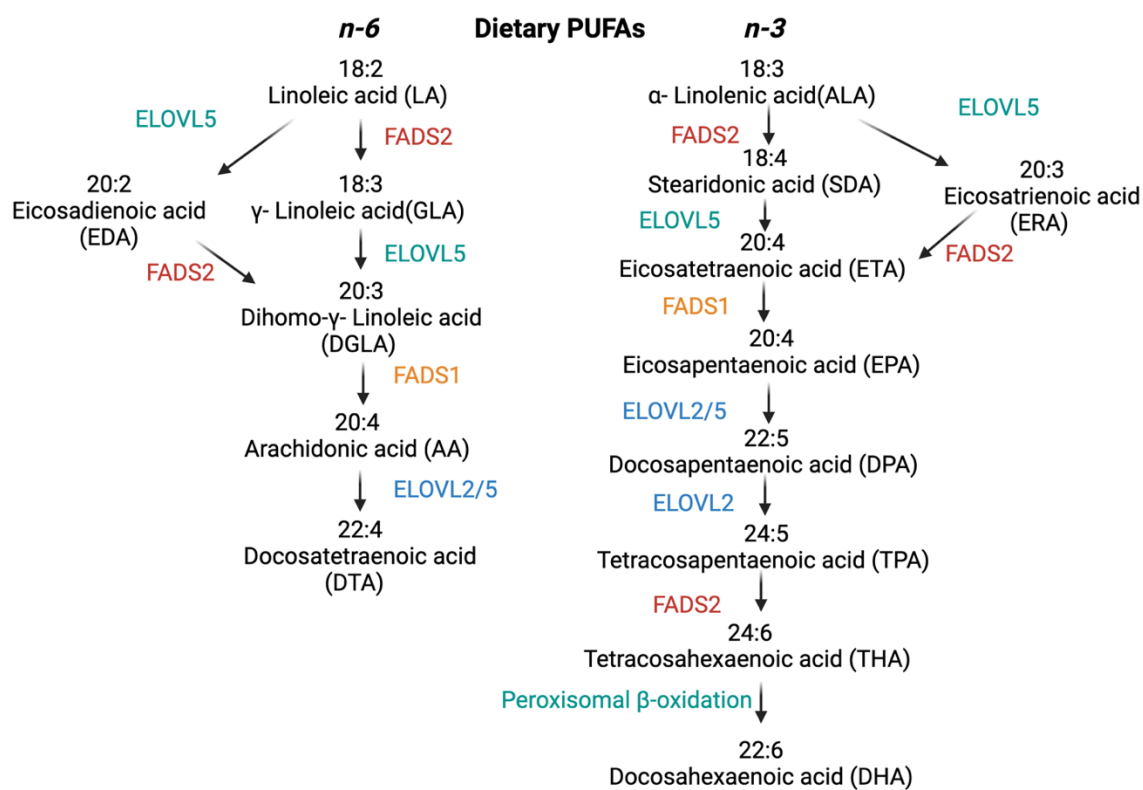


Figure 6 Metabolism of dietary PUFAs in cells to their derivatives before being transported to organelles. Dietary PUFAs (n-6 and n-3) are metabolised by desaturases (FADS), elongases (Elovl) and undergo peroxisomal-beta-oxidation to DHA, EPA and AA before being transported to different organelles by fatty acid transport proteins. PUFA: polyunsaturated fatty acids; DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid; AA: arachidonic acid. Image created by Bio Render®

1.8.2 Incorporation of PUFAs into the phospholipid membrane

The PUFA is then esterified by acyl-CoA synthetase (ACS) and incorporated into the sn-2 position of TAG or PL by acylation using acyltransferases, specifically lysophospholipid acyltransferases (LPLATs) [315, 319]. Phospholipids are made up of a hydrophobic tail and a hydrophilic head and are integral to the structure of cells and undergo remodelling and recycling through co-A independent transacylase transfer [320].

1.8.3 Synthesis of bioactive PUFA derivatives

Once stored as phospholipids, PUFAs can be synthesised to bioactive lipid mediators, (e.g. oxylipins) in a dynamic fashion through the release of glycopospholipids from the cell's phospholipid bilayer by calcium dependent or independent activity of phospholipase A2 (PLA2) which performs phospholipid diacylation [316]. PUFAs can then undergo recycling and remodelling through re-incorporation into the phospholipid bilayer. Alternatively, the oxylipins can be synthesised through the direct modification of PUFAs trafficked through the cells.

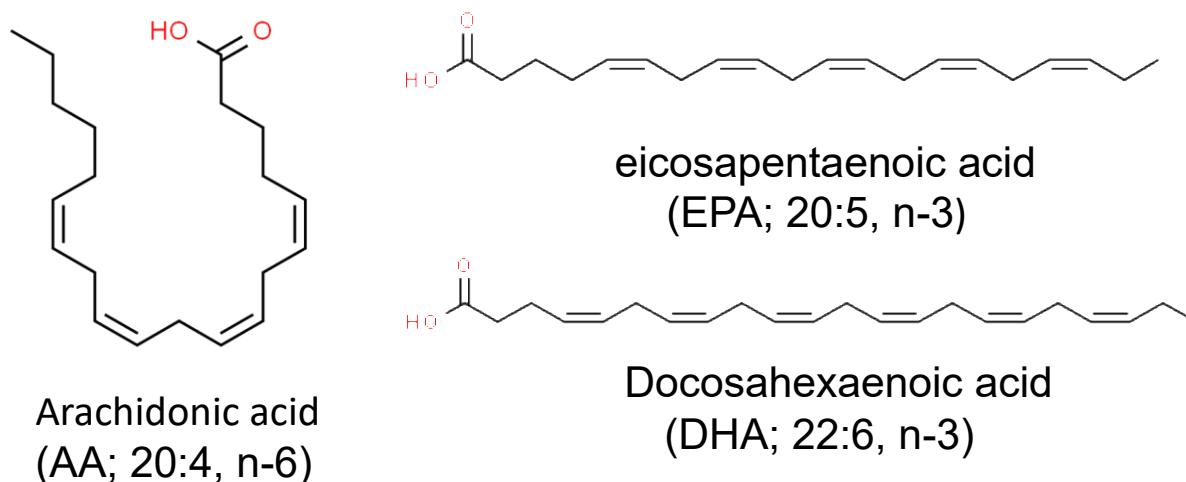


Figure 7 Main metabolic PUFAs structure: Arachidonic acid (AA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA).

The main metabolic PUFA is arachidonic acid (AA; 20:4, n-6), which makes up 15-20% of the lipid membrane and is derived from linoleic acid. The other predominant PUFAs are docosahexaenoic acid (DHA; 22:6, n-3) and eicosapentaenoic acid (EPA; 20:5, n-3) which are thought to be anti-inflammatory and derived from alpha-linoleic acid derivatives and dihomo- γ -linoleic acid (DGLA; 20:3, n-6) a linoleic acid derivative.

1.8.4 PUFA-derived Oxylipins

AA, EPA and DHA are all served as substrates for oxygenation by three sets of enzymes; cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) that act on different carbon positions of the acyl chain to produce a variety of free (non-esterified) oxylipin derivatives. The oxylipin family include eicosanoids, which are AA-derived 20 carbon metabolites that are tightly regulated due to their potency and short half-life in plasma [321]. Therefore, having been previously identified as important mediators of WAT turnover and inflammation, oxylipins and the subgroup of eicosanoids are of particular interest with their roles in obesity related T2DM and MASLD still to be fully defined. These oxylipins are able to activate different pathways including PPAR receptors, ligand activated transcription factors or signal through the G-protein-coupled receptors (GPCR) on the plasma membrane both in an autocrine and paracrine manner [316].

1.8.5 Cyclooxygenase (COX)-derived metabolites

Cyclooxygenase derivatives are the most well characterised PUFA-derived bioactive lipids and are catalysed by either COX-1 or COX-2 enzymes which are encoded by PTHS1 and PTHS2 genes. COX-1 is expressed at relatively constant levels while COX-2 is activated by

inflammatory cascades through cytokine, growth factors and hormone release [322]. The COX enzymes incorporate two oxygens to the acetyl chain which leads to the formation of the 5-carbon member ring structure at carbon 8-12 and the endoperoxidase bridge to form the structures known as prostanoids, a subgroup of oxylipins. AA, EPA, DHA and dihomo- γ -linoleic acid (DGLA) are catalysed by COX enzymes into the unstable PGH₁, PGH₂ and PGH₃ respectively via PGG₂. PGH derivatives are the intermediates for all further prostanoids; prostaglandins (D, E, F, G, H), prostacyclin (I) and thromboxanes (TXA and TXB) (**Figure 8**).

EPA is converted into series one prostanoids (PGD₁ and PGE₁), AA into series two prostanoids (PGD₂, PGE₂, PGF₂, PGI₂ and TxA₂) and DGLA into series three prostanoids (PGD₃, TxA₃) and as AA is the most abundant PUFA in the PL, the series two prostanoids are preferentially produced [323, 324]. These PUFA derivatives are produced by specific isomerases and synthases and are tissue dependent; for example thromboxane A₂ (TxA₂) is mainly expressed in macrophages and platelets, prostaglandin I₂ (PGI₂), known as prostacyclin is expressed in epithelial cells and prostaglandin F₂ α expressed at high levels in the uterus [322, 325]. These secondary prostanoids are often at nM concentrations within cells and have potent action on inflammation, pain and thrombosis [325].

The prostanoids are able to diffuse through the cell membrane to the GPCRs on the cell surface in an autocrine and paracrine manner. The double bonds that determine the prostanoids structural nomenclature enable the preferential binding of the individual to the specific receptors. The prostanoid receptors consist of five classes: Prostanoid receptor D (DP), Prostanoid receptor E (EP), prostacyclin receptor I (IP), thromboxane receptor (TP) and Prostanoid F receptor (FP) determined by the binding of the most potent ligand to the receptor, however there is also cross-reactivity [326].

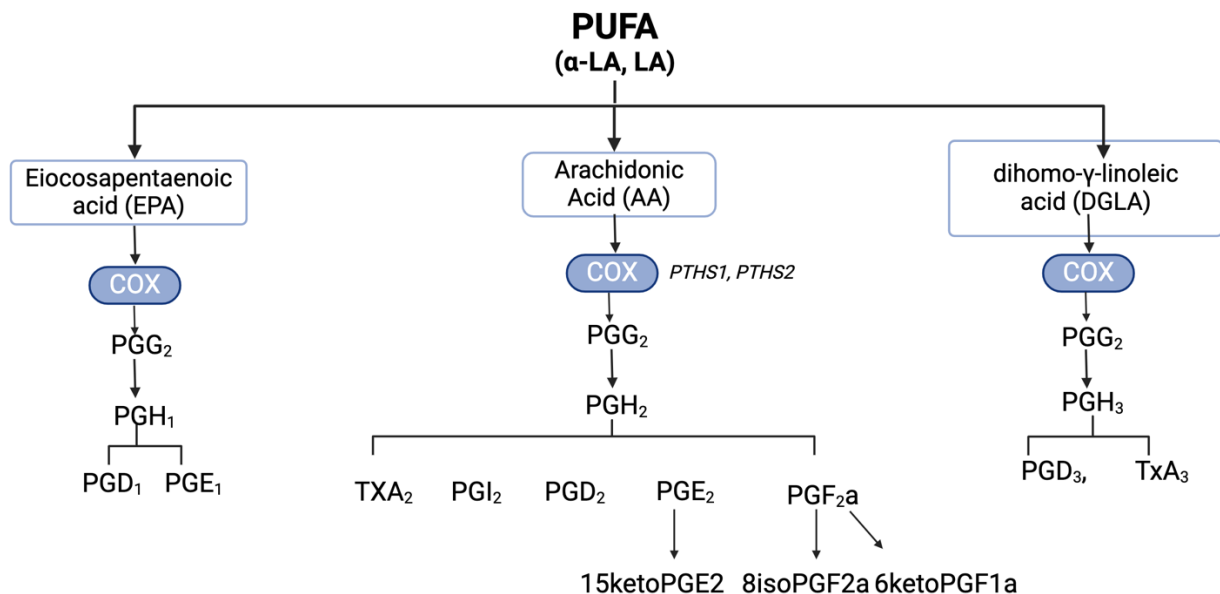


Figure 8 Pathway for cyclooxygenase (COX)-derived prostanoid. PUFAs are catalysed by either cyclooxygenase-1 or -2 encoded by the PTHS1 or PTHS2 gene to prostanoids. EPA is converted into series one prostanoids (PGD₁ and PGE₁), AA into series two prostanoids (PGD₂, PGE₂, PGF₂, PGI₂ and TxA₂) and DGLA into series three prostanoids (PGD₃, TxA₃). COX: cyclooxygenase; PUFA: polyunsaturated fatty acids; α-LA: alpha-linoleic acid; LA: linolenic acid; EPA: Eicosapentaenoic acid; AA: arachidonic acid; DGLA: di-homo-γ-linoleic acid; PG: prostaglandin; TX: thromboxane. Image created by Bio Render®

1.8.6 Prostaglandins and their roles in cell-signalling pathways

PGE₂ is the most potent and well characterised prostaglandin which is found in most cells in the human body. PGE₂ can activate EP4 receptors and upregulate the PKA pathway to induce lipolysis [208]. It has also been shown to inhibit PPARγ expression [327]; different concentrations have been implicated to explain why both inhibition and induction can occur by the same metabolite within cells. PGE₂ has also been shown to modulate angiogenesis with microsomal PGES-1-null mice having less vascular endothelial growth factor (VEGF). Furthermore, PGE₂ promotes macrophage polarisation to a M1 phenotype, rendering it a pro-inflammatory lipid mediator [315].

PGF_{2-α} is associated with hypertrophic cell growth, induction of interleukin synthesis and uterine contraction. It has also been identified as a PPARγ antagonist through several pathways, firstly through binding and activating the FP membrane receptor and triggering a Ca²⁺ flux which in turn inhibits the PPARγ expression through activation of calcineurin. A further pathway has been identified which results in phosphorylation of ERK1/2 via Ca²⁺/PKC/MAPK signalling pathway and leads to overexpression of Fra-1 (fos-related antigen-1) which inhibits adipogenesis via PPARγ phosphorylation [328-330]. On the other hand PGJ₂ (derived from PGD₂) is a strong PPARγ ligand at high concentrations whilst also

being the main prostanoid synthesised in mast cells and leukocytes with proinflammatory effects through DP1 and DP2 GCPRs[315].

Prostacyclin is the most important prostanoid produced by vascular endothelial (VEC) and smooth muscle cells (VSMC), enabling VSMCs relaxation and preventing platelet cell aggregation. PGI₂ receptor IP-null mice have accelerated atherosclerosis and platelet aggregation. PGI₂ has also been shown to be secreted from adipocytes and acts on PPAR γ both directly and indirectly (via cAMP and PK pathway or CEBP) [331, 332].

1.8.7 Thromboxanes and their role in cell homeostasis

Thromboxane has been extensively investigated as a potent stimulator of platelet aggregation and smooth muscle constriction and has been implicated in the pathogenesis of atherosclerosis and myocardial infarction. Aspirin is used to treat and prevent further myocardial infarctions with the main action through inhibition of TxA₂ from platelets but minimal changes to PGI₂ from endothelial cells. It is interesting to note that COX-2 inhibitors, which are a potent non-steroidal anti-inflammatory (NSAID) have been controversially shown to cause increased myocardial infarction risk, which is thought to be due to a shift in the balance between TxA₂ (anti-inflammatory) and PGI₂ (pro-inflammatory) oxylipins within tissue, thus highlighting oxylipins are integral to homeostasis within cells and their effects are the result of a fine balance of different PUFA derivatives and similar shifts maybe important in other oxylipin signalling pathways [316].

TxA₂ has also been shown to be released on LPS stimulation in the liver, specifically by the Kupffer cells, where they have a pro-inflammatory role [324] however, there is very little data on whether TxA₂ had any role in WAT in obesity and whether it is involved in T2DM or MASLD disease progression.

1.8.8 The role of Cyclooxygenase (COX)-derived metabolites in T2DM/MASH in humans

Human lipidomic studies evaluating the role of COX-derived metabolites in obesity-related T2DM suggest prostaglandins, specifically PGE₂, PGD₂, PGF₂ and 15-Keto-PGE₂ all increase in the plasma in line with insulin resistance with one human study also showing an increase in PGE₂ and PGD₂ in the urine with worsening metabolic control [333-335]. Furthermore, the prostaglandins PGD₂ and PGE₂ also increase in obesity associated-MASH progression [333, 336]. While the literature certainly suggests there is an increase in prostaglandins with worsening MASH and T2DM, resolvins and LTB₄ significantly decreased in the plasma of T2DM individuals compared to non-diabetic individuals [337]. Interestingly, exercising did not have an effect on the COX-derived metabolites in patients' plasma with MASLD [336]. While systemic prostaglandins appear to increase with IR and MASLD, there

is currently no human data on tissue-specific changes in the liver and adipose tissue for these metabolites in obesity.

1.8.9 Lipoxygenase (LOX)-derived metabolites

Lipoxygenases are a family of iron (non-heme) dioxygenases that catalyse PUFAs through hydroperoxidation to leukotriene, lipoxins and hydroxyeicosatetraenoic acids (HETEs) and hepoxilins. Lipoxygenases are mainly produced by bone marrow derived cells. There are 6 lipoxygenase genes (ALOX) that code for 4 groups of LOX enzymes (LOX-3E, 5-LOX, 12-LOX and 15-LOX) based on which specific carbon of AA, DHA, DGLA and EPA they are oxidising. LOX-derived oxylipins including HETEs and leukotrienes are able to move freely intercellularly with GPCRs [315]. AA is metabolised by LOX-5, LOX-15 and LOX-12 to 5-HEpETE, 15-HEpETE and 12-HpETE respectively. The primary product of LOX oxidation is an unstable hydroperoxide which is then reduced to its corresponding hydroxide by catalysing the product with glutathione peroxidases.

5-HpETE is metabolised by 5-LOX to LTA₄ and further to LTB₄, LTC₄, LTD₄ and LTE₄ at the nuclear membrane while 12-LOX produces 12-HETE with 12-HpETE as an intermediate. 15-LOX produces 15-HETE and 15-oxo-EETE and can also lead to the production of LTA₄ and LTB₄ which are pro-resolving as opposed to other metabolites that are pro-inflammatory.

LA is metabolised to 13-Hydroxyoctadecadienoic acid (13-HODE), DHA is metabolised via the intermediates 11-HpDHA and 14-HpDHA to 11-HDHA and 14-HDHA by 12-LOX and EPA to E-series resolvins or LTA₅ and LTB₅ by LOX-5 via the intermediate 5-HpEPE (**Figure 9**).

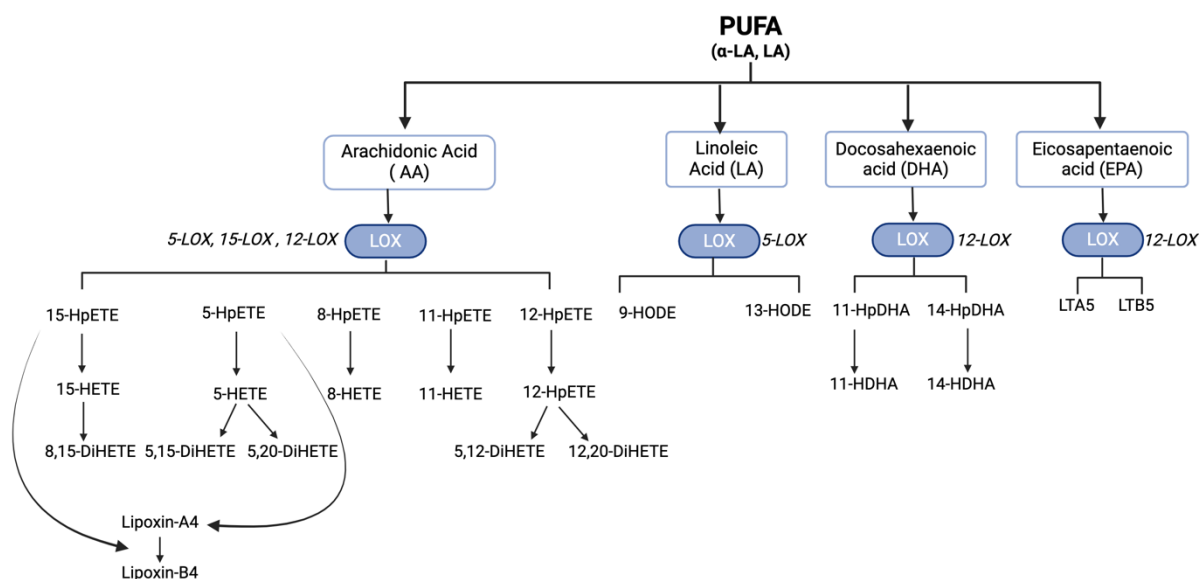


Figure 9 Pathway for lipoxygenase (LOX)-derived metabolites. Lipoxygenases catalyse PUFAs through hydroperoxidation to leukotrienes, lipoxins, hydroxyeicosatetraenoic acids and hepxilins. Depending on which specific carbon of AA, DHA, DGLA and EPA are oxidised determines which LOX enzymes (LOX-3E, 5-LOX, 12-LOX and 15-LOX) is used. PUFA: polyunsaturated fatty acids; DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid; AA: arachidonic acid; α-LA: alpha-linoleic acid; LA: linolenic acid; LOX: lipoxygenase; HODE: Hydroxyoctadecadienoic acid; HETE: hydroxyeicosatetraenoic acids; DiHETE: dihydroxyeicosatetraenoic acid. Image created by Bio Render®

1.8.10 Lipoxygenase (LOX) derivatives in obesity and Inflammation *in vitro* and *vivo* models.

12-LOX is the most well-studied enzyme which is found in human platelets and its precursor cells; megakaryocytes while 15-LOX is predominantly in monocytes and 5-LOX in neutrophils. Lipoxygenases have been implicated in the bronchoconstriction of smooth muscle in asthmatic patients by LTC₄ and LTD₄ release from mast cells in both large and small airways of humans. One of the main clinical uses of lipoxygenases have been anti-leukotriene agents for use by asthmatic patients.

In obesity-associated adipose tissue homeostasis, LOX derived metabolites are shown to facilitate activation of PPAR in early differentiation using the cell line 3T3-L1 pre-adipocytes. 12/15- LOX knockout mice were shown to be protected from insulin resistance and had normal adiponectin and anti-inflammatory cytokines when fed a HFD compared to the wild type. 12(S)-HPETE (the unstable hydroperoxide form of 12-LOX reaction) and 12(S)-HETE (reduced form) have also been shown to be pro-inflammatory by releasing cytokines, TNF-α and MCP-1, IL-6 and IL120p40 with a reduction in adiponectin in the cell line 3T3-L1 [316, 325, 338]

LOX-5 pathway products have also been implicated in obesity-associated low-grade inflammation in adipose tissue and adipocyte dysfunction through Nf-κB and cytokine release [339]. In HFD-induced MASLD/MASH mice 5-LOX is increased and fibrotic changes

are then attenuated when 5-LOX is inhibited [340]. LTB₄ has also been shown to promote lipid accumulation through PPAR- α signalling pathway and the BLT1 and BLT2 receptors [341] which may be contributing to the pathogenesis. Furthermore, ALOX3 and ALOX12 products hepoxilin A3 and B4 are pro-adipogenic, and are PPAR γ agonists, specifically targeting early differentiation. Interestingly, ALOX3 has been shown to co-localise with PPAR γ in the nucleus of the cell [342].

1.8.11 The role of Lipoxygenase (LOX)-derived metabolites in T2DM/MASH in humans

Most of the research to date has focused on using cell lines or animal models to investigate the link between oxylipins and metabolic syndrome. Recent lipidomic studies in humans with obesity have mainly focused on changes in plasma levels of oxylipins with a few studies that have measured tissue-specific levels. Patients were five times more likely to be obese with increased plasma concentrations of 5-HETE, 8-HETE and 12-HETE [343] and these metabolites in a further study were shown to increase in the plasma of individuals with obesity as systemic inflammation progressed [344]. There was also a global increase in LOX-derived oxylipins in visceral and subcutaneous fat during obesity-driven T2DM progression [345]. Fisk et al. on the other hand, has challenged these findings in a study of individuals that compared oxylipin levels in obese and lean individuals' subcutaneous fat and showed that 9-HODE and 13-HODE decrease with increasing insulin resistance, the difference in results may in part be due to this study population included a lean group, not included in the previously mentioned studies [346].

However, 9-HODE and 13-HODE were seen to increase with MASH progression in both the plasma [347, 348] and liver tissue [349] in three studies. Caussy et al. also showed that plasma AA- derivatives 12-HETE, 11,12-DiHETE and 14,15-DiHETE were associated with liver fibrosis [350] which was echoed by Puri with increases in 5-HETE, 8-HETE and 15-HETE observed in the transition from MASLD to MASH [351].

Therefore, as there appears to be some evidence, albeit conflicting, that there are oxylipin changes in both disease and tissue phenotypes, this strengthens the hypothesis that oxylipins are a potential mechanism for cross-tissue disease progression in T2DM and MASLD in humans and warrants further investigation of multi-tissue profiling in obesity.

1.8.12 Cytochrome P450 (CYP)-derived metabolites

Cytochrome P450 (CYP) enzymes are produced predominantly by the liver but are also produced by adipocytes, kidney, heart, skeletal muscle and the vasculature and are generally bound to the mitochondrial membrane and endoplasmic reticulum [352]. While cyclooxygenases and lipoxygenases have been extensively investigated the CYP pathways and their PUFA-derived metabolites and functions are less well described. CYP enzymes

are heme-containing monooxidases that catalyse the transfer of one oxygen atom to the substrate while reducing the other to water. This reaction requires the transfer of an electron to the heme iron by nicotinamide adenine dinucleotide phosphate (NADPH) [352]. The CYPs structure contain a β -sheet rich N-terminal domain and a large helix-rich C-terminal catalytic domain.

There are 57 functional CYP genes identified in humans with 50 coding for microsomal CYPs and 7 for mitochondrial isoforms and they have multiple important roles in catalysing reactions. The most well known is their role in drug and xenobiotics metabolism. Humans are constantly exposed to multiple sources of chemicals, either through diet, drugs, environmental pollutants and dietary additives to name a few, all of which can range from harmless to potentially toxic and therefore cellular mechanisms to ensure elimination of these chemicals are in place to prevent cellular toxicity. Drug and xenobiotic metabolism involve four phases that enable absorption, metabolism and cellular excretion of the chemical. Following influx into the cell, namely the hepatocyte, by transporter enzymes, the absorbed chemical undergoes oxidation by mainly CYP enzymes during phase I and phases II (conjugation) and III (extrusion) follow which results in excretion of the xenobiotics through bile, urine and faeces. CYPs are the major contributor to phase I oxidation in xenobiotic metabolism, they are also involved in catalysing a wide range of other cellular reactions [353].

CYP enzymes are also involved in lipid metabolism and catalyse PUFA-derived metabolites. Notably, CYP enzymes catalyse AA to its derivatives, of which there are 2 distinct pathways. The first is the epoxygenase pathway which is encoded predominantly by the CYP2J and CYP2C genes and generates epoxyeicosatrienoic acids (EETs); multiple isozymes and other CYPs including CYP 4X1 and CYP 2U1 are also epoxide producing. The second, is the ω -hydroxylase pathways which encodes predominantly CYP4A and CYP4F genes and generates 20-hydroxyeicosatetraenoic acids (20-HETE), but other CYPs including CYP 4X1 and CYP2U1 can also form HETEs. AA is not the only PUFA that is metabolised by CYP enzymes, LA is metabolised to epoxyoctadecamonoenoic acids (EpOMES), EPA to epoxyeicosatetraenoic acid (EpETE) and DHA to epoxydocosapentaenoic acid (EpDPEs) respectively [316, 353] (**Figure 10**).

As mentioned, EETs are generated through epoxidation of AA, (eg. CYP 2J2, CYP 2C8 and CYP 2C9 CYP 2S1 enzymes) which are localised to the ER. Four EET regioisomers (5,6-, 8,9-, 11,12-, 14,15-) are produced as cis-epoxides but the balance of what is produced is determined by the tissue type and microenvironment i.e. hormones, growth factors and transcription factors that determine the isoform that generates the product, for example CYP

2C8 metabolises AA exclusively to 14,15-EET and 11,12-EET in a 1.3:1 ratio in a region and stereospecific manner, while CYP 2C9 is less regional and stereoselective.[354] Overall, 67%-80% of all EETs produced are 11,12-EET or 14,15-EET regioisomers with each producing R,S or S,R enantiomers and it has been shown that the enantiomers can influence function; 11(R),12(S)-EET enables renal large vascular smooth muscle relaxation while 11(S),12(R)-EET activates the cardiac K_{ATP} pathway [315].

HETEs are produced mainly by CYP 4A and CYP 4F enzymes through inserting a hydroxyl group into the terminal carbon of AA. 20-HETE is the most abundant CYP-derived oxylipin in the liver and accounts for 50-75% of all eicosanoids. In a healthy state there is equilibrium between EET anti-inflammatory and 20-HETE pro-inflammatory state. Studies have shown that 20-HETE is elevated both in cirrhosis and in plasma in MASLD as well as other proinflammatory diseases including diabetes, pregnancy and obesity, with a shift in the balance from EETs to 20-HETE in these disease states [315].

Most work studying the CYP-sEH pathway use mouse or rat models however, the CYP enzymes and genes that code for them are species dependent, in mice the *cyp2j* locus has potentially 8 functional genes as it undergoes allelic expansion but this same gene *cyp2j* in rats has 3 functional genes, rendering the rat easier to study epoxygenase related mechanisms when using targeted gene deletion. The Behmoaras lab use a rat deficient in *cyp2j4*, the orthologue of human CYP2J2, which was shown to be widely expressed in the main rat metabolic organs making it ideal to study epoxygenases in obesity and metabolic disease, which is discussed in detail below [209].

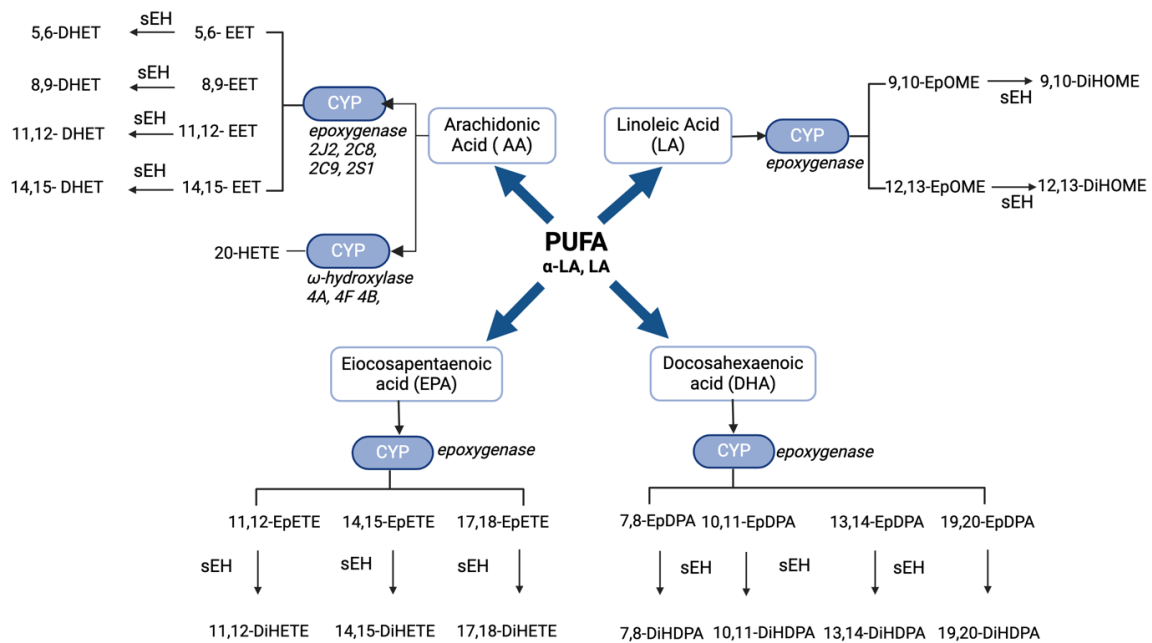


Figure 10 Pathway for Cytochrome-P450 (CYP)-derived metabolites. PUFAs are catalysed by CYP enzymes and undergo either deoxygenation or hydroxylation to their derivatives. HETEs are produced mainly by CYP 4A and CYP 4F enzymes while EETs are generated through CYP 2J and CYP 2C enzymes and their isomers. The epoxides (EETs, EpOMES, EpETEs, EpDPAs) are further metabolised by soluble epoxide hydrolase (sEH) to diols (DHETs, DiHOMES, DiHETEs, DiHDPAs). PUFA: polyunsaturated fatty acids; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; AA: arachidonic acid; α -LA: alpha-linoleic acid; LA: linolenic acid; CYP: Cytochrome-P450; EET: epoxyeicosatrienoic acids; EpOME: epoxyoctadecamonoenoic acids; EpETE: epoxyeicosatetraenoic acid; EpDPA: epoxydocosapentaenoic acid; DHET: dihydroxyeicosatrienoic acid; DiHOME: dihydroxyoctadecenoic acid; DiHDPAs: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid. Image created by Bio Render®

1.8.13 The role of epoxides in obesity and T2DM/MASH

The epoxides, EETs have been well described as anti-inflammatory over the last two decades, particularly in decreasing vascular inflammation and atherosclerosis [355]. Mouse models that result in EET overexpression either with increased expression of CYP2J2 or CYP2C8 or are EPHX2 KO mice, have decreased vascular inflammation and NF-KB signalling [356, 357] with an anti-inflammatory phenotype. Further mouse models with overexpression of EETs have been shown to reduced ER stress in heart failure [358] protect against diabetic cardiomyopathy [340] and diabetic nephropathy [359].

EETs have been shown to also have a role in insulin signalling in both hepatocytes and adipocytes cell lines and primary cultures. In hepatocytes EETs were found to attenuate insulin resistance and HEPG2 cell lines and adipocytes treated with exogenous EETs (and EpOMES) were shown to have improved insulin sensitivity through increased insulin stimulated phosphorylation of the insulin receptor and AKT [360].

The Behmoaras lab have also investigated the role of epoxygenases in obesity and metabolic syndrome using a *Cyp2y4*^{-/-} rat model fed a CAF (cafeteria) diet. This work

demonstrated the importance of the CYP-sEH pathway in regulating WAT homeostasis and glucose metabolism. Rats with *cyp2j4* deletion had a higher percentage increase in their body weight than the WT and had worsening glycaemic control with increased insulin levels. *Cyp2j4* was shown to be essential for healthy WAT adipogenesis and under metabolic strain, this deletion resulted in adipocyte dysfunction characterised by adipocyte hypertrophy and reduced PPAR γ and CEBP α . WAT from *cyp2j4*^{-/-} rats had increased infiltration of macrophages(CD68+) compared to the WT; suggestive of increased inflammation with adipokine modifications also detected including a reduction in adiponectin. Markers of increased ECM deposition were observed with higher levels of hydroxyproline and collagen type VI in the WAT of *Cyp2j4*^{-/-} rats compared to WT. While WAT homeostasis is clearly perturbed by *cyp2j4* deletion in nutritional excess, there was also evidence that changes in CYP-derived metabolism had systemic consequences. Hepatic lipid accumulation increased *de novo* lipogenesis and dysregulated gluconeogenesis were observed in the rats fed a high fat diet compared to their WT fed CAF diet [209].

Taken together these results suggest CYP metabolism is integral to WAT homeostasis and crosstalk between WAT and the liver. Reduction in epoxygenases in nutritional excess results in metabolic decline and hepatic steatosis coupled with a shift in the AA pathway to COX- and LOX- derived eicosanoids. These findings have led me to investigate whether similar perturbations are seen in human obesity and form an integral part of my hypothesis.

Other animal models have also corroborated these findings, In an ApoE^{-/-} KO mouse, HFD resulted in the suppression of EETs with a switch to increased 20-HETE [361]. While the Behmoaras model relied on a deletion to characterise epoxygenases in obesity, a CYP2J3 mouse model which results in the over expression of EETs restored AMPK activation and adiponectin production in mice fed a high fat diet [362]. Abraham and colleagues also demonstrated with a CYP2J2 transgenic mice, overproduction of EETs attenuates inflammation and restores adipogenesis in adipose tissue through increased adiponectin and cross-talk with HO-1, a stress response protein, and decrease in PPAR γ and CEBP α preventing terminal differentiation, maintaining small 'healthy' adipocytes [355].

Humans with MASH on biopsy coupled with HFD mouse model showed that EET levels in the liver increased but further over expression of EETs by disruption of *Ephx2* in the mouse model attenuated inflammation and fibrosis in both the liver and adipose tissue and improved glucose metabolism [363].

In summary, these studies suggest EETs are important modulators of inflammation, predominantly are pro-resolving, promote healthy adipogenesis, are insulin sensitising and improve glucose metabolism through multiple identified pathways, thus this has been a potential target for treatment in several pathological disease including T2DM and MASLD.

1.8.14 Cytochrome-P450 (CYP)-derived metabolites and their role in obesity-mediated T2DM/MASLD in humans

While mouse models and cell lines have convincingly portrayed EETs as pro-resolving mediators during HFD, EETs measured in humans have conflicting results. Increased EETs in human plasma were associated with insulin sensitivity in one study [364] while other studies suggested EETs increased with IR [365, 366]. The literature investigating their role in MASLD is also mixed, Wells et al. suggested serum EETs were increased in MASH and Kalveram and colleagues showed there was increased CYP-epoxygenase activity with hepatic steatosis. On the other hand, Arvind et al. showed serum EETs decreased with MASLD [363, 367]. EETs have also been shown to be low in the plasma of obese patients but increased in patients with obesity and cardiovascular disease[368]. This mixed literature base highlights the need for human tissue-specific and disease-specific studies and an area I aim to address with my research. The role of soluble epoxide hydrolase (sEH) in modulating EET levels as a treatment target will be discussed in the next section.

1.8.15 Soluble Epoxide function and physiological relevance

Following the formation of the epoxides, they are rapidly hydrolysed by soluble epoxide hydrolase to diols. There are several epoxide hydrolases in mammals, but the main body of research has focused on microsomal epoxide hydrolase (mEH) encoded by the gene EPHX1 and soluble epoxide hydrolase (sEH) encoded by EPHX2 genes. sEH and mEH have a two-step base-catalysed mechanism which adds water to the epoxide producing the 1,2-dihydroxy-fatty acids (DiHAs or diols).

sEHs are the dominant EH in most tissues in humans including the liver and are 125kDA dimer that are mainly found in the cytosol or peroxisomes[369]. Their structure contains the C-terminal which performs the EH activity and an N- terminal which is a phosphatase, the function of which remains elusive. It is an excellent scavenger in states of high production of EETs (e.g. ischaemic reperfusion) and readily clears EETs from the cytosol. mEH was 10 to 1000-fold slower than sEH at hydrolysing EETs but more recent research by Edin et al. has shown using *Ephx1^{-/-}* and *Ephx2^{-/-}* mouse models that in fact EPHX1 contributes considerably to EET hydrolysis as it couples with CYP to control basal levels but performs slowly in states of high EET production [370]. These mouse models also showed that inhibition of both sEH and mEH has improved clinical outcomes from ischaemia [370].

Mowoskey et al. also demonstrated that the two sEH enzymes' regulation is linked, as Ephx1 is knocked out Ephx2 levels increase and vice versa[371].

In recent years sEH inhibitors (SEHi) have been investigated for clinical use with the aim of increasing pro-resolving EETs and reducing diols in the targeted tissue. Due to the cross-over of sEH with mEH it is important to avoid a cross-reaction when designing the drugs; mEH can degrade toxins, carcinogens and mutagens with deleterious effects[372].

Mounting evidence in animal models have demonstrated the benefits of using sEHi to prevent disease progression in multiple pathologies. In metabolic disease, an sEHi prevent diabetic retinopathy by augmenting retinal VEGF pathways in mice [373] and several studies have shown the benefit of sEH depletion or inhibition on pancreatic islet cells which improved glucose homeostasis through modulating apoptosis pathways and islet cell size [374]. They have also been shown to be beneficial for cardiovascular disease, inhibition of sEH has been linked to reduced cholesterol and protected from atherosclerosis which was attributed to AMPK activation and SREBP inhibition [375] . sEHIs in a mouse model have also led to a reduction in myocardial infarctions through EET-PPAR γ pathways [376].

Furthermore, there is increasing evidence they have a role in neurological pathology. An sEHi in a mouse model of Alzheimer's disease reduced neuroinflammation [377] and inhibition of sEH in T2DM mice reduced cognitive decline by protecting hippocampal neurons [378]. Other neurological diseases including depression [379] and arthritis-induced pain [380] have also been studied with pre-clinical trials suggesting improvement of disease progression.

These promising results have led to human trials using sEHIs, yet human studies have not been as clear cut, sEH inhibition has been shown to be beneficial for diabetic retinopathy, and in reducing coronary artery disease [373, 381]. There has also been interest in its use for pain and currently a phase II trial is underway [382]. sEH inhibitors have also been trialled to reduce CSF inflammatory cytokines following subarachnoid haemorrhage. While the results showed decreased inflammatory cytokine profile, they were however, non-significant [383].

1.8.16 Soluble epoxide hydrolase and its role in Obesity and T2DM/MASLD

As mentioned above the use of sEHi as a therapeutic tool is becoming an increasingly popular area of research in several disease pathologies with diabetic retinopathy, pancreas function and cardiovascular disease all being positively modulated. As these are closely linked to obesity, metabolic syndrome and MASLD I next reviewed the currently literature to establish if there is a role in these diseases for sEH modulation. There have been increasing

numbers of *in vitro* and *in vivo* studies being undertaken. Firstly, total adipose sEH activity has shown to be increased in mouse models and 3T3-L1 adipocyte cell lines[384, 385] in nutritional excess and in an obese sEH KO mouse model, insulin resistance was attenuated, plasma glucose levels improved and pancreatic islet cells were found to be larger, possibly due to the increased vasculature[386].

Interestingly, both PPAR α and PPAR γ induce sEH activity in 3T3-L1 adipocytes [384] but this is likely tissue-specific; because in contrast cardiomyocytes in rats had a reduction in sEH protein levels with PPAR agonists[387].

Inhibition of sEH has been shown in both obese rat and mouse models to improve metabolic syndrome [388-390] Several obese mouse studies also demonstrated sEHi improved adipose tissue and liver ER stress, modulated inflammation and autophagy pathways and reduced serum triglycerides through browning of the WAT [360, 391, 392].

Hepatic fibrosis and cirrhosis have also been attenuated by sEH inhibition in obese mouse or rat models again through reducing ER stress, modulating mitochondrial function and systemic inflammation or blunting angiogenesis [393-396].

Despite studies showing benefits of SEHi *in vivo* and *in vitro*, there is conflicting human data and recent human trials of sEH inhibitors such as GSK2256294 decreased inflammation through a reduction in adipose tissue IFN- γ and Th1 T-cells in obese patients but demonstrated no improvement in insulin resistance [397]. Further human clinical trial using sEHIs also suggested there is no improvement in glucose metabolism and insulin resistance in obese individuals [398]. No trials to date have used sEHi to prevent or reverse MASLD . Therefore, further tissue-specific human studies would be useful to further delineate the impact of EETs, sEH and diols in obesity related MASLD and T2DM.

1.8.17 Soluble epoxide hydrolase (sEH)- derived Diols and physiological relevance

PUFA-epoxides Epoxyoctadecadienoic acid (EpODE), epoxyeicosatrienoic acid (EET), epoxydocosapentaenoic acid (EpDPA), epoxyoctadecamonoenoic acid (EpOME) and epoxyeicosatetraenoic acid (EpETE) are subsequently metabolised by sEH to diols dihydroxyoctadecadienoic acid (DiHODE), dihydroxyeicosatrienoic acid (DHET), dihydroxydocosapentaenoic acid (DiHDPA), dihydroxyoctadecenoic acid (DiHOME) and dihydroxyeicosatetraenoic acid (DiHETE) respectively. It was presumed that sEH-derived diols were inactive metabolites which were able to translocate across cells and into the extracellular fluid as waste products to be excreted. This view was challenged by the observation that patients with respiratory distress syndrome had high levels of 9,10-dihydroxyoctadecenoic acid (9,10-DiHOME) which when inhibited by sEH improved

clinically, with improvement in their gas exchange, reduced ventilation requirement and reduced mortality [315]. Further studies have shown that at low concentrations of 12,13-DiHOME inhibits, not potentiates, the respiratory burst of neutrophils [399, 400]. Furthermore, in zebrafish low concentrations of 12,13-DiHOME is essential to WNT signalling of progenitor cell and stem cell proliferation which improved vascular wall repair after caudal vein plexus ischaemia [401]. Kundu et al. used primary human monocytes treated with different isomers of DHETs with varying concentrations at micromolar doses to demonstrate they are essential to monocyte chemoattractant protein-1 (MCP-1)-induced monocyte chemotaxis [402] and 11,12-DHET regulates cAMP production in HEK393 cells [403]. Two studies have also identified 14,15-DHET as PPAR α agonist in COS-7 and HEpG2 cell lines [404, 405]. Therefore, these studies provide evidence that diols are, instead, important in several different pathways involved in inflammation and adipogenesis with specific isomers being both tissue specific and acting in a concentration dependent manner [400].

1.8.18 Diols and their role in obesity and T2DM/MASLD in humans

The majority of human studies focus on circulating levels of diols with conflicting results [343, 363, 367]. Several human studies of obesity and IR showed overall reduction in diols in both plasma and subcutaneous fat. Specifically 12,13-DiHOME [406, 407] 5,6-DHET and 14,15-DHET [381] measured in the plasma were inversely proportional to T2DM and IR [346]. Arvind and Wells on the other hand had conflicting results when measuring diols in hepatic steatosis with Arvind reporting a significant decrease with MASH but Wells suggesting an increase [363, 367]. Their role, therefore, in T2DM and MASLD pathogenesis in humans is still to be defined.

In summary, PUFA-derived bioactive lipids are involved in key glucose, lipid and inflammatory pathways in both the liver and white adipose tissue. Previous work by the Behmoaras lab using a *cyp2j4*^{-/-} rat model demonstrated perturbations in epoxygenases in rats fed a CAF diet led to obesity, metabolic decline and hepatic steatosis and this work alongside the current literature to date forms the principal argument for my hypothesis. Furthermore, human studies focusing on oxylipins pathways in obesity are conflicting, predominantly investigate only plasma oxylipin changes and lack tissue-specific involvement which highlights a gap in knowledge in this field. Importantly, dissecting the cross-tissue relationship of oxylipins in MASLD and T2DM could provide treatment options or potential biomarkers for disease management or prevention.

Table 4 Main research papers that have measured human oxylipins in metabolic syndrome, T2DM or MASLD. A summary of the main oxylipins changes found in metabolic syndrome, T2DM or MASLD categorised by COX, LOX or CYP-450 pathway. CYP: cytochrome P450; LOX: lipoxygenase; COX: cyclooxygenase.

Pathway	Author	year	disease cohort	number (N=) individuals	number of lipids quantified	Tissue	Findings
CYP							
	Fisk et al.	2022	Obese/lean	84	111	SC WAT	9-HODE, 9-oxo-ODE, 13-oxo-ODE, 12,13-DiHOME, 11,12-DHET, LXB4, HXA2, 9-HoTre, RvE1, 8-HDHA, 14-,HDHA, 15-HDHA ,17-HDHA, 20-HDHA decrease with obesity and IR, PGF2alpha
	Pickens et al	2014	Obese/lean	126		plasma	Decreased in obesity:9,10-DiHOME, 12,13-DiHOME, 14,15-DiHETE,17,18-DiHETE,9,13-oxoOD. Increased in obesity:9,10 EpODE, 9,13 HODE ,5-HETE,9-HETE,11-HETE,15-HETE,20-HETE,8,9-EET,11,12-EET,14,15-EET.
	Grapov et al.	2020	Obesity/Obese+T2DM	12		plasma	reduction in DiHOMes, DiHODEs, and EpODEs wth exercise
	Lynes et al	2017	Obesity/Obese+T2DM			plasma	12,13-DiHOME negatively correlated with IR and obesity
	Vasan et al		Non-T2DM/T2DM	2248		plasma	12,13-DiHOME inversely correlated with hyperlipidaemia and T2DM
	Xiao et al.	2023	Non-T2DM/T2DM	40	22	plasma	EET+DHET, EEQ+DiHETEs, EDPs+DiHDPA, 20-HETE, 20-HEPE, 22-HDHA all increase with T2DM
	Lemetire et al	2021	Non-T2DM/T2DM	1161	6	plasma	5,6-DHET and 14,15-DHET decrease with T2DM and IR
	Gangadhariah et al.	2017	Insulin Sensitivity	31	3	plasma	8,9 EET, 11,12-EET, 14,15-EET positively correlate with Insulin sensitivity in Hypertensive patients
	Kalveram et al.	2021	MASLD	40	130	serum,liver	sEH DiHOME:EpOME ratio decreases in liver tissue. EET, EDP and EEQ increase with steatosis
	Camacho-Munoz et al.	2021	MASLD	78	65	plasma	Fenofibrate reduced plasma DHET(5,6DHET, 8,9-DHET, 14,15-DHET). OM_3CA reduced PGE1, PGE2 and PGD1 and TBxB2. Increase prostacyclin, EPA, DHA and CYP (17-HDHA, 18-HEPE, 19,20-DiHDPA)
	Arvind et al.	2021	MASLD	80	8	serum	Total EET, 11,12-EET, 14,15-EET, Total DHET, 11,12-DHET and 14,15-DHET deasead in steatosis/MASH. EET+DHET lower in MASH EET:DHET ratio no change
	Gorden et. al	2015	MASLD	88	366	plasma, liver	20 metabolites significantly different between MASH and SS
	Wells et al.	2016	MASLD	15	21	liver	Total EET, Total DHET and Total EET+DHET increased in MASH: 14,15-EET and 20-HETE most significantly different . 14,15-EET:DHET no change
LOX							
	Moller et al.	2015	Obese/ lean	42	60	plasma	increased 5,8,12-HETE and 20-HDHA with inflammation
	Fisk et al.	2022	Obese/ lean	84	111	SC WAT	9-HODE, 9-oxo-ODE, 13-oxo-ODE, 12,13-DiHOME, 11,12-DHET, LXB4, HXA2, 9-HoTre, RvE1, 8-HDHA, 14-,HDHA, 15-HDHA ,17-HDHA, 20-HDHA decrease with obesity and IR, PGF2alpha
	Pickens et al	2017	Obesity/lean	123	62	plasma	5-HETE, 11-HETE,15-HETE increase with with obesity. Diols inversely associated with obesity.
	Jurado-Fasoli et al.	2021	obese/CVD	136		plasma	15-HeTrE, 5-HETE, 14,15-EpETrE and 8,12-iso-iPF2a-VI increase with metabolic synrome 14,15-DiHETre, 17-18-DiHETE, 19,20-DiHPA deasead with metabolic syndrome
	Heemskerk et al.	2015	Obese/Obese+T2DM	35	25	visceral, SC WAT	Upregulation of 5-LOX pathway in T2DM and obese
	Santoro et al.	2014	MASLD/T2DM adolescent	80	12	plasma	9-HODE, 13-HODE increasein MASH
	Hall et al.	2017	MASLD	23	34	liver	9-HODE and 13-HODE increase in MASH
	Feldstein et al.	2010	MASLD	73	13	plasma	9-HODE, 13-HODE AND 9-OXOODE, 13-oxoODE. Increase with MASH
	Puri et al	2009	Normal/ MASLD	125		plasma	5-HETE, 8-HETE, 11-HETE ,15-HETE increase with MASH
	Caussy et. al	2020	MASLD	427	187	plasma	11,12-DiHETE, 12-HETE, 14,15-DiHETE increase with liver fibrosis
COX							
	Tuomisto et al	2022	nonT2DM/T2DM	8292	545	plasma	8-iso-PGA1,12-HETrE positively associated with T2DM, unknown ecosanoid inversely associated with T2DM
	Miao et. al	2021	non-T2DM/T2DM/ Stroke	57		plasma	resolvins and LTB4 ratio significantly lower in T2DM
	Pawelzik et al.	2019	Obese/Obese+T2DM	45	2	urine	PGE2 and PGD2 increased in obesity /T2DM
	Tan et al	2020	Obese/Obese+ T2DM	40	13	plasma	PGF2a, PGE2 15-Keto-PGE2 and 13,14-dihydr-15keto-PGE2 all increase with T2DM and obesity
	Tans et al.	2020	Obese/Obese+T2DM	30	13	plasma	PGF2, PGE2, 15-Keto-PGE2, 13,14-dihydro-15-Keto-PGE2 all increase in obese+T2DM
	Loomba et al.	2015	MASLD	29	158	plasma	PGD2, PGE2, 15-HETE, 11,12diHETE, 14,15DiHETE, 20-COOOH AA, 12,13-diHOME, 12-HETE, all increase with MASH
	Csader et al.	2023	MASLD	38	16	plasma	HETE, HDHA, PEG2, IsoP: no change after exercise

Chapter 2: Hypothesis and Aims

Throughout chapter one I have shown that obesity is strongly associated with metabolic dysfunction and perturbed WAT homeostasis (increasing inflammation, hypertrophied adipocytes and abnormal extracellular matrix remodelling) [157, 408, 409]. Adipose tissue dysfunction leads to excess free fatty acid release and FFA uptake in other metabolic organs such as the liver which can result in hepatic lipotoxicity and the development of MASLD in prolonged nutritional excess [103]. Dysfunctional visceral WAT and macrophage-mediated inflammation have been associated with MASLD progression histologically [284, 410]. However, the interplay between adipogenesis, ECM remodelling and macrophage-mediated inflammation in WAT and progression of liver pathology is not fully elucidated and the dynamic interaction of metabolites and/or bioactive lipids between the two organs remain poorly understood.

I have also described how oxylipins can control every aspect of WAT homeostasis either orchestrating lipid metabolism and adipocyte differentiation by binding to nuclear transcription factors and regulating their activity [328, 411] or accompanying immune homeostasis and tissue inflammation [412, 413]. Furthermore, oxylipins also exert angiogenic properties by acting as endothelium derived hyperpolarising factors [414]. As oxylipins control metabolic, adipogenic, immune and angiogenic pathways in WAT homeostasis during obesity it is plausible that they are likely able to regulate crosstalk with other vital metabolic organs including the liver.

The previous work by the Behmoaras lab has investigated this hypothesis and demonstrated there is a link between WAT and liver tissue homeostasis through oxylipin signalling pathways using a *cyp2j4*^{-/-} rat model of obesity-induced MASLD. Specifically, *cyp2j4*^{-/-} rat fed on CAF demonstrated adipocyte dysfunction (decreased CEBPA and PPARY expression), raised WAT hydroxyproline levels, collagen VI deposition and CD68⁺ macrophage infiltration which correlated with lipid accumulation in the liver [209] suggesting that Cytochrome P450-derived epoxides are essential for healthy WAT homeostasis and perturbations can lead to obesity-mediated steatohepatitis. One of the limitations of the study was the lack of tissue-specific KO models, making the liver-intrinsic oxylipin pathways as a potential driver of steatohepatitis. The exact role of oxylipins in human metabolic syndrome has remained controversial. So far, the human studies are either restricted to plasma measurements, or to a single tissue (visceral/subcutaneous fat, liver biopsies) but with low-throughput or selected analysis of oxylipin species [347].

Therein, I hypothesise that oxylipins are associated with obesity-related disruption of WAT homeostasis in humans and can lead to the progression of MASLD and T2DM.

To investigate this hypothesis my project has been conducted using a cohort of patients with obesity undergoing bariatric surgery and non-obese controls. Paired omental white adipose tissue, whole blood and liver tissue were used to address the following aims

1. Understand the transition from adaptive to maladaptive WAT homeostasis in humans.

I have phenotyped the omental WAT during the progression to insulin resistance by investigating the changes of the three main features of homeostasis; adipogenesis, inflammation and ECM remodelling in omental WAT from obese and lean patients.

As the majority of T2DM patients (74%) in this cohort were prescribed Metformin, a biguanide that is a NICE recommended first line treatment for glucose control [49], I therefore aimed to establish whether Metformin was a confounding factor that directly affected the changes in WAT homeostasis that I had observed. I used patient-derived ex-vivo WAT cultures and SVF-derived cultures to investigate if adipogenesis, inflammation or ECM remodelling is modulated by Metformin.

2. Identify a potential link between obesity-related dysfunctional WAT and severity of MASLD

I have used paired liver and WAT biopsy samples from patients undergoing bariatric surgery and compared the severity of MASLD progression with markers of WAT dysfunction (adipogenesis, inflammation and ECM remodelling). The severity of MASLD was measured by analysing liver biopsies using the histopathology scoring system NAFLD Activity Score (NAS) and a novel verified AI software.

Through these two aims, I investigated the plausible association between WAT and liver phenotypes in obese patients progressing towards MASLD and T2DM. This required a comprehensive and methodical integration of clinical and cellular/molecular biology data using in vitro and ex vivo setup. This part of my thesis (Figure 11) set the foundation of the question asked in Aim 3: Since oxylipins are secreted bioactive lipids found in WAT, liver and plasma, do the oxylipin levels in each of these tissues/fluids correlate with WAT and liver phenotypes determined in Aim 1 and 2?

3. Understand the role of oxylipins in obesity-related T2DM and MASLD.

I characterised the PUFA-derived lipidome in obesity and obesity related T2DM in humans. I measured oxylipin levels in paired oWAT, plasma and liver samples using UPLC-MS/MS. I then aimed to determine if oxylipins are associated with maladaptive WAT dysfunction.

Finally, I compared WAT, plasma and liver oxylin signatures with MASLD severity to characterise their role in the crosstalk between adipose and liver tissue.

Through these 3 aims, I conducted a comprehensive profiling of oxylin during the dynamic phenotypic changes that occur in the WAT and liver of obese patients transitioning towards MASLD and T2DM. To my knowledge, this is the first quantification of these bioactive lipids in a multi-tissue context in human samples. The results obtained here in my thesis allowed (i) a better understanding of WAT-liver crosstalk in metabolic syndrome, (ii) clarification of oxylin dynamics in view of the current proposed therapies based on sEH inhibition.

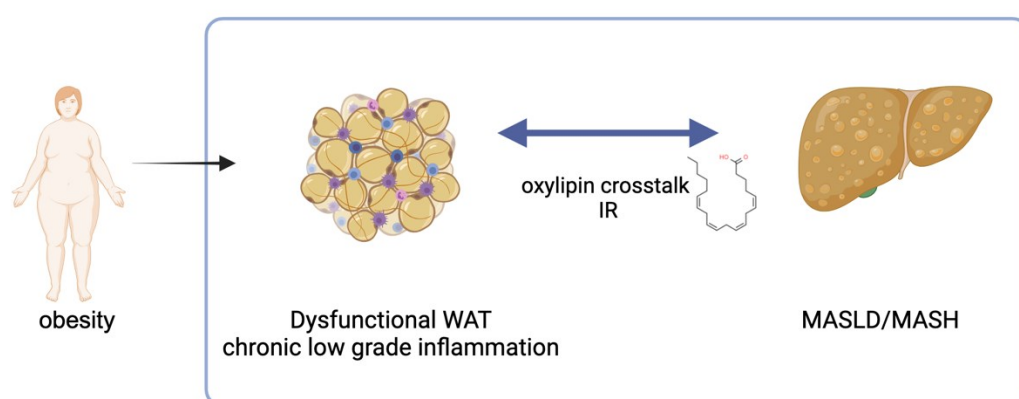


Figure 11: My hypothesis: oxylin are associated with obesity-related disruption of WAT homeostasis in humans and can lead to the progression of MASLD and T2DM. WAT: white adipose tissue; MASLD: metabolic-associated steatotic liver disease; MASH: metabolic-associated steatohepatitis; IR: insulin resistance. Image created by Bio Render®

Chapter 3: Methods

3.1 Patient Recruitment

Patients with obesity undergoing bariatric surgery (sleeve gastrectomy, Roux-en-Y gastric bypass or mini-bypass) and lean control patients undergoing upper gastrointestinal surgery (diagnostic laparoscopy, oesophagectomy or gastrectomy) at St Mary's Hospital and Hammersmith Hospital, Imperial College Healthcare NHS Trust London between June 2018-August 2023 were recruited under the ethical and Health Research Authority (HRA) approval (REC 19/WM/0229). Informed consent was obtained from all participants involved and the study complied with ethical guidance.

3.1.1 Inclusion / exclusion

All patients from both bariatric and UGI surgery must have capacity, provide fully informed consent and be over the age of 18.

Table 5 Inclusion and exclusion criteria for Bariatric and UGI surgery patients included in the study. BMI: body mass index; PPAR γ : Peroxisome proliferator-activated receptor gamma PPAR α : Peroxisome proliferator-activated receptor alpha.

Inclusion criteria	
Bariatric Services patients	UGI surgery services patients
obese or morbidly obese (BMI >30)	non-obese (BMI 18-25)
fit for surgical intervention	fit for surgical intervention
undergoing routine bariatric surgery	undergoing UGI surgery

Exclusion criteria
patients recruited from Bariatric Services and UGI surgery services
alcohol consumption >10g of ethanol per day.
viral Hepatitis, HIV, autoimmune, genetic or other metabolic causes of liver disease
abnormal clotting
on immunosuppressive medication and drugs that are known to precipitate hepatic steatosis
on insulin or PPAR γ / PPAR α agonists
evidence of cancer with metastatic spread or previous chemotherapy

Demographic and clinical data were collected from routine clinical tests undertaken as part of their care. Clinical data included past medical history, routine medications, alcohol consumption and smoking history. Routine clinical tests included BMI, glycated haemoglobin (HbA1C), liver function tests, lipid profiling, albumin and renal function and any available cross-sectional imaging results.

3.2 Sample collection

3.2.1 Omental and Subcutaneous WAT

Omental and subcutaneous white adipose tissue were collected at the time of bariatric surgery or upper gastrointestinal surgery by the operating surgeon using diathermy. 1g of omental and subcutaneous adipose tissue was snap-frozen for RNA, protein and lipid analysis and 250mg was fixed in 10% formalin for immunohistochemistry (IHC) analysis. A further 6g of omental adipose tissue sample was kept on ice and immediately used for stromal vascular fraction (SVF) isolation or *ex vivo* explant culture experiments (**Figure 12**).

3.2.2 Liver biopsy

The operating surgeon took a subcapsular liver biopsy using diathermy. The biopsy was dissected, and a cross-sectional piece was fixed in 10% formalin for histology, two 40mg pieces were stored in RNA later at -80°C. A further two to three 40mg pieces of liver were snap frozen on dry ice and stored at -80°C for lipid analysis.

3.2.3 Blood samples

Ten millilitres of blood were taken alongside the patient's routine pre-operative blood tests, either at the pre-operative clinic (non-fasted) or on the morning of surgery (fasted). The whole blood was then used for plasma isolation (**as described in section 3.3**).

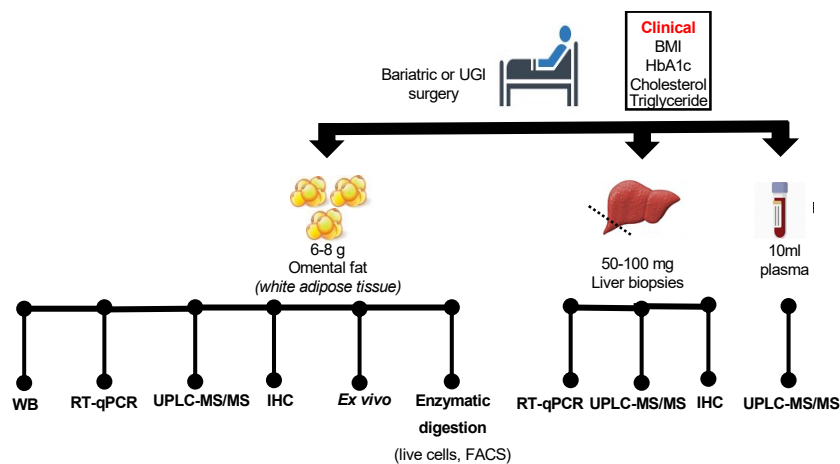


Figure 12 Study Design and schematic flowchart of the bariatric and upper gastrointestinal (UGI) surgery sample collection. Liver biopsies were only taken in N=41 bariatric surgery patients. IHC, immunohistochemistry; WB: western blot; SVF: stromal vascular fraction; RT-qPCR: real time quantitative PCR; UPLC-MS/MS: ultra-high performance liquid chromatography-tandem mass spectrometry; BMI: body mass index; HbA1c: glycated haemoglobin; FACS: fluorescence-activated cell sorting.

3.3 Plasma isolation from whole blood

Ten millilitres of whole blood were collected in Plasma- Ethylenediaminetetraacetic acid (EDTA) tube vacutainer (BD Vacutainer™, Cat#VS367873) and inverted to ensure

homogenisation. The vacutainer was then centrifuged at 2000 x g for 15 minutes at 4°C. The plasma is then removed and aliquoted into 1.5ml microtubes and stored at -80°C.

3.4 Tissue culture

Omental white adipose tissue was collected from patients undergoing bariatric surgery and transported in 20ml of cold Hank's Balanced Salt Solution. It was dissected for tissue culture experiments; either *ex vivo* explant culture or SVF isolation for SVF-derived adipocyte cell culture (**Figure 13**).

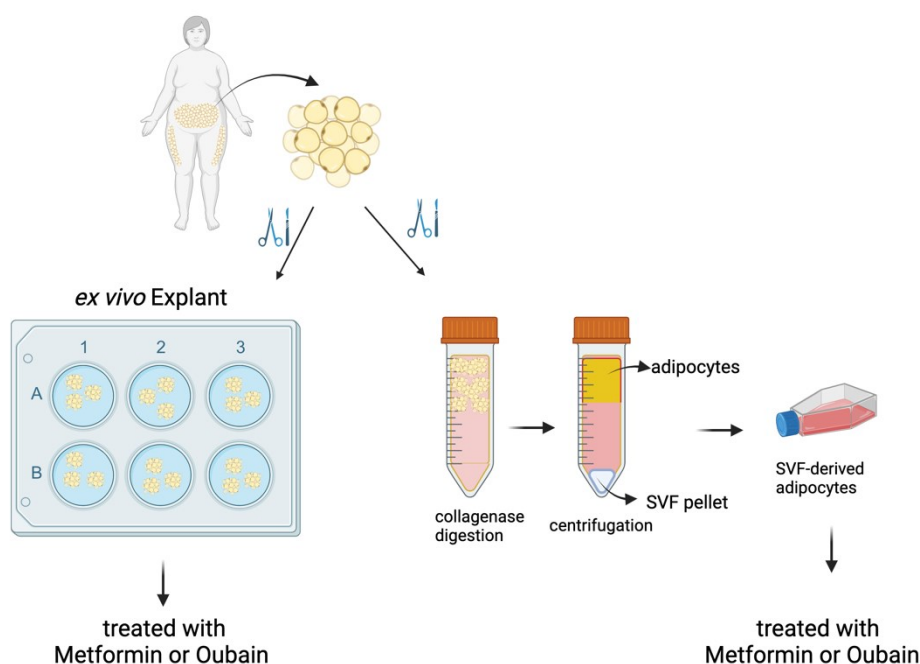


Figure 13 Schematic to represent the use of omental WAT collected from patients undergoing bariatric surgery. Tissue was either used for *ex vivo* Explant culture model following dissection or for SVF-derived adipocyte cell culture following isolation of the stromal vascular fraction from the oWAT by collagenase digestion and centrifugation. The two culture models were then used to investigate the effects of drugs on adipose tissue. SVF: stromal vascular fraction. Image created by Bio Render®

3.4.1 Stromal vascular fraction (SVF) isolation from bariatric patients

White adipose tissue collected from patients undergoing bariatric was transported in 20ml of cold Hanks' Balanced Salt Solution (Gibco™ HBSS, Fisher Scientific; 15420614). It was then cut into fine pieces using scissors and placed in a 50ml falcon with adipose tissue digestion buffer (10ml per 1g of oWAT). The oWAT in adipose digestion buffer (HBSS, 0.5% Bovine Serum Albumin (BSA), 10mM CaCl₂ and 4mg/ml collagenase type I (Sigma-Aldrich; C0130)) was then incubated at 37°C for 60 minutes. Once the tissue was completely digested, the suspension was then passed through a 200µm strainer (Pluri select; 43-50200-01) to remove remaining debris and centrifuged at 300 x g for 10 minutes at 4°C. The adipose layer was removed and the SVF pellet was resuspended in red cell lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl; Sigma-Aldrich; R7757) on ice for 3 minutes.

Following further centrifugation at 300 x g for 10 minutes, the SVF was used immediately for flow cytometry analysis or for SVF-derived adipocyte culture and differentiation.

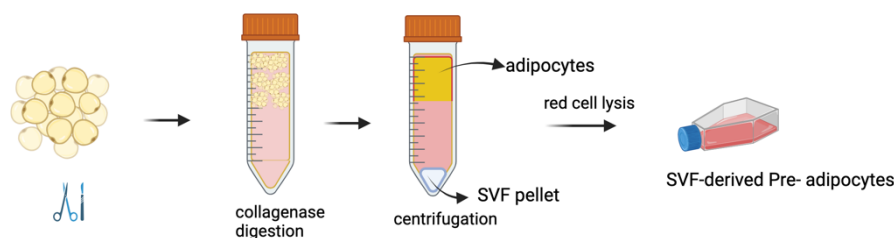


Figure 14 Isolation of the stromal vascular fraction from adipose tissue collected from patients undergoing bariatric surgery. oWAT was collected from patient undergoing bariatric surgery, it was transported in 20ml HBSS and then cut into fine pieces, digested using collagenase, strained through 200 μ m strainer and centrifuged. The stromal vascular fraction was pelleted and resuspended for red cell lysis, then re-centrifuged and resuspended in cell culture media for further cell culture. SVF: stromal vascular fraction. Image created by Bio Render®

3.4.2 SVF-derived adipocyte culture and differentiation

Following SVF isolation from patients' whole WAT, it was cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 20% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin in T25 until they reach confluence and passaged into larger T75 for expansion. This procedure favours the growth of multipotent stem cells capable of differentiating into adipocytes [415]. The cells were seeded by passage 5 to prevent alterations in function at a density of 800,000 cells/ml (**Figure 15**).

The stromal cells (pre-adipocytes) were then seeded into 96-well plate and treated with 50 nM, 100 nM, 500 nM and 5 μ M of Ouabain in Dulbecco's Modified Eagle Medium (DMEM) enriched with 20% Fetal Bovine Serum (FBS) for 24 h followed by FACs for cell viability assay (**Figure 15**).

Alternatively, the confluent pre-adipocytes were seeded into 24-well plate and differentiated with adipocyte differentiation media (Cell Applications Inc.; 811D). The adipose differentiation was performed using the protocol provided by Cell Applications and verified by oil-red-o staining and qRT-PCR for *ADIPOQ*, *FABP4* and *PPARG* (**Chapter 5 and Figure 15**).

At Day 6 of differentiation, the cells were washed with HBSS and treated with the drugs, either Metformin or Ouabain, in adipocyte maintenance media (Cell Applications Inc.; 811-M-250) for a) 24 hours (RNA harvested for qRT-PCR for adipogenesis genes or cells used for cell viability assay) or b) 7 days (protein harvested and PPAR γ Western Blot performed) (**Figure 15**).

All cultures treated with Metformin were done at a concentration of 100nM, 1µM, 1mM. Alternatively, the cells were treated with Ouabain at concentration of 50 nM, 100 nM, 500 nM and 5 µM.

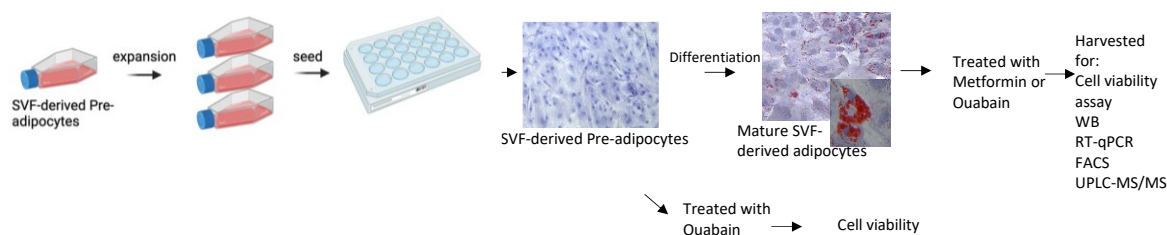


Figure 15 SVF-derived adipocyte cell culture model. Following the isolation of the SVF, the cells were cultured and expanded until confluence. The pre-adipocytes were then seeded and cultured in adipocyte differentiation media for 6 days. Mature adipocytes were treated with drugs (Metformin or Ouabain) and harvested for analysis (western blot, RT-qPCR, flow cytometry or mass spectrometry.) WB: western blot; SVF: stromal vascular fraction; RT-qPCR: real time quantitative PCR; UPLC-MS/MS: ultra-high performance liquid chromatography-tandem mass spectrometry; FACS: fluorescence-activated cell sorting

3.4.3 Ex-vivo WAT culture

Whole adipose tissue was collected at the time of bariatric surgery, weighed and immediately divided into 150mg pieces under sterile conditions using a scalpel. Three samples per well were incubated at 37°C 5% CO₂ with 3ml DMEM (Gibco; 4196S-039) enriched with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich; P4333) in 6-well plate. For Metformin-treated explants, the culture media was supplemented with Metformin (Sigma-Aldrich; Cat#317240) reconstituted in water at indicated concentrations (100nM, 1µM and 1mM). For Ouabain-treated explants, the culture media was supplemented with Ouabain reconstituted in 0.01% DMSO (Sigma) at indicated concentrations (50 nM, 100 nM, 500 nM and 5 µM). Explants were incubated for 48 hours and harvested for SVF isolation (to measure cell viability by FACS) or for total RNA extraction for qRT-PCR, protein extraction for WB or for UPLC-MS/MS (Figure 16).

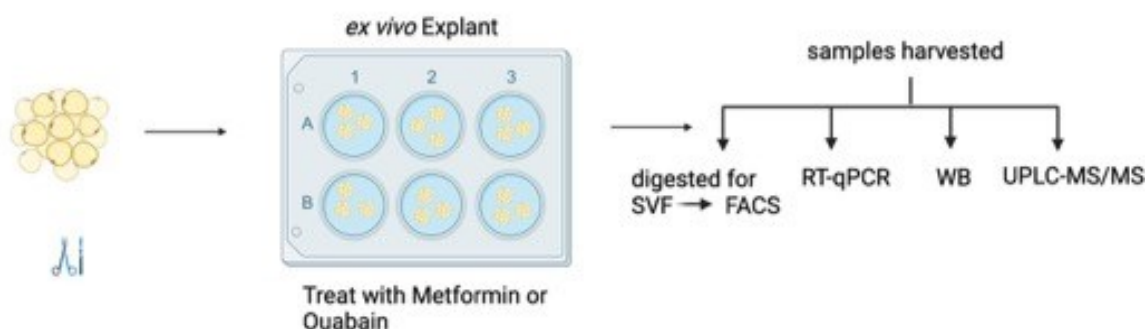


Figure 16 Omental WAT collected for ex vivo Explant model. oWAT was collected from patients undergoing bariatric surgery and dissected into 150mg pieces under sterile conditions. The explants were then cultured with maintenance media plus Metformin or Ouabain. The samples were then harvested and used for analysis (flow cytometry, RT-qPCR, western blot or mass spectrometry). WB: western blot; SVF: stromal vascular fraction; RT-qPCR: real time quantitative PCR; UPLC-MS/MS: ultra-high performance liquid chromatography-tandem mass spectrometry; FACS: fluorescence-activated cell sorting. Created by BioRender™

3.4.4 Viability testing

Viability of SVF-derived adipocyte cultures were assessed by adding 10% AlamarBlue (Invitrogen;919119) directly into the culture media of 96 well plate for 4 hours. Light absorbance was measured at 570nm using Multiscan Ascent™ (Thermo Fisher). The percentage of reduced AlamarBlue and cell viability were quantified by subtracting the background emission from cell emissions measured and plotted using a scatterplot.

3.4.5 Isolation of macrophages and non-adherent peripheral blood mononuclear cells

Human monocyte-derived macrophages (hMDM) were differentiated from buffy coats from healthy donors using gradient separation (Histopaque 1077, Sigma). Following Histopaque separation, peripheral blood mononuclear cells were resuspended in RPMI 1640 (Life Technologies) in T175 and the monocytes were selected following their adherence for 2 h at 37°C, 5% CO₂.

Non-adherent peripheral blood mononuclear cells were collected and washed twice with HBSS. Non-adherent peripheral blood mononuclear cells were seeded at 200,000 cells/ml and cultured for 5 days in media (RPMI 1640, 2-mercaptoethanol (50 nM, Sigma), HEPES (10 mM), non-essential amino acids, sodium pyruvate (0.2 mM, Thermo Fisher) and 20% foetal calf serum) in 96-well plates. The non-adherent peripheral blood mononuclear cells were then treated with control (0.01% DMSO) or Ouabain (Sigma, 03125), at 50 nM, 100 nM, 500 nM, and 5 µM for 24 hours and collected for viability using FACs.

The adherent peripheral blood mononuclear cell monolayer was washed twice with HBSS and monocytes were differentiated into human monocyte-derived macrophages for 5 days in macrophage differentiation media (RPMI 1640, 100 ng·ml⁻¹ macrophage colony-stimulating factor (M-CSF, PeproTech, 300-25-10UG) and 10% fetal calf serum). The macrophage purity was confirmed as previously described[416]. Cell dissociation buffer (Gibco, 13151014) was then used to passage the cells into 24-well plates using the manufacturers optimised protocol. They were then seeded at 500,000 cells/ml in RPMI 1640 and 10% fetal calf serum. The media was supplemented with control (0.01% DMSO) or Ouabain (Sigma, 03125), at 50 nM, 100 nM, 500 nM, and 5 µM for 24 hours and collected for viability using FACs.

3.5 Transcriptomic analysis

3.5.1 RNA extraction

Before RNA extraction, the surfaces were decontaminated using RNaseZap™ spray (Invitrogen; AM9780) to ensure no RNase would degrade the RNA extracted from the samples.

Whole adipose tissue (150mg), liver tissue (25mg) or cells were suspended in 1ml Trizol™ (Ambion Life Technology; 15596026) in a 2ml Eppendorf. Trizol™ is an acid-guanidinium-phenol based reagent, the low pH enables the separation of RNA from DNA. The oWAT and liver tissue then underwent mechanical homogenization to disrupt the tissue; a 5mm ball bearing was added to the 2ml Eppendorf and was shaken at 50Hz for 1-2 minutes using the Qiagen TissueLyser II. 200µl chloroform was then added to the solution and incubated at room temperature for 5 minutes followed by centrifugation at 12,000rpm for 20 minutes. The addition of the chloroform acts as a phase separation agent. The solution is split into the aqueous phase (containing RNA), Interphase and organic phase (containing proteins and DNA).

The aqueous phase containing the RNA was removed and the total RNA was extracted using RNeasy extraction kit (Qiagen; 74106) according to the manufacturer's instructions. This is a silica-column based extraction technique that enables the RNA to bind to the column, ensuring high quality pure RNA when eluted.

Following RNA extraction, the concentration and purity was measured using the NanoDrop™ 2000 Spectrophotometer ensuring 260:280 ratio >1.80 and a minimum concentration of 50ng/µl.

3.5.2 Complementary DNA synthesis

Complementary DNA (cDNA) was obtained using the Bio-Rad iScript kit (Bio-Rad, UK; 1708891). The iScript reaction mix contained a blend of oligo(dT) and random hexamer primers and the iScript Reverse Transcriptase was an enzyme derived from Moloney murine leukaemia virus (MMLV) and blended with RNase inhibitor.

TotalRNA and the iScript kit were thawed and kept on ice. 500ng-1µg of previously extracted total RNA in nuclease-free water was added to 4µl of 5x iScript Reaction Mix and 1µl of iScript Reverse Transcriptase. The microtube with the master mix was incubated in a thermal cycler firstly to allow for priming at 25°C for 5 minutes. Next, it was incubated for 20 minutes at 46 °C for the reverse transcription reaction and 1 minute at 95 °C to ensure RT inactivation. The cDNA is synthesised at 1:1 ratio with TotalRNA used in the master mix. Nuclease-free water is then added to the synthesised cDNA to ensure a final concentration of 2.5ng/ul.

3.5.3 Quantitative Reverse Transcription PCR (qRT-PCR)

Quantitative RT-PCR was performed on whole WAT (OM and SC), *ex vivo* cultured WAT, SVF-derived adipocytes and whole liver tissue. qRT-PCR quantifies gene transcripts by measuring SYBR®Green I dye that fluoresces when bound to nonspecific double-stranded

DNA (dsDNA). The fluorescence response is measured in a linear relationship with the polymerised chain reaction (PCR) product over 40 PCR cycles in real time. The SYBR®Green I dye excites at 497nm and emits at a maximum of 520nm. When unbound, it has very low background fluorescence and increases 1000-fold when bound to dsDNA, so the presence allows the quantification of accumulation of PCR products, therefore good primer design and purification are imperative. The melting curve should always be visualised to ensure there are no primer-dimers which are a result of the SYBR®Green I dye binding to non-specific dsDNA and not the desired target product.

Primers were designed firstly by acquiring the gene sequence available on ENSEMBL. Primer3 software was then used to design primers from the acquired gene sequence. To prevent genomic DNA amplifications the primers were designed across exons and the final amplified sequence product was less than 200bp long. The GC content was >55% for all primers. Candidate primers were then tested for nucleotide alignment using Basic Local Alignment Search Tool (BLAST NBCI) to discard any off-target priming. The primer sequences are provided in Table 6.

Quantitative Reverse Transcription PCR (qRT-PCR) reactions were performed using the ViaA 7 Real-Time PCR system (Thermofisher ViiA7) in a 384-well plates as technical triplicates. A total of 5ng of cDNA per sample was used for each reaction. Firstly, the Brilliant II SYBR Green QPCR Master Mix (Agilent; 600828) was thawed and kept on ice until required. 6.25µl of Brilliant II SYBR Green QPCR Master mix was added to 0.5µl forward primer (416nM final concentration) and 0.5µl reverse primer (416nM final concentration) plus 2.5µl of the sample. Nuclease-free PCR -grade water was added to ensure a total volume of 12µl.

ViiA 7 RUO Software was used for the determination of Ct values by using a two-step cycling protocol. The plate was first heated to 95°C for 10 minutes to active DNA polymerase followed by 40 cycles of 30 seconds at 95 °C and 1 minute at 60 °C in to enable annealing and extension of dsDNA. The thermocycler was set to detect fluorescence during these cycles.

The results were analysed using the comparative Ct method with each sample normalised to ACTIN or HPRT gene. For each sample the average of the technical triplicate Ct value for the gene of interest was normalised to the average of the technical triplicate for the housekeeping gene ($\Delta Ct = Ct \text{ gene of interest} - Ct \text{ housekeeping gene}$). A calibrator sample Ct was then chosen as the reference Ct and subtracted from the ΔCt of all samples ($\Delta\Delta Ct =$

ΔCt of samples - ΔCt of calibrator sample). The fold change of gene expression was then calculated using (Fold change = $2^{-\Delta\Delta\text{Ct}}$)[417].

Formula for comparative Ct method:

Fold change = $2^{-\Delta\Delta\text{Ct}}$

$2^{-\Delta\Delta\text{Ct}} = [(\text{Ct gene of interest} - \text{Ct housekeeping gene}) \text{ sampleA} - (\text{Ct gene of interest} - \text{Ct housekeeping gene}) \text{ calibrator sample}]$

Table 6 Human primer sequences for RT-qPCR

Human gene	Forward Primer (5'- 3')	Reverse Primer (3'-5')
<i>PPARG</i>	TTGCAGTGGGGATGTCTCAT	TTTCCTGTCAAGATCGCCCT
<i>FABP4</i>	AAACTGGTGGTGGAATGCGT	GCGAACTTCAGTCCAGGTCA
<i>ADIPOQ</i>	ATGGCCCCTGCACTACTCTA	CAGGGATGAGTTCGGCACTT
<i>LEP</i>	ACCAAGGTCTTCAGCCATCA	CTTGTGTTGCTGGGAGTTCC
<i>FASN</i>	GCCTTTGAAATGTGCTCCCA	GTGAACTGCTGCACGAAGAA
<i>COL1A1</i>	TCCGACCTCTCTCCTCTGAA	GTCTTTTGCTTCCTCCCACC
<i>COL6A1</i>	CGCTTCATCGACAACCTGAG	TCCAGCCCCTTCTTGATAGC
<i>ACTA</i>	TGCCTTGGTGTGTGACAATG	TCACCCACGTAGCTGTCTTT
<i>CD68</i>	CCTCCAAGCCCAGATTCAGA	CATTGTACTCCACCGCCATG
<i>IL1B</i>	GGAGAATGACCTGAGCACCT	GGAGGTGGAGAGCTTTTCAGT
<i>TNF</i>	TCCTTCAGACACCCTAAACC	AGGCCCCATTTGAATTCTT
<i>IL6</i>	AGTCCTGATCCAGTTCCTGC	AAGCTGCGCAGAATGAGATG
<i>IL10</i>	TGTTTTCCCTGACCTCCCTC	AGTGGTTGGGGAATGAGGT
<i>TREM2</i>	TGCGGAATCTACAACCCAT	GCATCCTCGAAGCTCTCAGA
<i>ALOX5</i>	ATGCCAACAAAACAGACCCC	TGGAACGCACCCAGATTTTG
<i>ALOX12</i>	TCTCCAACCCCAACACTGTT	GGAAGTTGAAGATGGGGTGC
<i>ALOX15</i>	CTGCAACTGGATCTCTGTGC	TCTTCCAGCTCTTCTTCCCG
<i>CYP2S1</i>	TGGGCATGGACAGGGTTAAT	GTTGCTTTGTGGCCCTTGTA
<i>CYP2J2</i>	GGAGAACGGACAGCCTTTTG	AGCTGGCATGTCTTTGAAGC
<i>CYP2C8</i>	TGACCCTGGCCACTTTCTAG	ATGAGGGTGGCAGAGAAACA
<i>CYP2C9</i>	GGGGCAGCTCTAACCTCTAG	CTGGGAGGCGGAGGTTATAG
<i>EPHX1</i>	ATGACTCTCCTGTGGGTCTG	GAGGAGATGATGGTGCCTGT
<i>EPHX2</i>	GAAAGATCAACCGCCCCATG	CCCACCTGACACGACTCTAT
<i>ACTIN</i>	ATGTGGATCAGCAAGCAGGA	CAATCAAAGTCCTCGGCCAC
<i>HRPT</i>	TGAGGATTTGGAAAGGGTGT	AATCCAGCAGGTCAGCAAAG

3.6 Histology

Liver, oWAT and scWAT were fixed in 10% Formalin for 24 hours and transferred to 70% ethanol until being processed and embedded in paraffin by Lorraine Lawrence.

3.6.1 oWAT immunohistochemistry and adipocyte area quantification

Haemotoxylin and eosin (H&E) staining was performed by Lorraine Lawrence and adipocyte mean areas were measured using 10X brightfield images taken on the Leica DM4B microscope. They were analysed using Image J Adiposoft plug-in [418]. The scale was set using a scale bar and the minimum and maximum areas were set to 20 and 20000 μm^2 respectively.

For CD68 immunostaining, the DAKO EnVision™+ System was used. This is a 2-step IHC technique that uses a horseradish peroxidase (HRP) labelled polymer conjugated with secondary antibodies.

Firstly, the 5 μm thick paraffin WAT sections were dewaxed in 100% xylene for 4 minutes (x2) and rehydrated in 100% ethanol for 4 minutes (x2). The slides were then placed under running water for 2 minutes.

As formalin fixing forms protein cross-links that mask antigenic sites it can give rise to false negative staining. By heating with a citrate-based solution the protein cross-links are broken, and the antigens and epitopes are unmasked. Therefore, Antigen retrieval with sodium citrate buffer (SCB) (1.47mg tri-sodium citrate in 500ml distilled H₂O corrected to pH 6) was carried out by heating the immersed slides in a microwave for 5 minutes (x4) at 800w.

The sections were then allowed to cool and rinsed in phosphate buffered saline (PBS) for 5 minutes (x2). Five drops of peroxide block were added to each slide and left to incubate for 10 minutes at room temperature in a humidified chamber to prevent any endogenous peroxide activity. The slides were then rinsed in PBS (x2) for 5 minutes. Non-specific proteins were then blocked by incubating the slides in Marvel milk block (1g milk powder in 10ml PBS) for 15 minutes. Next, the slides were incubated with primary antibodies against human CD68 (1:200 dilution: Bio-Rad; clone KP1, MCA5709) for 1 hour at room temperature in a humidified chamber. The slides were then washed in PBS for 5 minutes (x2).

The sections were further incubated with 2 drops of the horseradish peroxidase-labelled polymer for 30 minutes at room temperature (DAKO EnVision™+ System; Agilent Technologies (UK)) and then washed in PBS for 5 minutes (x2) on a shaker.

Diaminobenzidine (DAB) chromogen- substrate buffer (20 μl DAB in 1ml of DAB substrate) was then added to the sections and incubated at room temperature for 15 minutes until the

sections turned a light brown. Slides were then washed thoroughly in PBS for 5 minutes (x2). For nuclei staining the slides were dipped in Haematoxylin stain for 1 minute and rinsed in a water tank until the water ran clear. The slides were then dipped for 1 second in acid-alcohol (70% ETOH, 30% H₂O, 10ml HCL) to clean excess haematoxylin staining and rinsed in the water tank with running water for 5 minutes.

Next, the sections were first dehydrated with 100% ethanol for 3 minutes (x2) and further placed in xylene for 3 minutes (x2). Pertex®, a xylene-based permanent mounting medium, was used to fix the cover slip to the slide and left to dry for 24 hours.

The slides were visualized using the Leica DM4B microscope and 10 images were taken per section at 20x magnification. The number of CD68 positive crown-like-structures was then quantified for each section.

3.6.2 oWAT immunofluorescence

For immunofluorescence (IF) of Collagen type I and Collagen type VI staining, 5µm thick paraffin sections were dewaxed in 100% xylene for 4 minutes (x2) and rehydrated in 100% ethanol for 4 minutes (x2). The slides were then placed under running water for 2 minutes.

Antigen retrieval with sodium citrate buffer (SCB) (1.47mg tri-sodium citrate in 500ml distilled H₂O corrected to pH 6) was carried out by heating the immersed slides in a microwave for 5 minutes (x4) at 800w. The sections were then blocked with IF blocking buffer (1xPBS, 5% normal donkey serum, 0.3% TRiton™X-100) at room temperature for 1 hour. Slides were then incubated overnight with Anti goat-Type I collagen (Southern Biotech, 1:100; 1310-01) or Anti rabbit -Type VI Collagen (Abcam 1:200; clone: EPR17077, ab199720) diluted in antibody dilution buffer (1x PBS, 1% Bovine serum albumin (BSA), 0.3% TRiton™X-100) at 4°C in a humidified chamber. After washing with PBS for 5 minutes (x3), slides were incubated for 1.5 hours with Donkey Anti-Goat AlexaFluor555 (Abcam 1:100; ab150129) or Donkey anti-rabbit AlexaFluor555 (Invitrogen 1:400; A-31572) in a humidified chamber in the dark. The slides were then rinsed in PBS for 5 minutes (x3).

Slides were then mounted using VECTASHIELD with DAPI (abcam; H-1200-10) aqueous medium and kept at 4°C in the dark until imaged. To ensure that the antibodies were specific to Collagen type I and Collagen type VI positive controls were used (liver tissue for collagen type I and placenta for collagen type VI). Secondary-only staining was also performed for both secondary antibodies to ensure that there was no unspecific staining. (**Figure 17**)

Images were taken using epi-fluorescent Leica DM4B microscope at 10x magnifications and 5 images per section. Mean grey values were acquired for raw intensities (A.U.) using ImageJ software.

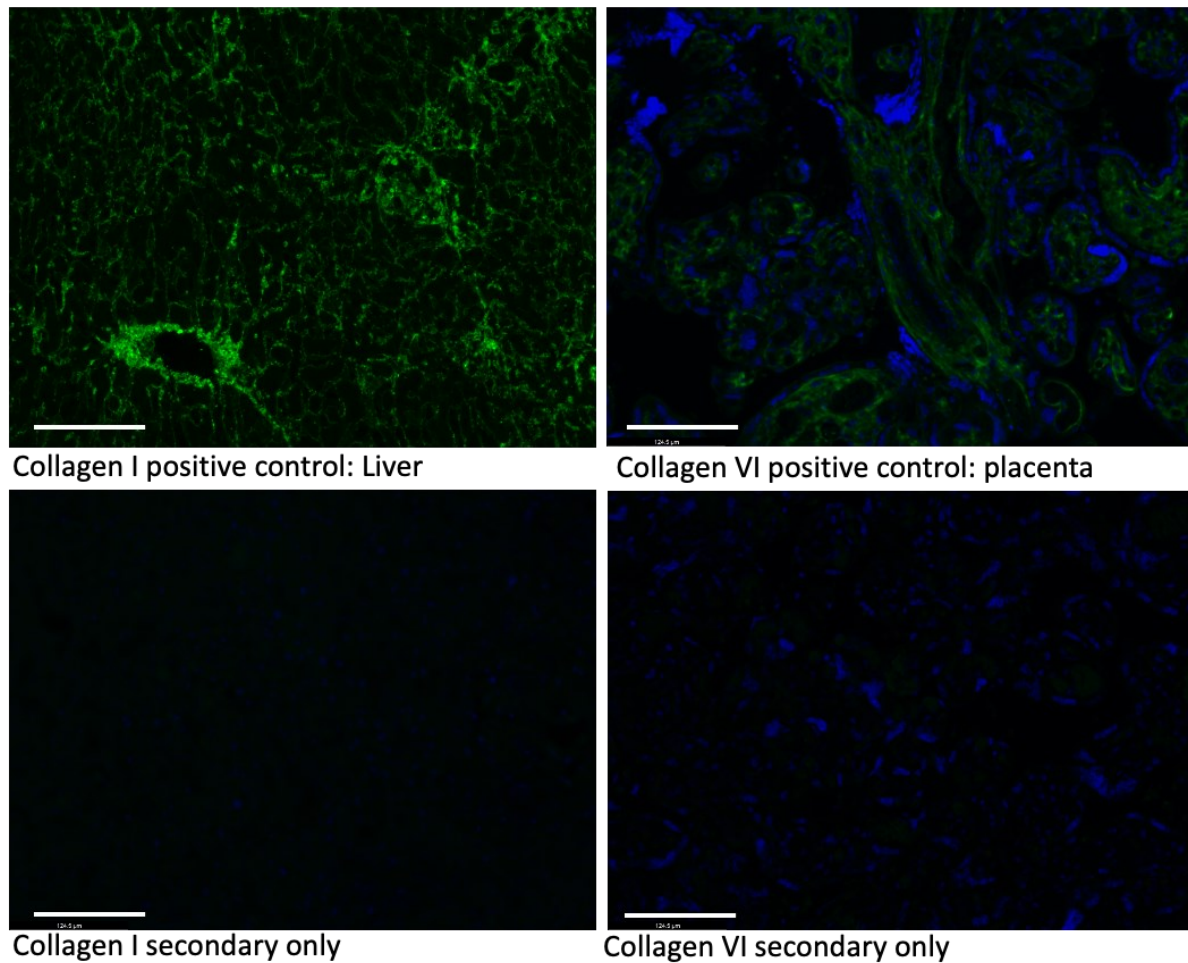


Figure 17 Collagen I and Collagen VI staining of positive controls (liver and placenta) and secondary only staining.

3.6.3 Liver Histology

Haematoxylin and eosin and Sirius Red staining of liver samples were performed as part of immunohistochemistry service provided at Imperial College London. Histopathologist, Professor Goldin, used the NAFLD Activity Score CRN and Kleiner fibrosis score system [66]. This was used to group the patients into 3 groups (0-2 =no MASH, 3-4= Intermediate (MASLD/MASH) and 5+=MASH). Liver biopsies were also scored using a verified software package that used an algorithm which analysed haematoxylin and eosin staining and Sirius red staining for histologic features of MASLD by quantifying (percentage) collagen deposition (collagen proportionate area; CPA), steatosis, ballooning and inflammation for each specimen described in depth here [419].

3.7 Protein analysis

3.7.1 Flow cytometry

Following SVF isolation from the whole WAT, the pellet enriched for the SVF was resuspended and used for (i) monocyte/macrophage detection and quantification and (ii) assessing cell viability in response to treatment with Metformin. All the following steps were performed on ice or at 4°C. Monocyte/macrophage detection and quantification was then assessed by staining with commercially validated antibodies CD14-PE (BioLegend; Cat#301806, clone:M5E2), CD45-FITC (BioLegend; Cat#304005, clone: HI30), CD206-BV-421 (BioLegend; Cat#321125, clone: 15-2), CD3-APC (BioLegend; Cat#317318, clone: OKT3). All antibodies were diluted 1:100 in 100ul FACS buffer (1%BSA, 5mM EDTA, 0,05% Sodium Azide in PBS) based on optimisation experiments performed previously by the Behmoaras lab and incubated at 4 °C for 30 minutes. SVF was then centrifuged at 300 x g for 5 mins and resuspended in Dead/Live stain diluted 1:200 (Thermo Fisher; L34975) based on manufacturers' recommendations and incubated for a further 10 minutes on ice. Cells were further centrifuged at 300 x g for 5 minutes and fixed in 1% PFA before flow cytometry. For measuring cell viability, SVF was stained with APC- Annexin V (1:30 dilution) and propidium iodide (PI) (1:50 dilution) (Biolegend;79998) based on manufacturers' recommendations. Annexin V and PI are used to identify apoptotic and necrotic cells. Annexin V is an intracellular protein that bind to phosphatidylserine (PS) in a calcium-dependent manner. When cells become apoptotic the PS migrates to the external membrane and is detected. To differentiate apoptotic from late apoptotic/necrotic cell PI staining is use as it passes at this stage to the nucleus and binds to the DNA which then fluoresces and is detected by the PE/Texas® Red channel.

All samples were acquired with at least 8,000 events per sample on a LSRFortessa flow cytometer (BD Biosciences, USA) and data were analysed with FlowJo software, version 10 (Tree Star Inc. Ashland, OR, USA).

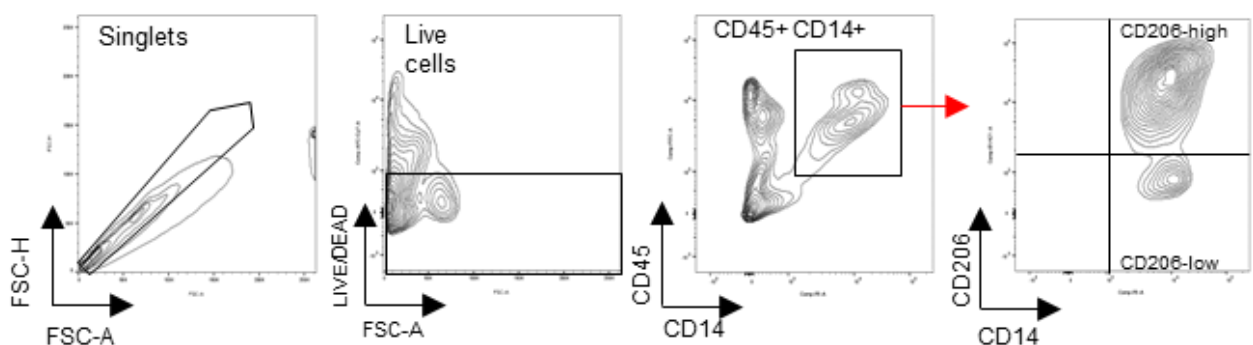


Figure 18 Gating strategy for monocyte/macrophage markers in SVF isolated from WAT. From left to right the panels indicate i) singlets, ii) live cells, iii) CD14⁺CD45⁺ cells iv) CD206⁺ cells.

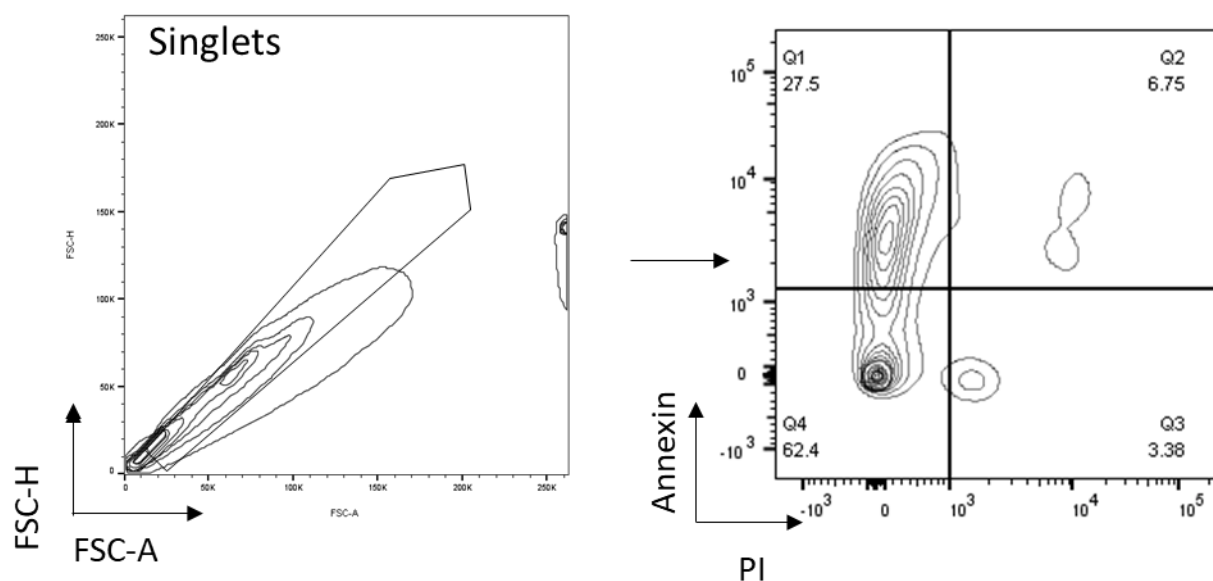


Figure 19 Representative gating strategy for PI and Annexin staining.

Table 7 Antibodies used for Flow cytometry assays.

	Clone	Company	Fluorochrome	Dilution
CD45	HI30	Biolegend	FITC	1 in 100
CD14	M5E2	Biolegend	PE	1 in 100
CD206	15--2	Biolegend	Brilliant Violet-421	1 in 100
CD3	OKT3	Biolegend	APC	1 in 100
Live/Dead stain		ThermoFisher	L/D far red	1 in 200
Annexin-V		Biolegend	APC	1 in 30
PI		Biolegend	PE/Texas Red	1 in 50

3.7.2 Western blotting

3.7.2.1 Protein Lysate preparation

Whole adipose tissue (150mg), liver tissue (20mg) or cells were suspended in 500μl Radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific; 89901) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1% protease inhibitor Cocktail (Thermo Fisher Scientific;78430) and 1% phosphatase

inhibitors (Cell Signaling Technology; 5870S) in a 2ml Eppendorf. The WAT and liver tissue then underwent mechanical homogenisation; a 5mm ball bearing was added to the 2ml Eppendorf and was shaken at 50Hz for 1-2 minutes using the Qiagen TissueLyser II. Protein lysates were then incubated at 4 °C for 2 hours followed by centrifugation for 20 min at 12,000 x g, at 4 °C and supernatants were used for Western blot analysis.

Total protein concentration was determined by Pierce™ Bicinchoninic Acid Kit (BCA) (Thermo Fisher Scientific; 23227) as per the manufacturer's instructions. The assay relies on colorimetric detection of BCA/copper complex following a 2-step reaction. Firstly, cuprous cation (Cu^{2+}) is reduced to Cu^{1+} by protein in an alkaline medium. Cu^{1+} then chelates with two molecules of BCA resulting in a purple colour reaction that is detected at 562nm.

The protein lysate was then denatured to enable the antibody of interest to access the specific epitope. 2X Laemmli buffer, which contains an anionic detergent sodium dodecyl sulfate (SDS), plus Betamercaptoethanol (1:50 dilution) was mixed with the protein lysate at 1:1 ratio. The mixture was boiled at 95-100°C for 5 mins and then centrifuged at 14,000 x g for 2 minutes before loading.

3.7.2.2 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast by using BioRad Mini Protean II stand. Each hand cast gel was made fresh using two polymerising agents (10% Ammonium Persulfate (APS) and the essential catalyst Tetramethyl ethylenediamine (TEMED)). Each resolving gel solution was made to the required percentage concentration ideal for the specific protein of interest depending on its molecular weight. Most resolving gels were cast at 10% (6.66ml ProtoGel 30%, 5.2ml 4x Resolving buffer, 7.94ml DW, 200ul 10% APS, 20ul TEMED) and the solution was immediately poured into the glass cassette up to three quarters of its capacity. The solution was allowed to stand for 30 minutes to ensure polymerisation was complete before ethanol was used to wash the top of the gel. The stacking gel was made at 4% SDS-PAGE (1.3ml ProtoGel 30%, 2.5 ml 4x Resolving buffer, 6.1ml DW, 100ul 10% APS, 20ul TEMED) and immediately poured over the resolving gel. A well-comb was then inserted to the top of the cassette to allow for 10 or 15 samples to be loaded. The stacking gel was then allowed to polymerise for a further 30 minutes.

The well-comb and cassette were removed from the cast gel, and it was placed into the electrode assembly holder. This was then placed into the tank forming an inner chamber and the tank was filled with running buffer (10x: 30.3g Tris (base), 144g Glycine, 10g (1%) SDS in 1L DW (4°C)). The first well was loaded with a protein-coloured ladder (5µl) (Amersham™

ECL™ Rainbow™ Marker -full range; GERPN800E: Sigma Aldrich) and the remaining wells were loaded with 25-50ug of protein lysate. The SDS-PAGE was then run at 80v for 30 minutes until the samples reach the interface of the stacking and resolving gel, it was then run at 180v for 1 hour. The gel was then removed from the assembly tank and removed from the cassette with a scalpel and spatula.

3.7.2.3 Membrane Transfer

The protein from the SDS-PAGE was transferred to Polyvinylidene Fluoride (PVDF) membrane (20µg porous diameter) using a BioRad Mini Protean II tank. A 'sandwich' was made, with the SDS-PAGE and membrane making up the middle. The outer layer was made up of a transfer buffer-soaked sponge that was mounted with three pieces of filter paper soaked in transfer buffer (100ml of 10X Transfer Buffer, 200ml Methanol, 700ml DW) either side of the SDS-PAGE and PVDF membrane. This was then tightly clamped using a cassette to ensure all bubbles were removed. The cassette was then placed into the transfer holder and placed into the tank with the gel facing towards the cathode and the PVDF membrane facing the anode. Ice cold transfer buffer was then added to the tank which was run at 300mA for 1 hour at 4°C.

3.7.2.4 Protein Detection

The membrane was carefully removed from the transfer holder and the rest of the 'sandwich'. It was then washed with TBS-T (100ml 10X TBS, 890ml DW 10ml, 10% Tween 20) and blocked in 15ml of WB blocking solution (5% BSA in 40ml TBST) for 1 h at room temperature on a shaker to prevent the binding of non-specific antigens to primary antibodies. After an hour, the solution was removed and then incubated overnight at 4 °C with primary antibodies either in 5% milk (0.75g milk in 15ml TBST) or 5% BSA (0.75mg BSA in 15ml TBS-T) depending on the manufacturer's instructions. Primary antibodies used were anti-PPAR γ (1:1000; Cell Signaling Technology; C26H12), anti-CEBP α (1:1000; Cell Signaling Technology; 2295S) and anti-actin- β (1:12,000; Santa Cruz; SC-47778) and anti-CD36 (1:1000; Abcam: EPR22509-4; ab252922) (**Table 7**).

The membrane was then washed three times for 10 minutes in TBS-T on a shaker and then exposed to an HRP-labelled secondary antibody for 1 h at room temperature. Secondary antibodies used were rabbit anti-mouse antibody (Dako; P0260; 1 in 6,000) or swine anti-rabbit antibody (Dako; P0217: 1 in 5,000) (**Table 7**). The immunocomplexes were visualized by the detection system SuperSignal West Pico PLUS Substrate (Thermo Fisher Scientific; 34580) which is an enhanced chemiluminescent HRP substrate that can pick up picogram to

femtogram levels of protein. The membrane was incubated with 1ml of the substrate for 2 minutes and then placed in a double-layered film inside a Kodak BioMax cassette. Once in the dark room a piece of Amersham Hyperfilm ECL® was placed on top of the membrane and exposed for up to a minute, correcting the exposure time by signal obtained for the individual proteins. The Amersham Hyperfilm ECL® was then developed using the Konica Minolta SRX-101A processor and signal was compared to the band size of the protein ladder reference.

Table 8 Antibodies used for IHC, IF and WB experiments. IHC: Immunohistochemistry, IF: Immunofluorescence, WB: Western Blot. CEPB α :CCAAT/enhancer-binding protein alpha; PPAR γ : Peroxisome proliferator-activated receptor gamma; AKT: protein kinase B.

Antibody	Clone	Company	Catalogue number	Isotype	Host	Primary or Secondary Ab	dilution
CD68	KP1	Bio-Rad	MCA5709	IgG1	mouse	primary	1 in 200
Type VI Collagen - terminal C	EPR17077	Abcam	ab199720	IgG	rabbit	primary	1 in 200
Collagen I donkey anti-goat	polyclonal	Southern Biotech	1310-01	IgG	goat	primary	1 in 100
AlexaFluor555 donkey anti-rabbit	polyclonal	Abcam	ab150130	IgG	donkey	secondary	1 in 100
AlexaFluro555	polyclonal	Invitrogen	A-31572	IgG	donkey	secondary	1 in 400
PPAR γ	C26H12	Cell signalling Technology	2435T	IgG	rabbit	primary	1 in 1000
CEBP α	polyclonal	Cell signalling Technology	2295S	IgG	rabbit	primary	1 in 1000
β -Actin	C4	Santa Cruz	sc-47778	IgG	mouse	primary	1 in 12000
CD36	EPR22509-40	Abcam	ab252922	IgG	rabbit	primary	1 in 1000
rabbit anti-mouse HRP	polyclonal	Dako	P0260	IgG	rabbit	secondary	1 in 6000
swine anti-rabbit HRP	polyclonal	Dako	P0217	IgG	Pig	secondary	1 in 5000
phospho Akt(Ser473)	D9EI	Cell signalling Technology	4060	IgG	rabbit	primary	1 in 200
Akt(pan)	C67E7	Cell signalling Technology	4691	IgG	rabbit	primary	1 in 200
CD206	15-2	Bio-Rad	MCA2155T	IgG	mouse	primary	1 in 1000

3.8 Lipid analysis

3.8.1 Oil Red O staining

Oil Red O is a water-soluble dye that detects lipid droplets. SVF-derived adipocytes cultured in chamber slides were firstly rinsed gently with PBS without disturbing the monolayer and then fixed using 10% Formalin. The slide was then incubated at room temperature for 60 minutes. After incubation 10% formalin was removed, the monolayer was washed with 2ml of sterile water and then incubated with 60% Isopropanol for 3 minutes at room temperature. The solution was then aspirated off and filtered Oil Red O solution (0.5% Oil Red O dye in 99% isopropanol; Sigma) was added to each well for 5 minutes. The Oil Red O dye was then aspirated and rinsed in a water bath until the water ran clear. Haematoxylin staining for nuclei was added to each well for 1 minute as a counterstain and then rinsed well in warm water, ensuring the monolayer never dried out, preventing disruption of vacuoles. The slides were photographed by light microscopy at 20x and 40x using Leica DM4B microscope.

Table 9 Solutions for the described protocols. DW: distilled water; SVF: stromal vascular fraction; DAB: diaminobenzidine; IF: Immunofluorescence; RIPA: Radio-Immunoprecipitation Assay; FACS: Fluorescence-activated cell sorting; PBS: Phosphate-buffered saline; TEMED: Tetramethylethylenediamine; EDTA: Ethylenediaminetetraacetic acid; BSA: Bovine serum albumin; APS: Ammonium persulfate.

Buffer name	Formulation
Adipose digestion buffer	HBSS, 0.5% BSA, 10mM CaCl ₂ and 4mg/ml collagenase type I
SVF-culturing media	DMEM enriched with 20% FBS and 1% penicillin-streptomycin
Explant culturing media	DMEM enriched with 10% FBS and 1% penicillin-streptomycin
Red cell lysis buffer	8.3 g/L ammonium chloride in 0.01 M Tris-HCl
Sodium citrate buffer	1.47mg tri-sodium citrate in 500ml DW corrected to pH 6 using 1N HCl
Marvel milk block	1g milk powder in 10ml PBS
DAB chromogen-substrate buffer	20ul DAB in 1ml DAB substrate buffer
1% Acid-alcohol	70% ETOH, 30% H ₂ O, 10ml HCL
IF blocking buffer	1xPBS, 5% normal donkey serum, 0.3% Triton-X-100
Antibody dilution buffer	1xPBS, 1% BSA, 0.3% TRitonX-100
RIPA buffer	25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS
FACS buffer	1% BSA, 5mM EDTA, 0,05% Sodium Azide in PBS
Resolving gel (10%)	6.66ml ProtoGel 30%, 5.2ml 4x Resolving buffer, 7.94ml DW, 200ul 10% APS, 20ul TEMED
Stacking gel (4%)	1.3ml ProtoGel 30%, 2.5 ml 4x Resolving buffer, 6.1ml DW, 100ul 10% APS, 20ul TEMED
10X Running buffer	30.3g Tris (base) 144g Glycine, 10g(1%) SDS in 1L DW(4°C)
10x Transfer Buffer	30.3g Tris (base), 144g Glycine in 1l DW (4°C)
1x transfer Buffer	100ml of 10X Transfer Buffer, 200ml Methanol, 700ml DW (4°C)
10X TBS	200ml 1M Tris (pH7.5) 24.2g, 97g NaCl in 1L DW
Oil red O solution stock solution	300 mg Oil Red O powder, 100ml 99% Isopropanol
Oil red O solution working solution	3 x Oil red O solution stock solution: 2x DW

3.8.2 Oxylin quantification

Metabolites were extracted from omental WAT, liver and plasma samples by solid-phase extraction and quantified by UPLC-MS/MS similar to what was previously described and outlined below [420].

Omental WAT

Frozen omental WAT (50 mg) was homogenized in 350µl ice-cold methanol by shaking with a steel bead for 5 minutes at 30hz. Samples were centrifuged at 1000 x g for 5 min at 4°C, and supernatants were mixed with 10µl internal standards [(PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11 (300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)]. The recovery of each was PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11 (300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)], 2.7 ml of water and passed through HyperSep Retain SPE columns (Thermo Fisher Scientific, Bellefonte, PA). The columns were washed with 2.5% methanol, 0.05% acetic acid, and then eluted with 1 ml of methanol and 1 ml of ethyl acetate into glass tubes containing 6µl of 30% glycerol in methanol. The eluates were dried under vacuum centrifugation and reconstituted in 50µl of 30% ethanol.

Plasma

For quantification of free/non-esterified plasma oxylin, 150µl of plasma was mixed with 450µl methanol and 10 µl of an internal standard solution [(PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11 (300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)]. The recovery of each was PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11 (300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)] mixed and centrifuged at 10,000g for 10 minutes. The supernatant was transferred to an Eppendorf tube and dried under vacuum centrifugation. Samples were reconstituted in 800µl ethyl acetate, passed through a phospholipid removal column (Cerex Maestro, 15 mg, Tecan). The column was washed with 500µl acetonitrile and dried under vacuum centrifugation.

Liver

For quantification of free/non-esterified liver oxylin, frozen liver biopsies were weighed and placed in a tube with 19µl HBSS/mg liver. Each tube received 1µl of 1mM TPPU in methanol. Liver tissue was homogenized using a TissueLyzer II (Qiagen) set to 30 Hz for 10 minutes. 10µl internal standard mix [(PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11 (300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)]. The recovery of each was PGE2-d9 (300ng), LTB4-d4 (300ng), 11,12-DHET-d11 (150ng), 11,12-EET-d11

(300ng), 15-HETE-d8 (300ng), AA-d11 (750ng)] was added to 100µl (5mg) of liver tissue, which was boiled for 5 minutes. Free oxylipins were immediately extracted by liquid: liquid extraction with 800µl ethyl acetate and dried under vacuum centrifugation. To determine “Total” oxylipins (free and sn-2 esterified), a separate 5mg of lysate was incubated with 3 Units of porcine pancreas PLA2 (Sigma-Aldrich; 6534) to hydrolyze sn-2-esterified oxylipins before identical liquid: liquid extraction.

Online chromatography of oxylipins were assayed on an Ultimate 3000 UHPLC equipped with an Xselect CSH C18, 2.1 × 50 mm, 3.5 µm particle column (Waters, Wilford, MA) and TSQ Quantiva tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Solvent flow rates were fixed at 350 µl/min with a cone gas flow of 125 l/h, desolvation gas flow of 650 l/h, a source temperature of 125°C, and a desolvation temperature of 400°C. The applied solvents were Solvent A = 51:40:9 acetonitrile-water-methanol (v/v/v) with 0.1% glacial acetic acid; Solvent B = 85:15 acetonitrile-methanol (v/v) with 0.1% glacial acetic acid [421]. All analytes were monitored simultaneously in a scheduled multiple reaction monitoring experiment as negative ions at parent ion-product ion mass/charge ratio pairs and retention times. Quantification was determined using multiple reaction monitoring and quantified by a blinded observer using TraceFinder (v4.1, Thermo Fisher Scientific).

Peaks for analytes with less than ~10 signal to noise were scored as below detection. For each sample and analyte, a relative response ratio (peak area of analyte/peak area of recovered internal standard (ISTD)) was determined and compared to a standard curve of relative response ratios of known concentrations of standards[422]. As a measure of data quality, I have listed the average signal-to-noise ratios for each analyte for both adipose tissue and liver (**Table 9**). To ensure the quantifications used in the statistical analysis are accurate and reproducible, I ran WAT samples (n=84) twice and found that 88% of the metabolites correlated significantly ($p < 0.05$).

71 oxylipins were measured in plasma, WAT and liver samples. Samples were excluded from the analysis if they were under the limit of detection. Metabolites undetectable in more than 50% of the samples were not included in the final analysis. Amongst those, 19, 24 and 27 metabolites were undetectable in liver, WAT and plasma, respectively. A further 2 samples were excluded from the liver and plasma analysis.

Table 10 Reference standards and internal standards used to assess oxylipin concentrations. Ionization masses (MS1 and MS2) in mass/charge ratio (m/z), retention time (RT), the minimum/maximum concentrations used in the standard curve (pg/ul injected) and reference internal standard are indicated. Analyte quantification was determined using standard curves determined based on indicated ISTDs. r² values of the derived standard curves are shown. Average signal to noise (S/N) ratios for adipose tissue and liver (free) are also shown. (Table created by M.Eldin)

x	Compound (abbreviation)	MS1 (m/z)	MS2 (m/z)	RT (min)	Low Standard (pg/ul)	High Standard (pg/ul)	ISTD used	r ²	Adipose S/N	Liver S/N
6-Keto-prostaglandin F1alpha	6-keto-PGF1a	369.2	163.1	2.39	0.1	120	PGE2-d9	0.998	1864	76
Prostanoic acid B2	TXB2	369.2	169.2	3.21	0.025	30	PGE2-d9	0.997	44	83
Prostaglandin B2	PGB2	333.2	175.2	5.21	0.1	120	PGE2-d9	0.996	48	42
Prostaglandin D2	PGD2	351.2	271.1	3.96	0.025	30	PGE2-d9	0.969	1025	519
Prostaglandin E2	PGE2	351.2	271.1	3.75	0.025	30	PGE2-d9	0.994	701	356
8-iso-prostaglandin F2 alpha	8-iso-PGF2a	353.2	193.1	3.21	0.05	60	PGE2-d9	0.995	45	BD
Prostaglandin F2alpha	PGF2a	353.2	193.1	3.65	0.05	60	PGE2-d9	0.996	47	69
Prostaglandin D3	PGD3	349.2	269.2	3.31	0.025	30	PGE2-d9	0.951	BD	57
Prostaglandin E3	PGE3	349.2	269.2	3.16	0.025	30	PGE2-d9	0.99	BD	47
11-dehydrothromboxane B2	11-dehydro-TXB2	367.2	161.3	3.73	0.025	30	PGE2-d9	0.967	BD	158
15-keto Prostaglandin E2	15-ketoPGE2	349.2	113.1	4.8	0.025	30	PGE2-d9	0.97	BD	BD
15-Keto Prostaglandin F2alpha	15-keto-PGF2a	351.2	219.2	3.85	0.025	30	PGE2-d9	0.998	BD	BD
19,20-dihydroxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid	19,20-DIHDPA	361.2	273.2	6.77	0.1	120	11,12-DHET-d11	0.998	14	143
16,17-dihydroxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid	16,17-DIHDPA	361.2	233.1	7.06	0.1	120	11,12-DHET-d11	0.998	16	1296
13,14-dihydroxy-4Z,7Z,10Z,16Z,19Z-docosapentaenoic acid	13,14-DIHDPA	361.2	193.1	7.18	0.1	120	11,12-DHET-d11	0.998	32	1477
10,11-dihydroxy-4Z,7Z,13Z,16Z,19Z-docosapentaenoic acid	10,11-DIHDPA	361.2	153.1	7.35	0.1	120	11,12-DHET-d11	0.996	18	1928
7,8-dihydroxy-4Z,10Z,13Z,16Z,19Z-docosapentaenoic acid	7,8-DIHDPA	361.2	189.1	7.75	0.1	120	11,12-DHET-d11	0.998	BD	170
7,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	17,18-DIHETE	335.2	247.1	5.89	0.1	120	11,12-DHET-d11	0.999	24	1338
14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	14,15-DIHETE	335.2	207.1	6.13	0.1	120	11,12-DHET-d11	0.999	22	710
11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	11,12-DIHETE	335.2	167.1	6.23	0.1	120	11,12-DHET-d11	0.978	BD	572
12,13-dihydroxy-9Z-octadecenoic acid	12,13-DIHOME	313.2	183.2	6.29	1	1200	11,12-DHET-d11	0.987	749	3435
9,10-dihydroxy-12Z-octadecenoic acid	9,10-DIHOME	313.2	201.1	6.5	1	1200	11,12-DHET-d11	0.97	3230	11700
14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatrienoic acid	14,15-DHET	337.2	207.2	6.75	0.1	120	11,12-DHET-d11	0.997	31	9736
11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	11,12-DHET	337.2	167.2	7.09	0.1	120	11,12-DHET-d11	0.997	138	4927
8,9-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid	8,9-DHET	337.2	127.3	7.4	0.1	120	11,12-DHET-d11	0.997	29	1019
5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid	5,6-DHET	337.2	145.1	7.84	0.1	120	11,12-DHET-d11	0.998	84	8540
19,20-epoxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	19,20-EpDPE	343.2	281.1	10.34	0.2	240	11,12-EET-d11	0.998	53	160
16,17-epoxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid	16,17-EpDPE	343.2	233.1	10.78	0.2	240	11,12-EET-d11	0.998	BD	21
13,14-epoxy-4Z,7Z,10Z,16Z,19Z-docosapentaenoic acid	13,14-EpDPE	343.2	193.1	10.89	0.2	240	11,12-EET-d11	0.998	BD	54
10,11-epoxy-4Z,7Z,13Z,16Z,19Z-docosapentaenoic acid	10,11-EpDPE	343.2	153	10.98	0.2	240	11,12-EET-d11	0.997	BD	936
7,8-epoxy-4Z,10Z,13Z,16Z,19Z-docosapentaenoic acid	7,8-EpDPE	343.2	189.1	11.17	0.2	240	11,12-EET-d11	0.997	BD	205
17,18-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	17,18-EpETE	317.2	259.1	8.94	0.1	120	11,12-EET-d11	0.998	BD	33
14,15-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	14,15-EpETE	317.2	207.1	9.42	0.2	240	11,12-EET-d11	0.998	BD	110
11,12-epoxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid	11,12-EpETE	317.2	167.1	9.5	0.2	240	11,12-EET-d11	0.978	BD	45
12,13-epoxy-9Z-octadecenoic acid	12,13-EpOME	295.2	195.2	10.34	0.5	600	11,12-EET-d11	0.984	1568	1695
9,10-epoxy-12Z-octadecenoic acid	9,10-EpOME	295.2	277.3	10.54	0.5	600	11,12-EET-d11	0.991	418	457
14,15-epoxy-5Z,8Z,14Z-eicosatrienoic acid	14,15-EET	319.2	219.3	10.52	0.2	240	11,12-EET-d11	0.998	73	262
11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid	11,12-EET	319.2	179.2	10.9	0.2	240	11,12-EET-d11	0.997	71	129
8,9-epoxy-5Z,11Z,14Z-eicosatrienoic acid	8,9-EET	319.2	151.1	11	0.2	240	11,12-EET-d11	0.996	22	81
5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid	5,6-EET	319.2	191.3	11.17	0.2	240	11,12-EET-d11	NA	BD	127
20-hydroxydocosa-4Z,7Z,10Z,13Z,16Z,18Z-hexaenoic acid	22-HDoHE	343.2	269.1	8.07	0.025	30	d8-15-HETE	0.997	BD	249
17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid	17-HDHA	343.2	281.2	9.04	0.025	30	d8-15-HETE	0.999	1872	197
12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	20-HEPE	317.2	243.2	7.08	0.025	30	d8-15-HETE	0.998	BD	27
18-hydroxyeicosa-5Z,8Z,11E,14Z,16E-pentaenoic acid	18-HEPE	317.2	161.4	7.27	0.025	30	d8-15-HETE	0.998	BD	28
13-hydroxy-9Z,11E-octadecadienoic acid	13-HODE	295.2	195.2	8.47	2.5	3000	d8-15-HETE	0.967	63600	7140
9-hydroxy-10E,12Z-octadecadienoic acid	9-HODE	295.2	171.2	8.59	2.5	3000	d8-15-HETE	0.974	11700	15700
20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	20-HETE	319.2	245.1	7.8	0.5	600	d8-15-HETE	0.99	BD	91
19-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	19-HETE	319.2	231.1	7.68	0.5	600	d8-15-HETE	0.987	BD	399
15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	15-HETE	319.2	219.1	8.87	0.1	120	d8-15-HETE	0.996	37900	1494
12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	12-HETE	319.2	179.2	9.47	1.25	1500	d8-15-HETE	0.973	6710	4105
11-hydroxy-5E,8Z,12Z,14Z-eicosatetraenoic acid	11-HETE	319.2	167.2	9.22	0.1	120	d8-15-HETE	0.998	4269	1744
8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	8-HETE	319.2	163.3	9.47	0.1	120	d8-15-HETE	9.63	407	167
5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	5-HETE	319.2	257.3	9.47	0.1	120	d8-15-HETE	0.997	2236	1603
Lipoxin A4	Lipoxin_A4	351.2	115.1	4.43	0.025	30	d8-15-HETE	0.996	90	30
Lipoxin B4	Lipoxin_B4	351.2	221.1	3.96	0.025	30	d8-15-HETE	0.998	27	BD
Leukotriene B4	LTB4	335.2	195.3	6.11	0.025	30	d4-LTB4	0.998	38	BD
20-carboxy arachidonic acid	20-carboxy-AA	333.2	271.2	7.31	0.025	30	d4-LTB4	0.99	252	123
Docosahexaenoic acid	DHA	327.2	283.2	12.69	15	18000	AA-d8	0.989	46583	78500
Eicosapentaenoic acid	EPA	301.2	257.2	12.28	15	18000	AA-d8	0.979	41750	121800
Linoleic acid	LA	279.2	261.2	12.86	15	18000	AA-d8	0.981	21900	124900
Arachidonic acid	AA	303.2	259.2	12.8	15	18000	AA-d8	0.978	103800	114900
Prostaglandin E2-d9	PGE2-d9	360.2	280.1	3.72						
11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid-d11	11,12-EET-d11	330.2	179.2	10.9						
11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid-d11	11,12-DHET-d11	348.3	167.1	7.02						
15-hydroxyeicosatetraenoic acid-d8	d8-15-HETE	327.2	226.1	8.77						
d4-leukotriene B4	d4-LTB4	339.2	197.1	6.08						
Arachidonic acid-d8	AA-d8	311.2	267.2	12.79						

3.9 Statistical analysis

Results were analysed using GraphPad Prism 9 software (GraphPad, USA) or IBM SPSS statistics UK version 29.0.0.0 (241). All data are expressed as the mean $\pm$ SEM. Firstly, normality of the data was tested using Kolmogorov-Smirnov and the Shapiro-Wilk tests.

3.9.1 Univariate Analysis

Statistical analyses for normally distributed grouped data were performed using parametric statistics either using student's t-test (two-way) or one-way Analysis of variance (ANOVA).

For non-normally distributed grouped data non-parametric statistics Mann-Whitney U test or Kruskal-Wallis tests were used, applying appropriate post-hoc tests. For rank-based non-parametric data the Jonckheere-Terpstra test was used to compare an ordinal independent variable and a continuous or ordinal dependent variable where the direction of the ordinal data is considered.

Pearson's correlation coefficient for normally distributed data or Spearman's correlation coefficient for non-normally distributed data were used to evaluate the association between two continuous variables. For non-normally distributed categorical data used in **Table 10** and **Table 12**, Fisher's exact test and Kruskal-Wallis tests were used. Statistical significance was determined as $p \leq 0.05$ after applying post-hoc tests.

3.9.2 Multivariate Analysis

Multivariate analysis for WAT, plasma and liver oxylipins were performed using linear regression modelling after the dependent variable was normalised by log₁₀ transformation of the data, confounding factors including age, sex, ethnicity and fasting status were included in the model. Liver tissues covariates included in the regression modelling were age, sex, ethnicity, CPA, fat, inflammation and ballooning percentage.

Following identification of commonly altered lipid species across tissues a predicted score was calculated, and diagnostic accuracy assessed using area under the operating curve (AUROC). Statistical significance was determined as $p \leq 0.05$.

3.9.3 UPLC-MS/MS analysis

For the heatmap generation of WAT and plasma oxylipins data were evaluated by applying the online metabolomics platform MetaboAnalyst 5.0[423]. Normalized (log₁₀ transformed) data was auto scaled and clustered using Ward. The area under the curve (AUC) was calculated by using the univariate model in MetaboAnalyst. For oxylipin analysis false discovery rate (FDR) adjusted p-values using the Benjamini-Hochberg formula have also been included.

Chapter 4: White Adipose tissue dysfunction in obesity

4.1 Introduction

Adipose tissue is a pivotal regulator of whole-body energy homeostasis. When there is prolonged overnutrition, the white adipose tissue (WAT) becomes progressively inflamed and dysfunctional [123, 157, 424]. This maladaptive state of the WAT can cause surplus lipid release and deposition within the liver and other tissues [425], potentiating insufficient insulin secretion from pancreatic β -islet cells, insulin resistance and development of T2DM [408].

4.2 Aims of chapter

In caloric excess white adipose tissue dysfunction is defined by three main features a) adipocyte hypertrophy, b) macrophage-mediated inflammation and c) ECM remodelling. To address my hypothesis that white adipose tissue dysfunction is thought to be a precursor to hepatic inflammation and fibrosis, I firstly aim to understand the transition from adaptive to maladaptive WAT by phenotyping the three features of dysfunctional oWAT in a lean and obese cohort.

It is well described that insulin resistance and T2DM are strongly associated with prolonged obesity and therefore the cohort of patients undergoing bariatric and upper gastrointestinal surgery were divided into three groups depending on their metabolic profile (lean patients, patients with obesity without T2DM and patients with obesity and T2DM). These groups were then used to characterise the cohort and phenotype the oWAT collected.

4.3 Results

4.3.1 Clinical characteristics and metabolic profile of the bariatric patient cohort .

Before phenotyping the oWAT clinical characteristics of the cohort were analysed. Eighty-eight patients undergoing bariatric surgery, and nine lean patients were recruited to the study. Patients with obesity were split into those with a diagnosis of T2DM (N=27) (as stated in clinical records or glycated haemoglobin ≥ 48 mmol/mol) and non-diabetic patients (N=61; no clinical diagnosis of T2DM and glycated haemoglobin < 48 mmol/mol) (**Table 11**). None of the lean patients were diabetic. There was no significant difference in ethnicity between the groups ($p=0.74$) but the lean cohort were significantly older ($p=0.01$) and had fewer females than the obese groups ($p=0.005$). As expected, patients with obesity were more likely to have co-morbidities associated with metabolic syndrome including MASLD/MASH, dyslipidaemia and hypertension (**Table 11**). Patients with T2DM were taking glucose lowering drugs including Metformin (74%), Gliclazide (11.1%), Empagliflozin (14.8%), Sitagliptin (7.4%) and Liraglutide (7.4%) (**Table 11**). Patients taking insulin were excluded from the study.

Table 11 Characteristics and metabolic profile of cohort. N=9 lean patients with BMI<25 and N=88 patients undergoing bariatric surgery with BMI>35 stratified into T2DM (glycated haemoglobin ≥48mmol/mol or clinical diagnosis documented) and non-T2DM (glycated haemoglobin <48 mmol/mol or no clinical diagnosis) BMI: body mass index; HbA1c: glycated haemoglobin; ACEi: Angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; MASH: metabolic dysfunction-associated steatohepatitis; MASLD: Metabolic dysfunction-associated steatotic liver disease.

	Lean (n=9)	Obese (n=61)	Obese + T2DM (n=27)	P-value
Age	61.1 ± 13.4	47.08 ± 13.71	52.96 ± 8.7	0.01
Sex (F)	4	55	24	0.005
Ethnicity				0.74
Asian / British Asian	1	6	4	
Black / Black Caribbean	0	5	2	
White	3	27	16	
Other	5	14	6	
Mixed	0	2	0	
BMI (m²/kg)	22.9 ± 3.7	45.78 ± 7.6	48.15 ± 7.8	<0.0001
HbA1C (mmol/mol)	39.7 ± 5.0	37.64 ± 4.5	60.33 ± 18.1	<0.0001
Cholesterol (mg/dL)	4.5 ± 1.3	4.8 ± 0.99	4.57 ± 1.09	0.43
Triglyceride (mg/dL)	1.2 ± 0.68	1.8 ± 2.4	2.07 ± 1.77	0.31
Medicines				
Metformin	0 (0%)	2 (3%)	20 (74%)	<0.0001
Gliclazide	0 (0%)	0 (0%)	3 (11.1%)	0.016
Empagliflozin	0 (0%)	0 (0%)	4 (14.8%)	0.012
Sitagliptin	0 (0%)	0 (0%)	2 (7.4%)	0.14
Liraglutide	0 (0%)	0 (0%)	2 (7.4%)	0.14
Statin	0 (0%)	7 (11.5%)	10 (37.0%)	0.007
ACE-i/ ARB	0 (0%)	15 (24.5%)	15 (55.5%)	0.0015
Aspirin	0 (0%)	3 (5%)	2 (7.4%)	0.78
Co-morbidities				
Hypertension	4 (44.4%)	16 (26.2%)	19 (70.4%)	0.046
Dyslipidaemia	0 (0%)	6 (9.8%)	10 (37.0%)	0.002
Ischaemic heart disease	1 (11.1%)	1 (1.6%)	1 (3.7%)	0.16
MASLD/MASH	0 (0%)	3 (4.9%)	8 (29.6%)	0.003

4.3.2 Adipocyte hypertrophy and hyperplasia during progression to T2DM in obese patients

The literature suggests that uncontrolled hypertrophic expansion of WAT is linked with metabolic dysfunction while hyperplasia-mediated adipocyte expansion is considered to counteract the metabolic decline caused by hypertrophy [128, 426, 427]. As such, adipose hyperplasia and hypertrophy are adaptive and non-adaptive biomarkers of WAT, respectively. As discussed in Chapter one, hyperplasia is defined as *de novo* adipocyte differentiation from precursor cells i.e. adipogenesis which is regulated by key transcription factors peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer-binding protein alpha (CEBP α). Therefore, I sought to study oWAT hypertrophy and hyperplasia in relation to T2DM by measuring firstly adipocyte size to address hypertrophy and whole tissue protein levels of PPAR γ and CEBP α as markers of hyperplasia.

Patients' H&E stained oWAT was used to quantify the average adipocyte area (μm^2) and was measured using adiposoft plug-in for ImageJ software. The oWAT of N=9 lean patients, N=61 patients with obesity and N=27 patients with obesity and T2DM were quantified. As expected, lean controls had smaller average adipocyte area than the patients with obesity ($r^2=0.24$, $p<0.0001$), with or without T2DM (**Figure 20**).

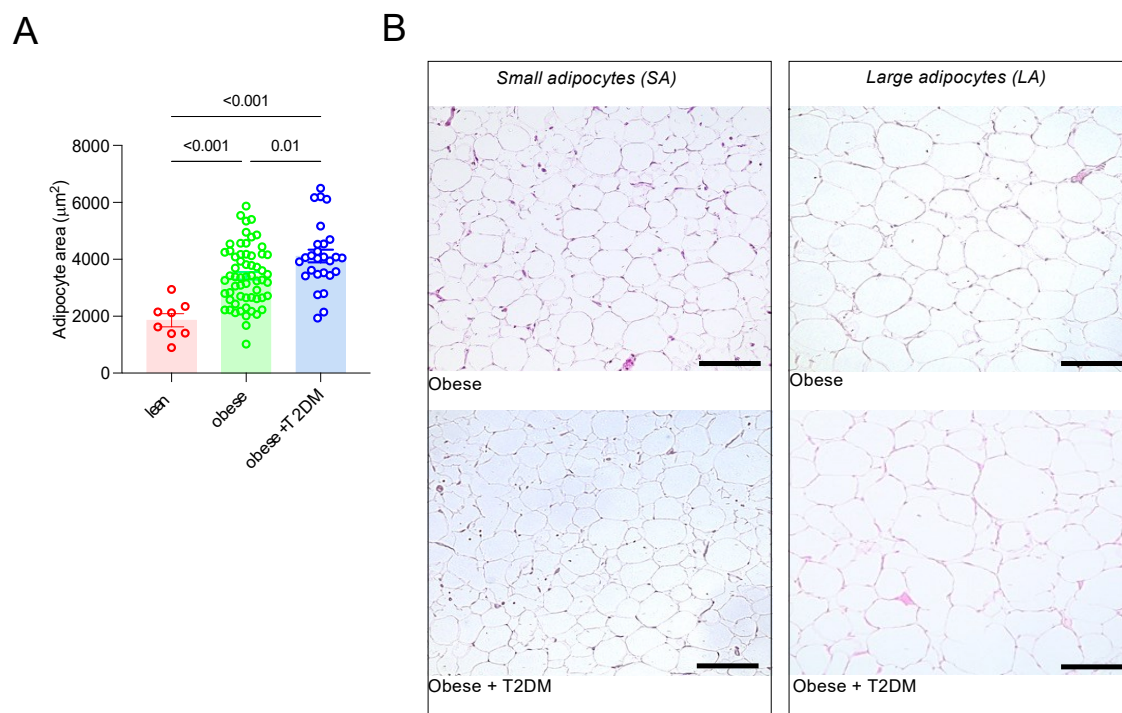


Figure 20 Adipocyte area in patients with obesity. (A) Average adipocyte area quantification in the WAT of lean patients (N=9), patients with obesity (N=61) and patients with obesity and T2DM (N=27). (B) Representative H&E staining of omental WAT with presence of small adipocytes (SA) and large adipocytes (LA) in patients with obesity with and without T2DM. Statistical significance was calculated using ANOVA with Tukey's multiple comparison test. Error bars represent SEM. Scale bars; 250 microns. T2DM: Type 2 diabetes mellitus; SA: small adipocytes; LA: large adipocytes.

BMI significantly correlated with mean adipocyte area ($r=0.35$, $p=0.006$) (**Figure 21**). Obese patients with T2DM had significantly larger adipocytes when compared to obese non-diabetic patients ($p=0.015$) (**Figure 20**). Glycated haemoglobin (HbA1c), a marker of glucose control over the previous 12 weeks, however, did not correlate with average adipocyte size ($r=0.18$, $p=0.09$) (**Figure 21**).

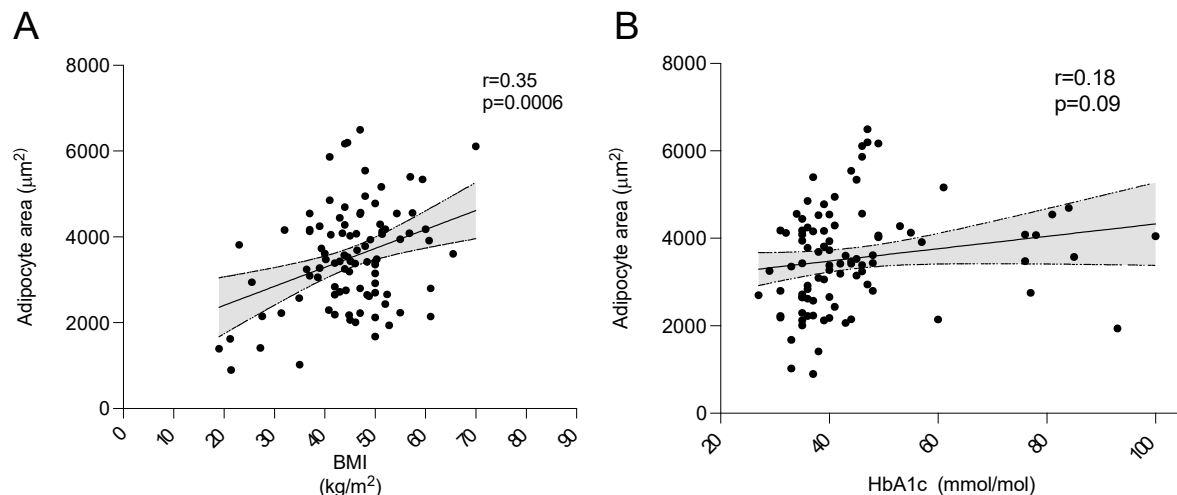


Figure 21 Correlation of adipocyte area with BMI and HbA1c (A) Correlation between adipocyte area (μm^2) and BMI (kg/m^2 ; left panel) and (B) adipocyte area (μm^2) and plasma glycated haemoglobin [HbA1c (mmol/mol); right panel]. Statistical significance was calculated using Pearson correlation coefficient). Error bars represent SEM. HbA1c: glycated haemoglobin; BMI: body mass index.

I next aimed to understand the relationship of hyperplasia with obesity and T2DM by focusing on two transcription factors extensively studied during adipogenesis. PPAR γ has been shown to be required for terminal adipocytes differentiation while CEBP α is crucial for WAT regeneration and expansion [428]. I quantified the protein expression of these two transcription factors in the oWAT using Western Blot. My aim was to establish if the size of the adipocyte was related to the protein levels of each transcription factor and whether this relationship was affected by the patient's metabolic profile. I therefore split the cohort of patients with obesity into patients with large or small adipocytes; I used the average adipocyte area (μm^2) measured by H&E staining for the patient's oWAT and then classified each patient's average adipocyte size as either large or small. As the median size of adipocytes in the obese cohort was $3500 \mu\text{m}^2$; mean adipocyte area above and below this threshold was considered large and small, respectively. I then measured the PPAR γ and CEBP α protein levels in 6 patients with obesity (N=3 large adipocytes, N=3 small adipocytes) and 6 patients with obesity and T2DM (N=3 large adipocytes, N=3 small adipocytes).

The results demonstrated that there was significantly more PPAR γ and CEBP α proteins expressed by small adipocytes when compared to large adipocytes in the oWAT and this was independent of T2DM status. (**Figure 22**).

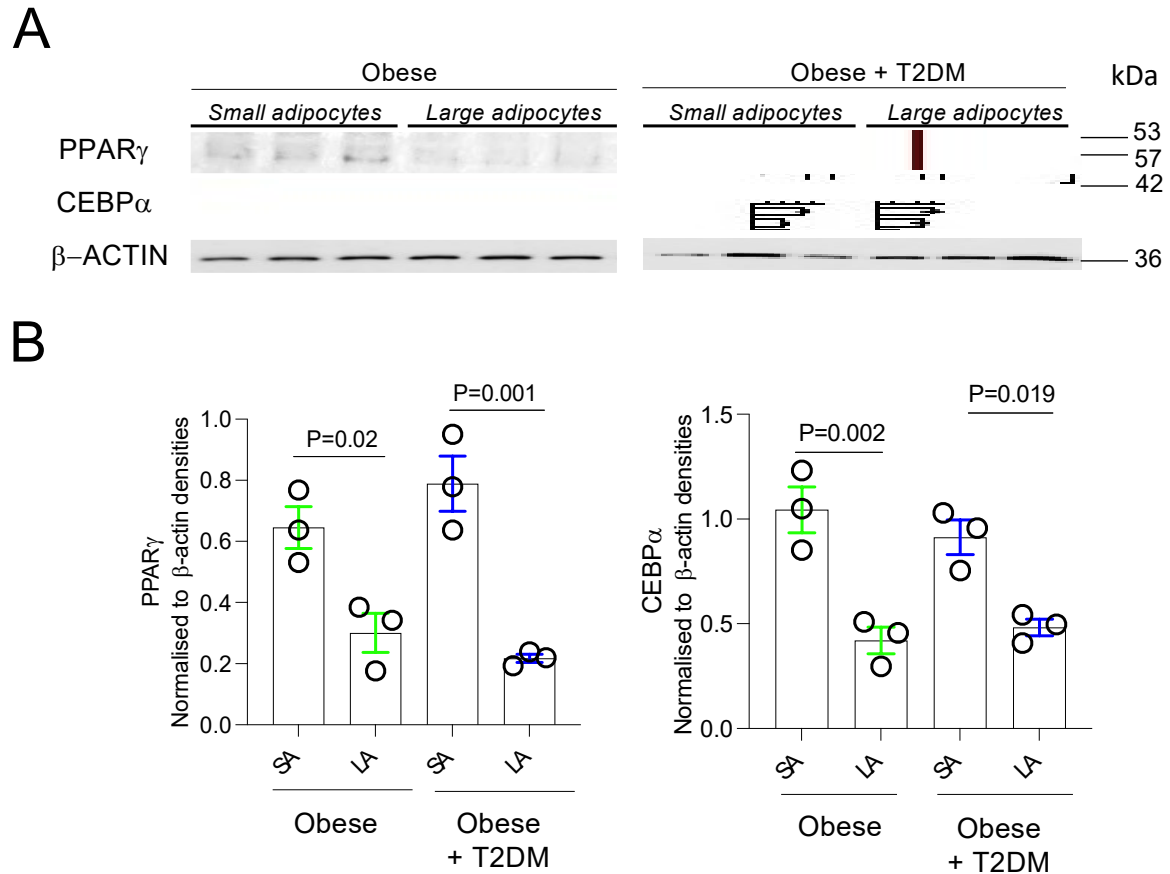


Figure 22 Adipogenesis in obesity **A)** Western Blot of WAT PPAR γ and CEBP α in patients with obesity with and without T2DM stratified according to the adipocyte size (N=3 per group). Non-specific band present on CEBP α blot of obese+T2DM patients at 48kpa. **B)** Relative PPAR γ and CEBPA densities normalised to β -actin densities. Statistical significance determined by student t-test. Error bars represent SEM. SA: small adipocytes, LA: large adipocytes. CEBP α : CCAAT enhancer-binding protein alpha; PPAR γ : Peroxisome proliferator-activated receptor gamma. T2DM: Type 2 Diabetes mellitus. Experiment carried out by T.Olona

4.3.2.1 Conclusion

In summary, adipocyte hypertrophy can clearly differentiate the obese and non-obese oWAT and adipocyte size distinguishes the WAT differentiation state with smaller adipocytes expressing more transcriptions factors associated with adipogenesis, independent of T2DM status.

4.3.3 Inflammation during progression to T2DM in obese patients

Monocytes and macrophages have been shown to be key players in chronic low-grade inflammation during obesity and their number has been associated with dysfunctional adipocytes and insulin resistance in the oWAT [123]. As the stromal vascular fraction contains the fibroblast-like precursors cells, inflammatory cells and epithelial cells, its isolation allows enriched analysis of the inflammatory effects in oWAT. I, therefore, isolated the SVF from the oWAT of patients with obesity (N=25) and patients with obesity and T2DM (N=10) to characterise the inflammatory profile of my cohort. Recent work has shown CD206⁺ cells have been described as adipocyte macrophage markers and work characterising monocytes and macrophages in oWAT by a previous PhD student showed that 80% of CD14⁺ monocytes were CD206⁺. Therefore, I characterised the WAT from obese patients by measuring the number of CD45⁺CD14⁺CD206⁺ cells in the SVF of patients with and without T2DM.

As described in the literature, there were significantly more CD45⁺CD14⁺CD206⁺ cells in the SVF of patients with obesity and T2DM when compared to patients with obesity without diabetes (7.5% versus 3% respectively, $p=0.0004$) (**Figure 23**), with CD206⁺ monocytes significantly correlating with glycated haemoglobin ($r=0.45$ $P=0.04$) (**Figure 23**).

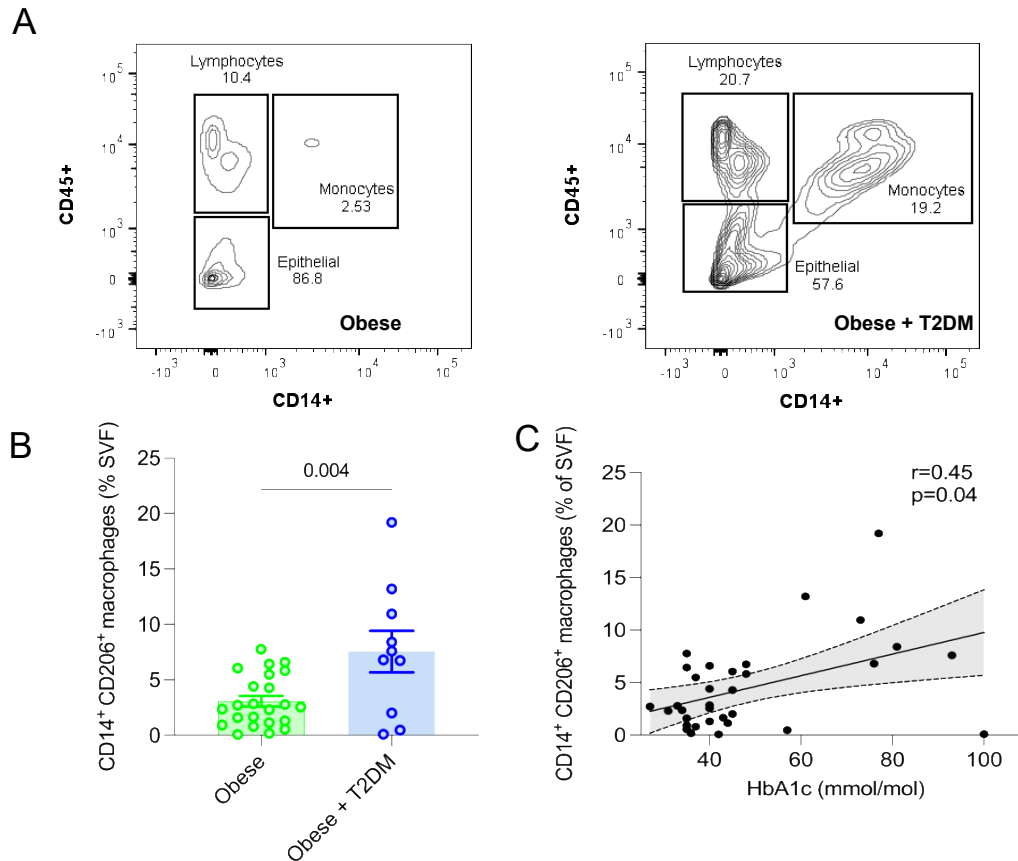
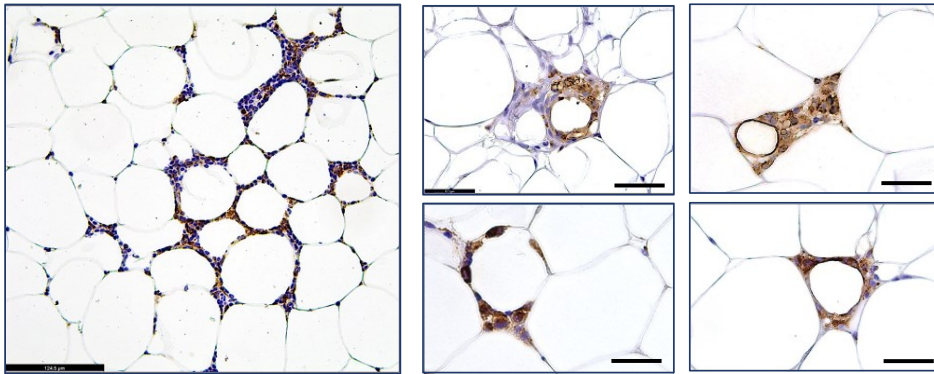


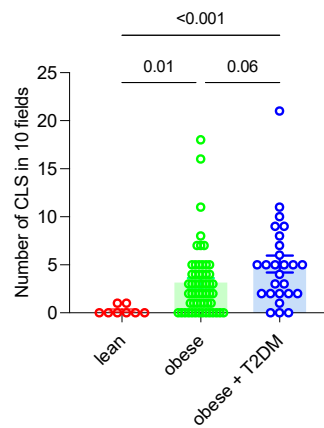
Figure 23 WAT inflammation during T2DM in patients with obesity. (A) Representative flow cytometry showing the increased proportion of monocytes/macrophages (CD45⁺, CD14⁺) in the SVF isolated from WAT of a patient with obesity and T2DM in comparison to a patient with obesity without T2DM. (B) Frequencies (%) of CD14⁺CD206⁺ cells in the SVF of patients with obesity (N=25) and patients with obesity and T2DM (N=10). Gating strategy for CD206⁺ cells in SVF represented in **figure 18 (methods chapter 3)**. (C) Correlation between CD14⁺CD206⁺ cell frequencies and HbA1c (mmol/mol) of patients with obesity (N=33). Error bars represent mean $\pm$ SEM. Statistical significance was calculated using Student's two tailed T-test (B) and Pearson's Correlation (C). SVF: stromal vascular fraction; T2DM: type 2 diabetes mellitus; HbA1c: glycated haemoglobin.

Clusters of macrophages with CD68 staining, a pan-macrophage marker, surrounding dying adipocytes have been shown to be more abundant in obese patients compared to lean individuals and are known as crown-like structures (CLS). To further characterise the inflammation of patient with obesity versus lean patients I used CD68 immunostaining to identify CLS within the oWAT of N=9 lean patient, N=56 patients with obesity and N=26 patients with obesity and T2DM. I found a significant increase in the number of crown-like structures in patients with obesity compared to lean patients (5 CLS versus 3 CLS versus 0.45 CLS in 10 fields; $p = 0.0001$) (**Figures 24**). There was a non-significant increase of CLS in patient with obesity and T2DM compared to non-diabetic patients with obesity. The number of CLS also correlated with increasing adipocyte size ($r = 0.29$, $p = 0.0004$) and HbA1c ($r = 0.30$, $p = 0.004$) (**Figure 24**).

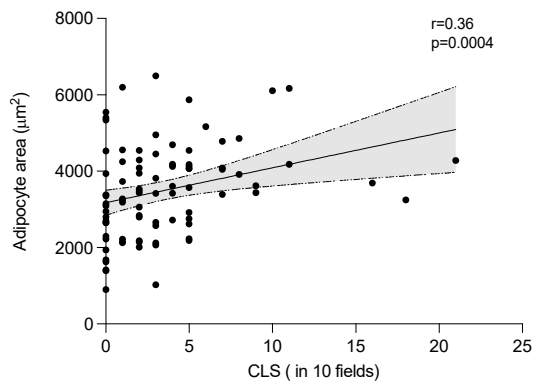
A



B



C



D

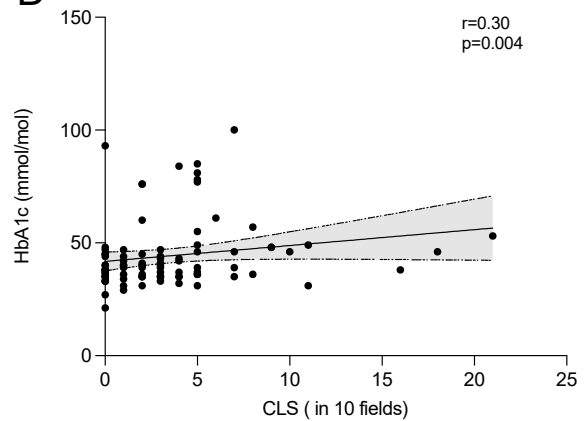


Figure 24 Crown-like structures in the oWAT (A) Representative IHC images for pan-macrophage marker CD68⁺ crown-like-structures (CLS) in the WAT of patients with obesity (N=5 patients). (B) Quantification of CLS in the WAT of lean (N=9), obese (N=56) and patients with obesity and T2DM (N=26). (C) Correlation between CLS and adipocyte area (um²) in the WAT of lean patients(N=7), patients with obesity (N=60) and patients with obesity and T2DM (N=25)(D) Correlation between CLS and HbA1c (mmol/mol) in the WAT of lean patients(N=7), patients with obesity (N=60) and patients with obesity and T2DM (N=25). Error bars represent mean $\pm$ SEM. Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison (A) and Spearman's correlation coefficient (C, D) scale bars; 124.5 microns.

Accordingly, *TREM2* has been identified in recent sc-RNA-seq studies as a macrophage marker of a newly identified lipid-associated macrophage subset localised to CLS, therefore I measured expression levels in our patient cohort (lean patients; N=7, obese; N=60 and patients with obesity and T2DM; N=25). *TREM2* expression levels were significantly increased in patients with obesity compared to lean patient (**Figure 25**) (lean versus obese versus obese with T2DM: $p=0.003$) but did not differ when grouped with obesity with or without T2DM. *TREM2* mRNA expression also correlated with BMI ($r=0.26$, $p=0.01$) but did not correlate significantly with increasing adipocyte size ($r=-0.06$, $p=0.53$) or HbA1c ($r=0.10$, $p=0.31$).

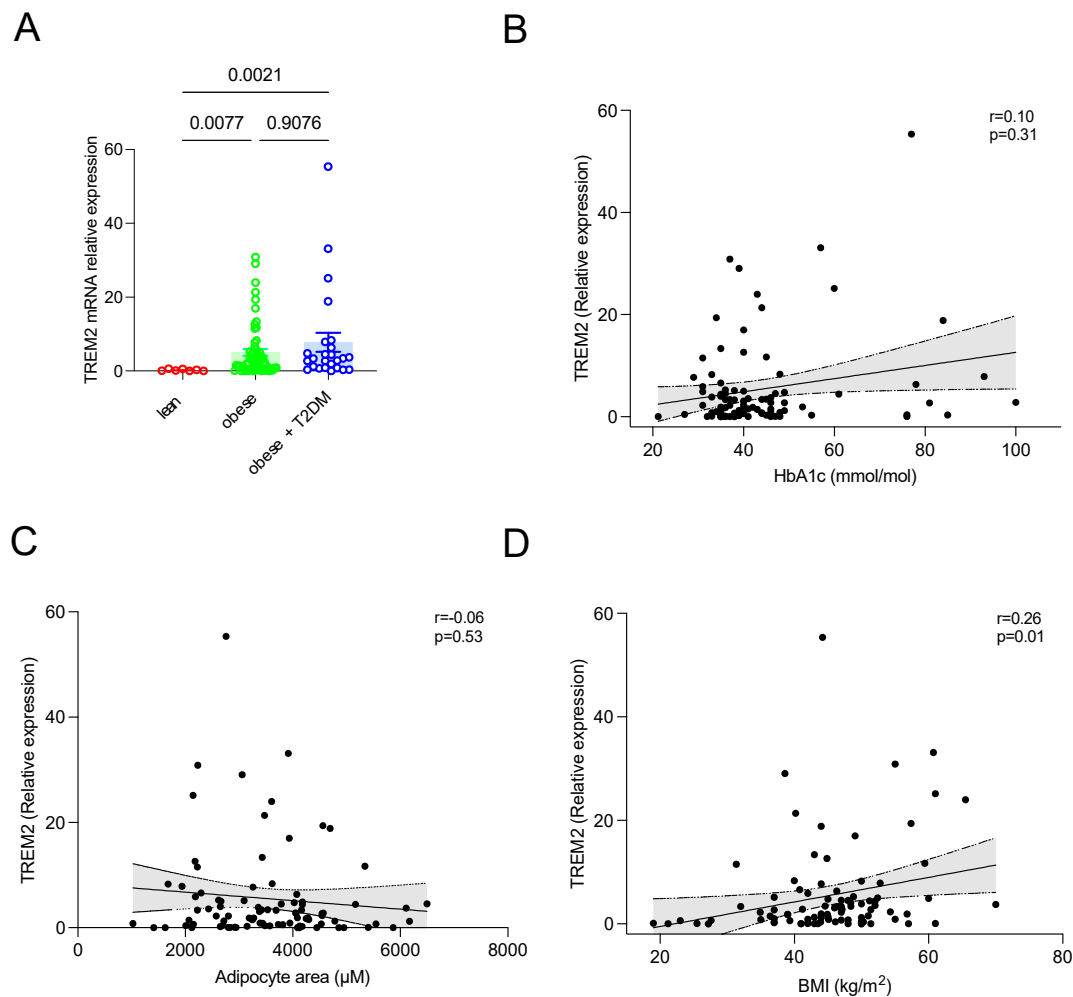


Figure 25 *TREM2* during progression to T2DM in WAT of patients with obesity. (A) *TREM2* mRNA expression in the WAT of lean patients (N=7), obese (N=60) and patients with obesity and T2DM (N=25). (B) Correlation between *TREM2* mRNA expression and HbA1c (mmol/mol) (N=90). (C) Correlation between *TREM2* mRNA expression and adipocyte area (μM) (N=90). (D) Correlation between *TREM2* mRNA expression and BMI (Kg/m^2)(N=90). Error bars represent mean $\pm$ SEM. Statistical significance was calculated using Spearman's correlation coefficient (B-D), Kruskal-Wallis test with Dunn's multiple comparison (A).

4.3.3.1 Conclusion

In summary there is significant increase in inflammatory markers in oWAT of patients with obesity compared to lean individuals. Furthermore, individuals with obesity and T2DM have significantly more monocyte/macrophages within the enriched SVF when compared to non-diabetic patients with obesity and clusters of crown-like-structures are associated with increasing adipocyte size.

4.3.4 ECM remodelling during T2DM in obese patients

Several studies have shown that ECM homeostasis is an important factor in WAT function, closely linked to adipocyte size and the inflammatory state of the WAT [195, 429]. Therefore, I firstly measured immunofluorescence of the most abundant collagen; Type I collagen in the oWAT in lean (N=7), patients with obesity (N=27) and patients with obesity and T2DM (N=12). There was an overall non-significant decrease between lean and obese oWAT ($p=0.21$). I also compared collagen I intensity between patients with obesity with and without diabetes and there was no significant difference as patients developed metabolic dysfunction ($p=0.95$). (**Figure 22**).

Type VI collagen has gained interest in the oWAT in recent years due to Khan et al. who demonstrated using a mouse model that collagen VI was closely related to metabolic status and inflammation in obesity. Therefore, I also measured fluorescence intensity of Type VI collagen in lean (N=7), patients with obesity (N=27) and patients with obesity and T2DM (N=12). Collagen type VI levels were significantly decreased in patients with obesity ($p=0.0017$) (**Figure 26**) however, there was no significant difference between obese oWAT with and without T2DM (**Figure 26**).

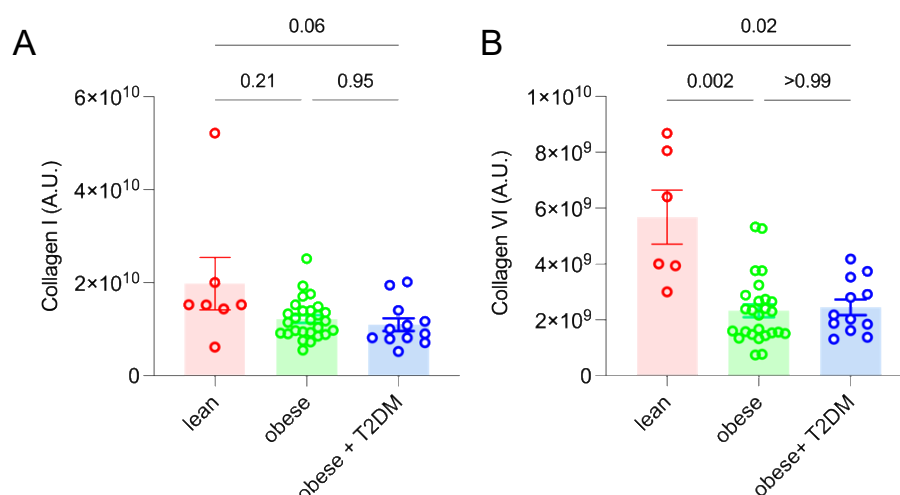


Figure 26 WAT ECM remodelling during progression to T2DM in patients with obesity. (A) Type I collagen fluorescence (top panel) raw intensities (A.U.) in lean (N=7), patients with obesity (N=27) and patients with obesity and T2DM (N=12). (B) Type VI collagen fluorescence (bottom panel) raw intensities (A.U.) in lean (N=7), obese (N=27) and patients with obesity and T2DM (N=12). Statistical significance was calculated using Kruskal-Wallis test followed by Dunn's multiple comparison; error bars are mean $\pm$ SEM.

Next, I aimed to establish if ECM changes were associated with the other key features of maladaptive WAT. Specifically, if collagen type I or VI deposition was related to adipocyte hypertrophy, inflammation and metabolic decline.

4.3.4.1 Metabolic status and collagen deposition

There was no significant difference in Type I collagen or Type VI collagen fluorescence intensity between lean patients and patient with obesity (with and without a diagnosis of T2DM). Glycated haemoglobin, a marker of average glucose control (HbA1c), did not correlate significantly with Type I and VI Collagen intensities in N=46 patients (Col I versus HbA1c ($r=0.014$ $p=0.48$); and Col VI versus HbA1c ($r=0.005$ $p=0.62$) respectively) (**Figure 27**). Taken together this suggests metabolic dysfunction is not associated with ECM remodelling.

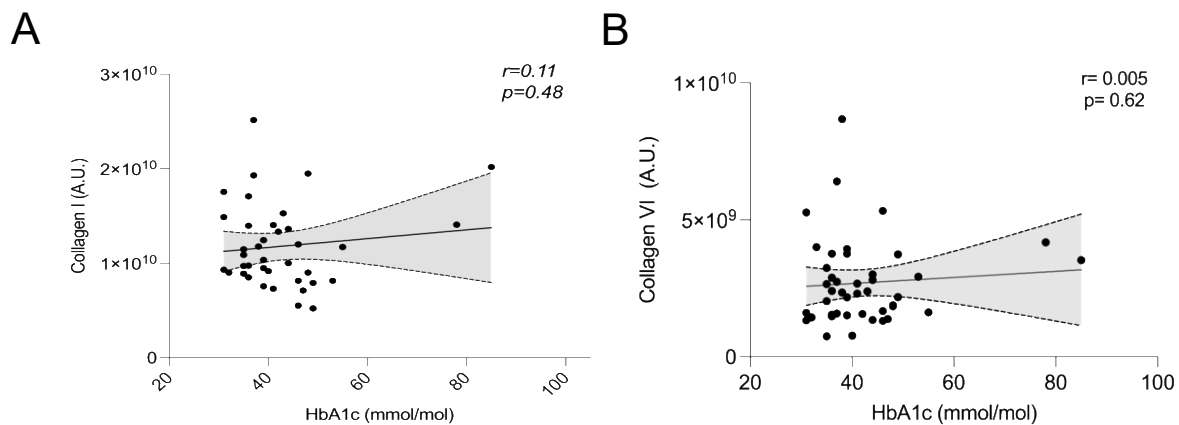


Figure 27 Collagen I and Collagen VI compared to HbA1c (A) Correlation between type I Collagen fluorescence intensities (A.U.) and HbA1c (mmol/mol) (N=46). **(B)** Correlation between type VI Collagen fluorescence raw intensities (A.U.) and HbA1c (mmol/mol) (N=46).

4.3.4.2 Adipocyte hypertrophy and relationship with collagen deposition

Firstly, I correlated collagen I and VI fluorescence intensities with average adipocyte size in N=46 patients. Both Type I collagen and Type VI collagen were found to be inversely correlated with adipocyte size ($r=0.55$, $p<0.001$ and $r=-0.37$, $p=0.016$ respectively) (**Figure 29**). The representative images of the three patient groups (lean, obese and obese plus T2DM) (**Figure 28**) show the heterogenous deposition of Collagen VI which appears in clusters, in contrast to Collagen I that has overall reduction with increasing adipocyte size (**Figure 28**).

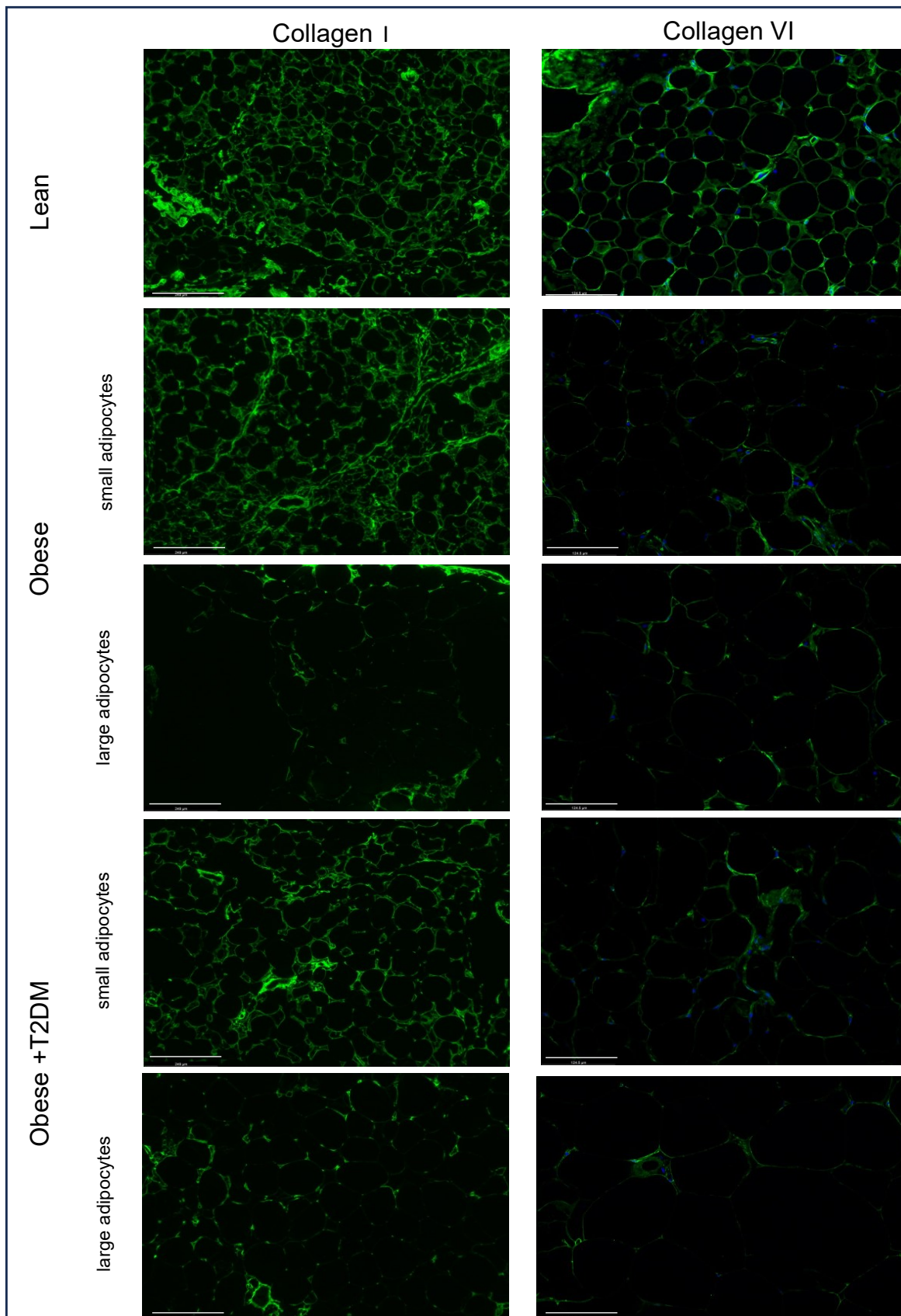


Figure 28 Collagen I and VI deposition in the oWAT. Representative immunofluorescence images for type I and type VI Collagen in lean patients or patients with obesity, with or without T2DM, with presence of small adipocytes or large adipocytes. scale bar 124.5 microns.

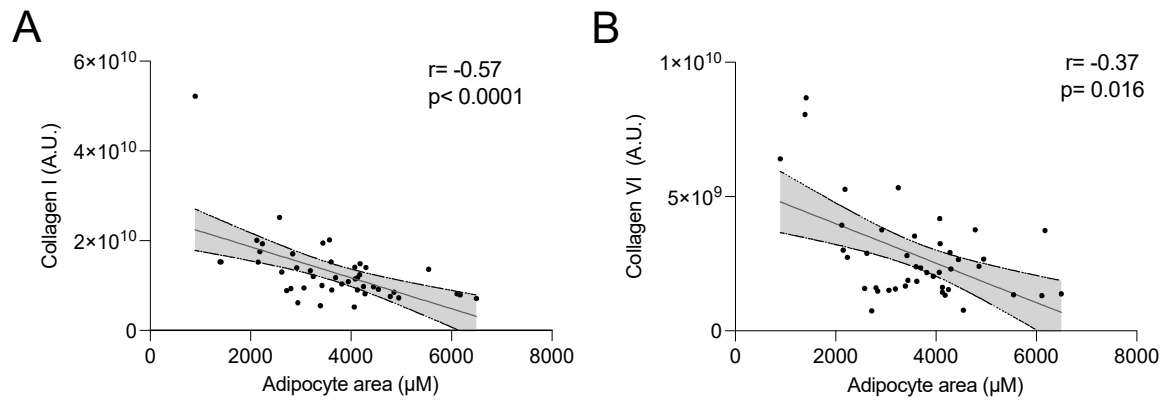


Figure 29 WAT ECM remodelling during T2DM in patients with obesity. (A) Correlation between type I Collagen fluorescence intensities (A.U.) and adipocyte area (μm^2) (N=46). (B) Correlation between type VI Collagen fluorescence raw intensities (A.U.) and adipocyte area (μm^2) (N=46). Statistical significance was calculated using Spearman's correlation coefficient; error bars are mean $\pm$ SEM.

4.3.4.4 oWAT Inflammation and the relationship with collagen transcripts

To assess whether inflammation was associated with ECM remodelling I correlated collagen transcripts COL1A1 and COL6A1 with the inflammatory marker transcripts CD68 and TREM2 (**Figure 30**) in N=46 patients. Both CD68 and TREM2 were not associated with COL1A1 ($r=-0.07$, $p=0.68$ and $r=-0.06$, $p=0.56$ respectively) suggesting it is not involved in inflammation in maladaptive WAT. COL6A1 transcripts on the other hand correlated positively with TREM2 levels ($r=0.47$, $p<0.0001$) and CD68 mRNA levels ($r=0.35$, $p=0.046$) in the WAT.

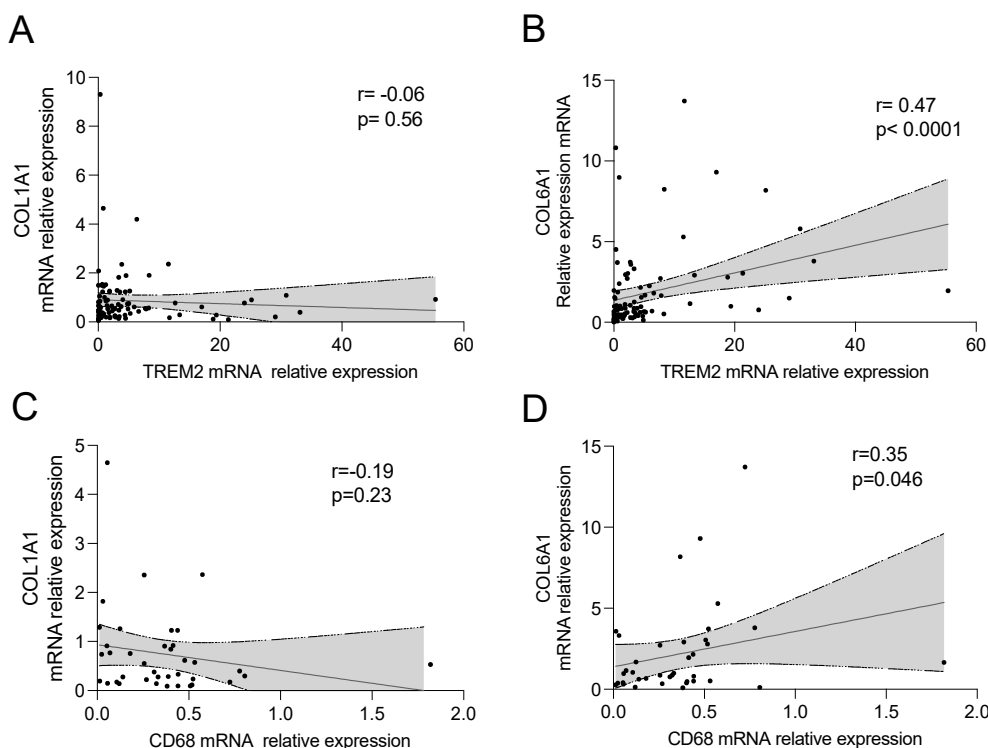


Figure 30 oWAT inflammation correlates with Collagen VI but not Collagen I. (A) Correlation between COL1A1 and TREM2 mRNA levels (N=91). (B) Correlation between COL6A1 and TREM2 mRNA levels (N=91). (C)

Correlation between COL1A1 and CD68 mRNA levels (N=40). (D) Correlation between COL6A1 and CD68 mRNA levels (N=40). Statistical significance was calculated using Spearman's correlation coefficient.

4.3.4.5 Conclusion

These results suggest that Type I collagen is more likely to regulate the structural integrity of the cell during adipogenesis, reducing as the adipocytes increase in size in maladaptive WAT. However, collagen VI deposition decreases with adipocyte size in keeping with maladaptive remodelling in obesity, paradoxically starts to increase when patients had evidence of more inflamed and metabolically unhealthy WAT, which I hypothesis might be the result of macrophage deposition.

4.3.5 Targeting adipose tissue macrophages in obesity and insulin resistance

The results above show that the inflammatory state of adipose tissue can be confidently assessed by measuring the resident macrophage numbers and CLS. The literature in murine models of high fat diet also showed, for the last decade, that macrophage-driven TNF and inflammatory cytokines correlate with adipose tissue dysfunction and associates with insulin resistance [149] However, in humans, the exact causal role of adipose tissue macrophages in insulin resistance have been more challenging to establish. For that reason, I hypothesised that existing medicines could have a role on the viability and/or function of adipose tissue macrophages and may help decipher their function in humans.

Cardiac glycosides are known to inhibit Na⁺/K⁺-ATPase and have been used as therapy for cardiac failure due to their inotropic effects on cardiomyocytes and arrhythmias such as atrial fibrillation. However, at certain doses they are cytotoxic and cause depletion of intracellular K⁺ and accumulation of intracellular Ca²⁺ and Na⁺. The monocyte cell line, THP-1 cells, demonstrated that cardiac glycosides can activate inflammasome pathways and induce pyroptosis in macrophages resulting in cell death [430]. In general, cells of the myeloid origin are sensitive to intracellular levels of potassium because of their enhanced ability to assemble the components of the inflammasome. The Behmoaras lab also showed that human macrophages treated with nanomolar concentrations of cardiac glycosides had compromised macrophage viability with increased caspase-1 activity and a dose dependent increase in IL1-β in the similar range to NLRP3 derived inflammasome activation along with caspase 3/7 and TNF-α release [207]. This work suggested that Ouabain induced macrophage cell death is associated with inflammatory and apoptotic caspases [207]. Therefore, my overarching aim was to use Ouabain to deplete macrophages in *ex vivo* human samples and observe the effects on WAT homeostasis. While Ouabain has a narrow therapeutic window and is toxic at certain thresholds, previous studies have demonstrated that nanomolar concentrations stimulate signalling cascades, specifically pro-inflammatory cytokine expression in macrophages ,and induce inflammasome-mediated cell death

(proptosis). Therefore, I chose to use a range of nanomolar concentrations for the experiments conducted using this cardiac-glycoside [207, 430, 431]. Interestingly, Ouabain's sensitivity toward mouse macrophages is relatively diminished compared to human macrophages and the main reason a solely human-centred setup was used [430].

4.3.5.1 Cardiac glycosides and their effect on macrophage viability

Firstly, I assessed the viability of human monocyte-derived macrophages (hMDM) and non-adherent human monocytes with either control (0.01% DMSO) or increasing nanomolar concentrations of Ouabain (50nM, 100nM, 500nM, 5 μ M) for 24 hours with N=6 donors. Cell viability was measured using annexin and PI staining and calculating percentage of cells with negative Annexin and PI staining.

Ouabain reduced viability of hMDM in a dose dependent manner (5 μ M >500nM >100nM >50nM) but did not significantly alter viability in non-adherent monocytes confirming the dose-dependent macrophage-specific cytotoxicity of Ouabain (**Figure 31**).

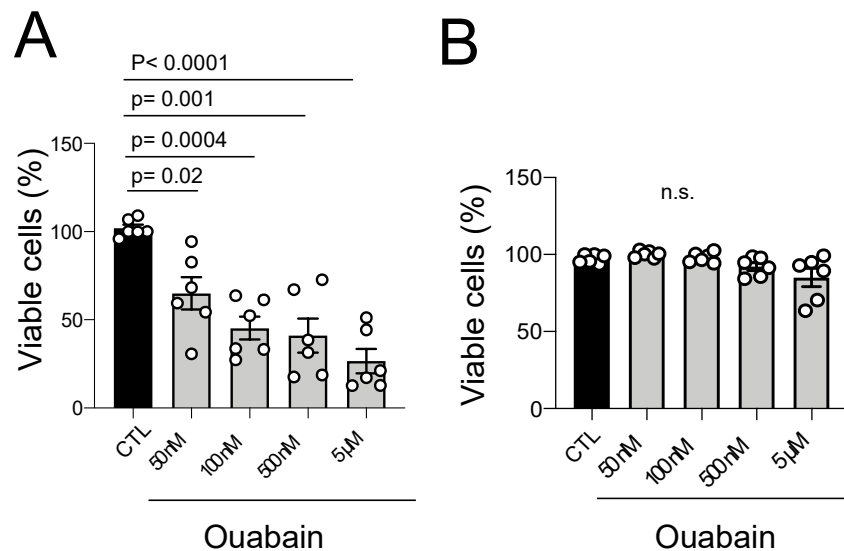


Figure 31 Ouabain induced macrophages specific cell death (A) Cell viability (percentage of cells with negative Annexin and PI staining) of hMDMs treated with Ouabain at increasing concentrations (50nM, 100nM, 500nM, 5 μ M) for 24 hours or control (0.01% DMSO) N=6 donors (B) Cell viability (percentage of cells with negative Annexin and PI staining) of non-adherent peripheral blood mononuclear cells treated with Ouabain at increasing concentrations (50nM, 100nM, 500nM, 5 μ M) for 24 hours or control (0.01% DMSO) N=6 donors. Error bars represent SEM. Statistical significance was calculated using a paired one-way ANOVA test followed by Dunnett's multiple comparison test. hMDM: human monocyte-derived macrophages.

To address whether depleting macrophages would have an impact on oWAT homeostasis I used N=6 *ex vivo* oWAT samples from patients undergoing bariatric surgery that were cultured either with control media (0.01% DMSO) or with increasing nanomolar concentrations of Ouabain (50nM, 100nM and 500nM) for 48 hours.

4.3.5.2 Macrophage specific depletion in the explants

The oWAT explants were stained with CD68 pan-macrophage marker and number of macrophages in 10 fields were counted in the control explant and explants treated with 50nM, 100nM and 500nM of Ouabain to corroborate the previous cell culture set up which suggested macrophages are depleted with low concentrations of Ouabain. Indeed, there was a marked reduction of CD68 macrophages in Ouabain treated oWAT versus control.

To further validate this finding, CD68 mRNA transcripts were measure and shown to be significantly reduced in the Ouabain treated explants compared to the control explants (N=6). CD206, a mannose receptor and macrophage marker has been shown to be highly expressed in WAT macrophages. A western blot showed that CD206 protein levels were also significantly reduced in N=2 explants treated with Ouabain compared to controls (Figure 32).

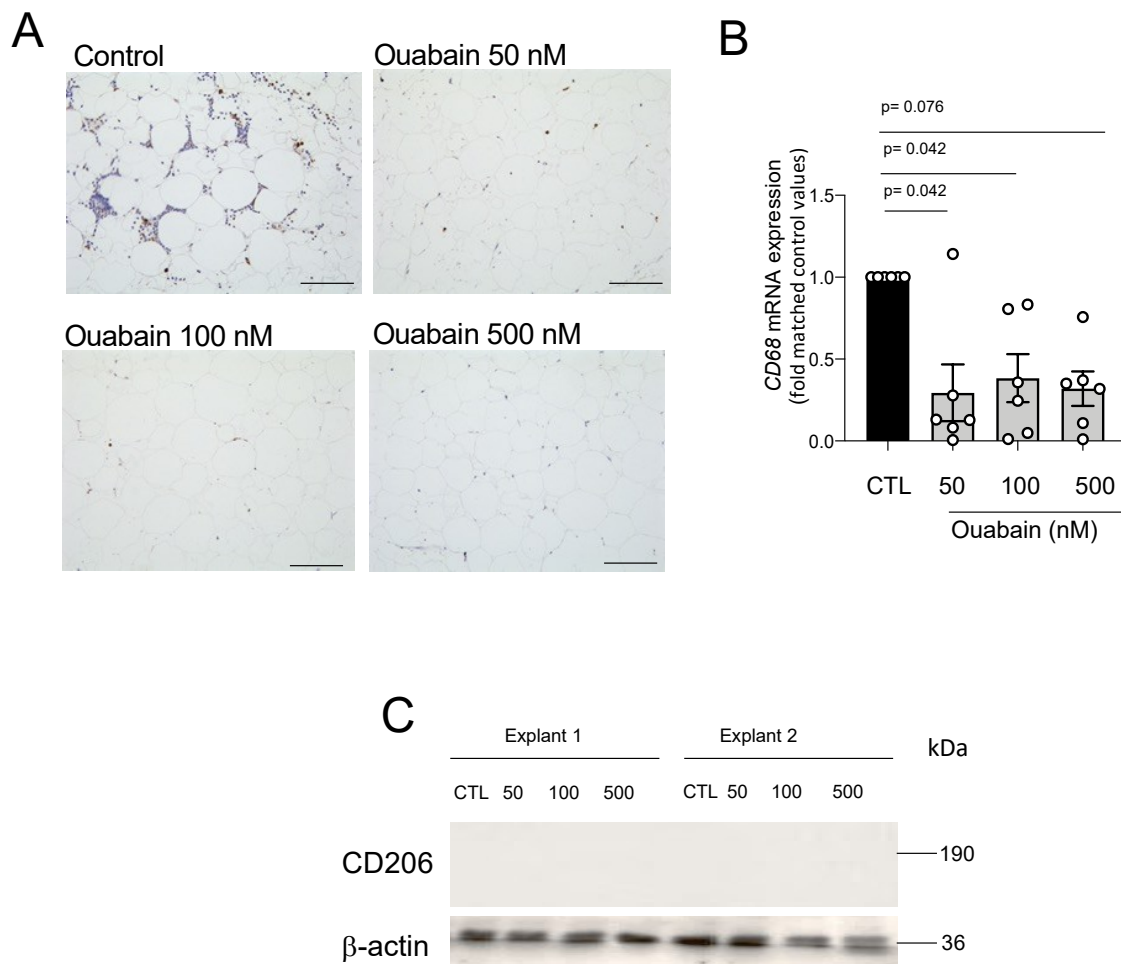


Figure 32 Macrophages are depleted in oWAT explants from patients undergoing bariatric surgery. (A) Representative pictures of CD68 immunostaining in human oWAT explants treated with Ouabain (50 nM, 100 nM and, 500 nM) for 48 hours. **(B)** Relative CD68 mRNA expression in human oWAT explants treated with Ouabain (50 nM, 100 nM and 500 nM) for 48 hours, n= 6 explants. **(C)** Representative CD206 western blots in human oWAT explants treated with Ouabain (50 nM, 100 nM and, 500 nM) for 48 hours. Error bars represent SEM. Statistical significance was calculated using a paired one-way ANOVA test followed by Dunnett's multiple comparison test. CD68 staining and western blot by T.Olona

To ensure that Ouabain induces macrophage-specific cell death I measured the viability of adipocytes firstly of SVF-derived pre-adipocytes which were treated with increasing (50nM, 100nM, 500nM and 5µM) concentrations of Ouabain for 24 hours in N=2 donors. Viability was again measured using percentage of negative annexin and PI staining and showed no significant increase in cell death (**Figure 33A**). Following this, I used SVF-derived adipocytes which were treated with 50nM, 100nM, 500nM Ouabain for 24 hours for N=2 donors and alamar blue staining demonstrated no or very little cell death even in high concentrations of Ouabain (**Figure 33B**).

Therefore, Ouabain does not affect pre-adipocytes nor mature adipocytes *in vitro* suggesting the effects of Ouabain appear to be macrophage specific.

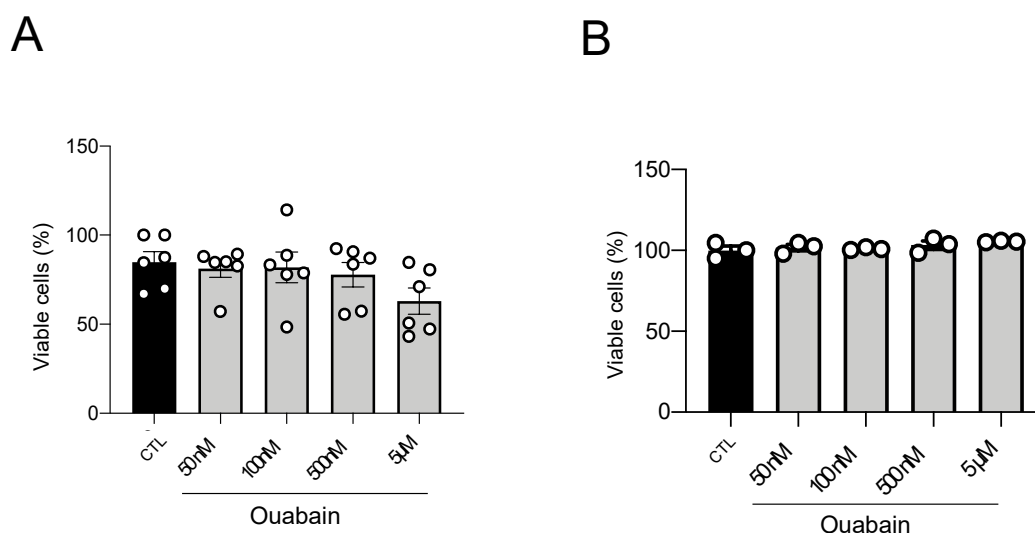


Figure 33

Cell viability in SVF-derived pre-adipocytes and adipocytes treated with Ouabain. **A)** Cell viability (percentage of cells with negative Annexin and PI staining) of SVF derived pre-adipocytes treated with Ouabain at increasing concentrations (50nM, 100nM, 500nM, 5µM) for 24 hours or control (0.01% DMSO) N=6 donors **(B)** Cell viability (alamar blue staining) of mature SVF-derived adipocytes treated with Ouabain at increasing concentrations (50nM, 100nM, 500nM, 5µM) for 24 hours or control (0.01% DMSO) N=6 donors. Error bars represent SEM. Statistical significance was calculated using a paired one-way ANOVA test followed by Dunnett's multiple comparison test. SVF: stromal vascular fraction.

4.3.5.3 Ouabain and its effect on WAT homeostasis

To characterise the WAT health, I incubated *ex vivo* explants N=6 with increasing doses of Ouabain (50nM, 100nM and 500nM) and control (0.01% DMSO) for 48 hours and investigated i) adipogenesis by measuring ADIPOQ mRNA transcripts and CD36 protein levels ii) insulin resistance by measuring pAKT and AKT protein levels. iii) ECM changes by quantified collagen type VI immunofluorescence due to the association observed in my human cohort with collagen type VI and inflammation.

4.3.5.4 Adipogenesis and insulin sensitivity in Ouabain treated oWAT explants

In order to assess how macrophage depletion affects adipogenesis and insulin sensitivity I first measured adiponectin transcript levels, an adipokine that has been associated with

insulin sensitisation and adipocyte differentiation. ADIPOQ mRNA transcripts were upregulated in the N=6 explants from patient with obesity that were treated with Ouabain compared to the control (**Figure 34A**).

CD36 is a fatty acid transporter that improves insulin sensitivity and promotes adipocyte differentiation, protein levels measured in N=5 explants were shown to be increased when treated with Ouabain suggesting improved insulin sensitivity and adipocyte cell turnover (**Figure 34B**).

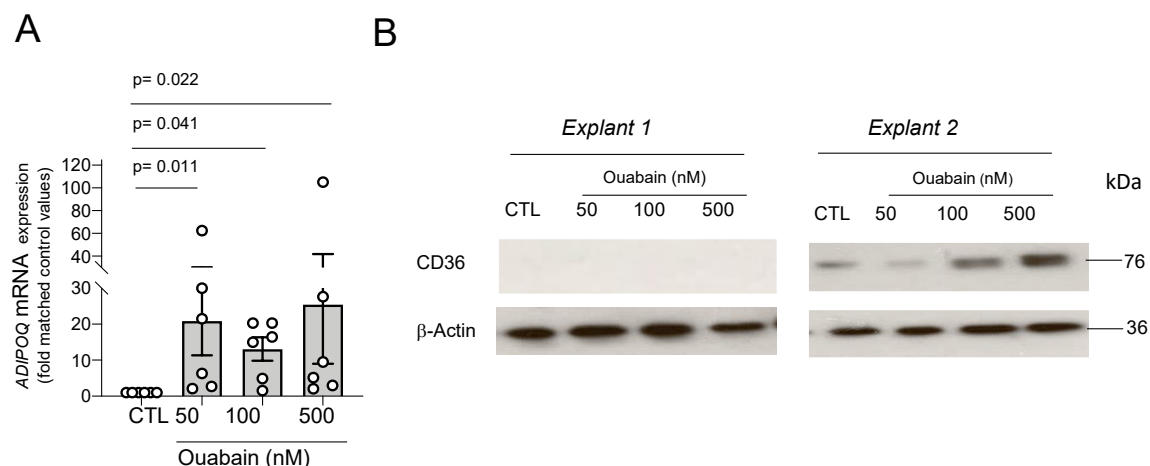


Figure 34 Adipogenesis in oWAT treated with Ouabain (A) Relative ADIPOQ mRNA expression in human oWAT explants treated with Ouabain (50, 100 and 500 nM) for 48 h; N = 6 explants. **(B)** CD36 Western blotting in human oWAT explants treated with Ouabain (50, 100 and 500 nM) for 48 h; representative of N = 5 explants. Error bars represent SEM. Statistical significance was calculated using a paired one-way ANOVA test followed by Dunnett's multiple comparison test. Western blot and rt-qPCR by T.Olona

Adipose specific insulin resistance was measured by comparing protein levels of AKTser473 phosphorylation to total AKT in N=4 explants. AKTser473 phosphorylation has been shown to be integral to insulin signalling pathways in adipocytes. Insulin binds to its receptor and activates proteins phosphoinositide 3-kinase (PI3K) which phosphorylates AKT at Ser473 stimulating GLUT4 enabling the translocation of glucose into the cell [432]. Protein levels of AKTser473/AKT were increased in explants (N=4) compared to control indicating improved insulin sensitivity when macrophages are depleted (**Figure 35**).

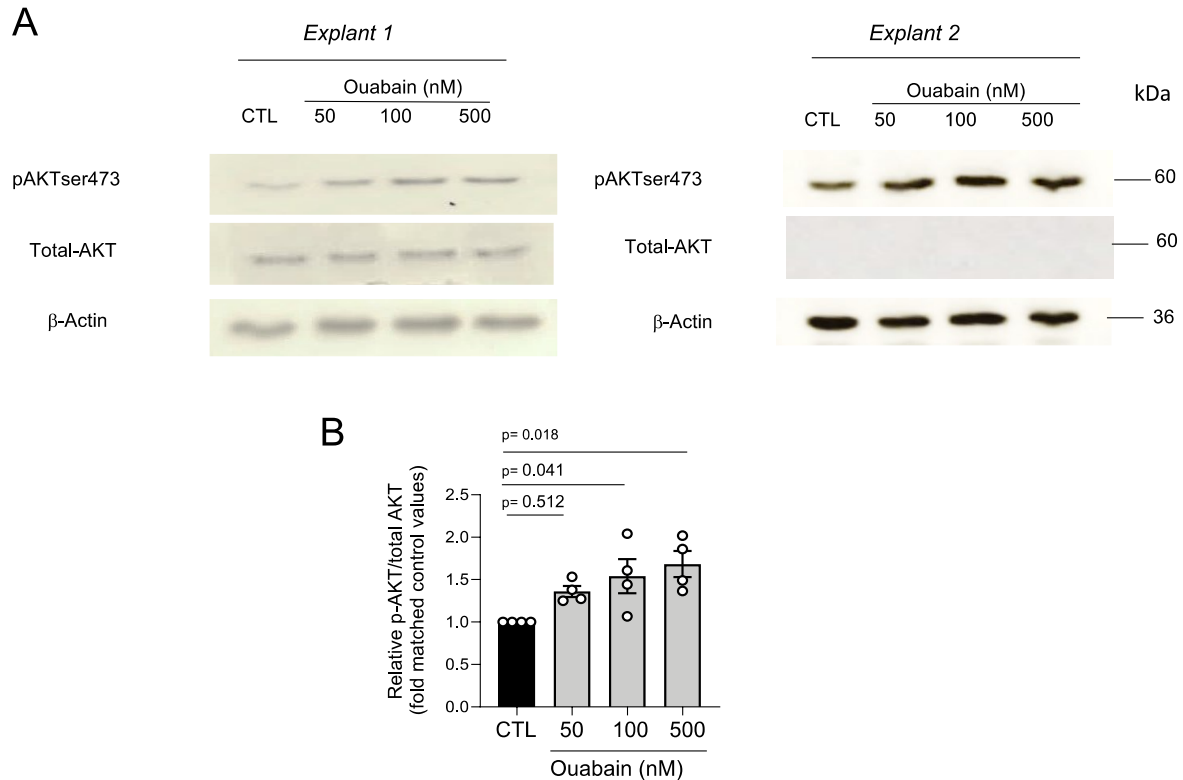


Figure 35 Insulin sensitivity after oWAT explants were treated with Ouabain. (A) Phospho-AKTser473, total Akt and β -actin western blot in human oWAT explants treated with insulin and Ouabain (50, 100 and 500 nM) for 48 h; N=4. **(B)** Analysis of relative levels of phospho-AKTser473 protein, normalized to the loading control (total AKT); N=4. Statistical significance was calculated paired non-parametric ANOVA followed by Dunn's multiple comparisons. Error bars represent SEM. Western Blot by T.olona

4.3.5.5 Collagen type VI deposition in Ouabain treated oWAT explants

Collagen type VI was found to be associated with increasing inflammation in oWAT as Collagen type VI immunofluorescence positively correlated with TREM2 and CD68 transcripts and appeared to increase in clusters. Therefore, I hypothesised that if macrophages were driving collagen type VI deposition, reduction of macrophages would result in a decrease in collagen type VI.

I measured Collagen type VI immunofluorescence in N=4 explants using mean grey intensities in oWAT explants treated with Ouabain for 48 hours at 100nM and compared this with control tissue. There was a downregulation of collagen type VI immunofluorescence, and this was reflected in a significant reduction of collagen type VI (COL6A1) mRNA transcripts when comparing control with 50nM, 100nM and 500nM of Ouabain in N=6 explants (**Figure 36**).

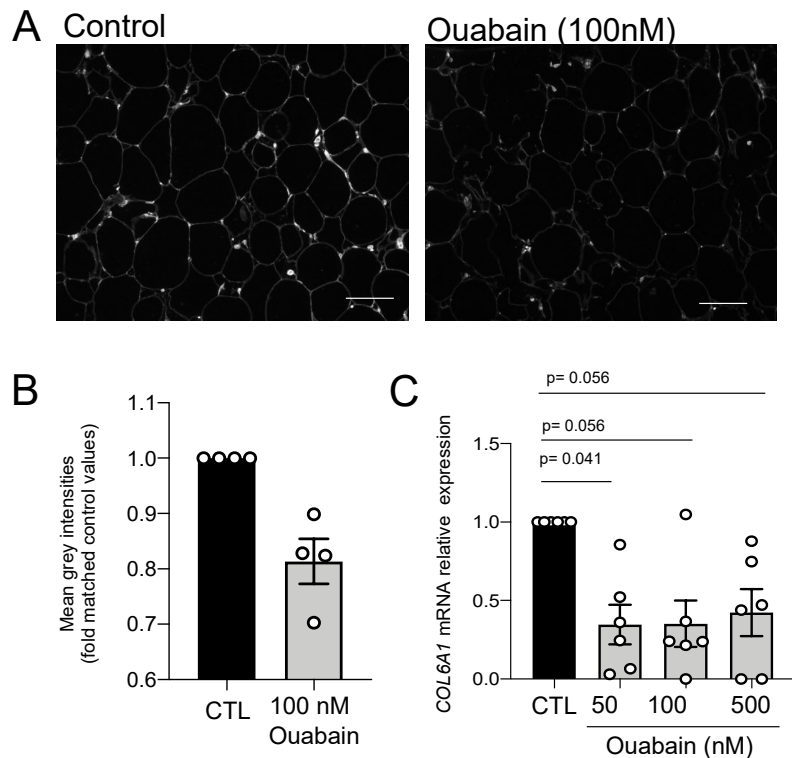


Figure 36 oWAT Collagen type VI levels after treatment with Ouabain. (A) Representative type VI collagen immunofluorescence pictures of human oWAT treated with Ouabain (100 nM) for 48 h. (B) Relative type VI collagen fluorescence. (C) Relative COL6A1 mRNA expression in human oWAT explants treated with vehicle (control) or Ouabain at indicated concentrations for 48 h; N = 4 explants for immunofluorescence and N = 6 explants for qRT-PCR. Statistical significance was calculated paired non-parametric ANOVA followed by Dunn's multiple comparisons or student's t-test. Error bars represent SEM. Scale bar = 50 μ m.

4.3.5.6 Conclusion

Ouabain has been shown to selectively deplete macrophages. Non-adherent monocytes, pre-adipocytes and mature SVF-derived adipocytes all maintained viability close to 100% after 24 hours of treatment with increasing concentrations of Ouabain. On the other hand, hMDM had marked cell death in a dose dependent manner when treated with Ouabain.

Furthermore, omental WAT *ex vivo* explants treated with control or increasing concentration of Ouabain were shown to have depletion of macrophages both by immunohistochemistry, showing reduced numbers of CD68 stained cells, and reduced mRNA transcripts of CD68. Protein levels of CD206 marker of adipocyte-associated macrophages were also decreased in Ouabain treated explants.

Adipocyte turnover and insulin sensitivity improved upon incubating explants with Ouabain as evidenced by increased ADIPOQ mRNA transcripts, increased protein levels of CD36 and increased AKTser473/AKT protein. Finally, collagen type VI also appeared to decrease with Ouabain therapy, with a reduction in collagen type VI immunofluorescence and COL6A1 transcripts. Taken together this suggests that macrophages are likely responsible for driving

inflammation, collagen type VI deposition and a reduction in adipocyte turnover all associated with maladaptive changes in oWAT during obesity highlighting them as a potential cell-specific drug target (**Figure 37**).

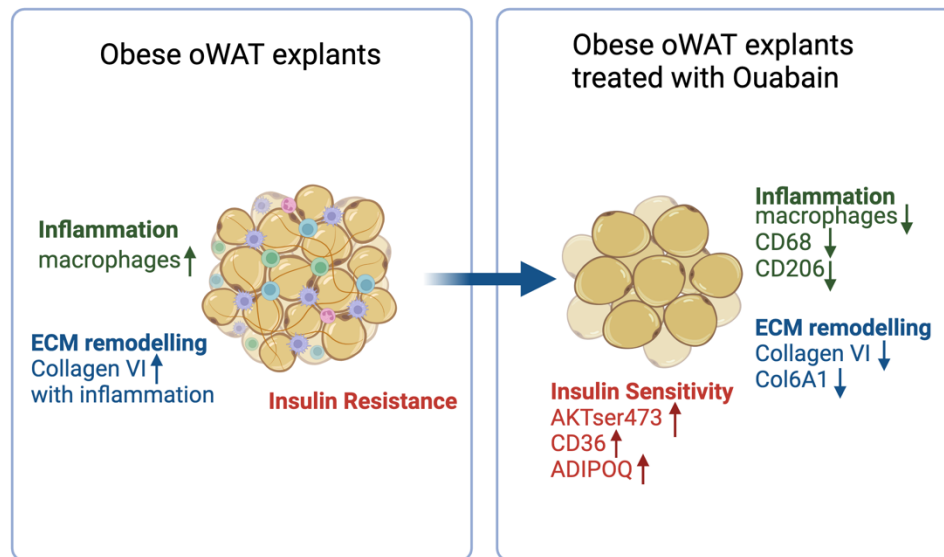


Figure 37 Schematic representing the effect of Ouabain treatment on *ex vivo* explants from patients undergoing bariatric surgery for obesity. Image created by Bio Render®

4.4 Discussion

The interplay between adipogenesis, inflammation and ECM remodelling in the oWAT and their relationship with insulin resistance is complex and not fully elucidated in humans. One strategy to dissect these is to focus on 'healthy' obesity, to understand the evolution of WAT homeostasis and progression to T2DM. My aim was to characterise a unique population of extremely obese patients and compare the metabolically healthy and the metabolically unhealthy with lean oWAT by using these three major component of WAT homeostasis: inflammation, fibrosis and adipogenesis [157].

I used glycated haemoglobin as a measure of metabolic health as this would give an account of glucose control over a longer period, rather than a snapshot of insulin sensitivity on the day of surgery, as often patient undergo pre-operative glucose optimisation in order to improve surgical outcomes.

4.4.1 Adipocyte hypertrophy versus hyperplasia

The association of adipocyte hypertrophy with declining metabolic status of oWAT has previously been demonstrated and is echoed in this cohort. Obese patients with a diagnosis of T2DM had significantly larger adipocytes than patients with obesity and preserved metabolic health and with lean patients. Previous studies have shown that adipocyte hypertrophy is closely linked with increased pro-inflammatory adipokine release and increased hypoxic stress which exacerbates metabolic dysfunction. On the other hand, smaller adipocytes have been shown to favour adipogenesis, thus reducing hypertrophic adipocytes and inflammatory factors which in turn leads to improved metabolic health.

Interestingly, this cohort also demonstrated that, independent of T2DM status, the size of the adipocyte determines the production of key factors involved in adipogenesis with smaller adipocytes having increased PPAR γ and CEBP α protein levels than large adipocytes that had loss of PPAR γ and CEBP α protein. A selective adipose tissue insulin receptor knockout mouse model also demonstrated, despite adipocyte specific insulin resistance, adipocyte size remained heterogenous and protected against obesity with no relative change in PPAR γ [433]. This has led me to hypothesise therefore, that hyperplasia itself is a compensatory mechanism driven by the oWAT to preserve homeostasis irrespective of systemic insulin resistance and alternative pathways or factors independent of adipocyte turnover, initially drive the metabolic decline of oWAT in obesity. However, as 70% of the obese cohort also take Metformin, these results could indeed be due to a confounding drug effect which I therefore shall explore in the next chapter.

Inflammation and macrophage infiltration increases as T2DM progresses

A robust indicator of WAT inflammation is the presence of mononuclear phagocytes [160, 161]. These macrophages perform functional roles in lipid metabolism and phagocytosis of dying adipocytes [434-436] and likely alter phenotype to their changing microenvironment. In keeping with previous studies, I confirmed a significant increase in the numbers of mononuclear phagocytes within the oWAT of T2DM obese patients as metabolic decline progressed. Indeed, there were increased numbers of CLS in obese patients compared to lean. CD45⁺CD14⁺CD206⁺ macrophages and TREM2 mRNA transcripts increased with HbA1c progression and obesity and the number of CLS increased as adipocytes increased in size.

I used the cardiac glycoside Ouabain a Na⁺/K⁺-ATPase blocker, to treat human monocyte-derived macrophages and *ex vivo* omental WAT explants and showed the importance of macrophages for WAT homeostasis. I showed that Ouabain even at low concentrations (50uM) leads to the death of macrophages which resulted in improved insulin sensitivity and adipogenesis in WAT explants [437].

While I didn't analyse surface markers and macrophage function in depth, recent studies have identified a number of WAT specific macrophage subsets with distinct functions, traditionally adipose tissue macrophages were thought to be M1-pro-inflammatory phenotype however this has been challenged in recent years and macrophage functions have been closely linked to glucose and lipid metabolism. Anti-inflammatory CD206⁺ macrophages have been identified in WAT and shown to be associated with systemic insulin control through prevention of growth and differentiation of adipocyte progenitor cells [163] and TGFβ signalling [163, 438]. A metabolically active macrophage subset has also been described in subcutaneous WAT, characterised as overexpressing specific lipid metabolism markers such as CD36, PLIN2 and ABCA1 [439].

Importantly, recent applications of single cell RNA-seq of WAT has demonstrated the plasticity of adipose tissue macrophages and has further challenged the role and classifications of these cell-types identifying multiple clusters of mononuclear phagocytes expressing distinct gene signatures unique to obese WAT, including TREM2⁺ and CD9⁺ macrophages contained within CLS [109]. The frequency of CD9⁺ and TREM2⁺ lipid-associated macrophages (LAMs) increase with obesity in mice and humans [113, 171] and these actively produce IL-1β and TNF in obese patients [113]. A further subset, unique to visceral WAT, of macrophages that co-express CD9⁺ and CD68⁺ have been described. [108] How these macrophage subsets modulate WAT homeostasis and the cell to cell interactions are still not fully understood but it is now accepted that macrophages are able to fuse to form

multinucleated giant cells which has been shown with live imaging to be superior to individual macrophages in phagocytosing oversized particles such as dying adipocytes or lipid remnants [169, 170]. While scRNA-seq has not differentiated between macrophages with different numbers of nuclei this could be achieved by cell-sorting before performing scRNA-sequencing and thus further delineating their role and relationship with LAMs [170].

These studies together with my results demonstrate that mononuclear phagocytes are incredibly versatile with a multitude of functions within the lean and obese WAT and have important roles in modulating lipid and glucose pathways and cell turnover. Due to this, macrophages in recent years have been the focus of potential human treatment targets for ameliorating obesity and IR. There is a plethora of literature using obese mouse models that show promising results for macrophage targeted treatments either through depletion, inhibition, genetic modification or using soluble molecules e.g. anti-TNF treatment that have led to improved WAT metabolic function and reduced weight gain summarised here [440].

However, to date no macrophage-mediated treatment target has been successful in humans. One observation against their use comes from patients with autoimmune disease. Individuals with obesity have been treated with steroids, anti-TNF and other immunomodulators for other auto-immune conditions for decades (including psoriasis, asthma, lupus and inflammatory bowel disease) however, there has been no global benefit to their metabolic status and in fact often anti-TNF treatment has been shown to cause weight gain [441]. In its defence, this could in part be due to the treatment of the underlying pathology improving the individuals' nutritional status. Steroids which are a popular treatment option in this group of patients are also widely known to increase IR and weight gain. This controversial viewpoint highlights the complexity of WAT dysfunction and the multi-functional roles that macrophages have and I hypothesise their role to modulate their function depending on their microenvironment is paramount to their success but paradoxically also the likely reason for their failure thus far in humans as a treatment target in obesity and metabolic decline.

4.4.2 ECM remodelling

While adipose tissue macrophages' role in adipocyte dysfunction is fairly well established, the exact consequences of ECM remodelling during the maladaptive transformation of WAT are complex and depend on many factors, including the fat depots (visceral vs. subcutaneous), different types of collagens and their distribution patterns [196, 199]. Each type of collagen is likely to be differentially influenced by the WAT microenvironment and while used commonly in the literature the term 'fibrosis' may be a broad misleading term to describe ECM remodelling in obesity [200].

Several other studies show that collagen abundance and 'fibrosis' decrease with adipocyte hypertrophy, suggesting the collagen matrix is key to maintaining WAT health by preventing unconstrained expansion [196, 201, 202]. Muir et al. have shown that human subcutaneous and visceral WAT 'fibrosis' is reduced (measured by sirius red staining, collagen transcripts and immunohistochemistry) with increasing HbA1c levels [202]. On the other hand, work by Gugliemi reported increased total fibrosis positively correlated with insulin resistance in obese omental tissue but there was no significant difference in collagen transcripts or adipocyte size with increased insulin resistance [203]. However, none of these studies delineated the roles of different types of collagens and evaluated them in context of other features of maladaptive oWAT.

Therefore, I performed a comparative quantification of type I and VI collagen levels in the oWAT of extremely obese patients with progressive metabolic decline and compared this with lean oWAT with the aim of identifying specific associations with individual elements of WAT adaption. I found that type I collagen inversely correlated with average adipocyte size, independently of T2DM status and WAT macrophage abundance. Whereas type VI collagen, while also decreasing with adipocyte size, also paradoxically increased in clusters and this correlated with WAT macrophage markers TREM2 and CD68.

These results suggest that ECM deposition can be a positive functional response i.e. collagen I's structural role but other proteins such as Collagen VI are closely associated with inflammation and maladaptive responses to obesity.

This differential function of type I and type VI collagens in WAT ECM remodelling are supported by other studies. Regarding type I collagen, HFD-fed MMP14^{+/-} mice were unable to undergo expansion of adipocytes due to a lack of collagen I [210]. Others studies corroborated my findings for collagen type VI and their role in inflammation; increased collagen VI deposition was associated with worsening metabolic function and WAT inflammation during chronic obesity [204-206].

Specifically, Khan et al has demonstrated that collagen type VI determines inflammation and metabolic health in mice [204]. Since macrophages have been shown to synthesise Col6 in lung fibrosis [442] (and given the association between the WAT Col6 levels and macrophages numbers in humans [207, 442]) it is plausible that Col6 is a macrophage-derived collagen that is a factor in driving maladaptive transformation of WAT.

To investigate this further I treated ex vivo explants with Ouabain in micromolar doses to deplete macrophages which resulted in a reduction of collagen VI deposition in the oWAT

explant, further reinforcing that inflammation during metabolic modifications in obesity is closely linked to collagen VI and a potential target for restoring WAT homeostasis [437].

While collagens have been studied here and by other groups, factors that influence collagen deposition including MMPs, growth factors, lamins and other components of extracellular matrix and their roles in WAT homeostasis and changes with metabolic decline are less well described. MMPs have been studied in rodent models with certain MMPs (MMP-3, -11, -12, -13 and 14) upregulated in visceral WAT in obesity while others were downregulated (MMP-7, -9, -16 and 24) [443] and limited data on human tissue shows MMP12 regulates adipocyte expansion and insulin sensitivity in subcutaneous human tissues [444]. MMP14 gene polymorphism has been associated with collagen turnover in human obesity [210] and MMP-9 correlated with insulin resistance in obesity in subcutaneous and omental fat [197] but the interplay with inflammation and other elements of the ECM is not well described and further research is needed.

4.4.3 Limitations

Despite strict exclusion criteria there was a significant difference in age and sex between the lean group and obese group in this cohort. In the lean cohort every effort was made to use patients with a normal BMI, no systemic features of malignancy and no exposure to chemotherapy or immunotherapy which resulted in a limited selection of patients. The difference in sex and age is in part due to the fact that 70% of bariatric surgery is undertaken on women while oesophageal and gastric cancers are more likely to be in men and have a generally older population making the selection criteria limited. In order to address this going forward, collection from benign laparoscopic abdominal surgery would in part mitigate these variables.

Furthermore, as patients required their glucose control to be optimised to undertake surgery, there is a relatively small number of patients with poorly controlled diabetes which may be the explanation for the non-significant differences observed when correlating HbA1c with adipocyte size and when comparing number of CLS, TREM2 transcripts and Collagen type I or Collagen type VI abundance between obese and obese patients with T2DM. A further explanation may lie with the effects of medications taken by predominantly diabetic patients. While we did not recruit patients taking insulin or PPAR γ agonists, a large proportion of the cohort take Metformin (74%) and there is very little robust data that shows the effect of Metformin on WAT biology and specifically on the transition of lean to obese, therefore, I have explored this in the following chapter.

4.4.4 Future work

I have explored WAT maladaptation due to obesity and T2DM in omental depots but it is well documented that the depot has both local and systemic effects and evolving literature also demonstrates the importance of brown WAT in metabolic homeostasis thus the role of other depots in the transition from 'healthy' to 'unhealthy' WAT should be explored.

Furthermore, I have also shown that *ex vivo* macrophage depletion makes AT healthier in humans, but whether this would also improve systemic insulin resistance and systemic glucose tolerance has not been addressed. One method to answer this question would be to locally deliver cardiac glycosides in an animal model and observe systemic affects.

However, as previously discussed mice do not harbour the same cytotoxic response in macrophages to cardiac glycosides and thus pigs maybe a more appropriate *in vivo* model.

As single-cell RNA-sequencing identifies new cell types their role and function in obesity should also be determined with cell-to-cell interactions further characterised utilising new techniques such as spatial transcriptomics. I would be keen to analyse macrophage sub-sets, specifically multinucleated macrophages coined 'adipoclasts' [170] and their relationship with ECM remodelling. While it is thought that they have a protective role in cell turnover, how they relate to collagen deposition and metabolic health has not been full elucidated.

4.4.5 Conclusions

In summary, oWAT maladaptive changes in prologued obesity are characterised by increasing adipocyte size, loss of PPAR γ and CEBP α proteins in hypertrophied cells and increasing numbers of macrophages (**Figure 38**). The structural integrity of the adipocyte is in part likely due to collagen type I which decreases as adipocytes hypertrophy. Collagen type VI on the other hand increases with macrophage markers and a reduction of collagen type VI in macrophage-depleted oWAT suggests collagen VI deposition is, at least, in part controlled by macrophages. Furthermore, macrophages appear integral to WAT homeostasis as depleting macrophages in explants using Ouabain, resulted in improved adipogenesis, adipocyte specific insulin sensitivity and a reduction in Collagen VI. Summarised in (**Figure 34**).

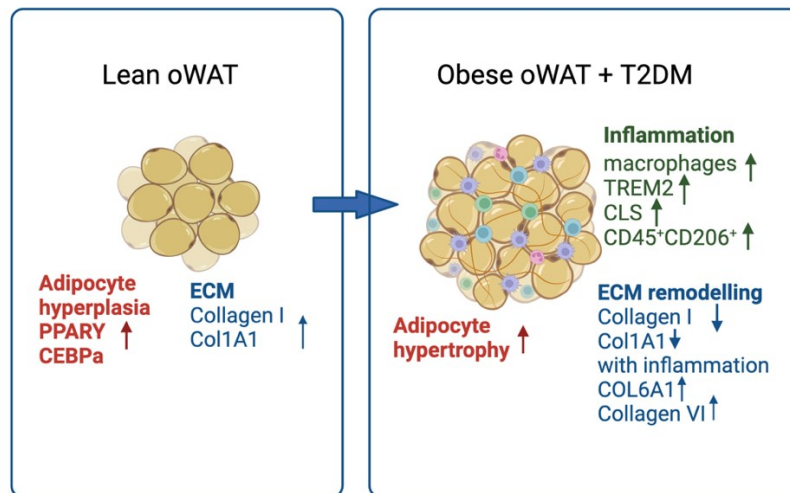


Figure 38 Changes seen in oWAT during obesity and progressive metabolic decline in the cohort of obese patients undergoing bariatric surgery and lean controls. Adipocyte hypertrophy, inflammation (increased numbers of macrophages, TREM2 expression, Crown-like-structures (CLS) and CD45⁺CD206⁺ cells), and ECM remodelling (decreased expression COL1A1 and collagen type I intensity, increased COL6A1 expression and collagen type VI intensity). oWAT: omental white adipose tissue; CLS: crown-like-structures; ECM: extracellular matrix; T2DM: type 2 diabetes mellitus. Image created by Bio Render®

Chapter 5: The effect of Metformin on white adipose tissue dysfunction.

5.1 Metformin

Metformin, a biguanide and an anti-glycaemic, is the first line treatment for patients with T2DM, it is also used as a treatment for polycystic ovarian syndrome and gestational diabetes. It is widely used due to its good safety profile and has relatively low side effects. It takes 24-48 hours to reach a steady state in the plasma and has a half-life of approximately six hours[445]. It is predominantly absorbed by the small intestines with relatively low bioavailability (40-60%) and higher concentration levels are observed in the portal vein and the liver compared to surrounding organs [446].

Metformin's mode of action is primarily to lower glucose levels and is known to be insulin sensitising. However, recent research has also suggested that it has pleotropic effects with weight loss and decreased appetite demonstrated in some studies. Further work has also shown Metformin can have beneficial effects on improving cardiovascular risk factors, lipid profiles, liver disease and even has a role in cancer prevention but these outcomes are disputed, with studies having conflicting results [447, 448].

While it is most widely used for systemic glucose control, there is no single molecular mechanism by which Metformin reduces circulating glucose instead it appears that it acts either through reducing glucose output by inhibiting hepatic gluconeogenesis, increasing glucose uptake in liver and skeletal muscles, sensitising GLP-1 signalling or reducing glucagon function [447].

Mitochondria are the primary organelle affected by Metformin and it was believed that Metformin acted principally on the liver through inhibiting the mitochondrial respiratory complex chain 1 leading to the activation of 5'AMP activated protein kinase (AMPK) as well as inhibiting glucagon-induced cAMP synthesis [447]. *In vitro* AMPK has been shown to improve insulin receptor function, improve glucose transport and reduce fatty acid synthesis through inhibiting ACC. While decreasing cAMP decreases protein kinase A (PKA) and thus reduces gluconeogenesis and increases glycolysis [446, 447, 449].

However, in recent years there has been controversy around the significance of AMPK-dependent function of Metformin due to the supraphysiological doses (millimolar) that have been used for the studies *in vitro* and *vivo* as opposed to micromolar concentrations observed in humans [450, 451]. Coupled with liver, intestinal and skeletal muscle specific AMPK knockout mice that still had lowered circulating glucose when treated with Metformin, [452] the AMPK-independent functions of Metformin have become increasingly apparent and

important. For example, Metformin has been shown to be insulin sensitising by increasing the activity of the insulin receptor and the insulin receptor substrate-2 which increases glucose uptake through GLUT-1, regulating insulin-mediated suppression of gluconeogenesis [447].

How Metformin modulates oWAT homeostasis during prologued obesity is relatively unknown. Studies on mice, zebra fish or using *in vitro* models with supratherapeutic concentrations have suggested Metformin is anti-inflammatory and reduces TNF- α and IL-6 levels [58, 446]. Adipogenesis has also been studied *in vitro* with high concentrations of Metformin suppressing adipogenesis [453-455]. Interestingly Chen et al. treated 3T3-L1 pre-adipocytes with varying dosing of Metformin (but still supratherapeutic) and demonstrated a dual effect with lower doses inducing adipogenesis and higher doses suppressing it [456]. Further studies are required at clinical doses to delineate Metformin's role in WAT function.

In my cohort of N=88 patients with obesity and N=9 lean patients, Metformin was prescribed and taken regularly by 74% of the obese patients with T2DM and 2% of the obese patients without T2DM. (**Table 1**). None of the lean patients were taking Metformin. Therefore, I wanted to establish whether Metformin was a confounding factor in the changes observed when phenotyping oWAT in obesity and T2DM in the previous chapter.

5.2 Aims of chapter

In order to address whether Metformin has a role in the oWAT phenotype I observed I hypothesised that Metformin could regulate adipogenesis, inflammation and ECM remodelling. To test this possibility, I used *ex vivo* cultured WAT and primary SVF-derived adipocytes from obese patients and cultured them in the presence and absence of Metformin.

5.3 Results

5.3.1 *Ex vivo* oWAT Explants and SVF-derived adipocyte culture patient characteristics

Omental WAT from seven obese patients undergoing bariatric surgery was used for *ex vivo* explant cultures as described in Chapter 3 (section 3.4.3). Six were non-diabetic and one patient had T2DM, with no one taking Metformin. They were predominantly female (5 female:2 male) aged 23-67 and had a mix of ethnicities with the aim to reflect the original cohort (Table 12). Omental WAT from one obese female patient undergoing bariatric surgery was also used for SVF-derived adipocyte culture as described in Chapter 3 (section 3.4.2). This patient was Asian, aged 42, non-diabetic and Metformin naïve (Table 12).

Table 12 Characteristics and metabolic profile of patients undergoing bariatric surgery who provided oWAT for N=7 oWAT explants and N=1 SVF-derived adipocyte cultures. T2DM defined as glycated haemoglobin ≥ 48 mmol/mol or clinical diagnosis documented in clinical notes. SVF: stromal vascular fraction; BMI: body mass index; T2DM: Type 2 Diabetes Mellitus.

	Ethnicity	Sex	Age	BMI (m ² /kg)	T2DM	Taking Metformin	HbA1C (mmol/l)
Explant 1	white British	F	67	51.4	Yes	No	49
Explant 2	white British	F	57	36.4	No	No	46
Explant 3	black Caribbean	F	54	40	No	No	48
Explant 4	black Caribbean	F	64	42	No	No	31
Explant 5	black British African	F	57	48	No	No	44
Explant 6	black Caribbean	M	59	48	No	No	41
Explant 7	other	M	23	43	No	No	39
SVF	Asian	F	42	46	No	No	40

5.3.2 *Ex vivo* oWAT Explants: viability after treating with Metformin

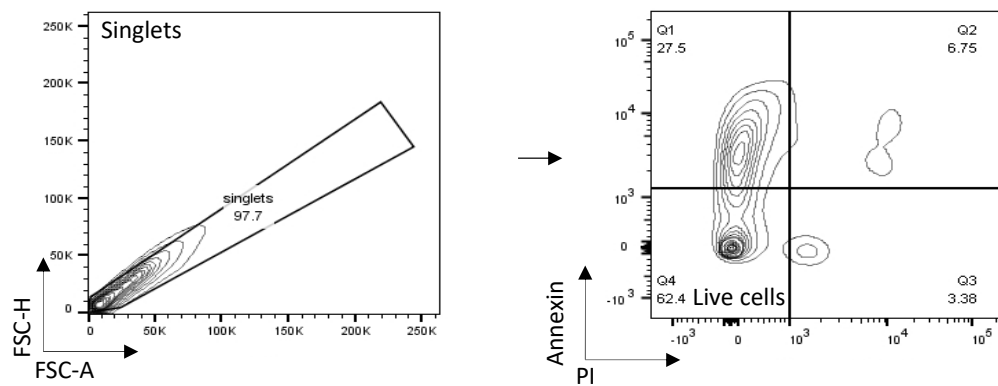
An *Ex vivo* explant model was first used to determine if Metformin regulates adipogenesis, inflammation and ECM remodelling of oWAT. The explant culture model is described in Chapter 3 (section 3.4.4) Briefly, three pieces of 150mg omental adipose tissue from each patient was dissected and cultured in the presence or absence of Metformin. Initially a range of concentrations of Metformin was used (100nM, 1uM, and 1mM) to establish if Metformin affected the viability of the explants.

Previous *in vitro* cultures had used concentrations as high as 1mM-10mM however human studies had shown that in patients taking Metformin at therapeutic dosing levels the plasma levels of Metformin are usually only 1- 20uM [457, 458]. Therefore, as organ uptake of C11 were measured and showed oWAT does not have high uptake of Metformin[458] the *in vivo* effect is likely to be more in the range of micromolar concentration in humans than

millimolar. To ensure that we used physiological doses the explants were culture in 100nM, 1uM and 1mM concentrations of Metformin.

To test viability of the explants, the SVF was isolated to enrich for the inflammatory cells and precursor cells vital for adipogenesis, inflammation and ECM remodelling. PI and annexin staining which identifies early apoptosis and necrosis clearly demonstrated that at the varying concentrations, the SVF cells were preserved (**Figure 39**). Metformin was therefore not found to be cytotoxic at these concentrations towards oWAT and all further experiments were conducted at 1µM Metformin due to it being estimated as the most physiological dose.

A



B

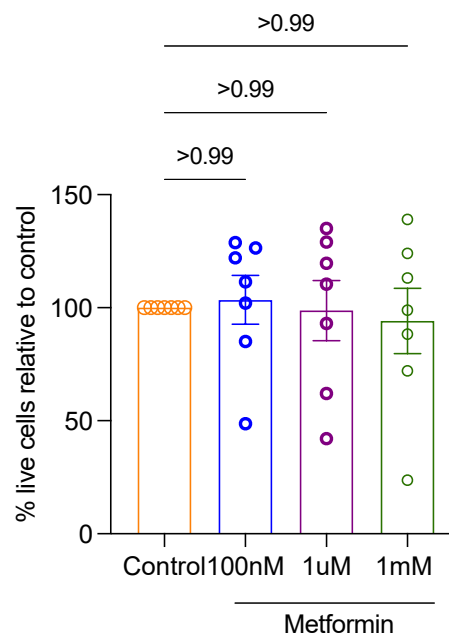


Figure 39 Metformin effect on SVF cell viability. A) Gating strategy for quantifying number of viable cells (PI/Annexin staining) in the SVF isolated from WAT explants (N=7) **B)** Percentage SVF cell viability following incubation for 48 hours in Metformin (100nM, 1µM and 1mM) compared to control (100%); One-way ANOVA with Dunnett's multiple comparison test was used for analysis, SVF: stromal vascular fraction; PI: Propidium iodide

5.3.3 *Ex vivo* oWAT Explants: Effect of Metformin on adipocyte differentiation

As previously explored, in prolonged obesity maladaptive WAT is characterised by hypertrophy of adipocytes while hyperplasia (i.e. adipocyte differentiation) is impaired. Therefore, key genes in adipogenesis pathway (**Chapter 1 Figure 4**) were measured in the explants cultured with Metformin for 48 hours. These included PPAR γ the master regulator of adipogenesis, essential for terminal differentiation as well as the gene involved in lipid metabolism and fatty acid transport ADIPOQ, which is a WAT-specific marker that is associated with mature adipocytes[459]. Both were measured alongside transcription factor FASN which has been shown to support PPAR γ and CEBP α in early differentiation [460]. CD36, a scavenger receptor that binds fatty acids and facilitates their transport into cells is important for adipose tissue energy storage and has also been shown to participate in adipocyte differentiation[461] and, therefore protein levels alongside PPAR γ and CEBP α were measured by Western blot.

Notably, 1 μ M Metformin-treated explants demonstrated upregulation of key adipocyte differentiation markers, *PPARG*, *FASN* and *ADIPOQ* expression levels (**Figure 40**). Furthermore, CD36 protein levels were consistently increased in 4 explants treated with (**Figure 40**) Metformin at 1 μ M.

Taken together, both expression of transcript markers of adipocyte differentiation and protein levels of marker CD36 were increased, this suggests that Metformin does indeed modulate adipogenesis positively. PPAR γ and CEBP α protein levels were also measured using Western blot however significant changes at 48 hours were not observed, which I hypothesise is likely due to the length of exposure to the drug. A prolonged culture of the explants with Metformin may have led to increased protein levels.

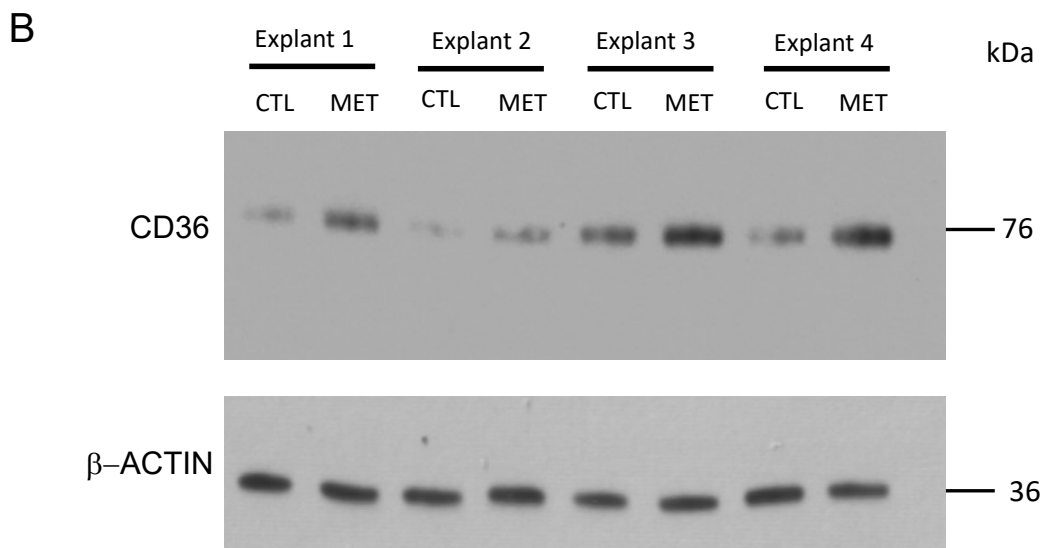
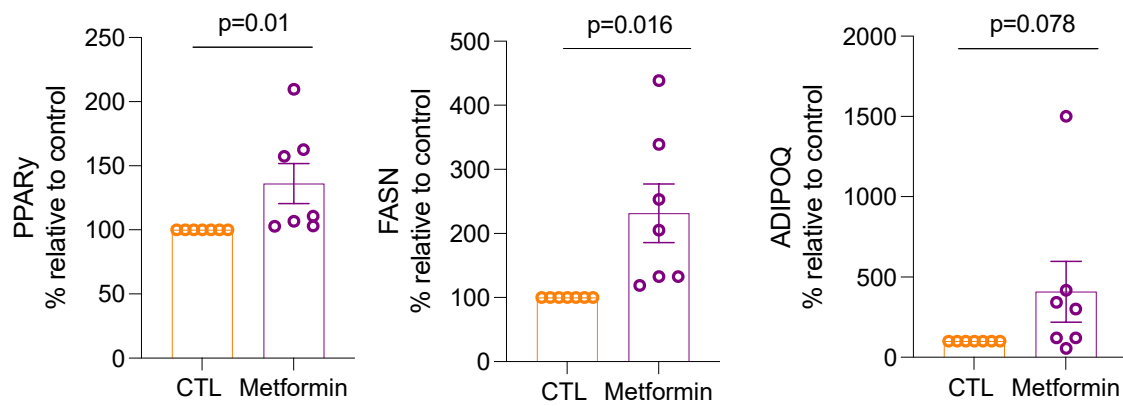


Figure 40 Metformin upregulates adipogenesis in WAT explants ex vivo (A) PPAR γ and FASN and ADIPOQ mRNA expression in oWAT explants treated with Metformin (1 μ M) for 48 hours (n=7 patients); **(B)** Protein expression of CD36 in oWAT explant (N=4) stimulated with Metformin (1 μ M) for 48 hours. Data are represented as mean $\pm$ S.E.M. Wilcoxon test were used; Ctrl, control.

5.3.4 *Ex vivo* oWAT Explants: BNP2 and PLN2 mediated PPAR γ activation by Metformin

A recent study isolated lung fibroblasts from patients with idiopathic pulmonary fibrosis and demonstrated that when treated with Metformin, there was an induction of lipogenic differentiation and activation of PPAR γ signalling through transcription factor BMP2; bone morphogenetic protein 2 [462] and PLN2; a lipogenic marker, adipose differentiation-related protein-2. Therefore, as this was in keeping with our findings that Metformin boosts adipogenesis, I measured BMP2 and PLN2 expression in the *ex vivo* explants to establish if a similar pathway was activated in the oWAT. There was no significant increase in transcripts at 48 hours, but an overall upward trend (**Figure 41**). Further work is needed to identify if this pathway or alternative pathways are modulated by Metformin as an adipogenic agent.

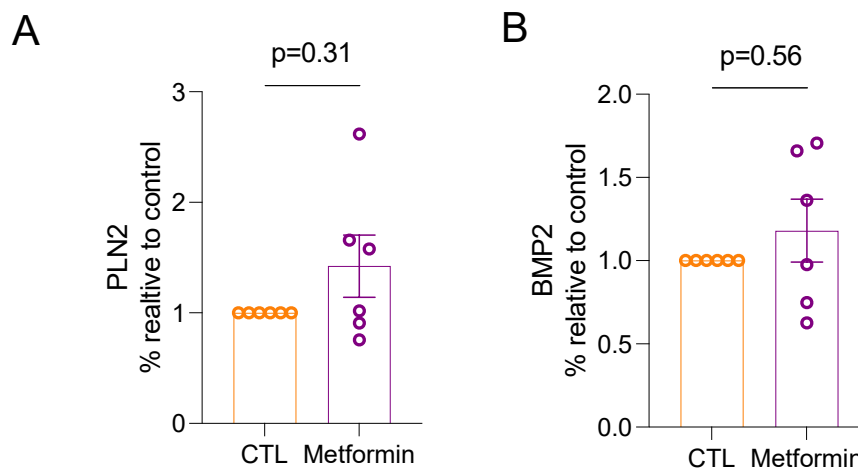


Figure 41 Adipogenesis Pathways activated by Metformin. (A) PLN2 mRNA expression in oWAT explants treated with Metformin (1 μ M) for 48 hours (N=6); (B) BMP2 mRNA expression in oWAT explants stimulated with Metformin (1 μ M) for 48 hours (N=6). Data are represented as mean $\pm$ S.E.M. Wilcoxon test were used; Ctrl: control; PLN2: perilipin 2; BMP2: bone morphogenetic protein 2.

5.3.5 Ex vivo oWAT Explants: Effect of Metformin on WAT Inflammation

Previous studies using mouse models suggest that Metformin improves inflammation through macrophage polarization away from a classic inflammatory M1 phenotype to an anti-inflammatory M2 phenotype with pro-inflammatory markers TNF alpha and IL-6 decreasing systemically [463].

As macrophages are the predominant immune cell implicated in maladaptive WAT in obesity, I first assessed whether Metformin influences the proportion of WAT macrophage using flow cytometry. The SVF was isolated and stained with CD14⁺CD45⁺CD206⁺ antibodies. The percentage of SVF-derived CD14⁺ or CD206⁺ cells did not differ between control and Metformin-treated samples (**Figure 42**).

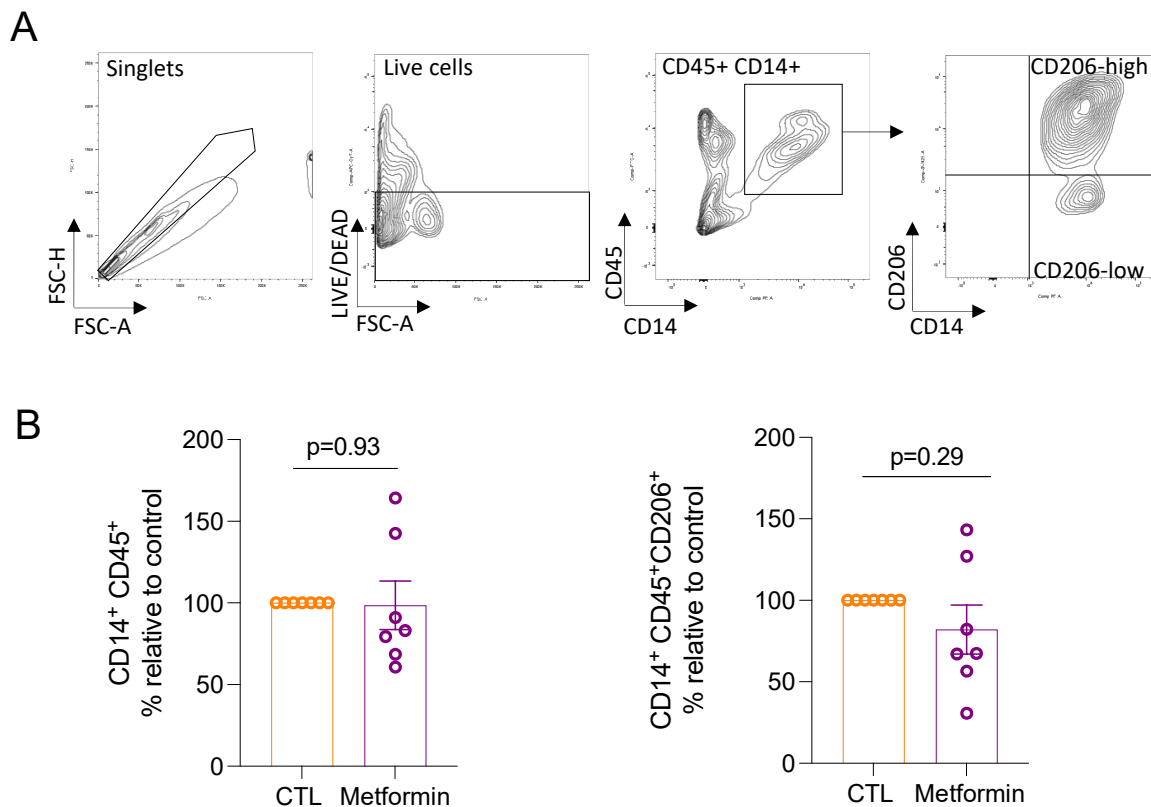


Figure 42 Inflammatory markers are not altered when oWAT explants are treated with Metformin 1 μ M.(A) FACS Gating strategy for monocyte/macrophage markers in SVF isolated from WAT; from left to right, the panels indicate (i) singlets, (ii) live cells, (iii) CD14⁺CD45⁺ cells, (iv) CD206⁺ cells. The arrows indicate the direction of gating. (B) Right panel: FACS analysis of CD14⁺CD45⁺ cells isolated from the oWAT of bariatric patients treated with Metformin 1 μ M for 48 hours. Left panel: FACS analysis of CD14⁺CD45⁺CD206⁺ cells isolated from the oWAT of bariatric patients treated with Metformin 1 μ M for 48 hours (N=7) Data are represented as mean $\pm$ S.E.M. and p<0.05 was assumed to be statistically significant when analysed with Wilcoxon test.

Following this I aimed to measure the expression of key inflammatory genes in the whole oWAT. mRNA of the oWAT explants showed no difference in CD68 transcripts and both pro-inflammatory (*TNF*, *IL6*, *IL1B*) and anti-inflammatory markers (*IL10*) were not significantly

altered between the control and the Metformin-treated explants after 48 hours of culture (**Figure 43**).

Taken together, flow cytometry antibody staining, and mRNA transcripts closely associated with inflammation, did not show a significant modulation in innate immunity by Metformin.

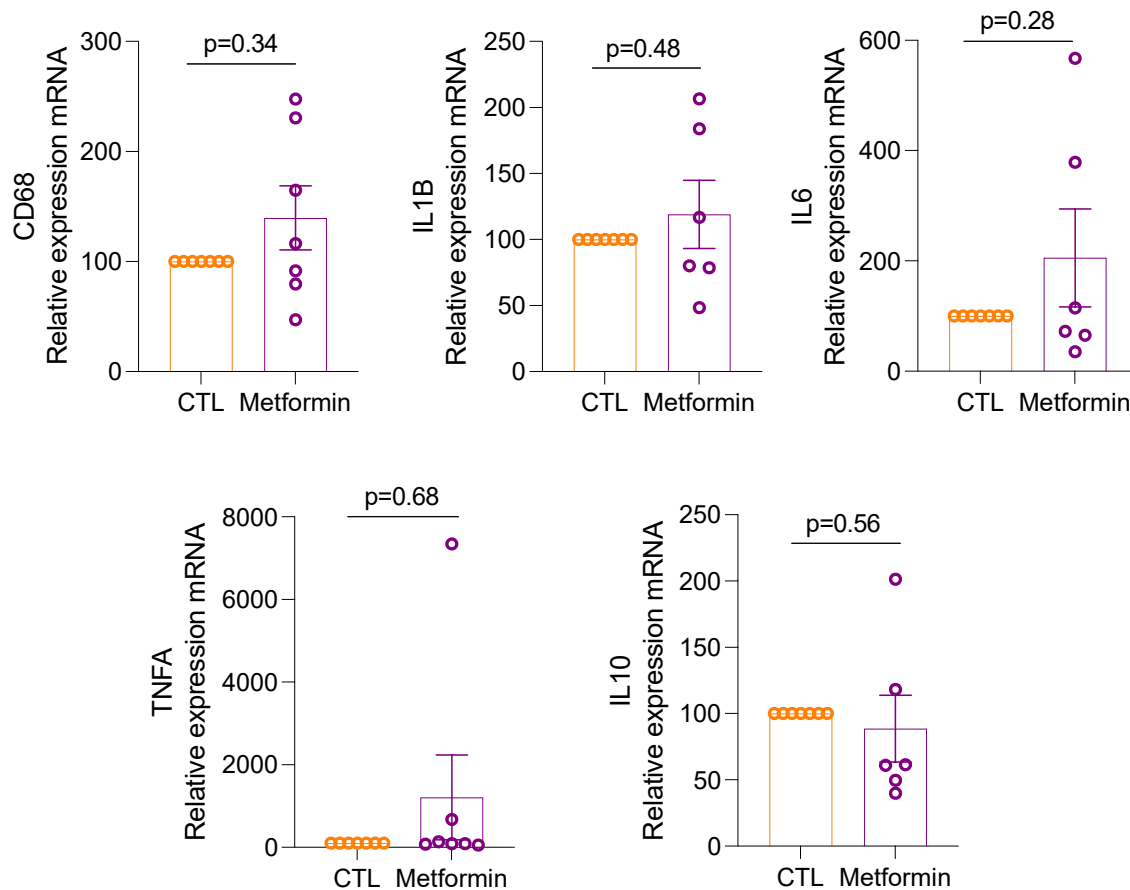


Figure 43 Explants treated with Metformin and its effect on inflammation mRNA expression of inflammatory pathway genes CD68, TNF, IL6, IL1B, IL10 (n=6) in oWAT explants treated with 1 μ M Metformin for 48 hours, relative expression compared to control. Data are represented as mean $\pm$ S.E.M. and $p < 0.05$ was assumed to be statistically significant when analysed with one sample t-test. Housekeeping gene: HPRT.

5.3.6 Ex vivo oWAT Explants: Effect of Metformin on ECM remodelling

Extracellular matrix remodelling has been recognised as a hallmark of white adipose tissue dysregulation and in the previous chapter I showed that collagen type I and VI decreased as adipocyte size increased with obesity. Studies suggest that Metformin is able to abolish TGF- β driven fibrogenesis in mouse models [464, 465]. Therefore, I measured the expression of genes closely associated with ECM remodelling pathways including *ACTA*, *COL1A1*, *COL6A1* and *TGFB* which were found to be similar between Metformin-treated explants and controls (**Figure 44**).

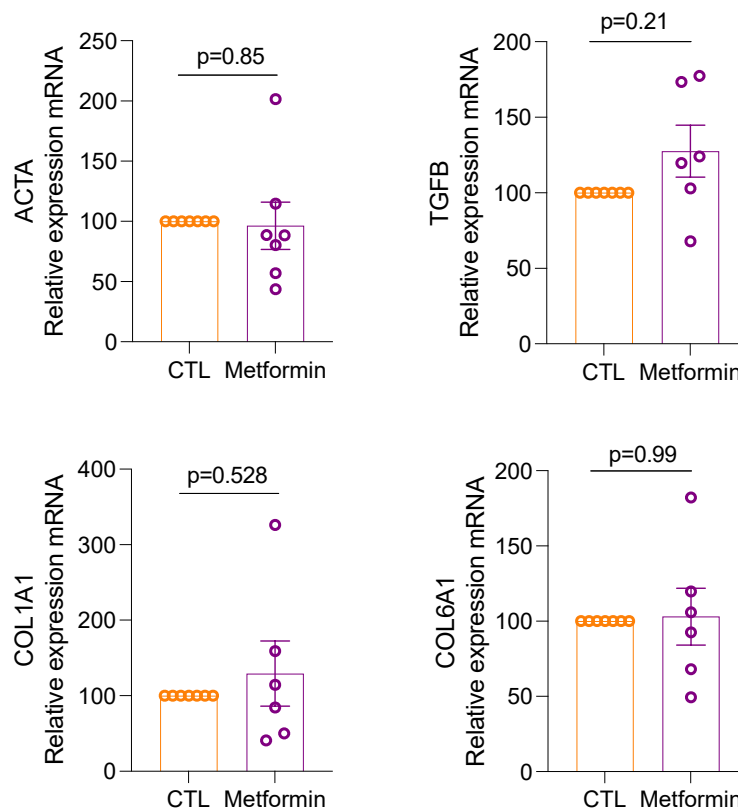


Figure 44 **Explants treated with Metformin and its effect on collagen deposition.** mRNA expression of ECM related genes *ACTA*, *COL1A1*, *COL6A1* and *TGFB* (n=6) in oWAT explants treated with 1 μ M Metformin for 48 hours, relative expression compared to control. Data are represented as mean $\pm$ S.E.M. and $p < 0.05$ was assumed to be statistically significant when analysed with one sample t test. Housekeeping gene: HPRT.

5.3.7 Conclusion

Taken together, these results suggest that clinically relevant doses of Metformin (1 μ M) regulate adipogenesis without having a significant effect on the inflammatory/fibrotic component of WAT pathophysiology during obesity (summarised in **Figure 45**).

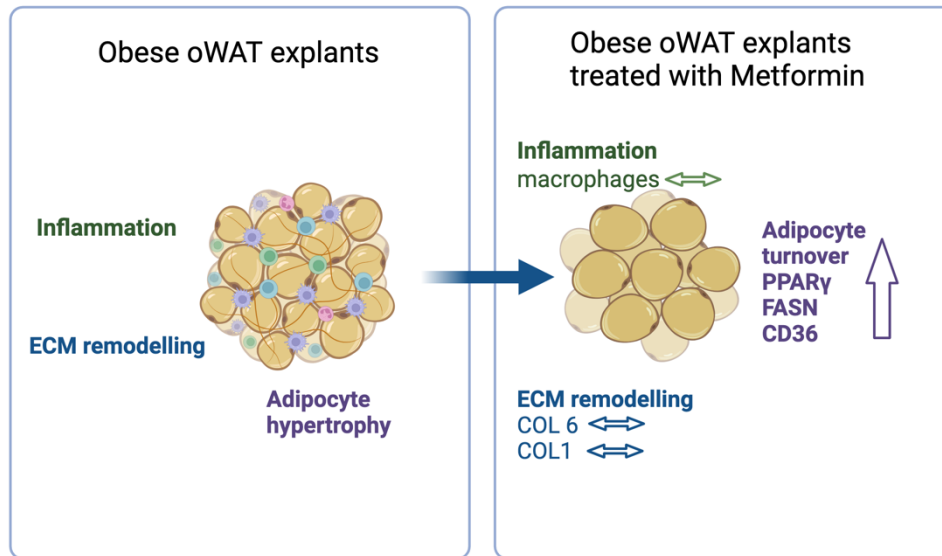


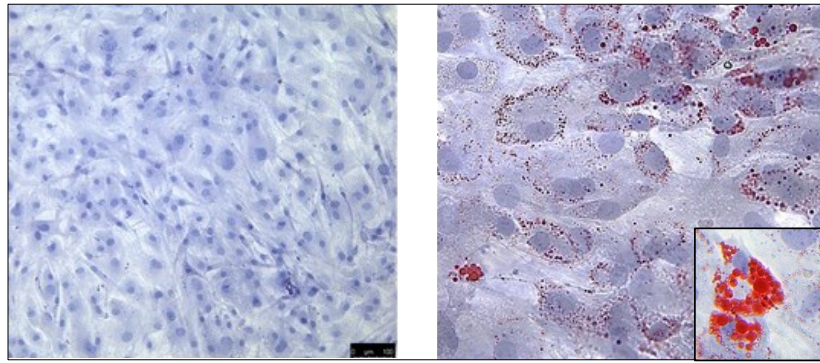
Figure 45 Changes seen in oWAT explants treated with Metformin 1 μ M. Adipogenesis transcription factors PPAR γ , FASN and CD36 increase with Metformin treatment. Inflammation and ECM remodelling are not modulated. oWAT: omental white adipose tissue; ECM: extracellular matrix; PPAR γ : Peroxisome proliferator-activated receptor-gamma, FASN: fatty acid synthase. Image created by Bio Render®

5.3.8 SVF-derived adipocyte culture model: viability after treating with Metformin

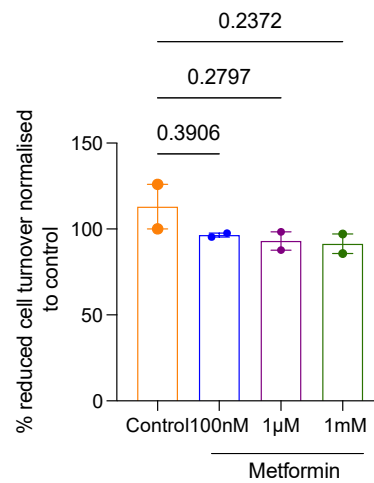
The *ex vivo* explants suggested that adipogenesis was modulated by 1 μ M Metformin and therefore I next aimed to validate these results using SVF-derived mature adipocytes isolated from the oWAT of the bariatric cohort patients as described in **methods 3.4.2 (Figure 46)**. Most of the literature studying the effects of Metformin on adipose tissue is performed using the mouse cell line 3T3-L1 and while an easy tool to study adipocyte differentiation and development they are unlikely to recapitulate the obese human adipocyte cell cycle and pathways accurately. Therefore, by using the SVF isolated from our bariatric cohort the aim was to use a more physiological set-up to address whether Metformin is adipogenic.

Firstly, the SVF was isolated from a patient undergoing surgery and cultured until confluent. Using adipocyte differentiation media I differentiated the SVF-derived precursor cells to mature adipocytes (**Chapter 3 Section 3.4.2**). Using Oil Red O staining I validated that the primary cells were able to differentiate into mature adipocytes (**Figure 46**). The representative images demonstrate the formation of lipid droplets as adipocytes mature over 7 days. At day 7 I measured the mRNA transcripts of transcription markers associated with adipocyte differentiation; there was a clear significant upregulation of FABP4, PPAR γ and ADIPOQ transcripts indicating that the adipocytes had differentiated to mature adipocytes (**Figure 46**).

A



B



C

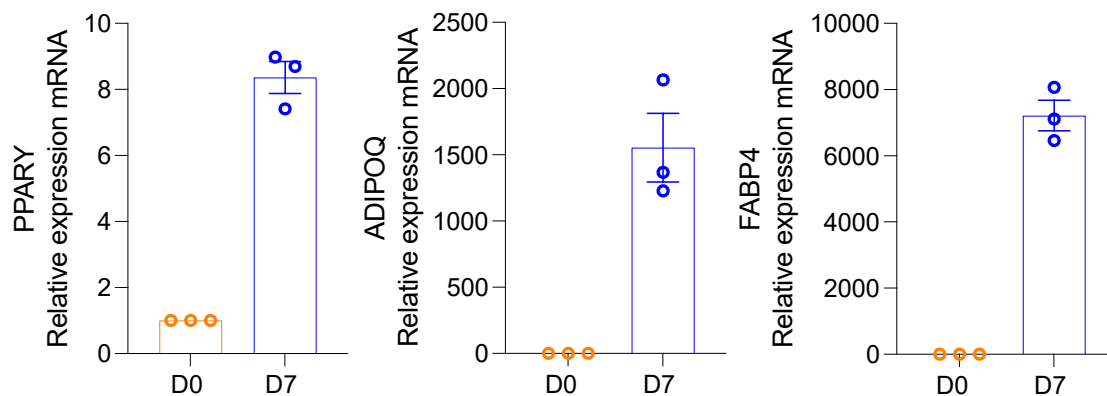


Figure 46 SVF-derived preadipocytes cultured in differentiation media for 7days. (A) Oil Red O staining in SVF-derived pre-adipocytes (left panel) and differentiated adipocytes (right panel) at 20x and inset 40x magnification. (B) Viability of SVF-derived adipocytes after differentiation using AlamarBlue following 24 hours incubation with Metformin (100nM, 1μM, 1mM). (C) mRNA expression of adipogenesis markers (ADIPOQ, FABP4, PPAR γ) at Day 0 for SVF-derived pre-adipocytes and differentiated adipocytes at day 7. relative expression to housekeeping gene HPRT. D0: day 0 D7: day 7 Data are represented as mean $\pm$ S.E.M. and $p < 0.05$ was assumed to be statistically significant when analysed with One-way ANOVA with Dunnett's multiple comparison test (B) or paired t-test (C). SVF: stromal vascular fraction.

5.3.9 SVF-derived adipocyte culture model: Metformin affects adipogenesis

The SVF-derived adipocytes were incubated for 24 hours with 1 μ M Metformin after 6 days of differentiation. Initially mRNA expression levels of *FABP4*, *ADIPOQ* and *LEP*, and PPAR γ adipogenic transcription factors that were also measured in the oWAT explants, were measured and were shown to be upregulated confirming the pro-adipogenic effect of Metformin (**Figure 47**).

PPAR γ protein levels measured by Western Blot were not significantly different between the SVF-derived adipocytes cultured in the presence or absence of Metformin for 24 hours after 6 days of differentiation. However, after a prolonged culture of 7 days with or without Metformin there was increased PPAR γ protein levels in adipocytes cultures with Metformin (**Figure 47**).

Interestingly, CD36, the scavenger receptor that was shown to be significantly increased in the explants was not significantly upregulated in the primary cell culture model. I hypothesis that the upregulation seen in the oWAT explants maybe the result of cell-to-cell interactions in the presence of Metformin, specifically the macrophages and adipocytes in the oWAT and therefore I was not able to observe the crosstalk between the macrophages and adipocytes in the SVF-derived adipocyte culture.

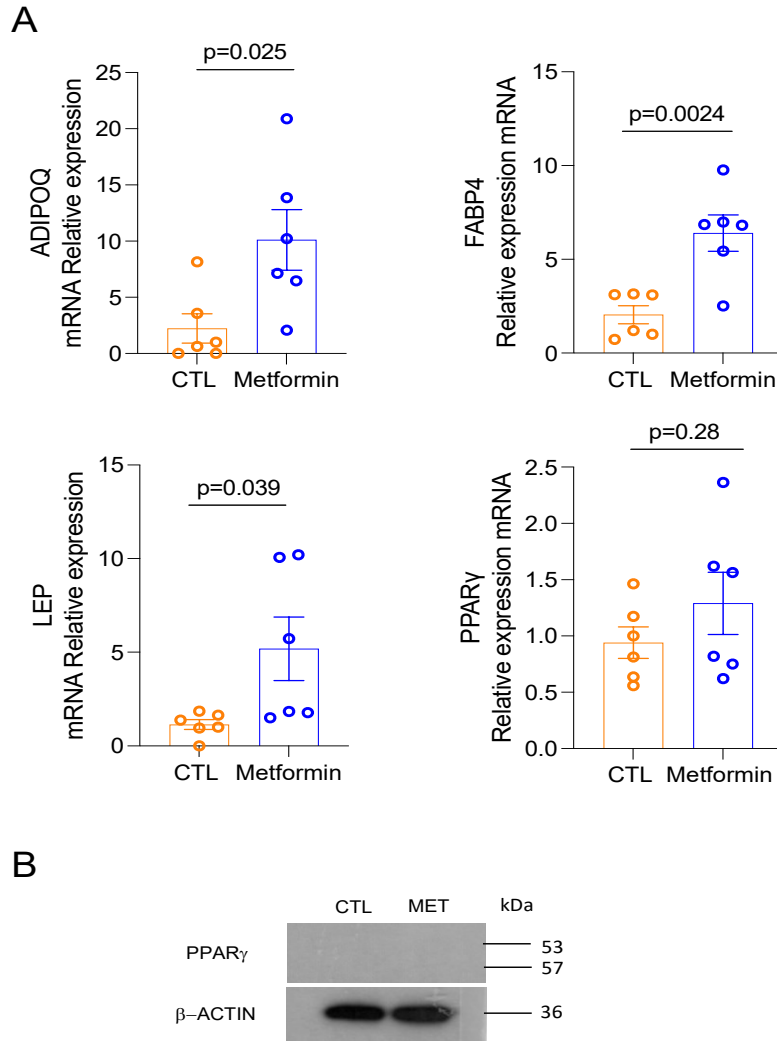


Figure 47 Metformin upregulates adipogenesis in SVF-derived adipocytes (A) ADIPOQ, FABP4, LEP and PPAR γ mRNA expression in SVF-derived adipocytes treated with Metformin (1 μ M) for 24 hours (N=2 donors) **(B)** Protein expression of PPAR γ in SVF-derived adipocytes treated with Metformin (1 μ M) for 24 hours. Data are represented as mean $\pm$ S.E.M. Wilcoxon test (A) were used; Ctrl, control; ADIPOQ: adiponectin; FABP4: fatty acid binding protein 4; LEP: leptin; PPAR γ : peroxisome proliferator activated receptor gamma.

5.3.10 Conclusion

In SVF-derived adipocyte culture model, Metformin was shown to be adipogenic both by significantly increasing the transcription factors FABP4, ADIPOQ and LEP and by increasing PPAR γ protein levels. This was in keeping with the *ex vivo* explant model that also demonstrated adipogenic boosting by Metformin at a transcript and protein level using 1 μ M Metformin. However, inflammation and ECM remodelling did not appear to be significantly altered when oWAT explants were treated with Metformin. The pathway which Metformin targets adipogenesis is yet to be fully established.

5.4 Discussion

The previous chapter investigated the interplay between adipogenesis, inflammation and extracellular matrix composition in oWAT with prologued obesity by comparing a lean and morbidly obese cohort with and without T2DM. As over seventy percent of my cohort were treated with Metformin, it was thus important I dissected the role of Metformin in oWAT homeostasis from the effects of prologued obesity and T2DM.

Therefore, I hypothesised that Metformin would modulate the three key elements of oWAT function and to investigate this further I used two experimental models. The first was to use oWAT *ex vivo* explants from the cohort of extremely obese patients not taking Metformin and treat with varying doses of Metformin for 48 hours. This enabled me to reproduce *in vivo* WAT function as closely as possible. I then measured key markers of adipogenesis, inflammation and ECM modification. I also used SVF-derived adipocytes isolated from extremely obese patients not taking Metformin and treated the cultures for 24 hours with 1 μ M Metformin to identify if the changes observed were adipocyte cell-specific.

5.4.1 Metformin increases adipogenesis in morbidly obese patients' oWAT

I analysed the effects of Metformin in human WAT explants and SVF-derived adipocytes to conclude that Metformin is not cytotoxic at both micro and millimolar concentrations. When used at 1 μ M, Metformin promotes adipogenesis during the treatment period (48h for explants and 24h for SVF-derived adipocytes). Although previous studies have suggested that Metformin downregulates transcription factors involved in adipogenesis and prevents lipid accumulation, these experiments were either performed in cell lines (mouse fibroblast cell lines, 3T3-L1 cell lines) or in presence of high concentration of Metformin (1mM-10mM) [453, 454, 466-468]. The concentration of Metformin used in these *in vitro* studies has in recent years been seen as controversial; they are often 100 times higher than the therapeutic concentrations measured in humans. As previously discussed, it has been shown that serum levels of Metformin peak at 20 μ M (range 1-8 μ M) when patients were treated with Metformin [457] and since WAT does not have high uptake after prolonged exposure to ¹¹C-Metformin [458], the *in vivo* effect of Metformin is likely to be in micromolar range in humans. Supra-pharmacological doses may alter the mechanism of action of the drug and be responsible for off-target and/or toxic effects [451]. In support of this, Chen et al. showed that Metformin has a biphasic response. When used at a concentration of 1.25mM, adipogenesis is enhanced but higher doses (5-10mM) supresses adipogenesis [456].

In addition, previous studies suggest the origin of the WAT can also play a role in its response to Metformin treatment, but this data is often conflicting. Epicardial adipose tissue treated with Metformin improved adipogenesis markers PPAR γ and adiponectin in keeping

with my results [455]. Adiponectin on the other hand was not altered in visceral WAT after patients were treated with oral Metformin for 3 months [469, 470]. Adipogenesis in subcutaneous WAT appears to be downregulated in human subcutaneous explants [469] and adiponectin either increased or did not significantly alter in other studies investigating subcutaneous WAT [471]. Further robust studies comparing adipose tissue depots would be useful to determine if there truly is a depot-specific effect exerted by Metformin.

The pathway through which Metformin boosts adipogenesis remains to be identified. In keeping with my findings, a recent study isolated lung fibroblasts from patients with idiopathic pulmonary fibrosis and demonstrated that when treated with Metformin, there was an induction of lipogenic differentiation and activation of PPAR γ signalling through BMP2 [462]. Therefore, I have measured expression of BMP2 and PLIN2 genes using RT-qPCR in Metformin treated explants and SVF-derived adipocytes. These experiments did not show a significant change but a general upward trend in BMP2 transcripts which could warrant further investigation.

5.4.2 Metformin does not alter other markers of oWAT dysfunction in human explants

I demonstrated that while adipocyte differentiation is modulated by Metformin, it does not influence inflammation. The SVF with enriched immune cells were isolated from the explants treated with Metformin. CD206⁺ macrophage numbers are increased in adipose tissue following prolonged obesity; however they did not alter with Metformin treatment.

Transcription factors for inflammation (M1 markers including IL-1b, IL-6 and TNF and pan macrophage marker CD68) and anti-inflammatory markers (IL-10) were measured and were not significantly different between explants treated with or without Metformin.

Jing et al. demonstrated TNF and IL-6 were downregulated in adipose tissue in obese mice, in contrast to my data [472] and the majority of further studies that have also investigated whether Metformin modulates inflammation were in *in vitro* cell lines such as 3T3-L1, macrophage/monocyte cell lines, primary cells or *in vivo* models using mice fed HFD [463, 473-479]. These *in vitro* cell lines or primary cells, while suggesting an anti-inflammatory effect [473, 478, 479] of Metformin do not fully recapitulate the inflammatory pathways of obese patients' oWAT. Very few *in vivo* models specifically investigated how Metformin modulates inflammation in adipose tissue. Instead these mouse models focused on the systemic effects or hepatic inflammation [474, 477]. Furthermore, these studies have again mainly used supratherapeutic doses in their experimental set-up so the relevance of using these results for analysing the effect of Metformin on human adipose tissue is difficult to interpret and suggest further *in vivo* data is needed.

Extracellular matrix deposition in Metformin treated explants did not significantly alter at a transcript level in my experiments. However, in the literature there is very minimal data available in adipose tissue, Luo et al. used *ob/ob* obese mouse model to show Metformin reduces excessive collagen deposition through AMPK suppression of TGF β in WAT [465] and a study in older patients treated with Metformin for 6 weeks suggested reduced TGF β in adipose tissue [480]. Lung fibrosis has also been shown to improve as Metformin attenuates TGF β in several myofibroblast models which again contrasts with my findings [481]. Taken together this could suggest a tissue or age specific effect of this drug on extra cellular matrix composition and warrants further investigation.

5.4.3 Limitations

While explants from *ex vivo* omental WAT is a good model for recapitulating oWAT function, the experiments achievable from each donor was limited by volume of tissue available at surgery and therefore I prioritised tissue for protein analysis, FACs and RNA extraction. Heterogeneity between donors is also an important confounding factor and thus by using 7 different donors I aimed to reduce this effect.

Obesity has been shown to slow differentiation and alter the pluripotency of SVF-derived adipocyte cultures [475]. Therefore, a challenge to these experiments was expanding the SVF-derived adipocytes at a large scale and at early passages for several different modes of analysis to be possible.

5.4.4 Future studies

Although I have established an adipogenic role of Metformin in T2DM patients' WAT, the cellular mechanisms of action by Metformin remain to be identified and an area of investigation I would like to take further.

It has been shown that Metformin's mechanism of action for glucose lowering and insulin sensitisation is at least in part AMPK-dependent through mitochondrial activation of AMPK which in turn inhibits hepatic gluconeogenesis. A previous study by Habinowski et al. has also suggested that Metformin blocks adipogenesis through AMPK inhibition in contrast to my results [454]. Therefore, it would be interesting to perform gain and loss of function of AMPK signaling in SVF-derived adipocytes using an AMPK inhibitor and transfected AMPK siRNA to establish if Metformin-driven adipogenesis is independent of AMPK signaling.

In recent years it has been elegantly shown that Metformin has pleiotropic effects in multiple tissues through several AMPK-independent pathways. I showed an upwards trend of BMP2 signaling pathway and increased PPAR γ in keeping with Kheirollahi et al. [462]. I would like to investigate their role in Metformin induced adipogenesis in WAT by stimulating with

rBMP2 or knockdown of PPAR γ in SVF-derived adipocytes to further evaluate these signaling pathways.

In chapter 7 I also address whether Metformin modulates oxylipin pathways that are altered in obesity mediated T2DM/MASLD. It is characterised that some oxylipins are PPAR γ ligands, and PPAR γ was also shown to augment soluble epoxide hydrolase activity [384]. Therefore, if there is an increase in PPAR γ with Metformin as described in this chapter, one would expect that Metformin could be having an indirect effect on oxylipin pathways. However, the results I present later (**Chapter 7, Figure 71**) show that Metformin does not change the CYP-derived oxylipins measured in oWAT explants treated with Metformin. This incongruity may be due to a species or tissue-specific effect as most data on PPAR γ -oxylipin interactions is from animal models or cell lines. Furthermore, the PPAR γ -oxylipin interaction is likely metabolite specific and I have focused on CYP-derived metabolites, therefore further work dissecting the effects of Metformin on the COX and LOX pathway would be of interest.

5.4.5 Conclusions

In summary, Metformin has been shown to upregulate adipogenesis in both explants and SVF-derived adipocyte culture, while having very little effect on inflammation or extra cellular matrix composition. This in part could explain the results described in the previous chapter which demonstrated no correlation with HbA1c and increasing adipocyte size when comparing oWAT from lean patients and oWAT from obese patients with and without T2DM. As most patients with T2DM were taking Metformin this may have enabled the oWAT to undergo hyperplasia, reducing the need for hypertrophic adipocytes, a mechanism which is considered beneficial to oWAT homeostasis and thus healthier oWAT. While Metformin has not mitigated oWAT dysfunction completely, it may have prevented a more extreme phenotype being observed.

These results focusing on the adipogenic role of Metformin emphasises the importance of WAT adipogenesis, inflammation and fibrosis when evaluating the effect of drugs already being use or repurposed. It also highlights an important question for future drug design or repurposing; is it more beneficial to have a drug that is stimulating adipogenesis to prevent adipocyte hypertrophy or a drug that dampens inflammation (e.g. targeting WAT macrophage viability) in the metabolically unhealthy WAT? One benefit of targeting adipogenesis is that the drug is likely to be specific to adipocytes, while finding a drug that targets only WAT macrophages without also damaging the hosts' global innate immune response is far more challenging and, to date, has not been achieved.

Chapter 6: Liver and white adipose tissue crosstalk in MASLD

6.1 Introduction

Through the last two chapters I have explored the changes obesity has on oWAT homeostasis focusing on the key hallmarks of obesity-associated oWAT dysfunction (adipogenesis versus adipocyte hypertrophy, inflammation and ECM remodelling) and the association with endocrine dysfunction and ultimately T2DM.

Prolonged overnutrition and obesity has been closely associated with detrimental effects on the liver which is recognised clinically as metabolic-associated steatotic liver disease. The spectrum of disease begins with lipid deposition within the liver, namely steatosis, and can progress to inflammation and fibrosis (MASH) and ultimately end-organ failure with cirrhosis and hepatocellular carcinoma.

It is widely believed that MASLD develops following prologued obesity leading to a ‘spill over’ of free fatty acids from the adipose tissue resulting in hepatic lipid accumulation [103]. There is an abundance of literature that shows a clear association with increasing visceral adipose tissue volume and progression of MASLD and it is well known that T2DM and MASLD are closely linked with faster progression of MASLD to MASH in patients with T2DM [482-485]. Furthermore, several studies show white adipose tissue macrophages increase as histopathological progression of liver disease occurs [284, 285, 287, 415].

Therefore, I hypothesised that the crosstalk between the oWAT and liver is important during disease progression following obesity and as oWAT becomes dysfunctional this leads to progression of hepatic inflammation and fibrosis. It is well known only 30% of people with hepatic steatosis develop inflammation and only 25% of those progress to fibrotic disease and cirrhosis but what mechanisms result in hepatic steatosis progressing to MASH and cirrhosis is not fully understood and therefore by understanding the crosstalk between WAT and liver disease this may identify key factors that result in increasing MASLD severity.

6.2 Aims of chapter

The aim of this chapter is to establish if WAT dysfunction correlates with progression of liver disease during prolonged obesity thus suggesting WAT homeostasis markers are up-stream regulators of MASLD progression.

In order to address this, my aims are:

1. Determine the extent of liver disease in the sub-cohort of bariatric patients using the semi-quantitative NAFLD activity score scored by an expert Histopathologist in MASLD as described in **methods section 3.6.3**.
2. Quantify the extent of inflammation, ballooning, fat and fibrosis in each liver biopsy for each bariatric patient recruited using a recently validated AI software as described in **methods section 3.6.3**.
3. Correlate the extent of WAT dysfunction phenotypes (adipocyte size, inflammation, ECM remodelling, adipokine expression) with progression of liver disease (NAS score and percentage inflammation, fat, ballooning and fibrosis on biopsy)

6.3 Results

6.4 Clinical characteristics and metabolic profile of the bariatric patient cohort

A sub-selection of N=41 patients recruited to the study had paired blood, oWAT and liver samples collected.

Patients with obesity were grouped by stage of liver disease as determined by the NAS scoring system into normal or non-MASLD (N=24), MASLD (N=14) and MASH (N=3). The clinical profile of this sub-cohort is summarised in **Table 13**. The average age ($p=0.33$) and sex ($p=0.54$) of patients were similar between the three groups and there was a similar spread of ethnicity ($p=0.09$). BMI was also similar between the three groups ($p=0.96$).

Five patients with normal liver biopsies (20%) were classified as T2DM but well controlled (HbA1c: 40.33 ± 6.1), five patients with MASLD (35.7%) were T2DM (HbA1c 45.08 ± 13.1) and two patients with MASH (66.7%) were classed as T2DM (HbA1c 57.33 ± 24.21) (as stated in clinical records or glycated haemoglobin ≥ 48 mmol/mol) (**Table 13**). This is in keeping with the literature that T2DM and MASLD are inextricably linked and patients with more advanced MASLD are at increased risk of T2DM [486]. There was no significant difference in other co-morbidities associated with metabolic syndrome (hypertension, dyslipidaemia) between the three groups in this cohort with obesity.

Patients with T2DM were taking glucose lowering drugs including Metformin (12.5% versus 29% versus 33%; non-MASLD versus MASLD versus MASH $p=0.33$), Gliclazide (0% versus 7% versus 33%; $p=0.04$), Empagliflozin (0% versus 7% versus 33%; $p=0.04$) and Liraglutide (0% versus 7% versus 0%; $p=0.038$) (**Table 13**). Patients taking insulin were excluded from the study. There was no significant difference in lipid profile (cholesterol and triglyceride levels) between the groups.

Table 13 Clinical characteristics and metabolic profiling of non-MASLD, MASLD and MASH among patients with obesity. Statistical significance calculated using Kruskal-Wallis test or Fisher's exact test. Non-MASLD: non-Metabolic dysfunction-associated steatotic liver disease; MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis; BMI: body mass index; HbA1C: glycated haemoglobin; ACE-i: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker.

	Non-MASLD <i>n</i> =24	MASLD <i>n</i> =14	MASH <i>n</i> =3	<i>p</i> value
age	49.7 ± 13.5	45.08 ± 13.1	56.33 ± 7.5	0.33
sex (F)	19	10	3	0.54
Ethnicity				0.09
asian/british asian	2	0	1	
black/ black caribbean	6	1	0	
white	14	8	1	
other	0	4	1	
mixed	2	1	0	
BMI (m ² /kg)	45.89 ± 6.7	46.68 ± 11.7	46.68 ± 11.7	0.96
HbA1C (mmol/mol)	40.33 ± 6.1	42.36 ± 12.11	57.33 ± 24.21	0.03
Cholesterol (mg/dL)	4.5 ± 1.3	4.8 ± 0.99	4.57 ± 1.09	0.45
Triglyceride (mg/dL)	1.2 ± 0.68	1.8 ± 2.4	2.07 ± 1.77	0.29
Drugs				
Metformin	3 (12.5%)	4 (29%)	1 (33%)	0.36
Gliclazide	0 (0%)	1 (7%)	1 (33%)	0.04
Empagliflozin	0 (0%)	1 (7%)	1 (33%)	0.04
Sitagliptin	0 (0%)	0 (0%)	0 (0%)	
Liraglutide	0 (0%)	1 (7%)	0 (0%)	0.38
Statin	5 (36%)	3 (21.4%)	1 (33%)	0.88
ACE-i/ ARB	11 (46%)	4 (28%)	2 (66%)	0.38
Aspirin	2 (8%)	1 (7%)	0 (0%)	0.87
Co-morbidities				
Hypertension	11 (46%)	7 (50%)	2 (66%)	0.78
Dyslipidaemia	3 (12.5%)	3 (21.4%)	1 (33%)	0.57
Ischaemic heart disease	1 (4.2%)	0 (0%)	0 (0%)	0.69
MASLD/MASH	0 (0%)	14 (100%)	3 (100%)	<0.0001

6.4.1 oWAT adipogenesis and MASLD progression

Adipocyte hypertrophy is a feature seen in progressive WAT dysfunction and prolonged obesity. The cohort of patients recruited for this study comparing lean and morbidly obese omental adipose tissue discussed in chapter 4 corroborated this with increased adipose hypertrophy occurring as patients became more obese and diabetic. The literature suggests MASLD severity correlates with increased visceral adipose tissue volume [484] and therefore I hypothesised that adipocyte size would also correlate with MASLD severity.

Firstly, when correlating adipocyte size with the individual elements of MASLD progression using AI software the percentage of fat within the liver positively significantly correlated with adipocyte size ($r=0.4$, $p=0.02$) but did not significantly correlate with percentage of fibrosis ($r=0.3$, $p=0.08$), inflammation ($r= -0.3$, $p=0.08$) nor ballooning ($r= -0.9$, $p=0.6$) (**Figure 48**).

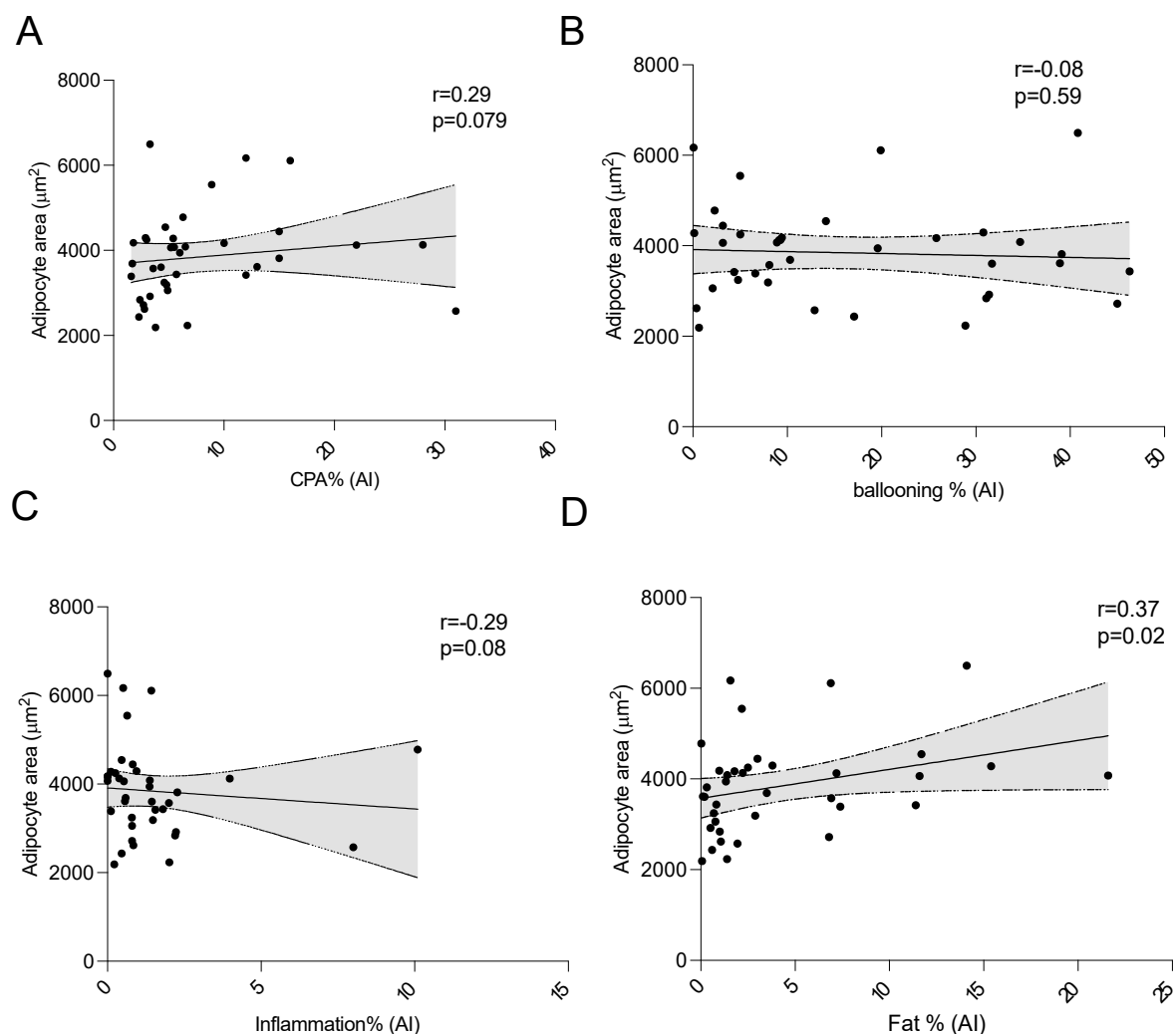


Figure 48 Adipocyte size correlates with percentage of fat in the liver. Correlation between oWAT adipocyte area (μm²) and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=37; statistical significance was calculated using Spearman's correlation coefficient. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

I correlated adipocyte size with overall NAS scores and fibrosis scores; there was no significant change in adipocyte size in patients with normal liver, MASLD or MASH ($p=0.26$) nor with progression of fibrosis (F0/1 versus F2 versus F3; $p=0.73$) (**Figure 49**).

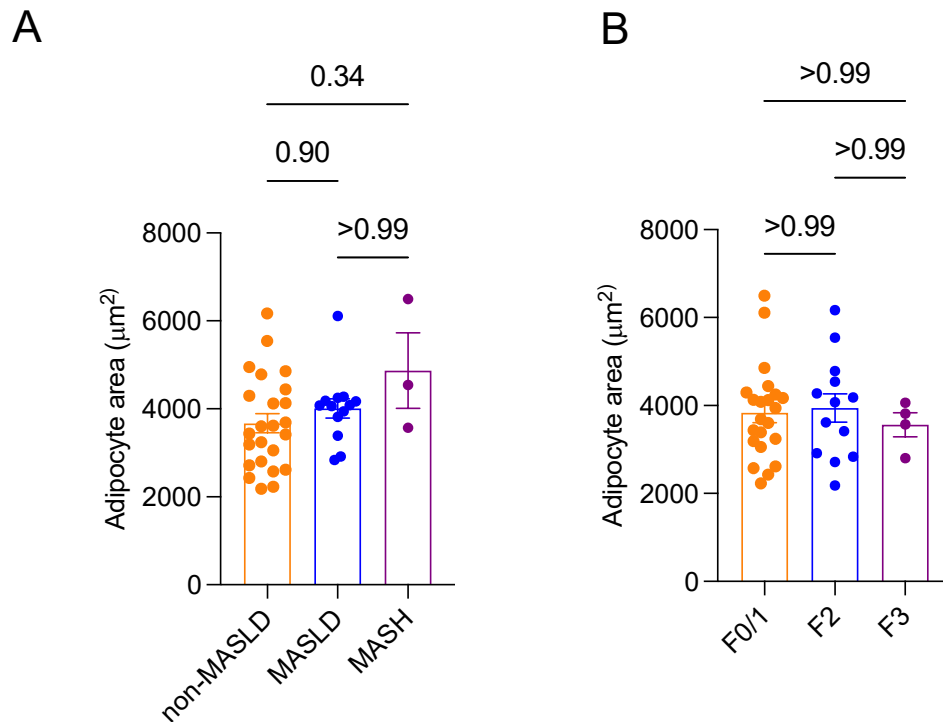


Figure 49 Adipocyte size and MASLD progression. oWAT average adipocyte area(μm^2) in obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=13), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=13, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

Next, I aimed to establish if transcription factor PPAR γ , the master regulator of adipogenesis, was associated with progression of liver disease. PPAR γ was shown at a protein level to decrease with increased adipocyte size in the cohort of patients in chapter 4.

There was a generalised negative correlation with fat percentage ($r=-0.29$, $p=0.07$) but no correlation of PPAR γ transcript levels with percentage of fibrosis ($r=0.17$, $p=0.11$), inflammation ($r=0.23$, $p=0.16$), ballooning ($R=-0.20$, $p=0.23$) (**Figure 50**).

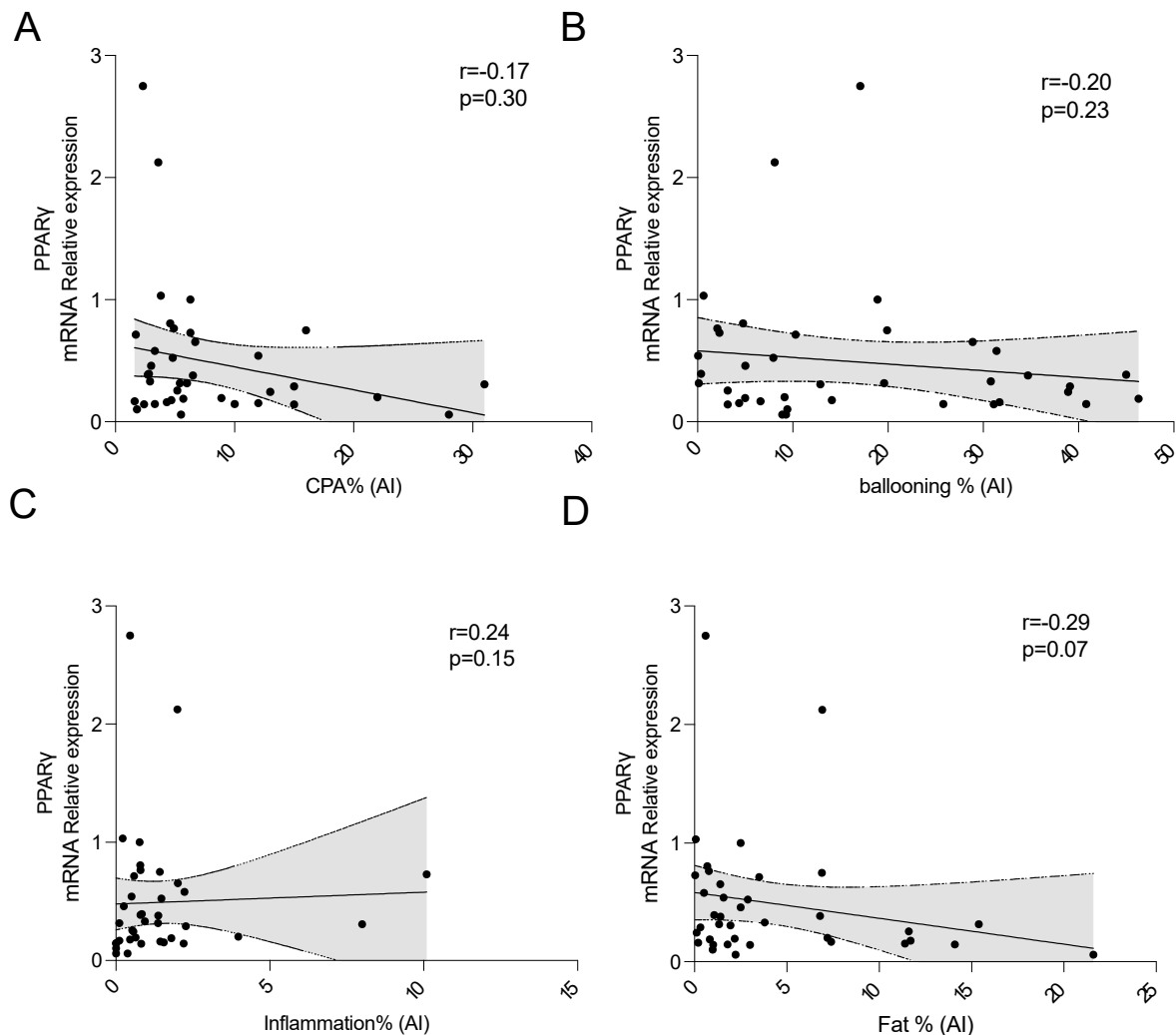


Figure 50 PPAR γ does not change with MASLD progression using AI. (A-D) Correlation between oWAT PPAR γ mRNA transcript levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

However, oWAT mRNA transcript levels of PPAR γ did not significantly differ between patients with non-MASLD, MASLD and MASH ($p=0.56$), they also did not change with progression of hepatic fibrosis ($p=0.61$) (**Figure 51**).

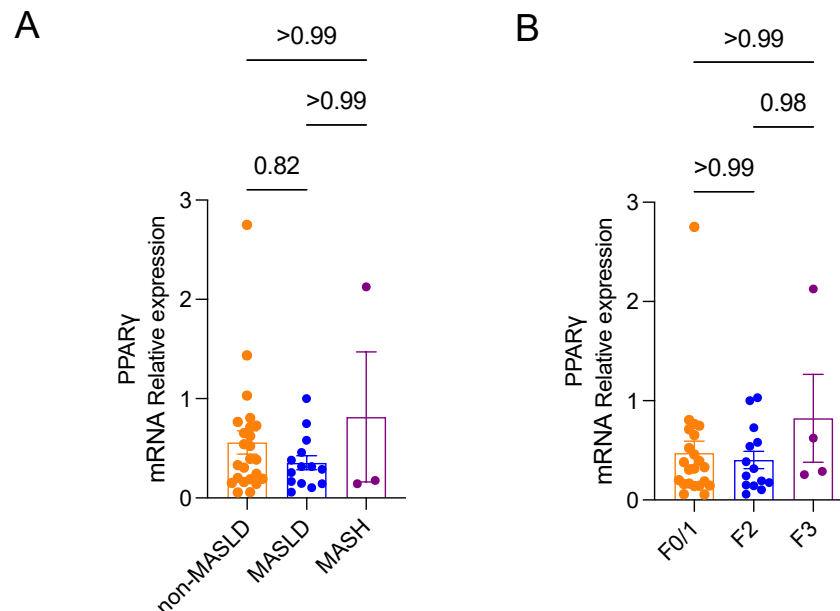


Figure 51 PPAR γ does not change with advancing MASLD scoring or fibrosis. PPAR γ mRNA levels in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis.

6.3.2 oWAT adipokine profile and MASLD

It has been demonstrated that adipokines released from the WAT are associated with MASLD severity. Adiponectin, a pivotal adipokine that is produced and secreted from adipocytes and is insulin sensitising, anti-inflammatory and pro-adipogenic, has been widely showed to decrease systemically with obesity and MASLD [311, 487-489]. However, the relationship between MASLD progression and oWAT levels of adiponectin is less well characterised. I hypothesised that ADIPOQ mRNA levels in the oWAT would decrease with MASLD progression [489].

However, ADIPOQ transcript levels measured in the oWAT did not alter between non-MASLD, MASLD and MASH phenotypes ($p=0.99$) and fibrosis groups ($p=0.42$) (**Figure 52**).

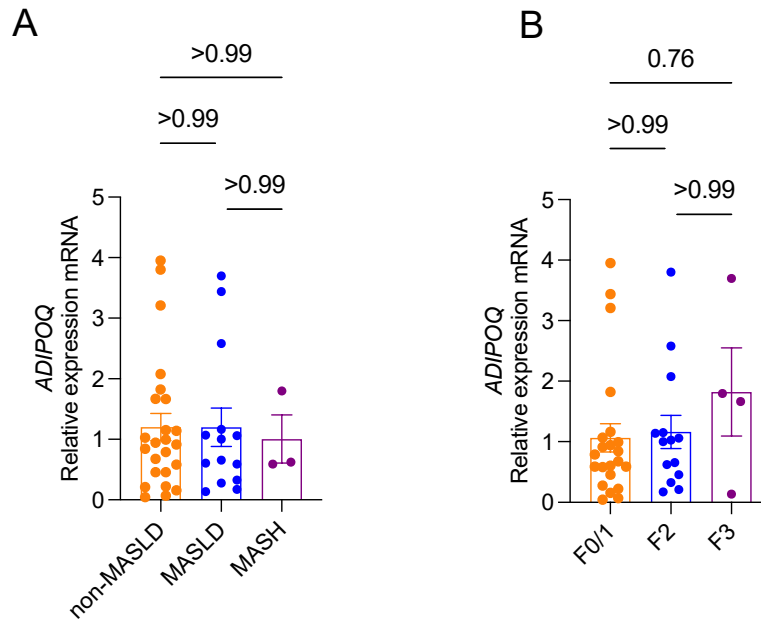


Figure 52 oWAT Adiponectin does not change with MASLD or fibrosis scoring. ADIPOQ mRNA levels in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

ADIPOQ transcripts also did not correlated with CPA percentage ($r=-0.19$, $p=0.24$), fat percentage ($r=-0.013$, $p=0.94$), ballooning ($r=-0.083$, $p=0.62$) nor inflammation ($r=0.16$, $p=0.35$) (**Figure 53**).

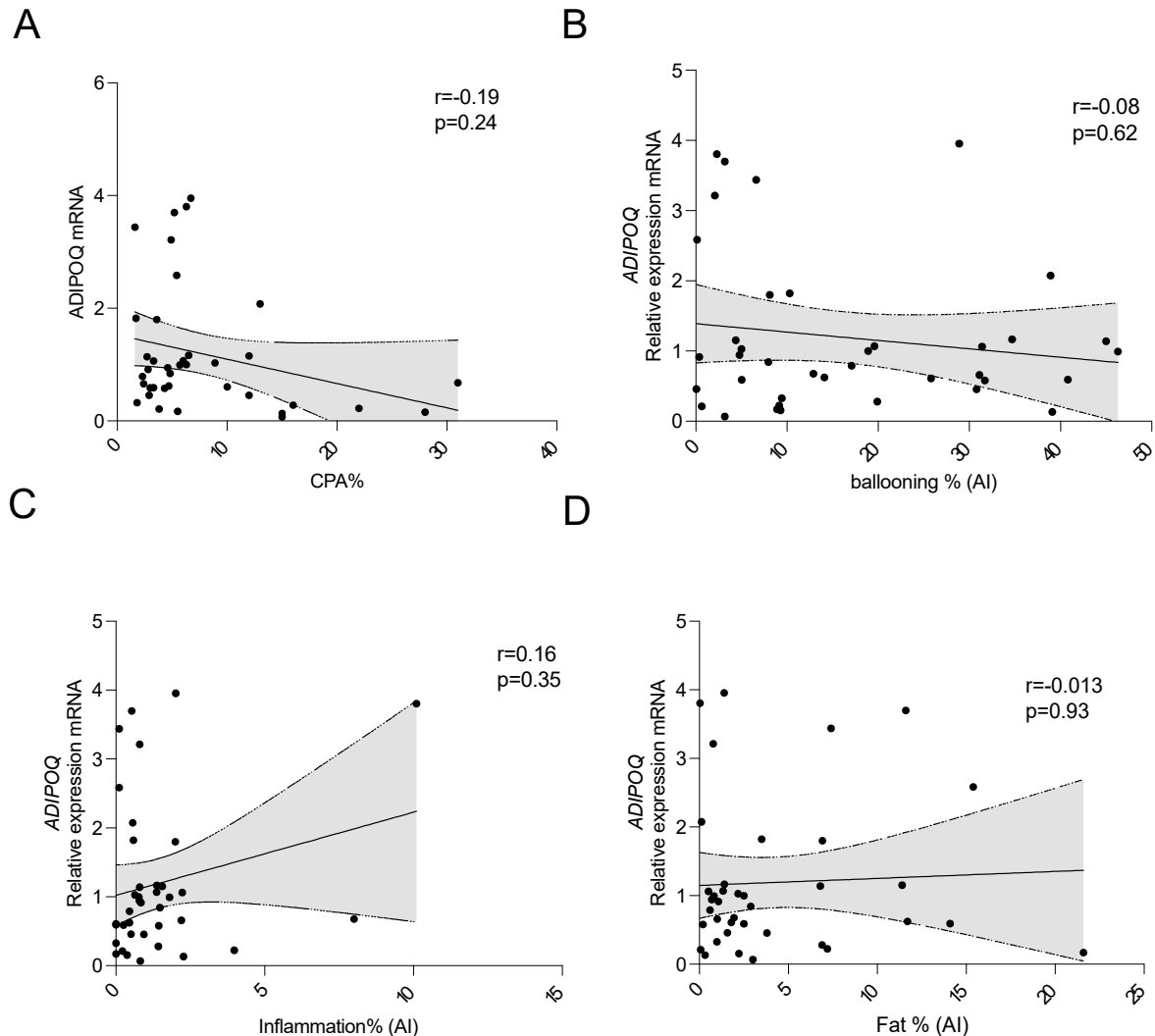


Figure 53 oWAT Adiponectin does not change with MASLD progression using AI scoring. (A-D) Correlation between oWAT ADIPOQ mRNA transcript levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

In summary, adipocyte hypertrophy positively correlated significantly with percentage of fat in the liver, PPAR γ had a generalised negative correlation with fat % but ADIPOQ mRNA transcripts do not significantly alter in line with severity of MASLD.

6.3.3 oWAT Inflammation and MASLD progression

Inflammation in the visceral WAT has been shown to mirror progression in MASLD.

Therefore, I aimed to explore if inflammation in oWAT of this cohort correlated with NAS scoring, fibrosis scoring and percentage of fat, inflammatory cells, fibrosis and balloon seen in liver biopsies.

It is widely accepted that during obesity and oWAT dysregulation there is an increase in crown-like-structures, which are made of macrophages that surround dying adipocytes. I hypothesised that if previous studies suggested inflammation in the WAT correlated with severity of liver disease, then the number of CLS would increase with severity of MASLD.

When comparing the number of CLS in 10 fields in non-MASLD, MASLD and MASH groups there was no significant difference in number of CLS in ten fields ($p=0.52$) and again no difference in number when comparing the extent of fibrosis ($p=0.67$) (**Figure 54**).

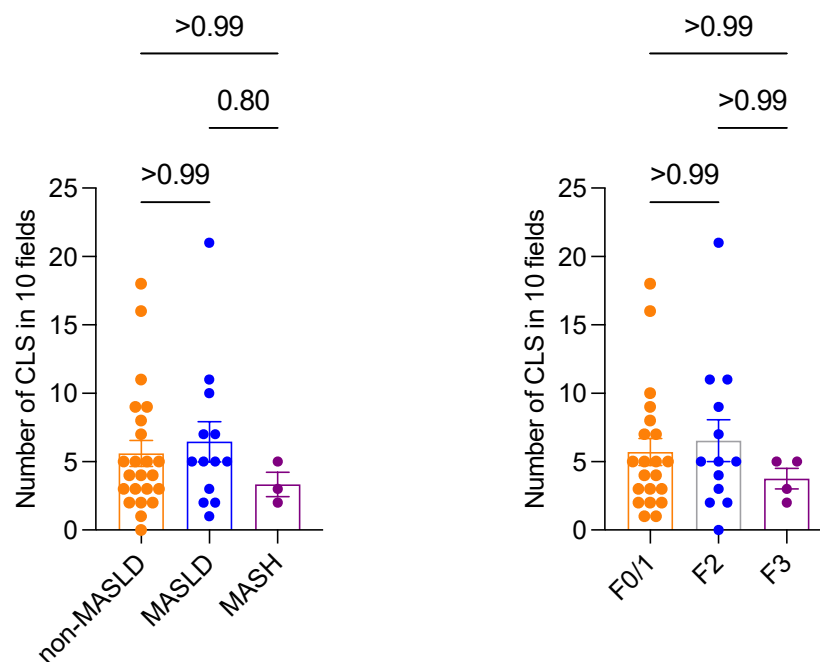


Figure 54 Crown-like-structures do not correlate with MASLD and fibrosis scoring. Number of crown-like structures (CLS) in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=23), 3-4 intermediate/MASLD/MASH (N=13), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=21, F2: N=13, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. CLS: crown-like structures. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

There was no significant correlation with the number of CLS and percentage of fibrosis ($r=-0.009$, $p=0.96$), fat ($r=-0.12$, $p=0.50$), ballooning ($r=-0.096$, $p=0.58$) nor inflammation ($r=-0.21$, $p=0.23$) (**Figure 55**).

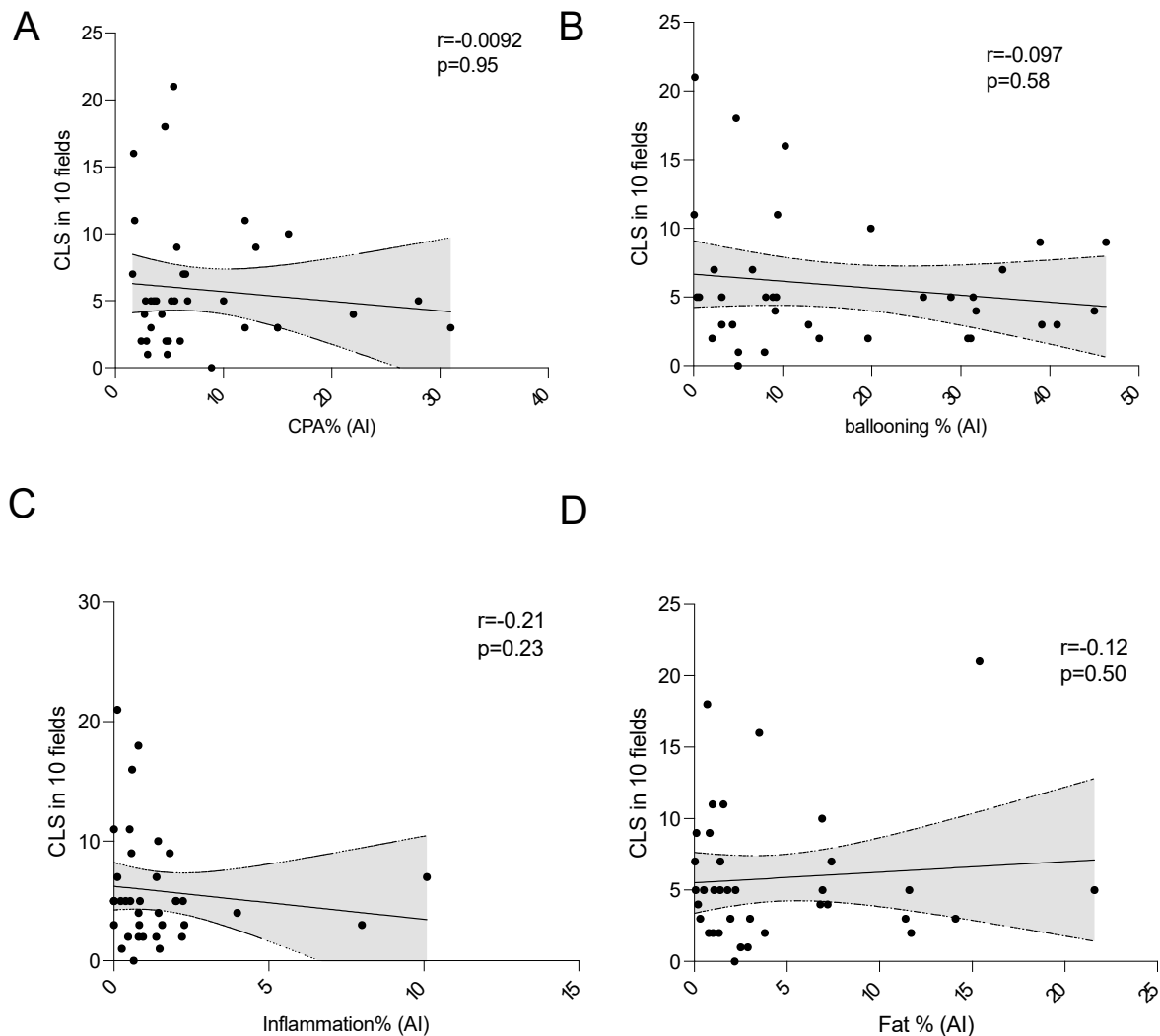
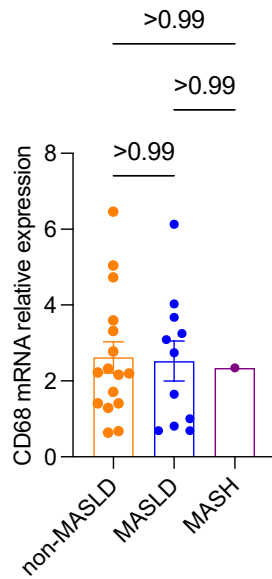


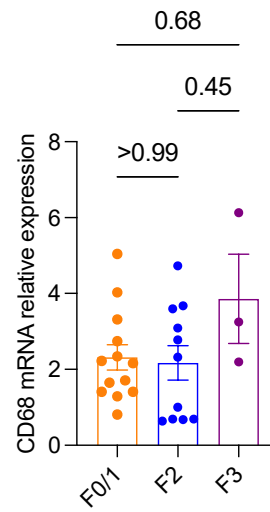
Figure 55 Crown-like-structures do not correlate with MASLD progression using AI. (A-D) Correlation between oWAT CLS in 10 fields and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=36; statistical significance was calculated using Spearman's correlation coefficient. CLS: crown-like structures; CPA%: collagen percentage area.

CD68 transcripts did not significantly alter when comparing non-MASLD, MASLD and MASH groups ($p=0.98$) and again when comparing different stages of fibrosis ($p=0.35$) (**Figure 56**). CD68 transcripts also did not correlate significantly with percentage of fibrosis ($r=0.076$, $p=0.71$), fat ($r=0.038$, $p=0.85$), ballooning ($r=-0.24$, $p=0.24$) nor inflammation ($r=-0.33$, $p=0.99$) (**Figure 56**).

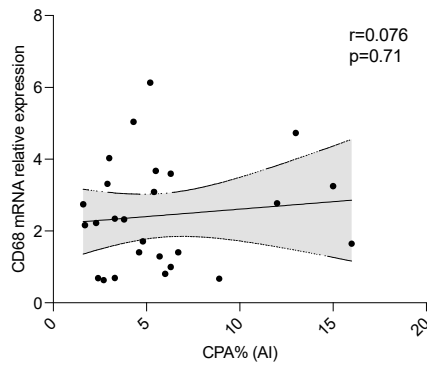
A



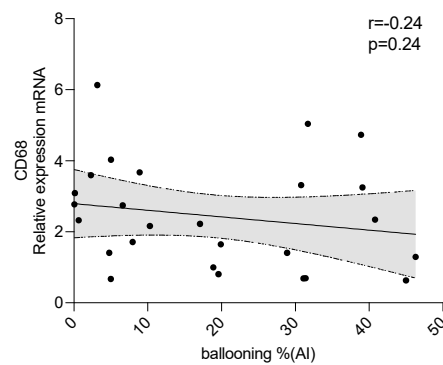
B



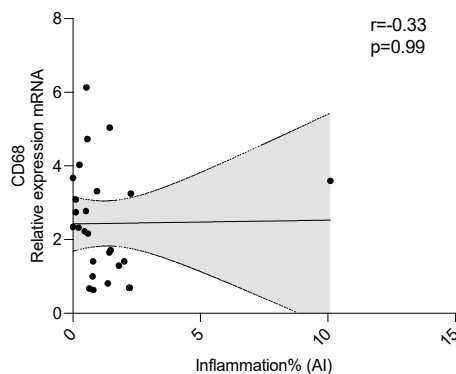
C



D



E



F

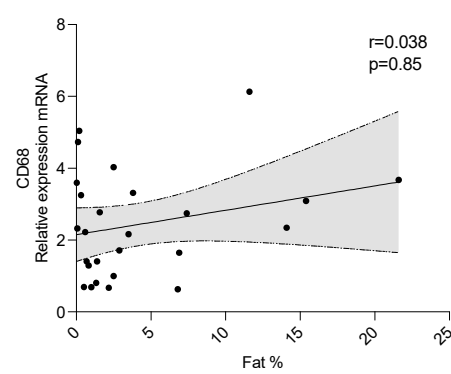


Figure 56 CD68 transcripts do not correlate with MASLD progression. CD68 mRNA levels in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). (C-F) Correlation between oWAT CD68 mRNA transcript levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=26; statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison(A-B). and Spearman's correlation coefficient(C-F). Error bars represent SEM. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

Single-cell RNA-seq techniques have enabled a further understanding of the complex dynamic inflammatory response in WAT in prolonged obesity with TREM2⁺ lipid-associated macrophages recently highlighted as a key subset of macrophages associated with metabolic dysfunction and WAT immune cell remodelling [109]. Therefore, I hypothesised that TREM2 mRNA levels in the oWAT would increase in tandem with liver disease severity. However, oWAT TREM2 mRNA levels did not significantly alter when comparing non-MASLD, MASLD and MASH groups ($p=0.78$) and again when comparing stages of fibrosis ($p=0.82$) (**Figure 57**).

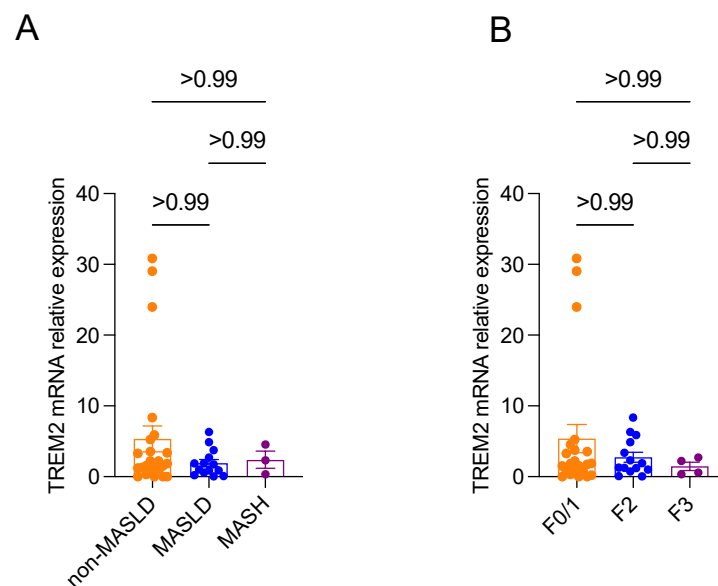


Figure 57 TREM2 transcripts do not correlate with MASLD and fibrosis scoring. TREM2 mRNA levels in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

oWAT TREM2 mRNA did not correlate significantly with percentage of fibrosis ($r=-0.19$, $p=0.25$), fat ($r=-0.06$, $p=0.70$), ballooning ($r=-0.07$, $p=0.68$) nor inflammation ($r=-0.27$, $p=0.11$) (**Figure 58**).

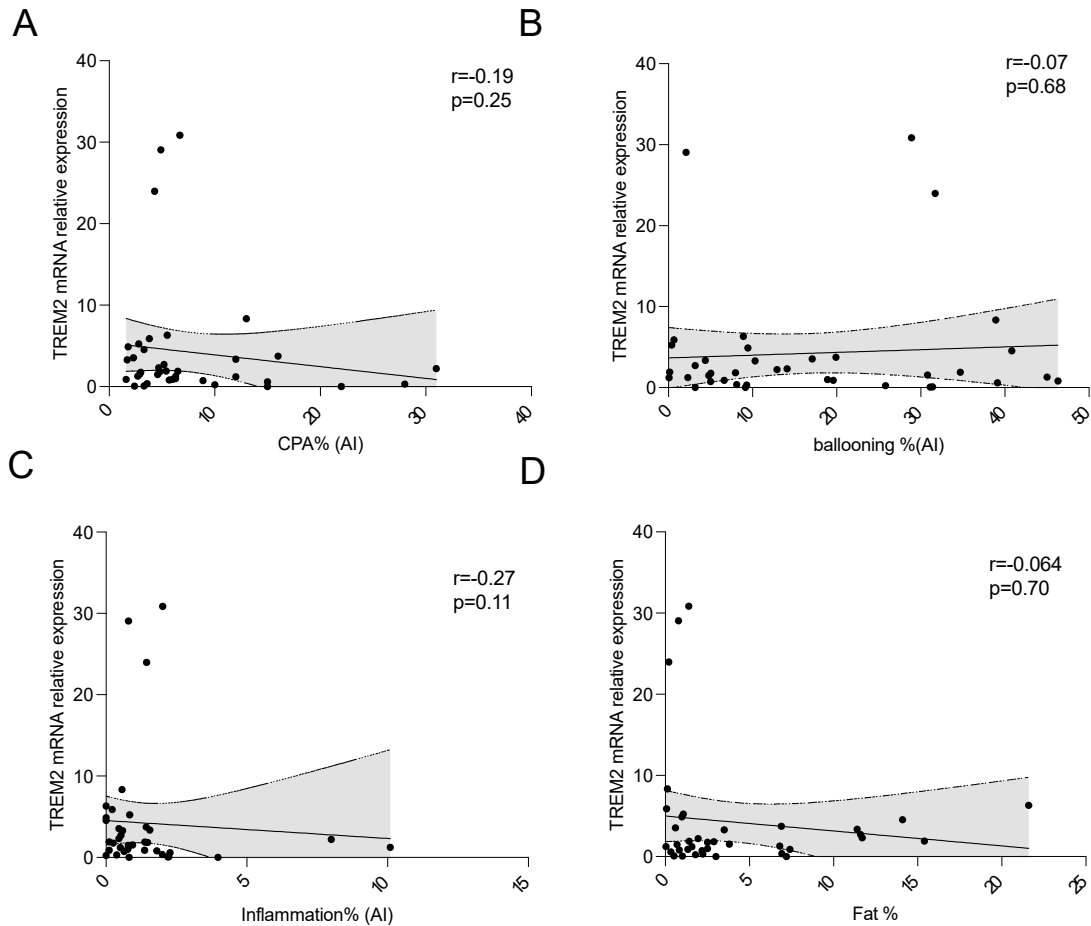


Figure 58 TREM2 transcripts do not correlate with MASLD AI scoring. Correlation between oWAT TREM2 mRNA transcript levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient. CPA: collagen proportionate area.

6.3.4 Conclusion

Overall, it appears that inflammation in oWAT measured by number of CLS and mRNA levels of TREM2 and CD68 does not associate with severity of MASLD.

6.3.5 ECM remodelling of oWAT and MASLD progression

ECM remodelling in obesity and WAT dysfunction and its relationship with MASLD progression is not fully elucidated. It is suggested that collagen I is the most abundant in the WAT with collagen VI in chapter 4 was shown to be associated with macrophage infiltration and increased with inflammation. Therefore, I aimed to characterise the relationship between collagen I and collagen VI in oWAT with MASLD severity and fibrosis progression.

Type I collagen raw intensities were negatively correlated with percentage of fat within the liver ($r=-0.37$, $p=0.042$) but were not associated significantly with percentage of ballooning ($r=0.22$, $p=0.19$), inflammation ($r=0.28$, $p=0.09$) nor fibrosis ($r=-0.11$, $p=0.50$) (**Figure 59**).

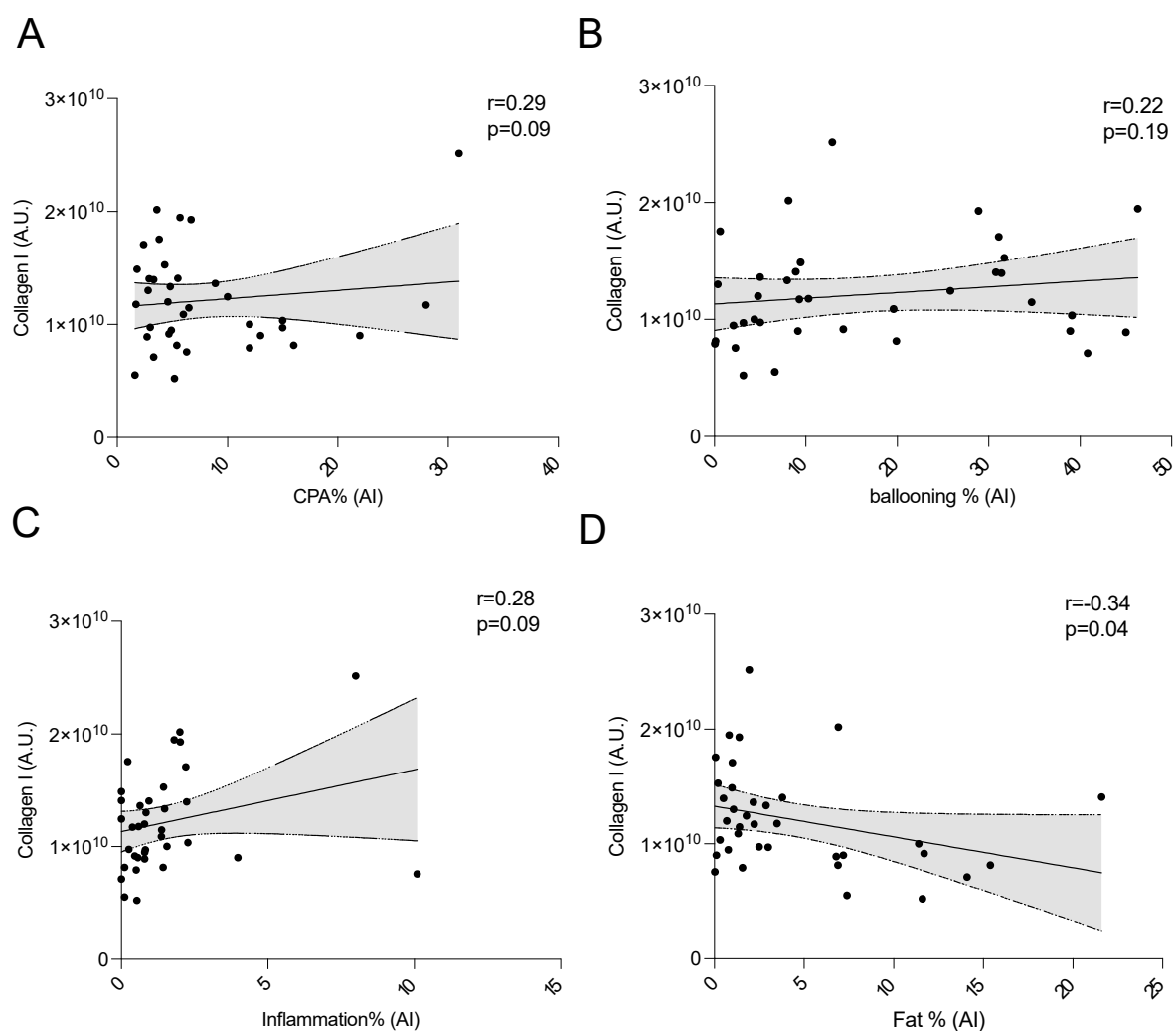


Figure 59 Collagen Type I intensity correlates with percentage of fat in the liver. (A-D) Correlation between oWAT Type I collagen fluorescence raw intensities (A.U.) and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=36; statistical significance was calculated using Spearman's correlation coefficient. CPA: collagen proportionate area.

Type I collagen raw intensities did not significantly change between fibrosis groups ($p=0.89$) nor non-MASLD, MASLD and MASH groups ($p=0.84$) (**Figure 60**).

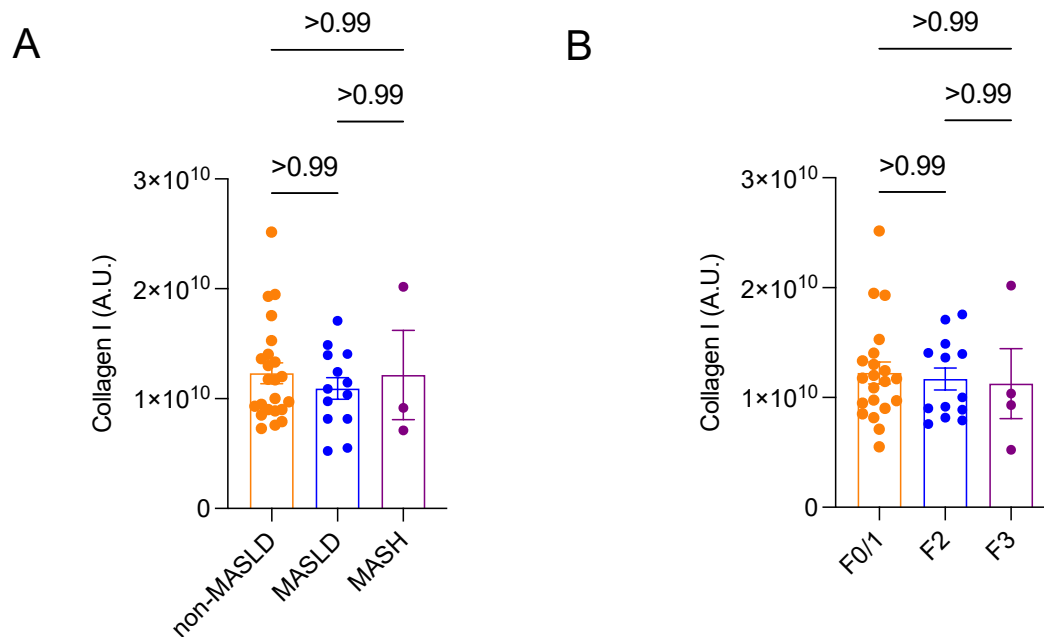


Figure 60 Collagen Type I intensity correlation with MASLD and fibrosis scoring. Type I collagen fluorescence raw intensities (A.U.) in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). Statistical significance was calculated using Kruskal-Wallis test with Dunn's multiple comparison. Error bars represent SEM. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

COL1A1 mRNA transcripts also did not differ significantly between NAS scoring ($p=0.64$) nor fibrosis ($p=0.7$). COL1A1 did not correlate with percentage of fibrosis ($r=0.2$, $p=0.23$), fat ($r=-0.04$, $p=0.8$), ballooning ($r=0.22$, $p=0.19$) and inflammation ($r=-0.03$, $p=0.88$) (**Figure 61**).

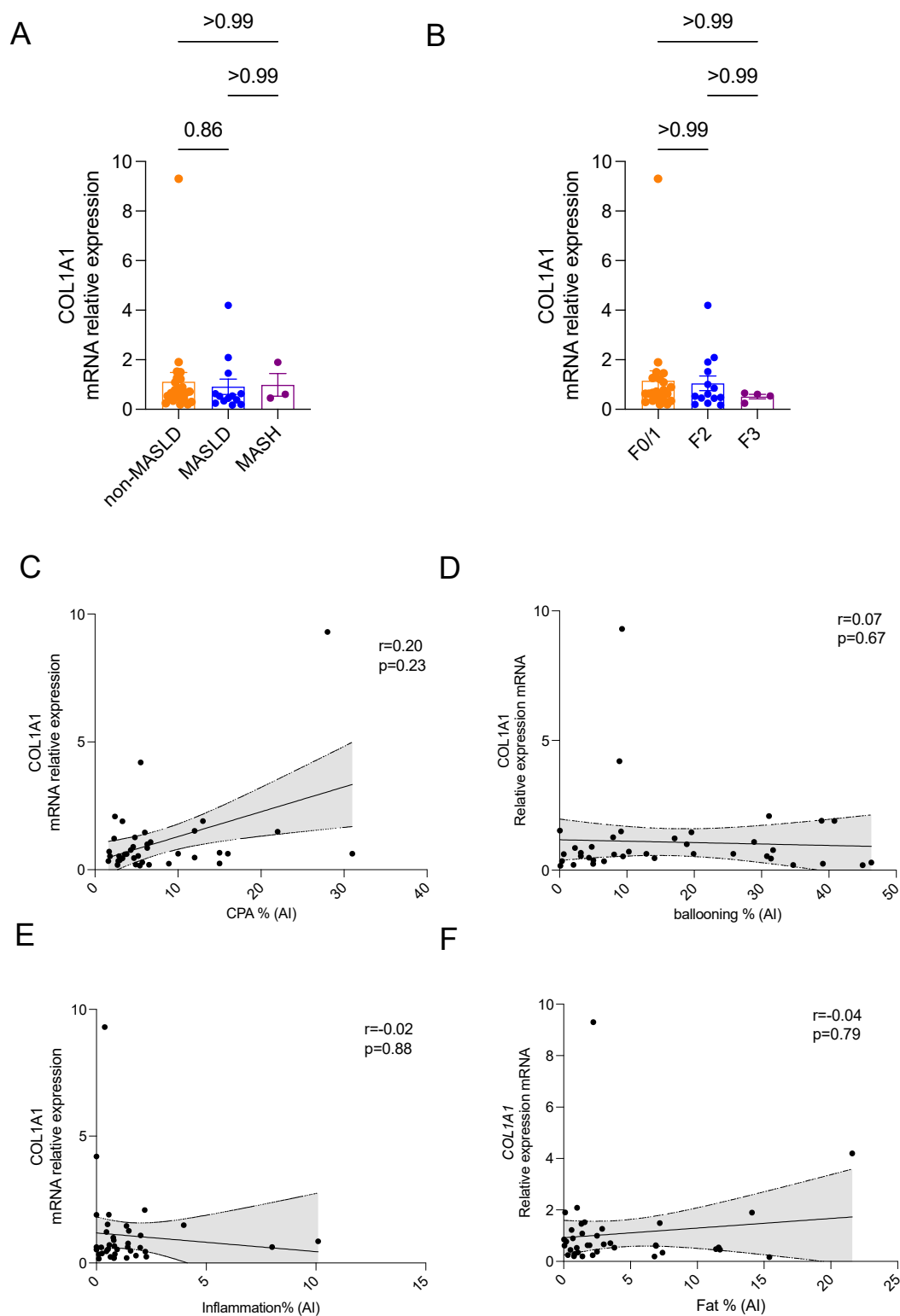


Figure 61 Collagen I transcripts in oWAT of obese patients do not correlate with MASLD. COL1A1 mRNA levels Type I collagen fluorescence raw intensities (A.U.) in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). (C-D) Correlation between oWAT COL1A1 mRNA transcript levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient (C-F) or using Kruskal-Wallis test with Dunn's multiple comparison (A-B). Error bars represent SEM. CPA: collagen proportionate area.

Collagen VI intensities negatively correlated with ballooning ($r=-0.37$, $p=0.029$), but not with percentage of fibrosis ($r=0.03$, $p=0.87$), fat ($r=-0.24$, $p=0.16$) and inflammation ($r=0.09$, $p=0.60$). Collagen VI intensities did not change with non-MASLD, MASLD and MASH groups ($p=0.56$) nor severity of liver fibrosis ($p=0.80$) (**Figure 62**).

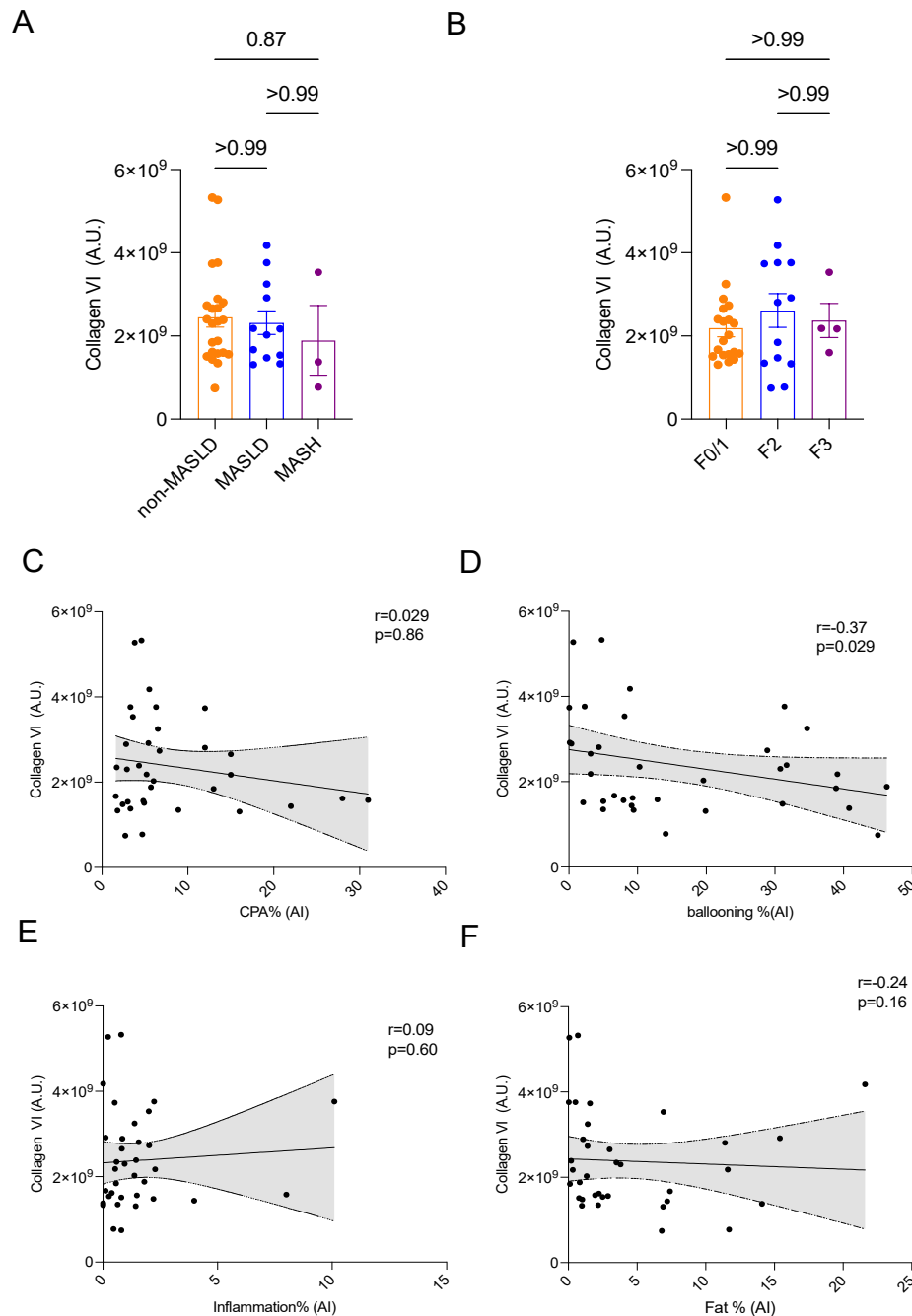


Figure 62 Collagen Type VI intensity correlation with MASLD progression. Type VI collagen fluorescence raw intensities (A.U.) in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). (C-D) Correlation between oWAT Type VI collagen fluorescence raw intensities (A.U.) and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient (C-F) or using Kruskal-Wallis test with Dunn's multiple comparison (A-B). Error bars represent SEM. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

COL6A1 transcripts did not correlate with percentage of fibrosis ($r=-0.16$, $p=0.33$), fat ($r=0.15$, $p=0.39$), ballooning ($r=0.12$, $p=0.49$) and inflammation ($r=-0.19$, $p=0.25$). There was also no significant difference between liver fibrosis groups ($p=0.43$) but there were borderline changes with NAS scoring ($p=0.05$) (**Figure 63**).

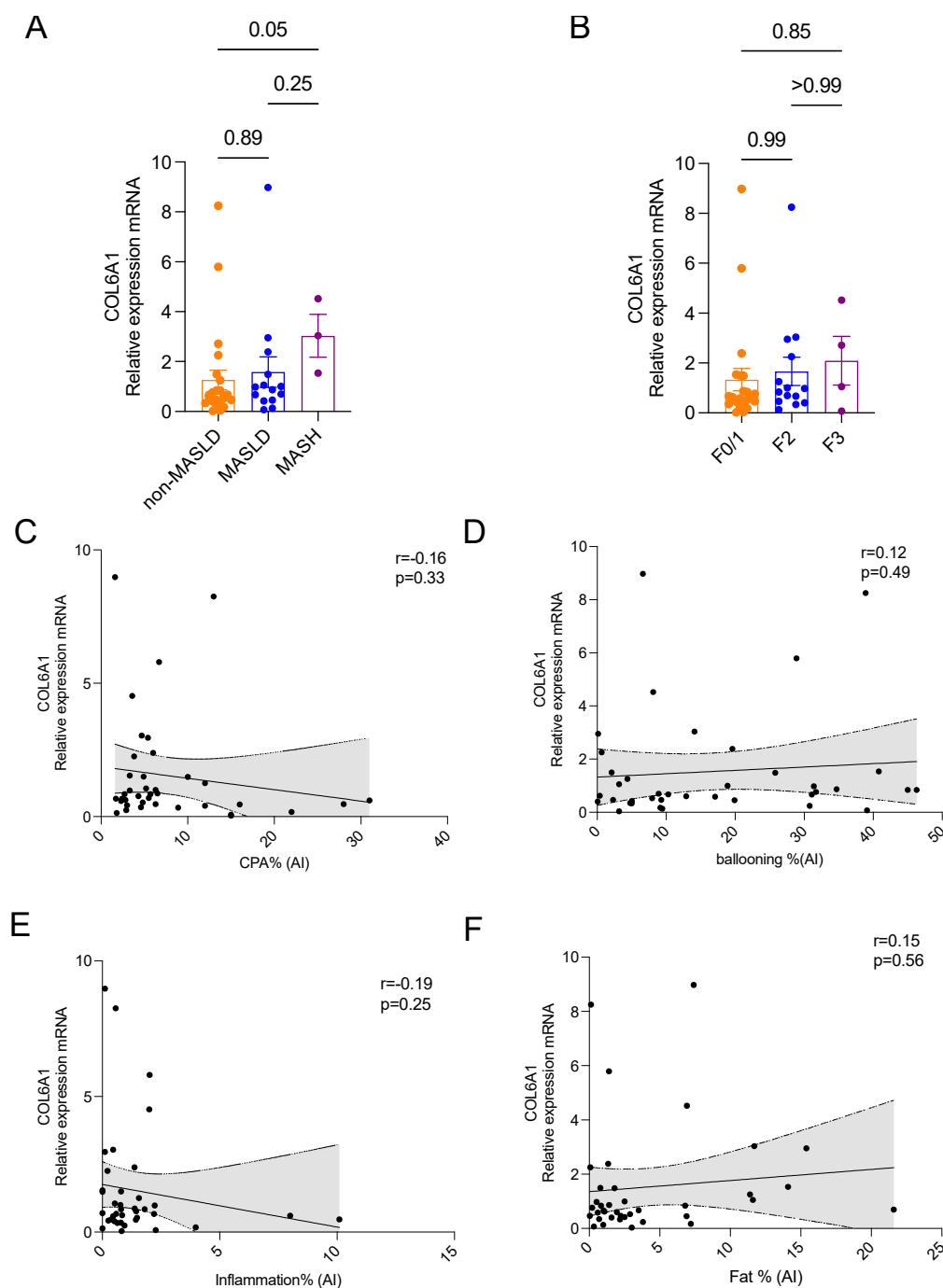


Figure 63 Collagen VI transcripts in oWAT of obese patients do not correlate with MASLD. COL6A1 mRNA levels in the oWAT of obese patients stratified into patients with (A) MASH [NAS scoring, 0-2 no MASLD (N=24), 3-4 intermediate/MASLD/MASH (N=14), 5+ MASH (N=3)] or (B) fibrosis score (F0-1: N=22, F2: N=14, F3: N=4). (C-F) Correlation between oWAT COL6A1 mRNA levels and markers of MASLD, measured by machine learning (CPA%, ballooning%, fat%, inflammation %) for each liver biopsy N=38; statistical significance was calculated using Spearman's correlation coefficient (C-F) or using Kruskal-Wallis test with Dunn's multiple comparison (A-B). Error bars represent SEM. CPA: collagen proportionate area; MASLD: Metabolic dysfunction-associated steatotic liver disease.

6.3.6 Conclusion

Adipocyte size correlated with accumulation of fat within the liver and PPAR γ had a generalised negative trend when correlated with MASLD progression. Collagen I and Collagen VI raw intensity values correlated with MASLD status show that they are inversely proportional to fat accumulation and ballooning respectively within the liver.

Other studies [482, 490] also demonstrate adipocyte size increases with hepatic ballooning and fat accumulation and strengthen the 'expandability hypothesis' which suggests adipose tissue regulates lipid flux to the liver; adipocytes have an expandability threshold that once reached, prevents the adipocyte from expanding further. At this point lipids are then released as FFA into the circulation and stored in the liver [103]. This threshold is thought to be unique to the individual but what determines it is yet to be defined. These results suggest it may in part be associated with ECM changes observed. Mechanistically, collagen I is described as a structural protein, and has been shown in mouse models on HFD to decrease in AT as the adipocytes expand [210] during obesity. Therefore, I propose that in obesity collagen remodelling initially enables the adipocyte to expand but as collagen decreases the adipocyte becomes unstable which results in lipid release and hepatic steatosis.

Despite comparing key factors that contribute to metabolic decline and WAT dysfunction with severity of liver disease, transcription factors, adipokines and inflammatory markers did not correlate with liver disease. As there appears to be no correlation with oWAT inflammation and MASLD progression this could be explained by the relatively early MASLD changes seen in this cohort of patients as only small numbers had evidence of fibrosis and no patients included had a histological diagnosis of cirrhosis.

6.4. Discussion

It is well established that adipose tissue and the liver are able to accommodate excessive lipid influx over time and adipose tissue is an excellent buffer for long term overnutrition. However, when AT loses this flexibility to store fat it is believed this leads to systemic release of free fatty acids and deposition within the liver often resulting in MASLD. This expandability limit is thought to differ at an individual level and likely dependent on other factors, yet to be defined [88, 102, 103].

The dysfunctional WAT that can no longer accommodate fat deposition becomes inflamed and metabolic decline often follows. The crosstalk between adipose tissue and the liver is therefore important in the development of T2DM and liver disease particularly as the progression of MASLD to MASH is shown to progress at a faster rate in patients with T2DM [491]. The pathological effects on liver tissue following the leakage of FFA from dysfunctional WAT has been shown in mouse models where lipolysis led to insulin resistance and ultimately MASLD [492]. The CYP2J4^{-/-} rat model used by the Behmoaras lab also demonstrated that as the rats became more obese, adipose tissue became inflamed and the obese rat developed hepatic steatosis [209]. Therefore, the aim of this chapter was to determine if the features of obesity-associated maladaptive human oWAT that I phenotyped in chapter four correlated with hepatic disease progression with the aim of identifying key factors that lead to MASH/cirrhosis. The NAS score and fibrosis grade was calculated by an expert histopathologist, and AI software was also used to calculate each marker of histological MASLD progression (fat, inflammation, ballooning and fibrosis) as a percentage of the sample analysed.

6.4.1 Adipocyte hypertrophy and MASLD progression

As the first maladaptive change I demonstrated in the oWAT was adipocyte hypertrophy in the progression from obesity to metabolic decline, I measured whether this correlates with MASLD severity using the four well established markers of histological disease progression; fat accumulation, ballooning, inflammation and fibrosis.

Hyperlipidaemia has been shown to closely link with adipocyte hypertrophy and systemic FFA release through the portal circulation [490] while visceral adipose tissue size has been linked to MASLD progression [482, 483, 485, 493, 494]. My results in part complemented these previous studies, as adipocyte hypertrophy correlated with percentage of fat within the liver of obese patients but not with other parameters of liver disease.

The CYP2J4^{-/-} obese rat model previously studied by the Behmoaras lab had increasing hypertrophied adipocytes and decreasing PPAR γ /CEBP that resulted in hepatic steatosis and *de novo* lipogenesis. [209] Similar to this study, while my cohort did not show significant

correlation with WAT PPAR γ transcripts and liver disease, there was a generalised downward trend. Work done by Medina-Gomez et al. demonstrated PPAR γ improves adipocyte tissue expandability and prevents hepatic lipotoxicity in a mouse model, however Medina-Gomez's study result could be due to PPAR γ activation that was observed in the hepatic tissue as opposed to improved visceral adipose tissue function [495]. Pioglitazone, a PPAR γ agonist has been extensively studied and has shown in several meta-analysis and systematic reviews to improve hepatic steatosis, but its effects on fibrosis are less robust and were not demonstrated in the pivotal PIVENS trial [75, 310]. As my data shows PPAR γ in oWAT has a non-significant negative correlation with hepatic steatosis and it doesn't correlate with MASH fibrosis/cirrhosis it supports these trials findings. Taken together these results suggests there could also be alternative pathways involved in crosstalk between the oWAT and the liver in fibrosis progression and I will investigate if oxylipins play a part in the next chapter.

6.4.2 oWAT Adipokines and the severity of MASLD

Adiponectin, a pivotal adipokine that is produced and secreted from adipocytes and is insulin sensitising, anti-inflammatory and pro-adipogenic, has been widely showed to decrease systemically with obesity and MASLD [311, 487-489]. As it is produced and secreted by adipocytes it is likely certain depots such as subcutaneous or omental, contribute to the systemic levels in varying amounts. Yoneda et al. demonstrated that systemic adiponectin decreased with increasing VAT, measured using computerised tomography, in MASLD [489]. The results from my cohort showed that there was no correlation with oWAT transcript levels of ADIPOQ and liver progression. It would be interesting to see if there are any changes with ADIPOQ mRNA transcript levels in subcutaneous WAT of my cohort. I postulate that it could be subcutaneous not visceral fat that is responsible for the changes in systemic levels of adiponectin observed in human studies. The literature to support this is conflicting, Nannipieri et al. reported no change in subcutaneous adiponectin levels but a decrease in visceral adiponectin mRNA levels with progression of MASLD[496], while two further studies suggested the reverse [497, 498]. Adiponectin is the most well characterised adipokine to be related to MASLD progression, other adipokines have also been studied including Leptin that increase with hepatic steatosis and therefore I would like to extend my work to include other significant adipokines.

6.4.3 Adipocyte inflammation and MASLD progression

There is extensive literature on hepatic inflammation in MASLD and an expanding literature base studying adipose tissue inflammation. However, there are limited studies that compare cross-tissue inflammatory changes with progressive obesity. My cohort appears to show

there is no correlation with visceral markers of inflammation in the oWAT including CLS, CD68 mRNA and TREM2 mRNA transcripts. In keeping with my results Boesch et al. recently also demonstrated no striking difference between different grades of liver disease and adipose tissue macrophage transcript signatures using sc-RNA-seq. The study did suggest there was a switch between vascular-related macrophage subsets and metabolically active subsets in MASH [288]. However, this was in a small number of patients and did not study simultaneously liver, adipose tissue and serum signatures in the whole spectrum of MASLD. These results, however, are in contrast to three studies, one of which is in children, that showed that increasing macrophage infiltration correlated with hepatic fibroinflammatory lesions [285-287] and du Pleuiss measured several key inflammatory genes in the visceral WAT that all correlated with histological severity of MASLD [284]. Furthermore, levels of IL-6, TNF and MCP-1 from visceral WAT macrophages and systemic levels of CCL2 corresponded with morphological changes of MASLD in obesity [283, 289-291]. I have used only three markers of inflammation (TREM2 and CD68 transcripts or number of CLS) which may have limited this analysis and therefore using a flow cytometry panel of markers of inflammation including pro and anti-inflammatory cytokines or analysing using transcriptomics as opposed to measuring individual genes might show significant correlation with hepatic steatosis and fibrosis. In conclusion, further in depth cross-tissue studies in humans are needed.

6.4.4 Adipose ECM remodelling and MASLD

Most human studies evaluating ECM changes in WAT in prolonged obesity focus on subcutaneous WAT and very few studies look at ECM remodelling in WAT and its relationship with MASLD progression and liver fibrosis. Beals et al. showed that 'fibrogenesis' (measured by collagen transcripts and collagen 1a turnover) within the subcutaneous AT was associated with obesity and MASLD and but did not study visceral WAT [499] and a recent study showed ECM-associated genes were increased in SAT from patients with hepatic fibrosis, but again did not study VAT [500]. Interestingly, Leven et al. also studied an obese cohort; they showed that COL1A1 transcripts measured in the visceral WAT did not correlate with MASLD or MASH but Sirius red, a global marker of fibrosis, positively correlated with MASLD and MASH [501].

My cohort, in contrast to these studies suggested collagen I and collagen VI in the oWAT inversely correlate with hepatic fat accumulation and ballooning with no association seen with fibrosis and inflammation. This would also be in keeping with chapter 4 that showed Collagen I and VI decreased with obesity and metabolic decline.

Taking Leven's findings and mine together, it poses the question, *is collagen deposition in the oWAT always counterproductive?* While Leven et al. results differ to mine, they highlight the importance of evaluating individual markers of oWAT ECM composition, and together these results suggest that in fact oWAT ECM remodelling is complex, different collagens appear to have different roles. Collagen I, as discussed in chapter 4 is likely more structural and integral to WAT homeostasis while collagen VI appears to be associated more with inflammation in the oWAT. Furthermore, the ECM remodelling seen in oWAT during obesity and metabolic decline appears to differ from the processes seen during the progression of liver fibrosis to cirrhosis where increased collagen deposition, predominantly collagen I and III, results in irreversible damage to the hepatic tissue driven by TGF- β signalling pathway [502]. This leads me to believe that further collagens and other ECM component such as MMPs in the oWAT also need to be studied in relation to hepatic fibrosis and these may vary depending on disease severity.

How are oWAT ECM modulations and adipocyte size linked to MASLD? The AT expandability theory suggests adipocytes are able to expand to a threshold at which point they can no longer store FFAs and instead lipids 'spill over' into the circulation with deposition into other metabolic organs including the liver [503]. This theory coupled with my results, leads me to hypothesis that the reduction in collagens alongside adipocyte hypertrophy renders the adipocyte unstable and in turn leads to FFA efflux and hepatic steatosis. It would be interesting to see if this relationship continues as MASLD progresses to cirrhosis.

6.4.5 Conclusions

In summary, there is evidence that adipocyte size increases in tandem with hepatic steatosis and Collagen I and Collagen VI deposition in the oWAT decrease as hepatic ballooning and steatosis progress which is in keeping with current theory that WAT lipid storage reaches a threshold at which excess FFA are then stored in liver. There was no significant correlation with hepatic inflammation nor hepatic fibrosis with markers of oWAT dysfunction. These results on one hand could suggest that disease progression in the liver evidenced by hepatic inflammation and hepatic fibrosis in prolonged obesity is in fact tissue specific, independent of oWAT function and depends purely on the local microenvironment. However, these findings could also be explained by the limitations of the cohort which I will discuss below and thus warrants further investigation. As obesity has been shown to cause multi-organ disease and early MASLD changes do correlate with oWAT dysfunction I believe that the latter is the most likely explanation.

6.4.6 Limitations

While there was a significant number of patients within this cohort with no-MASLD or MASLD on liver biopsy the number of severely fibrotic patients was small (n=4 for F3 and n=0 for F4). This in itself highlights the ability of the liver to accommodate to lipid accumulation; despite all the patients being classed as morbidly obese no patient was diagnosed with cirrhosis. As only small numbers had severe MASH and no patients had cirrhosis it does limit the power of the data for significant associations of oWAT changes with MASH/cirrhosis. Therefore, advanced liver disease needs further investigation.

Nevertheless, this work adds to the understanding of early changes in steatotic liver disease (steatosis and ballooning but no inflammation and fibrosis). Indeed, studying these early disease phenotypes may be able to identify important factors and triggers that drive more severe disease.

As this is a very heterogeneous population large numbers of patients are likely needed to identify significant changes in maladaptive oWAT that correlate with MASLD progression in obesity, and this should be considered when interpreting these results. Finally, two-thirds of the cohort comprised of women, and it is known oestrogen has effects on insulin resistance; therefore, ideally more males would be included in this cohort to reduce the possibility this is a confounding factor when interpreting these results. Future work would therefore have a larger patient cohort with more male recruits and should include more severe liver phenotypes, enabling direct comparison with end point markers for clinical trials i.e. fibrosis. Furthermore, we have not included lean patient liver tissue, and this study would benefit from including paired tissue from patients undergoing routine abdominal surgery with a normal BMI.

6.4.7 Future work

While my results show that adipocyte hypertrophy, collagen I and collagen VI all are associated with early changes in MASLD (ballooning and steatosis), I have analysed a relatively limited number of significant genes/ histological readouts for oWAT dysfunction and have only used histological readouts to analyse MASLD progression therefore, it would be interesting to perform a more extensive comparison of maladaptive oWAT and liver disease to identify if there are pathways that link the two tissues in prolonged obesity. I would also like to compare larger numbers of severe phenotypes, particularly increased numbers of patients with MASH fibrosis and cirrhosis to determine if this then demonstrates an association with oWAT inflammation. Therefore, I believe multi-tissue profiling using multi-omics is the next important step in identifying possible pathways or targets involved in oWAT and liver crosstalk.

Paired RNA-seq of oWAT and liver, either bulk or at a single cell level and proteomics could yield potential pathways involved in crosstalk. However, the difficulty with this design is that samples collected are often small, particularly from the liver, and thus limits multiple different analysis options from one patient.

Non-targeted lipidomics has been extensively performed on liver tissue for the whole spectrum of MASLD but relatively few studies analyse the changing lipidome across several tissues during MASLD disease progression and metabolic decline. Ooi et al. showed very little change between liver lipids and MASLD severity and again no correlation with visceral and subcutaneous lipid signatures and MASLD in a bariatric cohort [504]. The limitation of using non-targeted approach to lipidomics is that they are semi-quantitative [504]. Targeted lipid omics, such as focusing on the bioactive lipids, oxylipins, are more accurate and sensitive which may be beneficial when studying lipid species such as these that are potent at very low levels. Therefore, in the next chapter I aim to perform multi-tissue profiling of oxylipins focusing firstly on the transition from lean to obese and metabolically unhealthy and secondly on the progression of MASLD to MASH using plasma, oWAT and liver tissue from this cohort.

Chapter 7: Multi-tissue profiling of PUFA-derived oxylipins in obesity during transition to T2DM and MASLD

7.1 Introduction

Polyunsaturated fatty acid (PUFA) metabolism is central to adipose tissue health. PUFAs such as arachidonic acid (AA), linoleic acid (LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reside in cell membrane phospholipids or can be stored within triacylglycerols (TGs). PUFA-containing TGs increase during obesity in human WAT[505]. Upon cell activation, PUFAs are released and subsequently oxidized by three sets of distinct enzymes [lipoxygenases (LOX), cyclooxygenases (COX) and cytochrome P450 (CYP) family enzymes [321, 352] to produce oxylipins.

These short-lived soluble lipids have been shown to have a multifaceted role in controlling WAT homeostasis; oxylipins orchestrate lipid metabolism and adipocyte differentiation through binding to nuclear transcription factors and regulating their activity [328, 411] specifically PPARs [405, 412, 506-509], including PPAR γ [510, 511] an important transcription factor for adipocyte end differentiation. Oxylipins have also extensively been shown to accompany immune homeostasis and tissue inflammation and are involved in both pro-inflammatory and pro-resolving pathways. They control tissue resident macrophage maintenance through TLR signalling pathways, transcellular shuttling between cell types, known as transcellular biosynthesis and can undergo class-switching from proinflammatory to pro-resolving phenotype depending on the microenvironment [413]. Oxylipins have also been implicated in vascular endothelial homeostasis and activate cell signalling pathways involved in angiogenesis migration and proliferation, specifically CYP- derived oxylipins activate vascular endothelial growth factor (VEGF) a key factor in angiogenesis and coronary artery endothelial relaxation [414, 512].

Since WAT remodelling during obesity is defined by metabolic, adipogenic, macrophage-mediated inflammation and ECM changes [123, 128], it is highly likely that oxylipins therefore have significant roles in regulating WAT adaptations and are involved in crosstalk with other metabolic organs such as the liver. Furthermore, the crosstalk between the adipose tissue and the liver are an important feature of obesity-mediated T2DM and MASLD as previously discussed in Chapter 6, therefore tissue-specific oxylipin signatures could provide an understanding of the role of these bioactive lipids have during development of metabolic syndrome and liver disease.

While oxylipins have been studied in association with obesity/T2DM or MASLD/Metabolic dysfunction-associated steatohepatitis (MASH) in human patients [333, 335, 343, 345-348,

350, 364, 366, 367, 513-520], it has mainly been limited to the plasma. COX and LOX-derived oxylipins were generally found to increase during metabolic syndrome [335, 345, 347, 348, 366, 516, 520] however, to date, there is no study describing cross-tissue oxylipin dynamics in the context of evolving visceral WAT and liver phenotypes during obesity, T2DM and MASLD.

7.2 Aims of chapter

The aim of this chapter is to firstly characterise the PUFA-derived lipidome in obesity and obesity related T2DM in humans by measuring oxylipin levels in paired oWAT, plasma and liver samples using UPLC-MS/MS. Secondly, I aim to compare oWAT, plasma and liver oxylipin lipid signatures with MASLD severity to characterise their role in the crosstalk between adipose and liver tissue.

To address these aims I quantified 5 PUFAs and 71 PUFA-related oxylipin species in WAT, liver and plasma and correlated each species with;

- 1) lean and obese patients with and without T2DM to assess whether metabolic dysfunction is associated with specific oxylipin signatures in obesity.
- 2) WAT phenotypes (adipogenesis, adipocyte hypertrophy, macrophage infiltration, ECM remodelling) to determine if oxylipins are associated with maladaptive WAT dysfunction.
- 3) histopathological severity of MASLD (NAS score or percentage of ballooning, fat, inflammation, and collagen quantified in each biopsy using AI) to establish if the signatures observed are associated with liver disease.
- 4) I used the previously described *ex vivo* explant model to measure oxylipins in oWAT of bariatric patients in the presence and absence of Metformin to determine if Metformin modulates oxylipins as a large proportion of patients in the cohort are treated with this medication.

As described in the methods chapter any metabolite that was detected in less than 50% of patient samples was discarded from the analysis. I have summarised the analysis, which used a filtering approach, of oWAT and plasma oxylipins in obesity and T2DM in the flow chart below (**Figure 64**).

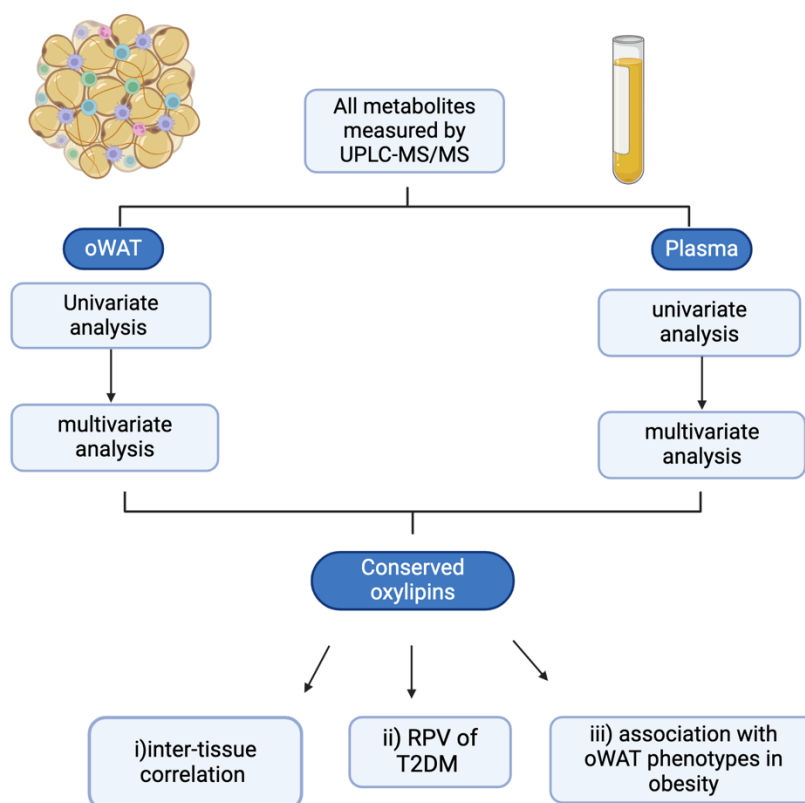


Figure 64 Flow chart to demonstrate filtering strategy when analysing oxylipins measured in the oWAT and plasma. RPV: relative predictive value; UPLC-MS/MS: ultra-performance-liquid-chromatography-mass spectrometry

7.3 PUFA-derived profiling in obesity and T2DM

7.3.1 PUFA-derived profiling in the oWAT

A primary focus of the overall project was to characterise how oWAT homeostasis is modulated by obesity and its relationship with metabolic decline and liver pathology, therefore I began by analysing how the oxylipin signature in adipose tissue changes with obesity. The oWAT from the cohort of patients characterised in chapter four was used to measure the oxylipins of N=88 obese (N=27 patients with T2DM and N=71 without T2DM) and N=9 lean patients using UPLC-MS/MS. Using the patient-derived omental WAT, I analysed 20-carboxy-AA, AA, LA, EPA and DHA levels, as well as 38 unesterified PUFA-derived oxylipins amongst which there were 19 CYP-derived, 10 LOX-derived and 9 COX-derived metabolites, including 7 readouts of sEH activity (epoxide:diol ratio); and reported the significant changes between lean, obese and diabetic obese groups (**Figure 65**).

7.3.2 Results: Univariate analysis

Firstly, I used a heatmap to review all the oxylipin species detected in the N=88 obese patients and N=9 lean patients (**Figure 65**). The top 10 oxylipins were further analysed

individually to visualise the difference between the groups (lean, obese and obese patients with T2DM) (**Figure 66**).

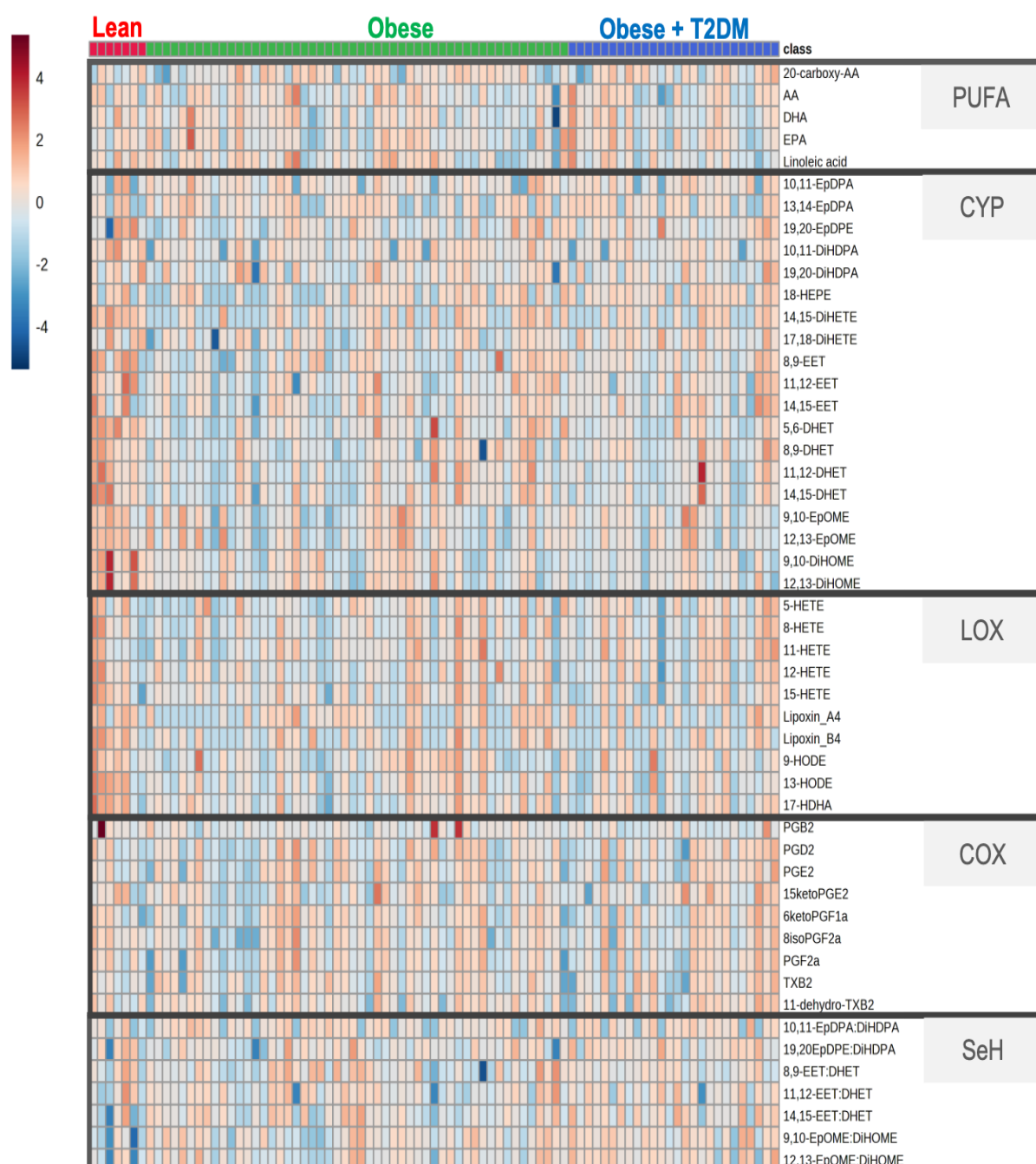


Figure 65 Heatmaps of metabolites measured in patients' oWAT. Heatmap showing PUFAs, CYP-, LOX-, COX-derived oxylipins, and sEH activity readout in lean patients (N=7), patients with obesity (N=52) and patients with obesity and T2DM (N=26). Heatmaps was generated on normalized data (log10 transformed) and clustered using Ward. PUFA: polyunsaturated fatty acid; CYP: cytochrome P450; LOX: lipoxygenase; COX: cyclooxygenase; T2DM: Type 2 diabetes mellitus.

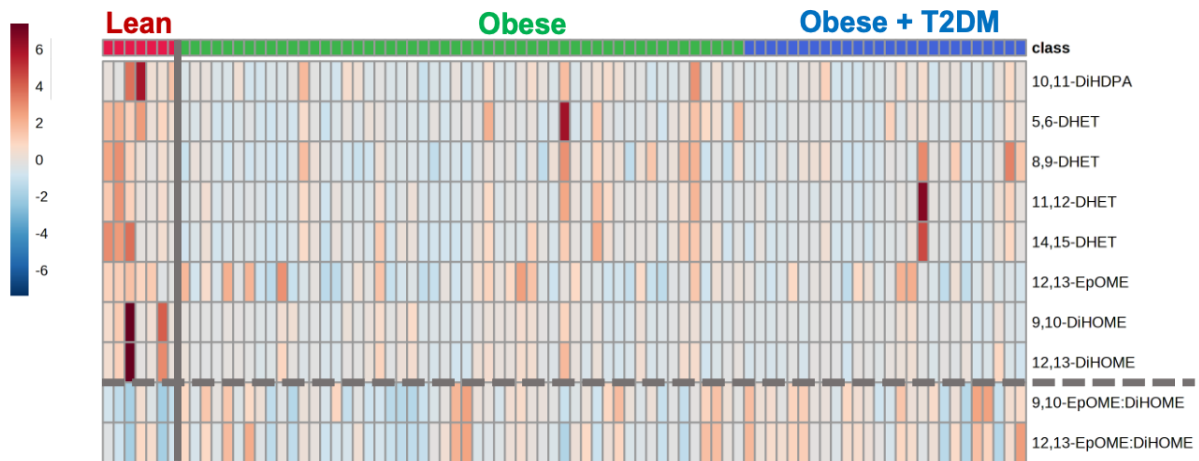


Figure 66 10 most significantly changing oxylipins (FDR <0.2). and sEH activity ratios (separated by a dashed line) determined by Kruskal-Wallis test (see also Table 13) Heatmap was generated on normalized data (log10 transformed) and clustered using Ward. sEH: soluble epoxide hydrolase.

While the heatmaps clearly demonstrate there is inter-patient variation in oxylipins there are still strikingly significant changes observed within the 8 CYP-derived metabolites decreasing with obesity (10,11-DiHDPAs, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 12,13-EpOME, 9,10-DiHOME, and 12,13-DiHOME), especially in AA, LA and DHA-derived fatty acid diols (**Figure 67**) in the oWAT. 9,10-EpOME:DiHOME and 12,13-EpOME:DiHOME ratios were also increased in patients with obesity, indicative of reduced sEH activity (**Figure 67**). The false detection rate (FDR) was calculated to be 0.026-0.2 for the top 10 significant oxylipin species measured in the oWAT (**Table 14**).

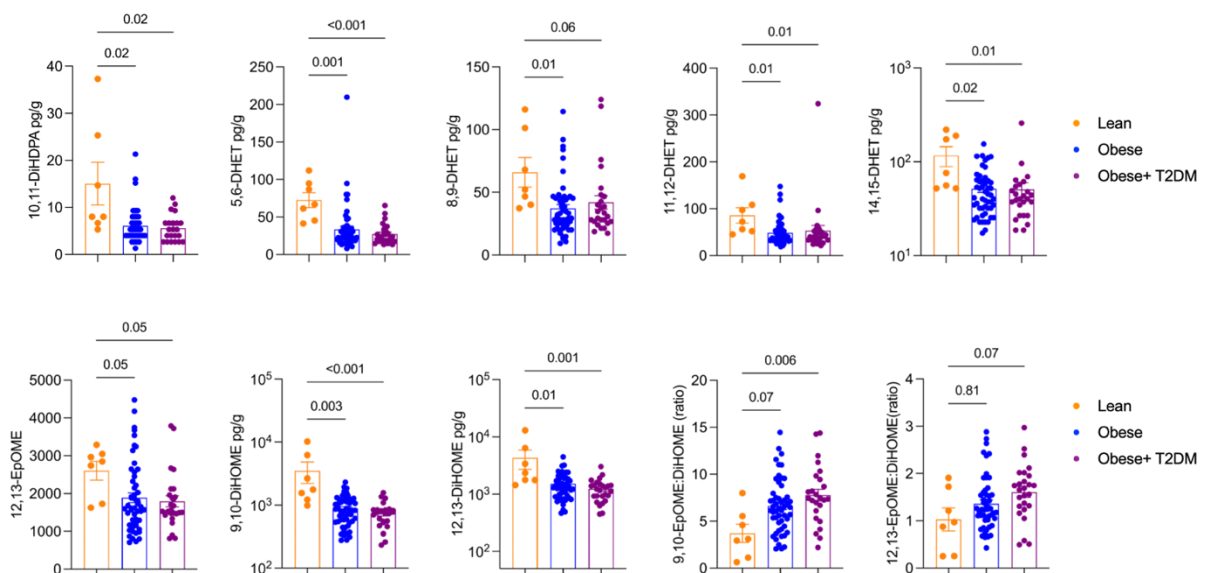


Figure 67 CYP-derived epoxides and diols measured in oWAT of lean patients (N=7), patients with obesity (N=52) and patients with obesity and T2DM (N=26). Significance was tested by Kruskal-Wallis test followed by Dunn's multiple comparison. CYP: cytochrome P450; T2DM: Type 2 diabetes mellitus.

7.3.3 PUFA-derived profiling in the plasma

As oxylipins are potent short-acting lipids tightly controlled at a cellular level, I wanted to establish if the finding described in the oWAT, namely a decrease in diols and an increase in epoxide:diol ratio, were tissue specific or whether there was a systemic effect. Therefore, next I performed similar univariate analyses using the patient-derived plasma. I detected and measured PUFAs, 20-carboxy-AA, AA, LA, EPA and DHA levels and 40 PUFA-derived oxylipins (22 CYP-derived, 12 LOX-derived and 6 COX-derived metabolites and 7 readouts of sEH activity) (**Figure 68**)

7.3.4 Results: univariate analysis

All the metabolites measured were represented in the heatmap in figure 68. The top 10 significantly changing oxylipins measured in the plasma were then summarised in a further heatmap (**Figure 68**). Each metabolite was compared (lean, obese and obese patients with T2DM) individually in figure 68 for the top 10 significant metabolites measured in the plasma. A further 2 oxylipins and sEH ratio measured in the plasma that were similarly observed to significantly change in oWAT were also included (**Figure 68**).

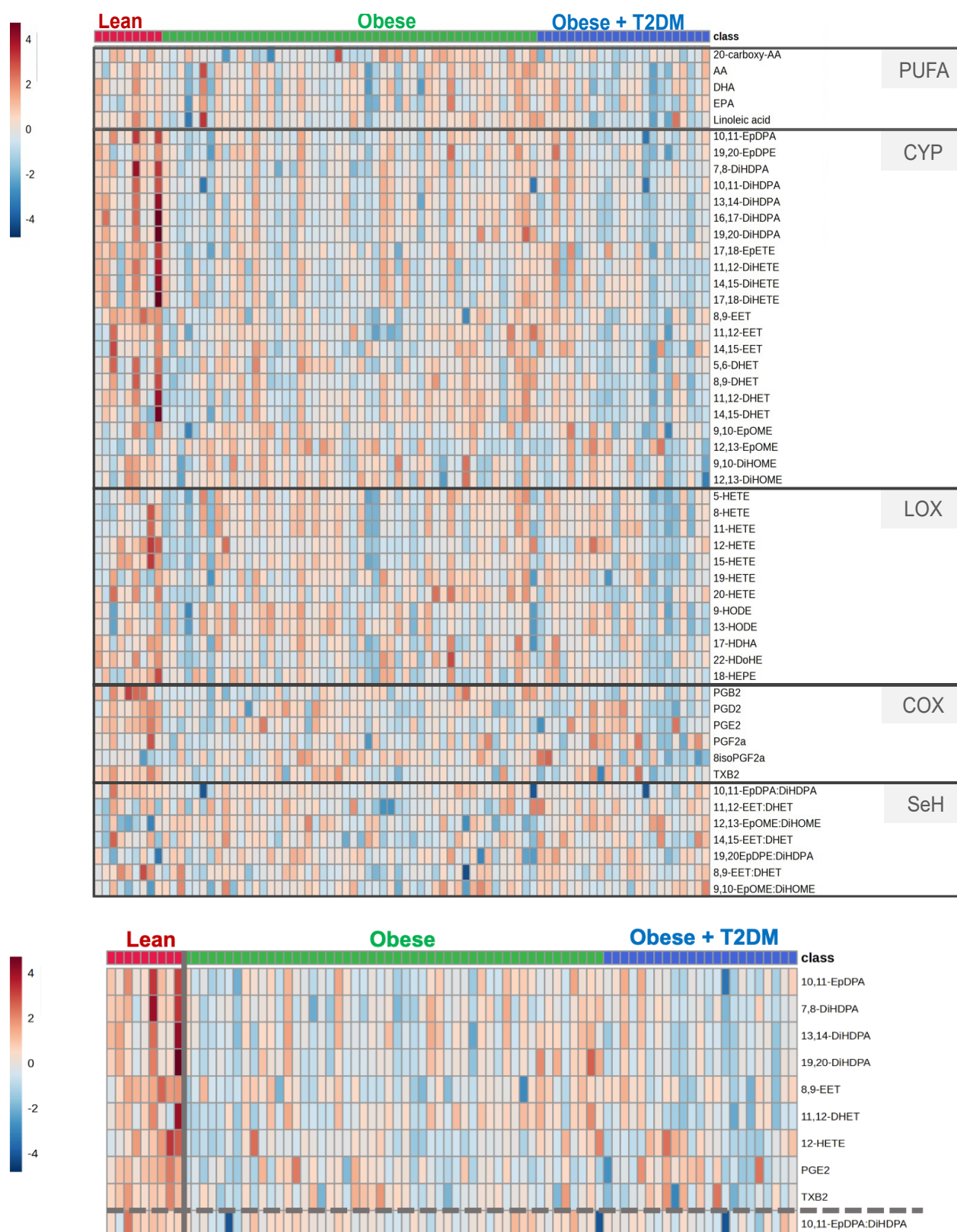


Figure 68 Heatmaps of metabolites measured in patients' plasma **(A)** Heatmap showing PUFAs, CYP-, LOX-, COX-derived oxylipins, and sEH activity readout in lean patients (N=9), patients with obesity (N=50) and patients with obesity and T2DM (N=25). **(B)** 10 most significantly changing (FDR<0.05) PUFA derived oxylipins and sEH activity ratios (separated by a dashed line) determined by Kruskal-Wallis test (see also Table 13) Heatmaps were generated on normalized data (log10 transformed) and clustered using Ward. PUFA: polyunsaturated fatty acid; CYP: cytochrome P450; LOX: lipoxygenase; COX: cyclooxygenase; T2DM: Type 2 diabetes mellitus; sEH: soluble epoxide hydrolase.

The most significant changes observed when measuring oxylipins in the plasma, like in oWAT, were mostly CYP-derived (including many DHA-derived oxylipins) and down-regulated in patients with obesity when compared to lean individuals. (**Figure 69**). These included 10,11-EpDPA, 7,8-DiHDPA, 13,14-DiHDPA, 19,20-DiHDPA, 8,9-EET, PGE2, TXB2 and 12-HETE and 3 CYP-derived epoxides that were also observed to change significantly in the WAT(5,6-DHET, 10,11-DiHDPA 11,12-DHET) (**Figure 69**). 12,13-EpOME:DiHOME and 19,20-EpDPE:DiHDPA ratios were also significantly increased, similar to the observations seen in WAT (FDR: **Table 14** range 0.005-0.1 in 13 plasma metabolites).

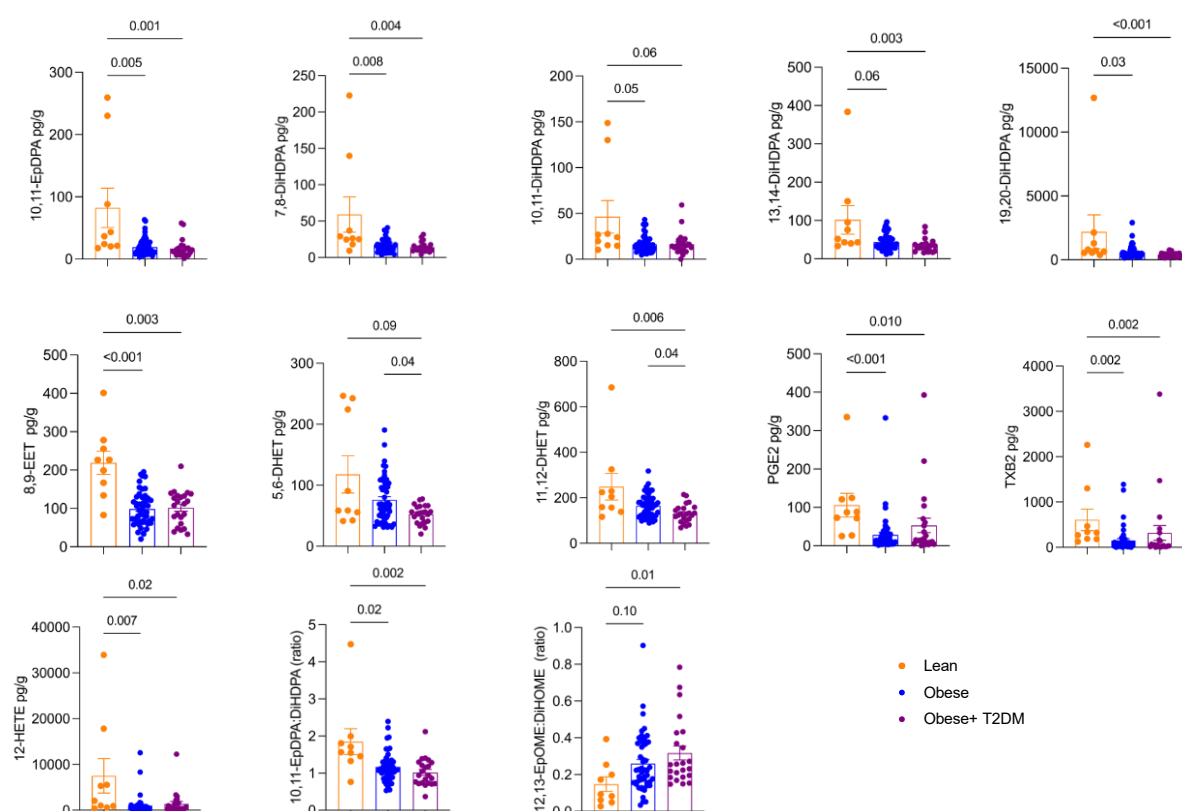


Figure 69 CYP- and COX-derived oxylipins in the plasma of lean patients (N=9), patients with obesity (N=50) and patients with obesity and T2DM (N=25). Significance was tested by Kruskal-Wallis test followed by Dunn's multiple comparison. CYP: cytochrome P450; COX: cyclooxygenase; T2DM: Type 2 diabetes mellitus; sEH: soluble epoxide hydrolase.

Table 14 . oWAT and plasma oxylipin quantification. Oxylipins were quantified in control (N=7), obese (N=52) and obese with T2DM patients (N=26). All significant metabolites decreased while epoxide:diol ratios increased with obesity and T2DM, except for 10,11-EpDPA: DiHDPA. Statistical significance was tested between the three groups using Kruskal-Wallis test; False Discovery Rate (FDR) calculated using Benjamini-Hochberg; n.d., not detected.

oxylipin	WAT		Plasma		oxylipin	WAT		Plasma	
	p-value	FDR	p-value	FDR		p-value	FDR	p-value	FDR
20-carboxy-AA	0.77	0.87	0.09	0.15	5-HETE	0.66	0.82	0.51	0.57
AA	0.81	0.90	0.14	0.22	8-HETE	0.43	0.71	0.81	0.83
DHA	0.74	0.86	0.17	0.26	11-HETE	0.58	0.78	0.93	0.93
EPA	0.93	0.97	0.62	0.66	12-HETE	0.51	0.74	0.008*	0.037
Linoleic acid	0.31	0.56	0.015*	0.055	15-HETE	0.28	0.53	0.051	0.10
10,11-EpDPA	0.54	0.74	0.0015*	0.020	19-HETE	n.d.	n.d.	0.50	0.57
13,14-EpDPA	0.52	0.74	n.d.	n.d.	20-HETE	n.d.	n.d.	0.045*	0.10
16,17-EpDPA	0.12	0.29	n.d.	n.d.	Lipoxin_A4	0.12	0.29	n.d.	n.d.
19,20-EpDPE	0.06	0.22	0.287	0.38	Lipoxin_B4	0.13	0.30	n.d.	n.d.
7,8-DiHDPA	n.d.	n.d.	0.004*	0.023	9-HODE	0.10	0.28	0.49	0.57
10,11-DiHDPA	0.014*	0.11	0.049*	0.10	13-HODE	0.046*	0.21	0.28	0.38
13,14-DiHDPA	n.d.	n.d.	0.004*	0.023	17-HDHA	0.1	0.28	0.16	0.25
16,17-DiHDPA	n.d.	n.d.	0.014*	0.055	22-HDoHE	n.d.	n.d.	0.015*	0.055
19,20-DiHDPA	0.39	0.69	0.001*	0.017	18-HEPE	n.d.	n.d.	0.27	0.37
17,18-EpETE	n.d.	n.d.	0.019*	0.057	PGB2	0.14	0.31	0.008*	0.037
18-HEPE	0.46	0.73	n.d.	n.d.	PGD2	0.74	0.86	0.017	0.057
11,12-DiHETE	n.d.	n.d.	0.043*	0.10	PGE2	0.86	0.93	0.001*	0.017
14,15-DiHETE	0.07	0.24	0.18	0.26	15ketoPGE2	0.60	0.78	n.d.	n.d.
17,18-DiHETE	0.15	0.32	0.08	0.14	6ketoPGF1a	0.89	0.95	n.d.	n.d.
8,9-EET	0.06	0.22	<0.0001*	0.005	PGF2a	0.95	0.97	0.22	0.31
11,12-EET	0.11	0.29	0.05	0.10	8isoPGF2a	0.54	0.74	0.021*	0.059
14,15-EET	0.61	0.78	0.55	0.60	TXB2	0.51	0.74	0.002*	0.020
5,6-DHET	<.001*	0.026	0.023*	0.059	11-dehydro-TXB2	0.41	0.70	n.d.	n.d.
8,9-DHET	0.018*	0.11	0.07	0.13	10,11-EpDPA:DiHDPA	0.22	0.43	0.003*	0.023
11,12-DHET	0.018*	0.11	0.004*	0.023	19,20EpDPE:DiHDPA	0.047*	0.21	0.047*	0.10
14,15-DHET	0.013*	0.11	0.07	0.13	8,9-EET:DHET	1.00	1.00	0.022*	0.059
9,10-EpOME	0.18	0.37	0.34	0.41	11,12-EET:DHET	0.74	0.86	0.34	0.41
12,13-EpOME	0.046*	0.21	0.42	0.50	14,15-EET:DHET	0.09	0.28	0.81	0.83
9,10-DHOME	<.001*	0.026	0.08	0.14	9,10-EpOME:DiHOME	0.006*	0.077	0.333	0.41
12,13-DHOME	0.002*	0.034	n.d.	n.d.	12,13-EpOME:DiHOME	0.026*	0.15	0.019*	0.057

7.3.5 Conclusions: conservation of 3 oWAT and plasma oxylipins and sEH ratio

Overall, both oWAT and plasma had a global decrease in measured diols and decrease in sEH activity, indicated by the increased epoxide:diol ratio. Notably, the commonly down-regulated oxylipins in obese WAT and plasma included 3 CYP-derived diols (5,6-DHET, 10,11-DiHDPA 11,12-DHET). Consistent with the decreased sEH activity in WAT, 12,13-EpOME:DiHOME ratio was similarly increased in the plasma of patients with obesity when compared to lean individuals (**Figure 69**).

7.3.6 Multivariate analysis of conserved oxylipins

The univariate analysis identified 3 CYP-derived diols and one sEH ratio that were altered in obesity in both oWAT and plasma. In chapter 4 I have previously described that the cohort had a larger proportion of females in the obese groups versus the lean group and the lean patients were slightly older, therefore it was important to determine if these were confounding factors that lead to the oxylipin signature observed. I performed multivariate linear regression analysis for each of the conserved metabolites and ratios. When metabolite measurements were adjusted for cofounders age, sex and ethnicity in the oWAT 5,6-DHET ($p=0.003$) 10,11-DiHDPA ($p=0.002$) and 11,12-DHET ($p=0.05$) and 12,13-EpOME:DiHOME ($p=0.021$) remain significantly altered in obesity (**Figure 70**). The plasma metabolites were adjusted for cofounders age, sex ethnicity and fasting status and 10,11-DiHDPA ($p=0.011$) and 12,13-EpOME:DiHOME ($p=0.03$) also remained significantly modulated in obesity (**Figure 70**).

A

5,6-DHET	R	R ²	Beta	t	Sig.
	0.541	0.292			
Age			0.002	0.196	0.055
Sex [Female=1 male=2]			0.073	0.231	0.025
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.015	-0.136	0.175
Obese[lean=1, obese=2]			0.104	-0.344	0.003*
Total: 85					

11,12-DHET	R	R ²	Beta	t	Sig.
	0.459	0.211			
Age			0.002	0.113	0.291
Sex [Female=1 male=2]			0.063	0.294	0.007
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.013	-0.165	0.12
Obese[lean=1, obese=2]			0.09	-0.232	0.05*
Total: 85					

10,11-DiHDDPA	R	R ²	Beta	t	Sig.
	0.506	0.256			
Age			0.002	0.208	0.062
Sex [Female=1 male=2]			0.079	0.018	0.873
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.015	-0.196	0.079
Obese[lean=1, obese=2]			0.106	-0.402	0.002*
Total: 85					

12,13-EpOME:DiHOME	R	R ²	Beta	t	Sig.
	0.299	0.089			
Age			0.002	-0.047	0.684
Sex [Female=1 male=2]			0.067	0.013	0.912
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.013	0.082	0.468
Obese[lean=1, obese=2]			0.096	0.295	0.021*
Total: 85					

B

5,6-DHET	R	R ²	Beta	t	Sig.
	0.341	0.116			
Age			0.002	0.035	0.764
Sex [Female=1 male=2]			0.071	-0.153	0.199
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.014	0.043	0.704
Fasted [Fasted=1 non-Fasted=2]			0.059	0.246	0.055
Obese[lean=1, obese=2]			0.099	-0.152	0.285
Total: 82					

11,12-DHET	R	R ²	Beta	t	Sig.
	0.435	0.19			
Age			0.001	0.047	0.671
Sex [Female=1 male=2]			0.049	-0.177	0.122
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.01	0.004	0.969
Fasted [Fasted=1 non-Fasted=2]			0.041	0.285	0.021
Obese[lean=1, obese=2]			0.069	-0.234	0.086
Total: 82					

10,11-DiHDDPA	R	R ²	Beta	t	Sig.
	0.523	0.274			
Age			0.002	0.282	0.01
Sex [Female=1 male=2]			0.08	-0.228	0.039
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.016	-0.076	0.464
Fasted [Fasted=1 non-Fasted=2]			0.068	0.07	0.551
Obese[lean=1, obese=2]			0.112	-0.34	0.011*
Total: 82					

12,13-EpOME:DiHOME	R	R ²	Beta	t	Sig.
	0.466	0.217			
Age			0.002	0.003	0.98
Sex [Female=1 male=2]			0.089	0.199	0.077
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.018	0.036	0.732
Fasted [Fasted=1 non-Fasted=2]			0.074	-0.289	0.018
Obese[lean=1, obese=2]			0.124	0.293	0.03*
Total: 82					

Figure 70 Multivariate analysis of CYP-derived diols conserved between oWAT and plasma. Linear regression tables for CYP-derived diols and 12,13-EpOME:diHOME measured in oWAT (**A**) and plasma (**B**), adjusted for age, sex and ethnicity; N=85 for oWAT; N=82 for plasma. Statistical significance was calculated using a linear regression model with normalization of dependent variable by log transformation of the metabolite. CYP: cytochrome P450.

The commonly down-regulated CYP-derived diols and up-regulated 12,13-EpOME:DiHOME ratio (summarised in **Figure 71**) in obese oWAT and plasma were further analysed in relation to (i) their inter-tissue correlation (i.e. WAT vs. plasma), (ii) their relative predictive value of T2DM (iii) their association with WAT phenotypes (**Figure 72-73 and Table 15**),

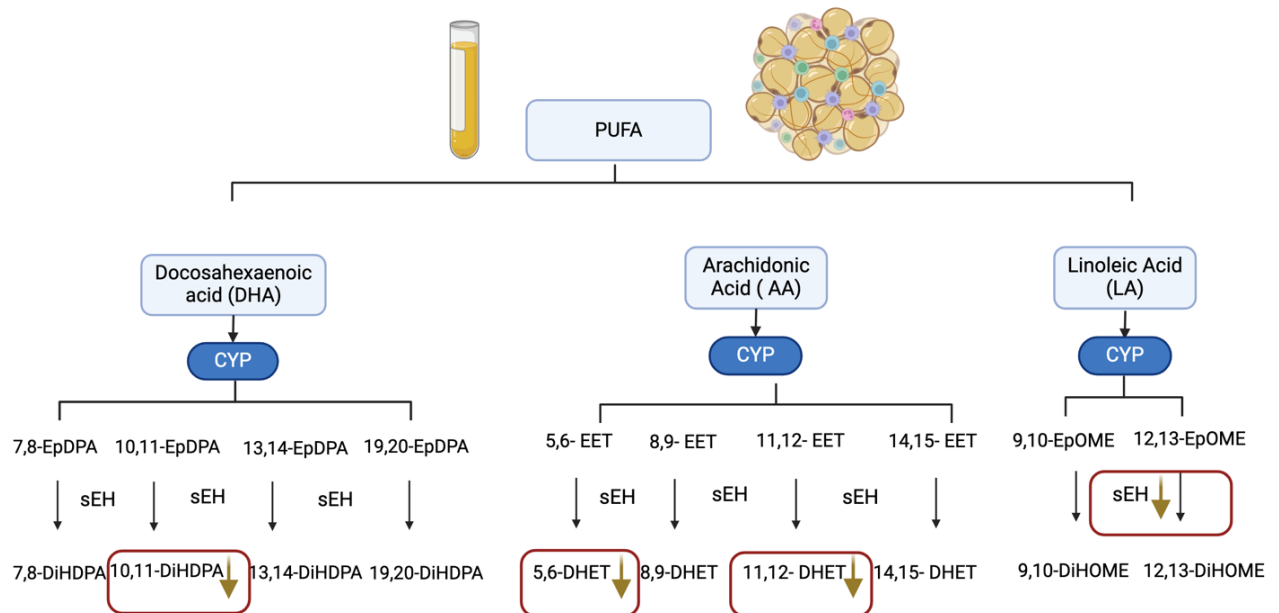


Figure 71 Schematic of PUFA derived CYP-sEH pathway. Significant decrease in diols (10,11-DiHDPA, 5,6-DHET and 11,12-DHET) and sEH (12,13-EpOME:DiHOME) in plasma and oWAT are highlighted in red. Image created by Bio Render®

7.3.7 Inter-tissue correlations of oxylipins

Inter-tissue analysis of the conserved metabolites aimed to establish if there was a cross-tissue effect; this would potentially provide a plasma biomarker or treatment target. The 3 diols measured in the WAT were shown to correlate significantly with the measurements in the plasma (10,11-DiHDPA; $r=0.41$, $p=0.0008$, 5,6-DHET; $r=0.4$, $p=0.0009$ and 11,12-DHET; $r=0.26$, $p=0.02$) though 12,13-EpOME:DiHOME ratio did not show correlation ($r=0.01$, $p=0.94$) (Figure 72).

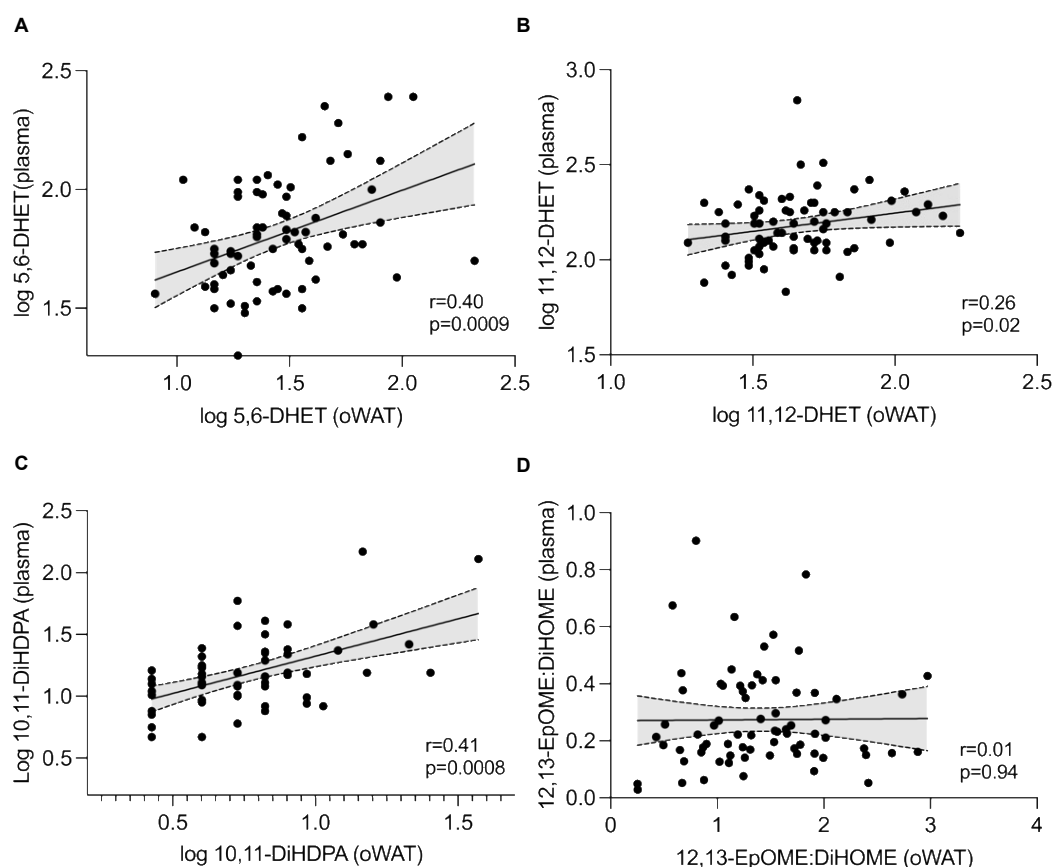


Figure 72 Correlation of the 3 CYP-derived epoxides (pg/g) and 12,13-EpOME:DiHOME ratio between plasma and oWAT (N=72) Significance was tested by Spearman's correlation coefficient. PUFA: polyunsaturated fatty acid; CYP: cytochrome P450

7.3.8 Relative predictive value of T2DM through plasma oxylipins

Furthermore, the conserved oxylipins and sEH ratio could be a useful tool if plasma oxylipins could be used to predict metabolic dysfunction. The area under the curve (AUC) was used to compare the T2DM diagnostic accuracy of 10,11-DiHDPA, 5,6-DHET, 11,12-DHET and 12,13-EpOME:DiHOME measured in the plasma. 11,12-DHET had the highest diagnostic accuracy (AUC 0.71) followed by 5,6-DHET (AUC 0.69) 12,13-EpOME:DiHOME (AUC 0.64) and 10,11-DiHDPA (AUC 0.54) (Figure 73).

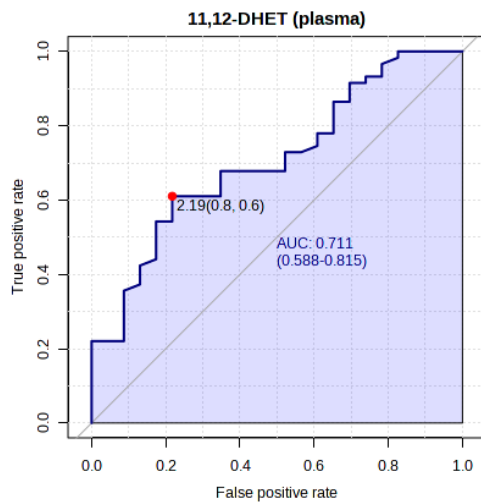
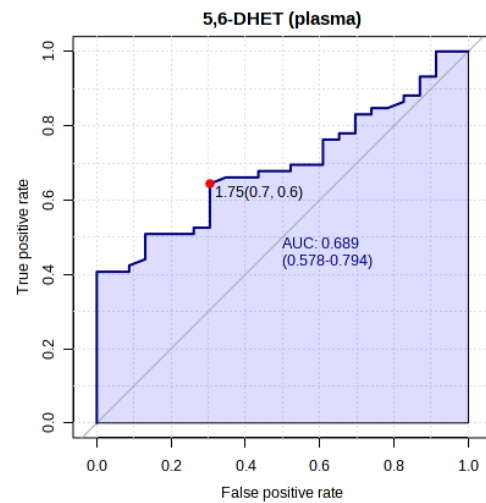
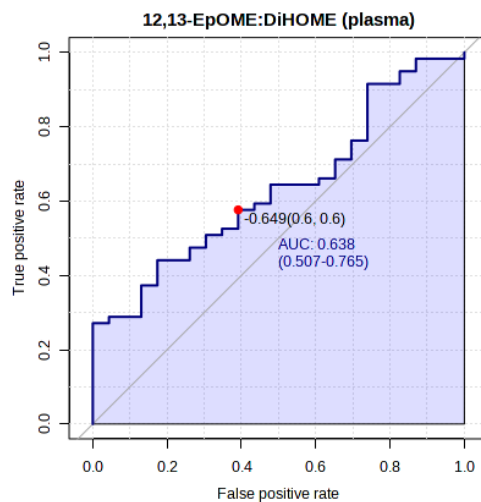
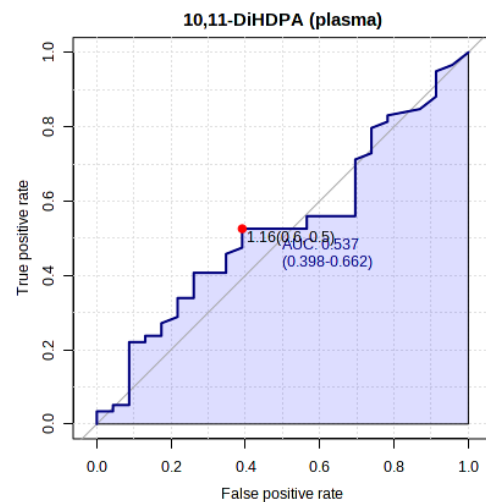
A**B****C****D**

Figure 73 AUC curves of 3 CYP-derived epoxides (pg/g) and 12,13-EpOME:DiHOME ratio for T2DM diagnostic accuracy (N=82). CYP: cytochrome P450; T2DM: Type 2 diabetes mellitus; sEH: soluble epoxide hydrolase; AUC: area under the operator curve.

7.3.9 Association of oWAT and plasma oxylipins with oWAT phenotypes

Oxylipins have been implicated in many important pathways involved in WAT homeostasis, whether as ligands for PPAR γ , a transcription factor critical for adipogenesis, or involved in pro-inflammatory or pro-resolving macrophage inflammation or as factors in angiogenesis. Therefore, I wanted to establish if the obesity-associated oxylipin signature seen in the oWAT and plasma is associated with oWAT dysfunction that I have described in chapter 4 and whether it could be a marker of pathological changes in the adipose tissue during the transition from lean to obese and metabolic decline. Clinical data (BMI, HbA1c) and phenotypic markers of adipose tissue (adipogenesis/hypertrophy, inflammation, ECM remodelling) were correlated with the 3 CYP-derived metabolites and 12,13-EpOME:DiHOME (**Table 15**) in both the oWAT and plasma.

Table 15 oWAT phenotyping and correlation with conserved oxylipins in oWAT and plasma. Correlation of oWAT (N= 83; left) and plasma (N=80; right) 10,11-DiHDPA, 5,6-DHET, 11,12-DHET and 12,13-EpOME:DiHOME with clinical (BMI, HbA1C) and oWAT (adipogenesis/hypertrophy, inflammation, ECM remodelling) phenotypes. Grey highlights denote significant associations. Significance was tested by Spearman's correlation coefficient.

	WAT oxylipins								Plasma oxylipins							
	10,11-DiHDPA		5,6-DHET		11,12-DHET		12,13-EpOME:DiHOME		10,11-DiHDPA		5,6-DHET		11,12-DHET		12,13-EpOME:DiHOME	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Clinical																
BMI (kg/m ²)	-0.155	0.179	-0.261	0.017	-0.175	0.113	0.073	0.513	-0.089	0.435	-0.083	0.466	-0.197	0.08	0.105	0.354
HbA1c (mmol/mol)	-0.072	0.534	-0.139	0.212	-0.135	0.224	0.138	0.213	0.102	0.373	-0.254	0.024	-0.144	0.206	0.047	0.683
Adipogenesis/hypertrophy																
Area (μm ²)	-0.188	0.103	-0.338	0.002	-0.382	<.001	0.24	0.03	-0.096	0.398	-0.327	0.003	-0.291	0.009	0.123	0.279
ADIPOQ mRNA	-0.048	0.677	-0.12	0.282	-0.271	0.014	0.003	0.98	0.076	0.510	0.029	0.797	-0.154	0.176	-0.007	0.953
PPARY mRNA	0.216	0.057	-0.037	0.742	-0.034	0.76	-0.043	0.697	0.171	0.132	-0.012	0.917	0.008	0.941	-0.084	0.457
Inflammation																
Crown-like structures (CLS)	-0.164	0.167	-0.097	0.393	-0.097	0.395	0.011	0.923	-0.066	0.571	-0.166	0.153	-0.03	0.798	-0.069	0.552
% mac SVF CD14 ⁺	0.163	0.446	0.048	0.818	0.217	0.287	0.458	0.018	0.179	0.393	-0.194	0.353	-0.138	0.512	0.131	0.533
CD68 mRNA	0.228	0.217	-0.163	0.348	0.158	0.364	0.379	0.025	0.146	0.409	-0.136	0.444	-0.165	0.352	0.061	0.732
TREM2 mRNA	-0.097	0.400	-0.09	0.418	-0.149	0.179	0.24	0.029	0.002	0.987	-0.034	0.766	-0.078	0.491	0.18	0.11
ECM remodelling																
Collagen I (a.u.)	-0.022	0.898	0.383	0.016	0.396	0.013	-0.259	0.112	0.125	0.456	0.19	0.132	0.311	0.012	-0.362	0.003
COL1A1 mRNA	0.171	0.134	0.33	0.002	0.335	0.002	-0.069	0.536	-0.220	0.052	-0.012	0.918	0.011	0.92	-0.175	0.121
Collagen VI (a.u.)	0.075	0.667	0.311	0.061	0.147	0.385	-0.311	0.061	0.227	0.183	0.122	0.346	0.267	0.036	-0.246	0.054
COL6A1 mRNA	0.142	0.214	0.132	0.236	0.045	0.687	-0.021	0.848	0.078	0.497	-0.021	0.855	-0.019	0.869	-0.005	0.966

Firstly, the most striking feature was that fatty acid diols significantly decreased as the size of adipocytes increased when measured in both WAT and plasma (**Table 15**) (5,6-DHET; oWAT $r=-0.338$ $p=0.002$, plasma $r=-0.327$, $p=0.003$ and 11,12-DHET; oWAT $r=0.382$ $p<0.01$, plasma $r=-0.297$, $p=0.009$).

Adipose tissue collagen I fluorescence intensity and COL1A1 mRNA transcripts decrease as 5,6-DHET ($r=0.383$, $p=0.016$, $r=0.33$, $p=0.002$ respectively) and 11,12-DHET ($r=0.396$, $p=0.023$, $r=0.335$, $p=0.002$ respectively) decrease in the oWAT. Collagen I was also shown to negatively associate with adipocyte size in chapter 4. Furthermore, 11,12-DHET in both oWAT and plasma significantly decreased with adipose tissue collagen I immunofluorescence intensity (oWAT $r=0.396$ $p=0.013$, plasma $r=0.335$, $p=0.002$) (**Table 15**). Taken together these results suggest diols decrease with oWAT dysfunction.

On the other hand, 12,13-EpOME:DiHOME associates significantly with oWAT inflammation (% of macrophages in SVF; $r=0.458$, $p=0.018$, CD68; $r=0.379$, $p=0.025$ and TREM2 mRNA levels; $r=0.24$ $p=0.029$) suggesting as oWAT inflammation increases 12,13-EpOME:DiHOME ratio increases.

7.3.10 CYP pathway enzymes in the oWAT

As 3 CYP-derived oxylipins and sEH ratio are significantly altered in obesity in both plasma and oWAT, this suggests a dysregulation in the CYP pathway which can derive from changes in the expression levels of CYP enzymes (*CYP2J2*, *CYP2S1*, *CYP2C8*, *CYP2C9*) and/or epoxide hydrolase enzymes (*EPHX1* and *EPHX2*).

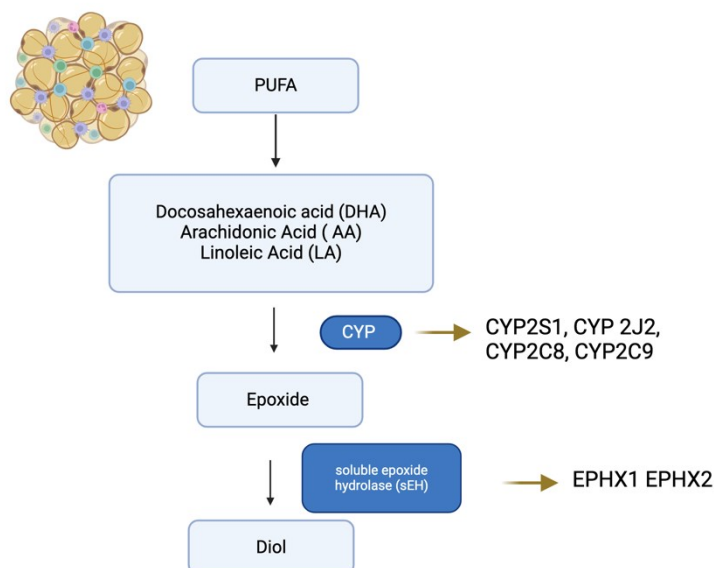


Figure 74. Schematic of PUFA derived CYP-sEH pathway including CYP and soluble epoxide hydrolase enzymes. CYP: cytochrome P450; PUFA: polyunsaturated fatty acid; sEH: soluble epoxide hydrolase. Image created by Bio Render®

I therefore, measured the expression of these key enzymes in the oWAT. No significant changes were found in the expression of *CYP2J2*, *CYP2S1*, *CYP2C8*, *CYP2C9*, *EPHX1*, *EPHX2* between lean, obese and diabetic obese groups (**Figure 75**). The results were similar for major LOX enzymes (*ALOX5*, *ALOX12* and *ALOX15*; **Figure 75**).

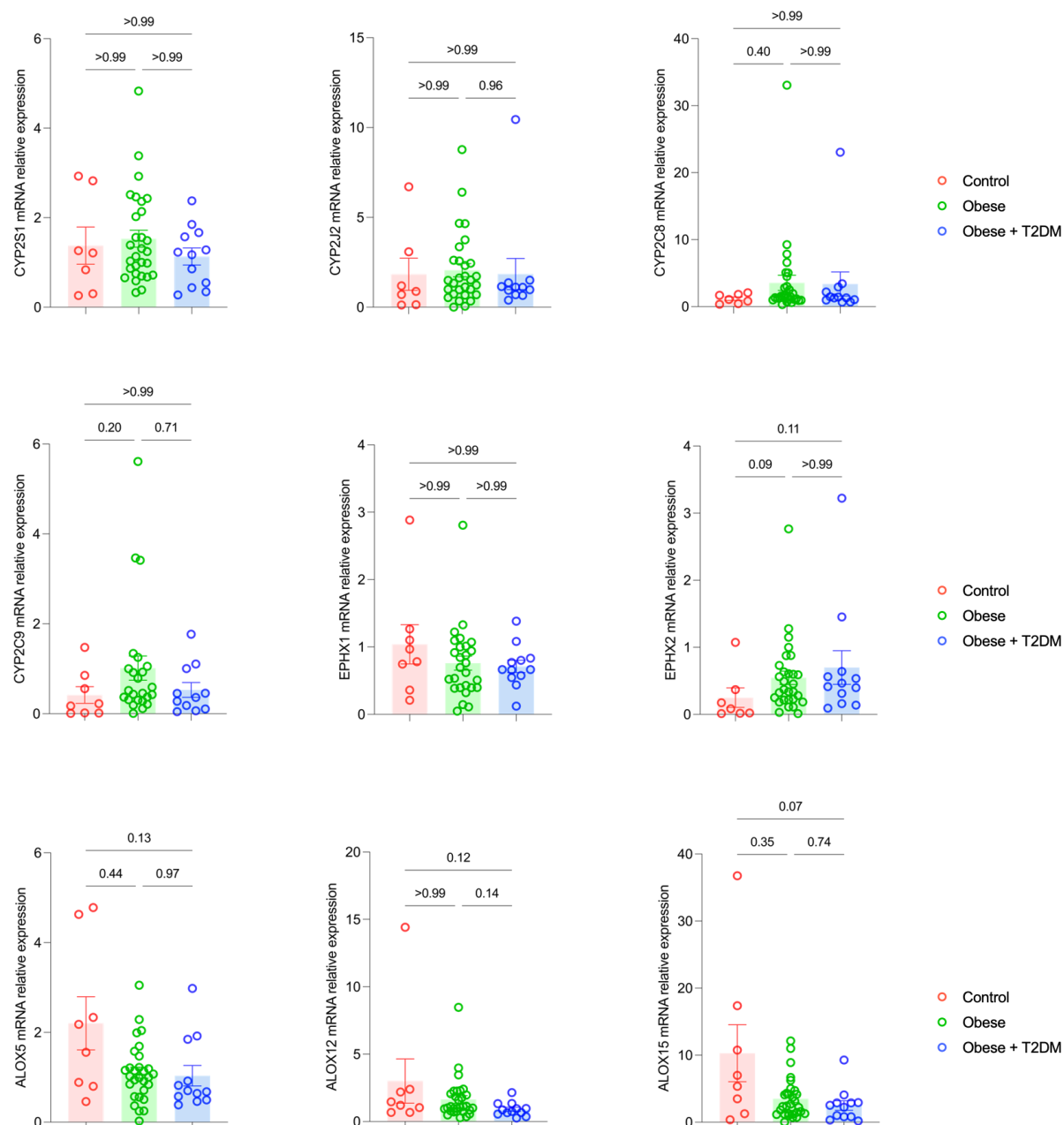


Figure 75 CYP, EPHX and ALOX enzyme expressions in lean, obese and obese/T2DM WAT of bariatric surgery patients. Expression of CYP2S1, CYP2J2, CYP2C8, CYP2C9, EPHX1, EPHX2, ALOX5, ALOX12, ALOX15 in the WAT of lean (N=8), obese (N=24) and obese /T2DM (N=11). Statistical significance by Kruskal-Wallis with Dunn's multiple comparison test. T2DM: Type 2 diabetes mellitus.

7.3.11 Metformin effect on oWAT oxylipins

Finally, a cofounding factor is the wide usage of Metformin in our bariatric cohort patients. Indeed, 74% of T2DM patients with obesity were prescribed Metformin (**Table 11**). In order to test whether Metformin can have an effect on WAT-derived oxylipin production, an *ex vivo* explant model was used (previously described in the methods chapter 3 and chapter 4 and 5). The 7 explants were cultured in the presence and absence of Metformin at 1 μ M and UPLC-MS/MS was then performed. With the exception of 5,6-DHET, there was no significant change in quantities of 10,11-DiHDPA and 11,12-DHET and 12,13-EpOME:DiHOME (Figure 76). Soluble epoxide hydrolase enzyme transcripts (EPHX1, and EPHX2) were also measured in the explants and no significant differences were seen, suggesting Metformin does not modulate the oxylipin signatures in the oWAT.

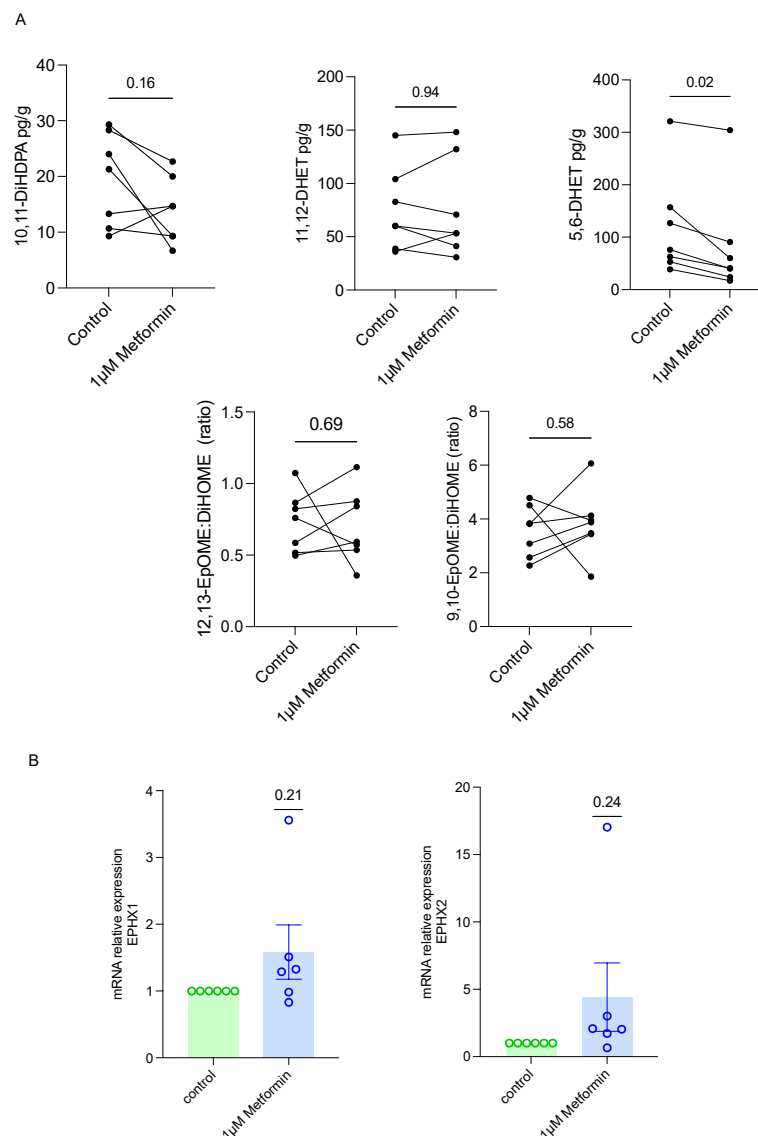


Figure 76 6. *Ex vivo* Metformin effect on fatty acid diols and epoxide:diol in explants isolated from bariatric surgery patients. (A) 10,11-DiHDPA, 11,12-DHET. 5,6-DHET, 9,10-EpOME: DiHOME and 12,13-EpOME:DiHOME levels in explants treated with or without 1 μ M Metformin (N=7). **(B)** Expression of EPHX1

and EPHX2 mRNA in explants treated with or without 1 μ M Metformin (N=7). Statistical significance measure by Wilcoxon matched pairs signed rank test (**A and B**).

7.3.12 Conclusions: Changes in the CYP-sEH pathway during transition to obesity and T2DM

PUFA-derived oxylipin species were measured in the oWAT and plasma of lean, obese and obese patients with T2DM and subjected to comprehensive univariate analysis which identified a significant oxylipin signature in obesity and metabolic syndrome. The analysis of oWAT and plasma oxylipins were further subjected to a filtering approach that included identifying (i) conserved associations between oWAT and plasma, (ii) significance retained after multivariate linear regression, (iii) association with markers of oWAT dysfunction (adipocyte size, ECM remodelling and inflammation) iv) and Metformin-independent association in oWAT.

Following the univariate and multivariate analysis (adjusting for age, sex, ethnicity, and fasting status) in both oWAT and plasma there was an overall decrease in primarily CYP-derived diols and decrease in sEH activity readout (epoxide:diol ratio) in obesity.

Specifically, there was a conserved decrease in 3 CYP-derived diols (10,11-DiHDPA, 11,12-DHET, 5,6-DHET) and decrease in sEH activity (12,13-EpOME:DiHOME ratio) between oWAT and plasma oxylipins in obesity. The 3 CYP-derived diols measured in the oWAT significantly positively correlated with the plasma levels, however sEH activity did not.

Next, I analysed the relationship of this oxylipin signature with the 3 CYP-derived diols in obesity with progression of WAT dysfunction. Adipocyte size increased with decreasing 5,6-DHET and 11,12-DHET in oWAT and plasma. Collagen I also decreased with 5,6-DHET and 11,12-DHET in the oWAT and 11,12-DHET in both oWAT and plasma.

As the 3 diols that were predominantly modulated in obesity and conserved between oWAT and plasma, were CYP derived, it was therefore important to establish if this was due to modulations in enzymes involved in the CYP-sEH pathways. However, CYP enzyme transcripts coding for the most highly expressed genes CYP2 J2, CY2 C8 and CYP2 S1, were not significantly altered in the oWAT of patients with obesity.

Furthermore, sEH activity was shown to decrease with worsening WAT homeostasis specifically, increased 12,13-EpOME:DiHOME ratio associated with inflammation (macrophage infiltration markers) but the enzymes that enable the reduction of epoxide to diols (EPHX1 and EPHX2) were measured by RT-qPCR and the transcript levels were unaltered in the oWAT suggesting a functional change in the activity of sEH.

As a large proportion of the obese cohort were prescribed and regularly taking Metformin, I endeavoured to establish if this medication was a confounding factor for observed results. I used an *ex vivo* explant model of obese oWAT and treated it with or without 1µM Metformin for 48 hours. Oxylipins were then measured and there was no generalised association between oxylipin profiles and Metformin treatment. There was no significant change in the conserved oxylipins (10,11-DiHDPA, 11,12-DHET, 12,13-EpOME:DiHOME ratio) except for 5,6-DHET. The sEH pathway enzymes EPHX1 and EPHX2 transcripts were measured in this explant model and did not significantly alter either with Metformin treatment. These results suggest Metformin does not modulate CYP-derived oxylipin pathways in obesity.

In summary, prolonged obesity leads to insulin resistance and T2DM, during this disease progression, the oWAT becomes dysfunctional. During this transition I measured oxylipins in oWAT and plasma found the oxylipin signature changed. Specifically, a reduction in CYP-derived diols was associated with adipocyte hypertrophy and sEH activity decreased as the oWAT became more inflamed. (**Figure 77**). These observed changes were seen in both plasma and oWAT of obese patients and the enzymes that metabolise this CYP-sEH pathway in both tissues were unaltered.

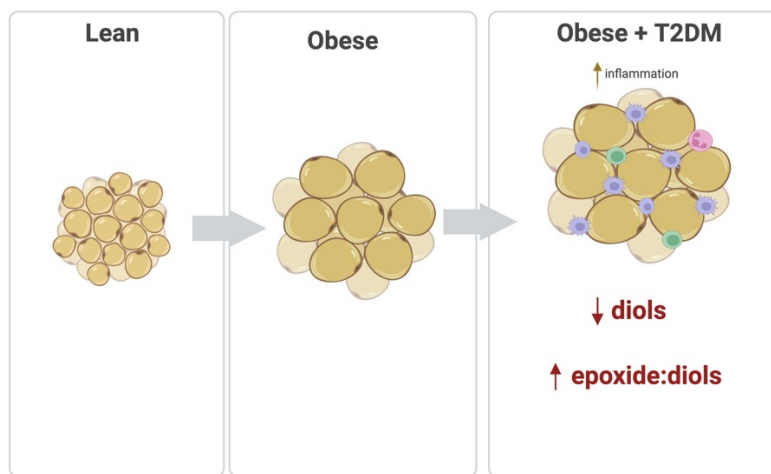


Figure 77 Schematic summary of CYP-sEH pathway evolution between lean and obese states in the oWAT and during oWAT inflammation. CYP: cytochrome P450; sEH: soluble epoxide hydrolase; T2DM: Type 2 Diabetes Mellitus. Image produced by Bio Render®.

7.4 Liver oxylipin profiling in obesity and T2DM/MASLD

Unhealthy WAT is often indicative of changes in the liver lipid content and progression toward MASLD. As dysfunctional oWAT associates with a specific oxylipin signature, namely the reduction in diols and increase in epoxide:diol ratio, the next aim was to investigate the liver oxylipin profiling within our bariatric cohort to potentially identify crosstalk between the oWAT, plasma and liver.

A subgroup of 41 patients from our patients with obesity undergoing bariatric surgery (**chapter 6 Table 13**) were sampled for liver biopsies paired with oWAT and plasma. PUFA/oxylipin profiling (20-carboxy-AA, AA, LA, EPA and DHA levels and 48 PUFA-derived oxylipins) of the cohort's liver samples was performed by UPLC-MS/MS. Liver biopsies were scored for existence of MASLD/MASH using the NAFLD activity score which encompasses steatosis, ballooning, inflammation and collagen deposition (see **Chapter 3 section 3.6.3**). The liver biopsies were also scored for percentage of ballooning, steatosis, collagen deposition (CPA) and inflammation using a verified AI scoring software. Unlike the oWAT and plasma, there were no liver biopsies taken from the control lean group, which needs to be taken into consideration when interpreting the liver tissue results in the next section.

Having used a filtering approach when analysing the oWAT and plasma which found a reduction in mainly CYP-derived diols, and an increase in epoxide:diol ratio in obesity and T2DM, the aims were;

1. Identify if diols and/or sEH were altered in liver tissue in obesity and T2DM.
2. Determine if there was a difference in signatures between total (free plus esterified) and free liver oxylipins in obesity and T2DM/MASLD.
3. Characterise the cross-tissue lipidome in MASLD namely liver, plasma and WAT tissue in obese individuals.

The strategy for answering these aims was undertaken using the analysis pathway outlined in Figure 78.

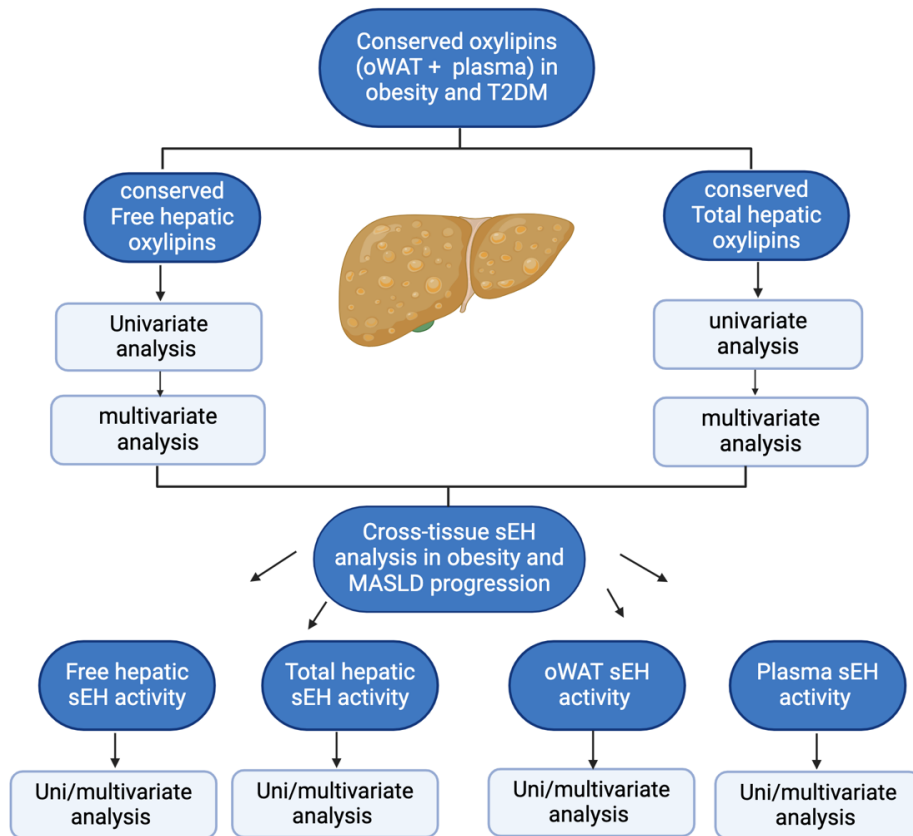


Figure 78 Flow chart to demonstrate filtering strategy when analysing hepatic oxylipins in obesity and i) T2DM and ii) MASLD. Total hepatic pool: free plus esterified; Free hepatic oxylipins: unesterified only. PUFA: polyunsaturated fatty acid; sEH: soluble epoxide hydrolase; MASLD: Metabolic dysfunction associated steatotic liver disease. Image produced using Bio Render®

7.4.1 Hepatic free diols in obesity and T2DM

oWAT and plasma had a global decrease in diols with obesity and T2DM, however there were no significant changes seen in free hepatic diols in obesity and T2DM. Specifically, when focusing on the conserved diols in the oWAT and plasma, 5,6-DHET, 10,11-DiHDPA and 11,12-DHET ($p=0.68, p=0.97, p=0.94$ respectively) were not significantly different between the patients with obesity and patients with obesity plus T2DM (**Figure 79**).

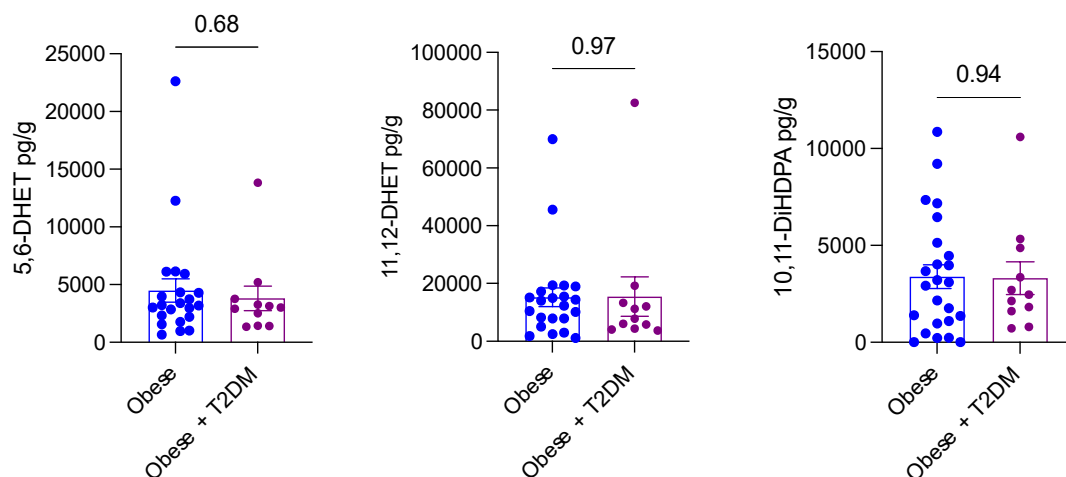
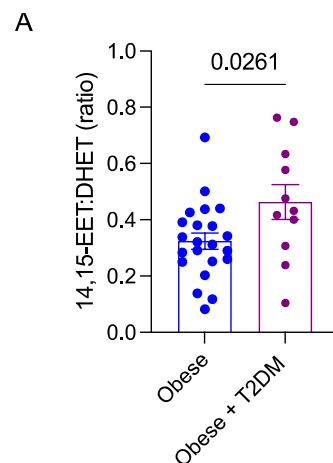


Figure 79 Hepatic CYP- derived oxylipins (5,6-DHET, 11,12-DHET, 10,11-DiHDDPA) in patients with obesity (N=23) and patients with obesity and T2DM (N=11). Significance was tested using Mann-Whitney. CYP: cytochrome P450; T2DM: Type 2 Diabetes Mellitus.

7.4.2 Hepatic sEH activity in obesity and T2DM

The sEH activity in the oWAT and plasma decreased in obesity when measuring several epoxide:diol ratios despite preserved EPHX1 and EPHX2 transcripts. I initially aimed to determine if this was also observed in the liver during progression from obesity to metabolic decline, free oxylipins were therefore measured using UPLC-MS/MS.

Consistent with this finding in the inflamed WAT, hepatic 14,15-EET:DHET ratio was significantly increased in obesity with T2DM ($p=0.003$) using univariate analysis. Multivariate analysis also suggested a decreased sEH activity in the liver in obesity and T2DM independent of the gender, age and ethnicity ($p=0.009$) (**Figure 80**).



B

Liver Free Metabolite: Log 14,15-EET:DHET	R	R Square	Standardized Coefficients Beta	t	Sig.
	0.551	0.303			
Sex [Female=1 male=2]			0.066	-0.014	0.932
Age			0.002	-0.173	0.347
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.017	0.329	0.053
T2DM [y=1 n=2]			0.062	-0.483	0.009*
Total 31					

Figure 80 Free hepatic 14,15-EET:DHET in obesity and metabolic decline. (A) sEH surrogate free 14,15-EET:DHET in liver biopsies stratified according to patients with obesity (N=22) and patients with obesity and T2DM (N=11) (B) Linear regression table for free 14,15-EET:DHET measured in liver adjusted for age, sex and ethnicity; N=31. Statistical significance was calculated using Mann-Whitney test (A) and a linear regression model with normalization of dependent variable by log transformation of the metabolite (B). sEH: soluble epoxide hydrolase.

7.4.3 Hepatic sEH enzyme expression

In keeping with the results observed in the oWAT, this decrease in hepatic sEH activity was not linked to changes in hepatic mRNA levels of *EPHX1* and *EPHX2*, the key genes coding for the sEH enzymes, both microsomal and soluble isoform expression were not significantly altered with T2DM in patients with obesity ($p=0.66$ and $p=0.69$) (Figure 81).

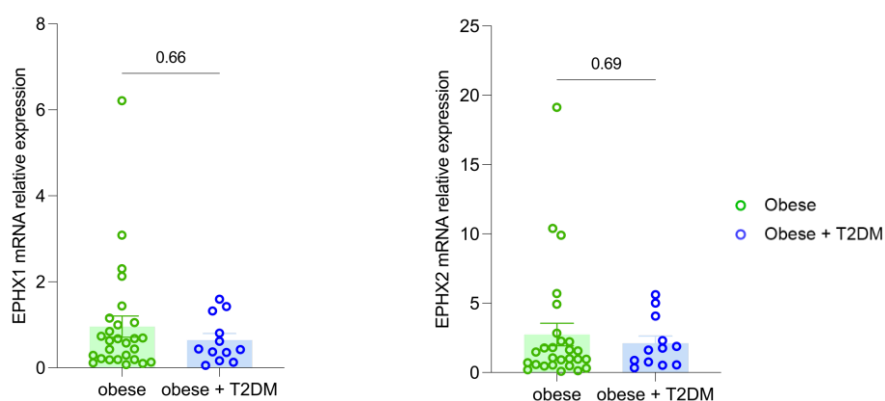


Figure 81 Expression of EPHX1 and EPHX2 in liver biopsies of obese (N=24) and obese /T2DM (N=12).

Statistical significance was tested by Mann-Whitney test. T2DM: Type2 Diabetes Mellitus.

7.4.4 Total oxylipins measured in the liver in obesity and T2DM

As previously described, oxylipins can reside in cell membrane phospholipids or can be stored within triacylglycerols (TGs) as esterified oxylipins until released by CYP-, COX- or LOX- enzymes producing unesterified oxylipins. UPLMS/MS of the plasma and oWAT previously discussed earlier in this chapter measured the unesterified (free) oxylipins. Therefore, by only measuring the free oxylipins important changes in stored oxylipin pools may be missed. Due to the technical difficulties of releasing the esterified oxylipins in oWAT and plasma, total (free unesterified plus esterified oxylipins) were not measured. However, it is possible by boiling the hepatic tissue followed by hydrolysis with exogenous PLA2 treatment to measure the total oxylipins in the liver tissue. Therefore, I aimed to determine if there was a significant difference in the oxylipin profiling in obesity and T2DM in total oxylipins (free plus esterified) when compared to free oxylipins only in hepatic tissue.

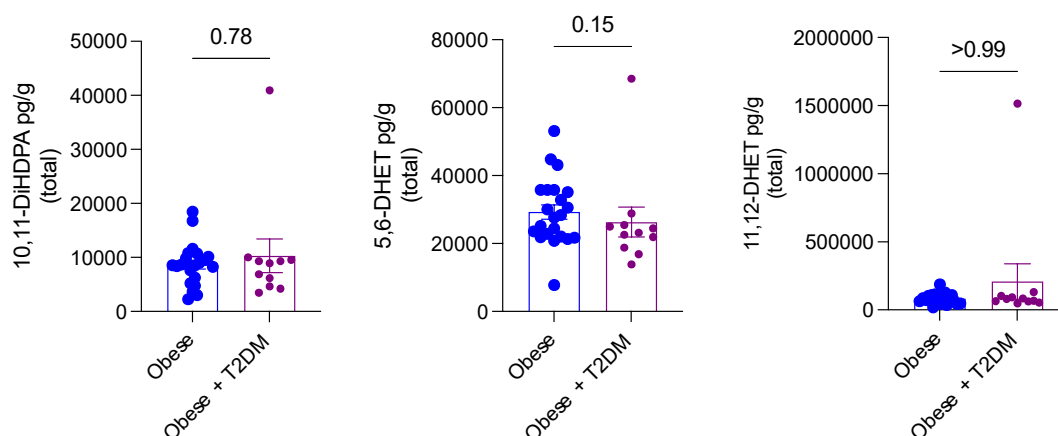


Figure 82 Hepatic CYP- derived total oxylipins (5,6-DHET, 11,12-DHET, 10,11-DiHDDPA) in patients with obesity (N=23) and patients with obesity and T2DM (N=11). Significance was tested using Mann-Whitney test. CYP: cytochrome P450; T2DM: Type 2 Diabetes Mellitus.

I firstly measured the total (free plus esterified) diols (10,11-DiHDDPA, 5,6-DHET and 11,12-DHET). However, there was no significant difference between total hepatic diols measured

in obese patients versus the obese patients with T2DM (10,11-DiHDEPA: $p=0.78$; 5,6-DHET $p=0.15$ and 11,12-DHET; $p=0.99$) (**Figure 82**).

When 14,15-EET:DHET was calculated based on total amounts (free plus esterified) of 14,15-EET and 14,15-DHET and compared in non-diabetics and T2DM patients with obesity there was no significant difference in sEH activity ($p=0.07$). A multivariate analysis model of total 14,15-EET:DHET which included the independent factors age, ethnicity and sex also did not show a significant change in obese patients with and without T2DM ($p=0.29$) (**Figure 83**).

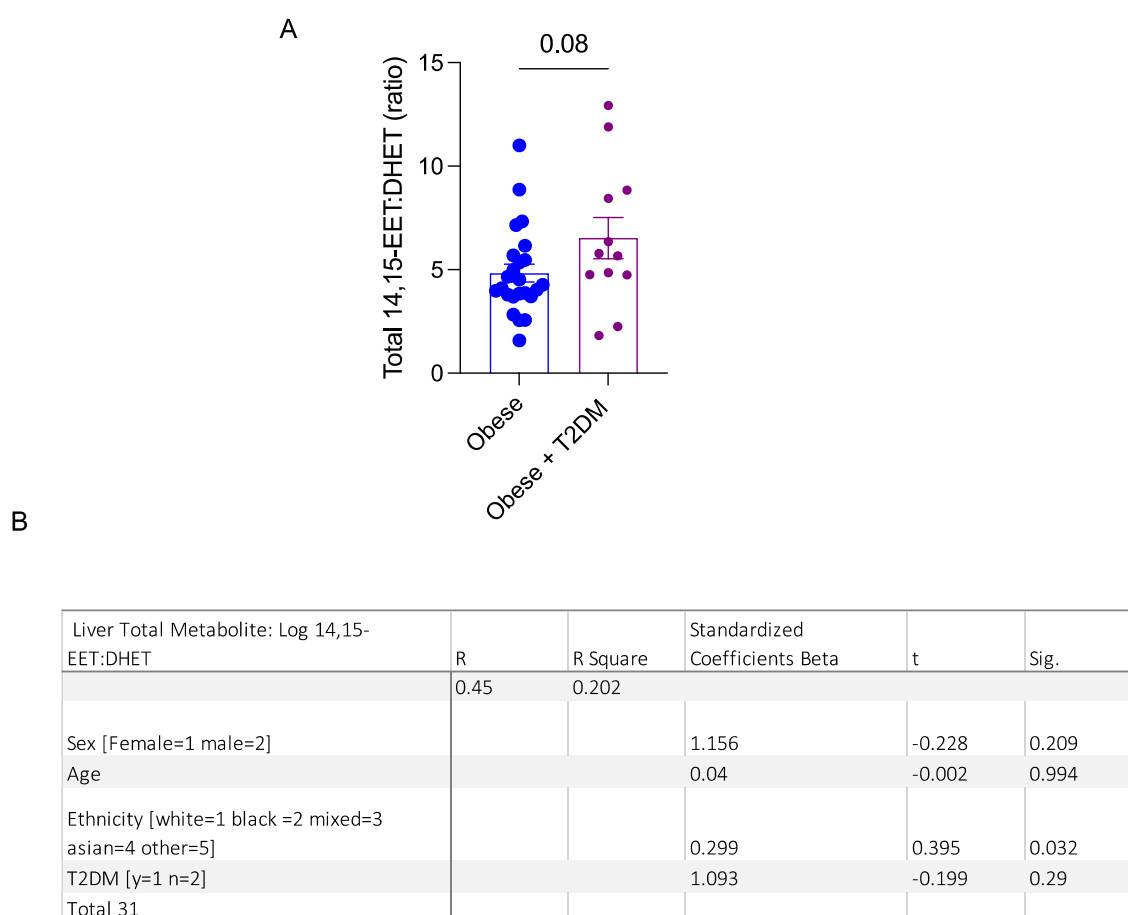


Figure 83 Total hepatic 14,15-EET:DHET in obesity and metabolic decline. A) sEH surrogate total 14,15-EET:DHET in liver biopsies stratified according to patients with obesity (N=22) and patients with obesity and T2DM (N=12) **B)** Linear regression table for total 14,15-EET:DHET measured in liver adjusted for age, sex and ethnicity; N=31. Statistical significance was calculated using Mann-Whitney test (**A**) and a linear regression model with normalization of dependent variable by log transformation of the metabolite (**B**) total 14,15-EET:DHET. sEH: soluble epoxide hydrolase; T2DM: Type 2 Diabetes Mellitus.

As free hepatic 14,15-EET:DHET ratio increases with T2DM in patients with obesity, similar to the oxylipin profile observed in the oWAT and plasma, the next aim was to establish whether sEH activity is altered in the spectrum of MASLD phenotypes in this cohort. The cohort was initially grouped based on the total NAFLD activity score (liver tissue with no

MASH (total score of 0-2), liver tissue with possible MASH (intermediate score 3-4) and liver tissue with MASH (score 5+).

Using univariate analysis of free 14,15-EET:DHET it was evident that sEH activity also decreased in MASH ($p=0.03$). It was then determined if this was associated with specific histological features i.e. steatosis, ballooning, inflammation or fibrosis. Free 14,15-EET:DHET decreased with increasing steatosis ($p=0.004$) and ballooning ($p=0.04$) but not inflammation and fibrosis (**Figure 84**).

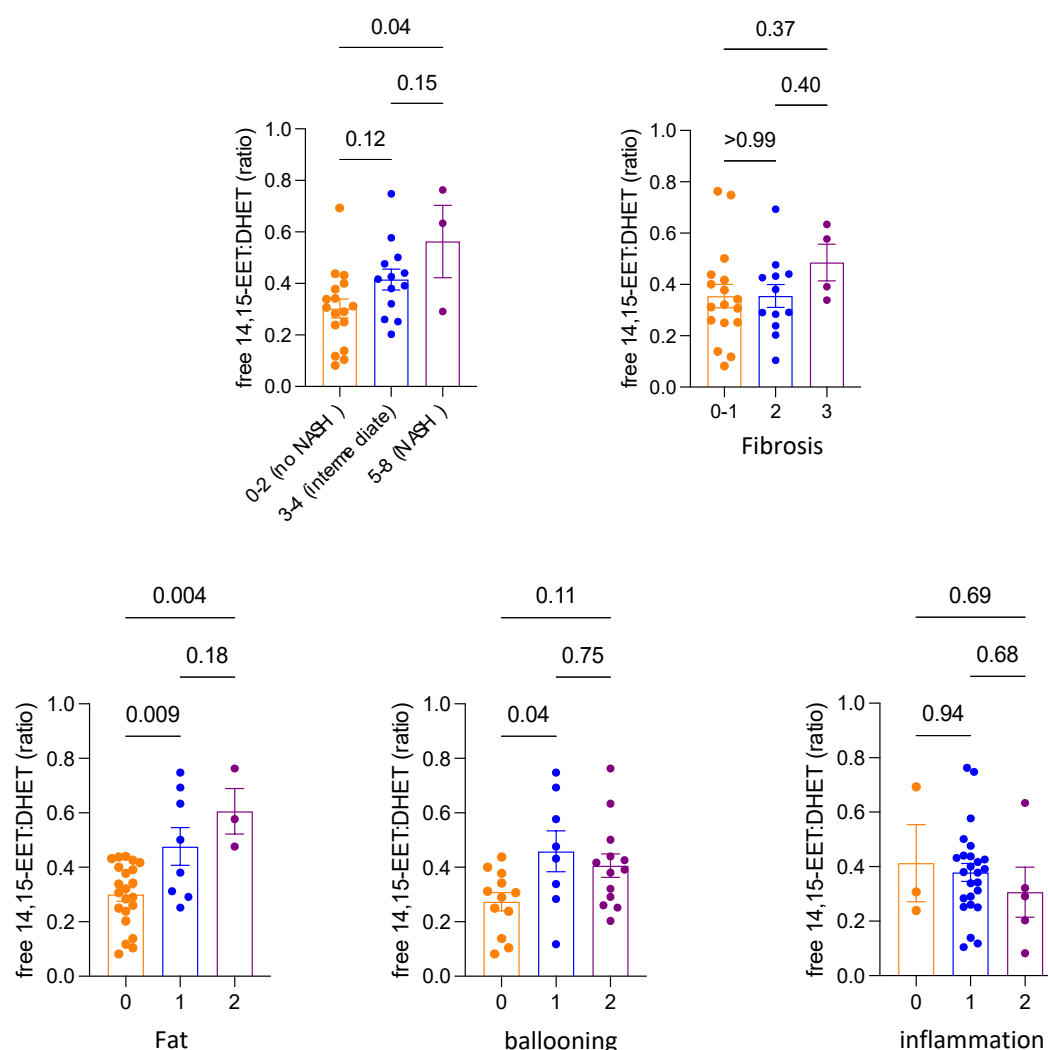


Figure 84 sEH surrogate free 14,15-EET:DHET in liver biopsies stratified according to patients with MASH [NAS scoring, 0-2 no MASLD (N=17), 3-4 intermediate/MASLD/MASH (N=13), 5+ MASH (N=3)] or fibrosis (F1: score 0-1 N=17, F2 : score 2 N=7, F3, score: 3 N=4) and ballooning score (0-2; score 0: N=12, score 1: N=8, score 2: N=13) steatosis score (0-3; score 0: N=22, score 1: N=8, score 2: N=3, score 3: N=0) and inflammation score (0; score 0: N=3, score 1: N=23, score 2: N=5). Statistical significance was calculated using Kruskal-Wallis with test with Dunn's multiple comparison. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

In contrast to free 14,15-EET:DHET, total (free and esterified) 14,15-EET:DHET levels were not significantly altered in MASH versus no MASH in the patients with obesity. When

comparing the individual features of MASH, again there was no change in the epoxide:diol ratio and thus sEH activity (**Figure 85**).

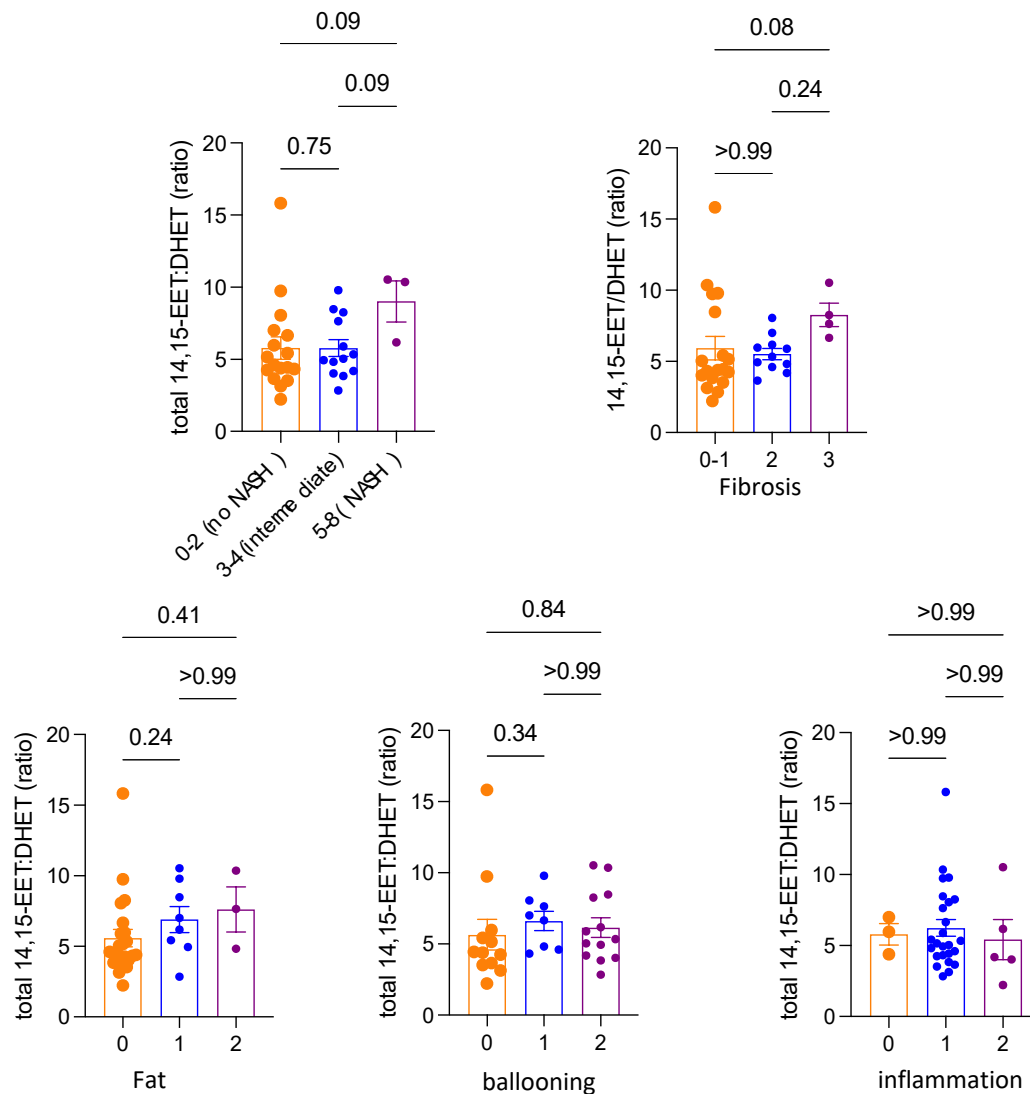


Figure 85 sEH surrogate total 14,15-EET:DHET in liver biopsies stratified according to patients with MASH [NAS scoring, 0-2 no MASLD (N=17), 3-4 intermediate/MASLD/MASH (N=13), 5+ MASH (N=3)] or fibrosis (F1: score 0-1 N=17, F2 : score 2 N=7, F3, score: 3 N=4) and ballooning score (0-2; score 0: N=12, score 1: N=8, score 2: N=13). Steatosis score (0-3; score 0: N=22, score 1: N=8, score 2: N=3, score 3: N=0). Inflammation score (0; score 0: N=3, score 1: N=23, score 2: N=5). Statistical significance was calculated using Kruskal-Wallis with test with Dunn's multiple comparison. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis

7.4.5 Comparing sEH activity in liver, plasma and oWAT with MASLD phenotypes

The positive association of free liver 14,15-EET:DHET ratio increasing in both T2DM and MASH phenotypes lead me to establish if global sEH activity in the liver, plasma and oWAT is altered in MASH. As the patients included in the study had predominantly early MASH changes, the use of semi-quantitative scores with the NAFLD activity score led to small numbers in MASH group (no MASH N=17, Intermediate MASH N= 13, MASH N=3). Therefore, by using validated software to quantify the percentage of inflammation, steatosis, ballooning and fibrosis in each biopsy it enabled analysis with spearman's correlation coefficient which focused on the early transition of MASLD to MASH and the relationship with the epoxide:diol ratio.

The correlation of different free hepatic epoxide:diol ratios with % CPA, ballooning, fat and inflammation in the liver tissue revealed significant positive associations (**Table 16**). Ballooning correlated significantly with epoxide:diol ratios including 10,11-EpDPA:DiHDPA, 11,12-EET:DHET, 14,15-EET:DHET. Fat % correlated significantly with 12,13-EpOME:DiHOME, inflammation with 19,20-EpDPE:DiHDPA and 17,18-EpETE:DiHETE and there was no sEH correlation with fibrosis (CPA%). Multivariate regression retained significance for all except inflammation with 19,20-EpDPE:DiHDPA and 17,18-EpETE:DiHETE when adjusted for age, sex and ethnicity (**Table 17**).

Table 16 sEH activity and markers of MASLD progression. Correlation of sEH surrogates, measured in each tissue (liver, plasma, WAT) by UPLC-MS/MS with markers of MASLD, measured by machine learning (**See Methods**; CPA%, ballooning%, fat%, inflammation %) for each liver biopsy (N=35). MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis; sEH: soluble epoxide hydrolase; CPA: collagen proportionate area; UPLC-MS/MS: ultra-performance-liquid-chromatography-mass spectrometry

		Liver metabolites:free		Plasma metabolites		WAT metabolites	
		R	p-value	R	p-value	R	p-value
CPA%							
<i>SeH</i>	10,11-EpDPA:DiHDPA	-0.27	0.14	-0.06	0.77	0.37	0.04
	8,9-EET:DHET	0.05	0.79	0.52	0.003	-0.10	0.59
ballooning %							
<i>SeH</i>	10,11-EpDPA:DiHDPA	0.38	0.030	0.02	0.94	0.14	0.47
	11,12-EET:DHET	0.45	0.009	0.05	0.79	-0.11	0.56
	14,15-EET:DHET	0.45	0.009	0.05	0.80	0.03	0.87
	19,20EpDPE:DiHDPA	0.16	0.379	0.41	0.02	0.36	0.04
fat%							
<i>SeH</i>	9,10-EpOME:DiHOME	0.23	0.21	0.43	0.02	0.17	0.36
	12,13-EpOME:DiHOME	0.36	0.05	0.23	0.21	0.14	0.45
	14,15-EET:DHET	0.34	0.06				
				0.20	0.28	0.27	0.12
Inflammation %							
<i>SeH</i>	19,20-EPDPE:DiHDPA	0.56	<.0001	-0.03	0.90	-0.17	0.35
	17,18-EpETE:DiHETE	0.38	0.03	n.d	n.d	n.d	n.d
	11,12-EET:DHET	0.30	0.10	0.28	0.13	-0.25	0.15
	9,10-EpOME:DiHOME	0.22	0.23	-0.06	0.77	-0.25	0.16
	12,13-EpOME:DiHOME	0.27	0.14	-0.21	0.27	-0.30	0.09

Table 17 Multivariate analysis of different free epoxide:diol measured in MASLD liver biopsies of bariatric surgery patients. Linear regression tables for 10,11-EpDPA:DiHDPA, 11,12-EET:DHET, 14,15-EET:DHET, 12,13-EpOME:DiHOME, 19,20-EpDPE:DiHDPA, 17,18-EpETE:DiHETE in MASLD liver tissue adjusted for confounding factors (age, gender and ethnicity; N=31). Markers of MASLD were measured by AI which calculated percentage of CPA, ballooning, steatosis and inflammation in individual liver samples (see methods). Statistical significance was calculated using a linear regression model with normalization of dependent variable by log transformation of metabolite activity. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis; sEH: soluble epoxide hydrolase; CPA: collagen proportionate area.

Free Log 10,11- EpDPA:DiHDPA	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.676	0.457	-0.029	-0.178	0.86
Age			-0.087	-0.49	0.629
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.148	0.874	0.391
CPA (%)			-0.135	-0.818	0.421
Fat (%)			0.444	2.729	0.012*
Ballooning (%)			0.438	2.47	0.021*
Inflammation (%)			0.157	0.915	0.369
Total 31					

Free Log 12,13- EpOME:DiHOME	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.61	0.372	-0.08	-0.454	0.654
Age			0.017	0.091	0.928
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.233	1.28	0.213
CPA (%)			0.293	1.647	0.112
Fat (%)			0.444	2.539	0.018*
Ballooning (%)			0.263	1.381	0.18
Inflammation (%)			0.172	0.937	0.358
Total 31					

Free Log 11,12-EET:DHET	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.674	0.454	-0.044	-0.266	0.793
Age			0.099	0.557	0.582
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.219	1.29	0.209
CPA (%)			0.116	0.701	0.49
Fat (%)			0.506	3.102	0.005*
Ballooning (%)			0.424	2.384	0.025*
Inflammation (%)			0.267	1.555	0.133
Total 31					

Free Log 14,15-EET:DHET	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.678	0.46	-0.042	-0.259	0.798
Age			0.065	0.37	0.715
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.109	0.644	0.526
CPA (%)			-0.078	-0.472	0.641
Fat (%)			0.523	3.222	0.004*
Ballooning (%)			0.466	2.636	0.014*
Inflammation (%)			0.066	0.385	0.704
Total 31					

Free Log 19,20- EPDPE:DiHDPA	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.519	0.27	-0.184	-0.969	0.342
Age			-0.331	-1.615	0.119
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			-0.115	-0.583	0.565
CPA (%)			0.164	0.856	0.4
Fat (%)			0.095	0.505	0.618
Ballooning (%)			0.107	0.523	0.606
Inflammation (%)			0.284	1.43	0.166
Total 31					

Free Log 17,18- EpETE:DiHETE	R	R ²	Beta	t	Sig.
Sex [Female=1 male=2]	0.471	0.222	0.243	1.242	0.226
Age			0.309	1.458	0.158
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]			0.166	0.82	0.421
CPA (%)			0.026	0.13	0.897
Fat (%)			0.085	0.434	0.668
Ballooning (%)			0.292	1.378	0.181
Inflammation (%)			0.296	1.444	0.162
Total 31					

Following the analysis of liver epoxide:diol ratios with MASLD progression, I aimed to investigate whether this relationship was also observed in the plasma and oWAT during the transition from MASLD to MASH and thus identify potential inter-tissue dynamics. When analysed according to the liver pathology criteria (ballooning, steatosis, inflammation and fibrosis), several epoxide:diol ratios were also increased in plasma and oWAT (**Table 16**) Fibrosis (CPA%) correlated with 8,9-EET:DHET in plasma, and 10,11-EpDPA:DiHDPA in the oWAT, while ballooning correlated significantly with 19,20EpDPE:DiHDPA in both plasma and oWAT and fat% correlated with 9,10-EpOME:DiHOME in the plasma (**Table 16**).

Multivariate analysis retained significance for CPA% with 8,9-EET:DHET and 14,15-EET:DHET and ballooning % for 19,20-EpDPA:DiHDPA in the plasma and 10,11-

EpDPA:DiHDPA in the oWAT. Age, sex and ethnicity were included in the regression model. The association of oWAT 19,20-EpDPA:DiHDPA and plasma 9,10 -EpOME:DiHOME with fat or ballooning were lost in multivariate analysis (**Table 18**).

Table 18 Multivariate analysis of different free epoxide:diol in oWAT and plasma measured in MASLD of bariatric surgery patients. Linear regression tables for plasma sEH activity (8,9-EET:DHET, 9,10-EpOME:DiHOME, 19,20-EpDPE:DiHDPA, 14,15-EET:DHET) and oWAT sEH activity (10,11-EpDPA:DiHDPA and 19,20-EpDPE:DiHDPA) adjusted for confounding factors (age, gender and ethnicity; N=31). Markers of MASLD were measured by AI which calculated percentage of CPA, ballooning, steatosis and inflammation in individual liver samples (see methods). Statistical significance was calculated using a linear regression model with normalization of dependent variable by log transformation of metabolite activity. MASLD: Metabolic dysfunction-associated steatotic liver disease; MASH: Metabolic dysfunction-associated steatohepatitis; sEH: soluble epoxide hydrolase; CPA: collagen proportionate area.

A

plasma: Log 8,9-EET:DHET	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.618	0.382			
Age			0.322	1.671	0.108
Sex [Female=1 male=2]			0.039	0.204	0.84
CPA (%)			0.083	0.446	0.66
Fat (%)			0.571	2.607	0.016*
Ballooning (%)			0.118	0.615	0.544
Inflammation (%)			0.302	1.502	0.147
Total 31			-0.299	-1.343	0.192

plasma: Log 9,10-EpOME:DiHOME	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.539	0.291			
Age			0.18	0.872	0.392
Sex [Female=1 male=2]			-0.156	-0.769	0.45
CPA (%)			-0.03	-0.15	0.882
Fat (%)			0.432	1.84	0.079
Ballooning (%)			0.325	1.581	0.127
Inflammation (%)			0.303	1.405	0.173
Total 31			-0.397	-1.662	0.11

plasma: Log 19,20-EpDPE:DiHDPA	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.519	0.269			
Age			0.179	0.855	0.401
Sex [Female=1 male=2]			-0.019	-0.091	0.928
CPA (%)			0.028	0.139	0.891
Fat (%)			-0.003	-0.012	0.99
Ballooning (%)			0.102	0.488	0.63
Inflammation (%)			0.567	2.59	0.016*
Total 31			-0.059	-0.243	0.81

plasma: Log 14,15-EET:DHET	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.708	0.501			
Age			-0.436	-2.521	0.019
Sex [Female=1 male=2]			-0.21	-1.239	0.228
CPA (%)			0.205	1.223	0.234
Fat (%)			0.527	2.679	0.013*
Ballooning (%)			0.335	1.946	0.064
Inflammation (%)			-0.059	-0.326	0.747
Total 31			-0.308	-1.537	0.138

B

oWAT: Log 10,11-EpDPA:DiHDPA	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.657	0.432			
Age			0.352	1.509	0.148
Sex [Female=1 male=2]			0.209	1.011	0.325
CPA (%)			-0.3	-1.082	0.293
Fat (%)			0.388	1.504	0.149
Ballooning (%)			0.199	0.792	0.438
Inflammation (%)			0.497	2.365	0.029*
Total 31			0.183	0.982	0.338

oWAT: Log 19,20-EpDPE:DiHDPA	R	R ²	Beta	t	Sig.
Ethnicity [white=1 black =2 mixed=3 asian=4 other=5]	0.479	0.229			
Age			0.172	0.85	0.403
Sex [Female=1 male=2]			0.4	1.977	0.059
CPA (%)			0.068	0.357	0.724
Fat (%)			-0.038	-0.196	0.846
Ballooning (%)			0.051	0.258	0.799
Inflammation (%)			0.155	0.761	0.454
Total 31			0.007	0.033	0.974

7.4.6 Conclusions

In summary, the hepatic diols (when both free and total were measured) did not decrease with obesity and T2DM, however this is probably affected by not having a lean control group. The increased epoxide:diol ratio observed in inflamed oWAT and plasma in obesity is conserved in the liver. Free hepatic 14,15-EET:DHET increases with obesity and T2DM, in contrast to total hepatic 14,15-EET:DHET.

Furthermore, several epoxide:diol ratios increase in the liver with MASLD progression including 10,11-EpDPA:DiHDPA, 11,12-EET:DHET, 14,15-EET:DHET, 12,13-EpOME:DiHOME. Interestingly, 12,13-EpOME:DiHOME, also a marker of oWAT inflammation in our obese cohort was increased with steatosis in the liver.

When comparing MASLD phenotypes with free hepatic, plasma and oWAT epoxide:diol ratios, there was evidence of a global decrease in sEH activity in MASLD across all three tissues.

Overall, these results show that while obesity is associated with reduced diols in plasma and oWAT, obesity-associated WAT inflammation and MASLD progression is associated with dampened sEH activity (**Figure 86**).

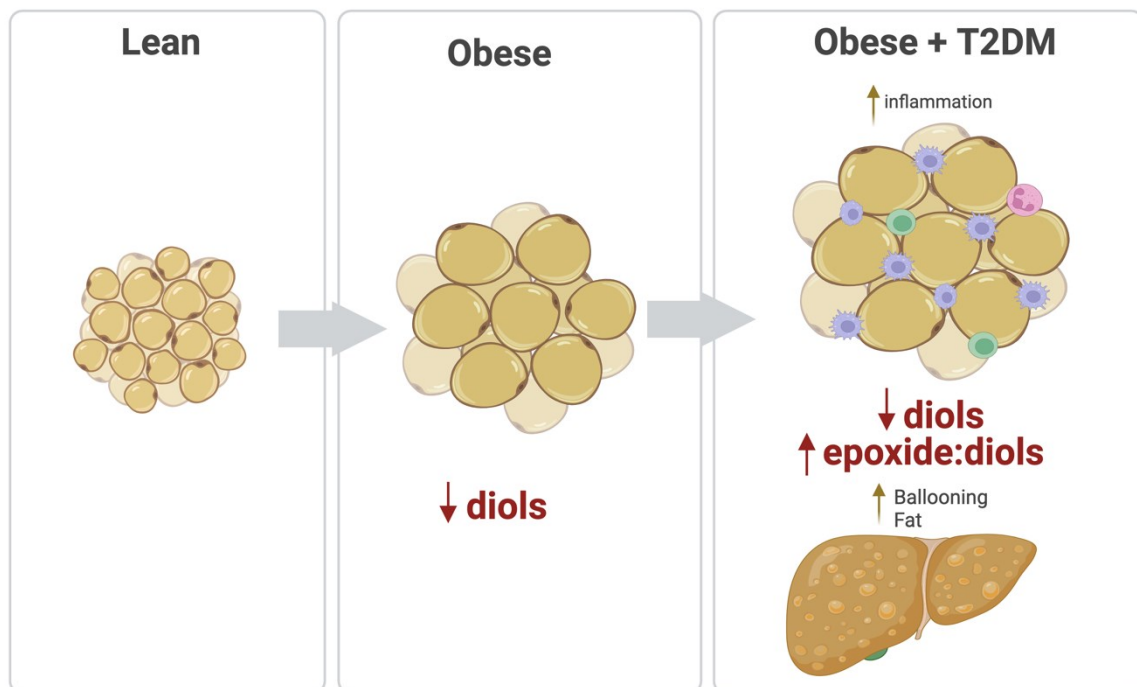


Figure 86 Schematic summary of CYP-sEH pathway evolution between lean and obese states in the WAT and during oWAT inflammation and hepatic steatosis. sEH:soluble epoxide hydrolase; CYP: cytochrome P450; T2DM: Type 2 Diabetes Mellitus. Image produced by Bio Render®.

7.5 Discussion

Oxylipins are oxygenated bioactive lipid mediators with intracellular and extracellular (via G protein-coupled receptors) signalling actions in tissues. Depending on the type of PUFA they are derived from and the type of primary oxidative enzyme, they can have pro-inflammatory or pro-resolving properties that are reported in the plasma, adipose tissue and liver [321, 324, 392, 521]. Oxylipins have also been associated with adipogenesis, specifically acting as PPAR γ ligands in adipose tissue, and angiogenesis in vascular endothelial cells [412, 512, 522]. Collectively, these findings suggest they could modulate oWAT, liver and plasma at multiple levels in obesity and progression of metabolic syndrome/MASLD. PUFA-derived oxylipins have previously been measured and shown to have a generalised increase in COX and LOX metabolites in MASLD and metabolic syndrome [342, 347, 349, 523] however, there is relatively limited data on the tissue specific roles of oxylipins in obesity and the progression of T2DM and MASLD in humans. Therein I decided to characterise the lipidome in omental WAT, liver and plasma in obese and lean individuals.

To establish whether oxylipins are associated with obesity and cross-tissue progression to metabolic syndrome and MASLD I used both the cohort described in chapter 4 (which includes lean and obese individuals oWAT and plasma) and the paired cohort of patients in chapter 6 (paired oWAT, plasma and liver tissue) undergoing bariatric surgery and measured PUFA-derived metabolites in all three tissues by UPLMS/MS. The paired cohort included patients with a spectrum of MASLD disease diagnosed by liver biopsy and patients from both cohorts were stratified into non-diabetic and T2DM (as previously described) and non-MASLD, MASLD and MASH enabling the analysis of PUFA-derived oxylipin lipid changes during disease progression.

7.5.1 Metabolic syndrome and the CYP-sEH pathway

Firstly, I characterised the changes in lipid species seen in obesity and metabolic decline which identified significant changes in the CYP epoxide/diol pathway in the WAT, liver and plasma of individuals with metabolic syndrome. CYP-derived diols, whether AA, LA or DHA-derived, were prominently reduced in the WAT and plasma of individuals with obesity when compared to lean individuals. Amongst these, reduced 10,11-DiHDPA levels were conserved between plasma and WAT and the association of diols with obesity were not affected by cofounders (including age, sex and ethnicity).

On the other hand, epoxide:diol ratios increase in the oWAT and plasma suggesting decreased sEH activity with progressively dysfunctional WAT in obesity; with another systemically conserved marker being 12,13-EpOME:DiHOME. The epoxide:diol profiles in the liver biopsied from patients with obesity corroborated these findings, suggesting an

overall reduced sEH activity (increased free 14,15-EET:DHET in liver tissue). Hepatic diols did not significantly change with obesity and T2DM, but this could be explained by not having a lean group for comparison.

Human studies to date have not focused on the transition from healthy oWAT to dysfunctional oWAT in obesity and the relationship with oxylipin species. CYP-derived diols were associated with progression of dysfunctional oWAT when correlating the unhealthy oWAT phenotype (adipocyte hypertrophy, ECM modulation and macrophage-mediated inflammation) with oxylipin species measured in obese adults. Specifically, 11,12-DHET decreased in both plasma and oWAT with adipocyte hypertrophy and ECM changes (reduction in collagen I). Furthermore, 12,13-EPOME:DiHOME in both plasma and oWAT increased with oWAT inflammation (macrophage infiltration) collectively suggesting oxylipins are significantly associated with oWAT dysfunction.

Overall, the results suggest that the fatty acid diol reduction may be associated broadly with obesity and reduced sEH activity (increased 12,13-EpOME:DiHOME in the oWAT and 14,15-EET:DHET in the liver) is indicative of metabolic dysfunction characterised by WAT inflammation.

The literature on CYP-sEH pathway association with human metabolic syndrome is contradictory [333, 343, 346, 350, 364, 367, 513-515, 517-519]. Epoxides have been positively linked to hypertension [364] and circulating diols were shown to decrease with exercise in obesity [524]. Overall, there has been converging evidence that supports my findings that the metabolic component of obesity is characterized by reduced diols and/or sEH activity. Firstly, lower proportions of CYP-derived diols negatively correlate with HOMA2-IR in the subcutaneous WAT [346] with specifically a decrease in 11,12-DHET, in keeping with the conserved oxylipins seen in oWAT and plasma during obesity in my cohort. Secondly, elevated plasma total 14,15-DHET have been suggested to be protective during early phases of diabetes with an inverse association of 5,6-DHET and 14,15-DHET with BMI [518]. Plasma levels of another diol, 12,13-DiHOME, has been inversely correlated with BMI, insulin resistance and circulating markers of liver function [406], a finding replicated by other recent studies [343, 346, 407, 525] and other diols, including 19,20-DiHDPHA, which has been shown to decrease with metabolic decline [515]. Finally, a human trial demonstrated sEH inhibition does not modify insulin sensitivity in patients with obesity and prediabetes [398] and moreover, loss of EPHX1 activity causes lipoatrophic diabetes syndrome [526].

Additionally, an obesity-induced reduction in diols and/or sEH activity has also been observed in the WAT using animal models of obesity. In cafeteria diet fed rats and high-fat diet induced obesity in mice, a generalized down-regulation of WAT CYP-derived epoxides

and diols (including 12,13-DiHOME) confirm the findings presented here in humans [209, 420, 527].

Until recently, diols were considered pathological byproducts of anti-inflammatory epoxides leading to pro-inflammatory and cytotoxic effects and disease progression, but Lynes challenged this by showing that 11,12-DiHOME improved metabolic control through browning of visceral fat. Large data in humans corroborated these findings and a recent mouse model that expressed 12,13-diHOME from transplanted brown AT improved cardiac function [365, 406, 525]. These studies, together with my results highlights the possibility of using diols as a potential therapy to prevent metabolic decline and oWAT dysfunction in obesity.

7.5.2 Biomarkers for predicating metabolic decline in obesity

The three conserved diols significantly correlated in both oWAT and plasma, therefore the potential for diols/sEH activity as biomarkers in determining metabolic decline was investigated. The overall diagnostic performance of the oxylipins to predict T2DM was strongest with 11,12-DHET which had an AUC 0.71. While this has a relatively strong positive predictive value, we currently have stronger clinical indicators readily available and therefore is likely not clinically useful currently in isolation. Dalle et al. used a panel of oxylipins to predict steatosis and MASH but as this included 23 oxylipins, it would not be a feasible test for clinical use [528]. However, a small panel of oxylipins that could identify patients that were at risk of further metabolic decline or using oxylipins to determine the positive predictive value in MASLD maybe a more useful tool [528]. It would be particularly beneficial if it could be used in conjunction with already available diagnostic markers to establish who will progress to more significant liver disease, currently an unmet clinical need.

7.5.3 Metformin effects on CYP-derived oxylipins

As previously explored in chapter four, Metformin is adipogenic but the pathway that is being modulated has not been identified. It has been shown oxylipins are PPAR γ ligands and PPAR γ is integral to adipocyte differentiation therefore, it was plausible that Metformin modulates the oxylipin signature and in turn adipogenesis. When treating explanted oWAT with Metformin there was no global change in either diols or epoide:diol ratios suggesting Metformin does not alter oxylipins in obesity. There is a paucity of literature that investigates this relationship to support my results with only Gorbratenkov et al. demonstrating COX-derived species decrease with Metformin treatment on astrocytes [529]. As 5,6-DHET did appear to decrease and with no previous data for comparison it therefore would warrant further investigation to establish whether this was a reproducible finding in isolation however,

overall, my results suggest Metformin most likely increase adipogenesis through alternative pathways that warrant further investigation.

7.5.4 CYP-sEH pathway and MASLD

As my results showed cross-tissue changes in the CYP-sEH pathway during obesity and metabolic decline, my next aim was to evaluate whether this was also observed during the progression of simple steatosis to MASH. Importantly, sEH activity was shown to decrease in all three tissues during progression of simple steatosis to MASH.

More generally, different epoxide:diol ratios (10,11-EpDPE:DiHDPA, 11,12-EET:DHET, 14,15-EET:DHET) correlated positively with ballooning and steatosis of the obese liver. Interestingly, 12,13-EpOME:DiHOME, was also associated with hepatic steatosis, suggesting that is a common marker of WAT inflammation and liver steatosis during obesity. While there was a strong relationship with ballooning and steatosis with reduced cross-tissue sEH activity, there was a weak relationship with liver fibrosis and inflammation. As this cohort comprised of individuals with predominantly early MASLD, I hypothesise more advanced liver disease may lead to sEH activity being negatively linked to hepatic inflammation and fibrosis.

Epoxides are commonly described in the literature as resolving/regenerative and anti-inflammatory mediators [356, 377, 530, 531] as opposed to diols [532] which until recently were seen as pathological and pro-inflammatory [373, 406]. As such, the inhibition of sEH activity has been associated with improved disease outcomes, mainly in animal models. Both obese rat and mouse models treated with sEH inhibitors (sEHi) have been shown to attenuate hepatic steatosis and fibrosis through blunting angiogenesis and inflammation and modulating endothelial reticulum stress and mitochondrial function [357, 360, 391, 394]. Furthermore, mouse models have shown improved glucose control with sEHIs acting on autophagy and inflammatory pathways. More specifically, soluble epoxide hydrolase inhibition on mice islet cells improved insulin secretion and glucose control by reducing apoptosis [374, 386, 533] and ER stress [360]. The benefits of sEHi in animal models is not restricted to metabolic disease with studies showing a reduction in myocardial infarctions though increasing angiogenesis as well as preventing cognitive decline in T2DM mice. Taken together these studies highlight multiple pathways that are modulated by sEH activity in several diseases associated with metabolic syndrome [378, 534].

These promising results have led to human trials using sEHi, yet human studies have not been as clear cut, sEH inhibition has been shown to be beneficial for diabetic retinopathy, acting on VEGF pathways, and in reducing coronary artery disease [373, 381]. My findings from a human cohort of individuals with obesity however, showed sEH activity was reduced

but epoxides themselves did not significantly alter with metabolic decline and hepatic steatosis, therefore by decreasing sEH activity further using sEHi in this context is unlikely to lead to beneficial effects. Indeed, sEH inhibitors in a randomised control trial of obese T2DM patients, while improving oxidative stress, did not appear to improve glucose or blood pressure control and these findings are supported by other studies showing similar results [397, 398, 518].

In summary, my findings, together with others [209, 343, 346, 406, 407, 518, 525-527] suggest that therapies based on sEH inhibition in metabolic syndrome should consider a potentially defective CYP-sEH pathway in metabolic tissues such as WAT and the liver of obese patients. However, this defective pathway may be different during complications of T2DM (retinopathy, nephropathy, cardiomyopathy) whereby an induction of the pathway (increased sEH activity) may occur in contrast to the decreased activity seen in metabolic decline and hepatic steatosis and thus are likely tissue- and context-specific [381, 518].

Therefore, if there is a defective CYP-sEH pathway in obesity what is the proposed mechanism? My results show oWAT dysfunction, specifically adipocyte hypertrophy correlates with a defective CYP-sEH pathway, therefore, the answer may lie in the pathological changes seen in oWAT during prolonged obesity. As previously described, “unhealthy WAT” in obesity is associated with reduced adipogenesis and increased adipocyte hypertrophy as opposed to healthy obesity that is associated with active adipogenesis. With this in mind, previous work has shown that rat epoxygenase *Cyp2j4* deletion accelerated adipogenesis and increased PPAR γ and CEBP α expression while *Ephx1* deletion in pre-adipocytes *in vitro* prevented adipocyte differentiation [209, 420, 526]. These studies suggest a pro-adipogenic role of diols and an anti-adipogenic role of epoxides. Taken together with my results in chapter 4 that demonstrated adipocyte hypertrophy led to decreased PPAR γ and CEBP α expression, decreased sEH activity and a defective CYP-sEH pathway is plausibly linked to decreased adipogenesis but the precise mechanism is yet to be determined. Thus, a further understanding of the relationship of oWAT adipogenesis with diols and sEH activity in adipose and hepatic tissue will be key to understanding their pathological role in obesity and their potential as treatment targets.

7.5.5 Limitations and Future work

While the results described show a conserved decrease in sEH activity in MASLD, the cohort comprises of only obese patients for the paired liver tissue with oWAT and plasma and therefore it would be important to include a lean control group hopefully strengthening these results. Furthermore, the MASLD cohort is predominantly early MASH with relatively low numbers of patients with MASH and fibrosis and no patients with cirrhosis. Therefore, by

including more patients with severe phenotypes it would establish whether sEH activity is modulated in severe cirrhosis. As there are relatively few diagnostic markers of early progressive disease in MASLD pursuing this association as a potential biomarker would indeed be an interesting possibility. However, the challenges of using oxylipins are described in the literature; there are inconsistencies between studies and the root of this issue is likely multifactorial. Firstly, studies have shown that oxylipins can be easily degraded, secondly methods for extraction and measuring each oxylipin varies between labs and as they are only found at very low levels within tissue, small variations can make significant changes in results [535, 536]. Thirdly, the majority of studies only measure unesterified oxylipins, which therefore poses the question, *are we missing significant changes by not measuring all pools of oxylipins?* My data suggests there is a difference between total and free oxylipins in the liver tissue but suggests that the free pool is the most clinically relevant with the statistically significant changes. However, this needs further exploration with some studies suggesting total oxylipins are more stable and therefore making them more reliable biomarkers. Nevertheless, the Zeldin lab, who performed the UPLMS/MS (and with whom we have collaborated) has been pioneering new extraction methods for total and free oxylipins and has demonstrated high levels of reproducibility in all three tissues. Therefore, further work to optimise extraction of these relatively unstable lipids may help with enabling their use as biomarkers.

While I have identified a cross-tissue oxylipin signature associated with both T2DM and MASLD progression, by expanding the paired cohort to include more severe phenotypes of both metabolic disease and MASH with fibrosis and cirrhosis and the inclusion of paired lean hepatic tissue with oWAT and plasma would be important to see if the relationship with sEH activity/ diols is conserved. Following this, performing functional studies could identify if increasing CYP-derived diols/sEH activity in MASLD and T2DM would attenuate disease progression.

I have also shown that the enzymes EPHX1 and EPHX2 that code for CYP pathway sEH were not altered in both obesity and MASLD, therefore the relative activities of sEH enzymes with respect to CYP epoxygenase activities taking place upstream needs to be investigated.

Furthermore, combining lipid omics with transcriptomics, could further delineate the pathways involved in modulating CYP- derived diols/sEH and identify targets that could be beneficial in prevention of disease progression in T2DM and MASLD.

7.5.6 Conclusions

Collectively, these data suggest that early metabolic complications of obesity are characterised by dysfunctional CYP-sEH pathway that manifests with reduced sEH activity

and diols in WAT and liver. Further studies need to clarify whether this is conserved in MASLD cirrhosis and whether modulating this pathway has beneficial clinical implications in T2DM and MASLD progression.

Chapter 8: General Discussion

Obesity is a huge health burden with increasing prevalence worldwide [1]. It has been closely linked to insulin resistance and metabolic syndrome and a central feature is the crosstalk between white adipose tissue and the liver. In prolonged obesity the white adipose tissue becomes inflamed and dysfunctional and this maladaptive state of the WAT can cause surplus lipid release and deposition within the liver and other tissues [425]. WAT dysfunction is associated with developing T2DM and MASLD, however, it is unknown exactly what drives the disease progression [103]. Oxylipins are bioactive lipids that have been shown to modulate WAT homeostasis at multiple levels *in vivo* and *in vitro*, but human studies remain controversial and restricted often to investigating only changes in the plasma [412, 414]. Therefore, this work aimed to characterise the crosstalk between the maladaptive oWAT and MASLD progression and determine if tissue-specific changes in oxylipins were associated with disease pathology.

This work revealed a novel significant change in the CYP-sEH pathway in WAT, liver and plasma of individuals with metabolic syndrome and MASLD. I characterised the changes seen in oWAT during the transition to metabolic syndrome and identified oWAT adipocyte hypertrophy and collagens correlated with features of MASLD; hepatic ballooning and steatosis but not hepatic inflammation or hepatic fibrosis [537].

8.1 oWAT phenotype in obesity and progression to T2DM

It is well characterised that AT in prolonged nutrition excess has maladaptive changes, firstly the adipocyte size increases, favouring hypertrophy over hyperplasia which results in metabolic consequences. Smaller adipocytes are associated with improved glucose metabolism while large adipocytes are associated with metabolic decline [131, 132]. My cohort has corroborated this and interestingly showed as the size of the adipocyte increased the protein levels of CEBP α and PPAR γ decreased, independently of T2DM status. I hypothesised that hyperplasia is likely due to be a compensatory mechanism in metabolic decline to preserve homeostasis. A mouse model on a HFD with selective adipocyte insulin resistance had a heterogenous population of adipocyte sizes and no relative change in PPAR γ [433] which suggests other mechanisms initially drive the metabolic decline in obesity. I also suggested this phenomenon could be in part due to a drug affect and have therefore investigated the role of Metformin in oWAT function as this was used by over seventy percent of the obese cohort.

Metformin has been shown in recent years to have beneficial effects on multiple disease pathways including being anti-carcinogenic, and anti-inflammatory and improves lipid profiles [447]. While the role of Metformin is relatively well described in the liver, very little is known

on how Metformin affects the WAT, particularly as most of the studies have been performed in cell lines using supratherapeutic doses [450, 451]. Using an *ex vivo* explant model for oWAT and SVF-derived adipocyte culture model I showed that Metformin at micromolar doses is in fact adipogenic (i.e. promoting adipogenesis) with increased CD36 protein levels and increased PPAR γ , FABP4 and FASN transcripts. Therefore, I believe that Metformin was likely contributing to the phenotype seen in my cohort and may have prevented a more extreme phenotype in the obese patients. Importantly, I also showed that inflammation and ECM remodelling were not modulated. This work did not identify a mechanism of action for Metformin and an area that would be interesting to explore further.

AT homeostasis relies on the immune cell population to perform important roles in lipid and glucose metabolism and phagocytosing of dying adipocytes. However, during obesity it has been shown that macrophages infiltrate the adipose tissue, with the literature describing it as a chronic low inflammatory state [175]. Recent single-cell RNA-seq has uncovered the complexity of this cell type with a new lipid-associated macrophage (LAMS) phenotype and unique VAT macrophages that express CD9⁺ and CD68⁺ but their functions are yet to be fully defined [113, 167]. In keeping with this previous work, I showed that oWAT inflammation increases in obesity, as evidenced by increases in CLS, TREM2 mRNA transcripts and CD45⁺CD14⁺CD206⁺ macrophages in T2DM which correlates with adipocyte hypertrophy. I used cardiac glycoside Ouabain, a Na⁺/K⁺-ATPase blocker, to deplete monocyte-derived macrophages in an *ex vivo* explants oWAT model and showed that this improved insulin sensitivity and collagen VI deposition decreased [207]. This work strengthens the recent interest in macrophages as a target for improving metabolic health in obesity which has gained traction in the last decade. However, no drugs to date have improved metabolic health and reduced weight gain in humans and controversially it has been observed that obese patients on immunomodulatory therapy for other autoimmune conditions continue to suffer from T2DM, MASLD and raised BMI [441]. Therefore, their use is still very much debated. As most literature in this field is from animal models and the dynamics of macrophage function in humans is complex and situation-specific, further functional studies in humans during obesity progression will help understand if this cell-type is a realistic target.

ECM composition in the WAT and its function remains relatively unknown. Some studies have suggested in obesity there is an overall increase in ECM deposition and described it as fibrosis [195, 200] but the literature is very conflicting with others suggesting 'fibrosis' decreases in obesity [202, 203]. My work showed that collagen type I and collagen type VI decreased with adipocyte hypertrophy and collagen type VI increased with inflammation. Collagen type I is likely integral to cell stability and necessary for adipocyte expansion.

Collagen type VI has been shown in mouse models to be associated with poor metabolic health and increased inflammation [204]. Taken alongside my work that showed Ouabain caused macrophage depletion and decreased collagen VI deposition it suggests collagen VI is part of the inflammatory response.

8.2 Crosstalk between oWAT and liver in obesity driven T2DM and MASLD

Animal studies, including a *cyp2j4*^{-/-} rat model on a CAF diet previously studied by the Behmoaras lab [207] have shown the connection between poor oWAT homeostasis, metabolic syndrome and progressive liver disease in obesity. However, animal models often do not recapitulate the spectrum of liver disease seen in humans. Therefore, as there is relatively little human work studying the crosstalk between oWAT and the liver in obesity, I aimed to determine if oWAT maladaptive responses were linked to MASLD/MASH. A strength of this work is the direct comparison of multiple features of progressive disease in paired liver and oWAT tissue in obese patients, which has not been comprehensively addressed in humans previously [482, 483, 485]. Adipocyte hypertrophy was found to positively correlate with hepatic steatosis and collagen VI and collagen I decreased as hepatic ballooning and steatosis progressed. There was no correlation of oWAT inflammation with liver disease. As collagens decreased while adipocyte size and hepatic steatosis increased, this strengthens my hypothesis discussed earlier that collagens are integral to adipose structure and likely leaves the adipocyte unstable in lipid excess which in turn results in deposition of FFA into the circulation and ultimately the liver, also complementing the ‘spill over’ theory in the literature [103]. I would like to characterise this relationship further with other ECM markers including metalloproteins, collagens and TGF- β pathways and how it relates to hepatic steatosis development.

8.3 CYP-sEH pathway during the transition to T2DM and MASLD

Oxylipins are potent lipid mediators that have been studied in several other disease including cardiovascular disease, pain, and hormonal pathways and depending on the specific PUFA-derived metabolite have been described as pro-inflammatory or pro-resolving as well as being involved in PPAR γ signalling and angiogenesis [324, 391, 512]. I measured the oxylipins during the progression to T2DM and MASLD. The strength of my analysis was the paired multi-tissue approach (measuring oxylipins in liver, plasma and oWAT) using both obese and lean patients, and as far as I am aware, this has not previously been undertaken.

During obesity, fatty acid diols decreased in the oWAT and plasma but there was no change in hepatic tissue levels. They highlight a previously unappreciated role of fatty acid diols in adipose tissue homeostasis which has been contradictory in the literature to date. However, there is emerging evidence diols do decrease during metabolic decline in obesity [346, 381]

and an sEH inhibitor, which decreases diols, did not improve insulin sensitivity, in a human trial [398].

When I compared levels of diols in the oWAT and plasma to the oWAT maladaptive changes characterised in the same cohort the diols were inversely correlated with adipocyte hypertrophy. An EPHX1 deletion, the enzyme coding for the epoxide to diol, in a pre-adipocyte cell culture prevented adipogenesis [526]. Taken together with my results, this suggests diols are integral to healthy adipogenesis and are likely influencing the balance of adipogenesis versus hypertrophy in obesity.

These results also suggested an overall reduced oWAT and liver sEH activity during metabolic syndrome and MASLD progression with epoxide:diol ratio increasing with oWAT inflammation and hepatic steatosis. This is in contrast to animal models that suggest inhibiting the sEH activity and increasing epoxides attenuates hepatic steatosis and glucose control [360, 393]. Interestingly the human trial using sEHi did not show improvement in insulin sensitivity as expected [381, 397, 398]. My results therefore can explain, in part, these findings. Epoxides were not significantly modulated in prolonged obesity in my cohort but as there were already low levels of sEH activity coupled with decreased levels of diols in the oWAT and plasma, reducing these further with the sEHi is likely to have further deleterious effects.

As I have demonstrated that CYP-derived oxylipins are modulated during obesity and some oxylipins have been shown to act on PPAR γ signalling [385] (which I also showed was upregulated by metformin in an explant model) this posed the question: are the oxylipin changes in the oWAT being driven by Metformin? Using the explant model treated with Metformin I measured the CYP-derived oxylipins and demonstrated that they are not significantly altered. This led me to suggest that Metformin is modulating adipogenesis but not through oxylipin-PPAR γ signalling.

8.4 Conclusions

This work has contributed to the understanding of obesity-mediated changes in human oWAT homeostasis and uncovered a conserved oxylipin signature involving the CYP-sEH pathway during the progression to T2DM and MASLD/MASH. Briefly summarised below

- 1) oWAT dysfunction in prolonged obesity and metabolic decline is characterised by adipocyte hypertrophy with a decrease in PPAR γ /CEBP α , macrophage-mediated inflammation and a decrease in collagen type I and type VI deposition.
- 2) Multi-tissue profiling of obese oWAT, liver and plasma show a decrease in predominantly CYP-derived fatty acid diols in oWAT and plasma with obesity while

sEH activity decreased with oWAT inflammation and hepatic steatosis during the progression to T2DM and MASLD.

- 3) Metformin has a pro-adipogenic action on human oWAT at physiological concentrations but does not alter oWAT inflammation or ECM composition using an *ex vivo* oWAT explant model.

Overall, adipogenesis has been altered at every level of investigation with adipocyte hypertrophy associated with deleterious effects in all tissues. I believe modulating this pathway potentially through increasing oWAT fatty acid diols or increasing sEH activity, may have beneficial effect on oWAT homeostasis and in turn hepatic function.

8.5 Limitations

The cohort used for this study comprised of patients undergoing bariatric surgery with obesity and lean controls undergoing upper gastrointestinal surgery. This group of patients, especially the patients with obesity, are often a multi-morbid population which is very heterogenous, and patients take multiple different classes of medications. To minimise this, patient on immunosuppression or insulin were excluded however, it is important to bear in mind other medications may also be influencing phenotypes presented.

Another limitation of the cohort was the lean patients were older and more likely to be male compared to the obese patients. Therefore, to draw direct comparison when analysing the crosstalk between oWAT and liver during progression of obesity, age and sex matched lean liver tissue would strengthen the results shown. Liver tissue was also not collected from the lean patients and therefore going forward including a lean group for the MASLD analysis would be of benefit.

8.6 Further work

I would like to extend this study to evaluate the more severe MASLD phenotype. The cohort of obese patients comprised predominantly of MASLD or MASH with no patients diagnosed with cirrhosis. This would benefit the results two-fold; firstly, oWAT inflammation did not correlate with hepatic disease progression in MASLD and hepatic inflammation and fibrosis did not appear to correlate with oWAT phenotyping. As I hypothesise this is due to the early stage of MASLD in this cohort, including more severe phenotypes would enable a further understanding of the oWAT and liver crosstalk in obesity in later disease. It would also establish if the oxylipin signature seen in this cohort is an early MASLD/ T2DM phenomenon or also present in MASH/cirrhosis. If this were to remain significant during MASH fibrosis and cirrhosis, it could be a potential target for therapeutic intervention or used as a possible biomarker. Currently there are no robust biomarkers with a high sensitivity and specificity for early MASLD/ MASH and no markers that are predictive of progression, currently patients

are re-assessed regularly to see if they have become fibrotic or cirrhotic and is therefore an unmet need clinically and would be an interesting avenue to pursue [77].

This work has focused on the omental WAT depot, but I have begun phenotyping a subgroup of patients from whom I also collected subcutaneous adipose tissue (scWAT) with the aim to conduct a similar analysis to that undertaken on the oWAT. I aim to determine if certain maladaptive features or lipid signatures are depot specific as the literature currently suggests. I would also like to identify the specific role scWAT has in disease progression in T2DM and MASLD though characterising the crosstalk between scWAT and the liver.

While I have undertaken multi-tissue profiling of oxylipins in obesity and characterised the CYP-sEH pathway changes seen in T2DM and MASLD progression, the next step would be to integrate this with other omics technology. I am currently using bulk-RNA-seq to characterise the transcriptome in paired oWAT and liver tissue in obesity which would complement the lipidomic profiling and identify signalling pathways that are being modulated with the aim to perform functional studies and identify potential mechanisms.

From a therapeutic angle, fatty acid diol supplementation in pre-clinical models of obesity, MASLD and MASH seem a logical continuation of the findings presented in this thesis. In fact, there is a growing literature about the protective effects of these metabolites (e.g. 12,13-DiHOME) but the cellular mechanisms through which they exert their effects remain largely unknown. Investigating the role of fatty acid diols in adipogenesis and conducting *in vivo* supplementation experiments will improve the translational research based on oxylipins beyond sEH.

As previously mentioned, there remains a clinical need to find non-invasive biomarkers that can be reliably used to stratify patients' stage of MASLD and I believe using a multi-omic approach would provide an opportunity to discover potential options. While there are novel drugs on the horizon with Resmetirone (a selective thyroid hormone receptor-agonist) recently been approved by the FDA in the US as a treatment option for MASH cirrhosis, there are currently no drugs licenced in the UK that can prevent progression and again this approach may provide new targets.

8.7 Final conclusions

I demonstrated that the CYP-sEH pathway is modulated during the transition from obesity to T2DM and progressive MASLD and adipogenesis is integral to oWAT health. Further investigation would determine if either of these were promising treatment targets. It also importantly demonstrates sEH is unlikely to be clinically useful in T2DM/MASLD.

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