

Sexual Dimorphisms in Fat Distribution: Investigating Oestradiol's Depot-Specific Effects on Preadipocyte and Macrophage Interactions

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*A thesis submitted for the degree of
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Abstract

Fat distribution is a key regulator of cardiometabolic disease risk, with biological females typically favouring fat storage in the gluteofemoral region, while males tend to store fat around the abdomen. Accumulation of gluteofemoral adiposity, particularly through hyperplastic expansion, is metabolically favourable and is thought to be promoted by the sex hormone oestradiol in females. Alterations in oestradiol concentrations, such as those occurring at menopause or during oestradiol therapy, are associated with shifts in fat distribution and subsequent changes in cardiometabolic risk. However, the mechanisms underlying these oestradiol-induced changes remain poorly understood and are complicated by the controversial literature surrounding the effects of oestradiol on adipose tissue expansion. Given the documented role of oestradiol in modulating the inflammatory phenotypes of other cells within the adipose tissue stromovascular fraction (SVF), particularly macrophages, it is essential to investigate how oestradiol may influence signalling between adipocytes and macrophages to promote depot-specific changes in adipose expansion, favouring gluteofemoral adipose tissue growth and abdominal adipose tissue regression. This thesis aimed to first characterize the depot-specific SVF population landscape using single-cell RNA sequencing (scRNASeq) on paired abdominal and gluteal adipose tissue biopsies. It then assessed oestradiol signalling in abdominal and gluteal preadipocytes using in-vitro models, and established an in-vitro co-culture system between preadipocytes and macrophages. This system facilitated the identification of oestradiol-primed communication signals that may modulate depot-specific expansion through RNA sequencing approaches. The results reveal unique transcriptional landscapes of abdominal and gluteal adipose tissue SVF and demonstrate that preadipocyte responses to oestradiol are highly depot-specific. Additionally, this work identified a range of candidate signals exchanged between preadipocytes and macrophages that may contribute to oestradiol-primed, depot-specific adipose tissue expansion. Future research should aim to functionally test these signals using the in-vitro systems developed here.

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Declaration

I hereby declare that no part of this thesis has been submitted for any degree at this or any other university. The work presented herein is entirely my own except where stated in the text and below:

- The human biopsies used for the single cell RNA sequencing dataset were taken and processed by Dr Andrea Van Dam of OCDEM, before undergoing library preparation by colleagues in Zurich. Sequencing and initial raw data processing was conducted by NovoNordisk.
- The fractionated adipose tissue dataset was generated by Dr Marijana Todorčević of OCDEM, but was left unanalysed and unpublished. All data analyses were performed only here.
- Measurement of oestradiol and relevant oestradiol metabolites in cell culture media supernatant was carried out at Manchester University NHS Foundation Trust Biochemistry Department, and Birmingham University Steroid Metabolome Analysis Core.

Supervision for this work was provided by Professor Fredrik Karpe, Dr Katherine Pinnick and Professor Jelena Bezbradica Mirkovic, and funding was provided by The Kennedy Trust, with support from the OxKen Studentship Programme.

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Abstract

Fat distribution is a key regulator of cardiometabolic disease risk, with biological females typically favouring fat storage in the gluteofemoral region, while males tend to store fat around the abdomen. Accumulation of gluteofemoral adiposity, particularly through hyperplastic expansion, is metabolically favourable and is thought to be promoted by the sex hormone oestradiol in females. Alterations in oestradiol concentrations, such as those occurring at menopause or during oestradiol therapy, are associated with shifts in fat distribution and subsequent changes in cardiometabolic risk. However, the mechanisms underlying these oestradiol-induced changes remain poorly understood and are complicated by the controversial literature surrounding the effects of oestradiol on adipose tissue expansion. Given the documented role of oestradiol in modulating the inflammatory phenotypes of other cells within the adipose tissue stromovascular fraction (SVF), particularly macrophages, it is essential to investigate how oestradiol may influence signalling between adipocytes and macrophages to promote depot-specific changes in adipose expansion, favouring gluteofemoral adipose tissue growth and abdominal adipose tissue regression. This thesis aimed to first characterize the depot-specific SVF population landscape using single-cell RNA sequencing (scRNASeq) on paired abdominal and gluteal adipose tissue biopsies. It then assessed oestradiol signalling in abdominal and gluteal preadipocytes using in-vitro models, and established an in-vitro co-culture system between preadipocytes and macrophages. This system facilitated the identification of oestradiol-primed communication signals that may modulate depot-specific expansion through RNA sequencing approaches. The results reveal unique transcriptional landscapes of abdominal and gluteal adipose tissue SVF and demonstrate that preadipocyte responses to oestradiol are highly depot-specific. Additionally, this work identified a range of candidate signals exchanged between preadipocytes and macrophages that may contribute to oestradiol-primed, depot-specific adipose tissue expansion. Future research should aim to functionally test these signals using the in-vitro systems developed here.

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List of Abbreviations

11βHSD		11 Beta-Hydroxysteroid Dehydrogenase
AcMo		Activated Classical Monocytes
ADSCs		Adipose-Derived Stem Cells
AGE		Advanced Glycation End Product
AKR		Aldo-Keto Reductase
ANGPTL4	. .	Angiopoietin-Like 4
APC		Adipose Progenitor Cell
APOE		Apolipoprotein E
ASAT		Abdominal Subcutaneous Adipose Tissue
ATM		Adipose Tissue Macrophage
BAT		Brown Adipose Tissue
BCHE		Butyrylcholinesterase
BMI		Body Mass Index
BSA		Bovine Serum Albumin
CD		Cluster of Differentiation (used for cell surface markers)
cDCs		Conventional Dendritic Cells
cMo		Classical Monocytes
CPM		Counts Per Million
CSF1		Colony Stimulating Factor 1 (Macrophage Colony Stimulating Factor)
CSF2		Colony Stimulating Factor 2 (Granulocyte-Macrophage Colony Stimulating Factor)
CYP19A1	. . .	Cytochrome P450 19A1 (aromatase enzyme)
DC		Dendritic Cell
DEG		Differentially Expressed Gene
DMEM/F12	. .	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
ERRs	Oestrogen-related Receptors
ER-α	Oestrogen Receptor Alpha (protein)
ER-β	Oestrogen Receptor Beta (protein)
ESR1	Oestrogen Receptor Alpha (transcript)
ESR2	Oestrogen Receptor Beta (transcript)
FA	Fatty Acid
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FDR	False Discovery Rate
FFA	Free Fatty Acid
FGSEA	Fast Gene Set Enrichment Analysis
GFAT	Gluteofemoral Adipose Tissue
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPER1	G Protein-Coupled Oestrogen Receptor 1 (formerly GPR30)
GPR30	G Protein-Coupled Receptor 30
GSEA	Gene Set Enrichment Analysis
HDL	High-Density Lipoprotein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-Fat Diet
HMDM	Human Monocyte-Derived Macrophage
HRT	Hormone Replacement Therapy
HSD	Hydroxysteroid Dehydrogenase
IDMS	Isotope Dilution Mass Spectrometry
IL	Interleukin (commonly followed by a number, e.g., IL6, IL1 β , etc.)
iMo	Intermediate Monocytes

LAM	...	Lipid-Associated Macrophages
LCMS	...	Liquid Chromatography Mass Spectrometry
LDL	...	Low-Density Lipoprotein
LPS	...	Lipopolysaccharide
M1	...	A subtype of macrophages often associated with inflammatory responses
M2	...	A subtype of macrophages often associated with anti-inflammatory responses
MAIT	...	Mucosal Associated Invariant T Cells
MBDFAT	...	Immortalised human preadipocyte depot-specific cell lines used in these experiments (female-derived)
MCP	...	Monocyte Chemoattractant Protein
M-CSF	...	Macrophage Colony-Stimulating Factor
MFe^{hi}	...	Iron-Regulating Macrophages
MHC	...	Major Histocompatibility Complex
MMe	...	Metabolically Activated Macrophage
MMP	...	Matrix Metalloproteinase
mNK	...	Mature Natural Killer
MOI	...	Multiplicity of Infection
Mo-Mac	...	Monocyte-Macrophage
ncMo	...	Non-Classical Monocytes
NFKB	...	Nuclear Factor Kappa B
NK	...	Natural Killer
PAT2 DFAT	...	Immortalised human preadipocyte depot-specific cell lines used in these experiments (male-derived)
PBMC	...	Peripheral Blood Mononuclear Cell
PBS	...	Phosphate-Buffered Saline
pDCs	...	Plasmacytoid Dendritic Cells
PPAR	...	Peroxisome Proliferator-Activated Receptor
PVMs	...	Perivascular Macrophages
qPCR	...	Quantitative Polymerase Chain Reaction

RIN	RNA Integrity Number
RIPA buffer	Radioimmunoprecipitation Assay buffer
RLT	RNA Lysis buffer
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SAT	Subcutaneous Adipose Tissue
scRNASeq	Single-cell RNA Sequencing
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SVF	Stromal Vascular Fraction
SWAT	Subcutaneous White Adipose Tissue
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming Growth Factor (often followed by a Greek symbol, as in TGF- β)
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor (often followed by a number, as in TLR4)
TMM	Trimmed Mean of Means
TNF	Tumor Necrosis Factor (commonly followed by a Greek symbol, as in TNF- α)
TPA	12-O-Tetradecanoylphorbol-13-acetate
TPM	Transcripts Per Million
Tregs	Regulatory T Cells
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
UMAP	Uniform Manifold Approximation and Projection
UMIs	Unique Molecular Identifiers
VAT	Visceral Adipose Tissue
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue

1

Introduction

1.1 Adipose Tissue and its Distribution in Relation to Metabolic Health

Adipose tissue is a specialised form of connective tissue with key roles in the regulation of energy balance. It stores excess energy in the form of triglycerides, housed within adipocyte lipid droplets. These lipids can be hydrolysed into free fatty acids (FFA) and glycerol, which are then released into the bloodstream to provide energy for other tissues during periods of increased demand, such as fasting or physical activity. This ability to store and release energy is vital for maintaining metabolic flexibility and energy homeostasis. Additionally, adipose tissue acts as an active endocrine organ, secreting a variety of hormones, such as leptin, which is critical for the central regulation of appetite and satiety, and adiponectin, which enhances insulin sensitivity. White adipose tissue (WAT), the most abundant form of adipose tissue, is responsible for energy storage and insulation, being located both subcutaneously and around the internal organs. In contrast, brown adipose tissue (BAT) contributes to energy expenditure through non-shivering thermogenesis, generating heat to maintain body temperature, especially in cold environments [1]. WAT also serves structural and mechanical functions, cushioning joints, and absorbing shock in pressure-bearing areas like the palms and soles [1]. This tissue

is composed of a diverse cellular community, including preadipocytes, endothelial cells, and immune cells, all of which contribute to its complex roles in regulating tissue homeostasis and metabolic responses.

In humans, WAT adipocytes reside in distinct anatomical depots, primarily comprising of gluteofemoral (GFAT), subcutaneous abdominal (ASAT) and intra-abdominal/ visceral (VAT) adipose tissue. These depots are differentially associated with metabolic risk, independent of total adiposity. Individuals who accumulate a higher proportion of adipose tissue in the upper-body depots (ASAT and VAT) exhibit a greater metabolic risk compared to those with preferential storage in the lower-body (GFAT). After adjusting for total fat mass, lower-body adiposity is associated with reduced cardiometabolic risk, suggesting that fat storage in this region exerts protective effects on metabolic health [2]. Notably, the relative expansion of each of these depots and its associated metabolic risk, is differentially influenced by plasma hormone profiles; particularly sex-steroid hormones. ASAT tends to expand in response to cortisol and androgens [3], meanwhile GFAT expands in response to oestradiol [4]. Sex-differences in these hormones and their depot-dependent responses, therefore, are key players in mediating sex differences in fat distribution and corresponding metabolic risk.

1.1.1 Depot-specific Lipid Handling

The differences in fat distribution and associated metabolic risk are thought to arise from the distinct developmental profiles of adipocytes and their unique microenvironmental niches within each depot, resulting in varying metabolic functionalities. ASAT and VAT exhibit a higher lipolytic turnover rate relative to GFAT, and are also more sensitive to catecholamine-stimulated lipolysis, leading to increased release of FFA into the circulation, particularly under conditions of fasting or stress [5]. This elevated FFA release may increase the risk of metabolic impairments including insulin resistance. Due to VAT's anatomical location, FFAs released from this depot are directly transported to the portal vein, resulting in a substantial hepatic FFA influx [6–8], which significantly raises the risk of

hepatic steatosis and insulin resistance. In contrast, GFAT acts as a longer term, metabolically safe ‘sink’ for energy storage. It has a slower lipolytic rate and is less sensitive to catecholamine-induced lipolysis, although is not thought to exhibit a greater capacity for de novo lipogenesis [9], as compared to abdominal tissue. The origin of the fatty acids (FA) being taken up, however does vary between ASAT and GFAT; ASAT shows a preference for chylomicron-derived FA, while GFAT shows enhanced uptake of very low density lipoprotein (VLDL) -derived FA [2]. This is of metabolic relevance, as lipodystrophic syndromes that are specifically associated with the loss of GFAT, are concurrently associated with increased plasma VLDL and heightened metabolic risk, highlighting the importance of GFAT-associated FA uptake from VLDL particles [2]. The unique lipid metabolic signature of GFAT therefore allows the efficient uptake of VLDL-derived FA, and retention of this FA, due to GFAT’s resistance to lipolysis. This makes GFAT a ‘stubborn’ FA storage site, even during weight loss, where lipid mobilisation from other depots is prioritised. Furthermore, GFAT expansion is associated with a more favourable adipokine profile and lower risk of metabolic disease [2]. Consequently, the greater storage capacity and reduced lipolytic activity of GFAT make it an ideal site for the effective sequestration of lipids, minimising the risk of FFA release and preventing lipid overflow into non-adipose, ectopic tissues [10].

1.1.2 Depot-specific Expansion Mechanisms

In response to surplus circulating lipids, adipose tissue can expand through either hypertrophy or hyperplasia. Adipocyte hypertrophy is the process by which there is an increase in cell size due to the accumulation of cellular lipid, meanwhile hyperplasia is a mechanism permitting an increased number of adipocytes following the proliferation and differentiation of preadipocytes. Hyperplasia is generally considered the more adaptive and metabolically favourable response to fat accumulation, as compared to hypertrophy, since smaller fat cells are regarded as more insulin sensitive and less inflammatory [11–13]. Hyperplasia allows for the effective buffering of excess lipids across newly differentiating cells, rather than

overloading existing lipid-filled cells. Adipocyte hypertrophy occurs in situations of demand for fat storage, with limited hyperplasia, leading to increased triglyceride content within already lipid-laden cells. Hypertrophy becomes deleterious when the cellular threshold for lipid storage is exceeded. At this point, adipocytes lose their capacity to store lipid effectively, leading to an inability to uptake FA from plasma lipoproteins, and the overflow of FFAs into the circulation. These FFAs may subsequently reach sites of ectopic fat storage, for example in the liver, contributing to the formation of hepatic lipid droplets, which can promote systemic metabolic dysfunction [14]. Enlarged adipocytes are also prone to endoplasmic reticulum stress due to the heightened demand for lipid processing, which activates inflammatory pathways, contributing to insulin resistance and adipocyte dysfunction. Moreover, hypertrophic adipocytes exhibit reduced secretion of beneficial factors such as adiponectin and IL-10, while increasing the secretion of pro-inflammatory cytokines including TNF- α and IL-6 [15]. Hypertrophy can also trigger fibrotic remodelling, characterised by the excessive deposition of extracellular matrix (ECM) proteins such as collagens, which stiffen the surrounding scaffold. This mechanical impediment restricts adipocyte expansion and therefore reduces their capacity for lipid storage. Fibrosis further impairs angiogenesis, creating a hypoxic environment that exacerbates cellular stress. Therefore, the ability of adipose tissue to expand and store lipids in response to nutrient flux is the crux to maintaining both adipose tissue health, and whole body metabolic health.

ASAT and VAT primarily favour hypertrophic expansion, whereas GFAT has a greater capacity for hyperplastic expansion [16–18]. Gluteal adipocytes may also possess a higher lipid storage capacity, allowing for a greater extent of hypertrophy before reaching their overload limit, as compared to abdominal adipocytes [19, 20]. This superior capacity for expansion, through increasing both cell size and cell number, allows GFAT to act as a safe buffer for lipid storage, providing a metabolic advantage when adiposity is favoured here, as opposed to ASAT.

1.1.3 Depot-Specific Inflammatory Profiles

As eluded to previously, the accumulation of fat in ASAT and VAT is associated with a more pronounced inflammatory profile relative to GFAT, contributing to differences in systemic inflammatory cytokine release and subsequent metabolic risk [21–24]. For example, measurement of cytokine concentrations in arterial and venous blood samples across ASAT and GFAT has revealed a greater release of IL-6 from ASAT, as compared to GFAT [25]. This data is supported by large-scale cohort studies, which associated inflammatory factors such as IL-6 and TNF- α with greater abdominal adiposity and reduced thigh adipose tissue [26]. Although it is traditionally thought that adipose tissue macrophages (ATMs) are the primary contributors to cytokine production in obesity-related chronic inflammation, it is unclear whether their phenotypes vary between ASAT and GFAT, and whether this contributes to differences in observed cytokine release [27, 28]. Existing literature has compared the abundance of ‘inflammatory’ and ‘anti-inflammatory’ macrophage populations between ASAT and GFAT, reporting a higher prevalence of inflammatory macrophages in GFAT [29]. However, this study used only a single marker to determine macrophage phenotype, and the chosen markers were based on murine models, which may not accurately represent human populations [30]. Other studies have reported no differences in inflammatory potential between the depots [31], or solely report on macrophage differences between VAT and subcutaneous adipose tissue (SAT) [22, 32], leaving a gap in understanding as to whether ASAT and GFAT show differences in inflammatory profiles, and if so, which cells are responsible for these differences.

Any existing depot-differences in inflammatory profiles, however, are likely exacerbated by metabolic variations in depot function, as ASAT is more susceptible to hypoxia, hypertrophy and cellular stress in response to excess nutrition, as compared to GFAT. Hypertrophy and adipocyte dysfunction can lead to necrosis, attracting local macrophages to scavenge cellular debris, which triggers inflammatory responses and contributes to the pathological release of cytokines [33]. This state of chronic inflammation may be further compounded by depot-specific differences in both

the rate of lipid turnover and the type of lipid species being released from each depot. Lipid exposure itself can influence macrophage polarisation; ASAT stores and releases more saturated FA species, while GFAT stores more unsaturated FA species [34]. Saturated FAs are known to induce a pro-inflammatory, M1 macrophage phenotype, while unsaturated FAs promote a more anti-inflammatory M2-like phenotype [35]. Although the relationship between FA storage, FA release and macrophage polarisation, in different depots has not been explicitly investigated, the existing differences in lipid dynamics and macrophage polarisation suggest that adipocyte depot-specific lipid handling likely influences macrophage polarisation in a depot-dependent manner.

1.2 Sexual Dimorphisms in Fat Distribution

1.2.1 The Evolutionary Need for GFAT Expansion in Females

Biological sex significantly influences the expansion of adipose tissue in different depots, including ASAT, GFAT and VAT. Women tend to accumulate fat in the lower-body, resulting in the classic ‘pear’ shape, while men tend to store fat around the abdomen, leading to an ‘apple’ shape. As discussed, these patterns contribute to sexual dimorphisms in metabolic risk. The preferential storage of lipid in GFAT, observed in women, begins at puberty, suggesting that GFAT expansion plays a favourable role in fertility. Evolutionarily, GFAT expansion in women likely evolved as a mechanism to allow the safe storage of a large amount of energy, to protect against energy deficits which could impede foetal growth during pregnancy. The male sex favoured the formation of muscle mass, allowing easier access to immediate nutrition, as there was no need for gestation-requiring long-term energy storage facilities.

Still, the modern food environment has changed since hunter-gatherer times, without sufficient time for the appropriate genetic compensation to increase fat storage capacity, particularly in males. This has resulted in an obesity crisis, and its compendium of metabolic complications. In Western societies, people

have largely unrestricted access to highly palatable, energy-rich foods. Ultra-processed foods in particular, are often low in fibre and high in sugars and fat, leading to overconsumption, positive energy balance, and the subsequent need for excess energy storage in adipose tissues. As a result, both sexes now need to accommodate significant fat storage, although males have a reduced capacity to do so, as compared to females.

1.2.2 The Role of Oestradiol in Modulating Fat Distribution

These sex differences in the ability to safely house adipose tissue in GFAT have manifested in differences in cardiometabolic disease risks, with men being at greater risk than women. Sex hormones are important players in these differences, as this disparity only emerges post-pubertally [36, 37] and dissipates at the menopause, coinciding with a surge, and drop in circulating oestradiol levels, respectively. These changes also coincide with changes in fat distribution; female puberty is associated with increased GFAT [38], while menopause is associated with increased VAT [39], which can be reversed with oestradiol treatment [40]. Further research has shown that plasma oestradiol promotes GFAT accumulation at the expense of VAT - for example, as seen in transgender women undergoing oestradiol therapy [41–44] and during hormone-replacement therapy (HRT) at menopause [45]. Interestingly, the use of oral contraceptives has not been reliably associated with changes in fat distribution [46–48], possibly due to the synthetic ethinyloestradiol used (as opposed to oestradiol in HRT), and also higher doses of progestin, which may have opposing actions to oestradiol [49, 50].

Evidence of oestradiol’s impact on fat distribution is also apparent in conditions relating to oestradiol deficiency, such as hypopituitarism (e.g in Sheehan’s Syndrome) [51], ovarian insufficiency [52], Turner’s syndrome [53], anorexia nervosa and hypothalamic amenorrhea [54], hysterectomy [55], aromatase inhibitor therapy [56], and hyperprolactinaemia [57]. These conditions are also frequently associated with increased metabolic risk.

Direct evidence for oestradiol’s effects on GFAT expansion in-vivo comes from human

studies using transdermal oestradiol patches, which specifically increase adipogenesis in the gluteofemoral depot [4]. This likely occurs through the recruitment of adipocyte progenitor cells, as transdermal oestradiol has been linked to an increase in the proportion of smaller, early-differentiated adipocytes, in GFAT but not ASAT [58]. A higher number of early differentiating cells has also been observed in premenopausal female GFAT as compared to male GFAT, further supporting the role of oestradiol in enhancing progenitor cell recruitment for adipogenesis in GFAT [16]. Mouse studies, although not able to accurately replicate human fat depot dynamics, support these findings, showing that adipose progenitor cell proliferation in response to a high fat diet (HFD) was dependent on plasma oestradiol and signalling through oestrogen receptor alpha ($ER-\alpha$) [59]. Meanwhile, knocking out $ER-\alpha$ results in reduced progenitor cell commitment to the WAT lineage, strongly suggesting that oestradiol can modulate adipocyte progenitor cell fate [60]. This may contribute to the broader actions of oestradiol on depot expansion. Oestradiol has also been implicated in the pathological expansion of GFAT in lipoedema, where GFAT may show abnormal expression of oestrogen receptors, leading to hypersensitivity and increased expression in response to oestradiol [61–63].

Although oestradiol promotes GFAT expansion, the effects of other sex hormones on fat distribution should also be considered. Testosterone may also contribute to sexual dimorphisms in fat distribution, having been shown to reduce the accumulation of both VAT and SAT [64]. Interestingly, its effects on VAT regression have been associated with its conversion to oestradiol - castrating male mice increases VAT mass, which is ameliorated with oestradiol treatment. Moreover, treating non-castrated mice with an aromatase inhibitor, which prevents the interconversion of testosterone to oestradiol, increased visceral adiposity, suggesting that circulating testosterone reduces VAT mass only when it is locally converted to oestradiol in VAT [64]. Meanwhile, the effects of testosterone on SAT are opposite to those of oestradiol, preventing the expansion of these depots. Human studies have further demonstrated that reduced testosterone is specifically associated with GFAT accrual, clearly highlighting its opposing role to oestradiol [65] in GFAT expansion.

Overall, oestradiol is a prime candidate for promoting female-associated GFAT expansion, and its associated reductions in metabolic risk. However, the mechanisms by which oestradiol regulates depot-specific adipose accumulation remain poorly understood, and are somewhat controversial.

1.3 Purported Effects of Oestradiol on In-Vitro Adipocyte Cultures

It has been established for over 20 years that preadipocytes and adipocytes express the oestrogen receptors necessary for responding to oestradiol [66]. However, their functionality and downstream effects in-vitro remain contentious, despite clear evidence of oestradiol's impact on adipose biology in-vivo. Studies have shown that oestradiol can either enhance [67–73], inhibit [74–79], or have no effect [80–83] on the proliferation and subsequent differentiation of preadipocytes. These contradictory findings underscore the complexity of oestradiol's roles, which appear to vary dependent on the specific in-vitro conditions.

One potential reason for these discrepancies is the lack of depot-specific adipocytes in many in-vitro models, since oestradiol may have opposing effects in ASAT and GFAT, inhibiting adipose expansion abdominally, while promoting gluteal expansion. In-vitro culturing of adipocytes is frequently conducted without considering the cellular depot of origin. For example in existing cellular models, primary adipocytes are often pooled from multiple different depots, cell lines in use may not be depot-specific (e.g 3T3-L1), the depot of origin may not be reported, or rodent cells, where depots are anatomically different to humans, may be used. This lack of specificity could obscure the effects of oestradiol in-vitro, particularly when primary samples are pooled from multiple depots, potentially masking depot-specific differences due to the effects of pooling. Studies directly comparing the effects of oestradiol on adipocytes from different depots are scarce, and the majority of these focus on comparing ASAT with VAT [84, 85], rather than ASAT with GFAT. Even within these comparisons, conclusions remain inconsistent, leaving the mechanisms behind oestradiol's depot-specific effects, largely unresolved.

Another explanation for these inconsistent findings may relate to the isolated monoculture conditions that are frequently used in-vitro. Whole adipose tissue houses cell types beyond those of the adipogenic lineage, including immune cells such as macrophages, which are known to play important roles in tissue homeostasis, turnover and expansion. In the studies where oestradiol has been shown to alter preadipocyte proliferation or differentiation, most used very early passage primary cells, where some immune components of the tissue were likely retained [67–69, 71]. Conversely, studies reporting no effect, or a negative effect of oestradiol, often used isolated preadipocytes or cell lines [75–79, 82, 83]. This suggests that cells other than those of the adipogenic lineage may be necessary for mediating oestradiol’s effect in adipose tissue. Given the substantial evidence supporting the role of macrophages in adipose tissue, and their phenotypic polarisation by oestradiol, it is crucial to consider the influence of oestradiol on communication between macrophages and adipocytes.

1.4 Macrophages in Adipose Tissue

1.4.1 Adipose Tissue Macrophages in Obesity and Pathology

Macrophages are the most abundant leukocytes in adipose tissue, increasingly recognised for their roles in tissue homeostasis, influencing remodelling, inflammation, and mitochondrial dynamics. M1 and M2 are two names assigned to anti- and pro-inflammatory (respectively) macrophage states. It is recognised that this division is not binary, but rather a spectrum of functional states between M1 and M2, that macrophages adopt based on local tissue signals. M1 and M2 nomenclature is retained here for simplicity only, and will be discussed later.

The deleterious role of macrophages in obesity-associated chronic inflammation is well-documented. In this context, macrophages form crown-like structures around hypertrophic, stressed and necrotic adipocytes, which promotes cytokine release and increases in basal tissue inflammation. Seminal research by Weisberg et al identified pathological changes in adipose tissue macrophage (ATM) composition

with obesity, noting an increase in macrophage content (from 10% to 50% cellular proportion), and a shift from an anti-inflammatory M2 phenotype, to a pro-inflammatory M1 phenotype [86, 87]. This macrophage increase was attributed to recruitment and infiltration of circulating monocytes, as opposed to the proliferation of existing resident macrophages, which typically exhibit an anti-inflammatory tissue-resident phenotype. As adipocytes undergo hypertrophy and cell death, the release of chemoattractants such as monocyte chemoattractant protein-1 (M-CP1), particularly in VAT [88, 89], drives monocyte recruitment. In response to tissue signals, these monocytes differentiate into cytokine-producing M1 macrophages [90]. This inflammation may be further perpetuated by adipocytes themselves, which produce pro-inflammatory mediators through processes such as hypoxia-induced cell death (often as a product of expansion without sufficient vascularisation) [91, 92], mechanical stress from hypertrophy [93], unfolded protein responses stimulated by excessive nutrient intake [94], and FFA-mediated stimulation of toll-like receptors (TLRs) [95–97]. Hyperglycaemia, often seen in obesity-induced type 2 diabetes mellitus (T2DM), may exacerbate inflammation by permitting glucose-mediated glycation and the subsequent formation of advanced glycation end products (AGEs) [98]. Both the activation of TLRs and the formation of AGEs activate inflammatory cascades, for example through NF κ B, further increasing cytokine production [98]. This ongoing cycle of inflammation promotes further monocyte recruitment, and an ensuing pathological spiral of obesity-associated inflammation. The resultant inflammation has systemic consequences, including insulin resistance across multiple organs [99, 100], altered hepatic glucose production [101] and the formation of cancer-promoting reactive oxygen species (ROS) [102, 103]. Additionally, this inflammation increases susceptibility to autoimmune and other inflammatory diseases such as rheumatoid arthritis, Alzheimer’s disease and depression [104–109]. Thus, cytokine production by macrophages has broad pathological consequences which warrant attention.

1.4.2 Adipose Tissue Macrophages in Tissue Homeostasis

Despite the pro-inflammatory roles of M1-like ATMs, other macrophage subtypes play important physiological roles in tissue homeostasis, regulating various functions relating to ECM, tissue growth, lipid turnover and mitochondrial exchange, to name a few. These roles are carried out by a variety of tissue-resident macrophage subtypes, which cannot be bluntly classified as either M1- or M2-polarised. They instead sit on their own tissue-specific spectrum, whereby traditional polarisation markers may not necessarily categorise their subtypes [30]. For example, CD206 is typically an anti-inflammatory macrophage marker, yet its deletion in adipose tissue has been shown to improve glucose homeostasis and suppress the proliferation of adipocyte progenitors, suggesting a different role in this context [110]. Furthermore, the CD206⁺ macrophage population in VAT of obese individuals has been shown to express other M1 and M2 markers, suggesting that CD206 may not be appropriate for delineating inflammatory and anti-inflammatory populations in humans [30]. This knowledge gap may further contribute to the observed higher abundance of ‘inflammatory’ macrophages in GFAT, which may be inappropriately defined according to existing markers [29]. This highlights the need for research to better characterise the roles and markers of different ATM populations, particularly across various adipose tissue depots, further questioning how they contribute to depot-specific tissue functions.

1.4.2.1 Adipose Tissue Macrophages in Obesity

In obesity, there is an infiltration of inflammatory CD11c⁺ monocytes which contribute to cytokine production in chronic inflammation. Meanwhile, resident ATMs adopt a ‘metabolically activated macrophage’ (MMe) phenotype, likely as a result of the nutritional stresses associated with obesity. This is evidenced by in-vitro treatment of macrophages with glucose, insulin, and palmitate, which is sufficient to trigger the MMe programme [111]. Adipocyte progenitors may also influence this inflammatory shift, as progenitors derived from obese or T2DM subjects show a reduced ability to activate M2-like phenotypes and secrete anti-inflammatory

factors, compared to equivalent cells from lean donors [112]. Physiologically, resident macrophage populations play important roles in lipid-handling (discussed further below), yet these functions are impaired in obesity and inflammation. There appears to be a dichotomy between beneficial lipid-handling functions and inflammation, with lipid-associated transcripts such as *PPARG*, downregulating inflammatory signatures in MMe populations [111]. As the need to clear dead adipocytes increases, MMes upregulate lysosomal exocytosis pathways alongside inflammatory signalling, potentially due to lysosomal stress [113, 114]. This shift diverts ATMs from their physiological lipid processing roles, leading to increased inflammation, which negatively impacts neighbouring adipocytes.

1.4.2.2 TREM2⁺ Macrophages

A popular line of recent research has focused on lipid-associated macrophages (LAMs), which are signified by expression of ‘triggering receptor expressed on myeloid cells 2’ (TREM2). In adipose tissue, these lipid-laden macrophages exhibit a downregulation of pro-inflammatory genes and upregulation of lipid handling transcripts, positively aiding regulation of lipid processing [115, 116]. In atherosclerotic plaques, however, TREM2⁺ LAMs may differentiate into a more inflammatory PLIN2/TREM1⁺ population, which abundantly express inflammatory markers including IL-1 β , chemokine CCL2 and TLR2 [117]. This highlights the need to investigate macrophages at the tissue-specific level. In adipose tissue, TREM2 expression enhances phagocytic and lysosomal functions, as well as lipid catabolism and oxidative phosphorylation. This signature plays a role in lipid-buffering; scavenging FFA species to prevent their release into the systemic circulation for ectopic deposition. Accordingly, population expansion is observed in response to lipolysis, weight loss, fasting and β_3 -adrenergic agonists, helping to buffer increased lipid mobilisation in these circumstances [118–120]. In chronic obesity, these macrophages aggregate around large adipocytes to reduce hypertrophic stress [116]. This contrasts adaptations to early obesity, which largely consists of elevations in inflammatory macrophages [121]. Deleting TREM2⁺ macrophages prevents

their accumulation around large adipocytes, increasing lipid trafficking demands on adipocytes, resulting in hypertrophy and inflammation [116], which is characteristic of the adaptations to early obesity. On a systemic level, deleting this population caused an increase in body fat accumulation and metabolic consequences such as elevated serum low-density lipoprotein (LDL), hyperglycaemia and hyperinsulinaemia [116]. Additional studies have shown that deleting ATM populations enhances tissue FFA release, underscoring the functional role of this population in lipid-trafficking [122], particularly during states of elevated lipid flux. In-vivo RNA and genetic data further support this; both TREM2 expression in adipose tissue and single nucleotide polymorphisms (SNPs) in the TREM2 gene are associated with body mass index (BMI) [123], further substantiating the function of TREM2⁺ macrophages in regulating adipose tissue expansion.

1.4.2.3 TIM4⁺ Macrophages

TIM4⁺ resident macrophages play a role in reverse cholesterol transport within adipose tissue, promoting the production of beneficial high-density lipoprotein (HDL) [124], and regulating overall lipid storage through the production of PDGF α [125]. They show a further role in suppressing the switch to M1-like phenotypes by suppressing NF κ B signalling and stimulating TLR4 internalisation [126]. Deleting this population has been shown to promote inflammatory phenotypes and obesity [126]. Obesity also causes the regression of this population, instead favouring the infiltrating, inflammatory CD11c⁺ cells [127]. This switch, encouraging the dominance of inflammatory macrophages over lipid-handling populations, likely contributes to postliminary dyslipidaemia and obesity-associated chronic inflammation.

1.4.2.4 Iron-Regulating Macrophages

Other ATM populations are specialised for regulating iron content, expressing heightened levels of iron-handling genes. These processes are well established in tissues such as the spleen, where macrophage phagocytosis allows the recycling of iron from erythrocytes to supply systemic iron demands [128]. Similar resident iron-cycling macrophages, the MFe^{hi} population, plays a role in buffering adipose

tissue iron concentrations [129, 130] to protect adipocytes. While iron is essential for cellular processes like adipocyte differentiation [131], excess iron is cytotoxic, especially in adipose tissue, where it can react with excess lipids to form ROS such as lipid hydroperoxides, leading to ferroptosis [132, 133]. Obesity also often leads to iron overload [134], which impairs the production of insulin-sensitising adipokines such as adiponectin, and promotes insulin resistance [135]. The buffering capacity of iron-regulating macrophages is therefore critical for dampening these metabolic consequences. Indeed, reducing adipose tissue iron content in obese mice leads to concurrent reductions in oxidative stress, insulin resistance and inflammation [136, 137]. Yet, in obesity, there is a relative decrease in MFe^{hi} macrophage populations, and a polarisation switch within this population. These cells exhibit increased expression of inflammatory genes, and reduced expression of iron-handling transcripts, which diminishes their iron-buffering capacity, and enhances their ability to produce inflammatory cytokines [111, 129], both of which then exacerbate obesity-associated inflammation. Moreover, intra-cellular iron itself has been shown to influence macrophage polarisation [138], suggestive of a bi-directional relationship between iron handling and macrophage function. Consequently, in obesity, macrophages may become less capable of buffering tissue iron fluxes, leading to the repartitioning of iron to adipocytes. This iron overload in adipocytes promotes cellular stress, inflammation and insulin resistance [129, 139, 140], underscoring the critical role of ATMs in regulating tissue iron fluxes for adipose tissue health.

1.4.3 Adipocytes Influence Macrophage Phenotype

Cross-talk between adipocytes and macrophages is indeed a bi-directional and complex interaction; adipocytes can also dictate macrophage function. Adipocytes influence monocyte infiltration and macrophage inflammatory functions in times of nutritional stress, largely mediated by stress-induced MCP1 release, changes in antigen processing and secretion of other metabolic or hormonal factors [141–143]. The release of these factors by adipocytes is further influenced by the microenvironmental nutrients and metabolic by-products to which they are exposed.

FA-treated adipocytes, for example, secrete high levels of fetuin-A, which acts as a chemoattractant to monocytes, subsequently promoting M1-like polarisation. In human obesity, HFD-fed mice, and *db/db* mice, fetuin-A levels are increased, further suggesting that fetuin-A is a lipid-induced factor [144]. Although depot-specific production of fetuin-A has not been investigated, given that ASAT is exposed to increased levels of dietary FFAs and has a higher rate of lipolysis compared to GFAT, there is an increased presence of microenvironmental FFAs. This may lead to a heightened release of fetuin-A from ASAT relative to GFAT, contributing to depot-differences in macrophage infiltration and polarisation. Furthermore, adipocyte-derived FA can directly affect macrophages. Studies have shown that FAs from adipocyte-conditioned media enhance lipid biogenesis in macrophages, likely promoting a tissue-resident phenotype [145]. Thus, differences in both dietary- and adipocyte-derived lipid within the adipose tissue microenvironment may contribute to depot-differences in macrophage polarisation and infiltration.

Interestingly, adipose-derived stem cell (ADSC) proliferation may also contribute to differences in macrophage polarisation. Preadipocytes share properties with ADSCs, which have been shown to promote multi-faceted anti-inflammatory activity at macrophages [146]. ADSC-conditioned media increases macrophage production of IL-10, while decreasing production of TNF- α [147]. This anti-inflammatory activity may lead to growth-restorative effects, as has been noted in other stem-like populations [148, 149]. Given the higher proportion of preadipocytes, and increased hyperplastic capacity of GSAT relative to ASAT, the presence of stem-like populations may further contribute to depot differences in macrophage polarisation. Functionally, the anti-inflammatory effects of ADSCs at macrophages are believed to improve systemic insulin sensitivity. Delivery of ADSCs derived from lean mice, to the adipose tissue of obese mice, remodelled the macrophage landscape, promoting anti-inflammatory polarisation and increased insulin sensitivity, which was mirrored in in-vitro transwell studies [150]. This may be mediated by preadipocyte- and ADSC-derived factors such as sirtuin-6, which modulates M2-like macrophage activation. Knocking out sirtuin-6 in adipose tissue led to glucose intolerance,

insulin resistance and a reduction in M2 macrophages in mouse adipose tissue. This was due to a reduction in adipocyte IL-4 production [151], since adipocyte-derived IL-4 activates PPARG in macrophages, to permit M2-like polarisation. Ablation of PPARG in myeloid cells promotes inflammation, and reciprocally impairs adipocyte lipid storage and metabolic function [152], further highlighting the complex interlinks between adipocyte function and macrophage polarisation.

1.4.4 Macrophages in Adipose Tissue Expansion

1.4.4.1 Roles of Macrophages in Adipose Tissue Extracellular Matrix Remodelling

ECM remodelling is essential for adipose tissue expansion, adapting dynamically to accommodate processes including proliferation, differentiation, migration, hypertrophy, adhesion, and apoptosis. The ECM consists of various proteins, including collagens, glycoproteins and proteoglycans, which provide structural support and elasticity for tissue integrity, and facilitate cell signalling through the sequestration and release of molecules such as growth factors. Macrophages play a pivotal role in regulating this scaffold, enabling both the breakdown and secretion of ECM proteins. They secrete matrix metalloproteinases (MMPs), which are the primary enzymes responsible for ECM degradation. Simultaneously, tissue inhibitor of metalloproteinases (TIMPs) inhibit the activity of these enzymes, tuning the balance of ECM production versus breakdown by fibrolytic macrophages [153]. Fibrolytic macrophage populations exhibit lower ECM production signatures compared to other macrophages [154], and they also orchestrate receptor-mediated and phagocytic uptake of ECM components, directing them towards lysosomal breakdown pathways [155]. These macrophages typically have anti-inflammatory characteristics, and knocking out receptors associated with these functions, such as stabilin-1, leads to an increase in inflammatory profiles and excess collagen deposition [156]. In agreement with this, pro-inflammatory macrophages exhibit a more pro-fibrotic phenotype, a subject well-researched due to their pathological role in obesity-associated adipose tissue remodelling. These cells have a higher production rate of fibrotic ECM

components including collagens, and also release cytokines such as CCL17 alongside other soluble factors which activate fibroblasts and preadipocytes to produce more ECM proteins [157, 158]. This pro-fibrotic activity has been demonstrated in conditioned media studies. In these studies, macrophages are cultured separately, and their media is then transferred to adipocytes or preadipocytes. Conditioned media from ATMs of obese donors was placed on cultured human preadipocytes, resulting in increased secretion of pro-fibrotic ECM components by the preadipocytes, and an impaired differentiation capacity [157]. Pro-inflammatory cytokine release also accentuates fibrosis, recruiting more pro-inflammatory immune cells and activating TLR4, which promotes ECM production and fibroblast proliferation and differentiation. Inhibiting TLR4 signalling in macrophages prevents obesity-associated WAT fibrosis and the upregulation of collagen genes, typically seen with HFD [159]. Further to this, recruited monocytes express specific integrin proteins, which gives them a high capacity to activate latent TGF- β stored on the ECM scaffold, further promoting fibrosis [160]. Abnormal deposition and activation of ECM proteins, particularly collagen, causes stiffness and impairs adipogenesis, subsequently triggering stress responses in adipocytes, reducing their lipid storage capacity and increasing insulin resistance [161]. In turn, this promotes ectopic fat deposition. Much of the research investigating macrophage contributions to fibrosis, however, focuses on other tissues, such as the liver in hepatic fibrosis, or the skin in wound healing, rather than on WAT specifically.

1.4.4.2 Roles of Macrophages in Adipose Tissue Angiogenesis

As well as modulating adipose tissue expansion by altering ECM dynamics, macrophages can regulate adipose tissue angiogenesis, subsequently determining the hypoxic load of the tissue. Their role to enhance angiogenesis is well established in tumour microenvironments, where they serve as major sources of angiogenic factors in response to hypoxia. ECM degradation by macrophages can further promote the release of ECM-associated VEGFA, a potent regulator of blood vessel formation, into its bioactive form [162], subsequently promoting angiogenesis. Similarities are

seen physiologically in adipose tissue, where macrophages are key sources of certain angiogenic factors such as PDGF [163]. For both tumour-associated macrophages and ATMs, a subset of LYVE1⁺ cells play a role in vascular remodelling. These cells are spatially located near vessels, and maintain the pro-angiogenic niche through producing factors such as PDGF, which promotes perivascular mesenchymal stem cell proliferation and endothelial tube formation [163–165]. This functional role has been further highlighted through depletion studies, whereby the removal of these macrophages reduces PDGF expression and adipose tissue vascular density [163, 166], inhibiting adipose tissue expansion. This inhibition is thought to stem from reduced PDGF secretion, which prevents pericyte detachment from vessels, thereby hindering vascular development in WAT. It was suggested that during times of nutritional overload, modelled by elevated glucose and hypoxia, PDGF secretion by macrophages increases, facilitating WAT expansion through PDGF-dependent vascular remodelling [163, 167].

1.4.5 Macrophages in Adipocyte Growth and Differentiation

1.4.5.1 Macrophages Modulate Adipocyte Differentiation

As well as modulating adipose tissue expansion through effects on ECM remodelling, ATMs may directly alter the proliferation and differentiation of preadipocytes and adipocytes. Research on this topic largely reports a pro-inflammatory and anti-adipogenic effect of macrophages on adipocytes, leading to the hypothesis that macrophages contribute to pathological inflammation in adipose tissue. These studies largely use conditioned-media systems, in which macrophages are typically polarised using inflammatory activators such as IL-1 β , tissue-type plasminogen activator (TPA), or lipopolysaccharide (LPS), to model M1-like macrophages. This phenotype may be further amplified by cellular activation from culturing on adherent plastic surfaces. Other protocols do not explicitly subject macrophages to a polarisation step, and may mitigate the macrophage colony-stimulating factor (M-CSF) -based differentiation steps which are important for inducing non-inflammatory

phenotypes. M-CSF is used to differentiate monocytes into macrophages, and is critical for systems that are modelling tissue-resident macrophage populations, such as those that exist in adipose tissue. M-CSF can also potentiate macrophage responses to M2-stimuli [168]. Thus, current studies are biased towards using inflammatory macrophages, which likely do not reflect the true phenotype of ATMs. Other issues arise from using conditioned media systems, which often cause nutrient depletion, imposing metabolic stress on the recipient cells. Many studies also do not filter the transferred media, leaving the possibility of transfer of the inflammatory-polarised macrophages over to the adipocyte population [169, 170]. Furthermore, macrophages are frequently stimulated using polarising agents such as LPS, immediately before incubation with conditioned media that is subsequently transferred to adipocytes, without a wash step in between [169, 170]. This may result in transfer of inflammatory polarising cytokines over to the adipocyte cultures. Unsurprisingly, much of the existing literature reports reduced differentiation capacities and reduced lipid storage in adipocytes, alongside elevated lipolysis, and increased inflammatory activation, after following conditioned-media or co-culture protocols [169–174]. In-vivo, this would likely result in impaired tissue expansion and subsequent lipotoxicity.

However, when macrophages have been polarised with anti-inflammatory factors, such as IL-4, the opposite effect is observed [173]. Anti-inflammatory macrophages enhance adipocyte function, for example in relation to metabolism and mitochondrial biogenesis [175]. Mouse knockout studies which have knocked out *TRIB1*, a factor important for M2-macrophage polarisation, reported reduced anti-inflammatory macrophage populations in adipose tissue, alongside a reduced capacity for adipose lipid storage and increased tissue lipolysis, resulting in hypertriglyceridaemia and insulin resistance. Restoration of these M2-like populations through bone-marrow transplant from wild-type mice, rescued the associated metabolic phenotype, strongly suggesting that anti-inflammatory macrophage populations play a critical role in regulating the adipocyte lipid storage capacity [176]. Moreover, in patients with lipoedema, a condition associated with excess adipocyte differentiation and lipid

storage in the lower-body, there is an increased abundance of M2-like macrophage populations. Treating ADSCs with conditioned media from lipoedema patient ATMs increases cellular lipid content, compared to ATM-conditioned media from control patients. This effect is suppressed when lipoedema patient macrophages are re-polarised away from an M2-like phenotype [177]. These findings indicate that macrophage polarisation significantly influences preadipocyte differentiation, with opposing effects seen in inflammatory, compared to anti-inflammatory, macrophages. Given the reported differences in cytokine secretion between GFAT and ASAT [25, 26], an M2-bias population in GFAT, secreting fewer inflammatory cytokines, may contribute to depot-differences in adipose tissue lipid storage and expansion capacity.

1.4.5.2 Macrophages Modulate Adipocyte Progenitor Proliferation

In addition to influencing adipocyte differentiation, macrophages impact preadipocyte proliferation. Hyperplasia is essential for promoting ‘healthy’ adipose tissue expansion, as it increases the lipid buffering capacity of the tissue, while reducing the need for hypertrophy. However, the literature investigating the effects of ATMs on preadipocyte proliferation is inconsistent, likely reflecting differences in macrophage protocols and the origins of preadipocytes. For instance, a population of CD206⁺ cells were found to suppress the proliferation of ADSCs in murine VAT. Depletion of this population increased ADSC proliferation, as measured by EdU incorporation and Ki67 staining [110]. Although CD206 traditionally marks M2-like cells, in VAT, this may not be the case [30], as other studies suggest that M2-like populations promote hyperplasia. One such study by Lee et al reported that an M2-like osteopontin (OPN) -positive macrophage population regulates the recruitment and proliferation of CD44⁺ (OPN receptor) preadipocytes at sites of adipocyte death. Preadipocyte proliferation occurred exclusively in this CD44⁺ population, which made physical contact with OPN⁺ macrophages. Knocking out OPN in macrophages prevented the recruitment and proliferation of progenitors, indicating that the OPN-CD44 interaction is critical for preadipocyte hyperplasia [178]. While transwell and conditioned media studies are commonly used to explore

cellular interactions, this axis emphasises that the lack of direct cell contact in these systems may impede the investigation of key physiological processes. Additionally, the specific cellular subtypes necessary to trigger these responses highlight the challenges of accurately modeling critical physiological exchanges in-vitro.

Hyperplastic effects of macrophages have also been observed in resident populations marked by other genes. A population of CX3CR1^{hi} macrophages in VAT, for example, have been shown to reduce ADSC senescence, facilitating hyperplastic expansion as an adaptive response to short-term HFD overfeeding [89]. The initial preadipocyte hyperplasia may increase tissue MCP1 levels, acting to stimulate macrophage proliferation [179]. In this way, growth-factor exchange is reciprocal. This has been modelled as a stable cellular circuit which is resistant to a threshold of system perturbations, allowing for the concurrent expansion of different populations (preadipocytes/fibroblasts and macrophages) in cases of tissue expansion, where the carrying capacity of the tissue increases. Contact-dependence was an important feature of this system; M-CSF-mediated contacts provided growth and survival benefits for both macrophages and fibroblast populations, and cells consuming, but not producing, growth factors displayed limited expansion [180]. This allows for the maintenance of a homeostatically robust system; macrophages maintain preadipocyte populations and vice versa.

However, long term nutritional stress, such as long-term HFD overfeeding, disrupts this balance, altering the overall cellular carrying capacity and abundances of each cell type within the tissue. With persistent overnutrition, adipocytes must accommodate an increased amount of lipid storage, initially by recruiting cells from the progenitor pool. As adipocytes expand, macrophages are faced with an increased burden of clearing dead and dying adipocytes, increasing cellular stress, fibrosis [181], inflammation and recruitment of circulating monocytes. This perturbs the polarisation of anti-inflammatory macrophage populations, such as the beneficial CX3CR1^{hi} macrophages [89], who can then no longer sustain preadipocyte hyperplasia. This leads to maladaptive senescence of ADSCs and diminished hyperplastic capacity, adding further stress to hypertrophic adipocytes,

a reduced tissue carrying capacity, and less growth factor availability for resident macrophage populations.

In this spiral of obesity-associated tissue adaptations, fibrosis further limits the system carrying capacity by mechanically impeding adipocyte expansion, impairing growth factor availability and limiting the formation of cell-cell contacts between adipocytes and macrophages. Fibroblast-like populations that sense these spatial restrictions further inhibit macrophage expansion [182], leading the system to adapt to a new pathologically stable state, characterised by an overrepresentation of fibrotic populations [180], which caps cell proliferation by restricting growth factor exchange. These studies underscore the importance of macrophages in modulating preadipocyte hyperplasia, particularly through contact-dependent growth factor exchange.

1.4.6 Issues in Existing Culture Models

Modelling these interactions in-vitro, however, presents significant challenges, particularly concerning the contact-dependence of growth factor exchange. Modelling adipocyte-macrophage communication using conditioned media and transwell systems excludes these interactions, omitting critical biological exchanges. For instance, direct contact through heparan sulphate proteins is necessary for mitochondrial exchange between adipocytes and macrophages, which may be important to support the survival of metabolically compromised cells [183]. Disrupting mitochondrial transfer from adipocytes to macrophages in murine adipose tissue resulted in decreased energy expenditure, and accentuated HFD-induced obesity, suggesting that this contact-mediated process plays a role in WAT homeostasis [183].

Cell-cell contact and motility, in themselves, mediated by adhesion proteins such as integrins, also affect macrophage polarisation [184, 185], and therefore need to be considered when designing in-vitro culture systems. The expression of integrins and adhesion proteins, such as ICAM1, change in response to the roughness of the surface on which macrophages are cultured, promoting an M2-like phenotype when cells are cultured on irregular surfaces. Changes in mechanotransduction and environmental sensing in response to rougher surfaces, further alters Rac protein

expression, again leading to M2-like polarisation [186, 187].

The hardness and stiffness of culture surfaces has also been shown to regulate macrophage polarisation. Plastic provides a hard and stiff surface for adhesion, promoting M1-like inflammatory properties [187], meanwhile elastic and less stiff surfaces, such as culturing upon another cell monolayer, or in 3D systems, or physiologically in whole tissue, may result in a more M2-like phenotype [187]. 3D culture systems further promote M2-like characteristics by altering the surrounding ECM architecture and the expression of actin proteins [188], reducing inflammatory cytokine production [189]. This highlights the limitations of using monoculture conditioned media-, and transwell-, systems to study biological interactions between macrophages and adipocytes.

Aside from depot, another critical, yet often overlooked factor, is the sex of the cells being cultured, which is infrequently reported. When considering the biological interaction between macrophages and preadipocytes, donor sex is relevant for both parties. Sex-dependent gene expression is known to influence preadipocyte responses, for example in adipose tissue expansion and production of inflammatory factors such as GM-CSF [190–192]. Male stromal cells intrinsically produce more GM-CSF, altering the polarisation of co-cultured immune cells to a more inflammatory phenotype, as compared to female cells [192]. Meanwhile, in macrophages, there are differences in basal inflammatory polarisation states, with male cells again showing heightened production of inflammatory mediators relative to female cells [193, 194]. This is particularly important in the context of obesity-associated chronic inflammation, where sex-differences in inflammatory responses are accentuated by factors relating to chronic nutritional stress such as FFAs and cytokines [193, 195, 196]. Murine bone-marrow transplant studies have confirmed this, demonstrating that male monocytes, when subject to HFD, were more migratory and inflammatory, when transferred to female mice, compared to female non-transferred monocytes [193]. Thus, innate cellular sex differences, independent of circulating factors, play a role in inflammatory responses which are relevant to co-culture systems.

Given the impact of these combined factors on macrophage-adipocyte cultures,

in-vitro models should strive to incorporate factors such as direct cell contact and sex-specific variables to more comprehensively model adipocyte-macrophage communications. In-vivo, other sex-specific factors, such as the hormone oestradiol, likely influence this preadipocyte-macrophage crosstalk. Considering oestradiol's anti-inflammatory effect at macrophages, and unresolved actions at preadipocytes, hormone-mediated crosstalk should also be investigated.

1.5 Oestradiol Reduces Inflammation

1.5.1 Systemic Anti-Inflammatory Activities

Oestradiol is regarded as having potent, systemic anti-inflammatory activities, acting at multiple organ systems to reduce the production of inflammatory signals [197]. These actions have been investigated in the context of neuroinflammation and neurodegenerative disease [198–200], arthritis [201], vascular inflammation [202], and leukocyte inflammatory activity [203], to name a few, and serum oestradiol levels correlate with C-reactive protein, a marker of systemic inflammation [204]. Oestradiol shows additional activity to promote inflammatory resolution, thereby shortening overall exposure to pro-inflammatory signals and having anti-inflammatory actions [205]. The direct effects of oestradiol are further evidenced by the dynamic fluctuations of anti-inflammatory activity which occur in response to changes in circulating oestradiol. For example, in pregnancy, where a surge in oestradiol is seen, there is a drop in systemic inflammatory markers, and inflammatory activity [203, 206, 207], while in menopause, when there is a drop in oestradiol, the opposite is true [208–211]. These data suggest that oestradiol has wide-reaching anti-inflammatory effects, particularly at cells, such as macrophages, which have immunoregulatory roles.

1.5.2 Anti-Inflammatory Activity At Macrophages

A substantial body of literature has highlighted the pronounced effects of oestradiol on immune cells, particularly macrophages. In-vivo studies indicate that oestradiol treatment promotes M2-like macrophage polarisation, leading to increased expression

of anti-inflammatory mediators such as IL-10, while simultaneously decreasing the expression of pro-inflammatory cytokines, like TNF- α [212, 213]. This anti-inflammatory effect has been observed in murine ATMs in-vivo, where oestradiol-induced M2 polarisation occurred independently of the hormone's anorectic effects [213]. Moreover, in-vivo oestradiol deprivation has been associated with heightened inflammatory activity. For instance, oophorectomy in pre-menopausal women increased the secretion of pro-inflammatory cytokines, including TNF- α and IL-1 β , from circulating blood monocytes. This heightened inflammatory response was reversed in women who received oestradiol treatment, while those who did not, continued to exhibit elevated inflammatory markers throughout the study period [214]. Even in the absence of direct oestradiol treatment, physiological fluctuations of oestradiol across the menstrual cycle have been found to influence serum cytokine profiles [215]. Furthermore, menopausal status has been associated with an impaired ability to polarise M2 macrophages [216]. Collectively, these studies underscore the role of circulating oestradiol concentrations, in-vivo, to drive macrophage towards an anti-inflammatory phenotype, thereby modulating inflammatory cytokine levels both locally and systemically.

In-vitro studies corroborate these findings, and provide insight into the mechanisms by which oestradiol regulates macrophage phenotypes. Consistent with the observation that menopausal status influences M2 macrophage polarisation, prior exposure to, or pre-treatment with oestradiol, has been shown to suppress macrophage inflammatory responses to stimuli, and promote M2 polarisation in both murine and human macrophage models [215, 217]. Similar effects were observed with an ER- α agonist, suggesting that ER- α , rather than ER- β mediates these responses [218]. The modulation of macrophage phenotypes by oestradiol occurs in a dose-dependent manner, and is conserved across the differentiation of monocytes to macrophages, which is further evident from birth, highlighting the robust nature of these responses [213, 219, 220]. Oestradiol's suppression of pro-inflammatory cytokines such as TNF- α and IL-1 β [221] is mediated through the inhibition of NF κ B activation

following ER- α engagement [222]. Additional in-vitro work suggests that ER- α activation regulates SOCS3 and STAT3 signalling pathways, to subsequently facilitate the resolution of inflammation, thereby reducing the duration of exposure to inflammatory cytokines [223]. Knockout studies have further demonstrated that ER- α , and not ER- β , expression in macrophages critically regulates oestradiol's effects on both intrinsic and LPS-stimulated cytokine production, primarily through the suppression of NF κ B signalling [224–226]. These effects hold true in-vivo, where myeloid-specific ER- α deficiency increases the production of inflammatory cytokines and results in metabolic impairment [226]. Still, other studies have hinted towards a role for GPER1, a G-protein-coupled oestrogen receptor, in mediating a rapid decrease in TLR4 expression in murine macrophages [227]. GPER1 responses are largely studied in the context of rapid membrane signalling, although longer-term transcriptional responses have been reported [228]. Given the prolonged nature of circulating plasma oestradiol, and the contrasting rapid signalling mediated by GPER1, the physiological implications of these GPER1-mediated oestradiol responses are unclear.

Interestingly, the immunomodulatory effects of oestradiol may be more pronounced in macrophages as compared to other immune cells. For example, T-cells in adipose tissue predominantly express ER- β , which is likely not responsible for mediating the anti-inflammatory effects of oestradiol at immune cells. Existing data suggest that T-cells might not be oestradiol-responsive [229, 230], or may even respond in a pro-inflammatory manner [231]. Other data suggest that T-cells rely on the oestradiol-response of other antigen presenting cells, namely macrophages, to respond to oestradiol [232], although this remains poorly understood [233], particularly in adipose tissue. Additionally, the effect of oestradiol on B-cell polarisation has been found to be mediated through an M2 macrophage population [234], with this population also potentially acting to suppress T-cell activation to further dampen the inflammatory milieu [235]. This suggests that the well-documented ability of oestradiol to polarise macrophages in adipose tissue may further regulate phenotypes of both adipocytes and other immune populations within the tissue.

1.5.2.1 Oestradiol in Monocyte Infiltration and Macrophage Differentiation

As well as reducing the inflammatory capacity of resident macrophage populations, oestradiol has been found to reduce the infiltration of inflammatory monocytes from the circulation, further reducing adipose tissue inflammation. At the individual monocyte level, oestradiol has been found to inhibit adhesive properties, preventing these cells from undergoing diapedesis and entering adipose tissue spaces [236, 237]. In agreement with this, serum oestradiol changes inversely correlate with ATM abundances [238], further evidenced in the menopause where a drop in serum oestradiol is accompanied by increased monocyte infiltration into adipose tissues [239]. Levels of secreted chemoattractants, such as MCP1, from adipose tissue depots correlate with tissue monocyte infiltration [240]. Interestingly, oestradiol may modulate the secretion of chemoattractants, such as MCP1 and MIF from within the adipose tissue, thereby reducing monocyte recruitment [241–244]. Furthermore, differential secretion of these chemoattractants has been reported between fat depots, potentially linking the differing activities of oestradiol on each fat depot with their differing inflammatory capacity and ability to recruit macrophages [240].

1.5.3 Oestradiol Actions On Macrophage-Adipocyte Communication

Given the contested effect of oestradiol at preadipocytes and adipocytes in-vitro, despite clear in-vivo effects, it is necessary to consider how in-vitro adipocyte monoculture systems might fail to accurately model the in-vivo microenvironment. The plethora of immune populations in adipose tissue may communicate through complex multi-population inter-cellular signalling to modulate tissue homeostasis. This may involve, for example, signalling across populations of T-cells, macrophages and natural killer cells, which likely all play a role in regulating adipose tissue biology, and may change in response to metabolic cues [86, 245, 246]. Given the body of literature on adipose tissue macrophages, and their aforementioned roles in adipose tissue biology and expansion, this work chose to establish a reductionist

system to investigate whether the lack of macrophages in in-vitro adipocyte culture systems may contribute to the inconsistency of the reported effects of oestradiol on depot-specific adipose tissue biology. Macrophages notably express ER- α , which is known to modulate the anti-inflammatory effects of oestradiol, potentially allowing them to govern the adipose tissue inflammatory microenvironment, and modulate the tissue expansion capacity in an oestradiol-responsive manner. For instance, in breast adipose tissue, whose expansion is oestradiol-dependent, macrophage infiltration is necessary for tissue growth [247]. Specifically, oestradiol-induced M2 macrophages support local stem cell hyperplasia [248, 249], possibly through the secretion of macrophage-derived Wnt ligands [250] and the activation of cyclins [251], although this mechanism has adverse implications in breast cancer. While some studies highlight the positive effects of oestradiol on preadipocyte proliferation in-vivo, the role of macrophages in this process remains underexplored, despite their potential to foster a growth-permissive microenvironment for preadipocytes. Therefore, the influence of oestradiol on resident macrophages may provide the critical link between oestradiol treatment and its positive metabolic effects on adipose tissue health, which may not be directly mediated by adipocytes.

1.6 Thesis Hypotheses and Aims

Overall hypothesis:

- Human fat distribution, in particular the sexual dimorphism of regional adipose tissue expansion, is driven by cell-cell communication between adipocytes and tissue resident macrophages in response to oestradiol, and its effects on the local inflammatory microenvironment.

Thesis Aims:

1. Use single-cell RNA sequencing data to characterise the distinct cellular composition of human abdominal and gluteal subcutaneous adipose tissue stromal vascular fractions, with a specific focus on preadipocytes and macrophages, highlighting any depot-differences.

2. Use depot-specific preadipocyte monoculture cell models to investigate if there is a direct effect of oestradiol on depot-specific preadipocytes in-vitro.
3. Establish a reductionist, depot-specific co-culture system for the investigation of communication between preadipocytes and macrophages.
4. Investigate the effects oestradiol and inflammatory stimuli, on communication between preadipocytes and macrophages, in-vitro, using depot-specific co-culture systems.

2

Materials and Methods

2.1 Cell Culture System Model

The majority of cell culture work presented in this thesis utilised in-house immortalised human preadipocyte cell lines, known as MBDFAT cells. These are cell lines, originating from paired subcutaneous abdominal and gluteal adipose tissue biopsies taken from a female donor. The preadipocytes were isolated and immortalised through lentiviral transduction with human telomerase and human papillomavirus type-16 E7 oncoprotein genes, as detailed previously [252]. After immortalisation, the cells were differentiated and subsequently subjected to ceiling culture methods, enriching for highly adipogenic cells, and de-fatting the cells for de-differentiation. These protocols are also described further elsewhere [253], and are widely employed in the relevant field of literature [254–257]. These depot-specific cell lines retain their depot-of-origin ‘memory’ despite immortalisation and in-vitro culturing processes, which is reflected by their inherent differences in metabolism and expression of developmental gene signatures [252].

For certain experiments, primary preadipocytes were used. These were derived from depot-specific subcutaneous adipose tissue biopsies, with preadipocytes isolated from the stromal vascular fraction (SVF). The primary cells were obtained following established protocols [252], cryopreserved in freezing media (foetal bovine serum

(FBS) + 10% DMSO), and used between passages 3 and 7 for this work. Fresh primary cell isolation was not performed for the presented experiments.

2.2 Culture and Maintenance of Preadipocytes and MCF7 Cells

Primary and immortalised human preadipocytes, as well as the MCF7 breast cancer cell line, were maintained in phenol-red free DMEM/F12 media (Gibco 11039021), supplemented with 10% FBS (Labtech), 100 $\mu\text{g.mL}^{-1}$ penicillin/ 100 $\mu\text{g.mL}^{-1}$ streptomycin (Invitrogen, 15140-122), 2mmol.L⁻¹ L-glutamine (Invitrogen, 25030-024) and 0.25 ng.mL⁻¹ human fibroblast growth factor (R&D Systems, 233-FB). This cocktail constituted the basal growth media formulation. The cells were plated into culture flasks, with media changed every 2 days, and cells passaged after reaching 80-90% confluence. For passaging, cells were washed with phosphate-buffered saline (PBS), without Mg²⁺ and Ca²⁺, before being lifted, using TrypLE™ Express lifting agent (ThermoFisher, 12604013).

Mycoplasma testing was conducted on cell media supernatant, quarterly. No mycoplasma contamination was found over the duration of the conducted work.

2.3 Adipogenic Differentiation of Preadipocytes

Preadipocyte differentiation into mature, lipid-laden adipocytes followed the standard protocol used within the research group [252]. After preadipocytes reached confluency, a 10-14 day differentiation period was followed, during which an adipogenic media cocktail was utilised. DMEM/F12 was the media base, with 17.5mM glucose, 15mM HEPES buffer (Gibco, 15630056), 100 $\mu\text{g.mL}^{-1}$ penicillin/ 100 $\mu\text{g.mL}^{-1}$ streptomycin (Invitrogen, 15140-122) and 2mmol.L⁻¹ L-glutamine added. As well as this, 130nM transferrin (Sigma, T8158), 17 μM pantothenate (Sigma, P5710), 33 μM biotin (Sigma, B4639), 10nM triiodothyronine (Sigma, T5516), 1 μM dexamethasone (Sigma, D2915) and 100nM human insulin (Invitrogen, 12585-014) were added throughout differentiation. For the first four days of

differentiation, 250 μ M IBMX (Sigma, I7018) and 4 μ M troglitazone (Sigma, T2573) were also added to the media.

2.4 Oestradiol Treatment

Powdered oestradiol (Sigma, E2758) was dissolved in ethanol, and stored as a 10mM stock at -20°C . For treatment, 1-10 μ L of stock was dissolved in 10mL sterile PBS to create a working stock solution. The final treatment concentration was achieved by adding the appropriate volume of the working stock to the culture media. Equivalent ethanol-treated controls were prepared, matching the dilutions used for oestradiol treatments. Subsequent analyses, explicitly measuring oestradiol in treated media, confirmed that it was being added consistently at the expected final concentration.

2.5 Primary Monocyte Isolation

Primary monocytes were obtained from human blood cones (NHS Oxford Blood Donor Centre). These were transferred to Falcon tubes and made up to 25mL with Hanks' Balanced Salt Solution (HBSS, Gibco, 14185045), containing 0.3mM EDTA (Santa Cruz Biotechnology, sc-203932). 20mL of Ficoll Paque Plus (GE17-1440-02) was pipetted into fresh Falcon tubes, and the 25mL of diluted blood solution was pipetted very slowly, drip-by-drip, onto the Ficoll using a 5mL stripette. Tubes were then centrifuged at 700g (without brake) for 25 minutes, at room temperature (RT). The 'fluffy' white layer containing peripheral blood mononuclear cells (PBMCs) was carefully collected using a sterile Pasteur pipette, made up to 50mL with HBSS 0.3mM EDTA, and then centrifuged at 500g for 10 minutes at RT. The supernatant was removed and the pellet was resuspended in 40mL HBSS 0.3mM EDTA, before being centrifuged again, as per the previous step. The pellet was resuspended and centrifuged a third time, before being resuspended in 1mL MACS buffer (HBSS, 3% FBS, 10mM EDTA), transferring the suspension to 5mL tubes.

The MagniSort™ Human CD14 Positive Selection Kit (Thermofisher, 8802-6834-74) was then used to isolate CD14⁺ monocytes. 200 μ L of MagniSort™ enrichment

antibody was added to the suspension, vortexed, and incubated at RT for 10 minutes. 3mL of MACS buffer was added before centrifuging at 400g for 3 minutes, at RT, followed by resuspension in 1mL MACS buffer. 300 μ L of MagniSort™ positive selection beads were then added. The suspension was vortexed and incubated for 10 minutes at RT. The volume was made up to 2.5mL by addition of MACS buffer, and the cells were resuspended. The tube was then fully inserted into the MagniSort™ magnet (ThermoFisher, MAG-4902-10), and incubated inside the magnet for 5 minutes. Following this incubation period, the supernatant, containing CD14⁻ cells, was then poured off, keeping the tube inside the magnet. The tube was then removed from the magnet and the cells were resuspended in 2.5mL MACS buffer. As before, they were inserted into the magnet again and incubated before the supernatant was poured off. The process was repeated a third time. CD14⁺ cells were then resuspended in 30mL Iscove's Modified Dulbecco's Medium (IMDM, ThermoFisher, 21980032) and counted, before centrifuging at 400g, for 5 minutes, at RT. Cells were then resuspended in freezing solution (90% FBS, 10% DMSO) at a density of 30 million cells.mL⁻¹, and aliquoted into cryovials for freezing at -80 °C before being transferred to liquid nitrogen. The typical purity of CD14⁺ monocytes isolated in this way is 80-90%. Following the 7-day, M-CSF-based differentiation protocol (described in Section 2.7), however, increases the population purity to 100% macrophages. This protocol was followed prior to any macrophage-based cell culture experiments.

2.6 Donor Sex Determination for Primary Macrophages

Due to the anonymising protocols within the NHS Oxford Blood Donor Centre, it was not possible to be informed of the sex of blood cone donors, prior to their use. Given the importance of knowing the donor sex for these experiments, it was necessary to determine this through other means. For determination of donor sex, the X- and Y-associated transcripts *XIST* and *DDX3Y* (respectively) were assessed for expression levels. For this, CD14⁻ cells, which would otherwise be discarded, were decanted

into a 50mL Falcon tube during the isolation protocol. This was topped up to 50mL with PBS, and cells were mixed well. From the suspension, 1mL was removed and transferred to an Eppendorf tube. The Eppendorf tube was spun down, at 300g for 5 minutes, and the pellet was subsequently resuspended in 500 μ L of TRIzol™ reagent (Ambion, 15596018) before freezing at -80 °C. Samples were then processed for RNA isolation, as outlined in Section 2.12, and qPCR expression of *XIST* (Figure 2.1A) and *DDX3Y* (Figure 2.1B) were determined. Assessing expression of these two transcripts provided a clear-cut method of determining donor sex. Following determination of donor sex, only female donors were utilised for culturing.

2.7 Primary Macrophage Differentiation and Polarisation

After thawing, 30 million macrophages were plated in a 10cm tissue culture dish, in 10mL RPMI (Gibco, 21875091), supplemented with 10% FBS (Labtech), 100 μ g.mL⁻¹ penicillin/ 100 μ g.mL⁻¹ streptomycin, and 100 ng.mL⁻¹ M-CSF (Biolegend, 574806). Differentiation took place over a 7-day culture period, with cells being fed an additional 5mL of media on day 5. This 7-day differentiation period is used to generate macrophages from the isolated monocytes, without explicitly skewing polarisation towards an M1- or M2-like phenotype [258]. A pilot experiment (reported in Section 5.2.2) investigated whether an inflammatory macrophage phenotype could be induced, using 40 ng.mL⁻¹ GM-CSF (Biolegend, 572903) instead of M-CSF for the 7-day differentiation period. As reported elsewhere though, GM-CSF and M-CSF -generated macrophages were not fully polarised to opposite states, and ideally require further polarisation inducers (e.g IL-4, LPS) to successfully induce M1- or M2-like phenotypes, respectively [258].

2.8 Primary Macrophage Co-Culture

For co-culturing, macrophages were first differentiated for 7-days with M-CSF, before being added to preadipocyte cultures, to reach a final ratio of 1:5 macrophages

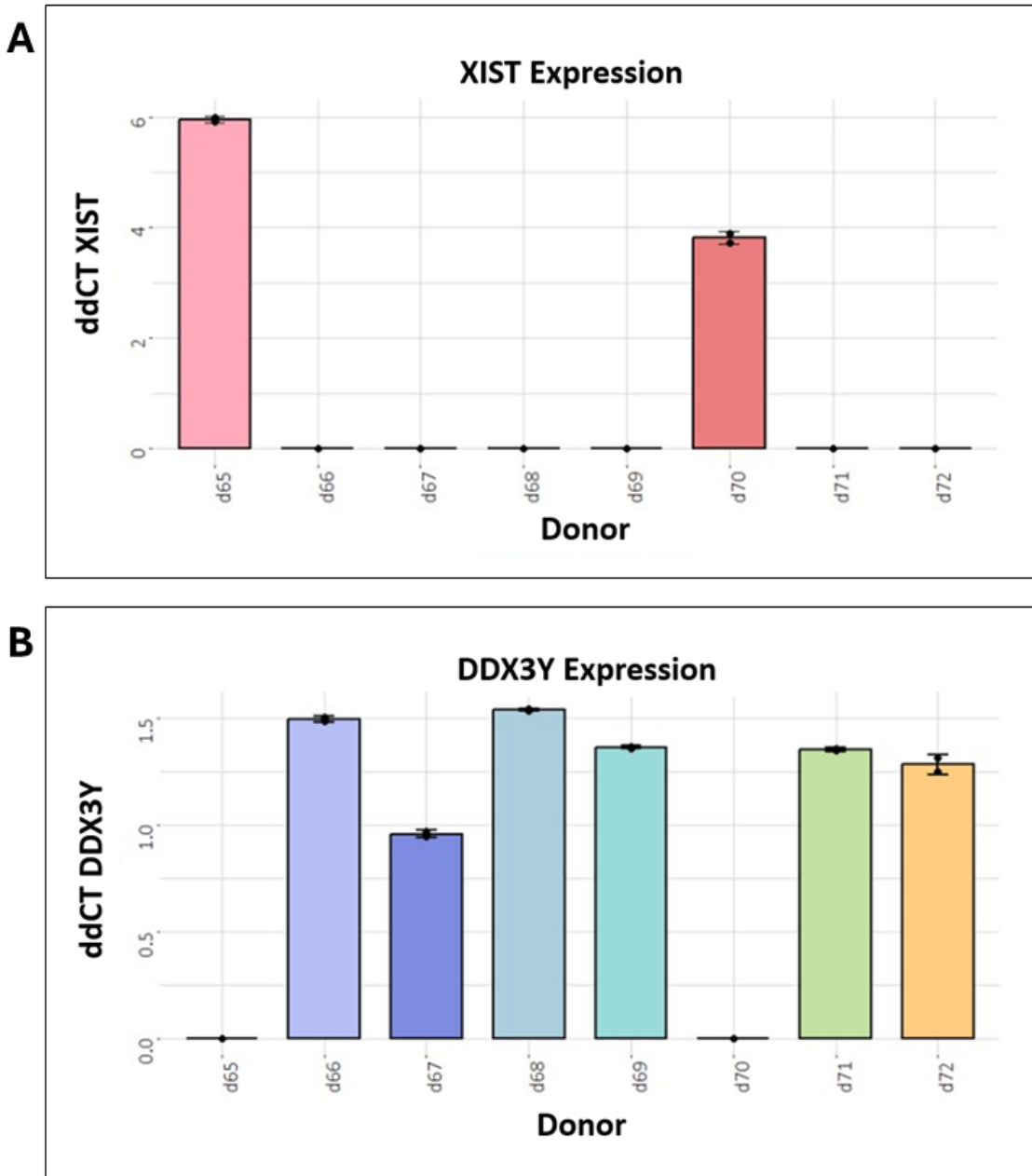


Figure 2.1: Example qPCR analysis, presenting sex-associated transcript expression from a set of monocyte donors. Cells were isolated as outlined in Section 2.5, and CD14⁺ cells were preserved for assessing expression of sex-associated transcripts. Donor number is presented on the x-axis, and $\Delta\Delta C_t$ values are presented on the y-axis. For each donor, 3 technical replicates were run. Data points represent technical replicate values, with error bars showing standard deviation. Coloured bars indicate the mean value across the technical replicates. Expression is shown for: **A)** XIST and **B)** DDX3Y.

to preadipocytes at the end of the co-culture period. This serves to mimic tissue physiology, where this ratio varies from 1:2-1:10, largely dependent on the obesity status of the adipose tissue, with obese adipose tissue containing a higher proportion

of macrophages than lean adipose tissue [86]. These calculations are generally based on mature adipocyte counts, rather than preadipocytes. Selecting an intermediate ratio allowed enrichment for macrophage-preadipocyte signalling without specifically modelling an obese adipose tissue environment. Direct contact co-cultures allowed preservation of cell-cell interaction signalling, which is increasingly recognised to play a role in adipose tissue physiology (e.g macrophage-adipocyte mitochondrial transfer [183]).

Preadipocytes were plated the day before macrophages were added (D-1 co-culture, D6 macrophage differentiation), with the seeding density being adjusted to allow preadipocytes to proliferate to reach a final number of 500,000 cells, (based on a doubling time of approximately 37 hours for both abdominal and gluteal preadipocytes), in a 6-well plate at the time of harvest (further detailed in Section 5.2.4). On D0 of the co-culture period (D7 macrophage differentiation), macrophages were removed from tissue culture dishes by incubating with cold PBS for 10 minutes at 4 °C, followed by gentle scraping to remove remaining adherent cells. Cells were spun down at 400g for 5 minutes at RT before being resuspended in media and counted. For co-culture, macrophages were diluted in phenol-red free DMEM F12 at 50,000 cells.mL⁻¹. 2mL of macrophage suspension was then added to each 6-well of preadipocytes, to give approximately 100,000 macrophages per well. These terminally differentiated macrophage cultures are unable to further proliferate, thus macrophages were plated at the desired final number to reach the intended co-culture ratio. For macrophage monoculture, cells were plated at a density of 1 million cells per 6-well.

2.9 Labelling Macrophages with BioTracker Dye

To label macrophages prior to co-culture, a membrane dye, BioTracker™ (Sigma, SCT106), was added. This is a lipophilic carbocyanine dye that can stably label cells in culture for prolonged periods. Cells were suspended at a density of 1 x 10⁶.mL⁻¹, and to this, 5 µL of BioTracker™ was added for each 1mL of suspension, as recommended by manufacturer protocols. The solution was subsequently mixed

by flicking, and incubated for 20 minutes, at 37°C in a water bath. The labelled cells were centrifuged at 300g for 5 minutes, before being resuspended in pre-warmed media to wash. The wash steps were repeated twice more, before the macrophages were added to monocultured preadipocytes/adipocytes.

2.10 Protein Collection and Quantification Assay

For obtaining cellular protein lysate, cells in 6-well plates were washed with PBS, transferred to ice, then harvested in 100 µL of ice-cold RIPA lysis buffer using cell scrapers. RIPA buffer (150mM NaCl, 50mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) was made in house, and cOmplete EDTA-free protease inhibitor and phosStop phosphatase inhibitor (Sigma, 4906845001, 4719964001) were added and dissolved in the buffer prior to use. The lysate was kept on ice and sonicated (5 second pulses for 15 seconds at 40kHz) before quantification.

The protein concentration of the lysate was quantified using the Bio-Rad DC protein assay kit (5000111). Protein standards were generated by serial dilution, whereby 4mg.mL⁻¹ bovine serum albumin (BSA, Sigma, A7030) in PBS was diluted in RIPA buffer. 5 µL of standard or sample were pipetted in duplicate onto a 96-well plate, before the kit reagents were added, as per the manufacturer guidance. Absorbance was then read at 650nm on the FLUOstar Omega Microplate Reader (BMG LabTech). Absorbance values were used to first create a standard curve, allowing the subsequent calculation of the protein concentration for each sample.

2.11 SDS PAGE and Western Blot

The lysate volume to obtain 20 µg of protein was calculated based on the protein quantification assay. This was made up to 23 µL with RIPA buffer, before the addition of loading buffer (4X Laemmli buffer (Biorad, 1610747) + 10% β-mercaptoethanol). Samples were aliquoted into qPCR tubes with loading buffer, then stored at -80°C, thereby preventing the need for freeze-thawing of samples. After removing from -80°C and thawing, samples were heated to 80°C for 8 minutes,

briefly centrifuged, then loaded into the gel. 4 μ L of protein ladder (Jena Bioscience, Blue-Eye, PS-104) was loaded at either end of the gel. Biorad Mini-PROTEAN TGX Stain-Free precast gels were used (BioRad 4568023, 4568033).

Gels were run at 85V in a tris-glycine-SDS running buffer for 2-3 hours, until the dye front reached the bottom of the gel, or the protein ladder was run to an appropriate place. Protein separation was confirmed by gel visualisation using a Biorad ChemiDocTM machine and Image Lab software.

Non-denaturing conditions were employed for membrane-receptor samples - this excluded sample boiling steps, instead allowing the sample to sit in lysis buffer for at least 30 minutes. As well as this, SDS, which is a strong denaturing reagent, was removed from buffers.

After imaging, gels were equilibrated in a trizma/glycine-based transfer buffer for approximately 15 minutes. 0.45 μ m polyvinylidene-fluoride membranes (Immobilon, IPVH00010) were cut to size and activated in methanol for 1-2 minutes before also being equilibrated in transfer buffer. Wet transfer tanks were assembled and protein transfer was run for 43 minutes at 85V. Membranes were then imaged to verify protein transfer, with files subsequently being used for total protein normalisation. Milk blocking buffer (5 % skimmed milk powder (VWR, 84615.0500) in TBS-T (1% tween-20 (Sigma, P1379-500ML) in tris-buffered saline)) was then added to membranes for 90 minutes at RT on a rocker. The blocking buffer was then removed and replaced with primary antibody, diluted in milk buffer. The membranes were incubated with the primary antibody dilution overnight at 4 °C. Further details on antibody products are provided in Section 4.2.2.1. The primary antibody dilution was then removed and membranes were washed in TBS-T, with the wash being changed every 5 minutes, four times. An appropriate species secondary antibody was diluted in milk blocking buffer, and incubated with the membranes for 90 minutes at RT with rocking. Washing was then repeated and the membranes were incubated for 2-3 minutes in ECL substrate (Biorad Clarity), with protein bands then being imaged on the Chemidoc system. Band intensities were quantified using the Image Lab software, and normalised to total protein content.

2.12 qPCR Gene Expression Assays

6-well plates of cells were washed in PBS before 500 μ L of TRIzol™ reagent was added. Cells were then scraped, and transferred to -80°C . Samples were thawed on ice, and 100 μ L of chloroform was added, followed by vigorous shaking for at least 30 seconds. The samples were left to settle for 5 minutes before centrifuging at 12000g, 4°C for 15 minutes. The aqueous phase of the solution, containing RNA, was transferred to elution tubes. An equal volume of isopropanol was added and samples were briefly vortexed before being incubated overnight at -20°C . Samples were then centrifuged at 12000g, 4°C for 30 minutes. The supernatant was removed and the remaining pellet was washed in 80% ethanol before centrifuging again (12000g, 4°C) for 15 minutes and repeating the process. After the second removal of ethanol, the RNA pellet was dried and resuspended in RNase-free water. RNA was then quantified using the spectrophotometer (NanoDrop, ND-1000).

cDNA synthesis was conducted using 600-800ng of the isolated RNA, and reverse transcription reagents (Life Technologies, 4368813), which were loaded into a Peltier Thermal Cycler (BioRad, Tetrad 2). The cycle parameters were set according to the reagents in use (25°C 10 minutes ; 37°C 120 minutes ; 85°C 5 minutes ; 4°C 10 minutes).

10mM tris hydrochloride was used to dilute cDNA 1:40. qPCR was conducted using TaqMan™ assay probe primers, purchased from ThermoFisher (Catalogue #4331182). A table of measured genes, and their assay identification numbers are provided in Appendix A.1. Primers were added to KAPA PROBE FAST qPCR Master Mix (2X) ABI PRISM (Kappa Biosystems, KK4707), and run on a Quantstudio 7 Flex (ABI) machine. Samples were pipetted in triplicate into 384-well plates for analysis. 6 μ L was transferred into each well (2.7 μ L diluted cDNA, 0.3 μ L selected TaqMan™ probe, 3 μ L Master Mix).

The $\Delta\Delta C_t$ relative quantification method [259] was used to calculate relative transcript expression. The ΔC_t value for each target gene was normalised to the geometric mean of 4 selected housekeeper genes for either primary or immortalised preadipocyte cell lines, based off Neville et al 2011 [260].

2.13 Immunofluorescent Antibody Staining

Cells were plated onto collagen-coated coverslips in 24-well plates prior to fixation for immunofluorescent staining. Coverslips were first sterilised in ethanol and left to dry before being lowered into the plate and washed with PBS. Collagen (Sigma, C4243) was then diluted in PBS and added to coverslips at $10\text{ }\mu\text{g.cm}^{-2}$ for 30 minutes at RT. The collagen solution was then removed and the coverslips were washed twice with PBS. Cells were then plated in media, onto the coated coverslips. Following cell culture, cells were washed with PBS then fixed by incubating with 4% paraformaldehyde (PFA, Santa Cruz Biotechnology, sc-281692) solution in PBS at 4°C for 10-15 minutes. Fixed cells on coverslips were stored in PBS, in 24-well plates with a parafilm-sealed lid, at 4°C .

For staining, PBS was removed from the coverslips and $100\text{ }\mu\text{L}$ of 0.1% Triton X-100 (Sigma, X100-500ML) was added for membrane permeabilisation, which was incubated at RT on the coverslips for 15 minutes. Two PBS washes followed. For blocking, $200\text{ }\mu\text{L}$ of 2-4% BSA solution was added, and coverslips were incubated at RT for 60 minutes. Primary antibody dilutions were made up in BSA solution, before being added to the coverslips and incubated overnight at 4°C . Control slides, incubated with just BSA solution were run in parallel. The primary antibody/BSA solution was removed and the coverslips were gently washed three times with PBS. The secondary antibody was then made up in BSA solution, at the dilution recommended by the manufacturer, as specified in Table 4.1. If required, cytoskeletal (Oregon Green 488-Phalloidin, ThermoFisher, O7466, dilution = 1:100) and nuclear (Hoechst 33342, ThermoFisher, 62249, dilution = 1:10,000) stains were added to the secondary antibody solution. Control solutions were made up, containing just these counterstains, in order to assess channel bleed-through. The appropriate antibody/counterstain solution was added to coverslips and incubated for 45-60 minutes at RT, protected from the light. The staining solution was then removed and coverslips were washed with PBS and stored, protected from light, at 4°C , or directly mounted for imaging.

For imaging, coverslips were removed from wells, and the adherent PBS droplet

was removed by dabbing the edge of the coverslip, at a 90 degree angle, onto a piece of tissue. The coverslips were then transferred, cells facing down, onto a small drop of mounting fluid (Vector, Vectashield H-100) on a glass slide. The coverslip was then sealed around the perimeter, onto the slide using nail polish. Slides were stored, protected from the light, at 4 °C. Imaging was performed using the Radiance 2100 laser scanning system confocal microscope (Bio-Rad), with images captured in the Laser sharp software (Bio-Rad) for confocal microscopy. For fluorescent imaging, the Nikon ECLIPSE Ci-S microscope was used in combination with the epi-fluorescent attachment set and DS-Fi3 camera (Nikon), alongside the corresponding NIS-Elements imaging software (Nikon) for image capture.

2.14 Proliferation Assays Measured by Cell Counting

For measuring cell proliferation by cell counting, 200,000 cells were plated into a T75 flask and cultured for 72-96 hours. Cells were then lifted, and 20 μ L of cell suspension was transferred into each chamber of a NexcelomTM Cellometer slide. Each chamber was counted three times, using the Cellometer Auto Bright Field Cell Counter (Nexcelom) and averaged. For within-chamber readings, if values were inconsistent, readings were repeated until similar counts were obtained. Each sample was counted using separate readings from at least three chambers.

2.15 Plasmid Isolation

For determining oestradiol binding activity to the cellular oestrogen response element (ERE), a reporter plasmid coupling the ERE to a luciferase reporter construct (Addgene, Plasmid #11354), was used. Luciferase signal was normalised to a Renilla control plasmid (Promega, E2241) which was constitutively active, and an eGFP plasmid (Addgene, Plasmid #54759) was also used to visually assess transfection efficacy. Addgene plasmids were provided as bacterial stabs, and required expansion and nucleic acid isolation, prior to use.

Bacterial work was conducted in close proximity to a lit flame. Bacterial stabs containing the desired plasmid were streaked across one portion of an Luria-Bertani (LB) agar plate, using a sterile loop. The LB agar contained the appropriate selection antibiotic, as determined by the antibiotic resistance gene on the plasmid - this was ampicillin for the ERE-TATA-Luc plasmid, and kanamycin for the eGFP plasmid. A fresh sterile loop was used to spread from streak 1, across the plate into an opposing direction. This was repeated for subsequent streaks, with the 4th streak orienting towards the centre of the plate. The LB agar plate was incubated overnight at 37°C to obtain single bacterial colonies. A single colony was isolated using a sterile loop, which was then inoculated into LB broth containing the appropriate selection antibiotic. The broth was loosely covered and left overnight in a shaking incubator at 37°C. A control broth tube was also incubated, to ensure that conditions were sufficiently sterile to prevent growth of undesired bacteria. A cloudy haze in the broth confirmed that bacteria were growing in the starter culture. Starter culture tubes were subsequently centrifuged at 7000g for 5 minutes, to pellet the bacteria. Remaining broth supernatant was removed. Pellets were stored at -20°C. To create glycerol stocks, pellets were resuspended in LB broth. 150 µL of 80% glycerol was added to each 350 µL of bacterial cell suspension.

Cultures were expanded to obtain sufficient plasmid DNA by inoculating a fresh overnight liquid culture using existing stocks. Qiagen midi-prep kits (Qiagen, 12243) were used to isolate plasmid DNA, as per the kit instructions. Plasmid DNA content was then quantified using the NanoDrop spectrophotometer. For each plasmid, DNA was diluted, using the recommended Qiagen kit elution buffer (EB) solution, to reach a dilution where the desired mass of DNA was obtained in 1 µL of diluent. Per electroporation, the mass of DNA was based on standard laboratory recommendations for the given vector (3 x ERE TATA Luc = 250ng ; pRL-TK = 10ng ; mEGFP-C1 = 250ng).

2.16 **Invitrogen Neon Electroporation-Based Transfection**

For electroporation, cells were trypsinised and counted. The desired number of cells was pipetted into a 1.5mL Eppendorf tube, which was then centrifuged at 300g for 5 minutes. The pellet was washed in PBS, and the process was repeated. All of the remaining PBS was removed and the pellet was resuspended in 9 μ L of Resuspension Buffer R (ThermoFisher, MPK1096). 1 μ L of the appropriate plasmid mix was added, and the Eppendorf tube was flicked to mix the contents. Using the Neon tips (ThermoFisher, MPK1096), the 10 μ L mixture was aspirated and inserted to the Neon transfection machine, into the plastic cylindrical cassette filled with Electrolytic Buffer E (ThermoFisher, MPK1096). Based on recommended and tested voltage conditions, for preadipocytes, the Neon Transfection equipment was set to 1150V, 20ms for 3 pulses. Following electroporation, the 10 μ L mixture was added to an appropriate volume of antibiotic-free media, which was mixed gently before being partitioned into 96-well culture plates.

2.17 **Lentiviral-Based Transduction**

Preadipocytes were seeded at 50-70% confluence in T25 flasks, and culture media containing 8 μ g.mL⁻¹ hexadimethrine (Merck Life Science, 107689-10G) was added. Viral particles were then thawed on ice and mixed thoroughly before being added to culture flasks. Multiplicity of infection (MOI) values of 1, 4 and 8, for both positive viral vector (SBI, TR455VA-P), containing a luciferase-coupled ERE, and an equivalent negative control viral vector (SBI, TR411VA-P) were used. A 'kill flask' was also seeded, containing no virus. Cells were incubated (37 °C, 5% CO₂) for 24 hours before media was changed to remove the viral particles from culture. As the viral reporter constructs contained a puromycin resistance element, the fresh media contained 2 μ g.mL⁻¹ puromycin (InvivoGen SAS, ant-pr-1). When the cells in the 'kill flask' had all died, remaining cells in the viral-infected flasks

were puromycin resistant, and therefore successfully transduced with the desired construct. These cells were expanded for luciferase reporter experiments.

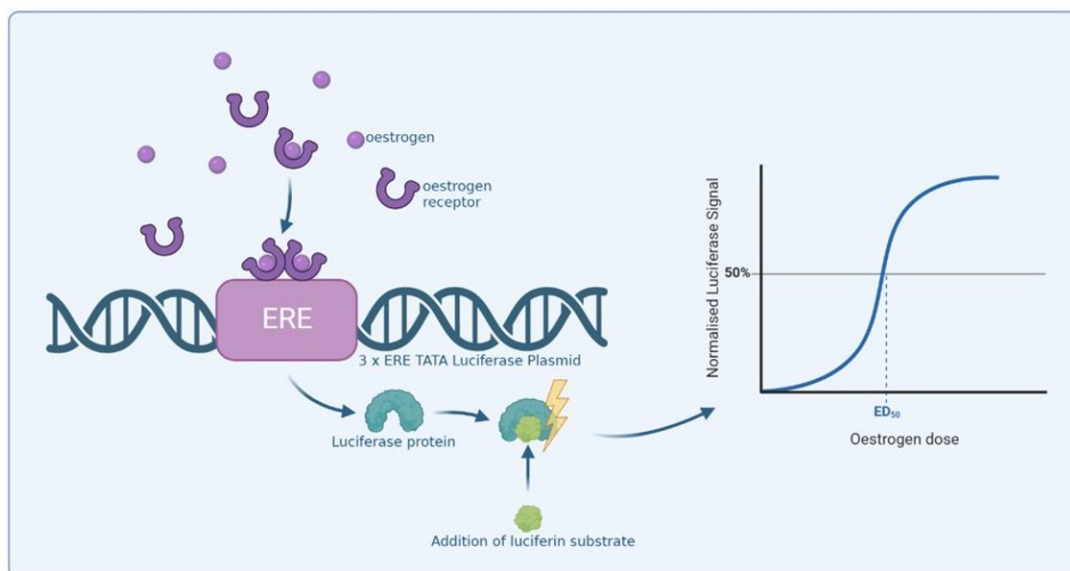


Figure 2.2: Schematic, illustrating the mechanism behind the luciferase-based assay system. Oestradiol binds to its associated receptor, which undergoes dimerisation. Dimerised, oestradiol-bound complexes bind to the transfected ERE, resulting in the production of luciferase protein. Subsequent addition of luciferin substrate generates a quantifiable signal from luciferase enzyme activity, indicating how much enzyme is present, and thus how much ERE has been activated.

2.18 Luciferase-Based Reporter Assay

The transduced or transfected cells were plated in 96-well plates with varying concentrations of oestradiol for the desired culture time. Mechanistically, this would allow for the binding of translocated oestradiol-receptor complexes to the transfected, or transduced ERE-TATA reporter sequence, then eliciting the transcription of the luciferase transcript, to produce the luciferin protein. A schematic illustrating these principles is shown in Figure 2.2. After this, media was removed and the cells were washed with PBS. Passive lysis buffer (Promega, E1941) was added and the plate was put on a shaker for 15 minutes before the lysate was frozen at -80°C . On thawing, the plate was put on a shaker for 15 minutes, and wells were scraped thoroughly. Lysate was transferred to 0.2mL qPCR tubes, which were centrifuged, before being

pipetted into a white plate for assay reading. LARII luciferase reagent (Promega, E1501) was made up according to manufacturer instructions by dissolving the lyophilised luciferase assay substrate in the provided buffer. This was subsequently stored, protected from the dark, at -80°C . The plate reader system tubing was primed with reagent before the assay was conducted on the luciferase setting, with injecting.

For the dual luciferase assay, which was used alongside the plasmid-based reporter assay, readings for each well were taken before injection, after the first injection, and then after the second injection. The first injection contained the luciferin substrate, which reacted with the luciferase enzyme, giving a signal intensity readout. Following this, the reaction was quenched with the Stop&Glo substrate, which also reacts with Renilla luciferase to provide an internal control reading for normalisation.

For the lentiviral reporter assay, a standard luciferase assay was used. Readings were taken for each well 1 second before injection, and subsequently for the next 11 seconds, at which point, it was evident that the reaction was stable and unchanged if measured for longer.

2.19 Statistics

ANOVA tests (1/2/3-way) were conducted in RStudio. Post-hoc pairwise t-tests were conducted across groupings, using a Bonferroni correction to account for multiple testing. Only post-hoc t-tests are presented graphically. ANOVA results are presented in the text.

2.20 Bulk RNA Sequencing Of Preadipocyte-Macrophage Co-Cultures

2.20.1 Harvesting and Staining Samples for Sequencing

For elucidation of the effects of oestradiol, inflammation and co-culture in abdominal and gluteal MBDFAT preadipocytes, a bulk RNA sequencing experiment was designed to investigate the transcriptional responses to these stimuli. For RNA

sequencing, three biological replicates were acquired for each sample, using different passages of preadipocytes, and for macrophages, a different (female) human donor. Co-cultures were set up, as outlined in Section 2.8, and equivalent preadipocyte monocultures were also plated. Following the addition of macrophage suspension, or a media equivalent for monocultures, to preadipocytes, 100nM oestradiol or ethanol vehicle control was added to each well. 10mM oestradiol stock (in ethanol) was diluted in PBS (1:1000) before being added to each well to obtain a final concentration of 100nM. Ethanol was diluted by an equivalent dilution factor and added to control wells. After 24 hours, 100 ng.mL⁻¹ of UltraPure LPS (InvivoGen SAS, tlrl-pektps), a specific activator of toll-like receptor 4 (TLR4), was added to half of the wells. After another 24 hours, live cells were stained and prepared for fluorescence-activated cell sorting (FACS), being sorted into lysis buffer.

For harvesting, cells were first washed with PBS and subsequently lifted using TrypLE™ Express before being added to PBS. Three technical replicates (3 x 6-wells) were pooled into one Falcon tube containing 9mL PBS, for each individual sample, to ensure sufficient RNA for sequencing. Samples were subsequently centrifuged at 300g, 5 minutes, at RT. Supernatant was removed and cells were resuspended in 100 µL FACS buffer (1% FBS, 10 µg.mL⁻¹ IgG human serum in PBS). Cell suspensions were then transferred into a 96-well U-bottom plate. Falcon tubes were washed with 100 µL Fc-block solution (1:100 dilution; BD Biosciences, 564219), which was subsequently added to the corresponding well in the 96-well plate and incubated with cells, on ice, for 20 minutes. Fc-block is a reagent specifically designed to reduce potential nonspecific background antibody staining caused by IgG receptors, thereby allowing for more specific population staining. Antibody dilutions were prepared during this incubation time (10 µL CD14-PE + 8 µL CD45-BV421 + 8 µL CD90-AF700 + 474 µL FACS buffer).

2.20.1.1 Antibody Surface Marker Selection

To separate preadipocytes and macrophages by FACS, specific cell-surface markers were necessary to distinguish each population for positive selection. As this is

not a commonly conducted protocol, it was necessary to establish which are the most appropriate cell surface markers for these cell types. Co-cultures were plated, as outlined Section 2.8, including LPS and oestradiol treatment to assess whether treatment altered surface marker staining. Staining was conducted, as outlined here, and live stained cell suspensions were analysed by flow cytometry on the LSRFortessa™ X-20 (BD Biosciences) to assess the expression of fluorescent markers. For macrophages, an antibody to the haematopoietic cell marker CD45 was trialled (BioLegend, 304031) as well as the monocyte/macrophage marker CD14 (ImmunoTools, 21620144). These markers showed clear population separation from CD14⁻CD45⁻ cells. CD34 has been used in existing literature to sort preadipocytes from primary tissue biopsies [261]. Using a CD34 antibody (BioLegend, 343509), however, did not mark preadipocytes well in this pilot experiment. An antibody to the fibroblast and stem cell marker CD90/THY1 (BioLegend, 328120) was then tested, performing much better than CD34 to mark preadipocyte populations. Therefore, populations were separated based on positive and exclusive staining on CD14 and CD45 for macrophages, and CD90 for preadipocytes.

After incubation with Fc-block, the 96-well plate was centrifuged in a pre-cooled centrifuge, at 300g, 4°C, for 5 minutes. Supernatant was carefully removed and cells were resuspended in 50 µL of the prepared antibody staining solution, or plain FACS buffer (for monocultures / unstained control pilot experiment). The plate was then incubated at 4°C, for 30 minutes, in the dark, with rocking. Following incubation, 150 µL of ice cold FACS buffer was added, before cells were spun at 300g, 4°C, for 5 minutes. Supernatant was removed, and cells were resuspended in 200 µL ice-cold FACS buffer before being spun again. This process was repeated a third time. The 200 µL cell suspension was then transferred to a 50mL Falcon tube through a 70 µM filter. The Falcon was washed with 200 µL of FACS buffer, which was subsequently transferred through the filter to wash any residual cells through. This was repeated, producing a 600 µL cell suspension. For co-culture suspensions, the 600 µL solution was transferred to FACS tubes, which were maintained on ice for transfer to the FACS facility at The Kennedy Institute of Rheumatology. For the

monocultured cells that were not undergoing FACS, 50mL Falcon tubes were spun down at 300g for 5 minutes, and the FACS buffer supernatant was removed. The cell pellets were subsequently resuspended in 350 μ L RLT buffer (Qiagen, 79216) and stored at -80 °C prior to RNA extraction. Co-cultured cells were sorted directly into 350 μ L RLT, by FACS (Aurora, Cytex Biosciences) separating macrophage and preadipocyte populations, before storage at -80 °C prior to RNA extraction. Due to FACS processing occurring in a tissue culture facility, fume hood access was not possible and therefore TRIzol™-based RNA extraction protocols could not be conducted. Qiagen RNeasy Mini kits (Qiagen, 74104) were used for RNA extraction, according to manufacturer protocols. DNase digestion was performed using the DNA-free kit (Life Technologies, AM1906), following manufacturer protocols. RNA quality was assessed using the TapeStation (Agilent, 4150) system to assess the RNA integrity number (RIN) (range = 8.4-10.0) and quantified using the NanoDrop Spectrophotometer (260/280 range = 1.91-2.23). Samples were sent to NovoGene for sequencing, to obtain 9G raw data per sample.

2.20.2 BioInformatic Analysis

Data was downloaded in its raw format. Integrity was checked by running the md5sum checking command. FastQC (public programme release available at <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC [262] packages were used to assess data quality, using metrics including the number of sample reads, per base quality score distribution, per sequence quality score, per base sequence content, per sequence GC content, per base N content, sequence length distribution, sequence duplication level, over-represented sequences and per sequence adapter content. Across the sequenced samples, mapping rates were consistent, averaging at 91.7%. Quality scores were also persistently high. Adapter contamination was low. The Trim Galore! package (public programme release available at https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to remove adapter sequences from reads and search for low-quality bases at the 3' read end, removing them until quality bases were reached. MultiQC was run again to

assess the effects of trimming on data quality. The Salmon package [263] was then used to map the clean reads to the transcriptome, using the human reference data set and annotations downloaded from Gencode (GRCh38.p14 (v44), available at https://www.encodegenes.org/human/release_44.html). Following indexing, transcript isoform quantification was performed with Salmon. Isoform-level and gene-level expression data were loaded into RStudio (Version 2023.06.1+524) using the TXimport package [264]. In RStudio, using the limma package [265], raw count data was used to calculate counts per million (CPM) and $\log_2(\text{CPM})$. Lowly expressed genes were filtered out using the edgeR package [266]. Between sample normalisation was performed using the trimmed means of M values (TMM) method, again in edgeR. After removing lowly expressed genes, gene count reduced from 62,266 to 16,789. Principle component analysis (PCA) and multi-dimension scaling (MDS) plots were used to visually analyse broad sources of variation between samples. This revealed one strong outlier sample, which was subsequently sent for re-sequencing. Re-sequencing removed this outlier. No additional outliers were identified. Differential gene expression analysis was conducted using limma. Weighted expression data was used to fit the linear models based on desired matrix comparisons. The tests used were standard Bayesian-moderated t-tests. A false discovery rate (FDR) adjustment of 0.05 was applied to all differential expression results. For gene set enrichment analyses, the fgsea package [267] in RStudio was employed, in conjunction with multiple pathway sets. These included canonical pathways from the C2 curated gene sets, including well-documented pathway maps such as KEGG and reactome pathways, alongside Hallmark Pathways, and Gene Ontology genesets (available at <https://www.gsea-msigdb.org/gsea/msigdb/>). Venn diagrams were generated using the ggvenn package [268].

3

Using Single-Cell RNA Sequencing Data to Map out Depot-Specific SVF Populations

3.1 Introduction

Adipose tissue, once perceived as a passive lipid reservoir, has transcended this simplistic notion, emerging as a metabolically active and dynamically regulated organ, with roles in metabolism, immunity, and endocrine signalling, which depend on cells aside from just adipocytes [269]. Traditional approaches for studying adipose tissue biology, including microscopy, cell culture, lineage tracing and fluorescence-activated cell sorting (FACS), have been instrumental in advancing existing knowledge. However, these methods often lack the unbiased ‘big picture’ insight which high throughput sequencing provides [270]. While bulk RNA sequencing has enabled the exploration of the molecular landscape of adipose tissue and its changes across different depots, sexes, and obesity statuses, it has been limited in its capacity to identify the specific cellular population contributors to the observed transcriptional changes. Although deconvolution algorithms have estimated shifts in cell population proportions from bulk RNA sequencing data, they are still unable to distinguish to what extent transcriptional variance is driven by changes in individual populations [271].

Single-cell RNA sequencing (scRNASeq) has become a transformative tool in its

ability to dissect the diverse cellular populations in adipose tissue, unravelling their distinctive gene expression profiles and roles within the tissue microenvironment. Unlike bulk RNA sequencing, scRNASeq enables the sequencing of individual cells, often through barcoded bead capture techniques that distinguish each cell before sequencing (Figure 3.1). By examining cellular transcriptional diversity at the single-cell level, scRNASeq offers a more nuanced understanding of how specific populations contribute to adipose tissue homeostasis and dysfunction. Although scRNASeq provides a snapshot analysis of the transcriptome, the plastic nature of adipose tissue populations allows for trajectory-based analyses that infer cellular dynamics over time. Additionally, interactome-based analyses can yield deeper insights into the interactions between different cell types within adipose tissue.

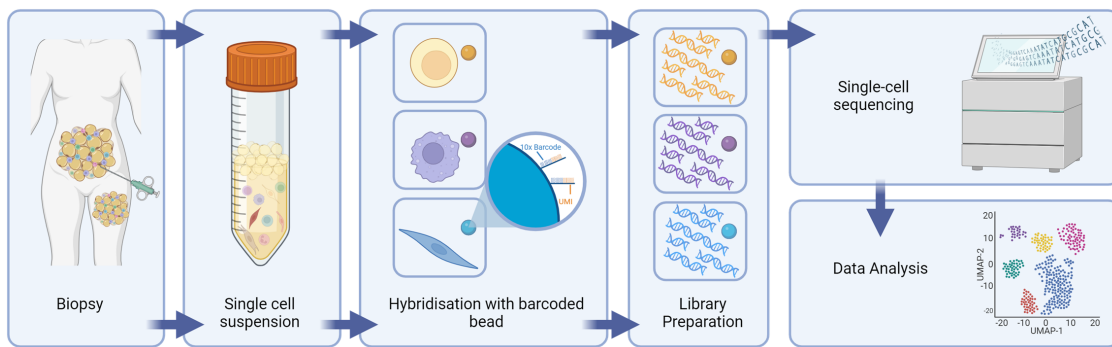


Figure 3.1: Schematic illustrating single cell RNA sequencing technology. Following biopsy, cells are suspended, and pipetted through a microfluidic chip at a limiting dilution, where they hybridise with a barcoded bead. Each bead contains a unique molecular identifier (UMI) and a 10x barcode. After bead capture, the cell is lysed and the bead dissolves. The 3' end of the released RNA binds to the poly-dT sequences coating the bead. The RNA hybridised with the bead is reverse transcribed to form cDNA. Each individual cell is associated with a single bead barcode, allowing the user to map the transcripts back to the original single cell. Oligos on each bead, however, have different UMIs, to uniquely mark each cDNA molecule. This allows the detection and elimination of PCR duplicates. Barcoded cDNA is used for library preparation using sequencing adapters and PCR amplification, followed by next generation sequencing.

However, scRNASeq of adipose tissue is technically challenging due to the large, lipid-filled nature of adipocytes. These cells are too large for microfluidic channels and are difficult to isolate in their entirety due to their buoyancy [272]. As a result, scRNASeq techniques in adipose tissue are restricted to either nuclei-based

sequencing or sequencing of the stromal vascular fraction (SVF). Although nuclei-based sequencing can encapsulate the mature cell fraction, it is less effective at recovering immune populations compared to scRNASeq [273]. Furthermore, it does not capture cytoplasmic RNAs, such as mitochondrial RNAs, which are biologically informative in the context of adipose tissue. Another limitation of nuclear RNA sequencing is its susceptibility to contamination from ambient RNA transcripts, necessitating extremely pure and high-quality nuclear preparations [274, 275]. On the other hand, sequencing the SVF circumvents some of these issues but omits the mature adipocyte fraction, which is of significant biological importance.

Research efforts utilising scRNASeq on murine and human adipose tissue have primarily focused on uncovering the transcriptional heterogeneity of adipocyte- and immune- derived populations, exploring how these cells contribute to tissue function, and how their roles vary by sex, age, and obesity status [276–280]. These studies have been instrumental in identifying baseline populations within adipose tissue, highlighting the dynamic changes in immune cell abundance in response to obesity, which was previously underappreciated [279, 280]. Despite these advances, many of these individual studies have been limited in their translational applications due to the small, specific cohorts being recruited. Most studies have included fewer than ten participants, often focusing on a single sex or adipose tissue depot, which has led to inconsistencies in the populations described across studies. To address this, the development of an integrated adipose tissue meta-map has been crucial for understanding inter-study relationships of cell populations, and the unique architecture of different white adipose tissue (WAT) depots [281].

Despite the unique capability of female gluteal adipose tissue to accommodate tissue expansion without increasing metabolic risk, its representation in scRNASeq studies is minimal. Select studies have compared abdominal visceral and abdominal subcutaneous tissues from paired samples, elucidating the harmful phenotypes associated with visceral adipose tissue (VAT) expansion [276, 280]. Others have investigated the heterogeneity of cultured preadipocytes from gluteal adipose tissue biopsies [282]. However, no studies to date have simultaneously examined both the

immune and preadipocyte populations in gluteal adipose tissue or compared these populations with those in metabolically detrimental central adipose tissue depots. Given the importance of adipose tissue immune cells (particularly macrophages) in tissue remodeling during expansion, inflammatory responses, and lipid turnover, it is essential to understand how these cells contribute to the metabolic benefits of female gluteal adipose tissue. A key area of interest is the interactions between macrophages and preadipocytes, particularly concerning how these interactions influence preadipocyte function, especially in response to factors altering fat distribution, namely oestradiol.

3.1.1 Chapter Aims

1. To first characterise the cellular populations within the SVF of abdominal and gluteal adipose tissue, with a particular focus on macrophage and preadipocyte populations, using paired tissue biopsies from a large cohort, including both men and women.
2. Investigate how these SVF populations differ between depots, and whether sex-specific differences exist.
3. Identify depot- and sex- specific cellular communication signals between macrophages and preadipocytes, which could contribute to sexual dimorphisms in fat distribution.

Overall, the study design would offer a pioneering insight into the SVF landscape of female gluteal adipose tissue, providing deeper understanding of how its metabolic advantages may be attributed to the individual cellular populations, their distinct phenotypes, and their interactions.

3.2 Subjects, Materials, Methods and Development

3.2.1 Sample generation for scRNASeq

Statement of acknowledgement: Participant recruitment and generation of SVF samples were performed by Dr Andrea Van Dam (OCDEM, Oxford). Library preparation was conducted by colleagues in Zurich [283], with sequencing and initial raw data processing at NovoNordisk. A brief description of this work is provided in Sections 3.2.1.2 and 3.2.1.3, for clarity. All subsequent analysis was performed by myself, as described in this chapter, and these data remain unpublished at the time of submission.

3.2.1.1 Participant Characteristics

Participants were recruited from the Oxford BioBank [284] based on the rs1812175 genotype, in the *HHIP* gene, for a study conducted by Andrea Van Dam. This genotype has previously been associated independently with fat distribution [285–288]. Differential gene expression analyses indicated no significant differences across the three recruited genotypes (TT, CC, TC). Consequently, the dataset was repurposed for analysis in this thesis. Study eligibility was limited to individuals with a BMI ($kg.m^{-2}$) between 22 and 30, with donor BMIs matched across genotypes to minimise confounding. Age was similarly restricted to a narrow range to facilitate appropriate genotype matching. While this homogeneity in age and BMI supported the original study’s genotype-focused design, it limited the ability to investigate the effects of metabolic variation on gene expression within this cohort. The proportions of donor contributions to the final dataset varied, but the cellular population clusters were evenly distributed across donors, suggesting that inter-donor variability was well-controlled in the clustering of samples (Figure 3.2A). To further account for potential variations in library size between samples, TMM normalisation methods, as detailed in Section 3.2.3.2, were applied across donors. The study population comprised 17 females and 15 males. Data on menopausal status was not documented at the time of biopsy. Additional participant characteristics are presented in Table

3.2B. Ethical approval was granted by the Oxfordshire Clinical Research Ethics Committee (08/H0606/107 + 5) and all participants provided informed consent.

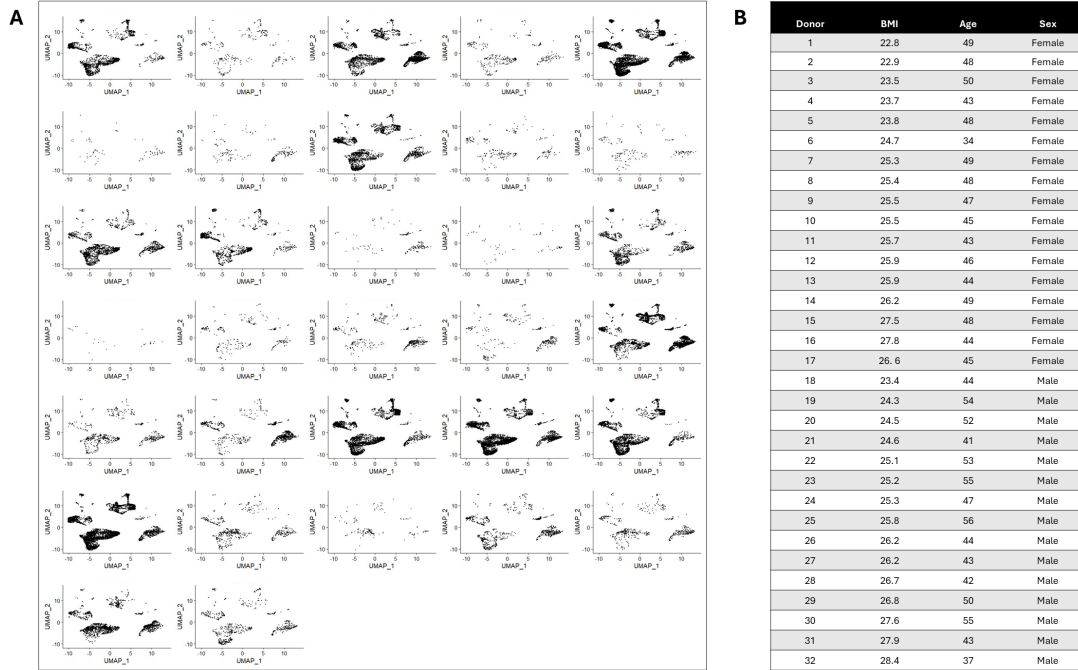


Figure 3.2: A) UMAP plots of cell clusters, divided by donor to show individual contributions across all 32 donors. Each plotted data point represents individual cells, and proximity of points represents transcriptional similarity of cells. Populations are further denoted in Figure 3.6A. Although total RNA contribution varied between donors, cluster distribution was broad, and there was no evidence of donor bias towards an individual population B) Table of donor characteristics, displaying BMI (kg.m^2), age and sex.

3.2.1.2 Sample Generation

Biopsy procedure methods are detailed briefly here, and a workflow summary is provided in Figure 3.3. Abdominal and gluteal subcutaneous adipose tissue samples were taken using needle aspiration, before transfer to gauze and immersion in Hanks Balanced Salt Solution (HBSS, Sigma, H9394) for washing. Collagenase digestion was performed using 1mg.mL^{-1} collagenase (collagenase H from *Clostridium histolyticum*, Roche, 11074032001) in HBSS, incubated for 45 minutes at 37°C . The sample was then centrifuged to separate out the mature adipocyte fraction and the SVF. The pelleted SVF was resuspended in 10mL HBSS, then filtered through a $250\mu\text{m}$ mesh, followed by a $100\mu\text{m}$ mesh. The sample was then centrifuged again before being resuspended in red blood cell lysis buffer, and incubated for 10

minutes at room temperature. The sample was pelleted and washed, before being transferred into freezing media, for storage in liquid nitrogen.

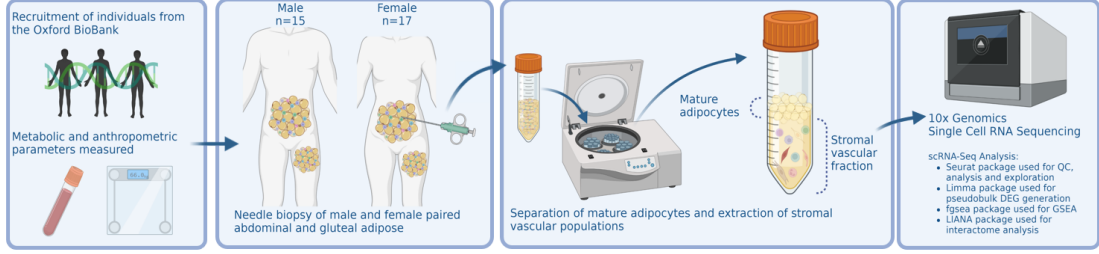


Figure 3.3: Workflow for generating single cell RNA sequencing dataset: Paired abdominal and gluteal subcutaneous adipose tissue biopsies were taken from 15 men and 17 women, recruited from the Oxford Biobank. Pertinent metabolic data for each individual were recorded at the time of biopsy. Samples were centrifuged to pellet the SVF. The pellet was then processed for single cell RNA sequencing, as detailed in Figure 3.1.

3.2.1.3 Library Preparation and Sequencing

Library preparation was conducted by colleagues in Zurich [283], using 10x Genomics Chromium Single Cell 3' Library and Gel Bead and i7 Multiplex kits. Illumina NextSeq500 was used for sequencing, using a 75-bp paired-end kit. Following alignment, and demultiplexing using Cell Ranger (10x Genomics), demuxlet [289] was used to generate the expression matrix.

3.2.2 Data Processing and Quality Control

The RStudio package Seurat [290] was used for quality control (QC), data normalisation and scaling, clustering and annotation of cell types. The RNA latency analysis was conducted using the scVelo package [291] in Python.

QC is critical for scRNASeq data, where captured cells can be stressed, broken or ruptured by bead systems, resulting in unreliable data which can be misinterpreted and lead to erroneous conclusions [292]. Broken, empty or multiple attached cells often have abnormal values for average gene expression, and thus gene expression per cell is used as a QC criterion. A value of 300-1000 RNA counts per cell is generally used as a minimum cutoff for inclusion, dependent on the sequencing depth achieved [293, 294]. In this dataset, a cutoff of 650 RNA counts per cell

was used, with a mean of 4368, indicating that the data was of good read depth (Figure 3.4A). Unique Molecular Identifiers (UMIs) were also used to filter out low quality cells. UMIs are short, random nucleotide sequences that are incorporated into individual RNA molecules during the initial stages of library preparation in scRNASeq. They serve as molecular barcodes uniquely associated with each RNA molecule originating from the same transcript. UMIs help to accurately quantify gene expression levels by distinguishing between genuine biological variation and technical noise introduced during library preparation and sequencing. Pruning cells based on UMI counts acts as an additional gene expression QC step. Lower UMI cutoffs are generally set at 500-1000 [293, 294], although cells within this range are widely regarded as low quality [293], and a cutoff of 1000 UMIs/cell was opted for, to increase the reliability of the obtained data. As shown in Figure 3.4D, this resulted in the exclusion of very few individual cells, further confirming that samples were of high quality. Another commonly used metric for QC is mitochondrial DNA content. RNAs coding for mitochondrially localised proteins are often enriched in broken cells due to the loss of cytoplasmic RNA alongside the retention of mitochondria within the cell [292]. Traditionally, across scRNASeq data, mitochondrial gene expression was thought to be generic across cell types, while ribosomal gene expression varied more widely [292]. However, more recent publications suggest that mitochondrial RNA content varies dependent on cell and tissue type, and that QC metrics should be carefully adjusted to suit individual tissue biology [295]. Although a cutoff of 5-20% mitochondrial RNA is generally used for QC pruning, other scRNASeq data sets of adipose tissue have identified populations of adipocyte progenitors with varying mitochondrial DNA content of up to 24% [280], potentially reflecting the distinct mitochondrial signature of this metabolically active tissue. Other papers have similarly reported adipocyte clusters that were enriched for mitochondrial processes [296]. Therefore, a mitochondrial RNA cutoff of 30% was used (visualised in Figure 3.4F).

A latency time analysis was also conducted in the context of QC, to provide insight into gene expression dynamics, therefore helping to identify ‘static’ populations

3.2.3 Data Analysis Methods and Development

3.2.3.1 Clustering Populations

Clustering for abdominal and gluteal samples was performed, combined, within Seurat, using the FindCluster and FindNeighbour functions, with the resolution being set as per standard thresholds (dims = 1:10, resolution = 0.5). Depots were pooled for clustering to allow like-for-like comparisons in downstream bioinformatic analyses. Subsequent subclustering was conducted on the monocyte/macrophage populations to resolve distinct macrophage varieties which were known to exist within the larger clusters that were originally identified. This is well-accepted, and frequently conducted in scRNASeq analyses, to provide better acuity within larger clusters of interest [298]. For example, *TREM2* expression, a lipid associated macrophage (LAM) marker [116], was observed in a subgroup of the initial macrophage cluster, suggesting that further clustering was necessary to discern smaller, distinct populations.

Population marker genes were identified using the Seurat FindAllMarkers function, and subsequently compared to existing published literature marker gene sets, to label each population identity.

3.2.3.2 Differential Gene Expression Analysis

For analysing differentially expressed genes (DEGs) comparing cluster-wide gene expression between conditions (e.g donor sex, depot), clusters were first pseudobulked to average gene expression across each cluster for each donor sample (e.g abdominal preadipocyte from donor 1). Conventional Seurat methods for differential gene expression analyses compare gene expression across each individual cell between the conditions of interest, which is biased towards highly expressed genes [299]. Conventional Seurat methods were unable to resolve certain lowly expressed DEGs which are known to be differentially expressed between abdominal and gluteal preadipocytes (e.g *HOTAIR* [300]). However, pseudobulk methods were able to resolve these low-expression DEGs. It is now widely accepted that pseudobulking scRNASeq data, followed by conventional RNAseq differential gene expression

analysis on individual populations, provides more reliable data for the identification of DEGs between conditions, due to an effective increase in sample depth [299] and reduced bias towards highly expressed genes.

To determine DEGs, pseudobulked count data was firstly filtered, removing lowly expressed genes, using the edgeR package [266]. Between-sample library normalisation was performed using the trimmed means of M (TMM) method, again in edgeR. This effectively accounted for differences in library sizes due to donor variation, allowing for estimation of relative RNA and the subsequent scaling of count data. Differential gene expression analysis was conducted using the limma package in RStudio [265]. Weighted expression data was used to fit the linear models based on desired matrix comparisons. Comparisons between abdominal and gluteal cells were controlled for sequencing run and donor sex when designing the model matrix. For sex- and depot- stratified analyses, matrices were designed using sample groupings: male abdominal, female abdominal, male gluteal, and female gluteal. The tests used were standard Bayesian moderated t -tests. A false discovery rate (FDR) adjustment was applied to differential expression analyses, filtering the results to genes surviving an adjusted p value threshold of 0.05. Downstream analyses primarily focused on results with $\log_{2}FC > 1$.

The fgsea package [267] in RStudio was used to investigate pathway enrichment from sets of DEGs. This was selected, as it provides high accuracy gene set enrichment analysis (GSEA) results, and utilises the output of limma-moderated t values rather than p values, $\log_{2}FC$ values, or gene rankings. This means that enrichment can be assessed on the directionality, magnitude, ranking and significance of the differential expression, which all dictate the given t value, rather than any one of these individual metrics. Canonical pathways from the C2 curated gene sets, including well-documented pathway maps such as KEGG and reactome pathways were downloaded, alongside Hallmark Pathways, and Gene Ontology genesets. These were taken from the Human Molecular Signature Database and used to assess pathway enrichment. For depot- or sex- specific investigations, individual gene groups from the desired condition, and their t values, were isolated before being run in fgsea.

3.2.3.3 Interactome Analyses

To investigate ligand-receptor interactions between preadipocytes and macrophages, the LIANA package in RStudio [301] was used to generate a database of hypothetical signal exchange across all cells within this dataset. Hypothetical signals are identified based on the expression levels of ligands and receptors across the annotated clusters, and compared to annotated ligand-receptor interaction pair databases. The LIANA package employs 16 different datasets from which to assess its predictions [301]. Interactions were subsequently filtered to select ligand-receptor pairs that were specific signals going from macrophages to preadipocytes, and also preadipocytes to macrophages. This generated two separate datasets, representing signalling in the two different directions. Identified gene sets of interest (e.g genes arising in differential expression analyses) were then mapped to these data, to extract the overlapping gene identities, which were both part of the interactome analysis, and differentially expressed across the conditions of interest.

3.3 Results

3.3.1 Defining Populations in Abdominal and Gluteal Subcutaneous Adipose Tissue SVF

To characterise the cellular populations within the SVF of abdominal and gluteal adipose tissue, cell clusters were generated, as discussed in Section 3.2, and visualised in a UMAP plot (Figure 3.6A), illustrating the spatial transcriptional relationships of cellular clusters. Following clustering, each population was defined and characterised based on published marker genes from existing literature (Figure 3.6B), discussed further below. The majority of cell types identified in this study have been previously reported in other published adipose tissue scRNASeq datasets, as discussed below in relation to cluster marker gene analyses. On average, adipocyte lineage cells (preadipocytes and APCs) comprised approximately 20% of the total biopsy cell populations. Immune cell clusters accounted for roughly 75% of this dataset, with the remaining cells primarily consisting of endothelial and smooth muscle cells.

While the proportion of vascular cells and in this dataset aligns closely with previous subcutaneous adipose tissue scRNASeq studies, adipogenic lineage cells were on the lower end of reported ranges, whereas immune cells were on the higher end, as compared to existing publications [281]. In the adipose tissue transcriptomic metamap published by Massier et al., fibroblast and adipogenic progenitors (FAPs) — which encompass the identified preadipocyte and APC populations here — ranged from approximately 15% to 80% of cells in subcutaneous adipose tissues [281]. Notably, however, many publications also encompass the mature adipocyte fractions using single nucleus RNA sequencing. When comparing to publications that investigate just the SVF, the presented data align more closely with existing literature [279, 281]. This was also observed in immune subpopulations; studies reporting the relative proportions of immune populations from adipose tissue SVF samples show better alignment with data presented here, as compared to data which include the mature adipocyte fraction [279, 281]. For example, non-obese individuals were found to exhibit the highest proportions of Natural Killer (NK) and T-cells in adipose tissue SVF, relative to other immune populations [279], which parallels the cellular proportions found in these data (further detailed in Appendix A.2). Additionally, the relatively low proportion of adipogenic lineage cells could be partially attributable to blood contamination in the biopsies, which may have increased the proportion of blood-derived immune cells. Inter-donor variability, however, was also evident (Appendix A.2), and should be taken into consideration. Variance in population abundance across donors is common feature of scRNASeq datasets, especially in relation to factors such as depots of origin, metabolic state, diet and sex [279, 281, 302–304].

secrete chemokines, proliferate, as well as phagocytose and present antigens to other immune cells. Following chemokine gradients to sites of inflammation, monocytes can infiltrate into tissues, including adipose tissue, before differentiating into effector cells - namely dendritic cells (DCs) and macrophages.

In adipose tissue, monocyte infiltration contributes to the local pool of ATMs and DCs. In obesity, chronic low-grade adipose tissue inflammation augments chemokine secretion from within adipose tissue, increasing the infiltration of monocytes [305]. Infiltrated monocytes that subsequently differentiate into macrophages and DCs in adipose tissue, tend to adopt inflammatory phenotypes, contributing to tissue inflammation, particularly in obesity. Obesity and weight loss are oppositely correlated with monocyte recruitment to adipose tissue and inflammatory status [141, 306–308]. As well as differentiating into macrophages, monocytes can reside in adipose tissue as they are, contributing to important tissue functions such as tissue vascularity [309]

Using unsupervised clustering, four populations of monocytes were identified in adipose tissue SVF.

Markers *CD14* and *FCGR3A*(*CD16*) clearly distinguished two clusters of classical monocytes (cMo) as well as two clusters of non-classical monocytes (ncMo), respectively. These have been well-documented in adipose tissue scRNASeq data [281]. An intermediate monocyte (iMo) population, as described in other scRNASeq datasets [310–312], was annotated from one of the non-classical populations. This cluster showed lower expression of *FCGR3A* as compared to the other non-classical population, and intermediate expression (comparing classical and non-classical populations) of various inflammatory markers including *TGFB1*, *IL7R*, *LGLALS3*, *TLR4*, and non-inflammatory markers such as *SIGLEC10*, *TLR2*, *SERPINA1*, *CXCL16*, *CDKN1C*, which parallels the populations described in existing literature [311, 313]. Further to this, expression of *CD179B/IGLL1* and *TNF- α* were higher than the other monocyte populations, and these had previously been noted as marker genes for the iMo populations identified by Dutertre et al and Gren et al [310, 314].

Between the two populations of classical monocytes, one showed elevated expression of key genes involved in adhesion, chemotaxis, and inflammation including *ICAM1*, *CCL3* and *TGFB1*, indicating that these may be infiltrating into tissue from the blood. They displayed highest expression of *DDR1* and *CD274*, which have been associated with monocyte infiltration and activation, respectively [315–318]. This suggested that this population was indeed ‘activated’, and thus this population was denoted ‘activated classical monocytes’ (AcMo).

3.3.1.2 Dendritic cells:

DCs respond to the extracellular milieu, sensing pathogen-associated molecular patterns in peripheral organs through the expression of pattern recognition receptors. Subsequent antigen binding, processing and lymphatic draining to lymph nodes allows DCs to present antigen on MHC surface molecules for T-cell activation. This allows DCs to initiate immune responses, acting at the interface between the innate and adaptive immune systems. Along with antigens, DCs express co-stimulatory molecules and secrete cytokines to direct T-cell activation depending on the signals received. This is especially important for T-helper cell polarisation and the induction of immune tolerance.

WAT DCs have been historically hard to distinguish [319] due to overlapping surface markers with other populations, such as activated macrophages and monocytes. ScRNASeq data thus provides a rare opportunity to definitively dissect out DC populations to investigate their transcriptome. In adipose SVF, three populations of conventional DCs (cDCs) were identified. These highly expressed *MHCII* and *CD11c*, which have roles in efficient antigen presentation. Plasmacytoid DCs (pDCs) were also identified, and these are important for type 1 interferon production [320]. All four populations expressed classic DC markers including *FLT3* [310]. While cDCs are the main resident DC population, pDCs are much rarer [321]. This corroborated with population abundances from this data.

The pDC population was distinguished from cDCs by the expression of well-defined marker genes including *IL3RA*, *IRF7*, *ITM2C*, *PLD4*, *NRP1/CD304*, *LILRA4* [310],

[312, 322, 323]. To separate out each cDC population, again, marker genes were compared to existing literature.

One cluster of cDCs showed specific expression of *CLEC9A*, *XCR1* and *CADM1*, which are all cDC1 markers [310]. Marker genes of this DC cluster mirrored those of a *CLEC9A*⁺ cDC1 population which have been noted in other adipose SVF datasets [279, 312, 323]. This population co-expresses monocyte marker genes such as *S100A8* and markers enriched for antigen processing, including *CD1C* and *CLEC10A* [279, 312, 323]. cDC1s favour stimulation of CD8⁺ T-cells, priming them against extracellular antigens and also effectively stimulating naive T-cell proliferation [312].

Another population of cDCs expressed elevated levels of the monocyte marker genes *CD14* and *ITGAM* [310]. Dutertre et al noted that cDC2s express less specific distinct DC marker genes, which often overlap with monocytes, suggesting that this cluster aligns with the cDC2 identity. Further evidence that these were cDC2s included high expression of *CD1C*, *FCGR2B*, *FCER1A*, *HLA-DRA*, *HLA-DQB1*, *HLA-DQA1*, which were cDC2 markers elsewhere [279, 312, 323]. This population differentiate from monocyte precursors and may accumulate in inflamed tissue, with a preferred ability to then stimulate CD4⁺ T-cells.

A third cDC population was distinguished by genes that are important in the early transition to DC maturity, such as the transcription factor *ID2*, and monocyte genes including *S100A8*, *S100A9* and *CD86*. Compared with other DC populations, this cluster showed lower expression of mature cDC marker genes such as *FLT3*, further supporting the notion that this was a less mature population [324]. Other literature found that this early population showed high expression of *TREM1*, *SIGLEC6* and *MARCKSL1*, which distinguished them from other DC subsets and macrophages [324, 325]. Similar expression was evident here, and thus this early cluster was labelled as ‘pre-cDC’. The SVF UMAP plot (Figure 3.6A) again supported this identity of pre-cDCs, as the cluster appeared, spatially, as an intermediate population between monocytes and cDC2s, likely representing a transitioning population.

3.3.1.3 T-Cells:

T-cells are important for immune homeostasis, critical for components of immune memory and self-tolerance. Following maturation, T-cells migrate to the periphery from the thymus, where each subset plays its individual role in tissue immune responses. When presented with antigen on MHCII molecules of antigen presenting cells and co-stimulatory molecules, CD4⁺ T-cells can divide rapidly and differentiate into effector T-cells, which secrete cytokines to direct other immune cells. CD4⁺ T-cells act as ‘helper’ cells, whereby they assist with B-cell maturation and activation of cytotoxic T-cells and macrophages. Different T-helper subsets secrete different types of cytokines to recruit specific immune cells. CD8⁺ T-cells have cytotoxic functions. Following recognition of antigen at MHC class I molecules, CD8⁺ T-cells can kill virally infected cells or cancer cells, and produce anti-viral cytokines to recruit other immune cells. Mucosal-associated invariant T-cells (MAIT) are also important for immediate pathogen responses, recognising bacterial metabolites, and responding through cytokine production to lyse infected cells. These initial effector cells are short lived, but a subset of memory cells persist, ready for re-encounter of the same antigen, and therefore providing long-term protection. While naive T-cells are important for recognising pathogens and inducing immune responses, T-reg cells have a role in maintaining tolerance to harmless or self-antigens by suppressing autoreactive T-cells that escaped thymic selection. They also shut off T-cell mediated immunity when immune responses are coming to a close. In adipose tissue, T-cells contribute to tissue inflammation, producing cytokines following initial stimulation by antigen presenting cells such as macrophages [326].

In this data, T-cells were defined through identification of published markers, including *CD3E*, *CD3D* and *IL7R*, which mapped to six clustered cell groups [313, 322, 327]. CD4⁺ T-cells were distinguished by expression of *CD4*. Two clusters of naive CD4⁺ T-cells were subsequently identified through expression of *CD2*, *CD28* and *CD40LG* [279]. These subpopulations differed in expression of markers linked to activation status, for example, one population expressed higher amounts of *TNF-α* and *MIR155HG*, while the other expressed greater *TCF7*, *CCR7* and

SELL [323, 328]. These have been linked to active and resting states, respectively, distinguishing two distinct population of T-cells ; NaiveCD4T and NaiveCD4T2. CD8⁺ T-cells expressed *CD8A*, as well as *CD8B* and *TRGC2* [328], marking two clusters which were subsequently divided into a more active cytotoxic population, and a naive population. Cytotoxic CD8⁺ T-cells had higher expression of genes relevant to cytotoxic function, including *GNLY*, *FGFBP2*, *GZMB*, *NKG7*, *KLRD1* and *GZMH*, while naive cells had greater expression of *CCL4L2*, *XCL2*, *YBX3*, *CRTAM*, *XCL1*, *RCAN2*, *IFNG* and *CCL3* [279, 323, 328].

Another population showed marker genes including *KLRB1*, *CEBPD*, *TLE1*, *NCR3*, *SLC4A10*, *GZMK* and *ZBTB16*, which mark MAIT cells [279, 329].

The population of T-reg was marked by genes including *CTLA4*, *FOXP3*, *IL2RA*, *TNFRSF18* and *ID3* [279, 328, 329].

3.3.1.4 NK-cells:

Natural killer (NK) cells play an important role in adipose tissue inflammation. They possess specialised killer cell immunoglobulin-like receptors (KIRs) and killer cell lectin-like receptors (KLRs), to identify MHC I molecules on target cells, allowing them to act through cytotoxic mechanisms to kill infected or cancerous cells. With adipose tissue chronic inflammation, macrophages secrete inflammatory cytokines, subsequently promoting NK-cell activation, proliferation, and further cytokine production [330].

In this SVF dataset, the broad marker *KIR2DL4* identified four different NK cell clusters [331, 332]. The two mature NK (mNK) populations were enriched for expression of classic NK genes including *FCGR3A*, *FGFBP2*, *PRF1*, *KLRF1*, *NKG7* and *GNLY* [279, 322, 329]. Comparing gene expression between the two populations of mNK cells revealed differences in the expression of *CD56/NCAM1*, likely relating to CD56-bright and CD56-dim cells for mNK and mNK2, respectively. Interestingly, they also differed significantly in the expression of mitochondrial genes, potentially relating to differences in mitochondrial content, fragmentation and fission, which are thought to regulate mNK metabolic responses and functional trajectories [333].

Another population expressed lower levels of these classical NK markers, but high levels of proliferation-related genes including *MKI67*, *TUBB*, *TOP2A*, *HMGB2* and *CDK1*, indicating that these were cycling NK cells [313, 322].

An immature NK (iNK) cell population was distinguished based on markers including *SELL*, *GZMK*, *NCAM1* and *EOMES* [279, 322, 329].

3.3.1.5 Macrophages:

As discussed in Section 1.4, adipose tissue macrophages (ATMs) play a critical role in tissue homeostasis, and significantly contribute to obesity-associated chronic inflammation, releasing pro-inflammatory molecules such as TNF- α from adipose tissue into the systemic circulation.

There are distinct populations of ATMs which have varying roles in tissue homeostasis and health. In recent literature it has become clear that macrophage populations substantially diverge from the traditionally accepted bimodality of the M1/M2 paradigm, especially in adipose tissue, where sets of metabolically unique populations exist. This includes lipid-associated macrophages (LAMs), which play critical roles in lipid buffering to prevent adipocyte hypertrophy and inflammation [116]. Another important population are perivascular macrophages (PVMs), also known as vascular associated macrophages (VAMs), which are enriched for expression of M2-like genes, such as *CD206* [334]. They surround blood vessels and have anti-inflammatory functions, also representing a self-renewing population derived from the embryonic yolk sac, as opposed to bone marrow-derived macrophages which differentiate from infiltrating monocytes [334].

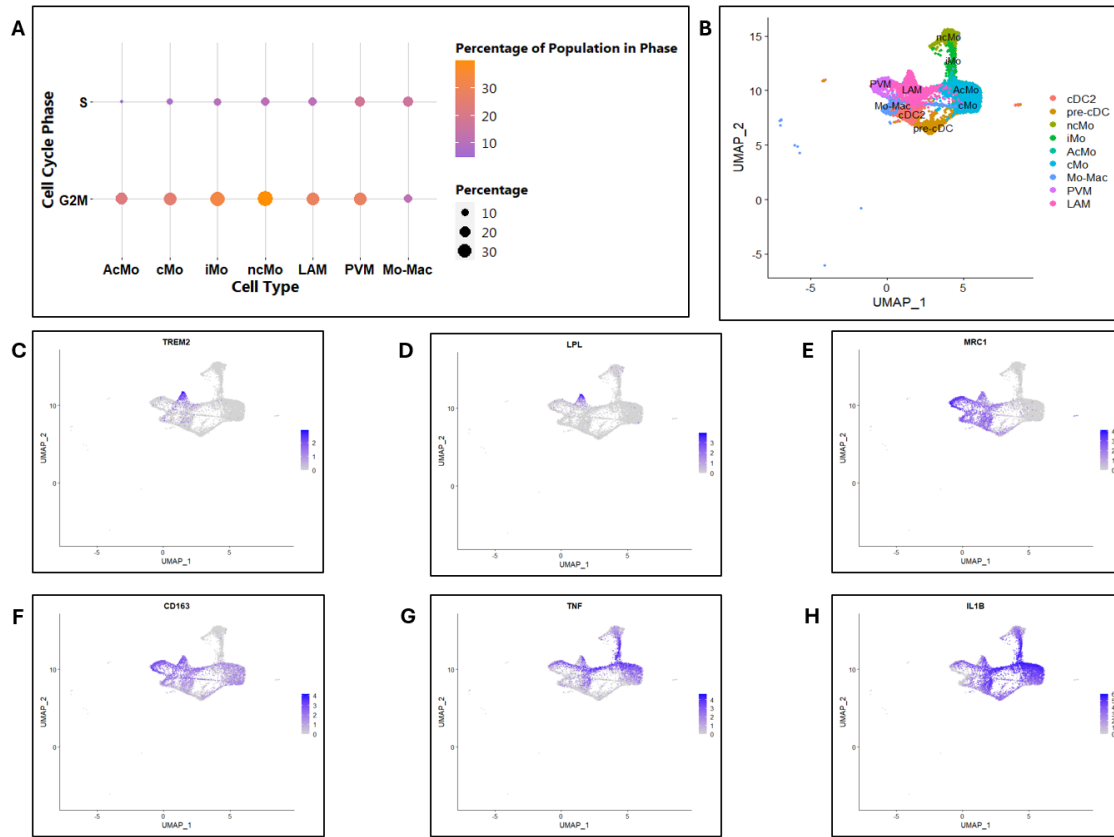


Figure 3.7: **A)** Cell cycle score analysis, calculating the proportion of macrophage subsets in each stage of the cell cycle, combining populations from both depots. Values are shown for G2M and S phases, with the remainder of cells being in the G1 phase. **B)** UMAP of macrophage populations, for reference when viewing the neighbouring feature plots. Feature plots illustrating gene expression across macrophage and monocyte populations from both abdominal and gluteal depots. Selected genes represent classic inflammatory ($IL1\beta$, $TNF-\alpha$) and anti-inflammatory ($MRC1/CD206$, $CD163$) markers [335], as well as transcripts associated with lipid associated macrophages (LPL , $TREM2$) [116]. Plots visualise expression for: **C)** $TREM2$ **D)** LPL **E)** $MRC1$ **F)** $CD163$ **G)** TNF **H)** $IL1\beta$.

In these data, macrophages were identified based on the expression of canonical markers such as $CD14$, $CD163$, $CD68$, $C1QC$, $VSIG4$, $MRC1$ and $CTSB$ [327]. Identified macrophages were divided into three subpopulations based on the expression of telling marker genes, as shown in Figure 3.7. These clusters were: LAMs, PVMs and a population of monocyte-macrophage (Mo-Mac) cells. The LAMs were distinguished by expression of $TREM2$, $CD9$, $FABP4$, $FABP5$, $APOE$, $APOC1$ [279] and $SPP1$ [313], as well as other transcripts associated with lipid metabolism including $LIPA$ [313] and $NPC2$. PVMs were an anti-inflammatory population, expressing higher levels of the M2 markers $MRC1$ ($CD206$), and

other anti-inflammatory population markers including *LYVE1*, *SELENOP*, *C1QA*, *LGMN* and *RNASE1* [279, 323]. Interestingly, when conducting a cell-cycle analysis on the dataset, it was evident that this tissue resident population was the most ‘self-renewing’ as compared to other macrophage and monocyte populations, having a greater proportion of cells in G2M and S phase (Figure 3.7A). The third population expressed less classical macrophage markers compared to the other two populations, and relatively higher levels of classical monocyte markers such as *HSPA1A*, suggestive of an intermediate cell population of partly differentiated cells [313]. This cluster showed elevated expression of *CXCL2* and *CXCL3*, which have been associated with Mo-Macs elsewhere [323]. They didn’t show increased expression of other traditional inflammatory macrophage markers such as *NFKB*, *CCL20* and *CCL3L1* [279] though, indicating that these were not a distinct inflammatory macrophage population [279].

3.3.1.6 B-cells and Plasma Cells:

B-cells importantly synthesise antibodies and can be found in both lymphoid and non-lymphoid organs, including adipose tissue. Their subsets and function in mice vary by depot and they are known to contribute to high-fat diet-induced adipose tissue inflammation by producing inflammatory cytokines and IgG [336]. Although human studies of adipose tissue B-cells are limited, Hagglof et al suggest that their population abundances increases during obesity, exacerbating obesity-associated metabolic consequences [337].

Plasma cells differentiate from B-cells following antigen exposure, and can also produce immunoglobulins and cytokines to combat infection. Unlike B-cells, plasma cells cannot proliferate further, but are able to secrete greater quantities of antibody. Again, these are found in human adipose tissue, and are more prevalent in visceral, as compared to subcutaneous depots, potentially contributing to inflammation in obesity [338].

In this adipose tissue SVF dataset, B-cells and plasma cells both expressed markers including *FCRL5*, *PUO2AF1* and *CD79A* [279, 327]. Plasma cells were marked

by expression of multiple immunoglobulin genes such as *IGLC2*, *IGHG1*, *IGLC3*, *IGKV4-1*, *IGLC1*, *IGHG3*, *IGHGP*, *IGHM*, *IGHV3-7*, *IGHV1-51*, *IGHA2*, *IGHG2* and *IGKC*, as well as the markers *MZB1*, *JCHAIN* and *PIM2* [327, 339]. B-cells were distinguished by expression of *MS4A1*, *CD19* and *CR2*, also showing higher expression of *CD79A* [279, 339].

3.3.1.7 Endothelial and Smooth Muscle:

Endothelial cells (ECs) and smooth muscle cells (SMCs) play an important role in regulating adipose tissue vascular function, which is vital for proper tissue oxygenation and endothelial transport. Their ability to mediate lipid influx is critical for normal tissue homeostasis; senescent ECs impair adipose tissue functions, increasing oxidative stress and inflammation while impairing lipolysis, adipokine secretion and insulin signalling due to defective fatty acid handling [340–342].

Vascular SMCs provide structural integrity to vessel walls and play an important role in regulating tissue oxygenation in adipose tissue. They can respond to factors released from adipocytes, including VEGF and oleic acid, which has been found to vary by depot, type 2 diabetes mellitus (T2DM) and obesity status, contributing to tissue inflammation [343].

ECs and SMCs were identified based on expression of published marker genes. For the EC population, this included *CD34*, *PECAM1*, *VWF*, *CD36* [339], *RBP7*, *PECAM1*, *CLDN5* [279], *MMRN2*, *ESAM* and *CDH5* [327], while for SMCs, marker genes such as *CD36*, *MYO1B*, *THY1*, *ACTA2*, *MYH11* [339], *POSTN*, *RGS5* [279], *KCNMB1* and *CNN1* [327] were identified.

3.3.1.8 Preadipocytes and Progenitor Cells:

Preadipocytes are immature adipocytes, capable of proliferating before they accumulate lipid and differentiate into mature cells. This proliferative capacity is important for lipid buffering, reducing the need for adipocyte hypertrophy and ectopic fat deposition, as detailed further in Section 1.1.2. Depot-specific proliferation capacities contribute to the ability of each depot to expand ‘safely’; subcutaneous preadipocytes have a greater proliferative potential compared to their visceral counterparts [344,

[345], increasing the amount of lipid that is able to be safely stored here. Several publications have discussed the presence of distinct preadipocyte populations in adipose tissue, as identified with scRNASeq [346], which differently contribute to processes including adipogenesis, proliferation, and fibrosis [281, 346, 347]. Differing abundances of these populations are seen between depots, and also in obesity and T2DM relative to lean individuals, with abundances further correlating with relevant metabolic parameters [276, 280, 281]. This suggested that metabolic disease may evoke specific remodelling of preadipocyte populations in a depot-specific manner.

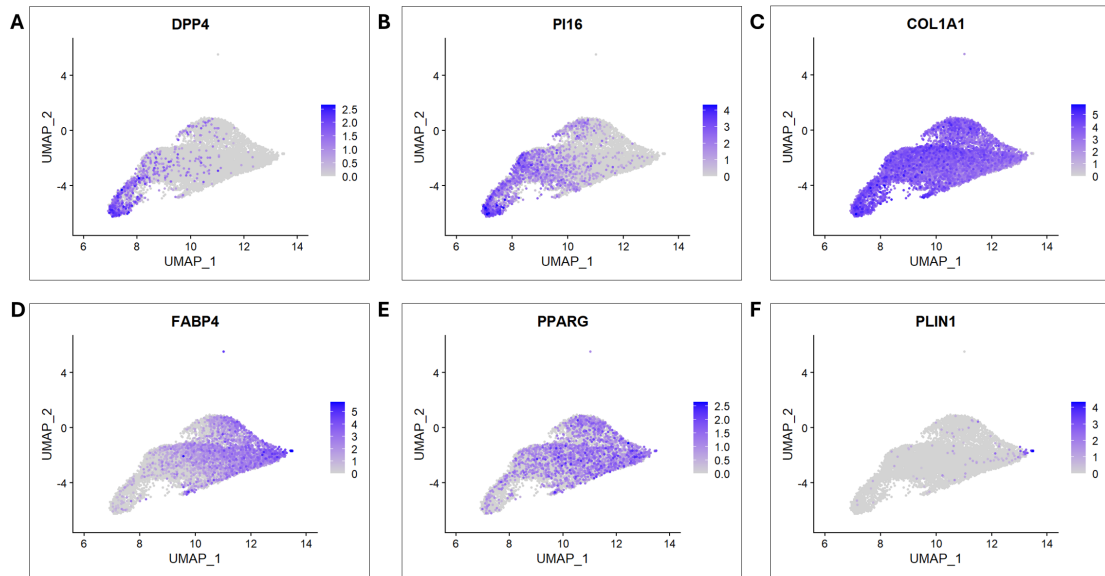


Figure 3.8: *FeaturePlot, showing expression of maturity-related marker genes across preadipocyte populations: A) FABP4 B) PI16 C) PLIN1 D) COL1A1 E) DPP4 F) PPARG. Expression is indicated by data point colours, further annotated in the colour legend of each plot. Markers were chosen based on their established roles in adipocyte differentiation and maturation (e.g PPARG, PLIN1, FABP4 [348]) or stemness and proliferation (e.g PI16 [348], DPP4 [349]), as well as differential expression across fractionated adipose tissue (further detailed in Section 4.2.2.5). Across each plot, cellular maturity increases from left to right.*

Preadipocytes were clustered into three distinct populations, and an additional population of precursory adipose progenitor cells (APCs) was also identified. Preadipocyte1, Preadipocyte2, Preadipocyte3 and APCs showed distinct transcriptional profiles, likely representing different stages in preadipocyte development. Different markers of maturity were expressed across these clusters, as shown in

Figure 3.8A-F. All populations were present in both depots, and in males and females. Although developmentally distinct populations of preadipocytes exist between depots, the transcriptional markers between them (e.g. *HOTAIR*) are lowly expressed. Therefore, in an unbiased clustering analysis, which utilises highly expressed genes to determine cell types, this is likely insufficient to separate cells which belong to the same broader cell type. All preadipocyte and APC populations expressed markers of preadipocytes including *PDGFRA* and also stem-like markers including *THY1/CD90*. Uniform expression of *PPARG* across the preadipocyte populations confirmed that these cells were committed to the adipocyte lineage. Lower expression of *PPARG* was evident in the APC population (Figure 3.8E), suggesting that these cells were in a less committed state. There was virtually no expression of mature cell markers such as *PLIN1* [350] (Figure 3.8F), confirming that these populations represented true preadipocytes.

APCs were marked by expression of stem-like markers associated with early progenitor cells, including *DPP4*, *PI16*, *CD55* and *COL1A1* (Figure 3.8A-B), while expressing relatively lower expression of committed preadipocyte markers such as *APOD* and *CXCL14* [277, 280, 281, 351–353]. Gene set enrichment analyses (GSEA), using Hallmark gene sets and comparing with the other preadipocyte clusters, revealed that this population was negatively enriched for adipogenesis (adjusted p value = 8.30×10^{-5} ; normalised enrichment score = -2.93), and positively enriched for mitotic spindle (adjusted p value = 0.018; normalised enrichment score = 1.96), relative to other preadipocyte populations, suggesting that this progenitor population is actively dividing and not primed for adipogenesis.

Preadipocyte1 and Preadipocyte3 appeared to be part of a continuum, with clustering dividing cells into a less and more mature population respectively. Preadipocyte1 showed elevated expression of markers including *MGP* and *WISP2*, relative to other preadipocyte clusters, as well as genes associated with repression of adipogenesis such as *H19* and *CTHRC1* [354–356], suggesting that these cells were a more immature population. Preadipocyte3 displayed markers of an increasingly committed cell type, which included *APOE*, *FABP4* (Figure 3.8D), *FABP5* and *CD36* [280], and

using a Hallmark gene set GSEA revealed enrichment for adipogenesis (adjusted p value = $4.58e^{-05}$, normalised enrichment score = 3.25) in this latter cluster.

Preadipocyte2 expressed markers associated with differentiation commitment (e.g. *ICAM1*, *CEBPB*, *CD44* [280, 348, 351]) and the clonal expansion immediately preceding differentiation [357] (e.g. *EGR1*, *JUN*, *FOS*, *MYC*, *CDKN1A*). This suggested that Preadipocyte2 were a population undergoing expansion, imminently before differentiating into mature cells. Marked expression of *ICAM1* has also been linked to perivascular niche preadipocytes, suggesting that this population may form close contact with ECs [351]. Interestingly, this population were enriched for Hallmark processes including TNF- α signalling (adjusted p value = $1.43e^{-05}$; normalised enrichment score = 2.50), interferon gamma response (adjusted p value = 0.001 ; normalised enrichment score = 1.91) and inflammatory response (adjusted p value = 0.002 ; normalised enrichment score = 1.89), suggesting that this population was also the most inflammatory of the identified preadipocyte populations.

3.3.1.9 Identifying Populations of Interest

As outlined in Sections 1.1.2 and 1.4, macrophages and preadipocytes play pivotal roles in maintaining adipose tissue health. Preadipocytes determine the tissue's carrying capacity by facilitating its expansion in response to nutritional stimuli. Their ability to proliferate influences the balance between hyperplasia and hypertrophy, enabling the safe storage of lipids within adipose tissue and diverting excess lipids away from non-adipose organs.

Immune cells are also crucial in regulating the inflammatory tone of adipose tissue, which impacts its lipid storage capacity, cytokine secretion, and insulin sensitivity. Macrophages, in particular, are key players, as their polarisation dictates the tissue's inflammatory status. This polarisation can be influenced by adipocyte dysfunction, nutritional overload, and other metabolic cues, placing macrophages at the intersection of adipocyte metabolism and inflammation, especially in obesity. Critically, macrophages relay inflammatory signals to other immune cells, amplifying

immune responses and cytokine production during inflammation. Additionally, they modulate adipose tissue function by regulating the growth factor niche for preadipocytes, thereby influencing the tissue's capacity for expansion. Thus, macrophages are central to the balance between adipose tissue inflammation and health, responding to metabolic disturbances and mediating communication between preadipocytes and immune cells to regulate both tissue inflammation and growth. Given the significance of the cross-talk between preadipocytes and macrophages, this work focuses on these specific populations within adipose tissue and how their interactions contribute to depot-specific tissue functions.

3.3.2 Preadipocyte and Macrophage Populations Show Depot Differences in the SVF Landscape

Having investigated the heterogeneity of macrophage and preadipocyte populations across the generic adipose tissue SVF, the next aim was to investigate how these populations vary between depots and sexes.

Preadipocyte populations exhibited varying abundances across sex-specific depots (Figure 3.9A). The less mature preadipocyte populations, specifically APCs and Preadipocyte1, were more prevalent in the gluteal depots, whereas Preadipocyte2 and Preadipocyte3 were more abundant in the abdominal depots. A cell cycle score analysis (Figure 3.9B) revealed a higher proportion of preadipocytes in the G2M or S phase in female abdominal and gluteal depots, as compared to male preadipocytes from both depots. This trend persisted even after stratification of the preadipocyte population by individual cluster. When comparing preadipocyte clusters between abdominal and gluteal depots, numerous DEGs were identified (Figure 3.9C), though the gene sets were largely similar across preadipocyte populations. Notably, genes including *CYP19A1*, *SHOX2* and *PI16* were top hits in the gluteal depots, relative to abdominal. *CYP19A1* encodes the aromatase enzyme, which is essential for the formation of oestradiol in adipose tissue through its ability to aromatise other steroids, such as testosterone. Differential expression of this transcript between depots likely contributes to greater local oestradiol production in gluteal adipose

tissue. *SHOX2* and *PI16* have been associated with adipogenesis and stemness of preadipocytes [348, 358]. Meanwhile abdominal preadipocytes demonstrated increased expression of *CD9*, *HAND2*, *IGFBP3* and *SOX9*; genes implicated in adipose tissue function and inflammation [359–363]. To capture the transcriptomic differences arising from variations in preadipocyte cluster abundance across depots, as well as to enhance statistical power, subsequent analyses were conducted by pooling the preadipocyte population clusters for each donor.

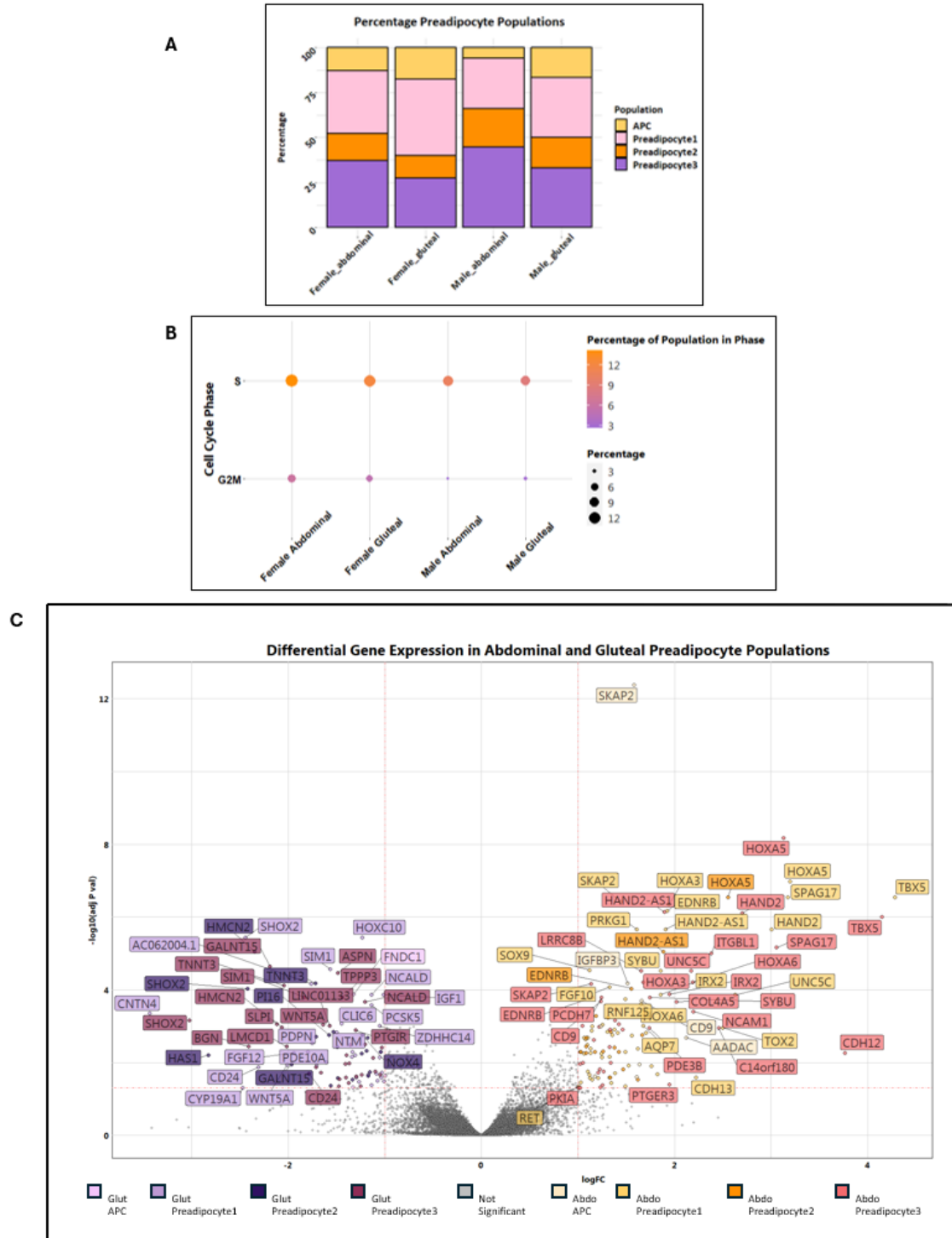


Figure 3.9: **A)** Preadipocyte population proportion abundances, calculated within each depot, and then compared across sex-divided depots. Percentage abundance of each preadipocyte population is calculated relative to the entire preadipocyte pool in a given depot. **B)** Cell cycle score analysis, calculating the proportion of preadipocytes from each depot, in each stage of the cell cycle. Values are shown for G2M and S phases, with the remainder of cells being in the G1 phase. Values are presented as an average across the pooled preadipocyte populations in each depot. **C)** Volcano plot, stratified by preadipocyte populations, showing differentially expressed genes between abdominal and gluteal preadipocytes. Log fold change (logFC) is presented on the x-axis, and $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Labels are coloured by the cluster in which they are differentially expressed, and also by which depot they are differentially expressed in.

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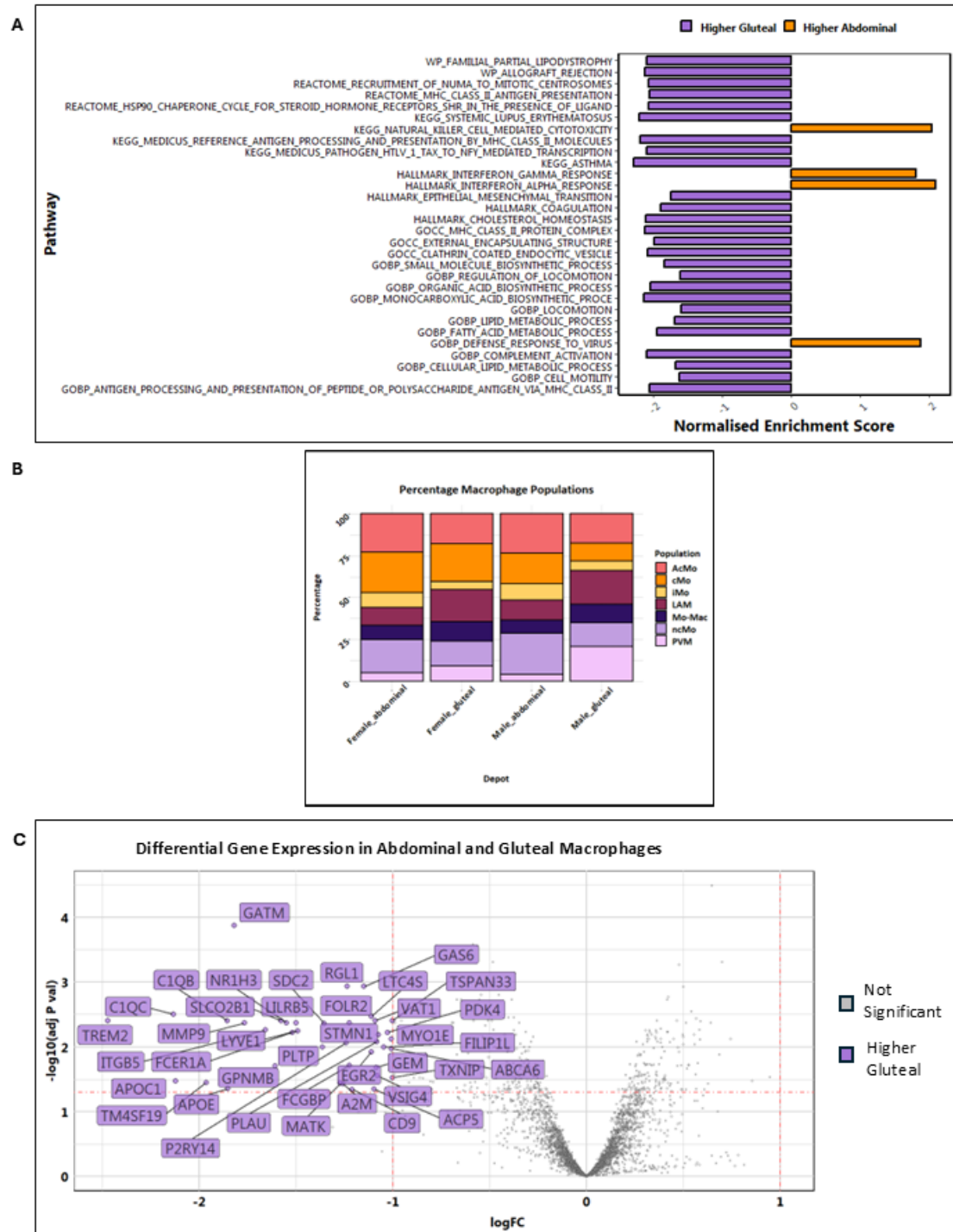


Figure 3.10: **A)** GSEA-based enrichment analyses, showing depot-differences in pathway enrichment, in macrophages. Positive enrichment score is indicative of abdominal enrichment, while negative enrichment scores are indicative of gluteal enrichment. **B)** Macrophage and monocyte-derived population abundances, calculated within each depot, and then compared across sex-divided depots. Percentage abundance of each macrophage and monocyte population is calculated relative to the entire macrophage and monocyte pool in a given depot. **C)** Volcano plot, showing differentially expressed genes between abdominal and gluteal macrophage and monocyte populations. Log fold change (logFC) is presented on the x-axis, and $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Labels are coloured by the depot in which genes are differentially expressed.

In macrophage populations, depot-specific differences in gene expression were also apparent. To better capture variations in macrophage abundance and the potential role of monocyte infiltration in shaping these differences, macrophage and monocyte populations were grouped for comparison. This approach, particularly useful given the relatively low number of macrophages, also improved the power of the dataset in the depot comparison analyses. DEGs between depots were enriched for lipid-processing pathways in gluteal populations (Figure 3.10A), characteristic of tissue-resident macrophages. Additionally, steroid hormone-related processes and MHC antigen presentation were enriched in gluteal macrophages, indicating enhanced responsiveness to steroid hormones and greater immunological activity compared to their abdominal counterparts, which is potentially of relevance in the context of oestradiol-mediated anti-inflammatory signalling. In contrast, abdominal macrophages were enriched for genes involved in cytotoxic processes, interferon production, and viral response, suggesting a predominance of inflammatory activity. Consistent with this, gluteal depots exhibited a higher proportion of anti-inflammatory macrophage clusters (e.g PVMs, LAMs) compared to abdominal depots (Figure 3.10B). Furthermore, key transcriptomic differences between abdominal and gluteal populations were associated with anti-inflammatory processes in the gluteal region, including *VSIG4*, *GAS6*, *LYVE1*, and *TREM2* (Figure 3.10C), further suggesting that these populations were polarised towards an anti-inflammatory phenotype. Genes that were differentially expressed, and upregulated in abdominal populations, did not reach the set cut off thresholds (log fold change > 1).

3.3.3 Depot-Differences in the Preadipocyte Transcriptome are Sexually Dimorphic

To further explore sex differences across preadipocyte populations, DEGs between abdominal and gluteal preadipocytes were stratified by sex. In the comparison of abdominal and gluteal preadipocytes stratified by sex, 181 ‘core’ depot DEGs were identified, while 269 DEGs were specific to males and 197 were specific to females

(Figure 3.11A). Pathway enrichment analyses indicated that the male-specific gene set was enriched for transmembrane transport and mitochondrial activity within the abdominal depot (Figure 3.11B). Volcano plots, focusing exclusively on sex-specific genes, further revealed DEGs involved in various lipid processing pathways (Figure 3.11C). Notable genes enriched in the abdominal depot included *BCHE* in females, associated with lipolysis and fatty acid utilisation [364], and *FABP5* in males, which has been linked to intra-cellular lipid accumulation [365]. In the gluteal depot, key transcripts included *CD24* in females, a gene promoting preadipocyte adipogenesis [366], and *MFAP5* in males, known to be upregulated in response to adipose tissue inflammation [367].

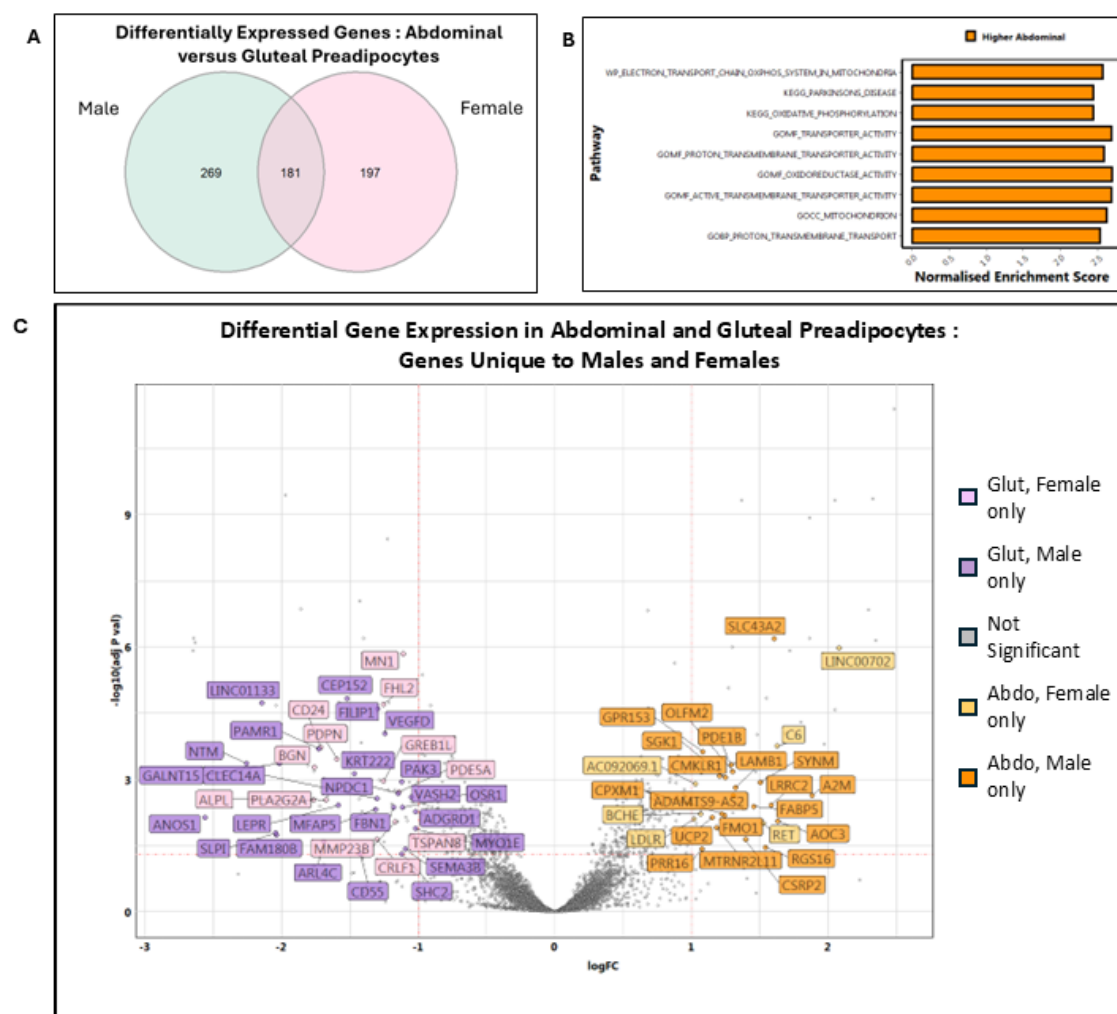


Figure 3.11: **A)** Venn diagram illustrating the number of differentially expressed genes between abdominal and gluteal preadipocytes, divided by which sex they are differentially expressed in. **B)** Pathway enrichment for genes that were uniquely differentially expressed in male abdominal versus male gluteal preadipocyte populations (genes, leftmost on the Venn diagram). No significant enrichment was evident for the female populations. **C)** Volcano plot showing differentially expressed genes between abdominal and gluteal preadipocyte populations, divided by sex. Log fold change (logFC) is presented on the x-axis, and $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Labels are coloured by the sex in which they are differentially expressed, and also by which depot they are differentially expressed in.

Gene (preadipocyte)	Sex	Depot	Type	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
LDLR	Female	Abdo	Receptor	ACTR2, ARPC5, HPSA8, LRPAP1	
CRLF1	Female	Glut	Receptor	CLCF1	
RET	Female	Abdo	Receptor	VEGFA, VEGFB	
PLA2G2A	Female	Glut	Ligand		ITGA4, ITGA5, ITGAV, ITGB1
CD24	Female	Glut	Ligand		SIGLEC10
BGN	Female	Glut	Ligand		LY96, TLR1, TLR2, TLR4
FBN1	Male	Glut	Ligand		ITGA5, ITGB1
MFAP5	Male	Glut	Ligand		NOTCH1
SLPI	Male	Glut	Ligand		CD4, PLSCR1
CD55	Male	Glut	Ligand		ADGRE2, CR1
LAMB1	Male	Abdo	Ligand		CD44, ITGA3, ITGB1
VEGFD	Male	Glut	Ligand		FLT1, ITGA4, ITGA5, ITGB1, NRP2
FABP5	Male	Abdo	Ligand		RXRA
SEMA3B	Male	Glut	Ligand		NRP1_PLXNA1, NRP1_PLXNA2, NRP1_PLXNA3, NRP2_PLXNA1, NRP2_PLXNA2, NRP2_PLXNA3
ANOS1	Male	Glut	Ligand		FGFR1, SDC2
A2M	Male	Abdo	Ligand		LRP1

Table 3.1: Interactome analyses, identifying ligand-receptor pairs that are differentially expressed between abdominal and gluteal preadipocyte populations (stratified by sex), which also have a corresponding macrophage receptor or ligand. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns.

To investigate how these DEGs may relate to intercellular interactions, preadipocyte DEGs were mapped to a filtered interactome dataset, as described in Section 3.2.3.3. Further investigation into the overlap between sex-specific depot DEGs and the identified interactome signals revealed a group of shared gene signals (Table 3.1). These therefore represent signals arising between preadipocytes and macrophages that are also differentially expressed between abdominal and gluteal preadipocytes, and specific to males or females. This transcriptomic overlap was particularly pronounced in the gluteal region, suggesting an enrichment for preadipocyte-macrophage communication in this depot. Notably, key ligand-receptor interactions included *CD24-SIGLEC10* signalling between preadipocytes and macrophages. This interaction has been shown to protect CD24-expressing cells from inflammatory clearance by macrophages [368], likely contributing to the promotion of an anti-inflammatory environment.

3.3.4 Depot-Differences in the Macrophage & Monocyte Transcriptome are Sexually Dimorphic

Similar to preadipocytes, the macrophage populations exhibited notable depot-specific differential gene expression, which was also influenced by sex (Figure 3.12A). Interestingly, the majority of differential gene expression between depots was observed in male macrophages, with males displaying almost ten times the number of DEGs as compared to females. This suggests that males exhibit greater depot-specific differences in the macrophage transcriptome. Pathway enrichment analyses in the male-specific genes (Figure 3.12B) revealed enrichment of inflammatory processes, such as interferon responses, in the abdominal depot, and an increase in anti-inflammatory processes, such as IL-4 responses, in the gluteal region, indicating a contrasting polarisation skew between these depots. Volcano plots further supported this observation, with top DEGs in male abdominal preadipocytes including interferon-regulated genes like *IFITM2* and *CD69*, while the gluteal depot showed enrichment of genes associated with anti-inflammatory and tissue-resident macrophages, such as *TREM2*, *LYVE1*, *GAS6*, and *CD163* (Figure 3.12C). These findings suggest that, in males, gluteal macrophages are more anti-inflammatory relative to their abdominal counterparts, whereas the depot contrast is less pronounced in females.

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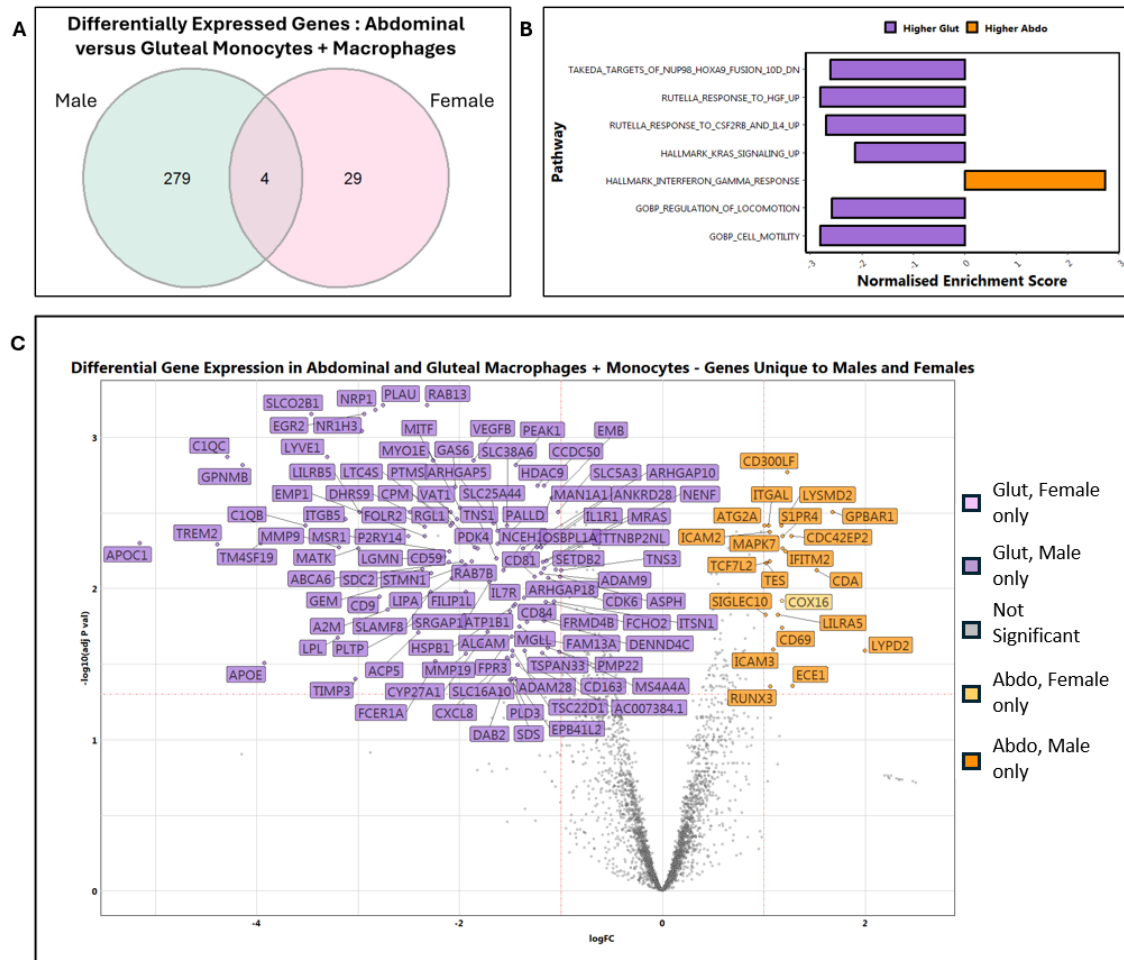


Figure 3.12: **A)** Venn diagram illustrating the number of differentially expressed genes between abdominal and gluteal macrophages, divided by which sex they are differentially expressed in. **B)** Pathway enrichment for genes that were uniquely differentially expressed in male abdominal versus male gluteal macrophage and monocyte populations (genes, leftmost on the Venn diagram). No significant enrichment was evident for the female populations. **C)** Volcano plot displaying the differentially expressed genes between abdominal and gluteal macrophage populations, divided by sex. Log fold change (logFC) in expression, comparing abdominal and gluteal macrophages, is presented on the x-axis, while $-\log(\text{adjusted } p\text{ value})$ is presented on the y-axis. Labels are coloured according to depot- and sex-specific enrichment.

Gene (macrophage)	Sex	Depot	Type	Corresponding Ligand (preadipocyte)	Corresponding Receptor (preadipocyte)
A2M	Male	Glut	Ligand		LRP1
ADAM9	Male	Glut	Ligand		ITGAV, ITGB1, ITGB5
ALCAM	Male	Glut	Ligand		CHL1, NRP1
APOE	Male	Glut	Ligand		ABCA1, LDLR, LRP1, LRP5, LRP6, SDC2
C1QB	Male	Glut	Ligand		C1QBP, LRP1
CXCL8	Male	Glut	Ligand		SDC2, SDC3
GAS6	Male	Glut	Ligand		AXL
LPL	Male	Glut	Ligand		CD44, LRP1
MMP9	Male	Glut	Ligand		CD44, IFNAR1, LRP1, RECK
PLAU	Male	Glut	Ligand		IGF2R, ITGA5, ITGB1, ITGB5, LRP1, PLAUR
PLTP	Male	Glut	Ligand		ABCA1
TIMP3	Male	Glut	Ligand		CD44
VEGFB	Male	Glut	Ligand		NRP1, RET
CD163	Male	Glut	Receptor	HMGB1, TNFSF12	
CD69	Male	Abdo	Receptor	LGALS1, MXRA5, MYL9	
CD81	Male	Glut	Receptor	C3, CD99, GPC3	
CD9	Male	Glut	Receptor	IL16	
FPR3	Male	Glut	Receptor	HEBP1	
GPXMB	Male	Glut	Receptor	ADAM10	
ITGB5	Male	Glut	Receptor	ADAM9, CCN1, COL18A1, COL4A2, EDIL3, ITGB3BP, LTBP1, LTBP3, MFGE8, PLAU, SERPINE1, TGFBI, TGFBI3, VCL	
LYVE1	Male	Glut	Receptor	VEGFC	
NRP1	Male	Glut	Receptor	ALCAM, C3, FGF2, FGF7, SEMA3E, VEGFA, VEGFB	
S1PR4	Male	Abdo	Receptor	GNAI2	
SDC2	Male	Glut	Receptor	ANGPTL4, APOE, FGF2, FN1, MDK, TGFBI, TNFSF13	
SIGLEC10	Male	Abdo	Receptor	CD24	
TREM2	Male	Glut	Receptor	ADAM10, APOE	

Table 3.2: Interactome analyses, identifying ligand-receptor pairs that are differentially expressed between abdominal and gluteal macrophage populations (stratified by sex), which also have a corresponding preadipocyte receptor or ligand. The macrophage differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on preadipocytes, are presented in the rightmost columns.

This difference was further corroborated by the interactome overlap analysis (Table 3.2). The analysis revealed macrophage interactions that promoted inflammation in abdominal cells, such as *CD69-MYL9* signalling [369], while anti-inflammatory signals were more prominent in the gluteal depot, including *CD163-HMGB1* [370] and *GAS6-AXL* interactions [371]. These findings suggest a clear dichotomy in macrophage function between the depots, with abdominal macrophages adopting a more pro-inflammatory phenotype and gluteal macrophages promoting tissue homeostasis and repair.

3.3.5 Sex-Differences in the Preadipocyte Transcriptome are Depot-Specific

Having compared transcriptomes between depots, stratified by sex, it was of further interest to directly compare transcriptomes between sexes within each depot, to elucidate the specific male-female differences in gene expression previously observed. This approach provided deeper insights into how sex-specific transcriptional differences are modulated by depot-specific contexts.

In preadipocytes, male-female differences in gene expression were more pronounced in the gluteal region (771 DEGs) compared to the abdominal region (428 DEGs) (Figure 3.13A). Enrichment analysis of the gluteal-unique gene set revealed several processes that were upregulated in female relative to male gluteal preadipocytes, including mitochondrial respiration, amino acid metabolism, and ribosomal processes (Figure 3.13B). These findings highlight the increased metabolic and protein synthesis activity in female gluteal preadipocytes. Conversely, in the abdominal depot, preadipocytes showed enrichment for DNA binding activities in males compared to females (Figure 3.13C), suggesting a differential role in gene regulation and transcriptional control between the sexes. A volcano plot showed the top DEGs between sexes within each depot (Figure 3.14), revealing several notable transcripts. In females, *SGK1*, which was specific to the gluteal depot, was identified as a key gene involved in promoting adipogenesis [372]. In contrast, *LPCAT2* emerged as a top hit for male gluteal preadipocytes and is known to enhance macrophage inflammatory responses [373]. These differential gene expression signatures underscore the functional divergence between sexes, particularly in how preadipocytes may influence local and systemic inflammation.

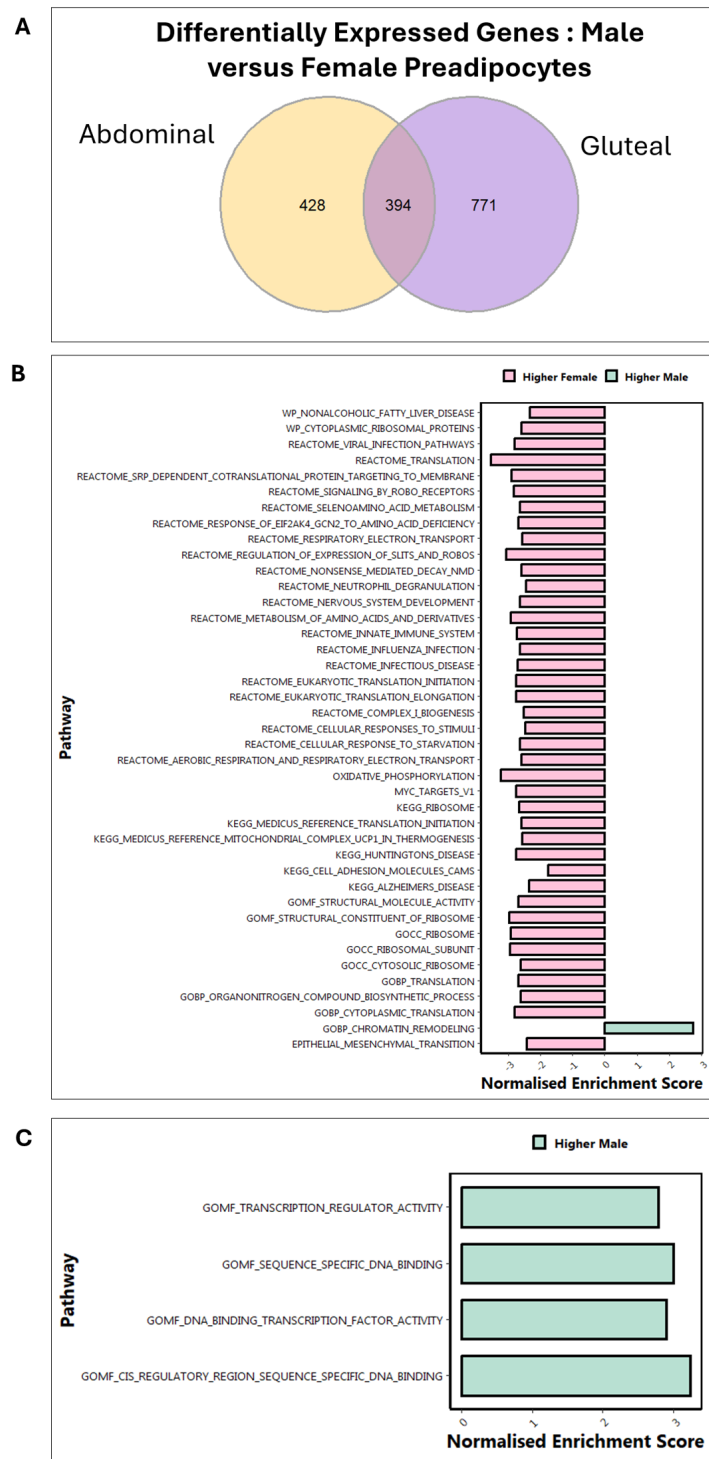


Figure 3.13: **A)** Venn diagram illustrating the number of differentially expressed genes between male and female preadipocytes, divided by which depot they are differentially expressed in. **B)** Enrichment analysis, displaying significant pathways enriched in the gluteal-specific genes that are differentially expressed between males and females. **C)** Enrichment analysis, displaying significant pathways enriched in the abdominal-specific genes that are differentially expressed between males and females.

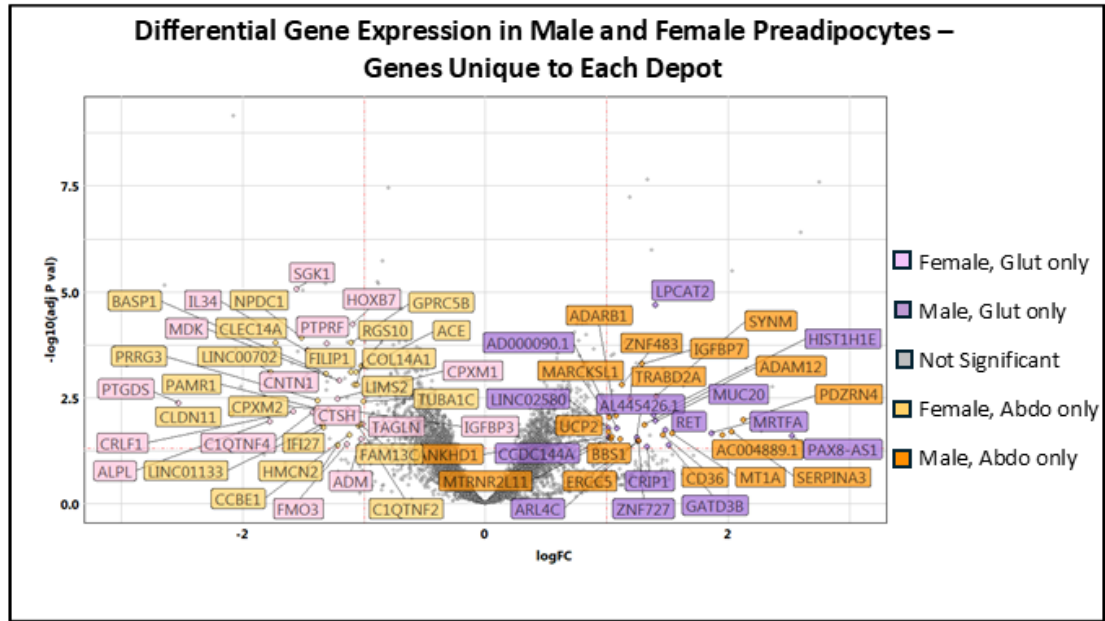


Figure 3.14: Volcano plot displaying the differential expressed genes between male and female preadipocyte populations, divided by depot. Log fold change ($\log FC$) in expression, comparing male and female preadipocytes, is presented on the x-axis, while $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Labels are coloured according to depot- and sex-specific enrichment.

Moreover, a subset of male-female DEGs that were specific to each depot included interaction signals between preadipocytes and macrophages. Notably, the majority of these interaction signals were found in the female gluteal population (Table 3.1), suggesting that this depot is highly primed for inter-cellular communication. Among the relevant signals, the *MDK-LRP1* axis was highlighted, which has been shown to induce cellular proliferation in recipient cells [374]. This finding implies that the female gluteal depot may be particularly conducive to enhanced cell signalling and interaction, potentially influencing depot-specific physiological outcomes.

Gene (preadipocyte)	Depot	Sex	Type	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
CD36	Abdo	Male	Receptor	S100A12, S100A8, S100A9, THBS1	
RET	Glut	Female	Receptor	VEGFA, VEGFB	
CRLF1	Glut	Female	Receptor	CLCF1	
COL14A1	Abdo	Female	Ligand		CD44
VEGFD	Abdo	Female	Ligand		FLT1, ITGA4, ITGA5, ITGB1, NRP2
ADAM12	Abdo	Male	Ligand		ITGB1, SDC4
ADM	Glut	Female	Ligand		ADRB2, GPR84
CNTN1	Glut	Female	Ligand		NOTCH1, NOTCH2
MDK	Glut	Female	Ligand		ITGA4, ITGB1, LRP1, NCL, NOTCH2, SDC2, SDC3, SDC4, SORL1

Table 3.3: *Interactome analyses, identifying ligand-receptor pairs that are differentially expressed between male and female preadipocyte populations (stratified by depot), which also have a corresponding macrophage receptor or ligand. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns.*

3.3.6 Sex-Differences in the Macrophage & Monocyte Transcriptome are Depot-Specific

When comparing macrophage populations between males and females, it became evident that the majority of differential gene expression was exclusive to the gluteal depot (Figure 3.15A), highlighting the distinct nature of this depot in terms of sex-specific variations. The volcano plot (Figure 3.15B) identified several key genes, including a cluster of highly differentially expressed *SEPT* genes in female gluteal macrophages. These genes encode for septin proteins, which have roles in suppressing inflammation [375], cytokinesis [376], phagosome formation [376], and also in promoting PPAR γ expression [377], likely further polarising cells towards an M2 phenotype [111] and aiding LAM formation [377]. Additionally, females showed enrichment of the *NRP1* transcript in abdominal macrophages, known for its role in macrophage-mediated protection against metabolic syndrome in adipose tissue [378]. In contrast, male macrophages in the gluteal region exhibited enrichment for *CIAO2A/FAM96A*, a gene involved in iron regulation. Knockdown of this transcript has been shown to promote M2-like macrophage polarisation, suggesting that its increased expression in male cells could contribute to a shift away from an anti-inflammatory phenotype [379]. Further analysis of the interactome data revealed a limited set of differentially expressed ligands and receptors (Table 3.4). Notably, *TNFSF13*, a member of the TNF superfamily found in male gluteal

macrophages, was identified as a significant ligand. TNFSF13 can bind to FAS on preadipocytes, which is involved in inducing apoptosis and can also activate NF κ B signalling to promote inflammation [380]. This interaction may lead to reduced survival of male gluteal preadipocytes, potentially contributing to a diminished expansion capacity of this depot.

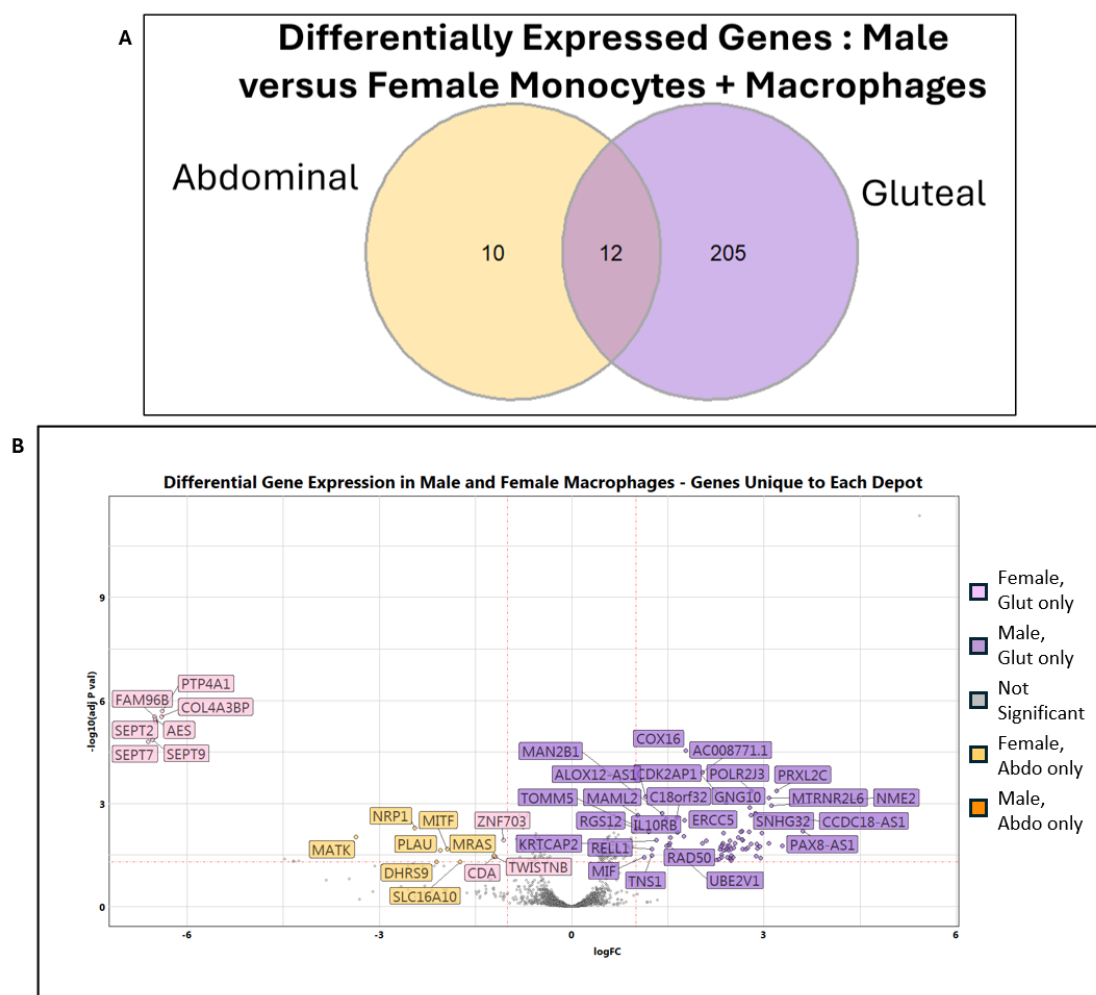


Figure 3.15: **A)** Venn diagram illustrating the number of differentially expressed genes between male and female macrophages, divided by which depot they are differentially expressed in. **B)** Volcano plot displaying the differential expressed genes between male and female macrophage and monocyte populations, divided by depot. Log fold change (logFC) in expression, comparing male and female macrophages, is presented on the x-axis, while $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Labels are coloured according to depot- and sex-specific enrichment.

Gene (macrophage)	Depot	Sex	Type	Corresponding Ligand (preadipocyte)	Corresponding Receptor (preadipocyte)
PLAU	Abdo	Female	Ligand		IGF2R, ITGA5, ITGB1, ITGB5, LRP1, PLAUR
MIF	Glut	Male	Ligand		ACKR3, CD44, CD74, EGFR, TNFRSF14,
TNFSF13	Glut	Male	Ligand		FAS, SDC2, TNFRSF14, TNFRSF1A
MAML2	Glut	Male	Ligand		NOTCH2, NOTCH3
NRP1	Abdo	Female	Receptor	ALCAM, C3, FGF2, FGF7, SEMA3E, VEGFA, VEGFB	

Table 3.4: Interactome analyses, identifying ligand-receptor pairs that are differentially expressed between male and female macrophage populations (stratified by depot), which also have a corresponding preadipocyte receptor or ligand. The macrophage differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on preadipocytes, are presented in the rightmost columns.

3.4 Discussion

Overall, this work offers an in-depth analysis of cellular and transcriptomic differences in the SVF of abdominal and gluteal adipose tissues, with a particular focus on preadipocyte and macrophage populations, as well as the hypothesised signalling interactions between them. Distinct depot- and sex-specific signatures were evident, revealing characteristics unique to the female gluteal depot, which had not previously been studied using scRNASeq. These findings may contribute to explaining this depot’s ability to expand adaptively in response to nutritional demands without increasing metabolic risk.

This work reveals that the female gluteal depot is characterised by a higher prevalence of less mature preadipocyte populations, such as APCs and Preadipocyte1, compared to the abdominal depot. This suggests that the gluteal depot maintains a larger reservoir of cells capable of further differentiation and expansion. In contrast, abdominal preadipocytes, were enriched for the more mature preadipocyte clusters and therefore potentially less adaptable. This depot-specific difference in preadipocyte maturation and abundance may contribute to the gluteal depot’s ability to accommodate increased fat without corresponding metabolic risks. These effects are likely augmented by differences in cellular proliferation, as reflected by the cell cycle score for mitotic phases in female preadipocytes. This was indicative of increased turnover rates in female cells, relative to their male equivalents. Given

the greater proportion of immature cells and increased proliferation in the female gluteal depot, this depot may be primed for healthy expansion, possessing a greater capacity for adaptive growth compared to the other sex-specific depots.

Transcriptomic analysis of preadipocytes supported this hypothesis, showing enrichment of adipogenesis-related genes such as *SGK1* in female preadipocytes, while male preadipocytes exhibited top hits associated with inflammation (e.g. *LPCAT2*). This suggests that female gluteal preadipocytes may promote fat expansion through enhanced differentiation, whereas male preadipocytes may be predisposed to creating a more inflammatory environment.

An intriguing finding was the significantly higher expression of *CYP19A1* (the aromatase enzyme) in gluteal preadipocytes compared to abdominal preadipocytes, suggesting increased local oestradiol production in the gluteal region. Interestingly, *CYP19A1* did not feature as a top hit in the sex-stratified analysis, indicating that its elevated expression in the gluteal depot likely contributes to oestradiol production in both male and female adipose tissues. However, the extent to which aromatase enzyme activity influences both local and systemic oestradiol concentrations remains unclear. In male mice, enhanced aromatase activity within adipose tissue has been shown to increase intra-tissue oestradiol levels without increasing circulating levels of oestradiol. Increased adipose tissue aromatase activity also resulted in reduced overall adiposity, improved insulin sensitivity and preadipocyte differentiation, and decreased infiltration of inflammatory macrophages [381]. These findings suggest that aromatase activity in adipose tissue has a profound capacity to regulate intra-tissue oestradiol concentrations, with downstream effects on tissue expansion and macrophage function.

The finding of increased gluteal *CYP19A1* expression, is particularly compelling given oestradiol's well-established role in promoting an anti-inflammatory macrophage phenotype [212]. Enhanced oestradiol production by preadipocytes in the gluteal depot could help explain the reduced inflammatory signature observed in this region relative to the abdominal depot, across both sexes, and may contribute to the increased stem-like signature that was observed in gluteal preadipocytes, which

showed elevated expression of stem-related genes such as *PI16* [348]. Macrophage data supported these findings. Gluteal macrophages harboured higher proportions of anti-inflammatory populations compared to the abdominal depot, and there was enrichment of genes related to lipid processing, steroid hormone responsiveness, and MHC antigen presentation, further pointing to M2-like phenotypes. This was suggestive of an enhanced oestradiol response capacity, arising alongside increased anti-inflammatory signatures, in a depot with an increased capacity to produce oestradiol. Thus, the gluteal macrophage population may exhibit an anti-inflammatory phenotype in response to oestradiol-primed polarisation, mediated by an enhanced production of oestradiol by gluteal preadipocytes. These anti-inflammatory populations may, in turn support local cellular proliferation [89, 382], thereby supporting healthy tissue expansion. This contrasts with abdominal macrophages, which showed a greater propensity for inflammatory processes, including interferon responses and viral defense mechanisms, which may skew this population towards an M1-like signature. Thus, this interplay between local hormone production and macrophage function may contribute to the unique metabolic environment of the gluteal depot. Further research is required to unravel how these molecular signatures contribute to the depot-specific function of adipose tissue, particularly in the context of tissue expansion and metabolic health.

The integration of interactome analysis with DEG sets provided further novel insights into the bidirectional communications between macrophages and preadipocytes, which may also be contributing to depot- and sex-specific differences in tissue expansion and inflammation. In the gluteal depot, there was substantial overlap in sex-specific DEGs related to preadipocyte-macrophage signalling, making this depot a prime focus for future research. Several targets have been identified, which should be investigated in future work. Notable targets include the CD24-SIGLEC10 axis in female gluteal tissue, which may facilitate a protective anti-inflammatory environment to support tissue expansion. Although not studied in adipose tissue, CD24 expression may increase in response to oestradiol [383, 384], thereby promoting the observed anti-inflammatory signalling following elevated

local oestradiol production by the aromatase enzyme. In the context of cancer, CD24-SIGLEC10 signalling between breast cancer cells and tumour-associated macrophages, has been found to dampen innate immune responses, creating an environment which is permissive of immune evasion and cellular proliferation [385]. Although deleterious in the context of cancer, this signalling may be permissive of anti-inflammatory hyperplastic expansion in the context of adipose tissue. In males, interactions such as TNFSF13-FAS may contribute to reduced preadipocyte survival, impacting depot expansion and function.

Overall, the observed differences in macrophage and preadipocyte populations between abdominal and gluteal depots, as well as between sexes, emphasise the importance of depot-specific microenvironments in fat distribution and metabolic health. The gluteal depot's ability to house a higher proportion of anti-inflammatory macrophages and less mature preadipocytes may contribute to its capacity for adaptive expansion without negative metabolic consequences. This may be further influenced by the heightened production of oestradiol in this depot, polarising macrophages towards this anti-inflammatory phenotype, to further support hyperplastic tissue expansion. In contrast, the inflammatory profiles and more mature preadipocytes in the abdominal depot could underlie the increased metabolic risks associated with abdominal obesity.

4

Assessing Adipose Tissue Oestrogen Responses

4.1 Introduction

Depot-specific differences in the stromal vascular fraction (SVF) landscape, as reported in the previous chapter, were indicative of differences in oestradiol production and responsiveness between abdominal and gluteal adipose tissue depots. This highlighted the elevated expression of aromatase (*CYP19A1*), the key player in oestradiol production, in gluteal preadipocytes, relative to abdominal preadipocytes. Oestradiol-responsive signalling targets were further identified in gluteal preadipocytes, as well as broad anti-inflammatory pathways in gluteal macrophage populations, potentially linked to oestradiol's known anti-inflammatory effects [386]. Given the importance of oestradiol in promoting the female profile of fat distribution, alongside these previous results highlighting the potential differences in oestradiol responses between depots, it was pertinent to directly examine these depot-specific oestradiol responses. This focus was particularly relevant in light of the conflicting literature regarding the effects of oestradiol on preadipocyte proliferation and differentiation, as discussed in Section 1.3.

4.1.1 Differing Forms of Oestrogens and Oestrogen Receptors Dictate Tissue Hormone Responses

Oestradiol is the primary form of oestrogen in humans, being the most active oestrogen, and also the most abundant form in pre-menopausal women. Its concentrations fluctuate significantly throughout the menstrual cycle, with a range of 20-750 pg.mL⁻¹, dropping to 10-20 pg.mL⁻¹ post-menopause [387], which is similar to concentrations found in males. Oestrone, a weaker oestrogen, circulates at levels of 50-100 pg.mL⁻¹ [388]. While this range remains relatively stable post-menopause [389], as oestradiol levels decrease, oestrone becomes the dominant circulating oestrogen. In males, oestrone levels are lower, typically under 20 pg.mL⁻¹. Oestriol, the weakest and least abundant oestrogen of the three, is predominantly present during pregnancy due to its placental production.

The aforementioned ‘potency’ of each form of oestrogen is defined by their differential binding affinities and actions on oestrogen receptors (ERs), namely ER- α , ER- β and GPER1. ER- α and ER- β mediate classical genomic signalling, initiating the transcriptional responses to oestradiol. In contrast, GPER1 primarily mediates non-genomic signalling, resulting in more rapid cellular responses [390].

When oestradiol binds to ER- α or ER- β , either in the cytoplasm or at the cell membrane, the receptor undergoes a conformational change, facilitating its dimerisation. These dimers can form either homo- or hetero- dimers, which translocate to the nucleus and bind to Estrogen Response Elements (EREs) on DNA, to directly influence transcription. This genomic response pathway can regulate gene expression involved in key processes such as cell proliferation, differentiation, and metabolism. These are the primary activities of ER- α and ER- β , although they both have additional actions through non-genomic signalling mechanisms. At the membrane, ER- α and ER- β can interact with other membrane-bound receptors to initiate rapid intra-cellular signalling such as MAPK/ERK and PI3K/AKT cascades, subsequently influencing processes such as cell survival, proliferation and metabolic regulation [390].

The balance between expression and activation of ER- α and ER- β is traditionally

considered to be important for the output effects of oestradiol, with these receptors having been proposed to exert opposing effects in certain cell types [391, 392]. Thus, it was necessary to examine receptor expression profiles, and their relative ratios, to determine the overall physiological effect of oestradiol in adipose tissue.

On the other hand, GPER1 (formerly known as GPR30), primarily mediates the rapid, non-genomic actions of oestradiol, through membrane receptor signalling. GPER1 is located at cell membranes, mediating rapid responses to oestradiol through G-protein-coupled pathways, activating second messengers such as phospholipase C and protein kinase A. This rapid signalling is important for acute cellular responses, such as changes in ion transport and enzyme activity.

Additionally, oestrogen-related receptors (ERRs), although not directly activated by oestradiol, can play a role in modulating oestrogen-mediated signalling. Being structurally similar to classic ERs, ERRs can interact with ER co-activators, potentially enhancing the effects of their signalling, or competing with them to bind transcriptional regulators. Thus, the transcriptional output of oestradiol may also depend on the activities of ERRs, and their ability to modulate classical ER signalling.

4.1.2 The Actions of Oestradiol in Adipose Tissue

Adipose tissue is a source and target of oestradiol, due to its expression of both aromatase and ERs, respectively. Aromatase is the enzyme which can convert dihydroepiandrosterone to estrone, and testosterone to oestradiol. Estrone can subsequently be converted to oestradiol through 17 β -Hydroxysteroid dehydrogenase (17 β HSD) activity, allowing adipose tissue to synthesise oestradiol via multiple pathways, and contribute to circulating plasma oestradiol levels. In pre-menopausal women, this contributes to a minority of total circulating oestradiol due to the dominance of ovarian oestradiol production. Meanwhile, in post-menopausal women, aromatase-mediated synthesis of oestradiol becomes the primary source of circulating hormone. Still, publications investigating adipose tissue aromatase activity have largely focused on how hormone production contributes to systemic oestradiol levels, without establishing the intra-tissue effects of hormone production; whether

aromatase activity significantly contributes to local oestradiol production, and how this subsequently influences adipose tissue metabolism.

Primary adipose tissue biopsy samples are reported to express the three primary ERs [390, 393–399], although not all literature has reported this full complement of receptors, suggesting that sample variation factors, such as donor sex and depot of origin, may contribute to the differential expression of ERs, likely influencing subsequent tissue responses [397]. This is further confounded by the lack of research into GPER1-mediated oestradiol signalling, specifically in adipose tissue, which is largely due to the more recent discovery of this receptor.

In-vivo, oestradiol profoundly affects adipose tissue biology. In humans, this is exemplified by the reductions in plasma oestradiol seen at the menopause, which occur alongside an increased prevalence of obesity and metabolic disease [400–402], which can be partly prevented using oestradiol replacement therapy in the form of hormone replacement therapy (HRT) [403]. With HRT, women are more insulin sensitive and less likely to develop type 2 diabetes mellitus (T2DM) [403]. These effects are thought to be partly mediated by the effects of oestradiol on the retention of lower-body subcutaneous adiposity, and reductions in central obesity, promoting a metabolically safe adipose tissue storage signature [2]. This oestradiol-driven fat storage signature contributes to the reduced risk of metabolic disorders in pre-menopausal women. These differences in fat distribution and metabolism between males and females, as a product of variations in oestradiol levels, curtail in pre-pubescent and post-menopausal individuals, underscoring the importance of oestradiol in mediating sex-specific metabolic regulation.

Still, the mechanisms dictating these in-vivo responses are unclear. Oestradiol evidently has opposing activities on abdominal and gluteal adipose tissues, likely having a context-dependent effect, which is scarcely reported in the current literature. Studies have reported the effects of oestradiol to promote lipolysis and fatty acid oxidation in-vivo, which was shown, for example by Eon et al, who compared the effects of oestradiol and vehicle treatment in ovariectomised mice [404]. Oestradiol treatment was associated with increased fatty acid oxidation, leanness and reduced

adipocyte size. The periovarian adipose tissue (a visceral depot) showed significant changes in depot weight, but the subcutaneous adipose tissue did not, further suggesting a context-dependent activity of oestradiol in-vivo, which has not yet been addressed. Murine studies are further complicated by the lack of depot translatability to humans, additionally highlighting the need for investigations of the depot-specific actions of oestradiol in humans. Although this dichotomy of effects of oestradiol on human adipose tissue depots has been acknowledged [405], there is scarce research attempting to explain the biology behind these differences. The relative expression of ER- α and ER- β has been suggested to play a role in mediating the depot-differential effects of oestradiol, but there is little functional evidence to support this hypothesis thusfar [405].

4.1.3 In-Vitro Studies Highlight Controversies Surrounding the Effects of Oestradiol in Adipose Tissues

In-vitro studies have also demonstrated the expression of ER- α , ER- β (transcript names *ESR1* and *ESR2*, respectively) and GPER1 in adipocytes [406, 407], however, not all studies consistently report the presence of these receptors, likely due to cellular variations, such as whether the cells were immortalised, whether they were male or female, their depot of origin, the degree to which the preadipocytes were differentiated, and their species of origin. The lack of standardisation of these factors across studies has complicated the interpretation of ER expression in adipocytes, likely contributing to the controversies surrounding oestradiol responses in adipose tissue.

Despite the general consensus of the presence of ERs in adipose tissue, its biological effects on in-vitro adipocyte cultures are highly contested. Studies have highlighted both pro- and anti- adipogenic effects of oestradiol (detailed more in Section 1.3), reporting on its effects at key adipogenic transcription factors including PPARG and CEBPA, which promote cellular differentiation, as well as effects on preadipocyte proliferation pathways. As eluded to in Section 1.3, these contradictions may relate to both cellular factors, as well as experimental specificities, such as the

timing and concentration of oestradiol treatments. To further complicate these interpretations, studies have typically focused on a singular, indirect measure of (monocultured) preadipocyte oestradiol response (e.g proliferation, transcriptional responses, adipogenesis), without prior direct assessment of ERE binding activity following oestradiol treatment. Such dose-response studies using reporter assays would allow the optimisation of concentrations used to elicit oestradiol responses in-vitro. Still, the in-vivo depot-specific adipose tissue concentrations of oestradiol have not yet been investigated, meaning that a standardised, physiologically relevant system that mimics in-vivo oestradiol concentrations, cannot yet be developed.

4.1.4 Chapter Aims

1. Determine ER expression profiles in abdominal and gluteal adipose tissues, as well as in depot-specific MBDFAT cell lines, to establish whether they are appropriate for investigating oestrogen responses in the proposed model system.
2. Investigate whether ER expression differs between depots, and preadipocytes as compared to mature adipocytes.
3. Establish whether differential expression of *CYP19A1* in abdominal and gluteal preadipocytes translates to quantifiable differences in oestradiol production and metabolism.
4. Explore the downstream effects of depot-specific oestradiol treatment in preadipocytes, investigating effects that relate to tissue expansion (e.g proliferation), as well as transcriptional responses.

4.2 Materials, Methods and Development

4.2.1 Oestradiol treatment - Optimisation Strategies

4.2.1.1 Oestradiol Vehicle and Storage

To examine the effects of oestradiol (17β -oestradiol) on preadipocyte and adipocyte cultures, cells were treated with oestradiol under tightly controlled conditions.

Prior to treatment, several factors were considered to ensure that the biological activity of oestradiol in culture media was preserved. First, the storage conditions for oestradiol were reassessed. Initially, oestradiol dissolved in DMSO (ApexBio, A8425-APE) was stored in the provided plastic tube, at -20°C , as per standard manufacturer recommendations. However, this protocol required repeated freeze-thaw cycles due to the high freezing point of DMSO. Additionally, steroid hormones, including oestradiol, are known to adsorb to plastic surfaces [408], potentially reducing the effective concentration of hormone being pipetted from the original container. To mitigate these concerns, powdered oestradiol (E2758, Sigma-Aldrich) was dissolved in ethanol, eliminating the need for freeze-thaw cycles, and stored in a glass container at -20°C , a method commonly considered optimal for the preservation of steroid hormones.

4.2.1.2 Oestradiol Background Levels in Media

Another key consideration was the potential background concentration of oestradiol or other oestrogenic compounds in the cell culture media, which could contaminate vehicle control media and confound the observed effects of oestradiol treatment. The primary sources of these oestrogenic compounds were phenol-red and foetal bovine serum (FBS). To eliminate the oestrogenic effects of phenol-red [409, 410], phenol-red-free media was utilised for all experiments involving oestradiol. Although FBS is also known to contain oestradiol, the specific concentration for the batch in use was initially unknown. Charcoal stripping is a common method employed to deplete oestradiol from FBS; however this procedure also removes essential growth factors, vitamins and metabolites, which are vital for cell culture. Consequently, it was necessary to quantify the oestradiol levels in both untreated and charcoal-stripped FBS, to determine the necessity of charcoal stripping.

For the charcoal-stripping process, 1L of FBS was incubated (with rocking) with 20g of dextran-coated charcoal (Sigma, C6241), at 4°C for 24 hours, following established protocols designed to reduce oestradiol concentrations to sub-detectable levels [411]. The mixture was subsequently filtered through $45\mu\text{m}$ filters to remove

charcoal residues, aliquoted into 50mL Falcon tubes, and stored at -80°C . Both charcoal-stripped and unstripped FBS from the same batch, were sent to Oxford University Hospitals' Biochemistry Department, for oestradiol quantification via indirect competitive immunoassays. The results indicated that the charcoal-stripped FBS contained 55pmol.L^{-1} of oestradiol, compared to 90pmol.L^{-1} in the unstripped FBS, representing a 39% reduction. Given that FBS was used at a 1:10 dilution in culture media, the background oestradiol concentrations were deemed physiologically insignificant (9pmol.L^{-1} in unstripped and 5.5pmol.L^{-1} in stripped FBS), based on standard reference ranges [412]. This was further confirmed through liquid chromatography tandem mass spectrometry (LCMS), performed by Manchester University's Division of Laboratory Medicine, which measured total media oestradiol concentrations as below detection limits, at $< 3\text{pmol.L}^{-1}$ (0.816pg.mL^{-1}). Considering that the experiments employed supraphysiological concentrations of oestradiol (1-100nM), this minimal background concentration was unlikely to affect the results. In fact, lot to lot variability may present a greater challenge for hormone response consistency, than the choice between stripped and unstripped FBS [411]. Therefore, unstripped FBS was ultimately selected for use, as it preserves the serum components essential for adipocyte function [413]. The same FBS lot number was used for all experiments, to prevent batch-to-batch variability effects.

4.2.1.3 Background Levels of Other Relevant Oestrogens

In addition to oestradiol, other oestrogens, primarily oestrone and oestriol, are present in FBS and human plasma, and therefore should be considered in this context. However, these hormones are less abundant in plasma and are considerably less potent, as compared to oestradiol, which is why they are rarely investigated when investigating oestradiol activity in culture. Oestrone binds to ER- α and ER- β with 10- and 50- fold lower affinity, respectively, than oestradiol, while oestriol has 10- and 333- fold lower binding affinity for these receptors [414]. Literature suggests that oestrone concentrations in unstripped, neat FBS can reach up to 12pmol.mL^{-1} (3244pg.mL^{-1}), a level comparable to the quantified oestradiol concentrations in

this study [411]. To assess the background levels of oestrogens in the cell culture media, supernatants were taken from abdominal and gluteal, preadipocyte and differentiated preadipocyte MBDFAT cell cultures. These cells were incubated with media for 48 hours, with or without oestradiol treatment. Alongside these samples, untreated control media, was sent for analysis. For each condition a full oestradiol metabolite panel was quantified.

Measuring oestrogens in cell media is challenging due to difficulties with ionisation and the typically low concentrations that are present. However, specialised extraction techniques combined with innovative IDMS (Isotope Dilution Mass Spectrometry) methods developed at the Birmingham University Institute of Metabolism and Systems Research are addressing these challenges. To ensure accurate measurements, phenol-red-free media was used for analysis. In the growth media containing FBS, oestrone levels were determined to be 0.266pmol.mL^{-1} . Although internal standards were not available for oestriol, values were estimated based on peak area. However, oestriol co-elutes with $11\beta\text{O}$ -hydroxyestradiol ($11\beta\text{OHE}_2$), making it difficult to differentiate between the two compounds. The peak areas for oestriol were notably low, substantially lower than those for oestrone and oestradiol. Given that the concentrations of oestrone and oestriol were extremely low in the growth media, and considering that both exhibit significantly weaker oestrogenic activity at ER- α and ER- β compared to oestradiol (the most potent and abundant oestrogen) [414], the presence of these compounds was not considered a concern for background oestrogenic effects.

4.2.1.4 Oestradiol Stability in Culture Media

The stability of oestradiol in these culture systems could also be assessed with aforementioned media analysis (Section 4.2.1.3), using cell culture supernatants. MBDFAT preadipocytes were incubated with media, as described in Section 4.2.3, as part of a larger experiment to assess oestradiol metabolism. Given that media changes were being conducted at 48-hour intervals for cell maintenance, the oestradiol concentration in cell media after 48 hours of culture was measured,

to ensure that it was stably present. The baseline oestradiol level in oestradiol-treated growth media was 12.24ng.mL^{-1} , and after 48 hours of culture, MBDFAT preadipocyte media supernatant concentrations were 12.12ng.mL^{-1} and 12.40ng.mL^{-1} for abdominal and gluteal preadipocyte respectively. This confirmed that oestradiol remained stable in this culture system for the intended duration.

4.2.1.5 Oestradiol Bioactivity in Culture Media

To verify the bioactivity of oestradiol in-vitro in the specific media system being used, a reporter assay, as described in Section 4.2.7.1, was conducted. This allowed the application of oestradiol to culture media, and assessment of subsequent activity of the ERE. Using MCF7 cells (a breast cancer cell line which is highly responsive to oestrogen) as a positive control, it was possible to confirm that oestradiol was bioactive in culture media.

4.2.2 Assessing Oestrogen Receptor Expression in Cell Culture Models

To determine ER expression, Western blots, immunofluorescent staining and qPCR methods were employed, following standard cell culture methods, as detailed in Section 2.2. For Western blotting and gene expression data, MBDFAT preadipocytes were plated at a seeding density of 200,000 cells per well, in a 6-well plate, and cultured for 48 hours, before harvesting in the appropriate lysis agent. Further details on these protocols are available in Section 2.11 (for Western blots) and Section 2.12 (for qPCR). Immunofluorescent staining was conducted at the equivalent relative seeding density, plating cells in 24-well plates containing a collagen-coated coverslip. Preadipocytes were cultured for 48 hours before fixation and staining, as detailed in Section 2.13.

4.2.2.1 Antibody Selection Optimisation

Antibodies for ER- α and ER- β are primarily tested and validated in breast cancer cell lines, such as MCF7 cells, where expression is particularly high. However, this can be problematic for cell types where expression may be lower, or different isoforms

may be present. Western blotting was therefore used to validate the specificity of commercially available antibodies, where literature was not available. Antibodies were tested in MBDFAT preadipocytes, differentiated MBDFAT preadipocytes, and MCF7 breast cancer cells. Details on the antibody dilutions, clones and origins for the products used in Western blotting are provided in Appendix A.3.

For ER- α , antibodies from ThermoFisher (PA1309 ; PA1308) did not produce a band at the expected molecular weight (Figure 4.1), while the GeneTex antibody GTX127978 resulted in a clear band at approximately 66kDa. Both denaturing and non-denaturing conditions were tested. This was necessary since some receptor was expected to be membrane-localised. Membrane-localised proteins have hydrophobic regions, which can aggregate under denaturing conditions, impairing their separation through SDS-PAGE blotting, and impairing protein band visualisation [415]. Stripping membrane receptors of their post-translational modifications with denaturing conditions can also impair antibody binding. Furthermore, membrane receptors have complex tertiary and quaternary structures, which can misfold under denaturing conditions, making it difficult for antibodies to recognise conformational epitopes. Using non-denaturing conditions improved the performance of PA1309 and PA1308 antibodies. The GTX127978 antibody produced a band under standard denaturing conditions, and thus non-denaturing conditions were not tested.

For ER- β , antibodies tested included PA1-311 (ThermoFisher), 14-9336-80 (ThermoFisher), MA524807 monoclonal (clone PPZ0506, ThermoFisher) and GTX134663 (GeneTex). A band for ER- β was anticipated at 55kDa. Antibodies PA1-311, 14-9336-80 and GTX134663 all produced bands at unexpected molecular weights, which did not match known isoforms or receptor dimerisation weights. Literature suggests that non-specific binding is a common issue for the performance of ER- β antibodies, advising that the validated monoclonal antibody clone PPZ0506 is selected for use [416]. This antibody did not produce bands in MBDFAT preadipocytes, confirming that previous banding was likely off-target, although a positive control was not used to confirm the specificity of this validated antibody.

For GPER1, antibodies AF5534-SP (BioTechne), NLS4271 (BioTechne) and GTX107748

(GeneTex) were tested across various cell types, including MCF7 cells, macrophages, MBDFAT preadipocytes and differentiated MBDFAT preadipocytes, but none produced the expected band at 42-44kDa. The GTX107748 antibody produced bands at approximately 50 and 63kDa (Figure 4.1). While this doesn't correspond to the expected isoform at 44kDa, a 60kDa isoform has been reported elsewhere [417]. Although the 60kDa isoform is normally of a lower proportion as compared to the 44kDa isoform, this larger isoform may be more prevalent in adipose tissue, where isoform expression has not been extensively characterised.

For CYP19A1, several antibodies were tested, comprising of NB100-1596SS (BioTechne), ab18995 (Abcam), GTX88826 (GeneTex) and MCA2077S (BioRad). The expected molecular weight approximates at 58kDa, although in adipose tissue a band at 51kDa has been observed [418]. The majority of tested antibodies failed to produce banding, despite total protein being imaged on each blot. The ab18995 antibody produced bands at approximately 50kDa and 30kDa, which may reflect specific binding. Although not yet documented in adipose tissue, UniProt data suggests that isoforms H0YLP1, H0YNJ7 and E7EPL6 approximate to 30kDa. Thus, a reliable CYP19A1 antibody could not be validated for use in MBDFAT cells, despite evidence for RNA expression of this enzyme using bulk RNA sequencing (described in Section 4.3.6).

4.2.2.2 Immunofluorescent Staining and Optimisation

The immunofluorescent staining protocol, detailed in Section 2.13, was followed for visualising ER antibody staining. The antibodies utilised for staining, following Western blot validation, alongside their paired secondary antibody, and their dilutions, are shown in Table 4.1.

Protein Target	Primary Antibody				Corresponding Secondary Antibody			
	ID, Supplier	Clone	Host, Isotype	Dilution for immunofluorescence	ID, Supplier	Dilution	Host, Isotype	Fluorophore
ER- α	GTX127978, GeneTex	Polyclonal	Rabbit, IgG	1:2000	A-21429, ThermoFisher	4ug/ml (1:500)	Goat, anti-rabbit, IgG	Alexa Fluor 555
ER- β	MA524807, ThermoFisher	Monoclonal, PP20506	Mouse, IgG2b	1:1000	A-21422, ThermoFisher	2ug/ml (1:1000)	Goat, anti-mouse, IgG	Alexa Fluor 555
GPER1	GTX107748, GeneTex	Polyclonal	Rabbit, IgG	1:500	A-21429, ThermoFisher	4ug/ml (1:500)	Goat, anti-rabbit, IgG	Alexa Fluor 555

Table 4.1: Validated primary antibodies and their paired secondary antibodies used in immunofluorescent staining protocols.

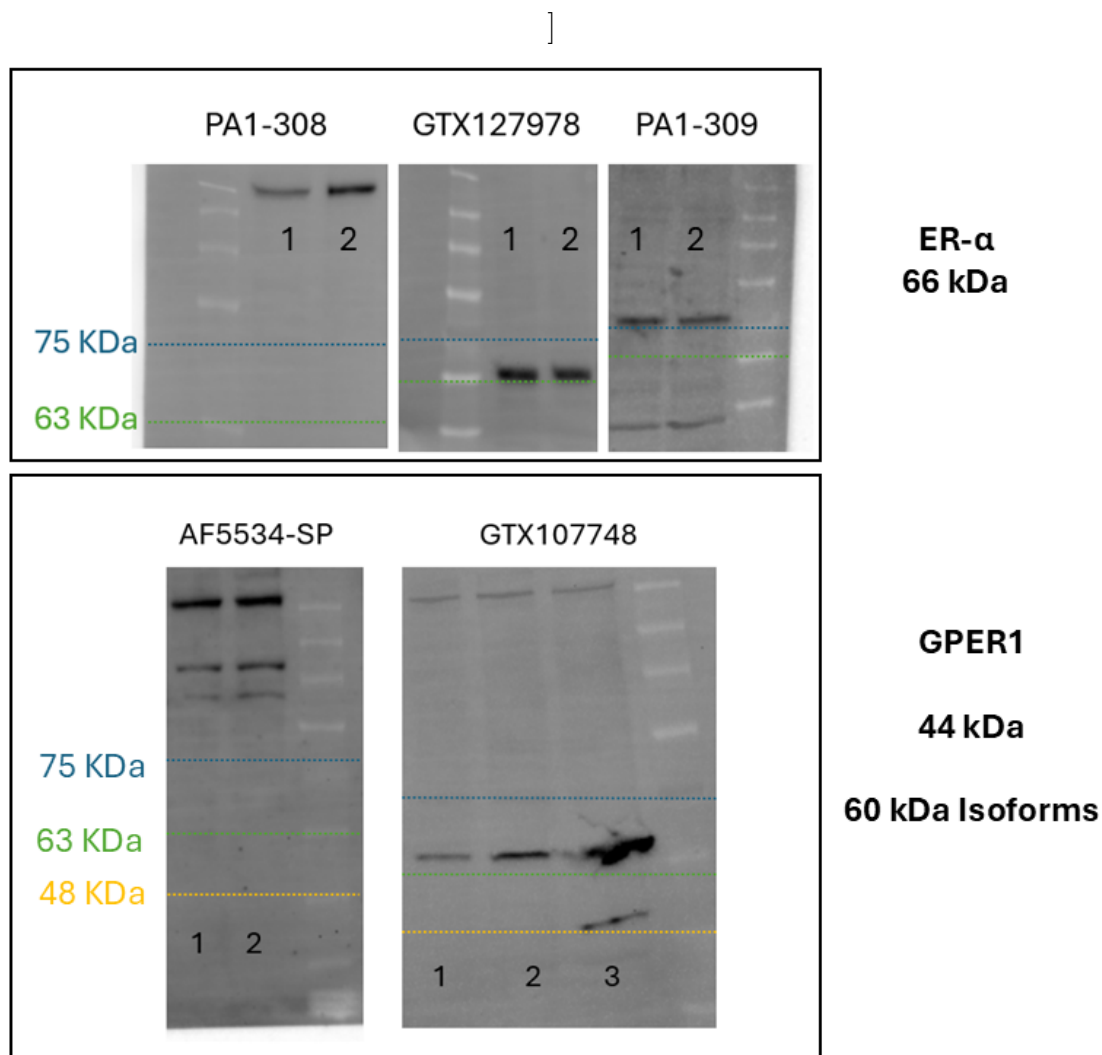


Figure 4.1: Antibody validation for $ER\alpha$ and $GPER1$, using Western blotting. GTX127978 for $ER\alpha$ and GTX107748 for $GPER1$ produced banding at expected molecular weights, where other commercially available antibodies failed to do so. Lanes are numbered, representing: 1 = abdominal MBDFAT preadipocytes, 2 = gluteal MBDFAT preadipocytes, 3 = MCF7 breast cancer cells.

Several stages of the protocol required optimisation. Staining solutions were made up into one mixture, including the secondary antibody, nuclear stain (Hoechst) and actin stain (Phalloidin). Advised incubation times differed for each component. The 20mM Hoechst 33342 nuclear stain solution (ThermoFisher, 62249) was advised to be diluted at approximately 1:10,000 and incubated at room temperature (RT) for 10 minutes. The respective values for Phalloidin (Oregon Green 488-Phalloidin, ThermoFisher, O7466) were 1:100 and 45-60 minutes RT. For secondary antibodies,

incubation time was also recommended for 45-60 minutes RT. In order to incubate for the longer period, the concentration of the Hoechst nuclear stain was reduced. Since the Hoechst stain was particularly bright when imaging, a couple of concentrations were tested with the 45-60 minute incubation period. A dilution of approximately 1:30,000 (667nM) worked well to preserve staining at the longer incubation time without bleeding into other channels.

Refinement was also made for the PBS washing steps. Particularly with the mature adipocytes, the high frequency of PBS washes often resulted in the loss and peeling of cells from coverslips. To prevent this, the number of PBS washes at each step was reduced from three to two, instead using a larger volume with each wash. Effort was also made to wash the cells particularly gently, avoiding harsh pipetting which causes shear stress. This helped cells stay adherent without increasing background signal or reducing the efficacy of washing. As well as this, the PBS solution was adjusted. Instead of using standard PBS, which is designed for lifting cells (without Ca^{2+} and Mg^{2+}) PBS containing Ca^{2+} and Mg^{2+} (ThermoFisher, 14080055) was selected for staining protocols, to optimise for cell adherence. PBS was at RT to avoid cell detachment associated with low temperatures.

Due to concerns of non-specific binding, the bovine serum albumin (BSA) concentration in the blocking solution was increased from 2% to 4% BSA, and a 0.1% BSA solution was also used in subsequent staining solutions.

4.2.2.3 Bulk RNA Sequencing of MBDFAT Preadipocytes

Bulk RNA sequencing was conducted in oestradiol- or control- treated MBDFAT preadipocytes, as part of a larger co-culture experiment. Further methodological details are specified in Section 2.20. In brief, abdominal and gluteal MBDFAT preadipocytes, were seeded at 130,000 cells in each well of a 6-well plate, to give a density of 500,000 cells per well at the end of the culture period. They were treated with 100nM oestradiol or vehicle control for 48 hours before being harvested in lysis buffer. They then underwent RNA extraction, and were sent

for sequencing. For the interest of this chapter, presented data are only from monocultured preadipocyte samples.

4.2.2.4 Single Cell RNA Sequencing

Receptor expression data across adipose tissue SVF populations, is taken from the single-cell RNA sequencing (scRNASeq) dataset detailed in Section 3.3.1.

4.2.2.5 Bulk RNA Sequencing Of Fractionated Adipose Tissue

To compare receptor expression across adipose tissue fractions (e.g SVF and mature adipocytes), scRNASeq data could not be utilised, as it included only the SVF, excluding mature adipocytes. Therefore, an alternative bulk RNAseq dataset, derived from abdominal and gluteal fractionated adipose tissue biopsies, was used to assess whether receptor expression varied between mature and SVF fractions, and between depots.

Paired abdominal and gluteal adipose tissue biopsies were taken from 10 participants, recruited from the Oxford Biobank in 2017 as part of a project investigating the function of the T2DM risk gene, KLF14 [419]. Participants were originally recruited based on the rs972283 genotype, (rs4731702 C/T is in 100% linkage disequilibrium with the ‘proxy’ SNP rs972283 A/G) encompassing individuals with AA and GG alleles. Genotype did not have a significant effect on the examined gene expression of interest (Appendix A.4). The samples were prepared by Marijana Todorčević and sequenced at the Wellcome Trust Centre for Human Genetics, Oxford. Samples from one participant were excluded from the analysis as a consequence of their outlying BMI value, and due to the male sex of the donor.

In brief, the subcutaneous abdominal and gluteal depots were sampled using standard needle biopsy techniques. Donor characteristics are provided in Table 4.2. Data for BMI ($kg.m^{-2}$) and WHR were available from baseline Biobank recruitment measurements (7-8 years prior to biopsy). For each donor, two samples per depot were generated - one for fractionation, and one for whole-tissue RNA sequencing. The biopsies were washed and collagenase treated (collagenase H from *Clostridium histolyticum*, Roche, 11074032001). For samples undergoing separation,

the collagenase digest was subsequently centrifuged at 300g for 5 minutes, separating out the following layers: oil, mature adipocytes, supernatant, SVF and red blood cells. The oil layer was first discarded, followed by the collection of the mature adipocyte layer, which was subsequently washed, and further purified from residual oil and washings through centrifugation. The supernatant was also discarded, allowing for the collection of the pelleted SVF, which was washed and re-pelleted before applying a red blood cell lysis buffer. After incubation, the sample was re-pelleted and washed again. Once isolated, each fraction was harvested in 500 μ L of TRIzolTM and stored at -80°C for subsequent RNA extraction. RNA was isolated using standard TRIzolTM extraction protocols, detailed in Section 2.12.

Donor	BMI at recruitment (kg.m^{-2})	WHR at recruitment	Age at biopsy (years)	Sex
1	24.61	0.84	39	Female
2	22.79	0.76	46	Female
3	26.69	0.75	45	Female
4	25.01	0.9	44	Female
5	22.83	0.71	42	Female
6	24.45	0.89	35	Female
7	24.39	0.77	34	Female
8	24.29	0.78	47	Female
9	23.52	0.83	45	Female
10	24.09	0.76	45	Female

Table 4.2: Table containing donor characteristics for fractionated adipose tissue biopsy samples. BMI (kg.m^{-2}) and WHR data were taken at time of recruitment to the Oxford Biobank. Age is reported relative to the date of biopsy.

Data are expressed as transcripts per million (TPM), due to the lack of raw sequencing data availability. This offers limited bioinformatic utility for differential gene expression analysis between genes, since raw library sizes are unavailable. Therefore, the data are used solely to demonstrate gene expression patterns across samples for individual genes. These results remain unpublished and have been exclusively analysed for this thesis.

4.2.3 **Analysing the Concentration of Oestradiol and its Metabolites in Cultured Media Supernatant**

To determine the concentration of oestradiol produced by MBDFAT preadipocytes and differentiated preadipocytes, 200,000 preadipocytes were plated per well in a 6-well plate, and cultured in growth media for 48 hours, after which the media supernatant was collected for analysis (for preadipocytes). For differentiated MBDFAT cells, preadipocytes were differentiated according to standard lab protocols (Section 2.3), before they were incubated with equivalent growth media to preadipocytes (with fibroblast growth factor omitted). Oestradiol cellular storage and uptake was also assessed by treating preadipocytes and differentiated MBDFAT preadipocytes with 10nM oestradiol or vehicle control for the 48-hour media-incubation period. The media concentration of oestradiol was initially measured through LCMS techniques, conducted at Manchester University's Division of Laboratory Medicine. For LCMS measurement, samples were analysed alongside a control growth media sample, although oestradiol-treated media was not analysed. For calculating the change in concentration of oestradiol in the cell culture media, the concentration of oestradiol in the control growth media was subtracted from that in the cell media supernatant samples. Relative concentrations of oestradiol per mg of protein were then calculated, following protein assays conducted on the cultured cells.

For a detailed assessment of the oestradiol metabolite panel, and an analysis of the relative oestradiol uptake and metabolism by abdominal and gluteal MBDFAT preadipocytes and adipocytes, samples were sent to the University of Birmingham Steroid Metabolome Analysis Core (SMAC), for analysis using a specially developed IDMS assay for steroid hormones. Samples included cell culture supernatant, similar to those sent to Birmingham for LCMS analysis. Concentrations of oestrone (E_1) and oestradiol were measured quantitatively, using internal standards. Meanwhile, values for oestriol (E_3) and an unidentified oestradiol metabolite (peak at 1.52 Mz) could not be calculated relative to internal standards, and are thus presented as raw peak area values. Oestriol is also noted to co-elute with 11- β OHE₂, preventing the distinction between these two chemicals. For the generation of samples, protocols

were similar to the previous experiment investigating oestradiol concentrations in media supernatant. Differentiated preadipocytes were, however, incubated with differentiation media, and therefore additional samples of differentiation media were sent for analysis. Control media samples with added oestradiol were also analysed, allowing the examination of the relative uptake and metabolism of oestradiol in cultured cells, as the control media samples were not incubated with cells, and contained the original amount of added oestradiol. For data analysis, the concentration of oestradiol or its metabolites in media samples was subtracted from the corresponding control media sample (with or without oestradiol), and normalised to cellular protein content, as before.

4.2.4 Evaluating the Effect of Oestradiol on Preadipocyte Proliferation

4.2.4.1 Cell Counting to Calculate Doubling Times

As detailed in Section 2.14, cell counting was conducted to evaluate proliferation. Specifically, 200,000 MBDFAT preadipocytes were seeded in a T75 flask. After 72-96 hours, cells were trypsinised and counted. Each slide chamber was counted at least 3 times, or until reliably consistent readings were obtained. An average count for each chamber was calculated. The exact times of plating and trypsinisation were noted to accurately calculate cell doubling times. Doubling time calculation was based on the formula [420]:

$$\frac{\text{duration} \times \ln(2)}{\ln\left(\frac{\text{finalcellnumber}}{\text{initialcellnumber}}\right)}$$

Following the calculation of doubling time for each treatment condition, a change in doubling time was calculated by subtracting the value of treated cells, with that of control cells. This was subsequently divided by the raw doubling time value, to give a relative change in doubling time, for each treatment. To validate this method for assessing cellular proliferation, MBDFAT proliferation was compared to that of primary preadipocytes, which grow slower as compared to immortalised cell lines. This comparison confirmed the validity of the counting method. Additionally,

MCF7 proliferation responses to oestradiol were assessed as a validation experiment, using identical protocols.

4.2.5 Evaluating the Transcriptional Responses to Oestradiol in Preadipocytes

To investigate gene expression mediated by oestradiol in MBDFAT preadipocytes, a range of oestradiol-responsive genes from the literature were selected. These selections were based on the magnitude of response, the cells in which a dose response had been documented, and the availability of TaqMan™ assays, for each gene. The chosen genes included *IGFBP3*, *B4GALT1*, *MYC* and *VEGFA* [421–429]. Literature on oestradiol response genes, however, primarily investigate breast cancer cells, and there is scarce publicly available transcriptome data for oestradiol-responses in preadipocytes. Selected genes, therefore, were largely not preadipocyte specific, although *VEGFA* is reported to be oestradiol-responsive in preadipocytes [427]. qPCR assays were performed as described in Section 2.12. MBDFAT preadipocytes were cultured in 6-well plates for 48 hours in standard growth media, with added oestradiol or vehicle control, before being harvested in TRIzol™.

4.2.6 Assessment of Oestradiol Uptake Using a Fluorescently Labelled Hormone-Based Assay

To ensure that oestradiol was entering cells, a fluorescently labelled hormone was used to track uptake and migration. Estradiol Glow (Jena Bioscience, PR-958S), is a fluorescently labelled 17β -oestradiol product, labelled with a low molecular weight red fluorophore. The product was diluted in sterile PBS, and added to cells at 10nM or 100nM. Varying incubation periods were trialled, and cells were viewed live, and then imaged after paraformaldehyde fixation and counterstaining for cytoskeletal and nuclear elements, following protocols outlined in Section 2.13. This label is suitable for histological fixation using paraformaldehyde, without significant loss of fluorescence intensity.

4.2.7 Assessing Oestradiol Responses with ERE-Reporter Assay Systems

4.2.7.1 Plasmid-Based Reporter Assay

To investigate the explicit oestradiol response in MBDFAT preadipocytes, reporter assays were employed. These measured the activation of EREs, therefore giving a readout of the formation, translocation and subsequent binding of oestradiol-receptor complexes. The first of these was a plasmid-based assay. Plasmids co-transfected into cells included an eGFP-expressing plasmid (AddGene, #54759), allowing the determination of transfection efficacy, and an ERE-TATA-Luc plasmid (AddGene, #11354), resulting in the production of luciferase protein in response to oestradiol. A Renilla luciferase plasmid was also transfected, for use as an internal control for normalisation of the Firefly luciferase signal (Promega, E2241). Plasmids were isolated and transfected into MBDFAT preadipocytes and MCF7 cells through NeonTM electroporation methods, as described in Section 2.16. 10,000 cells were plated per well, in a 96-well plate, left to settle overnight, and transfection efficiency was confirmed by visual analysis of GFP fluorescence. After treatment with a range of oestradiol concentrations for 48 hours, cells were harvested in lysis buffer and the dual luciferase assay was performed, as outlined in Section 2.18. Responses were also assessed at 1 hour, 6 hours, 24 hours, 48 hours and 72 hours (data not shown). Additional assays, using primary cells (methods detailed in Section 2.1), PAT2 DFAT cells [252] (another depot-specific immortalised cell line, similar to MBDFAT cells, but derived from a male donor), and differentiated preadipocytes (MBDFAT, PAT2 DFAT, primary cells), were performed, to establish whether the observed response was specific to MBDFAT preadipocytes. MCF7 cells were similarly transfected before treatment with oestradiol, and multiple lysates were prepared and stored at -80°C . Given the robust responses seen in this cell line, these lysates were used as a positive control, included in every assay plate to ensure reagent functionality.

During plate reading, three measurements were taken: before injection, after the injection of luciferase substrate, and after the injection of the quenching substrate/

Renilla substrate. As the Renilla luciferase was constitutively active, this served as an internal control. To calculate relative readings, the pre-injection value was subtracted from the post-injection value, and the blank (lysis buffer only) reading was subtracted from the sample readings. The Firefly luciferase signal was normalised to the Renilla luciferase signal by dividing the blank-corrected reading after the first injection by that after the second.

4.2.7.2 Lentiviral-Based Reporter Assay

A lentiviral-based reporter assay was also employed, to generate a stable cell line expressing the ERE-TATA-Luc construct (pGF2-ERE-rFluc-T2A-GFP-mPGK-Puro, TR4XXVA-P, SystemsBioSciences). Transduction methods are described in Section 2.17. For every assay, negative control vector (pGF2-mCMV-rFluc-T2A-GFP-mPGK-Puro, TR4XXVA-P, SystemsBioSciences) cells were cultured alongside the positive reporter cells, and subject to equivalent treatments. For assessment of oestradiol responses, 10,000 cells per well were plated in a 96-well plate, subject to serum starvation for 24 hours, and then treated with either oestradiol (10nM), PPT (1nM), fulvestrant (1nM), letrozole (2nM), or PGE2 (1 μ m) for 24 or 48 hours. Concentrations were based on existing literature, selecting doses that are reported to decrease/increase enzyme/receptor activity [430, 431]. Data is shown for 48-hour treatment, although an identical pattern of response was seen at 24 hours. Frozen MCF7 lysate from the plasmid-based ERE-TATA-Luc assay, treated either with control or 1nM oestradiol, was added to each plate, to ensure that reagents were working as expected and a positive signal was recorded. The luciferase assay was then performed, blank-corrected values were calculated, and treatment responses were expressed relative to control samples. The assay was also repeated with differentiated preadipocytes.

4.2.8 Using RNA Sequencing to Investigate the Depot-Specific Effects of Oestradiol

To investigate the absolute transcriptomic effects of oestradiol on MBDFAT preadipocytes, bulk RNA sequencing was conducted on preadipocyte monocultures following

treatment with oestradiol or vehicle control. Abdominal and gluteal MBDFAT preadipocytes were cultured in 6-well plates with 100nM oestradiol treatment, or vehicle control, for 48 hours. Following treatment, cells were subject to washing, incubation and filtering steps (described in Section 2.20), further acting as a control condition for the co-culture experiments described in the following chapter.

4.3 Results

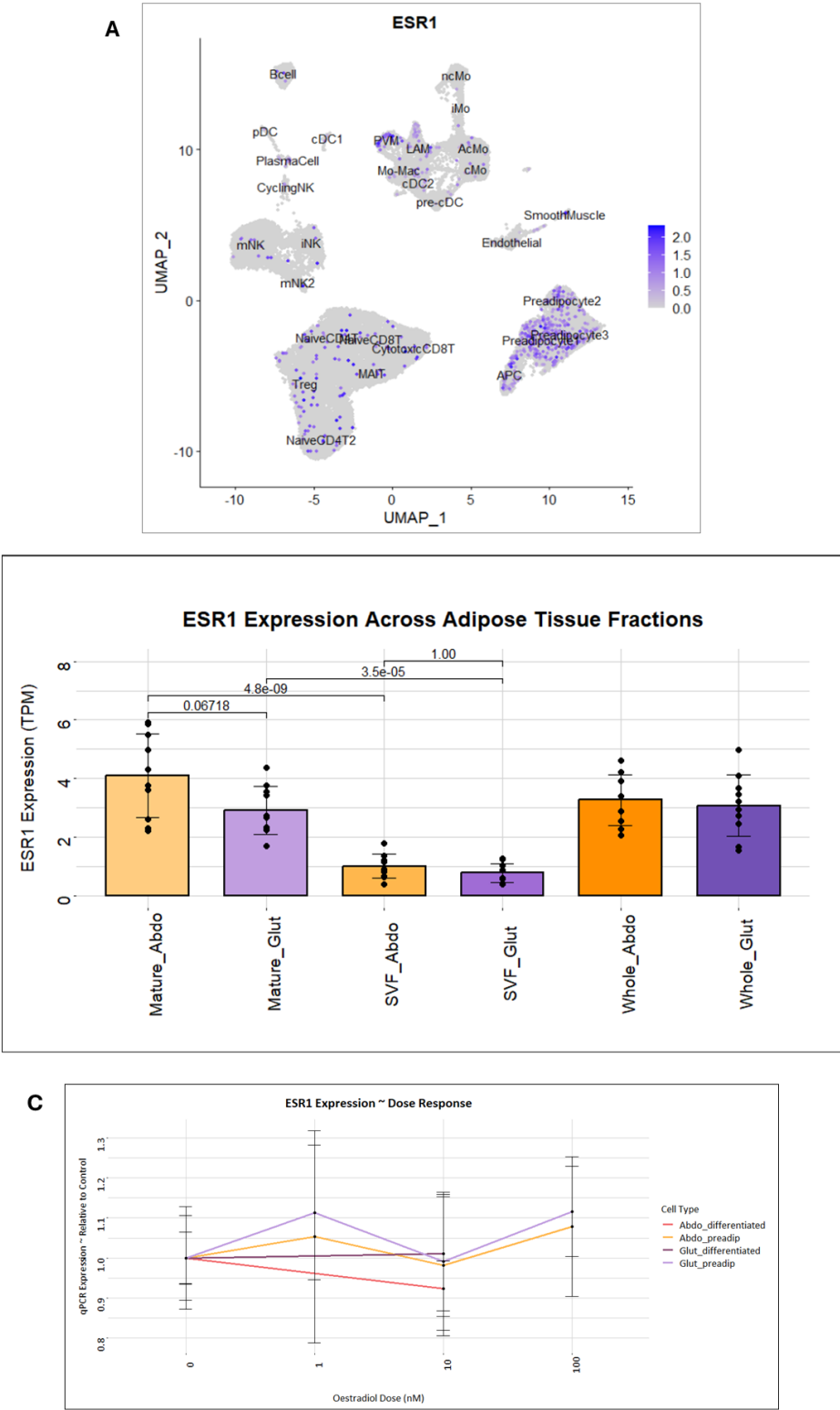
4.3.1 Oestrogen Receptors Are Expressed in MBDFAT Preadipocytes

Although the literature is largely in agreement that preadipocytes express ER- α [85], there is less consensus on the expression of ER- β , and limited data investigating the expression of GPER1. Furthermore, the specific MBDFAT cell line used in these experiments had not previously been used to investigate oestradiol-mediated responses. Given these points, it was necessary to investigate the expression of the cohort of ERs in the planned cell model, as well as in data extracted from adipose tissue biopsies, to understand whether MBDFAT cell expression accurately reflects physiological tissue expression patterns.

4.3.1.1 *ESR1* and ER- α

Looking at *ESR1* expression in scRNASeq data revealed predominant expression of this receptor in the preadipocyte fraction of primary biopsies, with some expression also seen in tissue-resident macrophage populations (Figure 4.2A). Expression across the preadipocyte population was heterogeneous, and no significant differences between abdominal and gluteal preadipocytes or between males and females were evident. Fractionated adipose tissue biopsy data confirmed *ESR1* expression in the adipose tissue SVF, but additionally revealed that expression was higher in the mature adipocyte fraction relative to the SVF (Figure 4.2B). A 2-way ANOVA test showed significant effects of depot ($p=0.0242$) and tissue fraction ($p=5.60e^{-13}$), but no interaction of these factors ($p=0.1468$), on *ESR1* expression. *ESR1* expression was unchanged by oestradiol treatment at 1-100nM in abdominal

and gluteal MBDFAT preadipocytes (Figure 4.2C). To confirm that MBDFAT cells were appropriate for studying preadipocyte oestrogen responses, receptor expression was examined in cultured MBDFAT preadipocytes, comparing protein expression to primary cells, as well as MCF7 cells, which served as a positive control. Relative to primary preadipocytes and MCF7 cells, MBDFAT preadipocytes showed less receptor expression for both depots (Figure 4.2D). The specificity of observed protein banding should be further confirmed using siRNA treated cells for *ESR1*. Immunostaining data revealed a predominantly nuclear expression pattern in abdominal (Figure 4.2E) and gluteal (Figure 4.2F) preadipocytes, which was less prominent as compared to differentiated preadipocytes (Figure 4.2G), macrophages (Figure 4.2H), and MCF7 cells (Figure 4.2I), supporting the fractionated tissue data and Western blot data, respectively. Immunostaining data could be further confirmed using isotype controls for the ER- α antibody. Representative unstained control images are presented in Appendix A.5.



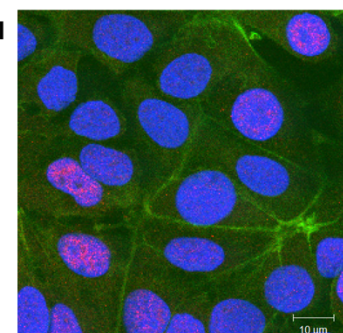
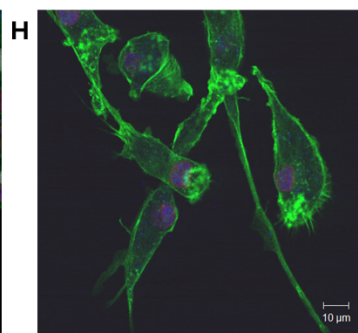
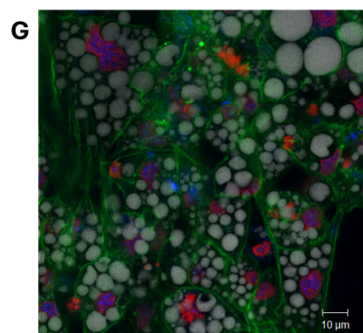
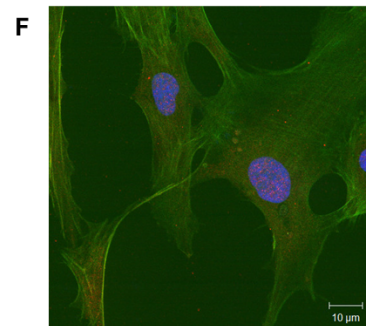
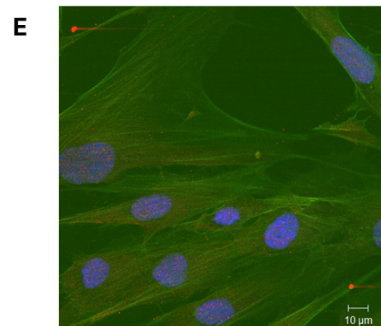
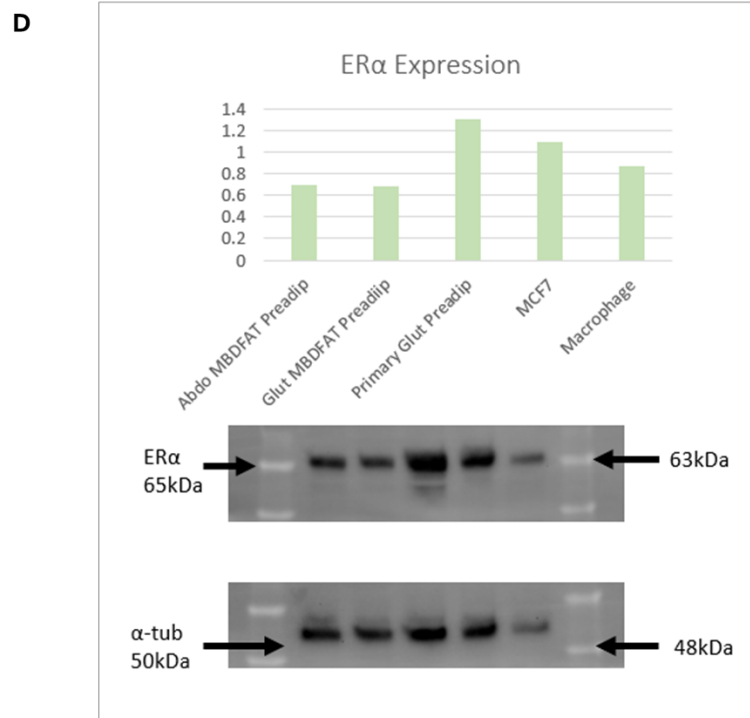


Figure 4.2: **A)** Featureplot showing *ESR1* expression in combined abdominal and gluteal adipose tissue SVF. Clusters are identical to those described in Section 3.3.1. Expression levels are indicated by colour, with grey being indicative of zero expression. **B)** Expression of *ESR1* across adipose tissue fractions (SVF and mature adipocytes), and whole tissue, in paired abdominal and gluteal biopsies ($n=10$). On the x-axis from left to right, sample data presented are: abdominal mature adipocyte fraction, gluteal mature adipocyte fraction, abdominal SVF, gluteal SVF, whole abdominal adipose tissue, whole gluteal adipose tissue. Post-hoc *t*-tests, with Bonferroni correction for multiple testing, are graphically presented. Bars represent the mean value. Data points are plotted in black. Error bars represent standard deviation. **C)** Change in *ESR1* expression (relative to control) with oestradiol treatment. For each experimental run ($n=5$), dose change in expression was calculated relative to control. Bars represent standard deviation, and points represent the mean value for each group. Responses for each cell type are plotted with a different colour line. **D)** Western blot for *ER- α* across abdominal and gluteal MBDFAT preadipocytes, primary gluteal preadipocytes, MCF7 cells and macrophages (left to right). Cells were cultured following protocols specified in Section 2.11. For bar graph presentation, pixel intensity was normalised to that of total protein for each lane. Housekeeping protein (α -tubulin) expression is also presented. Subfigures **E-I**) - Representative images, showing *ER- α* immunostaining (Alexa Fluor 555, red), alongside actin staining (Phalloidin 488, green) and nuclear staining (Hoechst, blue), imaged on the BioRad confocal microscope, detailed in Section 2.13. Representative staining control images are presented in Appendix A.5. Images are representative of a minimum of $n=3$. Staining is shown for: **E)** Abdominal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment; **F)** Gluteal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment; **G)** Abdominal MBDFAT differentiated adipocytes, cultured without oestradiol, for 10 days; **H)** Differentiated macrophages cultured for 48 hours without oestradiol treatment; **I)** MCF7 cells, cultured for 48 hours without oestradiol treatment.

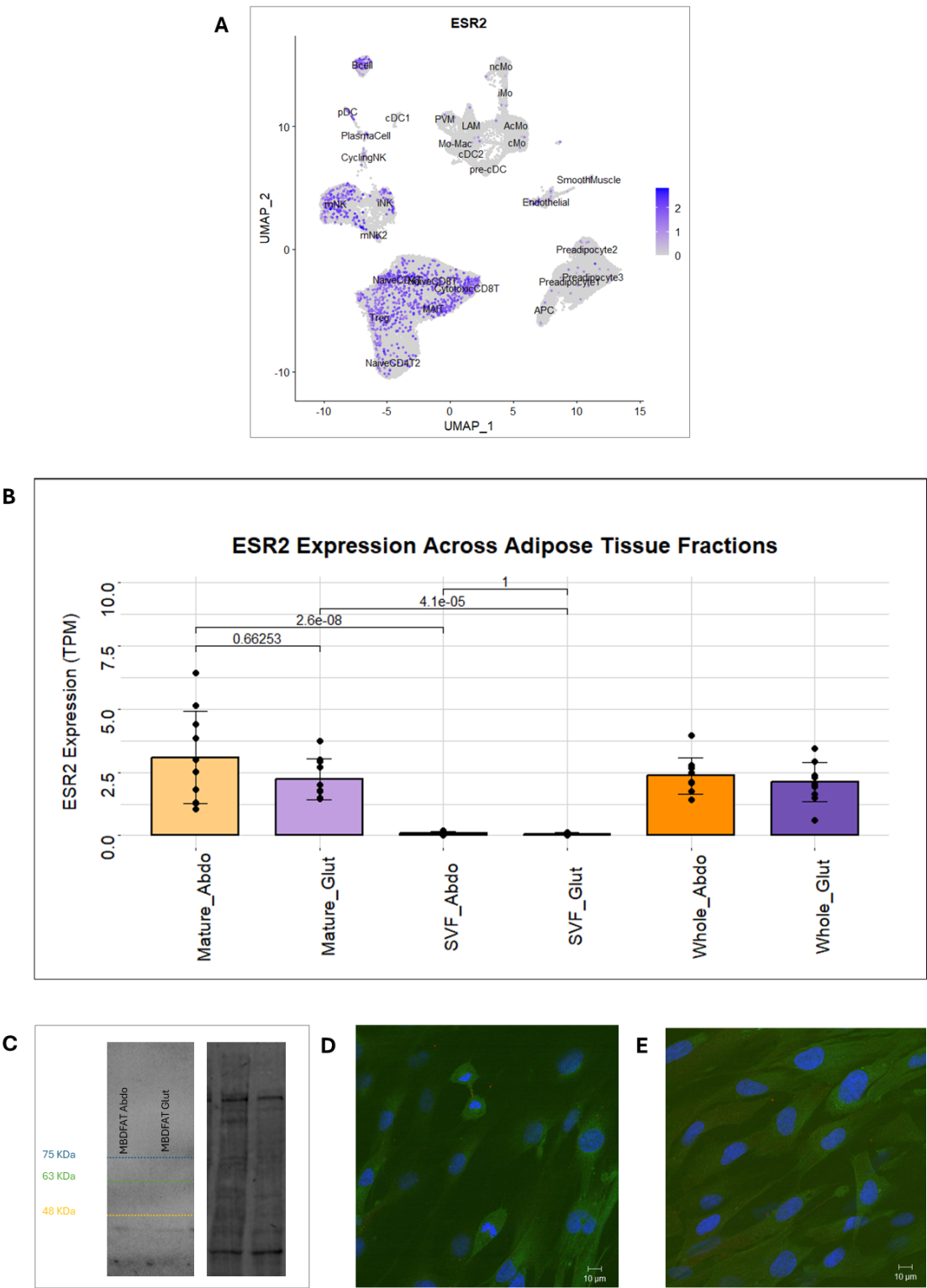


Figure 4.3: **A)** Featureplot showing *ESR2* expression in combined abdominal and gluteal adipose tissue SVF. Clusters are identical to those described in Section 3.3.1. Expression levels are indicated by colour, with grey being indicative of zero expression. **B)** Expression of *ESR2* across adipose tissue fractions (SVF and mature cells), and whole tissue, in paired abdominal and gluteal biopsies ($n=10$). On the x-axis from left to right, sample data presented are: abdominal mature adipocyte fraction, gluteal mature adipocyte fraction, abdominal SVF, gluteal SVF, whole abdominal adipose tissue, whole gluteal adipose tissue. Post-hoc *t*-tests, with Bonferroni correction for multiple testing, are graphically presented. Bars represent the mean value. Data points are plotted in black. Error bars represent standard deviation. **C)** Western blot for ER- β (molecular weight = 59.2kDa) in abdominal and gluteal MBDFAT preadipocytes using the previously validated antibody. Total protein is shown in the rightmost figure. **D - E)** Representative images, showing ER- β staining (Alexa Fluor 555, red), alongside actin staining (Phalloidin 488, green) and nuclear staining (Hoechst, blue), imaged on the BioRad confocal microscope, detailed in Section 2.13. Representative staining control images are presented in Appendix A.5. Staining is shown for: **D)** Abdominal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment; **E)** Gluteal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment.

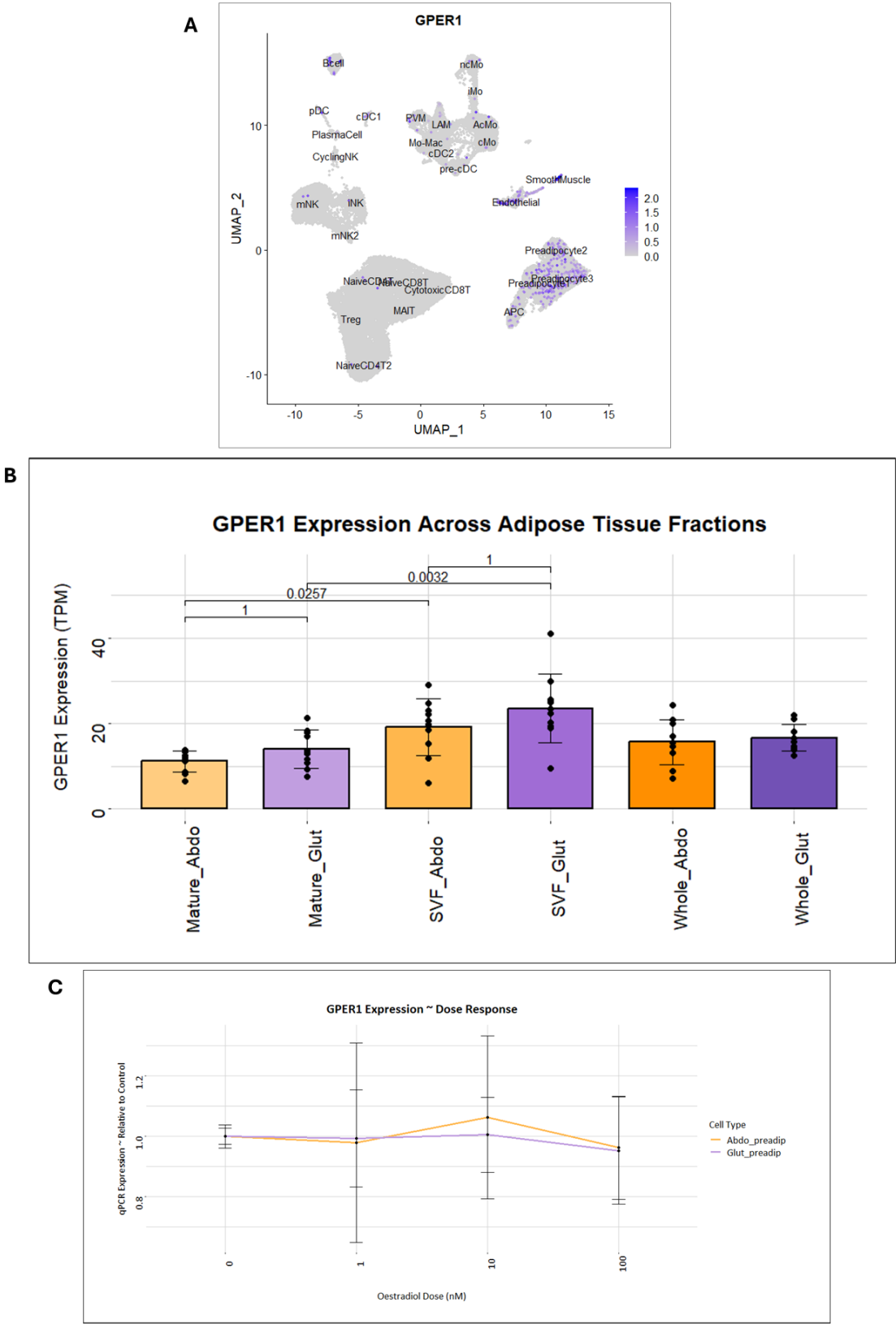
4.3.1.2 *ESR2* and ER- β

In adipose tissue SVF, scRNASeq data revealed that *ESR2* showed predominant expression in T-cell and NK-cell populations, with very little expression in preadipocytes (Figure 4.3A). This was confirmed in fractionated adipose tissue data, which showed low expression in SVF, and significantly higher expression in mature adipocytes (Figure 4.3B). A 2-way ANOVA test showed a significant effect of tissue fraction ($p=3.42e^{-12}$), but no effect of depot ($p=0.118$), and no interaction of these factors ($p=0.354$) in relation to *ESR2* expression. In MBDFAT preadipocytes, qPCR data revealed almost undetectable expression of *ESR2* (data not shown), which was confirmed in bulk RNA sequencing data presented later (Section 4.3.6), as well as Western blot and immunostaining data (Figure 4.3C-E). This suggested that in adipose tissue, mature adipocytes are the primary expressors of *ESR2*.

4.3.1.3 GPER1/GPR30 Expression

scRNASeq data indicated that *GPER1* expression was predominantly localised to preadipocytes, endothelial cells and smooth muscle cells within the adipose tissue SVF, with expression being heterogeneous across these populations (Figure 4.4A). In

fractionated adipose tissue biopsy data, *GPER1* expression was significantly higher in the SVF compared to mature adipocyte fractions for both depots, although there were no significant differences between depots (Figure 4.4B). A 2-way ANOVA test showed an effect of tissue fraction ($p=1.93\text{e}^{-05}$), but no effect of depot ($p=0.0519$), or depot x tissue fraction interaction ($p=0.6072$) on *GPER1* expression. In MBDFAT preadipocytes treated with oestradiol doses of 1-100nM, no change in *GPER1* expression was evident in either depot (Figure 4.4C), but expression was present, as confirmed in later bulk RNA sequencing datasets (described in Section 4.3.6). Western blotting revealed that GPER1 was expressed in both abdominal and gluteal preadipocytes, with expression being lower than MCF7 cells (Figure 4.4D). Banding was observed around 60kDa. Although this receptor is recorded as a 40-50kDa protein, literature has confirmed the existence of 60kDa and 80kDa isoforms [417], which could explain the banding in the presented blot. It has been noted elsewhere that heavier GPER1 bands are often observed due to interactions with other proteins, as well as glycosylation and other extensive carbohydrate processing that occurs during biogenesis and cellular transport [417, 432]. The manufacturer data sheet similarly highlighted this higher molecular weight [433]. The specificity of the observed Western blot expression should be further confirmed using siRNA treated cells for *GPER1*. Immunostaining imaging, to assess receptor localisation, revealed nuclear, cytoplasmic and membrane expression of GPER1 in MBDFAT abdominal (Figure 4.4E) and gluteal (Figure 4.4F) preadipocytes. Immunostaining data could be further confirmed using isotype controls for the GPER1 antibody. Representative unstained control images are presented in Appendix A.5.



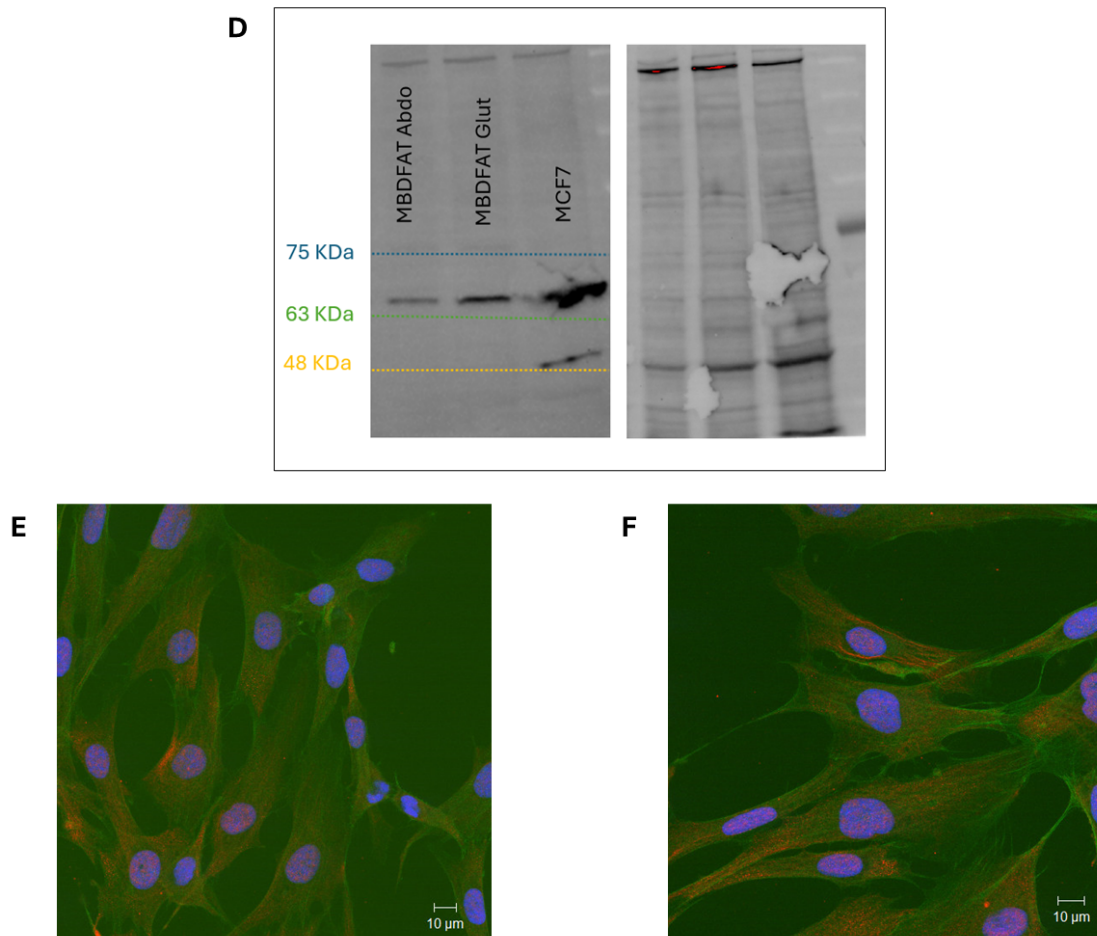


Figure 4.4: **A)** Featureplot showing *GPER1* expression in combined abdominal and gluteal adipose tissue SVF. Clusters are identical to those described in Section 3.3.1. Expression levels are indicated by colour, with grey being indicative of zero expression. **B)** Expression of *GPER1* across adipose tissue fractions (SVF and mature cells), and whole tissue, in paired abdominal and gluteal biopsies ($n=10$). On the x-axis from left to right, sample data presented are: abdominal mature adipocyte fraction, gluteal mature adipocyte fraction, abdominal SVF, gluteal SVF, whole abdominal adipose tissue, whole gluteal adipose tissue. Post-hoc *t*-tests, with Bonferroni correction for multiple testing, are graphically presented. Bars represent the mean value. Data points are plotted in black. Error bars represent standard deviation. **C)** Change in *GPER1* expression (relative to control) with oestradiol treatment. For each experimental run ($n=4$), dose change in expression was calculated relative to control. Bars represent standard deviation, and points represent the mean value for each group. Responses for each cell type are plotted with a different colour line. **D)** Western blot for *GPER1* in abdominal and gluteal MBDFAT preadipocytes, and MCF7 cells, which were cultured according to protocols specified in Section 2.11. Total protein blot is shown in the rightmost part of the figure, while antibody-probed gel imaging is shown on the left, with molecular weights indicated in coloured lines. Subfigures **E-F)** - Representative images, showing *GPER1* immunostaining (Alexa Fluor 555, red), alongside actin staining (Phalloidin 488, green) and nuclear staining (Hoechst, blue), imaged on the BioRad confocal microscope, detailed in Section 2.13. Representative staining control images are presented in Appendix A.5. Images are representative of a minimum of $n=3$. Staining is shown for: **E)** Abdominal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment; **F)** Gluteal MBDFAT preadipocytes cultured for 48 hours without oestradiol treatment.

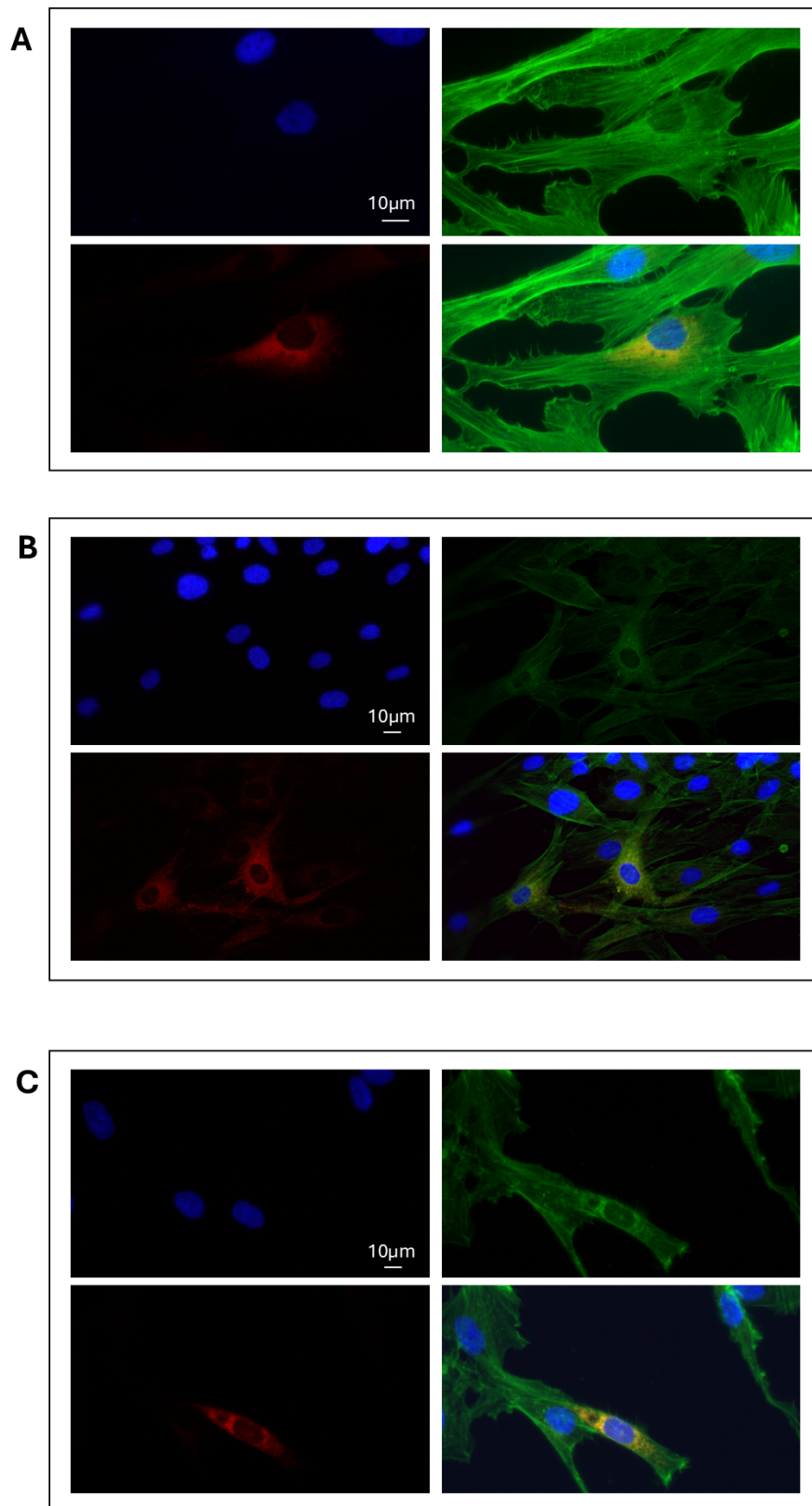


Figure 4.5: MBDFAT preadipocytes treated with 10nM of fluorescently labeled oestradiol (red) for 6 hours. As described in Section 4.2.6, cells were imaged after fixation and counterstaining for actin (Phalloidin-488, green) and nuclear elements (Hoechst, blue). In each subfigure, the top left panels show Hoechst-stained nuclei (blue), the top right panels display cytoskeletal actin, stained with Phalloidin (green), and the bottom left panels show the fluorescently labeled oestradiol (red). The bottom right panels present composite images combining all three colour channels. A small amount of bleedthrough from the red to the green channel was observed, but none in the reverse direction. This was confirmed using control samples treated with fluorescent oestradiol without the Phalloidin stain, and samples treated with Phalloidin without fluorescent oestradiol. Images are representative of three experimental runs, over which results were consistent. The images show **A)** Abdominal MBDFAT preadipocytes, **B)** Gluteal MBDFAT preadipocytes, and **C)** Abdominal MBDFAT preadipocytes.

4.3.1.4 Fluorescent Oestradiol Uptake

Oestradiol entry into MBDFAT preadipocytes was evaluated using a fluorescently labeled oestradiol compound. After 6 hours of treatment with 10nM of the compound, oestradiol uptake was predominantly extra-nuclear (Figure 4.5). The uptake pattern was heterogeneous across cells, with only a small minority of cells (less than 1%) showing substantial oestradiol influx. No obvious differences in fluorescent oestradiol entry were observed between depots (Figure 4.5A-C). This variability in uptake was unlikely to be linked to ER- α or GPER1 expression, as immunofluorescence studies revealed relatively uniform expression of these receptors. These findings raise questions about the factors that regulate extracellular oestradiol uptake in preadipocytes.

4.3.2 The Production and Metabolism of Oestrogens by Adipose Tissues

In fractionated adipose tissue, the expression of *CYP19A1* was investigated, to determine whether oestradiol production differs across depot and tissue fraction. A 2-way ANOVA indicated a significant effect of depot ($p = 3.44 \times 10^{-6}$) and tissue fraction ($p = 2.73 \times 10^{-8}$), but no interaction effect between these variables ($p = 0.07$) on *CYP19A1* expression. Expression was higher in adipose SVF, for both depots, as compared to mature cells, with gluteal SVF showing significantly higher

expression than abdominal SVF (Figure 4.6A). This was consistent with findings from preadipocyte scRNASeq data, where gluteal preadipocytes, which reside within the SVF, expressed significantly higher *CYP19A1* as compared to abdominal preadipocytes. To understand whether these differences related to functional differences in oestradiol production, the concentration of oestradiol in the cell culture media supernatant from cultured MBDFAT preadipocytes was measured (Figure 4.6B). A 2-way ANOVA indicated a significant effect of depot ($p=1.99\text{e}^{-04}$), a significant effect of differentiation ($p=6.27\text{e}^{-07}$), and a significant effect of depot x differentiation interaction ($p=1.99\text{e}^{-03}$) on oestradiol production. These depot- and differentiation- dependent differences in oestradiol production in cultured cells mirrored the differences in *CYP19A1* expression in fractionated tissue data; gluteal preadipocytes produced more oestradiol than abdominal preadipocytes, and preadipocytes produced more oestradiol as compared to differentiated preadipocytes. After treatment with the same concentration of oestradiol (10nM) for equivalent periods of time, the differentiated preadipocytes displayed a lower concentration of oestradiol in the cell culture media than the preadipocytes (Figure 4.6C). A 2-way ANOVA indicated no significant effect of depot, a significant effect of differentiation ($p=1.22\text{e}^{-09}$), and a significant effect of depot x differentiation interaction ($p=0.0423$) on oestradiol production. This suggested that uptake and metabolism of oestradiol may be greater in differentiated preadipocytes, relative to non-differentiated preadipocytes, who preferably produce oestradiol.

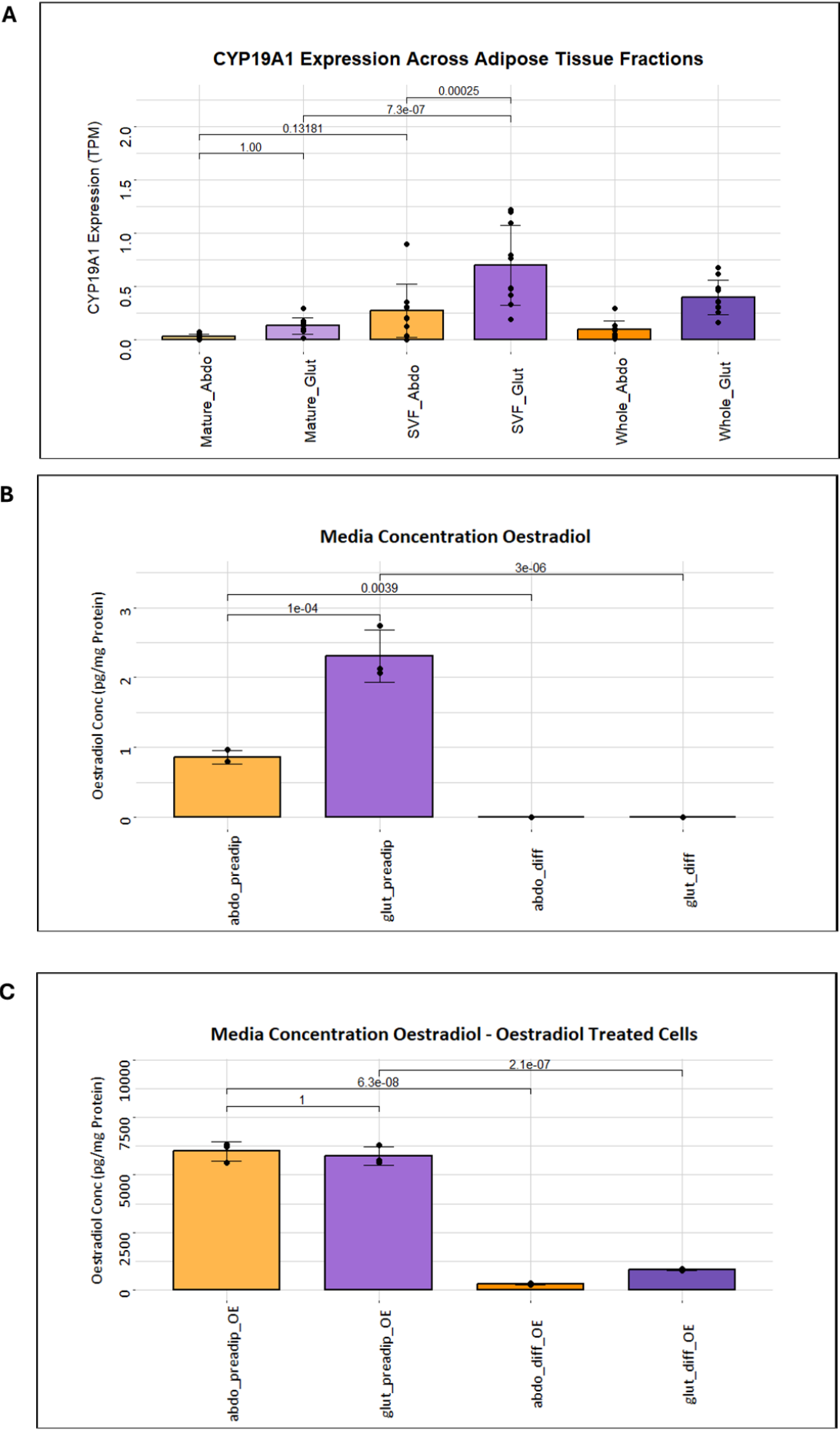


Figure 4.6: For all presented data, a 2-way ANOVA was conducted to assess the effect of depot and cell maturity/adipose tissue fraction, and the interaction of these factors. This was followed-up with post-hoc t-tests, with Bonferroni correction for multiple testing, which are presented on the graphs. The graphs show bars as mean group values, individual data points plotted in black, and error bars for standard deviation. **A)** Expression of CYP19A1 across adipose tissue fractions (SVF and mature cells), and whole tissue, in abdominal and gluteal biopsies ($n=10$). On the x-axis from left to right, sample data presented are: abdominal mature adipocyte fraction, gluteal mature adipocyte fraction, abdominal SVF, gluteal SVF, whole abdominal adipose tissue, whole gluteal adipose tissue. **B)** Media supernatant concentration of oestradiol, measured by LCMS, from cultured MBDFAT cells ($n=3$). On the x-axis, from left to right, the presented groups are: abdominal MBDFAT preadipocytes, gluteal MBDFAT preadipocytes, abdominal MBDFAT differentiated preadipocytes, gluteal MBDFAT differentiated preadipocytes. Preadipocytes and differentiated preadipocytes were cultured without phenol red. Media was incubated with cells for 48 hours before analysis. Values are blank-corrected. **C)** Media supernatant concentration of oestradiol, measured by LCMS, from cultured MBDFAT cells, treated with oestradiol ($n=3$). On the x-axis, from left to right, the presented groups are: abdominal MBDFAT preadipocytes + oestradiol, gluteal MBDFAT preadipocytes + oestradiol, abdominal MBDFAT differentiated preadipocytes + oestradiol, gluteal MBDFAT differentiated preadipocytes + oestradiol. Preadipocytes and differentiated preadipocytes were cultured without phenol red, in the presence of 10nM oestradiol. Media was incubated with cells for 48 hours before analysis. Values are blank-corrected to a control sample which was not treated with oestradiol.

To further investigate differences in oestradiol metabolism, media supernatant samples were collected, as before, and sent for an in-depth analysis of oestradiol metabolites, looking at 9 different compounds. In these samples, oestradiol, oestrone, oestriol, and an undefined peak at 1.52 Mz were identified. Baseline oestradiol concentrations in oestradiol-treated media were also recorded to evaluate relative production and uptake of oestrogens following oestradiol-treatment. Following treatment with oestradiol, a 2-way ANOVA indicated no significant effect of depot, a significant effect of treatment ($p=0.0102$), and no significant effect of depot x treatment interaction on oestradiol concentration. Post-hoc t-tests showed that this was insignificant for each depot, indicating that oestradiol may increase its own uptake in differentiated preadipocytes, but not in a depot-specific manner (Figure 4.7A). Oestrone production was also measured. A 3-way ANOVA showed a significant effect of depot ($p=4.87e^{-05}$), a significant effect of treatment ($p=4.14e^{-06}$), a significant effect of differentiation ($p=2.48e^{-03}$), a significant effect of depot x

treatment interaction ($p=8.06e^{-03}$), no significant effect of depot x differentiation interaction ($p=0.318$), a significant effect of treatment x differentiation interaction ($p=1.03e^{-03}$), and a significant effect of depot x treatment x differentiation interaction ($p=3.02e^{-02}$) on oestrone concentration. Notably, there was increased production of oestrone with oestradiol treatment in gluteal preadipocytes (Figure 4.7B), further suggesting that overall oestrogen synthesis and conversion pathways may be greatest in the gluteal SVF. Gluteal production of oestrone was significantly different from abdominal production, only in preadipocytes treated with oestradiol (adjusted p value = 0.00055). Depot differences in differentiated preadipocytes, and control-treated preadipocytes were not significant after corrections for multiple testing. Oestriol concentrations in media supernatant were not different from that of control media (data not shown). The novel metabolite, producing a peak at 1.52 Mz, was highest in media from gluteal differentiated preadipocytes (Figure 4.7C). A 3-way ANOVA indicated a significant effect of depot ($p=3.54e^{-04}$), no significant effect of treatment ($p=0.0657$), significant effect of differentiation ($p=6.85e^{-10}$), no significant effect of depot x treatment interaction ($p=0.586$), no significant effect of depot x differentiation interaction ($p=0.079$), no significant effect of treatment x differentiation interaction ($p=0.204$), and no significant effect of depot x treatment x differentiation interaction ($p=0.780$), on the metabolite concentration. There were significant differences between preadipocytes and differentiated preadipocytes. The physiological implications of the production of this metabolite are not known.

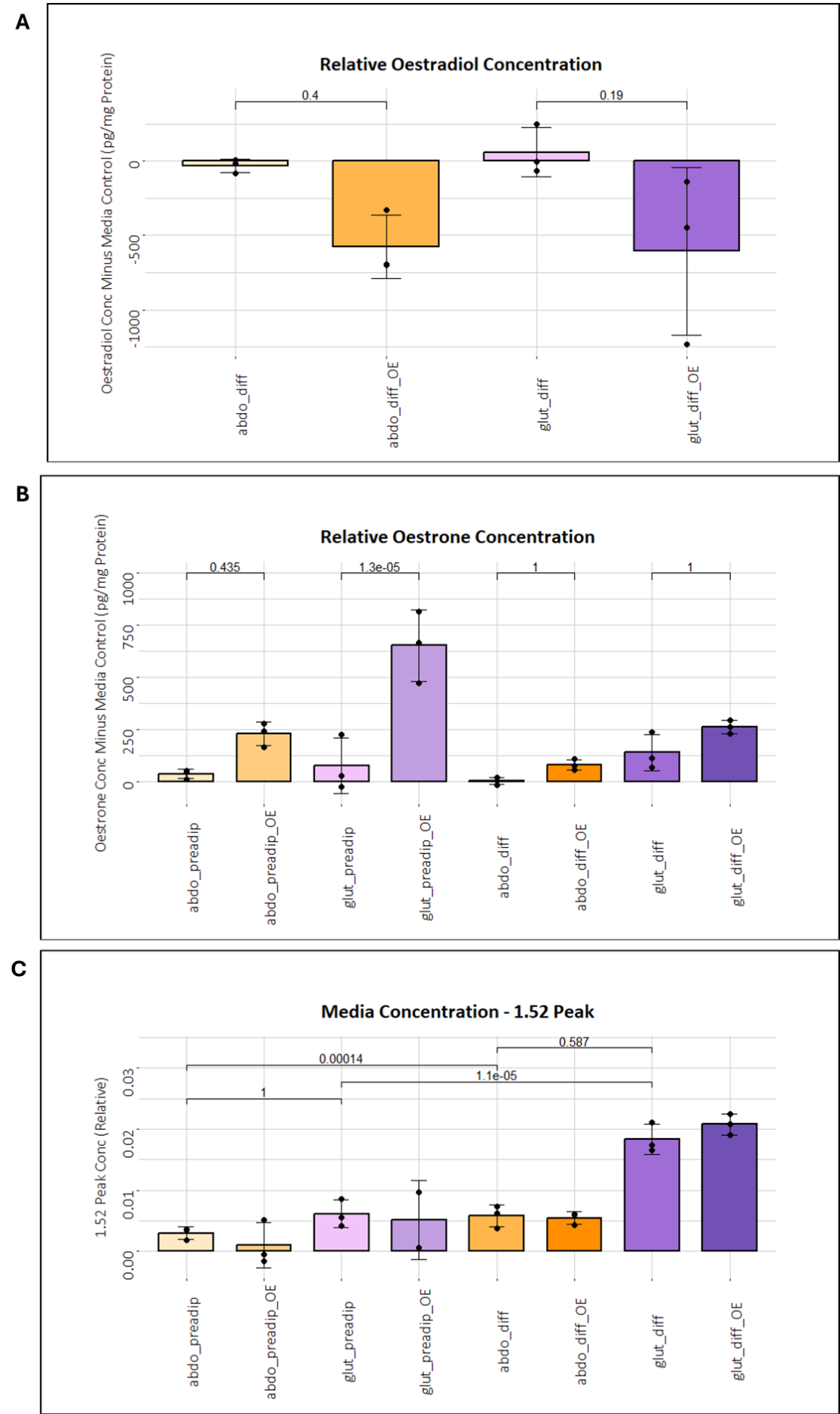


Figure 4.7: *Relative media supernatant concentrations of oestrogens measured using IDMS for abdominal and gluteal MBDFAT preadipocytes and differentiated preadipocytes (n=3), with oestradiol or vehicle control treatment. Control media samples, with or without oestradiol treatment, were also analysed, and used to blank-correct the presented values. This allowed the assessment of uptake or production of hormone, relative to the initial media start value. Media was incubated with cells for 48 hours. For all presented data, a 2-way (Subfigure A) or a 3-way (Subfigure B and C) ANOVA were conducted to assess the effect of depot, treatment, cell maturity, and the interaction of these factors. This was followed-up with post-hoc t-tests, with a Bonferroni correction for multiple testing, which are graphically presented. Results from the ANOVA are presented in the text only. The graphs present bars as the mean group values, individual data points are plotted in black, and error bars represent standard deviation. Data presented show concentrations for: A) Oestradiol: On the x-axis, from left to right, the presented groups are: abdominal MBDFAT differentiated preadipocytes, abdominal MBDFAT differentiated preadipocytes + oestradiol, gluteal MBDFAT differentiated preadipocytes, gluteal MBDFAT differentiated preadipocytes + oestradiol. B) Oestrone: On the x-axis, from left to right, the presented groups are: abdominal MBDFAT preadipocytes, abdominal MBDFAT preadipocytes + oestradiol, gluteal MBDFAT preadipocytes, gluteal MBDFAT preadipocytes + oestradiol, abdominal MBDFAT differentiated preadipocytes, abdominal MBDFAT differentiated preadipocytes + oestradiol, gluteal MBDFAT differentiated preadipocytes, gluteal MBDFAT differentiated preadipocytes + oestradiol. C) Undefined 1.52Mz peak: Sample ordering is identical to Subfigure B.*

4.3.3 MBDFAT Preadipocytes Do Not Show Proliferation Responses to Oestradiol

Given the reported effects of oestradiol on preadipocyte hyperplasia [69, 71], proliferation responses in MBDFAT preadipocytes were assessed following oestradiol treatment. Calculated cell doubling times revealed no proliferation response in either abdominal or gluteal MBDFAT preadipocytes in response to oestradiol (Figure 4.8). MCF7 cells exhibited greater proliferation in response to oestradiol at 1nM, 10nM and 100nM, relative to control treatment. When comparing cell type proliferation responses, 2-way ANOVA tests indicated an effect of treatment ($p = 8.52e^{-06}$), cell type ($p = 3.85e^{-10}$), and a treatment x cell type interaction ($p = 3.79e^{-04}$). Post-hoc testing, revealed that a significant effect of treatment was only present for MCF7 cells. This finding served as a positive control, validating the proliferation assay functionality.

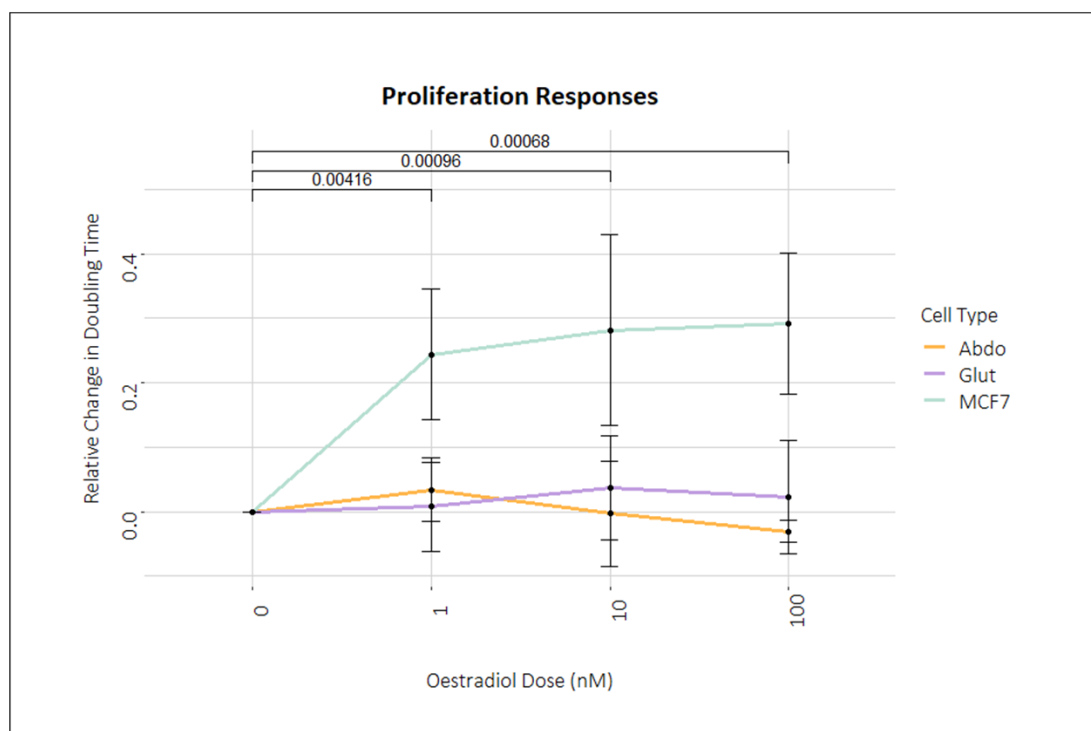


Figure 4.8: Oestradiol dose response showing relative change in cell doubling times for abdominal and gluteal MBDFAT preadipocytes ($n = 5$) or MCF7 cells ($n=3$). Cell type is indicated by line colour, according to the right-hand key. Cells were treated with oestradiol for 48 hours, with the respective oestradiol dose presented on the x-axis, and the relative change in doubling time (calculated relative to control treatment for each group), presented on the y-axis. Methods for cell culture and count methods are detailed in Section 2.14 and Section 4.2.4.1. Post-hoc t-tests, with Bonferroni correction for multiple testing, are presented for MCF7 cells.

4.3.4 MBDFAT Preadipocytes Do Not Show Changes in Oestradiol-Mediated Transcription in Reported Oestradiol-Sensitive Genes

To investigate transcriptional responses to oestradiol, literature-reported oestradiol response genes were assessed in response to varying concentrations of oestradiol in MBDFAT preadipocytes. Neither abdominal nor gluteal MBDFAT preadipocytes showed changes in gene expression in response to oestradiol (Figure 4.9). Direct oestradiol-response genes in adipocytes and preadipocytes, however, are scarcely reported, making gene selection somewhat difficult.

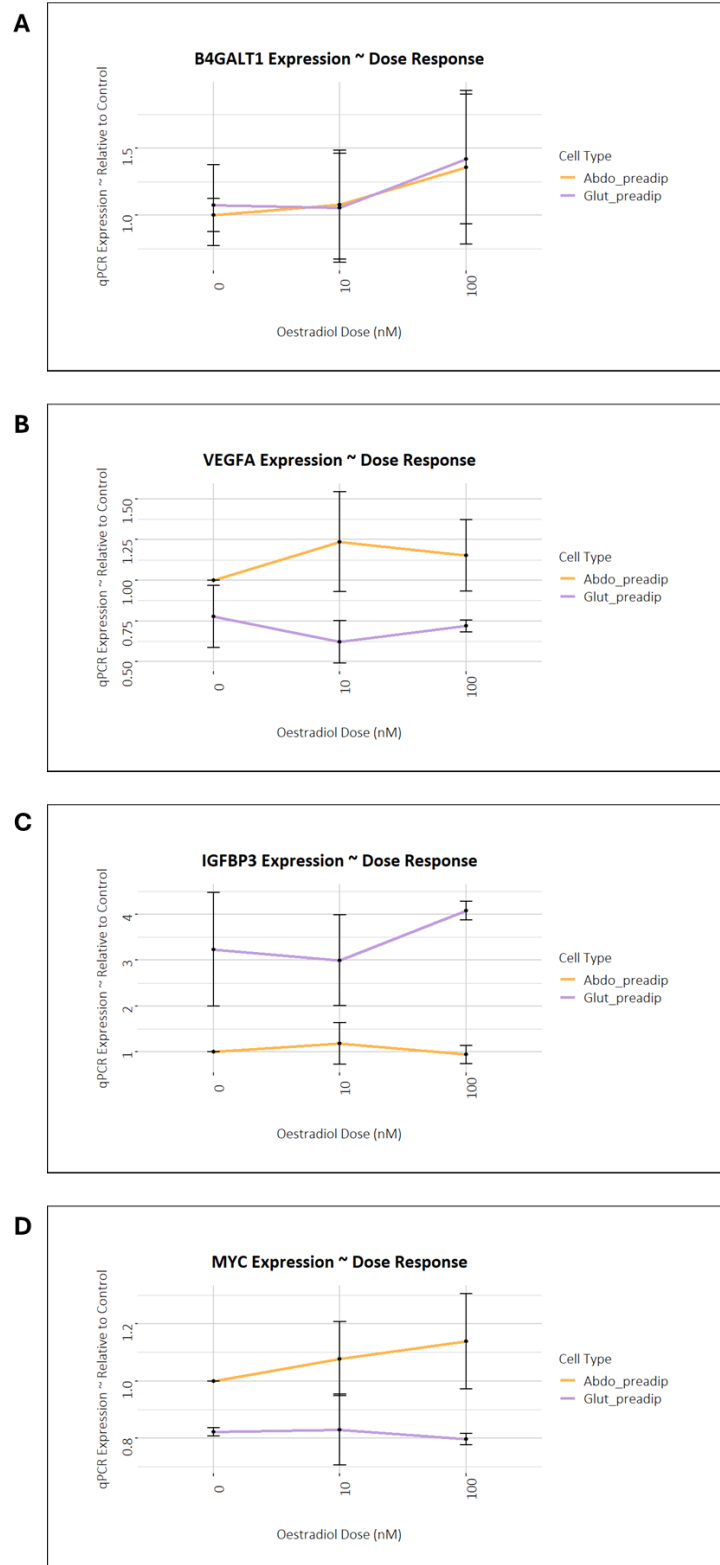


Figure 4.9: Literature-sourced oestradiol responsive genes, measured by qPCR and presented as dose responses to oestradiol in abdominal and gluteal MBDFAT preadipocytes ($n=3$). Abdominal preadipocytes are represented in the orange line, and gluteal preadipocytes are presented in purple. Expression was calculated as $\Delta\Delta C_t$ values, normalising to the geometric mean of three housekeeping genes (*UBC*, *18s*, *PGCK1*), whose expression was unaffected by oestradiol treatment. Change in expression for each dose was calculated relative to the abdominal control sample. Data points are shown as mean values, with error bars representing standard deviation. A 2-way ANOVA was conducted for each gene, to assess the effect of depot, treatment and the interaction of these factors. Genes presented are: **A)** *B4GALT1* (no significant effects); **B)** *VEGFA* (significant effect only for depot, $p = 0.00107$); **C)** *IGFBP3* (significant effect only for depot, $p = 0.000102$); **D)** *MYC* (significant effect only for depot, $p = 0.000455$).

4.3.5 Reporter Assays Could Not Confirm ERE Activity in Response to Oestradiol in MBDFAT Preadipocytes

To further investigate transcriptional responses to oestradiol, without having to select individual genes to report on, reporter assays, assessing activity of the ERE, were employed. A plasmid-based luciferase reporter assay system did not show ERE responsiveness following oestradiol treatment (1-1000nM) in abdominal or gluteal MBDFAT preadipocytes (Figure 4.10A). To confirm functionality of the assay, MCF7 breast cancer cells were similarly transfected with the reporter plasmid, and showed an expected response (Figure 4.10A). This assay was repeated in PAT2 DFAT preadipocytes (a male depot-specific preadipocyte cell line), primary preadipocytes, and differentiated preadipocytes for each of these preadipocyte sources. No responses were noted in any of the transfected differentiated preadipocyte cells (data not shown). Although successful electroporation was confirmed through eGFP visualisation, given the higher molecular weight of the ERE reporter plasmid relative to the eGFP plasmid, and the documented difficulty with transfection of preadipocyte cell lines [434–436], selective transfection of the smaller eGFP plasmid was possible. Therefore, it was sensible to move to a system permitting the stable expression of reporter plasmid, using antibiotic selection to ensure positive selection of successfully transduced cells. MBDFAT preadipocytes were transduced with a lentiviral reporter vector, containing an ERE-TATA-Luc element, also conferring

puromycin resistance, to allow for antibiotic-mediated cell selection. Following selection, the MBDFAT stable reporter cell line, tested as both preadipocytes and differentiated preadipocytes, were treated with a range of oestradiol concentrations (1nM-1uM), an ER agonist (PPT), an ER antagonist (fulvestrant), and an aromatase inhibitor (letrozole). ER antagonists were used to elucidate any baseline receptor activation which may increase ERE signal without extrinsic application of oestradiol. Aromatase inhibitors were similarly used to assess whether aromatase was contributing to a baseline oestradiol response. Luciferase signal for the reporter cells was not different from that of the empty vector control in MBDFAT preadipocytes (Figure 4.10B) or MBDFAT differentiated preadipocytes (Figure 4.10C). MCF7 lysate in these plates confirmed reagent functionality, but a positive MCF7 control stable cell line was not generated to confirm reporter functionality, leaving the possibility that the reporter construct was non-functional.

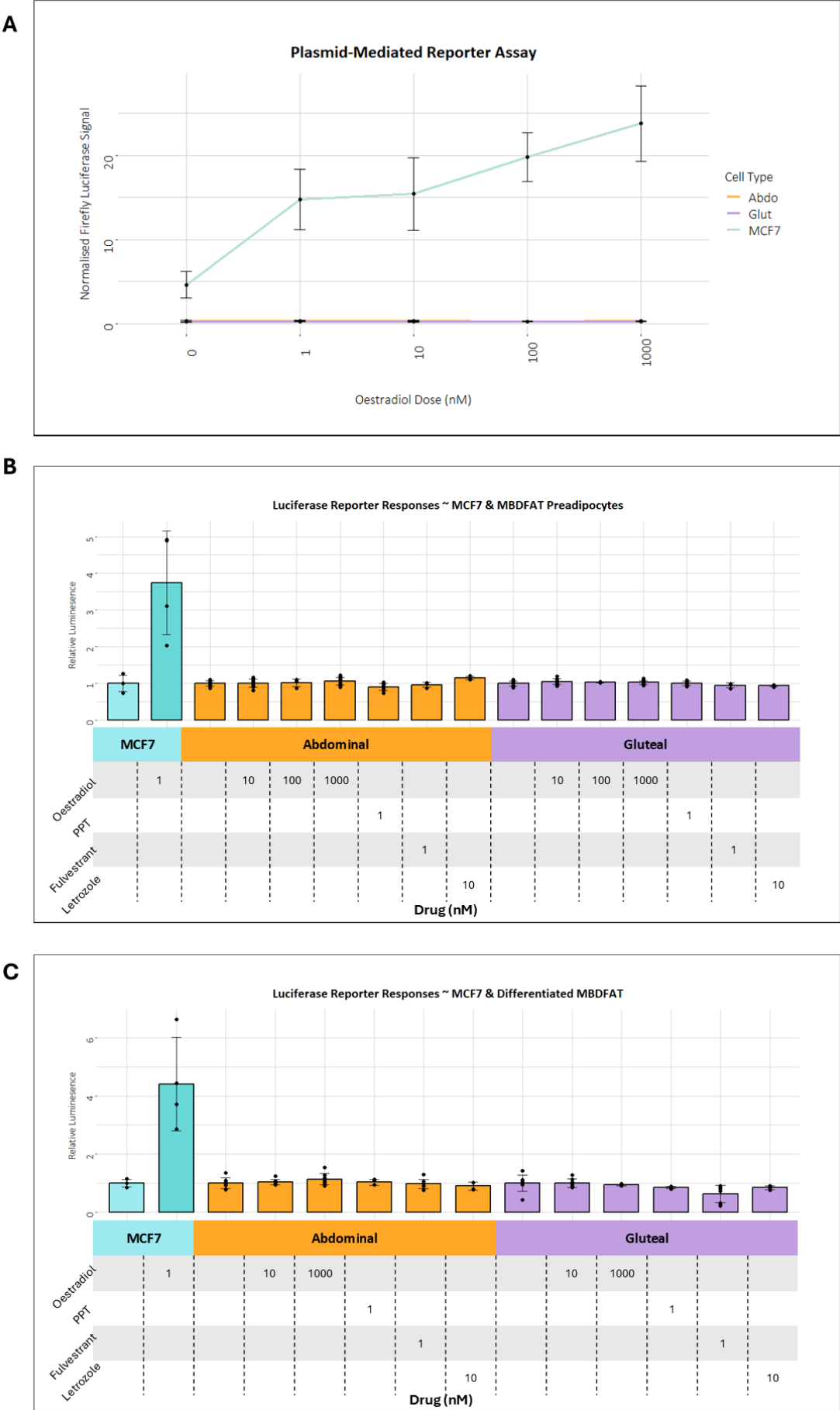


Figure 4.10: **A)** Dose response to oestradiol in the plasmid-based ERE-TATA-Luc reporter system, in MCF7 cells and abdominal and gluteal MBDFAT preadipocytes ($n=3$). Doses from 0-1000nM oestradiol were used. Data points are presented as mean values, with standard deviation presented as error bars. **B-C)** Response to oestradiol in the lentiviral-based ERE-TATA-Luc reporter system, in MCF7 cells, abdominal and gluteal MBDFAT cells ($n=3$), across a range of chemicals which modulate oestradiol responses. This included (left to right, per group) oestradiol doses (1-1000nM), PPT (1nM, ER α agonist), fulvestrant (1nM, ER antagonist), letrozole (10nM, aromatase inhibitor). Doses and chemicals are indicated on the lower axes. 4 technical replicates were carried out per condition. Bars show mean group values. Individual data points are plotted in black. Error bars show standard deviation. For MBDFAT stable reporter cells, values were not different from the negative control empty vector cells (not presented). Data from MCF7 cells are shown in blue, abdominal MBDFAT reporter cells in orange, and gluteal MBDFAT reporter cells in purple. Values for each reading were subtracted from the blank control well reading, then normalised to the control treatment value for each cell type. **B)** Assay conducted in MCF7 (plasmid transfected cells) and stable reporter MBDFAT preadipocytes. **C)** Assay conducted in MCF7 (plasmid transfected cells) and stable reporter MBDFAT differentiated preadipocytes.

4.3.6 The Oestrogen-Induced Transcriptome in MBDFAT Preadipocytes is Depot-Dependent

To gain a more comprehensive understanding of the transcriptional responses of MBDFAT preadipocytes to oestradiol, bulk RNA sequencing was employed. This powerful global approach allowed for the exploration of depot-specific differences in gene expression and oestradiol responsiveness, rather than limiting the analysis to individual depot comparisons. By capturing this broader transcriptomic landscape, it was possible to specifically assess how oestradiol affects preadipocytes from the two depots, differently. This is important given that, physiologically, these depots respond in different ways to oestradiol, with oestradiol increasing gluteal adiposity, and suppressing the expansion of abdominal adipose tissue. Using this approach facilitates the identification of genes that may be regulated in opposite directions in each depot, by oestradiol, which are statistically more likely to be flagged using these methods, as compared to analysis of a single depot.

Baseline comparisons between the depots, analysing transcriptional differences between abdominal and gluteal MBDFAT preadipocytes, highlighted distinct transcriptional landscapes. Notable top hits in gluteal preadipocytes included

genes that have been associated with oestrogen responses elsewhere, such as *HOTAIR*, *KLF14*, and *LITAF* [437, 438], as well as genes involved in lipid storage and adipogenesis, such as *TPD52* [439] and *PIEZO2* [440] (Figure 4.11A). Importantly, 56% of the DEGs identified in the scRNASeq dataset (described in Section 3.3.2), between abdominal and gluteal preadipocytes, were also differentially expressed between abdominal and gluteal MBDFAT preadipocytes (Figure 4.11B), demonstrating that the identified DEGs are reflective of real depot DEGs. Different sequencing depths and filtering steps likely contribute to the surplus of genes seen with bulk sequencing (right hand side of Venn). Between abdominal and gluteal MBDFAT preadipocytes, there was also notable enrichment for steroid synthesis pathways in the gluteal depot, relative to the abdominal depot, as well as pathways relating to antigen presentation and inflammation (Figure 4.11C).

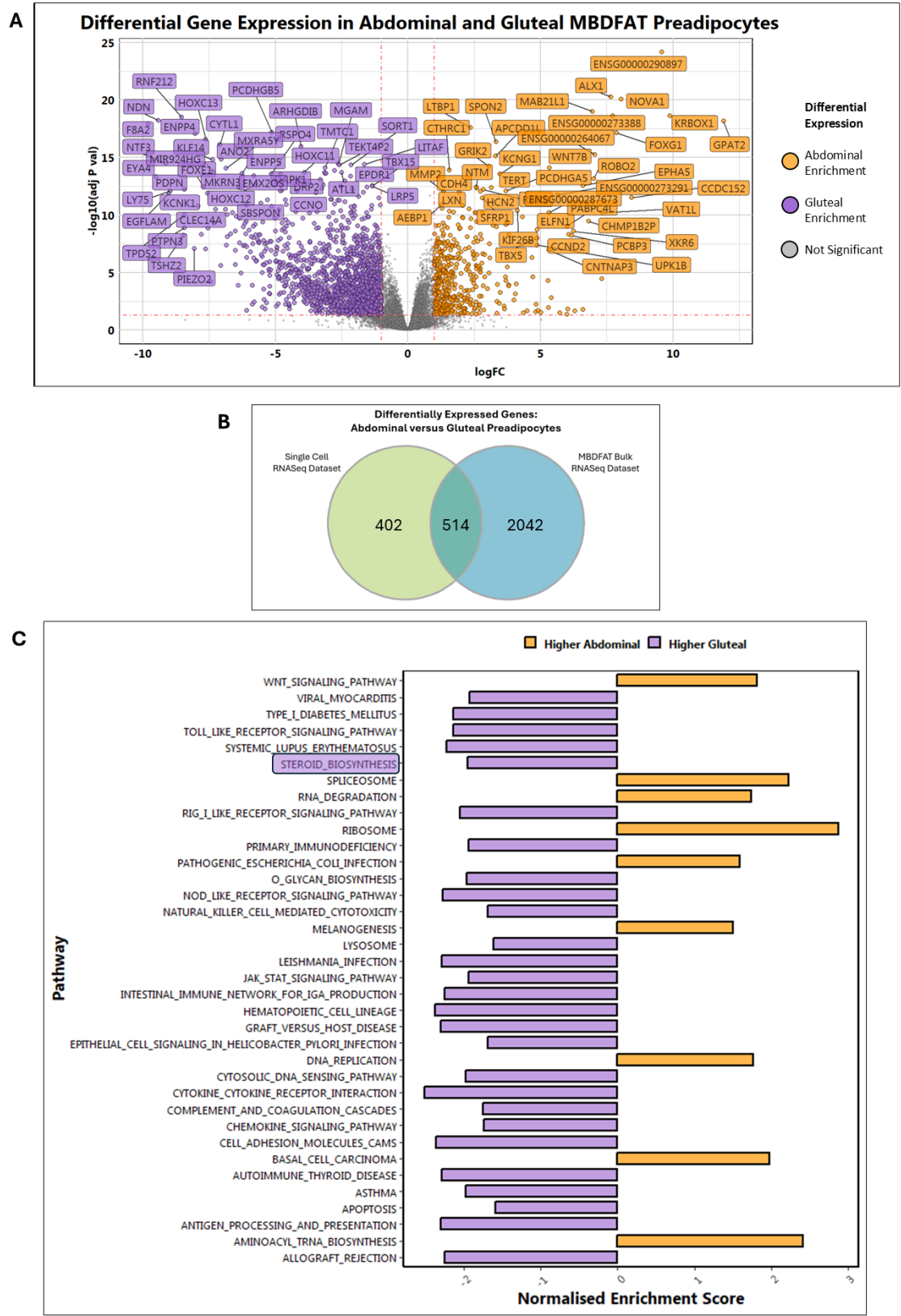


Figure 4.11: **A)** Volcano plot illustrating differentially expressed genes between abdominal and gluteal MBDFAT preadipocytes ($n=3$). Log fold change ($\log FC$) in gene expression is shown on the x-axis, and $-\log(\text{adjusted } p \text{ value})$ is presented on the y-axis. Thresholds of $\log FC = 1$ and adjusted $p\text{-value} < 0.05$ were applied, so that only points fulfilling these criteria are coloured. Positive log fold change indicates higher expression in abdominal preadipocytes, while negative log fold change indicates higher expression in gluteal preadipocytes. This is also illustrated by point colour, as indicated in the graph legend. **B)** Venn diagram showing the overlap of abdominal versus gluteal preadipocyte DEGs, comparing genes identified in the scRNASeq dataset, with those identified in the bulk RNA sequencing of MBDFAT preadipocytes. An adjusted p value threshold of 0.05 was applied to the gene sets. **C)** Pathway enrichment comparing untreated abdominal and gluteal MBDFAT preadipocytes ($n=3$), cultured for 48 hours. Pathways shown are only those surviving adjusted p value thresholds < 0.05 . Positive enrichment scores indicate pathways enriched in abdominal preadipocytes. Negative enrichment scores indicate pathways enriched in gluteal preadipocytes. This is further shown by bar colour, as indicated in the graph legend.

Further investigating the specific steroid hormone biosynthesis pathway differences between abdominal and gluteal MBDFAT preadipocytes revealed differences in expression at many stages of the steroid biosynthesis pathway (Figure 4.12). Gluteal preadipocytes showed enrichment of pathways for the synthesis and inter-conversion of steroid hormones, meanwhile abdominal preadipocytes showed upregulation of pathways relating to progesterone metabolism.

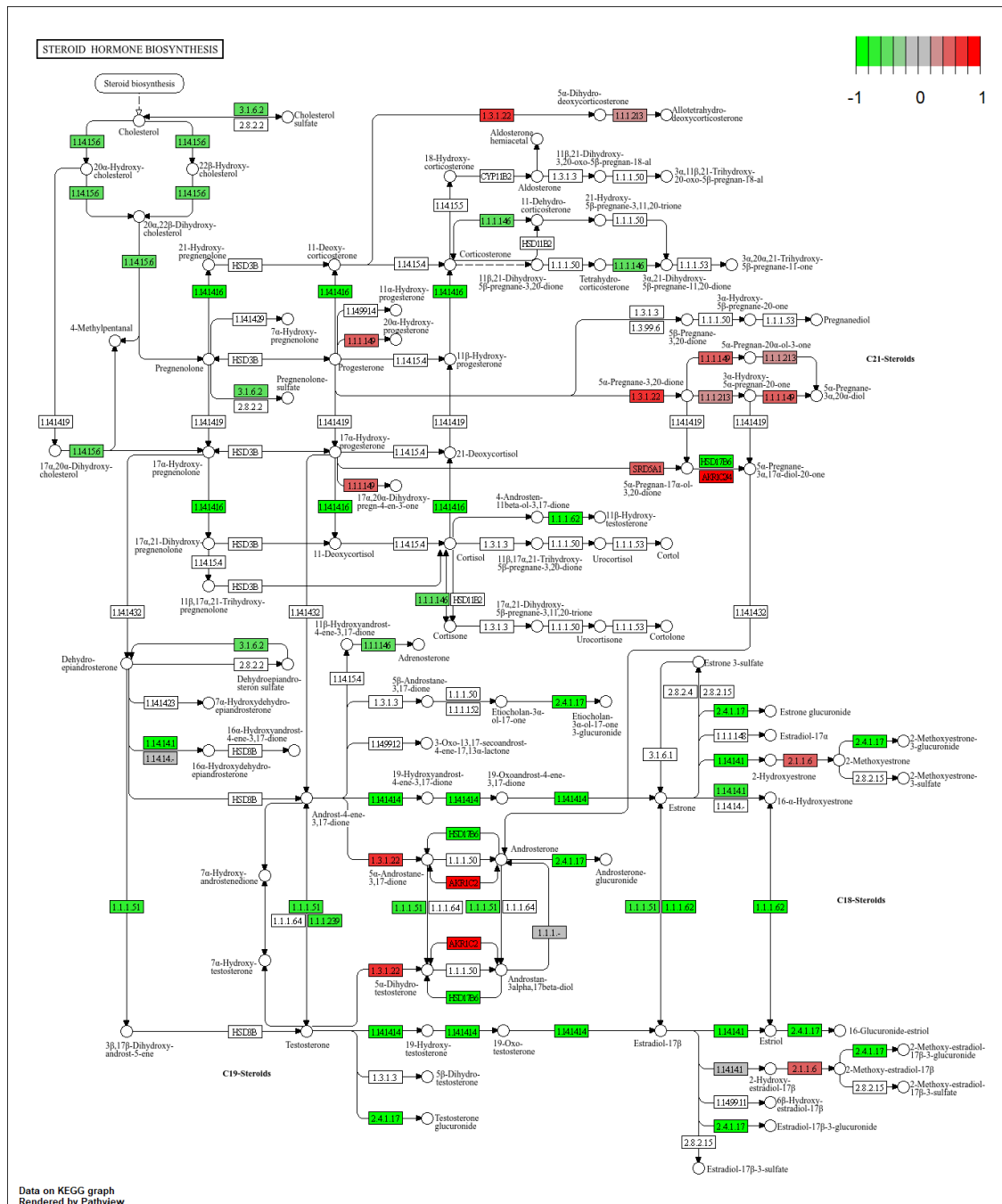


Figure 4.12: Changes in gene expression in abdominal compared to gluteal MBDFAT preadipocytes, for KEGG steroid biosynthesis pathways. Abdominal enriched genes are shown in red, and gluteal enriched genes are shown in green. FGSEA was conducted on DEGs from abdominal compared to gluteal MBDFAT preadipocytes, and Pathview analyses are used here to visualise DEGs, specifically looking at those involved in steroid hormone biosynthesis.

Subsequently, the abdominal and gluteal preadipocyte comparison was run, dividing by treatment-specific transcriptional responses. This largely revealed genes

that were affected by oestradiol to a small extent, in the opposite direction in abdominal and gluteal depots, thereby bringing out these depot-differences, in the context of oestradiol treatment. 1512 depot DEGs were differentially expressed with both oestradiol and vehicle treatment, representing a ‘core’ set of depot-specific genes (Figure 4.13A). 571 DEGs were only differentially expressed with oestradiol treatment, and 473 were only differentially expressed with vehicle treatment. Gene set enrichment analyses (GSEA) on stratified gene sets revealed pathways that were enriched for the 571 genes that were differentially expressed uniquely with oestradiol treatment, and no pathway enrichment for the vehicle-control-associated genes. These pathways included enrichment for glycolysis in abdominal preadipocytes and late oestrogen response in gluteal preadipocytes (Figure 4.13B). Top hits included *SCD5* and *METRNL*, which were differentially expressed with oestradiol treatment in gluteal and abdominal preadipocytes respectively (Figure 4.13C). *SCD5* has been implicated in adipocyte differentiation and lipid storage [134], while *METRNL* has a role in adipose tissue beiging and insulin sensitisation [441]. These results suggest that oestradiol responses in adipose tissue are not uniform but may be partly intrinsically specific to the depot from which the cells originate.

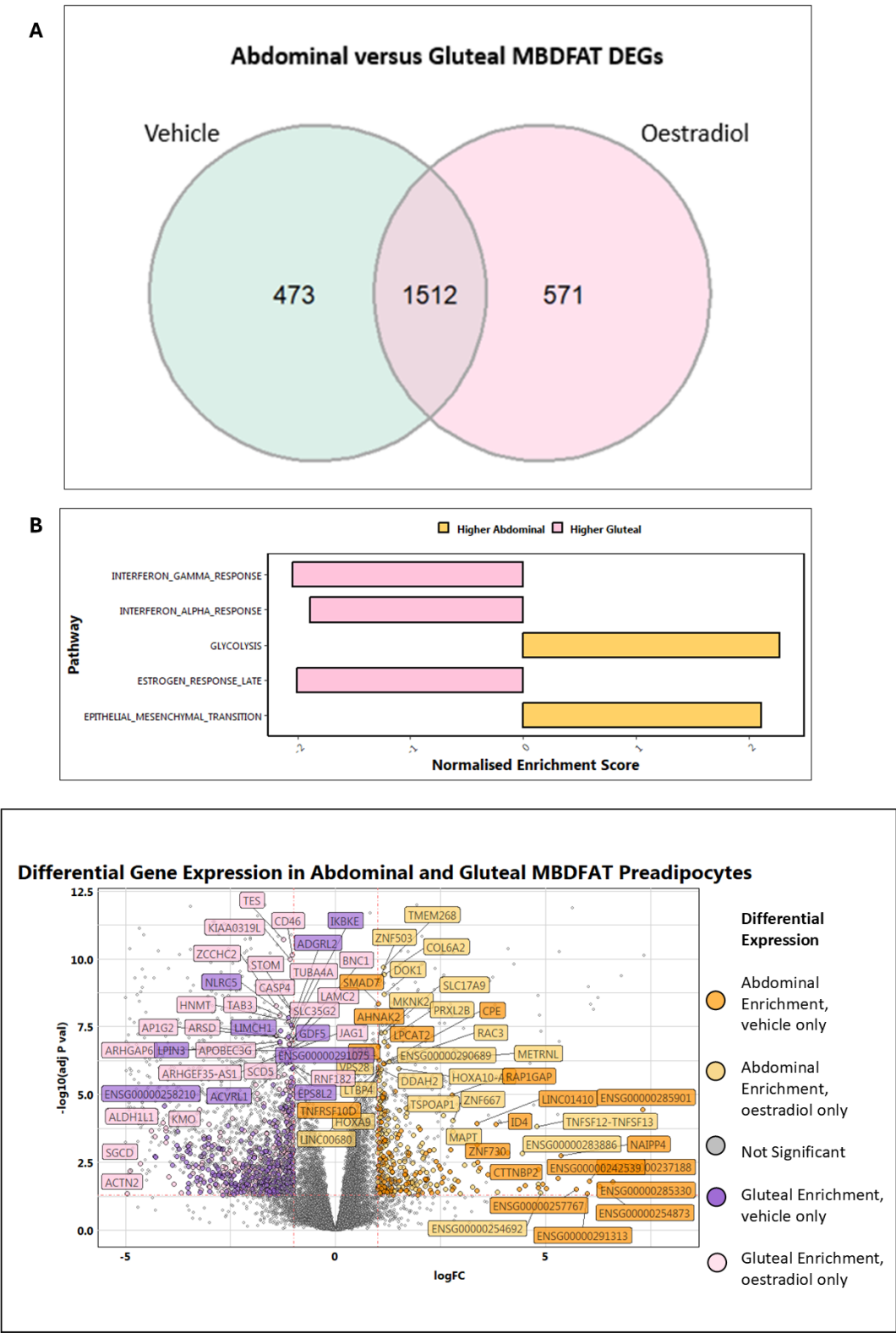


Figure 4.13: **A)** Significantly differentially expressed genes (adjusted p value < 0.05) between abdominal and gluteal MBDFAT preadipocytes, split by oestradiol treatment. MBDFAT preadipocytes were treated with 100nM oestradiol for 48 hours ($n=3$). Genes that are differentially expressed between abdominal and gluteal preadipocytes across both conditions are presented in the centre of the Venn diagram. Genes exclusive to each treatment are presented on the right and left sides of the Venn diagram, for vehicle control and oestradiol treatment, respectively. **B)** Pathway enrichment for the 571 genes differentially expressed only with oestradiol treatment. Only pathways surviving an adjusted p value threshold < 0.05 , are presented. **C)** Volcano plot, overlaying differentially expressed genes between abdominal and gluteal MBDFAT preadipocytes. Only genes that were uniquely differentially expressed (either with vehicle or oestradiol treatment) are coloured. Genes that would present in the centre of the preceding Venn diagram are not coloured, highlighting genes that are specific to treatments. Log fold change ($\log FC$) is presented on the x -axis and $-\log(\text{adjusted } p \text{ value})$ is presented on the y -axis. Only points with a log fold change > 1 and adjusted p value < 0.05 are coloured.

Directly comparing oestradiol-treated MBDFAT preadipocytes (from either depot) to vehicle-control treated cells, revealed very few differentially expressed genes after applying an FDR adjustment threshold of 0.05, highlighting the need for depot-specific comparisons. In abdominal preadipocytes, for example, there was only one transcript that was significantly upregulated with oestradiol treatment (ENSG00000258210). When running GSEA analysis (using Hallmark and KEGG pathways), which uses the t -statistic output from differential testing results, however, pathways were significantly affected by oestradiol treatment, even with application of adjusted p value thresholds, suggesting that processes were differentially regulated between conditions, but the statistical power may not have been sufficient to detect these changes at the individual gene level. In abdominal preadipocytes several processes were lower with oestradiol treatment (Figure 4.14A). These included pathways relating to inflammatory signalling (TNF- α signalling, interferon alpha signalling, inflammatory response, IL-2 signalling) and hormone responses (early oestrogen response, androgen response). Meanwhile, gluteal preadipocytes upregulated pathways relating to oxidative phosphorylation and ribosomes in response to oestradiol (Figure 4.14B). These results suggested that oestradiol responses are present in these systems, but that the statistical power of the dataset may not be sufficient to uncover these in themselves.

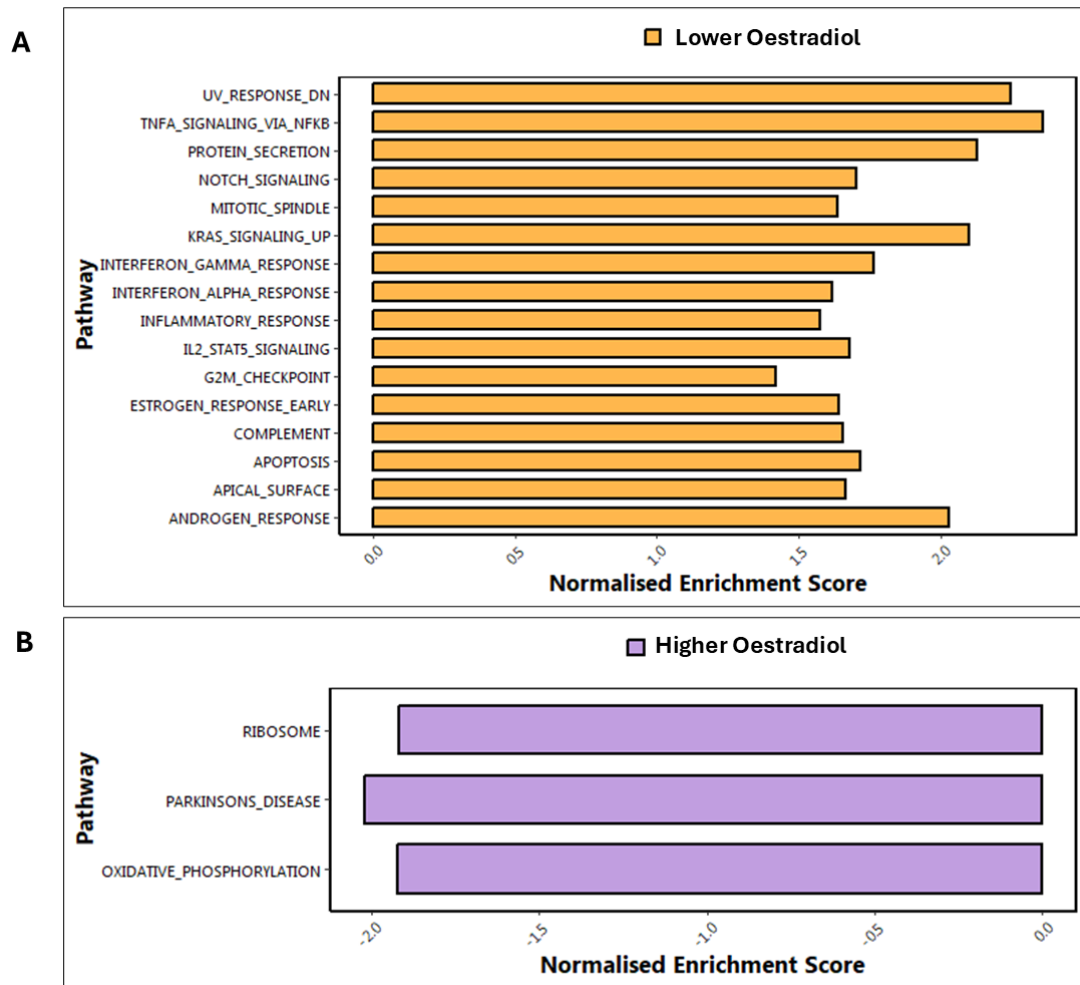


Figure 4.14: A-B) Pathway enrichment for MBDFAT preadipocytes treated with 100nM oestradiol for 48 hours, compared to vehicle treated cells ($n=3$). Pathways shown are only those surviving adjusted p value thresholds < 0.05 . Positive enrichment scores indicate pathways that were lower with oestradiol treatment, while negative enrichment scores were those that were increased by oestradiol treatment. Bar colour is indicative of which depot the pathways are enriched in (orange = abdominal, purple = gluteal) **A)** Pathways in abdominal MBDFAT preadipocytes **B)** Pathways in gluteal MBDFAT preadipocytes.

4.4 Discussion

4.4.1 Oestrogen Receptor Expression and Localisation

This work confirmed the expression of *GPER1*, *ESR1*, and *ESR2* in adipose tissue, further establishing the unique presence of *ESR2* in mature adipocytes, and not preadipocytes. Notably, *GPER1* emerged as the most abundantly expressed ER in adipose tissue SVF, a finding supported by GTEx expression data, reporting higher

expression of *GPER1*, compared to *ESR1*, in subcutaneous adipose tissue (reported as 15.25 versus 2.07, respectively) [442]. These results highlight the overlooked role of *GPER1* in previous studies, which primarily focus on ER- α activity in adipose tissue, investigating its genomic effects rather than the rapid signalling cascades mediated by GPER1. The short half-life of GPER1 (approximately 30 minutes) [443] compared to ER- α (approximately 6 hours) [444], might partly explain differences in transcript abundance, with GPER1 likely having a higher receptor turnover rate. Furthermore, expression of ER- α varies with oestradiol treatment, through changes in receptor phosphorylation, which subsequently regulates proteasome-resistance and therefore stability [444]. This may preserve protein expression levels without altering transcript abundance. In-vivo transcriptomic data, therefore, may not provide an accurate comparison of ER expression, given the differences in receptor dynamics.

Subcellular ER localisation has not been widely reported on in adipose tissue. These data demonstrate that ER- α localisation is predominantly nuclear. While this might suggest active ligand-bound receptors that have translocated to the nucleus as part of traditional hormone responses, studies have reported the nuclear expression profile of ER- α may remain nuclear, independent of hormone stimulus [445]. GPER1 staining was more diffusely distributed across subcellular compartments in preadipocytes, likely reflecting its ability to localise to multiple cellular organelles including the plasma membrane, golgi, endoplasmic reticulum, and the nucleus [417, 446, 447]. The specific location of GPER1 has been suggested to depend on cellular resting state and the temporal kinetics of receptor translocation [446], but the functional implications of GPER1 localisation are not yet understood [446].

In immune populations, *ESR1* was expressed in anti-inflammatory resident macrophage populations (PVMs & LAMs), and not in the more inflammatory populations, supporting data showing that ER- α expression decreases in response to M1 stimuli [216]. This may allow anti-inflammatory macrophages to better respond to oestradiol, subsequently exerting additional anti-inflammatory effects. GPER1 was less abundantly expressed in immune populations, while *ESR2* was largely localised to

natural-killer cell, and T-cell populations. Differences in relative receptor expression in cell types across the SVF may be responsible for differing responses to oestradiol.

4.4.2 Oestradiol Metabolism in Adipocytes and Preadipocytes

Having established the presence of ERs in the proposed model system, and in cell types across adipose tissue SVF, the production and metabolism of oestrogens was subsequently assessed. Differential production and metabolism of oestrogens between abdominal and gluteal depots was evident in both preadipocytes and differentiated preadipocytes. Preadipocytes produced higher levels of oestradiol, without additional oestradiol treatment, and produced higher levels of oestrone after treatment with oestradiol, as compared to differentiated preadipocytes. The differentiated preadipocytes/mature adipocytes may therefore be acting to metabolise or store oestradiol, consistent with the hypothesis of a local endocrine loop whereby preadipocytes produce oestradiol, which may be further metabolised by mature adipocytes. Gene look-ups in the fractionated tissue dataset revealed that relevant oestradiol metabolising genes (e.g. *CYP1B1*, *COMT*, *SULT1E1*, *ABCB1*) were not upregulated in mature cells relative to SVF. This suggested that mature cells were more likely acting as an oestradiol sink, internally storing the hormone, rather than actively metabolising it. Oestradiol storage, in the form of fatty acid esters, has been shown to increase with oestradiol concentration in both visceral and subcutaneous adipose tissues [448, 449], as investigated across states of differential female serum hormone profiles (pregnancy, pre-menopause, early post-menopause, late post-menopause). Interestingly, the esterification rate for oestradiol is greater than that of dehydroepiandrosterone (DHEA) and cholesterol, while the hydrolysis rate of these esters was much slower, suggesting that adipose tissue may act to selectively sequester free oestradiol [450]. The subsequent release of oestradiol partially depended on hormone-sensitive lipase activity, suggesting that intra-cellular oestradiol storage and release is regulated dynamically in relation to triglyceride storage [450]. Given the slower lipolytic turnover of gluteal adipose tissue, relative to abdominal adipose tissue, it is likely that gluteal adipose tissue is able to sequester

more local oestradiol, further exacerbated by the heightened oestradiol production in this depot. Still, there is very little understanding of the physiological implications of intra-tissue oestradiol production and storage, particularly concerning how this may differ from externally-derived oestradiol in adipose tissue. Further research is therefore necessary to investigate these grounds.

Oestrone production increased with oestradiol treatment across all conditions. Yet, expression of the 17β HSD2 enzyme, which is the primary enzyme permitting the conversion of oestradiol to oestrone, was low across all samples. This suggested that oestrone production may be mediated by alternative pathways. This could include the aromatisation of androstendione to oestrone, through CYP19A1 activity, or the conversion of oestrone-sulphate to active oestrone, through steroid sulphatase (STS) activity. The activity of alternative steroid-metabolising enzymes could also contribute to this process. A summary of these pathways is shown in Figure 4.15. Given that oestrone production was higher in gluteal, relative to abdominal samples, it seems plausible that the increased oestrone production may be mediated by aromatase (CYP19A1) activity, whose expression is higher in gluteal relative to abdominal adipose tissues. However, since oestradiol is not known to stimulate aromatase activity, further research is needed to elucidate the mechanism behind this increased production and its physiological implications.

4.4.3 Preadipocyte Functional Responses to Oestradiol

Despite the observed depot differences in oestradiol production and metabolism, functional oestradiol responses in preadipocytes were less clear. Proliferation assays, response gene data, and ERE reporter assays were unable to highlight oestradiol responses in MBDFAT preadipocytes. These results might indicate that cultured MBDFAT preadipocytes do not rely on classical ER- α -mediated genomic responses, and instead primarily utilise non-genomic pathways, for example through GPER1, to mediate oestradiol signalling; consistent with the finding of abundant GPER1 expression. Still, the interpretation of these results is somewhat clouded by the lack of literature highlighting specific oestradiol response genes, the difficulties

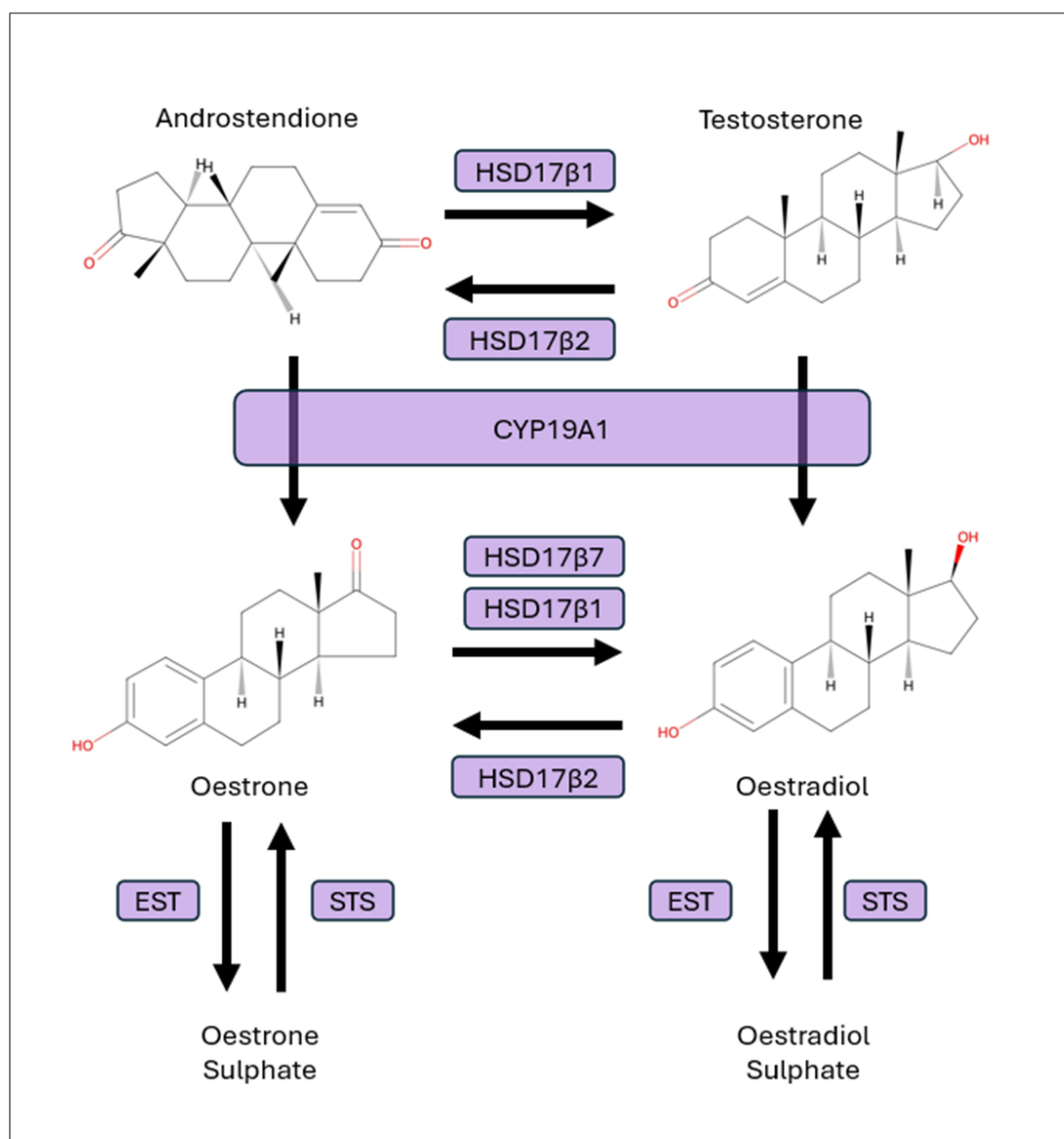


Figure 4.15: Schematic illustrating oestradiol metabolism pathways and relevant enzymes involved in these processes.

associated with transfecting preadipocytes, and also the lack of positive control data for the lentiviral mediated reporter assay. For example, the investigated oestradiol response genes (*B4GALT1*, *VEGFA*, *MYC* and *IGFBP3*) were not responsive to oestradiol, or differentially expressed between depots, in the bulk RNA sequencing data, highlighting the lack of literature in this field. Alternatively, there may be specific co-factors, necessary for oestradiol-mediated genomic signalling (e.g p160 steroid co-activators [451]), which may not have been abundant in the tested

culture system, or there may have been increased abundance of co-repressors (e.g. NCoR, SMRT [451]). The factors needed for oestradiol responses may be reliant on other cell types which are known to respond to oestradiol, such as macrophages [386], which may exert indirect, secondary effects of oestradiol at preadipocytes, as speculated in Section 1.5.3.

Other genes, such as *ESRRs* may also be important, specifically in the context of depot-specific physiological oestradiol responses. There is scarce literature on the effects of these genes in adipose tissues, yet *ESRRA* and *ESRRG* were lowly expressed in adipose tissue. Fractionated tissue data and scRNASeq data showed that *ESRRA* was expressed in the SVF, but did not show depot differences. Meanwhile *ESRRG* was expressed only in mature adipocytes, and was significantly higher in gluteal cells, as compared to abdominal cells ($p = 8.48 \times 10^{-6}$), suggesting that this may play a role in depot-specific oestradiol responses. Still, there is very little literature exploring the role of *ESRRG* in adipose tissue, and research that does exist largely relates to brown adipose tissue thermogenesis [452, 453], making it difficult to interpret the downstream impacts of differential depot expression of *ESRRG*. Given that *ESRRG* was only expressed in mature cells, it was also unlikely to be mediating the hyperplastic effects of oestradiol that are observed in-vivo. Again, this suggested a need to investigate other cell types within adipose tissues, generally excluded from in-vitro systems, that may provide the missing link in oestradiol signalling, explaining the discrepancies of effects seen in-vitro versus in-vivo.

4.4.4 Preadipocytes Show Depot-Specific Transcriptional Responses to Oestradiol

Overall, depot-differences between abdominal and gluteal MBDFAT preadipocytes were suggestive of gluteal enrichment for inflammation-related pathways and steroid hormone biosynthesis pathways. Investigating some of the gene sets involved in the gluteal enriched inflammatory pathways, suggested that this depot may be enriched for an ‘immunological signature’, which may not necessarily be conducive to inflammation. In the ‘cytokine receptor interaction’ pathway, for example,

several anti-inflammatory cytokines, or context-specific cytokines are enriched, including *CSF1* (*M-CSF*), *CXCL10*, *CXCL12*, *IL4R*, *IL20RB*, *IL19*, *IL10RB*, amongst others. Pathways relating to cell adhesion molecules, asthma, autoimmune thyroid disease, and several other inflammation-related pathways supported this conclusion, showing enrichment of immunological signatures which may not directly relate to inflammation, potentially suggesting an enrichment for preadipocyte communications with immune cells.

Also of relevance was the steroid hormone biosynthesis pathways enriched in gluteal preadipocytes, including genes such as *DHCR24*, *CEL*, *LIPA*, *HSD17B7*, *LSS*, *TM7SF2*, *FDFT1*, *MSMO1*, *DHCR7*, *SC5D*, *SOAT1*, *CYP27B1*, *UGT1A7*, *STARD8*, *FOXO1*, *CYP26B1*, *CYP21A2*, *CYP11B-AS1*, *CYP27B1* and *CYP19A1*, which largely relate to cholesterol synthesis and steroid hormone inter-conversion. Of particular note are *HSD17B7* and *CYP19A1*, which are responsible for conversion of oestrone and testosterone to oestradiol (respectively). Oestradiol treatment can stimulate *HSD17B7* expression, subsequently contributing to a feed-forward mechanism which amplifies oestradiol production [454]. This loop may operate in gluteal preadipocytes, whereby aromatase-mediated production of oestradiol stimulates the expression of *HSD17B7*, further increasing oestradiol production through the conversion of oestrone, with oestrone production also being elevated by the conversion of androstendione to oestrone by aromatase. This would result in a high basal production of oestradiol by preadipocytes, potentially mitigating the effect of externally applied oestrogens; the intrinsic production and sequestration of oestrogens may be internally activating oestradiol receptors, contributing to a high basal tone of oestradiol response. Most work within this field, however, is targeted at the external application of oestrogens, and has not yet investigated the relative contributions of internal hormone production. Intrinsic, concentrated cytoplasmic oestradiol production may be better located to mediate effective hormone responses, and may be further important for determining depot-specific oestradiol responses, which transcriptomically become evident when combining depot- and oestradiol-specific comparisons.

Indeed, when splitting the depot DEGs by treatment, pathway enrichment suggested a depot-specific effect of oestradiol to reduce gluteal inflammation. With oestradiol treatment, inflammatory and interferon-related pathways were higher in abdominal, relative to gluteal, preadipocytes.

These depot-differences were also evident on the individual gene level. When comparing oestradiol-treated preadipocytes, genes including *SCD5*, which promotes lipid storage, were enriched in gluteal preadipocytes, while *METRNL*, which decreases lipid storage, was enriched in abdominal depots.

The transcriptional responses to oestradiol only became apparent when comparing depots - this may explain some of the previous negative experimental findings, highlighting the need to study oestradiol in a depot-specific context in adipose tissues. It also suggested that the direct effects of oestradiol are likely subtle in preadipocyte in-vitro monoculture systems, and may require additional tissue factors to be better elucidated. Despite the small changes in gene expression in response to oestradiol, there were stark differences in pertinent oestradiol-responsive genes (e.g. *HOTAIR*) and oestradiol production, between abdominal and gluteal tissues, highlighting the need for future work to establish to what extent depot-differences in gene expression are driven by differences in depot-production of oestradiol. As local oestradiol production likely influences whole tissue biology, and not just the preadipocytes residing within adipose tissue, attention was subsequently aimed at addressing how other cells within adipose tissue may be responding to oestradiol. As previously mentioned, macrophages are distinctively polarised by oestradiol treatment [212], and may be responding to the production of oestradiol by preadipocytes. This interaction could therefore alter macrophage polarisation in a depot-specific manner (as a product of depot-differences in oestradiol production) and subsequently impact the tissue's inflammatory microenvironment, potentially affecting preadipocyte and adipocyte health, expansion, and turnover. The following chapter will seek to establish how interactions between macrophages and preadipocytes may be modulating depot-specific biology, particularly in the presence of oestradiol.

5

Preadipocyte-Macrophage Crosstalk

5.1 Introduction

5.1.1 Macrophages in Adipose Tissue Inflammation

Macrophages are essential regulators of adipose tissue homeostasis, mediating diverse functions such as inflammation and extracellular matrix (ECM) turnover. Increasing evidence highlights the importance of interactions between macrophages and adipocytes, particularly in the context of obesity-induced adipose tissue inflammation [86]. In this setting, macrophages play a pivotal role in chronic inflammation through the secretion of pro-inflammatory cytokines. Reciprocal interactions with adipocytes likely aggravate this inflammation, as adipocytes secrete inflammatory mediators, such as IL-6 and MCP-1 in response to macrophage-derived cytokines, further contributing to increased inflammatory monocyte recruitment, inflammatory polarisation, and an enhanced inflammatory milieu, creating a vicious cycle that maintains a chronic inflammatory environment in the context of adipose tissue inflammation.

The majority of current literature investigating the crosstalk between macrophages and adipocytes or preadipocytes, has focused on solely measuring inflammatory markers as the investigative output [95, 455], rather than analysing other relevant molecular players that may be modulating tissue metabolism. By primarily targeting

research towards inflammatory processes, there is a gap in understanding as to how specific molecular signalling between macrophages and adipocytes may contribute towards other aspects of tissue homeostasis, such as proliferation and expansion, despite abundant evidence that macrophages have roles in these processes [456]. This physiological signal exchange is especially important in adipose tissue, where macrophages exhibit a unique spectrum of functional heterogeneity, which cannot be broadly classified by traditional M1- and M2- phenotypes, even in circumstances of diet-induced obesity and chronic inflammation [457–459]. This is of particular relevance in humans systems, where murine macrophage population markers are often presumed to be conserved in human macrophage populations. Existing literature suggests that this might not be the case, since well-characterised murine markers of anti-inflammatory macrophages, such as CD206, do not translate to human differences in inflammatory macrophage abundance in certain depots [30]. Thus, there is a need to examine the depot-specific microenvironmental niches of co-culture systems, expanding these analyses beyond just inflammatory profiling.

5.1.2 Limitations of Existing Co-Culture System Studies

Furthermore, existing co-culture systems that are used to study macrophage-adipocyte interactions may inadvertently skew the understanding of this crosstalk, especially in the context of inflammation. Most studies rely on transwell or conditioned media systems, which tend to bias macrophages toward a more M1-like, pro-inflammatory phenotype [460], potentially amplifying the observed inflammatory signalling between adipocytes and macrophages. However, studies utilising direct-contact co-culture systems tend to establish anti-inflammatory environments [183, 461], suggesting that physical contact between macrophages and adipocytes is essential for mediating the beneficial functions of adipose tissue macrophages. This is likely due to the macrophages' roles in the clearance of dead or dying adipocytes, during which they secrete pro-adipogenic factors that limit inflammation and promote tissue regeneration [462]. Moreover, non-inflammatory uptake of lipid and cellular debris from apoptotic adipocytes by macrophages has been shown to

promote *PPARG* expression in macrophages [462, 463], which is further associated with anti-inflammatory macrophage polarisation and healthy adipocyte adipogenesis [462, 463]. These findings underscore the importance of local cellular interactions in fostering anti-inflammatory and metabolically advantageous tissue responses. Hence, developing a direct-contact co-culture system to explore the molecular mechanisms underpinning adipocyte-macrophage communication is critical for understanding adipose tissue function in both physiological and pathological contexts.

Most co-culture studies to date have employed differentiated adipocytes rather than preadipocytes in their systems, which limits the ability to explore how hyperplastic tissue expansion may be modulated by interactions with macrophages. Hyperplasia plays a key role in adipose tissue expansion and health, and macrophages are known to regulate the secretion of growth factors that influence this process. While some recent studies using single-cell RNA sequencing (scRNASeq) data have provided insights into how molecular signals between SVF populations change with obesity [279], there has been little effort to functionally characterise these signals. This has created a gap between bioinformatics-based hypotheses regarding signal exchange and the limited functional understanding offered by traditional tissue culture approaches, which predominantly focus on inflammation.

5.1.3 Depot-Specific Macrophage Functions

Depot-specific differences in macrophage infiltration and polarisation have been reported in the literature, particularly in relation to abdominal subcutaneous and visceral adipose tissue depots [22]. Studies suggest that visceral macrophages adopt a more pro-inflammatory phenotype, which may impair neighboring adipocyte function through specific signalling pathways [22]. However, research characterising the differences in cellular communication between abdominal and gluteal macrophage populations remains sparse, both in the context of inflammation, and also in the context of physiological signal exchange. As these depots are oppositely associated with metabolic risk, it is pertinent to establish how the residing macrophage

populations contribute to these depot-specific metabolic phenotypes, especially when considering their roles in physiological tissue homeostasis, turnover and expansion.

5.1.4 Oestradiol and Depot-Specific Adipose Tissue Function

Another key consideration when investigating depot-specific signal exchange, is oestradiol. Oestradiol is known to promote gluteal adipose tissue expansion, and to increase anti-inflammatory signatures in macrophages [386]. Macrophages show additional, metabolically relevant responses to oestradiol, such as lowered cholesterol content, and increased cholesterol efflux [464], which likely reduces their inflammatory potential further, and promotes healthy tissue responses. Murine studies using fat grafts further underscore the metabolic benefits of oestradiol, as the absence of this hormone leads to increased macrophage infiltration, adipocyte hypertrophy, and fibrosis of tissue grafts [465]. These responses are characteristic of obesity-associated adipose tissue inflammation and metabolic dysfunction [465]. These findings suggest that oestradiol plays a protective role in adipose tissue by reducing hypertrophy and inflammation.

In the gluteal depot, preadipocytes produce higher levels of oestradiol compared to their abdominal counterparts, creating a local microenvironment that is rich in oestrogens, as discussed in Section 4.3.2. Given the central role of macrophages in adipose expansion, for example through their impacts on progenitor cell proliferation [178], and their beneficial responses to oestradiol [386], it is pertinent to examine the effects of co-culturing macrophages and gluteal preadipocytes, where local oestradiol production by preadipocytes may polarise neighbouring macrophages. It's subsequently of interest to assess whether their responses may contribute to oestradiol-driven depot-specific tissue expansion, specifically through the release of signals that promote adipogenesis or proliferation. Understanding the oestradiol-mediated interactions between macrophages and preadipocytes is therefore essential for elucidating the mechanisms behind sex-specific fat distribution, particularly in terms of promoting healthy, hyperplastic expansion in gluteal depots.

A significant limitation of current co-culture systems is their lack of physiological translatability. Most studies mitigate the importance of direct cell-cell contact by employing transwell or conditioned media approaches, which may not accurately reflect the in-vivo signalling landscape. Another critical factor often overlooked in these studies is the sex of the cells used, which is rarely reported. This is particularly important when investigating oestradiol responses in macrophages, as sex-specific differences in oestrogen receptor expression and inflammatory responses have been observed [196]. Additionally, comparisons of male and female macrophage responses to oestradiol reveal distinct metabolic phenotypes. For instance, oestradiol selectively reduces cholesterol ester content and lipid loading in female macrophages, but not in males [466], emphasising the need to consider donor sex in macrophage studies, and also to report this alongside the subsequent findings.

5.1.5 Chapter Aims

1. Establish a co-culture system between depot-specific preadipocytes and macrophages, to further the understanding of depot-specific macrophage functioning and how macrophages may play a role in adipose tissue expansion.
2. Investigate the transcriptional changes in preadipocytes, induced by co-culture with macrophages. Using bulk RNA sequencing allows an unbiased approach to assess these responses, moving beyond the inflammation-focused stance of the current literature.
3. Characterise the gene expression responses of co-culture systems to pertinent stimuli including oestradiol, and inflammation, mediated by lipopolysaccharide (LPS).
4. Explore whether gene expression changes induced by co-culture or these stimuli are likely to translate to altered preadipocyte-macrophage crosstalk, using a ligand-receptor interaction database, derived from a scRNASeq dataset of adipose tissue SVF.

5.2 Methods

5.2.1 Optimisation of Co-Culture Incubation

To explore crosstalk between preadipocytes and macrophages, co-culture systems were established, as described in Section 2.20. In brief, 130,000 preadipocytes were plated in per well in 6-well plates on D-1, with 100,000 human monocyte-derived macrophages (HMDMs), differentiated with 100 ng.mL⁻¹ macrophage-colony stimulating factor (M-CSF) for 7 days, subsequently added to the plates on D0. Preadipocytes and macrophages were plated at a density that resulted in a final ratio of 5:1 preadipocytes to macrophages. The differentiated macrophages do not proliferate in culture, and thus the initial seeding density was based on the MBDFAT preadipocyte doubling time over the course of the culture period for this experiment. A pilot co-culture experiment was conducted to evaluate the influence of co-culture incubation time, with cells being visually assessed and harvested for qPCR analysis at 24, 48, 72 and 96 hours co-culture incubation timepoints. Based on preadipocyte density, and expression of markers relating to inflammation and macrophage abundance, the 48-hour timepoint was selected. At this timepoint, preadipocytes were not overly confluent and macrophages remained viable. After 24 hours of co-culture, significant differences in gene expression, compared to the other later timepoints, were seen for several measured genes, suggesting that a period longer than 24 hours was necessary for the system to equilibrate. Significant, but small changes in the expression of specific inflammation-related genes (e.g *IL1B*, *IL6*, *IL10*) was suggestive of an anti-inflammatory effect of co-culture incubation time (Figure 5.1A-C). *CD14* expression also increased at 72 hours (Figure 5.1D), although due to the unseparated cell samples being used, it was unclear which cell type this expression originated from. It has been noted elsewhere that co-culture of macrophages and preadipocytes promotes the formation of *CD14*⁺ preadipocytes [467], which could contribute to *CD14* expression in this system. As this modelling system was not specifically seeking to model an anti-inflammatory biased system, the 48-hour timepoint was selected. This allowed time for the system to equilibrate,

without concerns of preadipocytes becoming overly confluent, and would also prevent the need for media changes during the co-culture incubation time.

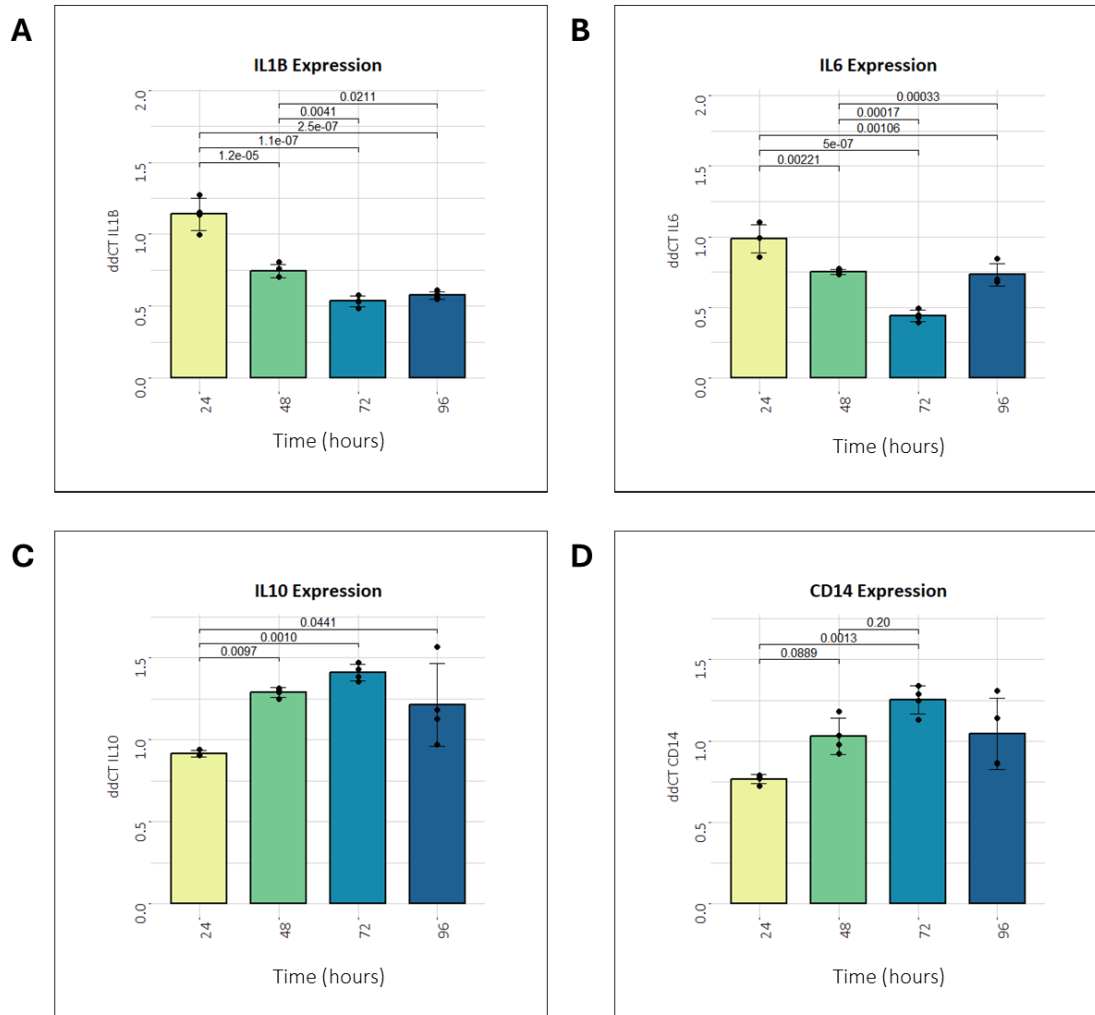


Figure 5.1: Gene expression, measured by qPCR, in co-culture systems. Gluteal MBDFAT preadipocytes were co-cultured with differentiated HMDMs for 24-, 48-, 72- or 96-hours. Macrophages were not separated from co-cultured preadipocytes, and thus RNA from co-culture samples represents pooled macrophage and preadipocyte RNA. Preadipocytes were plated in 6-well plates, to reach a final density of 500,000 cells per well. 100,000 HMDMs were added to each well to retain the 5:1 ratio of preadipocytes to macrophages. Standard co-culture protocols, outlined in Section 2.20 were followed. Data is presented as $\Delta\Delta C_t$ values. Several housekeeping genes were tested for determination of stability in co-culture, and genes *UBC*, *B2M*, *PPIA*, and *GAPDH* were selected for use. Data points are presented in black ($n=2$), coloured bars represent the mean values, and error bars show standard deviation. Where 1-way ANOVA tests have indicated significant effects of co-culture time on gene expression, post-hoc Bonferroni-corrected *t*-tests were conducted, and the associated corrected *p* values are presented graphically. Genes presented are: **A)** *IL1B*, **B)** *IL6*, **C)** *IL10*, **D)** *CD14*.

5.2.2 Optimisation of Treatments Applied to Co-Cultures

To investigate the anti-inflammatory effects of oestradiol treatment, an inflammatory stimulus was introduced to the co-culture system. In pilot experiments, granulocyte-macrophage colony-stimulating factor (GM-CSF) -polarised macrophages were tested as a means to introduce an inflammatory macrophage state. However, the anticipated changes in inflammatory and polarisation markers (e.g TNF- α , IL-1 β , CD206) were not observed. Consequently, LPS (InvivoGen, LPS-EK Ultrapure, Catalog code: tlrl-pektps) was trialled to elicit a stronger inflammatory response. The LPS used was derived from *E. coli* K12 strain, acting as a specific TLR4 ligand. At 100 ng.mL⁻¹ LPS and 24 hours of stimulation, the standard dose used for macrophage inflammatory stimulation [468], there was an effective increase in inflammatory markers in both preadipocytes and macrophages (differentiated in the presence of M-CSF). Differentiating macrophages in the presence of M-CSF allowed for the generation of unpolarised (M0) macrophages, for those that weren't being stimulated with LPS.

5.2.3 Cell Choices in Co-Culture Systems

All experiments utilised HMDMs that were differentiated for 7 days in the presence of 100 ng.mL⁻¹ of M-CSF. For preliminary experiments, using qPCR analysis, PAT2 DFAT cells (described briefly in Section 4.2.7.1) were cultured. These are an in-house generated depot-specific cell line, but unlike MBDFAT cells, these were derived from a male donor. These cells were used in co-culture systems, as many of the CD14⁺ cells initially received from the NHS blood bank, which had been stored as frozen CD14⁺ stocks, were derived from male donors. PAT2 DFAT cells were therefore used to retain a single-sex system.

Given the relevance of this work, specifically to female-based systems, and the reported differences in the inflammatory properties of male- and female-derived macrophages [386], determining the donor sex was crucial to allow for the selection of female donor monocytes. For subsequent experiments, using bulk RNA sequencing to investigate transcriptional responses, CD14⁺ monocytes were freshly isolated,

using the methods described in Section 2.5. Using the CD14⁺ discarded cells, donor sex was determined prior to culture, through quantification of sex chromosome-associated transcripts via qPCR (described in Section 2.6). Female monocytes were subsequently differentiated, and co-cultured with female MBDFAT preadipocytes. Using a female-based system was of greater physiological relevance to the oestradiol treatment being studied.

For visualisation of macrophage and adipocyte populations in co-culture, a BioTracker™ membrane dye was added to macrophages prior to co-culture. These cultures were used for imaging purposes, and not for qPCR or bulk RNA sequencing. Further methods detailing the specifics of the use of the BioTracker™ dye, are provided in Section 2.9. Labelled macrophages co-cultured with preadipocytes were visualised using light microscopy, and the cell types were identified with membrane dye visualisation through fluorescent imaging.

5.2.4 Co-Culture Timecourse

In the optimised system, preadipocytes were plated at 130,000 per well, in 6-well plates. 100,000 differentiated macrophages per well were added the following day (D0), alongside 100nM oestradiol or vehicle control. 24 hours later, 100 ng.mL⁻¹ LPS or vehicle control was added to the system, and co-cultures were harvested another 24 hours later.

For preliminary qPCR experiments, cells were harvested in TRIzol™, and standard RNA extraction procedures were followed, illustrating bulk gene expression changes in co-culture (see Section 2.12). For sequencing, macrophage and preadipocyte co-cultured cells were separated following staining for the surface markers CD14, CD45 and CD90. Cells were subsequently sorted by fluorescence-activated cell sorting (FACS), into Qiagen RLT buffer for harvesting. Only the sorted preadipocyte populations were sequenced, following RNA isolation. Detailed protocols are described in Section 2.20, and the workflow is visualised in Figure 5.2.

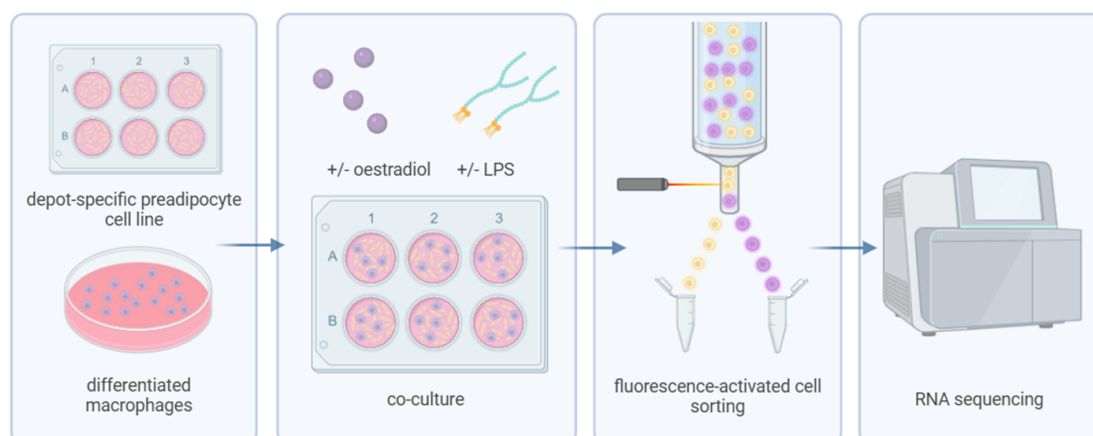


Figure 5.2: Workflow, demonstrating how co-culture samples for RNA sequencing were generated and processed. In brief, preadipocytes were plated on D-1, and M-CSF-differentiated macrophages were subsequently added on D0, along with oestradiol or vehicle control, at a density which would give a 1:5 ratio of macrophages to preadipocytes. 24 hours later, 100ng.mL^{-1} of LPS was added to half of the wells. After another 24 hours, cells were stained for population markers and co-cultures underwent FACS, before being harvested in lysis buffer. RNA extraction was performed on the isolated preadipocyte population, before samples were sent for sequencing.

5.2.5 Cell Sorting and Population Gating

As described earlier, three positive markers were used to separate the two cell populations present in the co-culture systems. As MBDFAT preadipocytes have not been sorted by FACS before, it was necessary to first establish an appropriate marker, using the existing research that was available, followed by pilot flow cytometry experiments. In a pilot flow cytometry experiment of co-cultures, CD14 and CD45 were selected to mark the HMDM populations, while CD34 was tested as a preadipocyte marker, as this has been used elsewhere [261]. The CD34 marker was not sufficient to positively mark the preadipocyte population, and also marked some of the macrophage populations, making it unsuitable for use (pilot flow cytometry plots are shown in Appendix A.6). A CD90 antibody was subsequently selected, as a fibroblast cell marker. This stained preadipocytes sufficiently to allow positive selection of both the macrophage and preadipocyte populations (successful FACS plots are shown in Appendix A.7). FACS gating and sorting was conducted with the help of Jonathan Webber at the Kennedy Institute of Rheumatology.

For gating populations, a forward scatter area (FSC-A) and side scatter area (SSC-A) gates were first used to exclude dead cells and cellular debris. FSC-A versus forward scatter height (FSC-H) were subsequently used to exclude doublets. Gating was then based on CD14 and CD45, allowing the identification of two distinct populations; a CD14⁺CD45⁺ population, and a CD14⁻CD45⁻ population. This latter population did not show positive CD34 staining in the pilot experiment, but did show positive CD90 staining in the later pilot experiment, allowing for positive preadipocyte selection using the CD90 marker. 250,000 preadipocytes per condition were positively selected, and sorted into lysis buffer for RNA extraction.

5.2.6 RNA Sequencing Analysis

RNA sequencing analysis was conducted from raw reads, using the packages and methods specified in Section 2.20.2. For gene set enrichment analyses (GSEA), where multiple analogous pathways were identified (e.g

GOBP_CELLULAR_RESPONSE_TO_REACTIVE_OXYGEN_SPECIES and GOBP_RESPONSE_TO_REACTIVE_OXYGEN_SPECIES), for ease of visualisation, only one representative pathway is presented. Volcano plots and subsequent analyses of differentially expressed gene (DEG) sets (e.g isolating signals for the interactome, examining overlapping DEG sets) were restricted to genes with $\log_{2}FC > 1$ or $\log_{2}FC < -1$, with an adjusted p -value threshold of 0.05.

To identify macrophage-preadipocyte signals of interest, the interactome data, generated from the scRNASeq data set was drawn upon (see Section 3.2.3.3). Identified gene sets from these bulk RNA sequencing data were assessed for overlap with the interactome signal database, allowing for the assessment of ligand-receptor interactions in co-culture systems, which were specifically modulated by a given treatment.

5.3 Results

5.3.1 Establishing a Co-Culture System to Investigate Oestradiol-Primed Crosstalk Between Preadipocytes and Macrophages

5.3.1.1 Co-Culturing Macrophages and Preadipocytes Results in The Formation of Complex Contacts Between Cell Types

HMDMs marked with a membrane dye, appeared viable when co-cultured with MBDFAT preadipocytes from either abdominal or gluteal depots. Moreover, these macrophages formed extensive direct contacts with preadipocytes, which may have been indicative of a more tissue-resident phenotype [469]. They did not exhibit the classic ‘fried-egg’ morphology that is associated with M1 inflammatory-polarised populations [469]. Representative images of these morphological phenotypes are presented in Figure 5.3A-C.

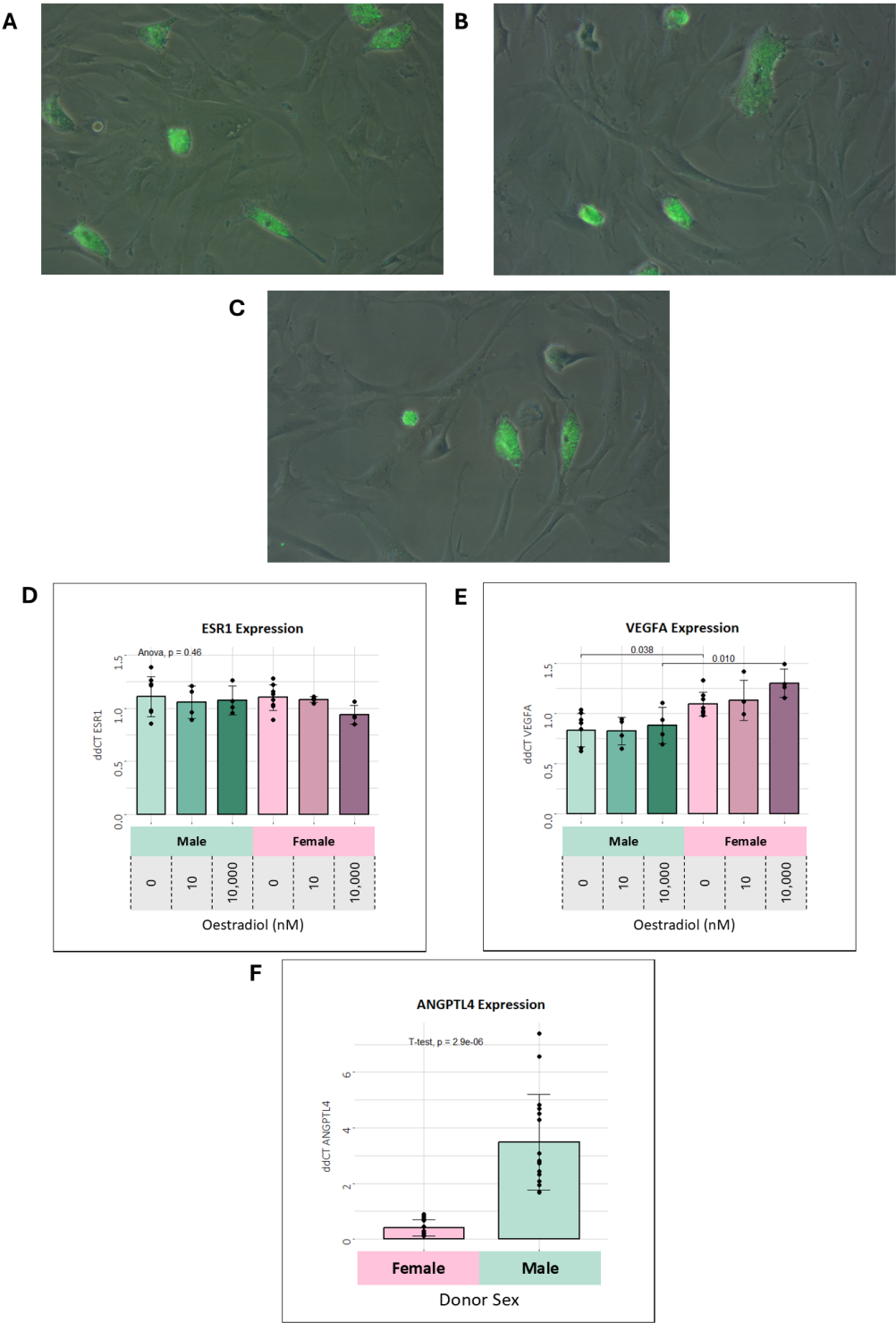


Figure 5.3: **A-C)** Representative images of HMDMs, differentiated for 7-days with M-CSF, co-cultured with MBDFAT preadipocytes. Macrophages were labelled with green BioTracker™ membrane dye, and imaged on D1 of co-culture **D-E)** Gene expression in male and female macrophages, treated with vehicle or oestradiol (100nM or 10,000nM). Cells were differentiated for 7 days with M-CSF, before being plated into 6-well plates, and treated with oestradiol or vehicle for 24 hours. qPCR assays were conducted for: **D)** *ESR1* and **E)** *VEGFA*. Data points are presented in black, with bars showing the mean value, and error bars representing standard deviation. Data are presented from one male and one female donor. 2 vials from each donor were brought up, and cultured on separate occasions, and 3 technical replicates were run. 2-way ANOVA statistics were conducted to assess intergroup differences, followed by post-hoc Bonferroni-adjusted pairwise t-tests. **F)** *ANGPTL4* expression in male and female macrophages. Data points are presented in black, with bars showing the mean value, and error bars representing standard deviation. Data are presented from one male and one female donor. 2 vials from each donor were brought up, and cultured on separate occasions, and 3 technical replicates were run. T-test statistics were conducted to assess intergroup differences.

5.3.1.2 HMDMs Show Sexual Dimorphisms in Oestradiol Responses

From the scRNASeq data, it was understood that adipose tissue macrophages, particularly the tissue resident populations, expressed *ESR1*. It was necessary to confirm that the HMDMs being cultured similarly expressed this receptor. A qPCR gene expression analysis showed that HMDMs expressed *ESR1*, and this was not different between male and female macrophages, or with oestradiol treatment (Figure 5.3D). *ESR2* was not expressed (data not shown).

A reporter assay to assess oestradiol responses, as conducted with preadipocytes in Section 4.3.5, would have been useful to determine whether HMDMs respond to oestradiol. Macrophages, however, are specialised in detecting and eliminating foreign material including cytosolic DNA - like that derived from plasmids or viral vectors. Introduction of foreign DNA results in inflammatory activation [470], and the degradation of the foreign DNA, thereby preventing the expression of the desired gene [471]. This makes them notoriously hard to transfect through plasmid-mediated mechanisms [471]. Meanwhile, standard viral-mediated gene transfer protocols are not appropriate as the macrophages are terminally differentiated and not actively dividing [471]. Viral vector introduction can also result in immunogenic responses, such as interferon production, subsequently inhibiting the introduction of the foreign

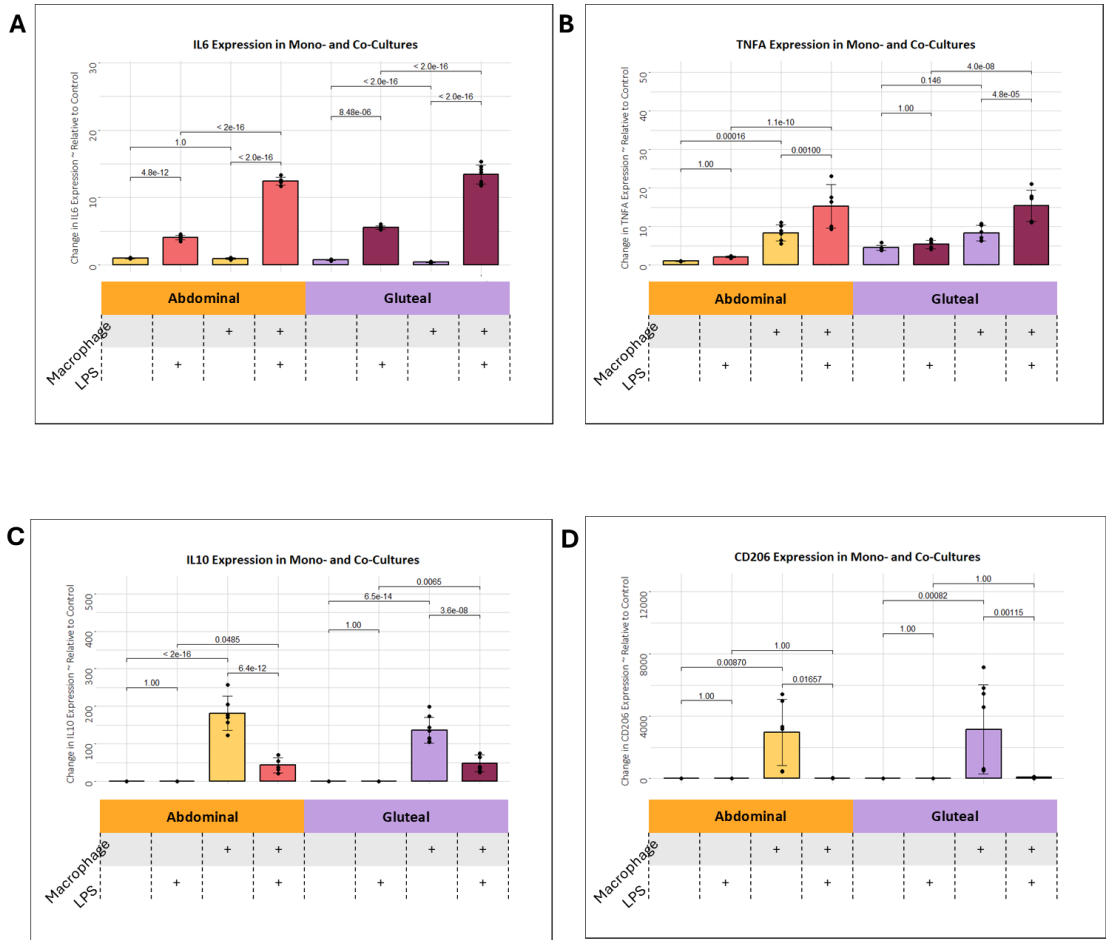
DNA [471]. As reporter assays were not readily achievable, subsequent investigation of oestradiol responses focused on investigating the expression of oestradiol-responsive genes. There is a broader body of literature assessing macrophage responses to oestradiol (as compared to preadipocyte responses), allowing for a more reliable gene selection. Expression of *VEGFA* and *ANGPTL4* have been reported to be responsive to oestradiol in murine macrophage populations [212]. An oestradiol dose-response for gene expression in both male and female macrophages was therefore assessed. Female macrophages expressed higher *VEGFA* compared to male macrophages (Figure 5.3E), and expression of *VEGFA* trended to increase with oestradiol treatment only in female macrophages. Although *ANGPTL4* is known to be an oestradiol-responsive gene in macrophages [212], its expression in HMDMs did not change with oestradiol treatment. However, donor-specific effects were evident, which may have been mediated by sex, as this has been reported elsewhere [472] (Figure 5.3F). The male donor macrophages showed significantly higher *ANGPTL4* expression as compared to the female donor macrophages.

5.3.1.3 LPS Induced Greater Gene Expression Changes in Co-Culture as Compared to Oestradiol

qPCR gene expression assays were conducted on co-cultured and monocultured preadipocytes to assess the anti-inflammatory effects of oestradiol, with or without LPS treatment. Genes pertinent to macrophage and preadipocyte inflammatory responses (e.g. *IL6*, *TNF- α* , *IL1 β* , *IL10*, *CD206*), as well as oestradiol-responsive genes (e.g. *ANGPTL4*, *VEGFA*) were assessed. There was a clear effect of LPS on gene expression (Figure 5.4), which was also co-culture dependent. *IL6* expression increased in response to LPS, with a more pronounced effect in co-cultures (Figure 5.4A). *TNF- α* (produced by both macrophages and preadipocytes) was increased by LPS stimulation (Figure 5.4B). Anti-inflammatory genes, including *IL10* and *CD206*, decreased following LPS treatment in co-cultures (Figure 5.4C-D). Both of these genes were higher in unstimulated co-cultures, as compared to preadipocyte monoculture, likely reflecting their macrophage-derived production. *VEGFA* expression increased with LPS, and this effect was more pronounced in co-cultures (Figure

5.4E). Although *ANGPTL4* was initially investigated as an oestradiol-responsive gene in macrophages [212], it was unexpectedly found to be responsive to LPS, particularly in abdominal co-cultures (Figure 5.4F). As *ANGPTL4* plays a role in suppressing lipoprotein lipase (LPL) activity, a subsequent analysis of *LPL* expression was run, showing a somewhat inverse pattern to that of *ANGPTL4*, whereby expression was suppressed by LPS (Figure 5.4G). *LPL* was higher in co-cultures as compared to monocultures. It wasn't clear whether this expression was due to macrophage-based expression, or whether preadipocytes increased *LPL* expression in response to co-culture conditions.

These interesting responses to LPS and co-culture warranted further investigation, in an unbiased manner, to assess whether the observed changes were indeed top LPS- and co-culture response genes, or whether these were a handful of many genes being regulated by these conditions. It was also necessary to investigate whether the gene readouts were preadipocyte-derived. To this end, bulk RNA sequencing was conducted on preadipocytes sorted after co-culture.



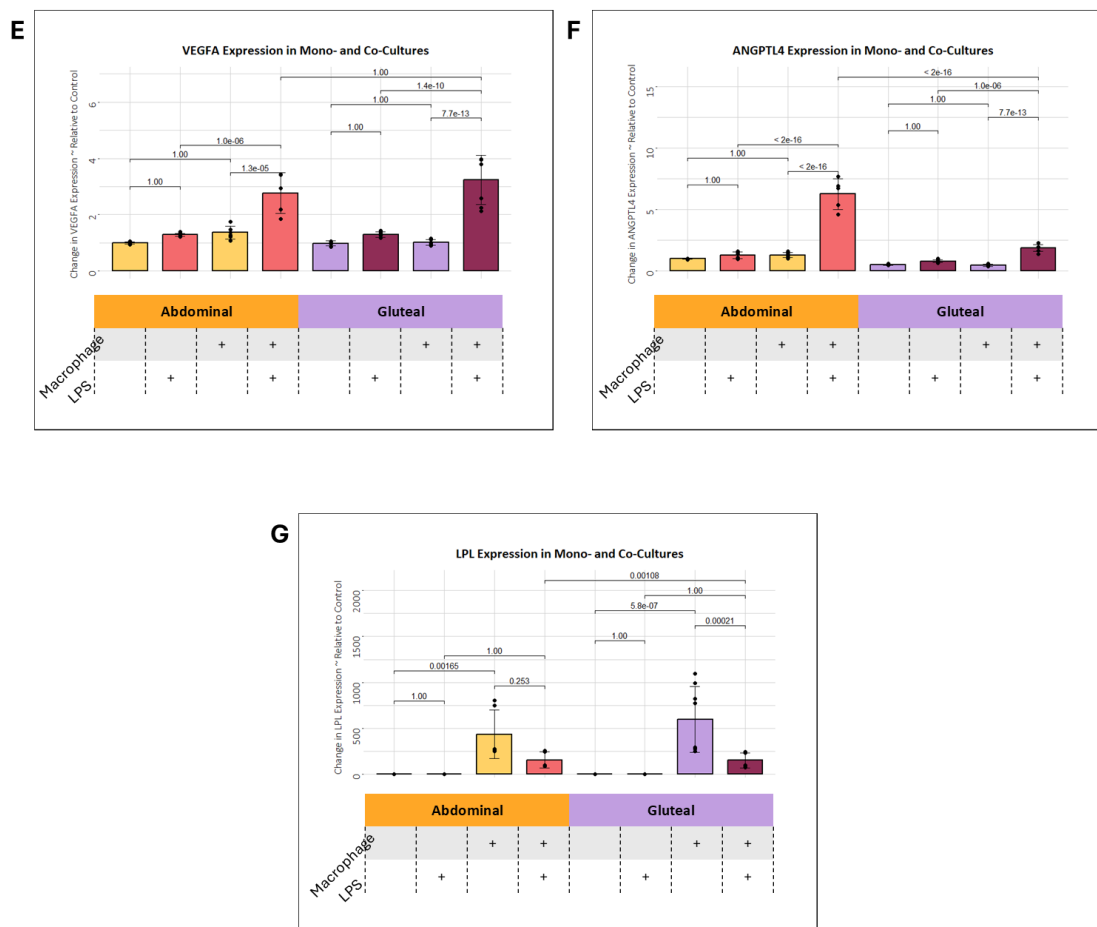


Figure 5.4: Expression of genes, measured by qPCR, in PAT2 preadipocyte monoculture and co-culture in response to 24 hours of 100ng.ml^{-1} LPS treatment. Macrophages were not separated from co-cultured preadipocytes, and thus RNA from co-culture samples represents pooled macrophage and preadipocyte RNA. Data for each condition, for each experimental run, is normalised to the abdominal vehicle control monoculture condition, presented in the leftmost column of each graph. Sample conditions are labelled on the x-axis, and fold change in expression is shown on the y-axis. 2-way ANOVA statistics were conducted to assess intergroup differences, followed by post-hoc Bonferroni-adjusted pairwise t-tests. Data is presented for 2 biological replicates, and 3 technical replicates. Data points are shown in black, error bars represent standard deviation, and bars show mean group values. Expression is shown for: **A)** IL6 , **B)** TNFA, **C)** IL10, **D)** CD206, **E)** VEGFA, **F)** ANGPTL4, **G)** LPL.

5.3.2 Bulk RNA Sequencing Co-Cultures Reveals Broad Gene Expression Patterns Mirroring Initial qPCR Assays

After sequencing preadipocytes from monocultured and co-cultured cells, it was necessary to investigate the transcriptional variance between samples. A multidimensional

mensional scaling (MDS) plot was created to show the transcriptional variation between samples, with each point representing a sample. The distance between two points reflects their transcriptional similarity. In this unsupervised analysis, the plot separated the samples based on treatment group, showing that there were no batch effects or sample outliers (following the re-sequencing of one sample), as shown in Figure 5.5A. Sample-to-sample proximity was largely related to depot and LPS treatment, meanwhile, there were minimal differences seen with oestradiol, suggesting that depot and LPS were the main factors modulating transcriptional variation in this system. Analysis of DEGs across conditions (adjusted p value < 0.05) indicated that depot, LPS, and co-culture had a profound effect on preadipocyte gene expression signatures (Figure 5.5B). With LPS, for example, there were over 10,000 cumulative DEGs. *ANGPTL4* expression was assessed, to investigate whether its expression patterns corroborated with the initial qPCR data, acting as an internal validation, to check the similarity with the preliminary qPCR co-culture data. This showed similar responses to LPS treatment in both mono- and co-cultured preadipocytes across abdominal and gluteal depots (Figure 5.5C), as compared to the original qPCR expression data (Figure 5.4F).

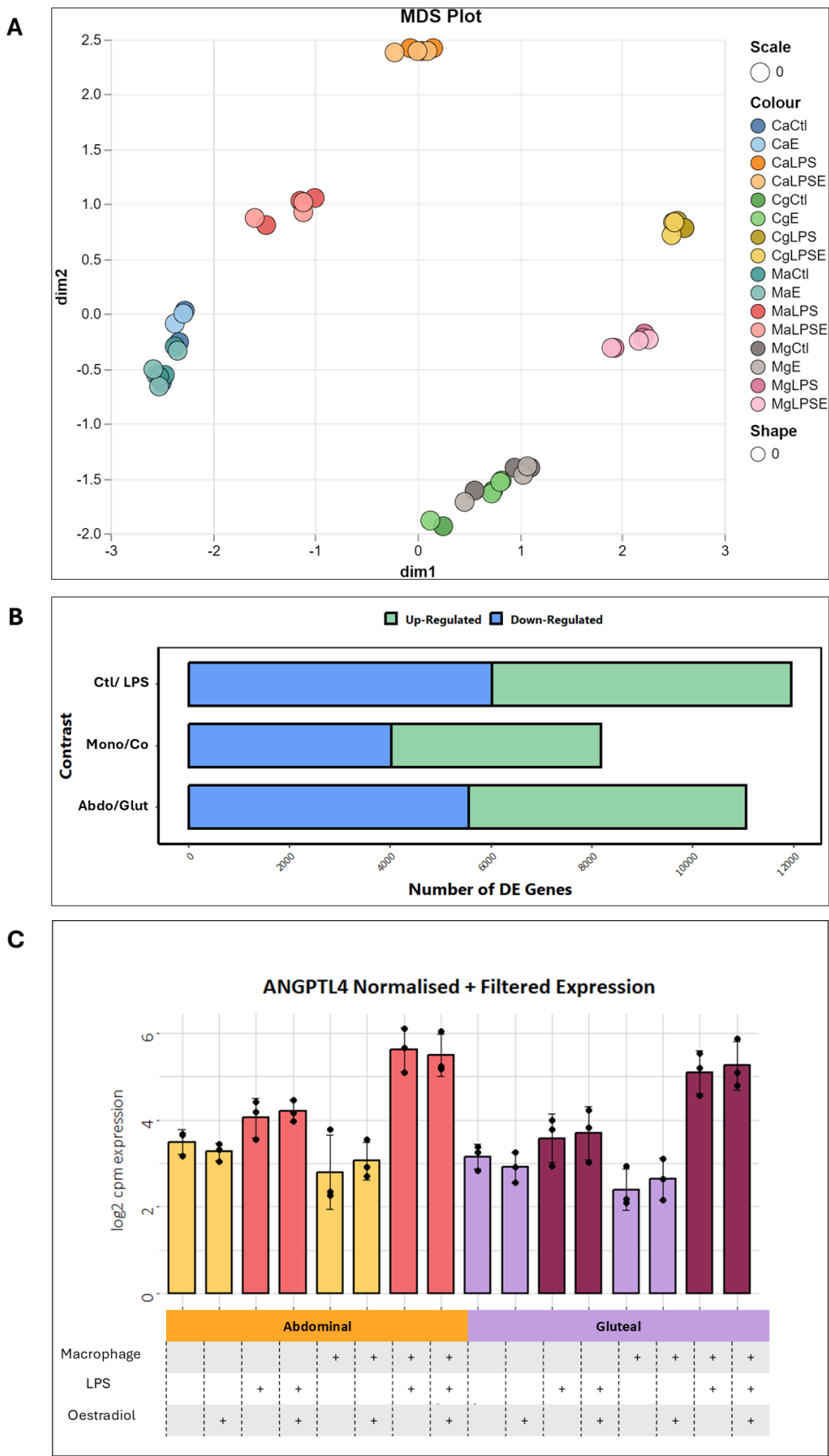


Figure 5.5: **A)** MDS plot showing the transcriptional clustering of samples sent for sequencing. Dimension1 (dim1) and dimension2 (dim2), representing the factors relating to the greatest sources of transcriptional variance, largely separated samples by depot, LPS treatment and co-culture/monoculture, indicating that these are the main dimensions modulating transcriptional differences. Sample identifiers for MBDFAT preadipocyte samples displayed in the MDS plot:

CaCtl = Co-cultured abdominal preadipocytes, vehicle control treatment;

CaE = Co-cultured abdominal preadipocytes, oestradiol treatment;

CaLPS = Co-cultured abdominal preadipocytes, vehicle control treatment + LPS;

CaLPSE = Co-cultured abdominal preadipocytes, oestradiol treatment + LPS;

CgCtl = Co-cultured gluteal preadipocytes, vehicle control treatment;

CgE = Co-cultured gluteal preadipocytes, oestradiol treatment;

CgLPS = Co-cultured gluteal preadipocytes, vehicle control treatment + LPS;

CgLPSE = Co-cultured gluteal preadipocytes, oestradiol treatment + LPS;

MaCtl = Monocultured abdominal preadipocytes, vehicle control treatment;

MaE = Monocultured abdominal preadipocytes, oestradiol treatment;

MaLPS = Monocultured abdominal preadipocytes, vehicle control treatment + LPS;

MaLPSE = Monocultured abdominal preadipocytes, oestradiol treatment + LPS;

MgCtl = Monocultured gluteal preadipocytes, vehicle control treatment;

MgE = Monocultured gluteal preadipocytes, oestradiol treatment;

MgLPS = Monocultured gluteal preadipocytes, vehicle control treatment + LPS;

MgLPSE = Monocultured gluteal preadipocytes, oestradiol treatment + LPS.

B) Numbers of differentially expressed genes comparing control with LPS treatment, monoculture and co-culture, as well as abdominal versus gluteal preadipocytes **C)** *ANGPTL4* expression across conditions from the RNA sequencing dataset, following library size normalisation. Data points are shown in black ($n=3$), mean group expression is shown in coloured bars, and error bars represent standard deviation. Sample conditions are shown in the x-axis matrix.

5.3.3 Oestradiol Only Elicits Depot-Specific Responses in Co-Cultured Preadipocytes

As eluded to in Section 4.3.6, in order to unveil the effects of oestradiol on preadipocytes, depot-specific comparisons were necessary, comparing the abdominal and gluteal transcriptome, stratified by oestradiol and vehicle treatment. This was also true in co-cultures, revealing the genes that were affected by oestradiol to a small extent, mostly in the opposite direction in abdominal and gluteal depots, thereby bringing out the depot-differences.

When comparing co-cultured abdominal and gluteal preadipocyte gene expression, many DEGs were identified. This analysis was run firstly comparing abdominal and gluteal preadipocytes that were treated with vehicle, and then comparing abdominal and gluteal preadipocytes that had been treated with oestradiol. Investigating the overlap of these DEG sets identified 585 DEGs between depots that were exclusive to vehicle treatment and 472 DEGs, uniquely differentially expressed with oestradiol treatment (Figure 5.6A). Additionally, 1399 DEGs were differentially expressed both with oestradiol and with vehicle treatment, representing a ‘core’ set of depot-specific genes (Figure 5.6A, shown in the centre of the Venn diagram). Enriched processes for each of the DEG sets, unique to either oestradiol or vehicle were subsequently investigated (Figure 5.6B). This showed that processes relating to ECM, TGF- β signalling and certain developmental signatures were enriched in oestradiol-treated abdominal preadipocytes compared to gluteal preadipocytes, while gluteal oestradiol-treated preadipocytes showed enrichment for immunological processes (e.g interferon alpha, allograft rejection), and adipogenesis. With vehicle control, enriched processes included ECM breakdown in gluteal preadipocytes.

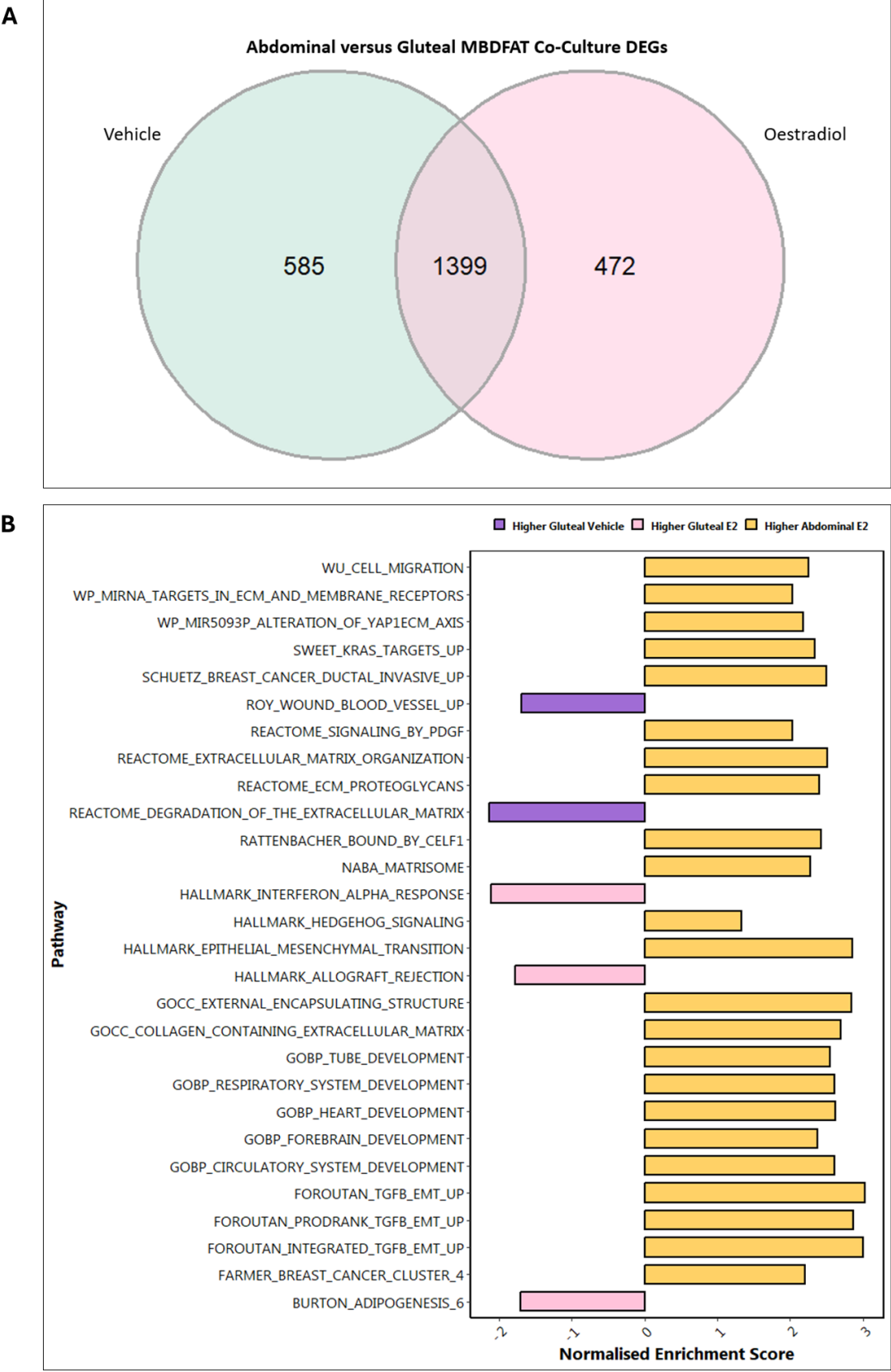
Top hits for DEGs (Figure 5.6C), supported the pathway analysis, with epithelial-mesenchymal transition- and TGF- β - related genes arising as top hits in the abdominal depot with oestradiol treatment (e.g collagens, *TGFBI*, *FSTL3*, *WNT5A*, *SPARC*). A selection of these transcripts have been further associated with the suppression of adipocyte hypertrophy and the suppression of adipogenesis (e.g *WNT5A* [473]; *SPARC* [474]). Meanwhile, in the gluteal depot, top hits included *CSF1*

(M-CSF), relating to immunological signatures and M2 macrophage polarisation, as well as *GDF5*, *CRYAB*, *GRK5*, and *ACVRL1* which have been implicated in adipogenesis [475–478]. Upregulation of M-CSF was particularly noteworthy, as this factor was used to differentiate CD14⁺ monocytes into macrophages. M-CSF treatment has also been found to potentiate responses to M2 stimuli [168].

Investigating the overlap of the identified, significant DEGs, with targets from the interactome analysis (Table 5.1), revealed a set of candidate genes that may modulate depot-specific oestradiol signalling between preadipocytes and macrophages. In the abdominal depot, this included targets such as *RAMP2*, *SPARC*, and *COL5A1*, which were DEGs with oestradiol treatment, and have been associated with adipocyte functions. All three of these transcripts have been associated with functions relating to reducing adipocyte size and white adipose tissue (WAT) volume [474, 479–481], with *RAMP2* having also been associated with M2 macrophage function [482, 483] through its activity in adrenomedullin signalling. In the gluteal depot, identified receptors included *ACVRL1* and *S1PR3*, which have been associated with both macrophage polarisation and accumulation, as well as adipogenic functions. Preadipocyte ligands such as *ANGPTL1*, *ANGPTL2* and *CSF1* were also identified, and these have been associated with M2 macrophage phenotypes [484, 485].

With vehicle treatment, there were no enriched pathways in the abdominal depot. Top unique DEGs in the abdominal preadipocytes included transcripts related to cellular differentiation, such as *MIR31HG* [486], *WNT16* [487], and *WNT10B* [488], which were enriched for convergence on FABP4 signalling (Figure 5.6C). There was no overlap with the interactome analysis in the vehicle condition, suggesting that oestradiol plays a significant role in enhancing preadipocyte-macrophage interactions. In the gluteal depot, there was significant pathway enrichment for ECM degradation (Figure 5.6B), which was further supported by the volcano plot, illustrating top hits relating to collagens and matrix metalloproteinase genes (Figure 5.6C). Other top hits included genes relating to suppression of preadipocyte commitment (e.g. *SOX6* [489], *LEF1* [490, 491]). Similar to abdominal

preadipocytes, vehicle-treated conditions showed minimal overlap with ligand-receptor pairs in macrophages, suggesting that oestradiol enhances depot-specific preadipocyte-macrophage communication (Table [5.1](#)).



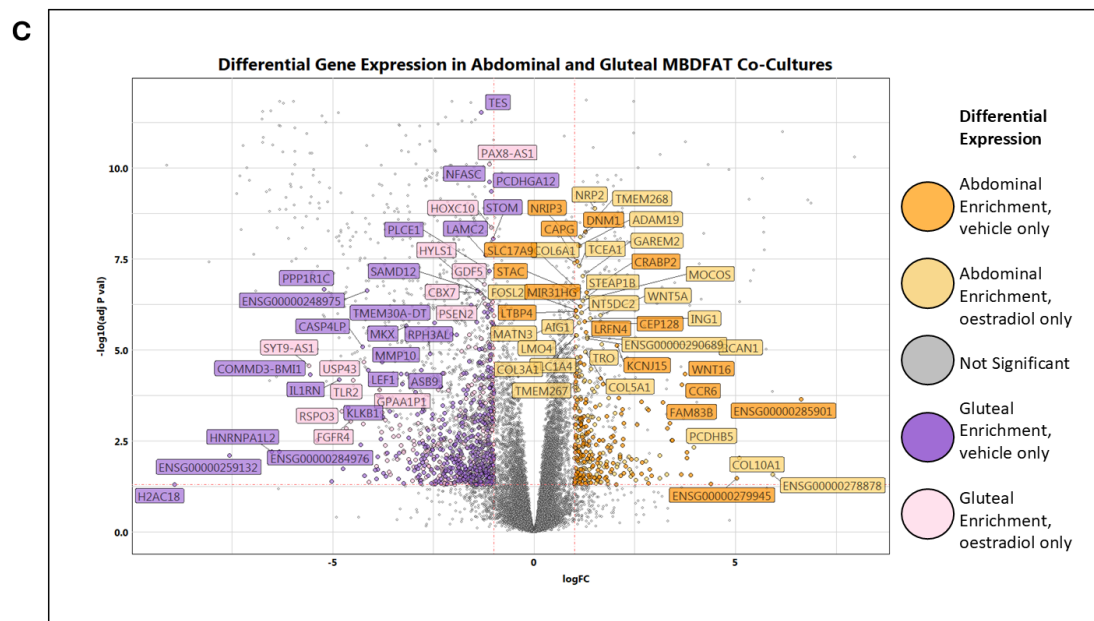


Figure 5.6: **A)** Significantly differentially expressed genes (adjusted p value < 0.05) between abdominal and gluteal co-cultured preadipocytes, split by oestradiol treatment condition. **B)** GSEA analysis. Gene sets, unique to each treatment, were taken separately from the preceding Venn diagram and assessed for enrichment in abdominal and gluteal co-cultured preadipocytes. Presented pathways survived significance thresholds (adjusted p value < 0.05). Normalised enrichment score is shown on the x -axis, with negative values representing gluteal enrichment and positive values representing abdominal enrichment. Pathways enriched in oestradiol or vehicle control treatment are differentiated by the bar colours. **C)** Volcano plot showing top genes differentially expressed between abdominal and gluteal co-cultured preadipocytes, which are unique to either oestradiol or vehicle control treatment. Log fold change ($\log_{FC}$) is presented on the x -axis, and $-\log(\text{adjusted } p\text{-value})$ is presented in the y -axis. Label colour is used to show treatment condition and depot. Coloured points represent genes that show $\log_{FC} > 1$, and adjusted p value < 0.05 . Grey points are those which do not meet these criteria, or are not specific to treatment (i.e part of the ‘core set’ of depot-specific genes). $\log_{FC}$ represents depot comparisons, and thus the direction of change with oestradiol or vehicle treatment is not shown.

Gene (preadipocyte)	Depot Enrichment	Treatment Enrichment	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
EDNRB	Gluteal	Vehicle	GNAI2	-
LDLR	Gluteal	Oestradiol	HSPA8 ; LRPAP1 ; APOE ; ACTR2 ; ARPC5	-
ACVRL1	Gluteal	Oestradiol	TGFB1	-
RAMP2	Abdominal	Oestradiol	ADM	-
S1PR3	Gluteal	Oestradiol	GNAI2	-
TNFRSF14	Gluteal	Oestradiol	MIF, TNFSF13, TNFSF14	-
SEMA4A	Gluteal	Vehicle	-	LILRB2; PLXND1; PLXNB2; NRP1_PLXNA2; NRP1_PLXNA1; NRP1_PLXNA3
ADAM10	Gluteal	Vehicle	-	IL6R; GPNMB; TREM2; TSPAN14; CD44; NOTCH2; NOTCH1; TSPAN17; AXL
ANGPTL2	Gluteal	Oestradiol	-	LILRB2 ; TLR4; LILRB3; ITGA5_ITGB1
ANGPTL1	Gluteal	Oestradiol	-	LILRB2 ; LILRB3
COL6A1	Abdominal	Oestradiol	-	CD44 ; ITGA3_ITGB1
CSF1	Gluteal	Oestradiol	-	CSF3R ; SIRPA ; CSF2RA
ADM	Gluteal	Oestradiol	-	GPR84 ; ADRB2
SPARC	Abdominal	Oestradiol	-	ENG ; FGFR1
FGF2	Abdominal	Oestradiol	-	CD44 ; NRP1 ; SDC4 ; SDC3 ; SDC2; FGFR1 ; GPC4 ; FGFR1
ANGPT1	Gluteal	Oestradiol	-	ITGA5; ITGB1
COL5A1	Abdominal	Oestradiol	-	SDC3
SEMA3B	Gluteal	Oestradiol	-	NRP1_PLXNA1; NRP1_PLXNA2; NRP1_PLXNA3; NRP2_PLXNA1; NRP2_PLXNA2; NRP2_PLXNA3
RSPO3	Gluteal	Oestradiol	-	SDC4
FST	Gluteal	Oestradiol	-	BMP2
ADAM12	Abdominal	Oestradiol	-	SDC4; ITGB1
TNFSF9	Gluteal	Oestradiol	-	HLA-DPA1
WNT5A	Abdominal	Oestradiol	-	FZD2; LDLR; FZD1 ; ADRB2; RYK

Table 5.1: Table listing the significant genes from the DEG analysis ($\log_{2}FC > 1$, adjusted p value < 0.05), comparing depot with and without oestradiol treatment in co-cultured preadipocytes, which were also candidate signals in the interactome analysis. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns. Only genes that were differentially expressed, unique to each treatment, and part of the preadipocyte-specific interactome dataset are shown.

5.3.4 Treatment with Both Oestradiol and LPS, Only Elicits Depot-Specific Responses in Co-Cultured Preadipocytes

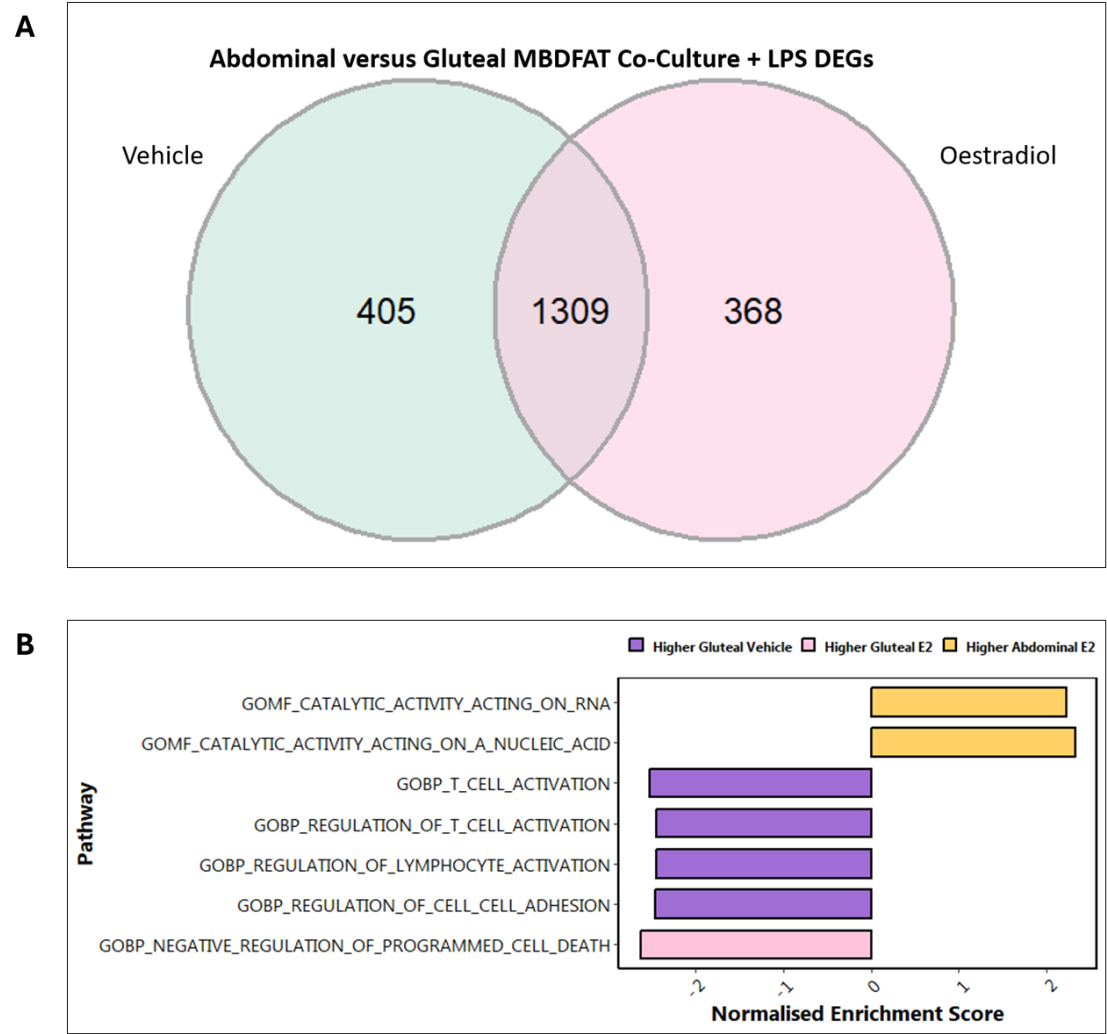
As previous literature has illustrated that the anti-inflammatory effects of oestradiol are more evident with the co-application of inflammatory stimuli [386], it was necessary to repeat the previous analysis, investigating the effects of depot in the context of oestradiol treatment, but with additional LPS stimulation. This analysis revealed 368 DEGs between abdominal and gluteal co-cultured preadipocytes treated with LPS alongside oestradiol, and 405 DEGs with LPS and vehicle control (Figure 5.7A). These unique DEG sets across abdominal and gluteal preadipocytes were

enriched for several processes (Figure 5.7B). Abdominal preadipocytes treated with oestradiol were enriched for catalytic activity processes. Notably, gluteal cells treated with LPS and vehicle control were enriched for lymphocyte activation processes, suggestive of increased inflammatory cell activation. Meanwhile their LPS + oestradiol-treated counterparts were enriched for negative regulation of programmed cell death, suggestive of reduced apoptotic burden with oestradiol.

Top gene hits for abdominal preadipocytes treated with LPS and oestradiol included protocadherin proteins, which are crucial for mediating cell-cell contact [492], and collagens, which are integral to ECM formation (Figure 5.7C). Carboxypeptidase E (*CPE*) was another significant DEG, which is involved in peptide hormone biosynthesis. In gluteal preadipocytes exposed to LPS + oestradiol, several DEGs, including *MET*, *EMC10*, *GRK5*, and *CRYAB*, have been linked to increased fat mass, adipocyte lipid accumulation, and differentiation [475, 493–495]. *CHRD*, a BMP2 antagonist [496, 497] was also enriched in this geneset, as well as genes associated with stem markers and lineage determination (*CD55* [498, 499], *ARGHAP18* [500]). A carboxylesterase enzyme, *CES4*, involved in lipid metabolism and steroid ester regulation, was another top hit [501, 502]. *CD55* was identified in the interactome analysis, interacting with macrophage receptors such as *ADGRE2* and *ADGRE5* (Table 5.2), which are linked to macrophage differentiation [503] and suppression of NFkB-induced inflammation [504]. The *JAG1* ligand for macrophage Notch receptors was also identified in this analysis, with the presence of Notch receptors being important for reducing M1 polarisation [505].

With LPS + vehicle control, abdominal preadipocyte top hits did not show clear functional enrichment. For example, genes that have been reported as both pro- and anti- adipogenic were identified (e.g *MIR31HG*, *ID3* [506, 507], *SHMT2* [508], *ZFAS1* [509], *KLF6* [510, 511]). The interactome analysis identified ligand-receptor interactions which haven't been specifically studied in adipose tissue, such as FABP5-RXR (preadipocyte-macrophage). FABP5 knockdown in adipose tissue reduces inflammation [512], and RXR signalling silencing in macrophages has a similar effect [513], although this interaction itself is not known to promote these

functions in adipose tissue. In gluteal preadipocytes, cells with LPS + vehicle control showed increased expression of genes associated with adipogenesis and inflammation, including *HOXC10* [514], *LTF* [515], *PLA2G5* [516]. PLA2G5 plays a role in releasing unsaturated fatty acid (FA) and preventing M1 macrophage conversion. The interactome analysis further identified ligands and receptors associated with M2 macrophage phenotypes (Table 5.2), including *APOE* and *TREM2* [517], as well as *MXRA5* and *AXL*.



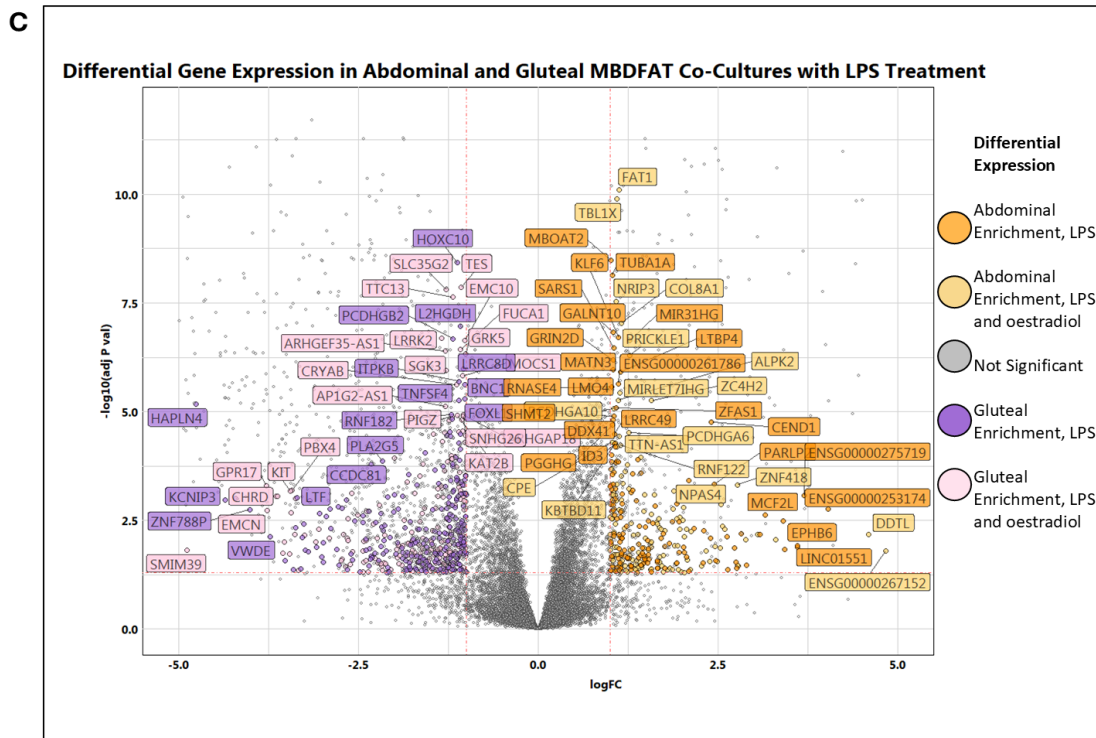


Figure 5.7: **A)** Significantly differentially expressed genes (adjusted p value < 0.05) between abdominal and gluteal co-cultured preadipocytes treated with LPS, split by oestradiol treatment condition. **B)** GSEA analysis. Gene sets, unique to each treatment, were taken separately from the preceding Venn diagram and assessed for enrichment in abdominal and gluteal co-cultured preadipocytes. Presented pathways survived significance thresholds (adjusted p value < 0.05). Normalised enrichment score is shown on the x -axis, with negative values representing gluteal enrichment and positive values representing abdominal enrichment. Pathways enriched in oestradiol or vehicle control treatment are differentiated by the bar colours. **C)** Volcano plot showing top genes differentially expressed between abdominal and gluteal co-cultured preadipocytes, which are unique to either oestradiol or vehicle control treatment. Log fold change ($\log FC$) is presented on the x -axis, and $-\log(\text{adjusted } p\text{-value})$ is presented in the y -axis. Label colour is used to show treatment condition and depot. Coloured points represent genes that show $\log FC > 1$, and adjusted p value < 0.05 . Grey points are those which do not meet these criteria, or are not specific to treatment (i.e. part of the 'core set' of depot-specific genes with LPS treatment). $\log FC$ represents depot comparisons, and thus the direction of change with oestradiol or vehicle treatment is not shown.

Gene (preadipocyte)	Depot Enrichment	Treatment Enrichment	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
TGFB3	Gluteal	Vehicle	TGFB1	-
PLXNA4	Abdominal	Oestradiol	FARP2, SEMA6B	-
C3	Gluteal	Vehicle		ITGAX; C3AR1; NRP1; C5AR2; LRP1; ITGAM; CR1; CD46; CD81
APOE	Gluteal	Vehicle		TREM2; LRP1; ABCA1; SORL1; SDC2; LDLR; SCARB1
SPARC	Abdominal	Vehicle		FGFR1; ENG
MXRA5	Gluteal	Vehicle		PILRA; AXL ; SIGLEC7; PILRB; CD69
FABP5	Abdominal	Vehicle		RXRA
ANG	Abdominal	Vehicle		PLXNB2
CD55	Gluteal	Oestradiol		ADGRE2; CR1; ADGRE5
JAG1	Gluteal	Oestradiol		NOTCH1; NOTCH2; CD46

Table 5.2: Table listing the significant genes from the DEG analysis ($\log FC > 1$, adjusted p value < 0.05), comparing LPS-treated abdominal and gluteal co-cultured preadipocytes with and without oestradiol treatment, which were also candidate signals in the interactome analysis. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns. Only genes that were differentially expressed, unique to each treatment, and part of the preadipocyte-specific interactome dataset are shown.

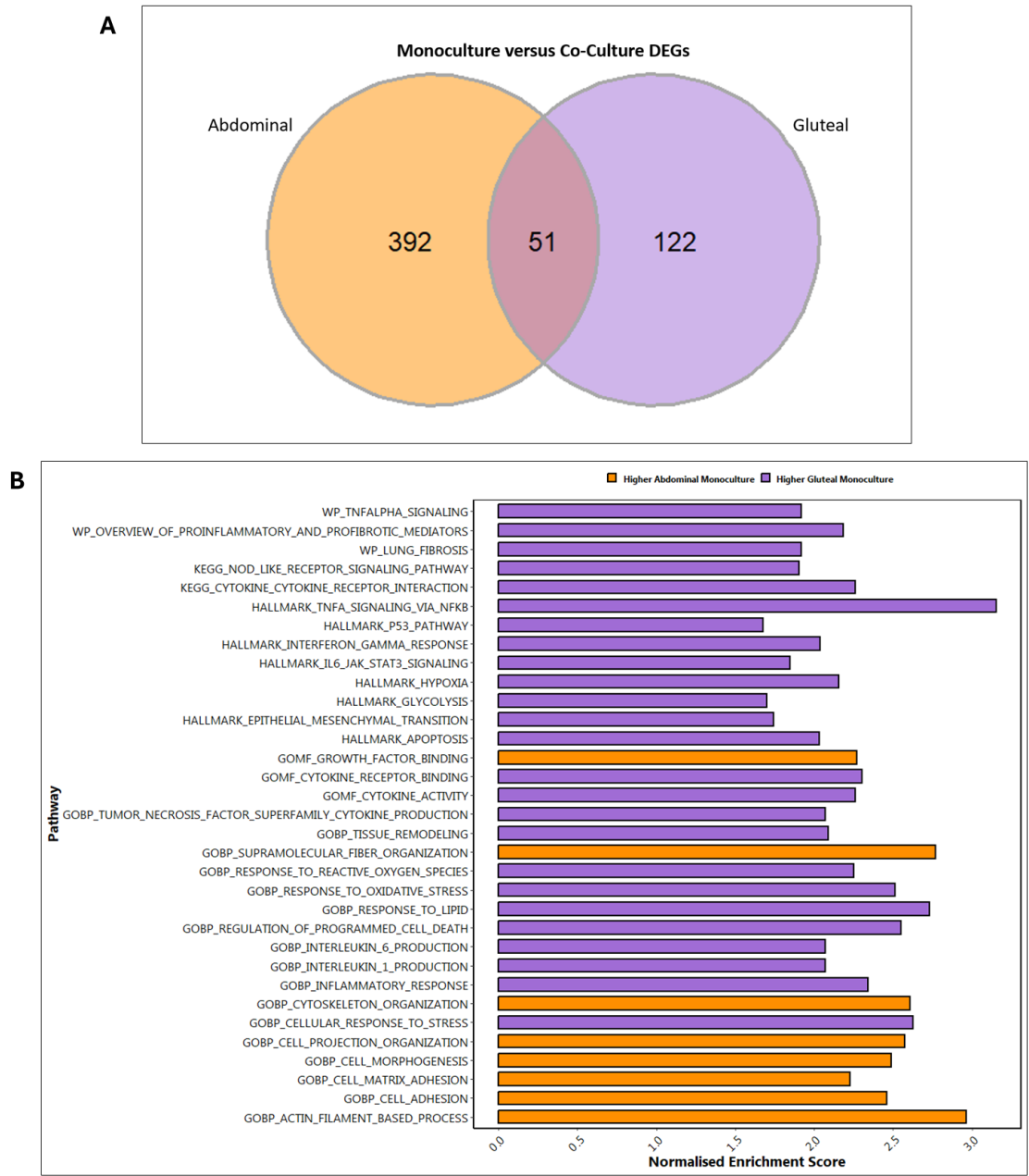
5.3.5 Macrophage Co-Culture Induces Depot-Specific Signatures in Preadipocytes

To understand how preadipocytes respond to co-cultured macrophages and to discern depot-specific differences, it was pertinent to compare gene expression profiles of preadipocytes in monoculture versus co-culture, and further analyse these profiles by depot. This approach allowed the determination of whether each depot creates a distinct niche for macrophages, and if the presence of macrophages influences preadipocyte phenotypes. This analysis identified 122 DEGs uniquely differentially expressed between gluteal preadipocytes in monoculture and co-culture, and 392 DEGs uniquely differentially expressed in abdominal preadipocytes, again comparing monoculture and co-culture (Figure 5.8A). Notably, GSEA revealed significant enrichment of inflammatory pathways in gluteal monoculture relative to co-culture (Figure 5.8B), while abdominal monoculture was enriched for processes relating to cell adhesion, morphology and cytoskeleton.

In abdominal preadipocytes, top hits for DEGs that were differentially expressed between mono- and co-culture, and unique to this depot, revealed targets of interest (Figure 5.8C). Top genes enriched in abdominal monoculture included transcripts associated with lipid accumulation and adiposity (e.g *ABCC6* [518], *CYGB* [519],

MYOZ2 [520], *TMEM200A* [521], *COL5A3* [522], *KMO* [523], *CD36* and *ANAX10* [524–527]), as well as genes associated with inflammation and insulin resistance (e.g. *IL18* [528–530], *EDN1* [531], *CAMK2N2* [532], *CD36*, *ANAX10* [527, 533–535]). *ESR1* was also higher in monoculture relative to co-culture, suggesting that the presence of macrophages may dampen oestrogen signalling in the abdominal depot. In co-culture, upregulated genes exhibited contrasting functions compared to those in monoculture (Figure 5.8C), such as reduced lipid accumulation and adipogenesis (e.g. *SELENOP* [536], *LYZ* [537]), enhanced beiging (e.g. *NEAT1* [538]), and decreased inflammation (e.g. *SELENOP* [539], *NEAT1* [540]). The interactome overlap analysis (Table 5.3), identified *MXRA5* as an upregulated ligand in abdominal preadipocyte co-culture. *MXRA5* acts at macrophage receptors including AXL, SIGLEC7 and PILR β , which have all been associated with anti-inflammatory macrophage responses [541–545].

In gluteal monoculture, GSEA analysis highlighted an upregulation of inflammatory pathways (Figure 5.8B). Top DEGs included key inflammatory mediators such as *IL6*, *CCL2*, *CXCL3*, *CSF2* (*GM-CSF*), *CXCL8*, *CXCL1*, *CXCL6*, and *IL1 β* , (Figure 5.8C) among others implicated in regulating inflammatory responses (e.g. *CCR6*, *MIR3142HG* [546] and *IER3* [547, 548]). Monocultured preadipocytes were also enriched for genes involved in reducing differentiation and suppressing adipocyte expansion (e.g. *STC2* [549], *SOD2* [550], *LIF* [551]). In co-culture, opposing genes, associated with decreasing inflammatory responses, were seen (e.g. *SERPINB4* [552], *PLA2G7* [553]). The interactome analysis revealed an enrichment for a ligand acting at *LILRB2* (Table 5.3), known for its anti-inflammatory functions in monocyte-derived cells [554].



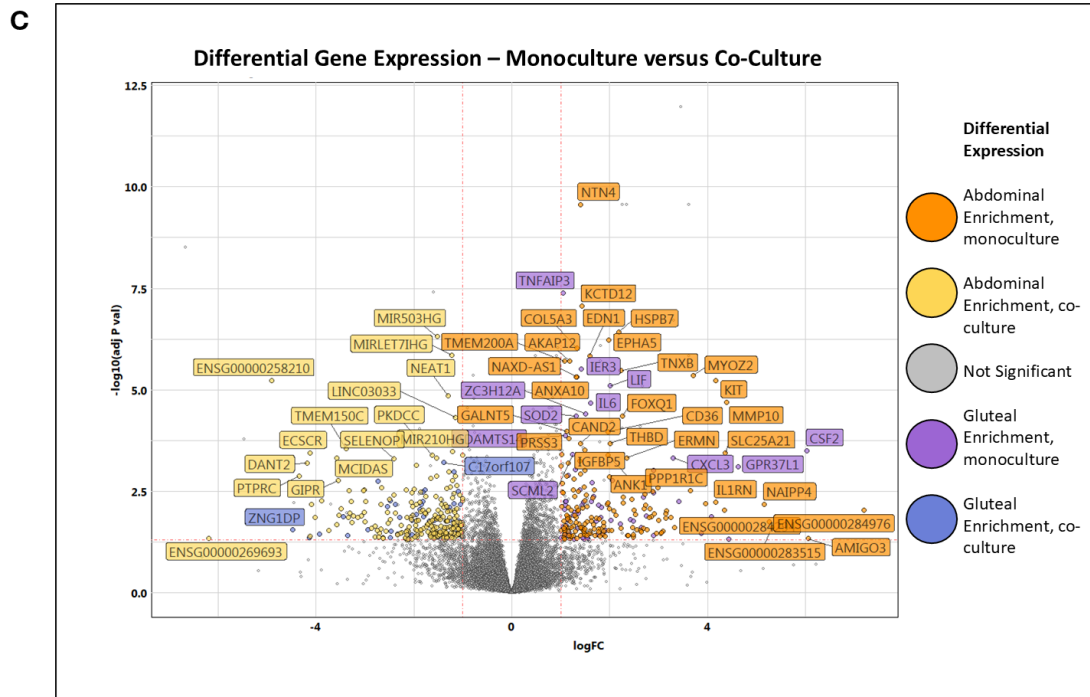


Figure 5.8: **A)** Significantly differentially expressed genes (adjusted p value < 0.05) between monocultured and co-cultured preadipocytes, split by depot. **B)** GSEA analysis. Gene sets, unique to each depot, were taken separately from the preceding Venn diagram and assessed for enrichment in monoculture and co-culture conditions. Presented pathways survived significance thresholds (adjusted p value < 0.05). Normalised enrichment score is shown on the x-axis, with negative values representing co-culture enrichment and positive values representing monoculture enrichment. Pathways enriched in abdominal or gluteal cells are differentiated by the bar colours. **C)** Volcano plot showing top genes differentially expressed between monocultured and co-cultured preadipocytes, which are unique to either abdominal or gluteal preadipocytes. Label colour is used to show condition and depot. Log fold change (logFC) is presented on the x-axis, and $-\log(\text{adjusted p-value})$ is presented in the y-axis. Coloured points represent genes that show $\log\text{FC} > 1$, and adjusted p value < 0.05 . Grey points are those which do not meet these criteria, or are not specific to treatment (i.e part of the ‘core set’ of mono/co-culture-regulated genes). LogFC represents mono/co-culture comparisons, and thus the direction of change with depot is not shown.

Gene (preadipocyte)	Depot Enrichment	Treatment Enrichment	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
CD36	Abdominal	Monoculture	S100A8; S100A9; S100A12; THBS1	
EDNRB	Abdominal	Monoculture	GNAI2	
CD40	Abdominal	Monoculture	TNFSF13B; LTB;	
MXRA5	Abdominal	Co-culture		PILRA; AXL; SIGLEC7; PILRB; CD69
SEMA3D	Abdominal	Monoculture		PLXND1; NRP2_PLXNA2; NRP1_PLXNA2; NRP2_PLXNA1; NRP2_PLXNA3; NRP1_PLXNA3; NRP1_PLXNA1
COL5A3	Abdominal	Monoculture		SDC3
CCL2	Gluteal	Monoculture		CCR1; CCR2; CCR5
SEMA4A	Gluteal	Co-culture		LILRB2; PLXND1; NRP1_PLXNA2; PLXNB2; NRP1_PLXNA1; NRP1_PLXNA3
PTGS2	Gluteal	Monoculture		ALOX5

Table 5.3: Table listing the significant genes from the DEG analysis ($\log FC > 1$, adjusted p value < 0.05), comparing mono- and co-cultured preadipocytes, which were also candidate signals in the interactome analysis. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns. Only genes that were differentially expressed, unique to each treatment, and part of the preadipocyte-specific interactome dataset are shown.

5.3.6 LPS induces Depot-Specific Gene Expression Signatures in Co-Cultured Preadipocytes

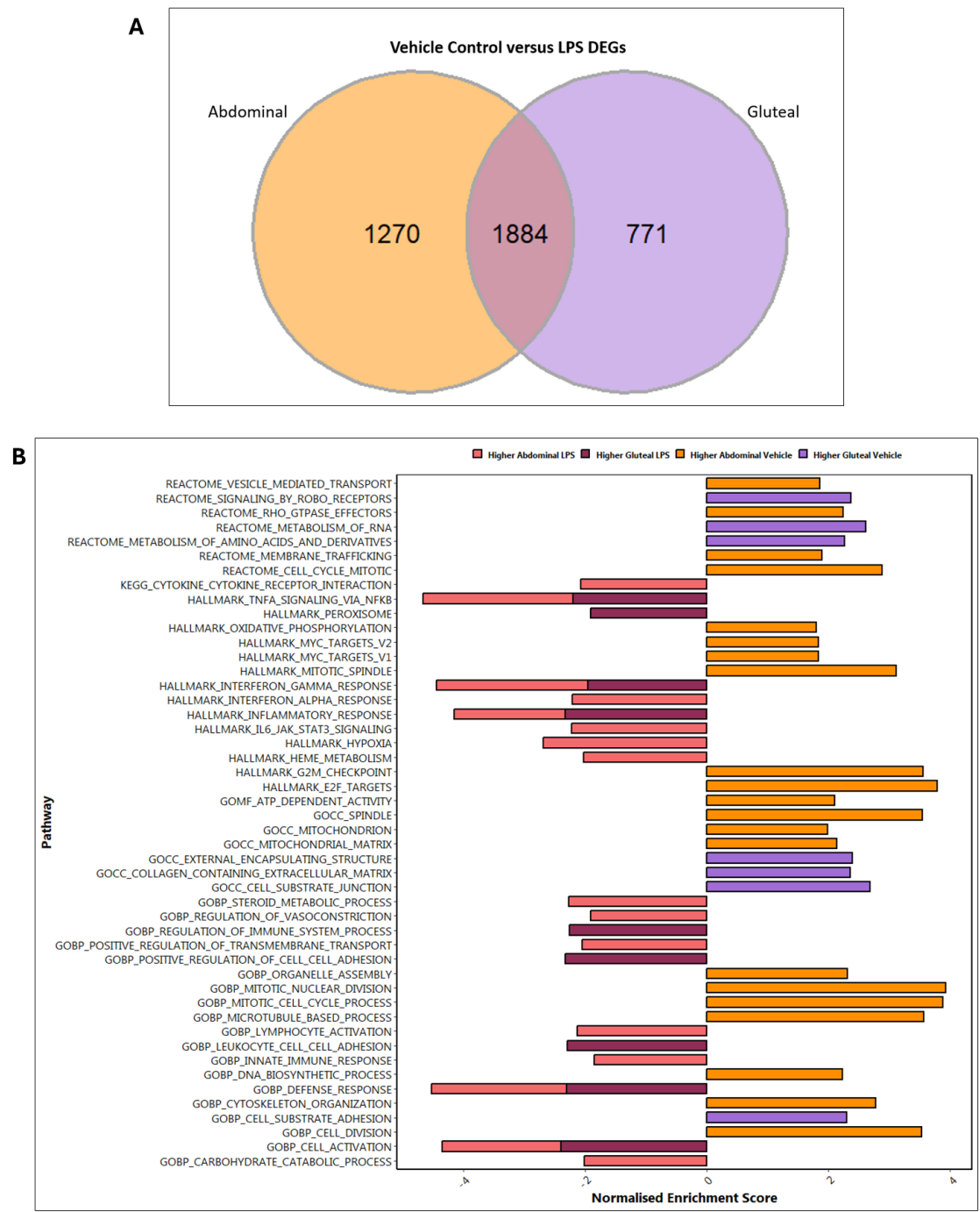
To explore how LPS influences depot-specific macrophage interactions and gene expression in preadipocytes, LPS treated cells were compared to vehicle control in abdominal and gluteal co-cultured preadipocytes. In abdominal preadipocytes, LPS treatment led to the unique differential expression of 1,270 genes, while for gluteal co-cultured preadipocytes, there were 771 unique DEGs (Figure 5.9A). In abdominal preadipocytes, LPS treatment regulated a range of processes. Notably, LPS suppressed markers of mitosis and cell division, as well as oxidative phosphorylation and membrane transport. Meanwhile LPS enhanced processes related to inflammation, hypoxia, vasoconstriction and immune activation (Figure 5.9B). In gluteal cells, there were few processes suppressed by LPS, and processes enhanced by LPS solely related to inflammation and immune cell activation (Figure 5.9B). In abdominal co-cultured preadipocytes treated with LPS, key DEGs (shown in Figure 5.9C) related to processes including inflammation (e.g *NFKB*, *AZI2* [555], *IL7*, *AEBP1* [556], *TNFRSF6B*, *CXCL13* [557], *IL7R*), lipid-induced cytotoxicity and oxidative stress, as well as reduced viability (*TNFRSF10B* (*DR5*), *TXNIP*,

HAND2-AS1 [558–560]). Other enriched genes have been associated with reduced insulin sensitivity, as well as changes in lipid accumulation and WAT mass (e.g. *AEBP1* [561, 562], *ACLS5* [563], *TPD52* [564], *CXCL13* [565], *IL7* [566], *SOX6* [567]). Ceramide synthesis genes, important for macrophage polarisation and insulin sensitivity (*CERS4* [568, 569]) were also upregulated with LPS. Interactome analyses (Table 5.4) further highlighted enrichment for inflammatory processes, through ligands and receptors such as *CXCL12*, *CD44*, *LDLR*, and *TNF α -FAS* signalling [570–573].

Meanwhile, LPS downregulated several genes involved in cell cycle, mitosis and proliferation (e.g. *TACC3* [574], *UBE2C* [575], *RCC1* [576], *NUSPA1* [577], *CKAP2* [578], *CDCA7L*, *STMN1* [579], *CCNA2*, *RAP2B* [580], *CDK1* [581], *KIF22* [582], *CDCA8*), as well as genes involved in lipid accumulation and differentiation (e.g. *FR2L1* [583], *FZD2* [584], *OXCT1* [585]). Interactome analysis supported these data, highlighting signals associated with proliferation and adipogenic function (e.g. *HBEGF-CD9* [359, 586]), as well as M2 polarisation (*RXR α* , *FDZ2* [587, 588]) and adhesion contacts (*EFNA5-EPHB6* [589]).

In gluteal co-cultured preadipocytes, LPS treatment predominantly upregulated genes associated with inflammatory signalling (e.g. *CCR6*, *CASP8* [590]), *NLRP3*, *IL16* [591], *KDSR* [592]) (Figure 5.9C). The interactome analysis corroborated this, highlighting ligand-receptor interactions involved in inflammation and macrophage polarisation (Table 5.4), such as *SLIT2/APP* [593, 594], *IL16*, and *CD44-HA* signalling [595] and *ACKR3* (*CXCR7*) [596].

Conversely, LPS treatment downregulated genes related to lipid storage and proliferation (Figure 5.9C), including *CAVIN1* [597], *SUN1* [598, 599], *NID2* [600, 601] and *DEPTOR* [602]. The interactome analysis supported this (Table 5.4), showing enrichment for *JAG1/NOTCH* signalling and *LAMA2* signalling, both of which are associated with adipogenesis [603–605]. The downregulation of *A2M/LRP1* by LPS, involved in macrophage proliferation and M2-like polarisation, further reflects an LPS-induced inflammatory signature [606].



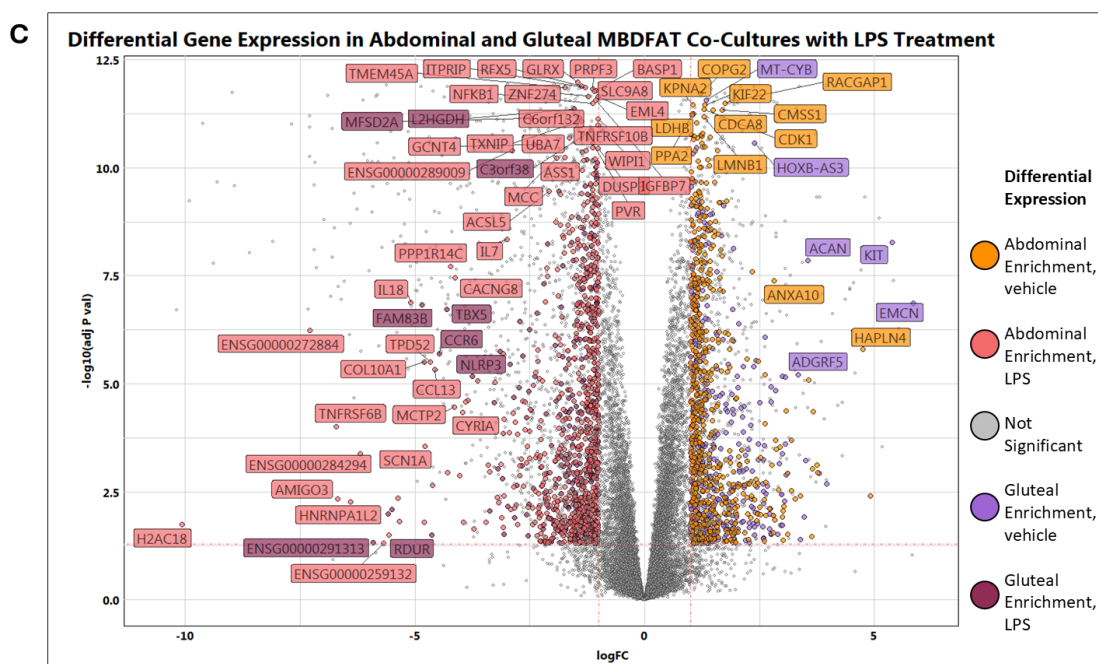


Figure 5.9: **A)** Significantly differentially expressed genes (adjusted p value < 0.05) between LPS and control treatment, split by depot. **B)** GSEA analysis. Gene sets, unique to each depot, were taken separately from the preceding Venn diagram and assessed for enrichment in LPS- and control- treated co-cultures. Presented pathways survived significance thresholds (adjusted p value < 0.05). Due to the extreme number of enriched pathways, for presentation purposes, only the top pathways for each relevant process, from each pathway gene set, are shown. Normalised enrichment score is shown on the x -axis, with negative values representing LPS enrichment and positive values representing vehicle enrichment. Pathways enriched in abdominal or gluteal cells are differentiated by the bar colours, which also show treatment condition. **C)** Volcano plot showing top genes differentially expressed between LPS and control treatment, which are unique to either abdominal or gluteal preadipocytes. Log fold change ($\log FC$) is presented on the x -axis, and $-\log(\text{adjusted } p\text{-value})$ is presented in the y -axis. Label colour is used to show treatment condition and depot. Coloured points represent genes that show $\log FC > 1$, and adjusted p value < 0.05 . Grey points are those which do not meet these criteria, or are not specific to treatment (i.e. part of the ‘core set’ of LPS-regulated genes). $\log FC$ represents LPS/control comparisons, and thus the direction of change with depot is not shown.

Gene (preadipocyte)	Depot Enrichment	Treatment Enrichment	Corresponding Ligand (macrophage)	Corresponding Receptor (macrophage)
CD44	Abdominal	LPS	VEGFA; VCAN; HBEGF; MMP9; LGALS9; PSEN1; ADAM10; VIM; LAMB3; PKM; SPP1; HGF; LPL; TIMP2; VCAN; FN1; LAMB2; TIMP3	-
CD9	Abdominal	Vehicle	HBEGF; IL16	-
LDLR	Abdominal	LPS	ARPC5; ACTR2; LRPAP1; HSPA8	-
FAS	Abdominal	LPS	TNF; TNFSF13; CALM1	-
AGTR1	Abdominal	LPS	HRAS	-
TNFRSF14	Abdominal	LPS	TNFSF13; TNFSF14; MIF	-
ERAP1	Abdominal	LPS	NUCB2	-
SDC2	Gluteal	Vehicle	CXCL8; TGF β 1; APOE; TNFSF13; HGF; FN1	-
GPC1	Gluteal	Vehicle	VEGFA; NRG1; APP	-
ACKR3	Gluteal	LPS	ADM; MIF; CXCL12;	-
PDGFRA	Gluteal	LPS	PDGFC	-
NOTCH3	Gluteal	Vehicle	PSEN1; MAML2; JAG1	-
IL6R	Gluteal	Vehicle	ADAM10; ADAM17	-
RPSA	Gluteal	Vehicle	APP; LAMB2	-
CXCL12	Abdominal	LPS	-	ITGA5; CD4; ITGA4; SDC4; CXCR4; ITGAV; ITGB1
MFAP5	Abdominal	Vehicle	-	NOTCH1
HMGB1	Abdominal	Vehicle	-	CD163; THBD; TLR2; TLR4; HAVCR2; CXCR4
TIMP3	Abdominal	Vehicle	-	CD44
FABP5	Abdominal	Vehicle	-	RXRA
SEMA4A	Abdominal	LPS	-	LILRB2; PLXND1
SFRP1	Abdominal	Vehicle	-	FZD2
EFNA5	Abdominal	Vehicle	-	EPHB6
LAMA2	Gluteal	Vehicle	-	CD44; RPSA;
F10	Gluteal	Vehicle	-	ITGAM; F3
SDC2	Gluteal	Vehicle	-	PTPRJ
HAS2	Gluteal	LPS	-	CD44
SEMA3D	Gluteal	Vehicle	-	PLXND1; NRP1_PLXNA1; NRP1_PLXNA2; NRP2_PLXNA3; PLXND1
JAG1	Gluteal	Vehicle	-	CD46; NOTCH1; NOTCH2
IL16	Gluteal	LPS	-	CCR5; CD4; CD9
SLIT2	Gluteal	LPS	-	APP; PLXNA1
A2M	Gluteal	Vehicle	-	LRP1

Table 5.4: Table listing the significant genes from the DEG analysis ($\log_{FC} > 1$, adjusted p value < 0.05), comparing LPS and control treated co-cultured preadipocytes, which were also candidate signals in the interactome analysis. The preadipocyte differentially expressed gene is presented in leftmost the ‘Gene’ column, and its corresponding ligands or receptors, expressed on macrophages, are presented in the rightmost columns. Only genes that were differentially expressed, unique to each treatment, and part of the preadipocyte-specific interactome dataset are shown.

5.3.7 LPS Robustly Affects Multiple Steroid Synthesis Pathways in Preadipocyte Co-Culture

Given that steroid hormones play a crucial role in fat distribution, and that steroid hormone pathways were differentially affected with LPS treatment (Figure 5.9B), it was necessary to explore how LPS affects steroid synthesis in preadipocyte-macrophage co-cultures. Analysis of DEGs in response to LPS treatment revealed significant alterations in enzymes involved in steroid synthesis. Specifically, LPS

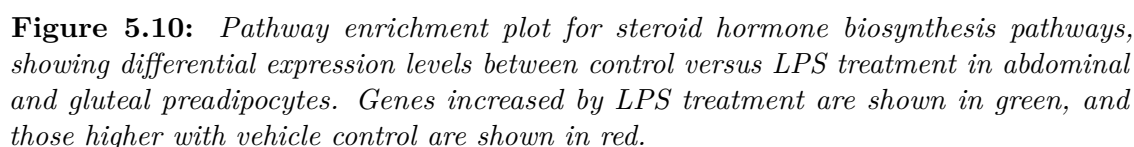
treatment led to the upregulation of enzymes involved in synthesising cholesterol precursors for steroid hormones, such as *STAR* and *CYP11A1*, as well as those promoting cortisol formation, like *HSD11B1*. Conversely, enzymes responsible for converting these intermediates into sex-steroids, including *AKR1C3*, *SRD5A1*, *STS*, *HSD17B4*, and *AKR1C2*, were downregulated. This pattern suggests that LPS treatment may be shuttling cholesterol intermediates towards the generation of cortisol, rather than favouring sex-steroid synthesis. Pathview analyses supported this shift in steroid synthesis pathways (Figure 5.10). Furthermore, receptors involved in steroid responses, such as *GPER1* and *AR* were downregulated with LPS treatment, while the glucocorticoid receptor, *NR3C1*, was upregulated. This indicates that LPS-treated co-cultured preadipocytes are more inclined to produce and respond to cortisol, while producing and responding to sex-steroids less.

5.4 Discussion

Overall, this chapter established and characterised a contact-dependent co-culture system between macrophages and preadipocytes, allowing for an in-depth investigation of depot-specific transcriptional responses to relevant stimuli. Leveraging the previously generated database of adipose tissue SVF ligand-receptor interactions, derived from scRNASeq data, this system also enabled the exploration of how macrophage-preadipocyte signalling changes in response to oestradiol and LPS.

5.4.1 Co-Cultures Treated with Oestradiol Showed Depot-Specific Lipid Storage Signatures

Oestradiol treatment revealed distinct depot-specific transcriptional signatures in abdominal versus gluteal co-cultured preadipocytes, highlighting opposing processes that may underlie differential fat storage patterns in response to oestradiol. When LPS was introduced to the co-cultures to assess the anti-inflammatory effects of oestradiol, specific depot-enriched signatures were maintained. Given, however, that these responses were not explicitly evident when comparing vehicle with oestradiol



treatment, further work should seek to establish whether other tissue factors are necessary to elicit more prominent oestradiol responses in preadipocytes.

5.4.1.1 Oestradiol May Increase Abdominal Adipocyte Lipolysis

In abdominal preadipocytes, compared with gluteal preadipocytes, oestradiol treated cells showed downregulation of genes involved in hypertrophy and inflammation, while genes related to lipolysis and M2 macrophage function were upregulated. Of the DEGs with oestradiol, *RAMP2*, encoding an adrenomedullin receptor, emerged as a significant target. Previous studies have documented adrenomedullin's anti-inflammatory role and its capacity to induce M2 macrophage polarisation, supported by knockout models demonstrating reductions in M2 macrophage populations in adipose tissue lacking adrenomedullin signalling [482, 483, 607]. Furthermore, adrenomedullin has been implicated in enhancing beige adipocyte formation in WAT [483] and promoting lipolysis in adipocytes [608, 609], with adrenergic stimulation amplifying these effects [610]. Heterozygote knockout mice of *RAMP2* show impaired metabolic homeostasis, elevated cytokine production, and increased WAT volume and hypertrophy [479]. The upregulation of *RAMP2* in abdominal preadipocytes could thus play a role in driving depot-specific lipolysis, particularly in the context of oestradiol, which has been linked to sex-specific differences in lipolytic responses to adrenergic stimulants such as epinephrine [611]. These findings suggest that depot-specific oestradiol responses, in combination with adrenergic signalling, may contribute to the preferential reduction of abdominal fat stores in females. *RAMP2* was also present in the interactome analysis, as macrophages and monocytes can produce adrenomedullin, to subsequently act at the preadipocyte *RAMP2* receptor [612]. These interactions with macrophages may therefore enhance the lipolytic function of the female abdominal adipose tissue with oestradiol. Additionally, SPARC, a preadipocyte-derived ligand significantly elevated in abdominal preadipocytes with oestradiol treatment, may contribute to reduced adipose tissue accumulation by reducing adipocyte size [480] and promoting preadipocyte proliferation [474]. The lack of significant ligand-receptor interactions in vehicle-treated conditions further suggests that oestradiol enhances the crosstalk between macrophages and preadipocytes, potentially facilitating these depot-specific responses. This should be further tested by explicitly comparing

these identified depot-specific responses relating to adipose tissue expansion (e.g. lipolysis, adipogenesis, hypertrophy) across abdominal and gluteal co-cultured and monocultured preadipocytes, with and without oestradiol treatment. This would help to confirm how these culture conditions directly affect downstream tissue expansion processes. Furthermore, it would be of additional benefit to sequence the macrophage component of the co-culture systems, to thoroughly investigate which macrophage targets may also be differentially regulated by the culture conditions being tested. Downstream analyses can then assess the roles of each of the ligand-receptor interactions of interest, individually, using targeted in-vitro approaches, such as knockout studies.

5.4.1.2 Oestradiol May Increase Gluteal Adipocyte Fat Storage

In the gluteal preadipocytes, compared with abdominal preadipocytes, oestradiol treatment was associated with immune-related and adipogenic processes, contrasting with the ECM-related genes and genes associated with the suppression of preadipocyte commitment, that were enriched in the vehicle-treated condition. Pathways indicative of enriched immune processes included anti-inflammatory genes such as *CSF1* (*M-CSF*), which was one of the top gluteal-enriched genes unique to oestradiol treatment in co-culture. M-CSF is a well-established M2 macrophage polarising factor, and was used in these co-culture systems to differentiate monocytes. It would be interesting to further explore whether this production of M-CSF by preadipocytes is sufficient to induce monocyte differentiation, by co-culturing undifferentiated monocytes with either abdominal or gluteal preadipocytes, with or without oestradiol. Subsequent assessment of M2 marker genes, for example through FACS, could then inform on whether monocytes are polarised in a particular direction following depot-specific co-culture and oestradiol treatment. This would further add to the evidence established here, that oestradiol facilitates the generation of an anti-inflammatory microenvironment within the gluteal depot. This anti-inflammatory environment could support healthier adipose tissue expansion, aiding processes such as preadipocyte proliferation, commitment and differentiation [485].

This signature hasn't been directly investigated in other literature, but research into lipodema, characterised by an increased gluteal fat mass which is particularly oestradiol-sensitive [61], has been informative. In these patients, there is an increased M2-like macrophage signature in gluteal adipose tissue [177], alongside increased oestradiol sensitivity and increased gluteal adiposity [61], strongly suggesting that these responses may be interlinked.

In the presented data, enrichment of processes relating to tissue expansion in the oestradiol treatment condition, further suggested that oestradiol may selectively enhance gluteal adipose expansion. This pro-adipogenic signature, exerted with oestradiol, was further retained with the application of LPS. Interestingly, this analysis also highlighted a carboxylesterase enzyme gene, involved in lipid metabolism and steroid-ester formation, likely contributing to the regulation of oestradiol esterification in gluteal fat [501, 502], thereby influencing local hormone storage and metabolism. These findings align with prior reports suggesting that oestradiol plays a role in depot-specific lipid storage by modulating both adipogenesis and inflammation in a context-dependent manner.

The outlined pro-adipogenic and anti-inflammatory activities from oestradiol treatment in gluteal preadipocytes, may be heightened by communication with macrophages, as the interactome analysis identified several relevant targets. This included *ACVRL1*, associated with cell proliferation through TGF- β receptor activity [478], which may be activated by M2 macrophage production of TGF- β [613]. With the additional application of LPS, this signature was still present, as genes such as *CD55*, a stem-like marker [498, 499], were also DEGs with LPS treatment. CD55 interacts with macrophage receptors such as ADGRE2 and ADGRE5 (Figure 5.7C), which are known to play roles in macrophage differentiation [503] and suppression of NF κ B-induced inflammation [504]. This suggests that CD55 may contribute to maintaining an anti-inflammatory macrophage population in the gluteal depot, even in the presence of inflammatory stimuli like LPS, potentially safeguarding the depot's adipogenic and lipid storage functions. This was further supported by the increased expression of *JAG1*, which acts at macrophage Notch receptors.

Knockout studies of Notch1 and Notch2 in adipose tissue macrophages lead to M1-like polarisation and impaired metabolic responses [505], indicating that JAG1 activation could promote an M2-biased macrophage population, further contributing to the maintenance of an anti-inflammatory environment.

Overall, these data suggest that oestradiol may play a role in regulating depot-specific fat storage and inflammation mechanisms, with potential opposing effects on lipolysis and fat storage in abdominal versus gluteal preadipocytes. Given, however, the subtlety of the direct effects of oestradiol treatment seen in MBDFAT preadipocytes in-vitro, further research is needed to establish whether other tissue factors are necessary to better elucidate tissue responses. Since mature adipocytes showed greater expression of the oestrogen receptors ER- α and ER- β , compared to cells within the SVF (explored in section 4.3.1), preadipocyte differentiation may be an important factor contributing to tissue oestradiol responses. Future work should therefore seek to characterise how oestradiol responses may change over a differentiation timecourse.

Oestradiol's ability, however, to promote M2 macrophage polarisation [212, 386] likely contributes to adipose tissue health. This is of particular relevance considering the increased production of oestradiol in the gluteal depot, which likely contributes to the beneficial metabolic signature observed with gluteal adipose tissue expansion [2]. These findings provide a mechanistic foundation for understanding how oestradiol contributes to the distinct adipose tissue distributions observed in females, a topic that remains underexplored in the current literature.

5.4.2 Preadipocytes Exert Depot-Specific Gene Expression Signatures in Response to Co-Culturing

Interestingly, preliminary qPCR data from co-cultures suggested that co-culture periods of 24 hours harboured a more inflammatory signature than co-cultures which were incubated for 48 hours or longer. This indicated that increasing the co-culture duration may provide time for the dampening of inflammation in the surrounding microenvironment. This is of particular relevance, given that many

existing co-culture studies, only use a 24-hour co-culture duration, potentially biasing these results towards a more inflammatory environment, and explaining the anti-adipogenic or pro-inflammatory effects that have been observed [170, 171, 174]. This is further highlighted by the frequent use of inflammatory-polarised macrophages in co-culture systems [169, 170]. Few studies have assessed the unbiased effects of co-culturing non-activated macrophages with preadipocytes, particularly in direct-contact systems, and thus the presented data uniquely highlighted the decreased inflammatory environment induced with increased co-culture duration. These preliminary data were supported by the subsequent sequencing data, where the comparison of mono- and co-cultured preadipocytes enabled the identification of macrophages' influence on preadipocyte phenotypes in a depot-specific context. GSEA firstly suggested enrichment for actin-based processes in monocultured abdominal preadipocytes, while top DEGs related to abdominal adiposity. Enrichment for actin-based processes may reflect the cytoskeletal changes that precede hypertrophy and lipid accumulation, suggesting that abdominal monocultured preadipocytes may be skewed towards hypertrophy, as compared to co-cultured cells. Interestingly, *ESR1* was also higher in monoculture relative to co-culture, suggesting that the presence of macrophages alters oestradiol signalling in the abdominal depot. Differential regulation of depot receptors, in this way, may contribute to depot-specific oestradiol responses, which haven't been observed in in-vitro monoculture systems, potentially due to the missing macrophage component which regulates this differential depot expression. With co-culture, top DEGs in the abdominal depot were related to anti-inflammatory and beiging processes in adipose tissue. A particular transcript of interest was *NEAT1*. This transcript is frequently loaded into extracellular vesicles [614], and has been shown to promote the polarisation of M2 macrophages [540], suggesting that co-culturing M-CSF-polarised macrophages with preadipocytes, may exert anti-inflammatory responses in both cellular parties, partly mediated by this exchange of long non-coding RNA-loaded vesicles. This signature was further supported by the interactome analysis, which identified *MXRA5*, acting at macrophage receptors including AXL, SIGLEC7

and PILR β , which may induce anti-inflammatory macrophage responses [541–545]. These results reinforce the hypothesis that when exposed to M-CSF-polarised macrophages, abdominal preadipocytes promote signalling that may protect against inflammation and encourage healthier adipose tissue function.

In gluteal preadipocytes, there was overwhelming enrichment seen for inflammatory responses in the monocultured cells, suggesting that co-culture suppresses inflammation. Additionally, transcripts associated with reduced adipocyte differentiation and suppression of expansion (e.g. *STC2* [549], *SOD2* [550], *LIF* [551]), were upregulated in monoculture, pointing to macrophages' role in promoting gluteal fat storage. This signature was supported by the genes which were upregulated in co-culture, including *SERPINB4* and *PLA2G7*, associated with decreased adipose tissue inflammatory responses [552, 553], further underscoring the anti-inflammatory properties of macrophage-preadipocyte interactions. The suppression of inflammatory signalling in the gluteal depot may thus facilitate tissue expansion by reducing fibrosis, thereby permitting for healthy mechanical tissue expansion and storage, in response to lipid. In summary, these findings demonstrate that co-culturing preadipocytes with M-CSF-polarised macrophages attenuates inflammation, particularly in the gluteal depot, while promoting signatures of adipogenesis and lipid storage. The depot-specific responses observed here suggest that macrophage-preadipocyte interactions are critical in shaping the inflammatory and expansion capacities of adipose tissues, with potential implications for understanding regional adiposity and its metabolic consequences. The ability of macrophages to mediate depot-specific phenotypes highlights their integral role in maintaining tissue-specific adipose function, particularly in regulating inflammation and facilitating healthy fat expansion, supporting the notion that macrophage-preadipocyte crosstalk is essential for the maintenance of depot-specific adipose functions.

5.4.3 LPS Modulates Depot-Specific Signatures in Co-cultured Preadipocytes

In response to LPS, depot-unique transcriptional signatures in co-culture were suggestive of distinct inflammatory responses in each depot.

5.4.3.1 LPS Impairs Abdominal Adipose Tissue Expansion Mechanisms

In the abdominal depot, LPS treatment significantly enhanced inflammatory responses while simultaneously activating pathways related to hypoxia, vasoconstriction, and immune activation, and suppressing oxidative phosphorylation and mitotic processes. Pathway enrichment analyses were supported by top DEGs, and the interactome analysis, revealing key target genes associated with inflammation, oxidative stress and cell cycle. The cell cycle signature was particularly striking, originally revealing over 100 enriched pathways associated with cell division and mitosis (parsed for presentation purposes). This strong cell cycle suppression signature suggests that LPS impairs the hyperplastic potential of co-cultured abdominal preadipocytes, likely reducing this depot's ability to expand and store fat effectively.

A diminished fat storage capacity is well-established as metabolically harmful, as it leads to FA spillover, lipotoxicity, and ectopic fat deposition, as adipose tissue cannot adequately sequester circulating FA [615]. Thus, this newly identified link between inflammation and reduced abdominal preadipocyte hyperplasia, may have deleterious consequences in the context of obesity-associated chronic inflammation. Chronic-inflammation may therefore potentiate metabolic dysfunction by preventing abdominal adipose tissue expansion. Moreover, this signature may be further worsened by inflammation-induced upregulation of ANGPTL4 and downregulation of LPL. LPL is the critical enzyme permitting the hydrolysis of FAs in circulating lipoproteins, for subsequent uptake into adipose tissue, preventing spillover and ectopic fat deposition [616]. ANGPTL4, a key suppressor of LPL activity, was upregulated under inflammatory conditions, inhibiting LPL function and likely further limiting FA uptake into adipose tissue.

The inflammation-mediated upregulation of ANGPTL4 and downregulation of LPL represents a novel finding with profound implications for understanding how obesity-associated chronic inflammation contributes to metabolic disease. These results uniquely highlight the LPS sensitivity of abdominal preadipocytes, revealing that inflammation disrupts both fat storage mechanisms and the mitotic capacity of these cells. Reduced adipose tissue uptake of circulating triglycerides would subsequently increase plasma triglyceride levels, promoting spillover and ectopic fat deposition. These findings therefore provide pioneering insights into how inflammation exacerbates metabolic dysfunction and suggests potential therapeutic targets to mitigate the harmful effects of chronic inflammation on adipose tissue function.

5.4.3.2 LPS Promotes Gluteal Adipose Tissue Inflammation

In the gluteal depot, processes and top DEGs increased by LPS treatment largely related to immune activation and inflammation, while downregulated pathways were indicative of ECM changes. The extent of metabolic pathway changes in the gluteal depot, did not parallel that of the abdominal depot, suggesting that, although LPS increased inflammation, it may not be subsequently impacting metabolic processes to the same extent as in the abdominal depot, suggesting that the gluteal depot may have a greater resistance to metabolic perturbation in response to inflammation, as compared to the abdominal depot. Given that the abdominal preadipocytes were particularly sensitive to changes in hyperplastic function, the gluteal depot may be better primed to combat these changes, allowing gluteal preadipocytes to continue hyperplastic functioning in the face of inflammation, thereby permitting healthy adipose expansion.

5.4.3.3 LPS Diverts Steroid Synthesis Pathways

Further to this, LPS critically impacted steroid synthesis pathways in abdominal preadipocytes, in a pattern that likely favoured cortisol generation and cortisol sensitivity, over sex-steroid synthesis and sensitivity. Given cortisol's role in promoting abdominal fat accumulation at the expense of gluteal fat [617], these findings suggest that LPS may promote greater abdominal adiposity through

cortisol generation, despite an impaired lipid storage signature in this depot. These findings also suggest that inflammatory stimuli can upregulate 11 β -hydroxysteroid dehydrogenase (11 β HSD) expression [618]. Interestingly, LPS has also been found to augment 11 β HSD expression in macrophage populations, and also promote M1 phenotypes [619], suggesting that inflammation and 11 β HSD expression may be intrinsically linked in multiple cell populations. Furthermore, downregulating the formation of beneficial steroids like oestradiol may further impact the detrimental effect of elevated cortisol. In this way, inflammatory stimuli may be affecting fat distribution on multiple levels, affecting regulatory steroid synthesis, as well as fat storage capacity, to finally promote unhealthy abdominal adipose expansion with a reduced tissue expandability. Physiologically, this would lead to an inability to effectively buffer circulating triglycerides, promoting the deposition of ectopic adipose tissue, predisposing to further metabolic disease.

Given these observations, future research should focus on the functional outcomes of preadipocyte responses to LPS, oestradiol and co-culturing, for example by measuring adipogenesis, lipolysis and cellular proliferation. Additionally, investigating the activity of relevant steroid synthesis enzymes will be crucial for understanding how inflammation modulates steroid production and responses, and whether this disrupts the activity of beneficial sex hormones such as oestradiol. The identified interactome targets should be further probed, to understand how these signals between macrophages and preadipocytes may be contributing to depot-specific tissue function, particularly in response to oestradiol and inflammatory stimuli.

6

Conclusion and Future Directions

6.1 Research Questions

Overall, this thesis firstly aimed to characterise adipose tissue stromal vascular fraction (SVF) populations, using single-cell RNA sequencing (scRNASeq) data to compare populations across different depots and sexes. This enabled a pioneering investigation into their transcriptional signatures. These novel insights highlighted the unique transcriptional biology of the female gluteal depot, which harboured a higher proportion of anti-inflammatory macrophage populations, and a greater abundance of immature preadipocyte populations (described in Section [3.3.2](#)), which likely contribute to the cellular hyperplastic pool. Understanding the underlying biology of this depot is essential for elucidating the molecular mechanisms that promote the female pattern of fat distribution and its associated metabolic protection.

This work subsequently aimed to investigate oestradiol responses in-vitro, using depot-specific preadipocyte cell line models. The complex and often contradictory findings within the current literature, regarding oestradiol's effects on monocultured preadipocytes, were somewhat validated by the difficulty associated with measuring preadipocyte oestradiol responses. To elucidate these effects, it was necessary to compare the depot-specific transcriptomic landscape, in order to highlight the subtle

and opposing effect of oestradiol on abdominal and gluteal preadipocytes. RNA sequencing analysis (described in Section 4.3.6) of oestradiol-treated preadipocytes, sourced from both abdominal and gluteal depots, therefore provided a fresh understanding of the molecular players that may mediate the depot-specific preadipocyte responses to oestradiol. Given the roles of macrophages in modulating adipose tissue homeostasis, and their well-documented oestradiol responsiveness [386], it was subsequently pertinent to examine how macrophage-preadipocyte crosstalk may further alter the depot-specific responses to oestradiol.

The next point that was addressed, therefore, was the establishment of co-culture systems for the investigation of depot-specific preadipocyte-macrophage communication. This revealed valuable observations regarding the potential intricate mechanisms by which macrophage-preadipocyte signalling varies across different fat depots, informed by signalling data, derived from in-vivo tissue biopsies. The identified interaction targets therefore offered an unprecedented perspective on how adipose tissue distribution may be regulated by macrophage-preadipocyte signalling (described throughout Section 5.3), particularly in response to oestradiol and also inflammatory signals. This has far reaching implications for understanding the drivers of sexual dimorphisms in regional adiposity, and their subsequent influences on metabolic health.

6.2 Depot-Specific Responses to Oestradiol and Macrophage-Preadipocyte Interactions

A key theme throughout this thesis has highlighted the contrast in responses of abdominal and gluteal preadipocytes in their production of, metabolism of, and responses to, oestradiol, investigating the molecular players likely contributing to these differences. The elevated production of oestradiol by gluteal preadipocytes, as compared to abdominal preadipocytes (explored in Section 4.3.2), may critically contribute to the increased anti-inflammatory macrophage signature that was observed in this depot. This may further promote adipose expansion through the regulation of the anti-inflammatory microenvironment, since this work also

showed that lipopolysaccharide (LPS), an inflammatory stimulus, suppressed cellular proliferation pathways in abdominal preadipocytes. Indeed, co-culturing preadipocytes and macrophages was further observed to decrease inflammatory responses in preadipocytes, as compared to monocultured cells (explored in Section 5.3). Moreover, this signature may be permissive of tissue expansion, particularly in the gluteal region, where there was significant downregulation of genes associated with the suppression of adipocyte differentiation and tissue expansion (e.g *STC2* [549], *SOD2* [550], *LIF* [551]), in adipocyte co-culture as compared to monoculture. Oestradiol treatment could further promote this anti-inflammatory signature, as the expression of key anti-inflammatory genes, such as *CSF1/M-CSF*, was increased in the gluteal depot with oestradiol treatment, alongside transcripts associated with fat metabolism and storage, including *CES4*. Meanwhile, opposite signatures, associated with decreased lipid accumulation, were upregulated in abdominal preadipocytes in response to oestradiol. Thus, oestradiol treatment in co-culture systems revealed a set of mechanisms by which oestradiol may modulate transcriptional signatures relating to sexual dimorphisms in fat storage. This suggested that the interplay between macrophages and preadipocytes creates depot-specific responses to oestradiol, demonstrating that adipose tissue health and function are not solely determined by adipocytes and preadipocytes but are also significantly shaped by macrophage-mediated signalling.

6.3 Inflammatory Responses and Their Impact on Adipose Tissue Function

This thesis further investigated the impact of inflammatory stimuli (discussed in Section 5.3), namely LPS, on macrophage-preadipocyte co-cultures, to reveal depot-specific differences in inflammatory responses, which have implications concerning tissue expansion capacity. Notably, abdominal preadipocytes exhibited a heightened sensitivity to LPS, as compared to gluteal preadipocytes, showing significant changes in processes relating to oxidative stress and hyperplasia, following LPS treatment. This response likely contributes to a suppression of cellular proliferation, that

biologically, would impair healthy tissue expansion mechanisms. LPS stimulation also influenced steroid hormone responses in this depot, potentially compromising the ability of preadipocytes to respond to sex-steroids, and promoting cortisol responsiveness. This shift in hormonal response signature may subsequently favour a central obesity phenotype due to the associations between cortisol and central obesity, providing a further mechanism by which inflammatory stimuli may hinder metabolic health, promoting central adiposity accumulation that can contribute to systemic metabolic disease. This may occur in spite of a reduced capacity for abdominal lipid storage, as LPS treatment resulted in increased expression of *ANGPTL4*, a transcript that has key regulatory effects on fat storage, especially in co-cultures. This uniquely highlighted an interaction between LPS-mediated macrophage activation, and adipocyte lipid metabolism.

The influence of LPS on metabolic processes was less prevalent in gluteal preadipocytes, which appeared to exhibit more resilience to LPS. Their transcriptional responses indicated an ability to maintain fat storage capacity and metabolic responses, in face of inflammatory stimuli. This depot-specific resilience to LPS may protect the gluteal depot against the metabolic effects of inflammation, helping to maintain gluteal fat as a metabolically protective depot. There was no evidence of altered steroid responses in gluteal preadipocytes treated with LPS, further suggesting that this depot may also retain its steroid hormones responsiveness. *CYP19A1* expression did not decrease with LPS treatment in gluteal preadipocytes, and thus the continued production of oestradiol in this depot may aid the retention of M2-polarised macrophages, despite inflammatory stimuli, aiding the preservation of the gluteal anti-inflammatory signature. This likely associates with the positive metabolic profile arising with the accumulation of gluteal adiposity, which appears to be less likely to contribute to chronic inflammation, such as that associated with obesity, as compared with the accumulation of abdominal adiposity. These observations align with studies linking lower-body fat distribution to improved metabolic health [620, 621], and suggest that inflammatory stimuli differentially impact the metabolic function and expansion capacity of abdominal and gluteal fat depots.

6.4 Broader Implications for Adipose Tissue Function and Metabolic Health

The insights gained from this thesis contribute to a deeper understanding of the biological underpinnings of regional adiposity, particularly the roles of oestradiol and macrophage-preadipocyte crosstalk. Depot-specific transcriptional responses to oestradiol and inflammation contribute to the understanding of sex-specific fat distribution patterns and their relevance to metabolic health. Women's preferential accumulation of gluteal fat, and the maintenance of this adiposity, and its resistance to inflammatory perturbation, likely serves a protective role against metabolic dysregulation. Moreover, these results also highlight the importance of considering the macrophage-mediated effects of depot-specific adipose tissue biology, particularly in the context of oestradiol. The co-culture system revealed that macrophages are not passive bystanders but active regulators of preadipocyte fate and function. Macrophages drive anti-inflammatory states in co-culture systems, further contributing to signatures relating to lipolysis and fat storage, in response to oestradiol or stressors such as LPS. These interactions are critical for understanding how inflammation and hormonal signals converge to regulate fat storage and distribution, with significant implications for therapeutic approaches targeting metabolic disorders such as obesity, type 2 diabetes mellitus, and cardiovascular disease.

6.5 Future Directions

The majority of this research employed cell culture systems and bioinformatics to investigate oestradiol responses in preadipocyte cell lines and explored how co-culture systems may modify these responses. Given that the MBDFAT cell lines utilised for the majority of this work exhibit lower ER- α expression compared to primary preadipocytes and differentiated preadipocytes, it would be valuable to replicate this study using primary preadipocytes and differentiated preadipocytes to determine whether they exhibit stronger transcriptional responses to oestradiol. Additionally, measuring intra-tissue concentrations of oestradiol and other relevant

steroid hormones across different depots would help confirm that the in-vitro media measurements accurately reflect the depot-specific in-vivo production of oestradiol. Building upon the findings from the interactome analysis and bulk RNA sequencing of co-culture systems, future research should aim to validate the functional relevance of candidate preadipocyte-macrophage communication signals. This could be achieved by modulating the activation of these signals in-vitro and assessing the phenotypic outcomes (e.g proliferation, differentiation, lipid accumulation). Further investigations should then explore how this inter-cellular communication operates in-vivo to influence fat distribution, particularly in response to changes in oestradiol concentrations or varying metabolic states.

Moreover, the observed link between steroid synthesis pathways and inflammatory stimuli warrants further exploration. Future studies should assess the concentrations of a comprehensive panel of steroid hormones, along with their receptors and relevant enzymes, in abdominal and gluteal adipocyte monocultures and co-cultures, with or without inflammatory stimuli. It would be valuable, also to repeat this analysis using tissue biopsies, across donors of different metabolic status (which should incorporate those with obesity-associated chronic inflammation), with a range of plasma oestradiol concentrations, and ideally also recruiting participants of differing cortisol status (e.g patients with Cushingoid disease). This approach would help clarify how depot-specific intra-tissue hormone production is modulated by macrophages, inflammation, and oestradiol, and how these factors contribute to variations in fat distribution phenotypes.

6.6 Conclusion

In conclusion, this thesis provides a novel perspective on the role of macrophage-preadipocyte interactions in depot-specific responses to oestradiol and inflammation, revealing their molecular and cellular underpinning mechanisms. By integrating macrophages into a preadipocyte culture system, these results reveal that macrophages likely play an essential role in shaping depot responses to these stimuli, which likely has functional implications relating to fat distribution and

metabolic health. These findings pave the way for future research to explore the molecular targets identified here, potentially informing therapeutic strategies that can modulate macrophage-preadipocyte signalling to promote healthy adipose tissue expansion, and fat distribution, to help combat obesity-related metabolic disorders.

Appendices

A

Appendix

A.1 qPCR Probe Assay Identification Codes

Gene	Assay ID
18s	Hs99999901_s1
ANGPTL4	Hs01101123_g1
B2M	Hs00187842_m1
B4GALT1	Hs00155245_m1
CD14	Hs02621496_s1
CD206	Hs00267207_m1
CYP19A1	Hs00903411_m1
DDX3Y	Hs00965254_gH
ESR1	Hs01046816_m1
ESR2	Hs001100353_m1
GAPDH	Hs99999905_m1
GPB1	Hs01922715_s1
IGFBP3	Hs00181211_m1
IL1B	Hs01555410_m1
IL6	Hs00174131_m1
IL10	Hs00961622_m1
LPL	Hs00173425_m1
MYC	Hs00153408_m1
PGK1	Hs99999906_m1
PPIA	Hs99999904_m1
TNFA	Hs99999043_m1
UBC	Hs00824723_m1
VEGFA	Hs00900055_m1
XIST	Hs01079824_m1

Figure A.1: Genes and Assay Identification Numbers for TaqMan™ qPCR Primers

A.2 scRNASeq Biopsy Cluster Proportions

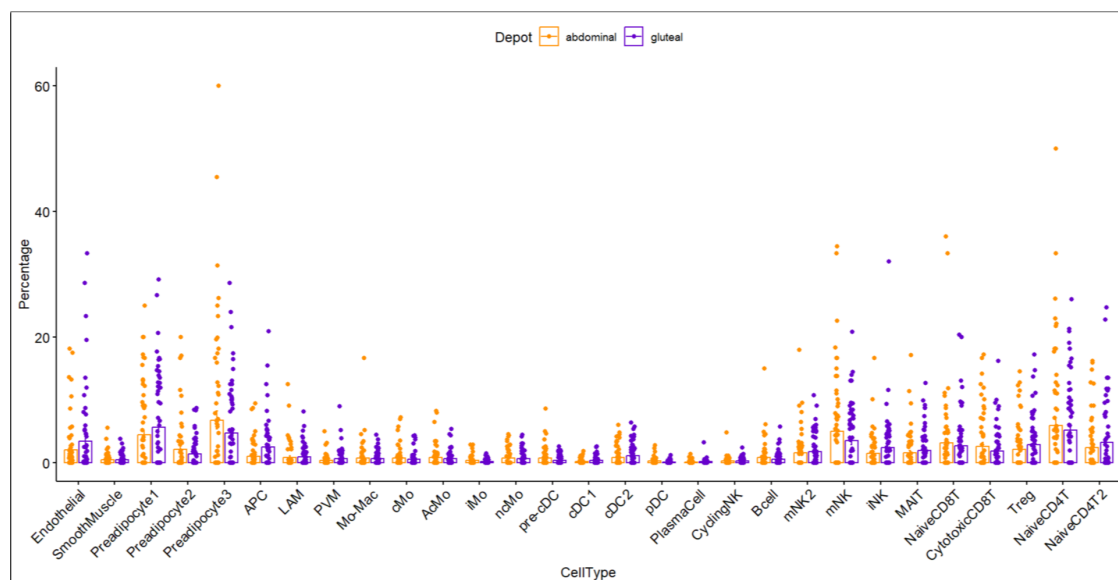


Figure A.2: Bar graph presenting cellular cluster proportions across each donor biopsy, displaying the proportion of cell types in each biopsy. Abdominal and gluteal biopsies are presented separately, with abdominal data points presented in orange, and gluteal in purple.

A.3 Detailed Information for Antibody Products Used in Western Blotting Protocols

Protein	Antibody ID, Supplier	Clone	Host, Isotype	RRID (if available)	Dilution for WB
ER- α	PA1-309, ThermoFisher	Polyclonal	Rabbit, IgG	AB_325814	1 μ g/mL
	PA1-308, ThermoFisher	Polyclonal	Rabbit, IgG	AB_325813	1ug/ml
	GTX127978, GeneTex	Polyclonal	Rabbit, IgG	AB_2885670	1:2000
ER- β	PA1-311, ThermoFisher	Polyclonal	Rabbit, IgG	AB_325597	1:1000, 1ug/ml
	14-9336-80, ThermoFisher	Monoclonal, MC10	Mouse, IgG2a kappa	AB_2572943	1:300, 1:1000
	MA524807, ThermoFisher	Monoclonal, PPZ0506	Mouse, IgG2b	AB_2717280	1 μ g/mL
	GTX134663, GeneTex	Polyclonal	Rabbit, IgG	AB_2887320	1:1000
GPER1	AF5534, BioTechne	Polyclonal	Goat, IgG	NA	1ug/ml
	NLS4271, BioTechne	Polyclonal	Rabbit, IgG	NA	1:1000, 1ug/ml
	GTX107748, GeneTex	Polyclonal	Rabbit, IgG	AB_1240855	1:500
CYP19A1	NB100-1596SS, BioTechne	Polyclonal	Rabbit, IgG	NA	1 ug/ml 1:1000
	Ab18995, Abcam	Polyclonal	Rabbit, IgG	NA	2ug/ml
	GTX88826, GeneTex	Polyclonal	Goat, IgG	NA	1:2000, 0.25ug/ml
	MCA2077S, BioRad	Monoclonal, H4	Mouse, IgG2a	NA	1:250
α -tubulin	Ab15246, Abcam	Polyclonal	Rabbit, IgG	NA	1:2000

Figure A.3: *Further specifics, detailing the antibody products used in Western blotting protocols.*

A.4 Effect of Genotype on Examined Genes of Interest

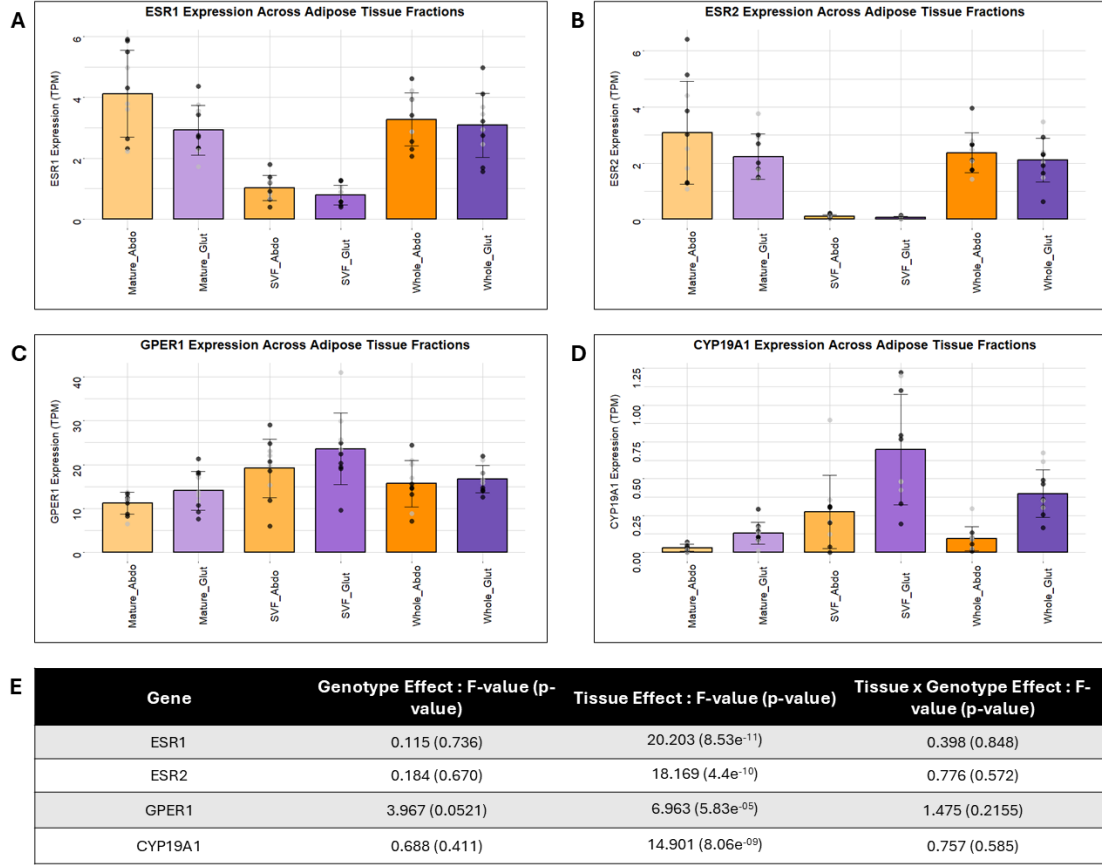
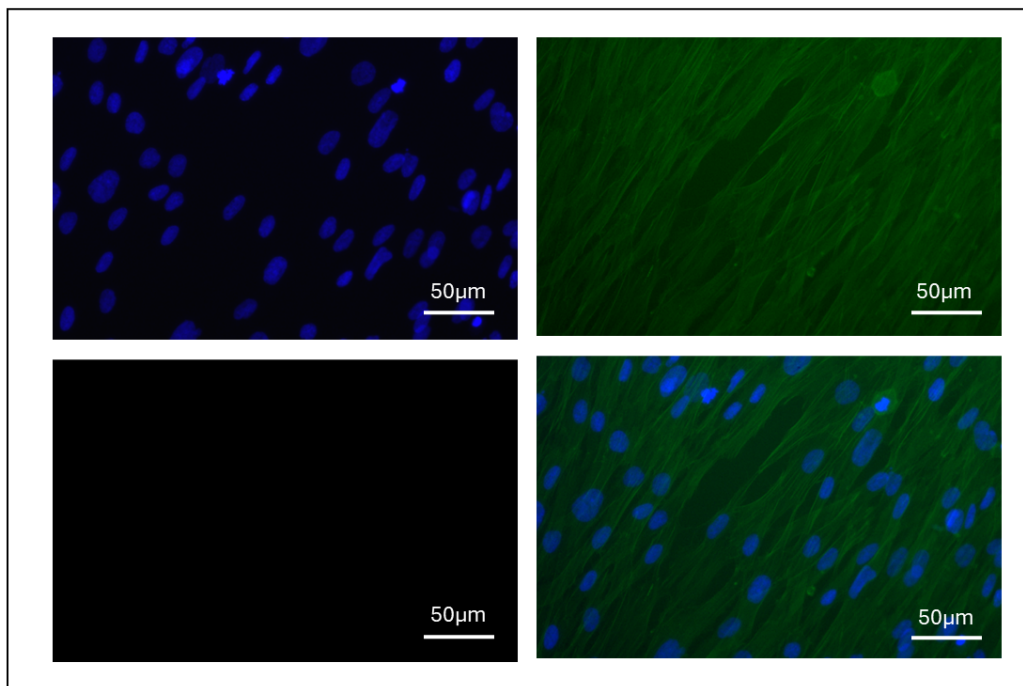
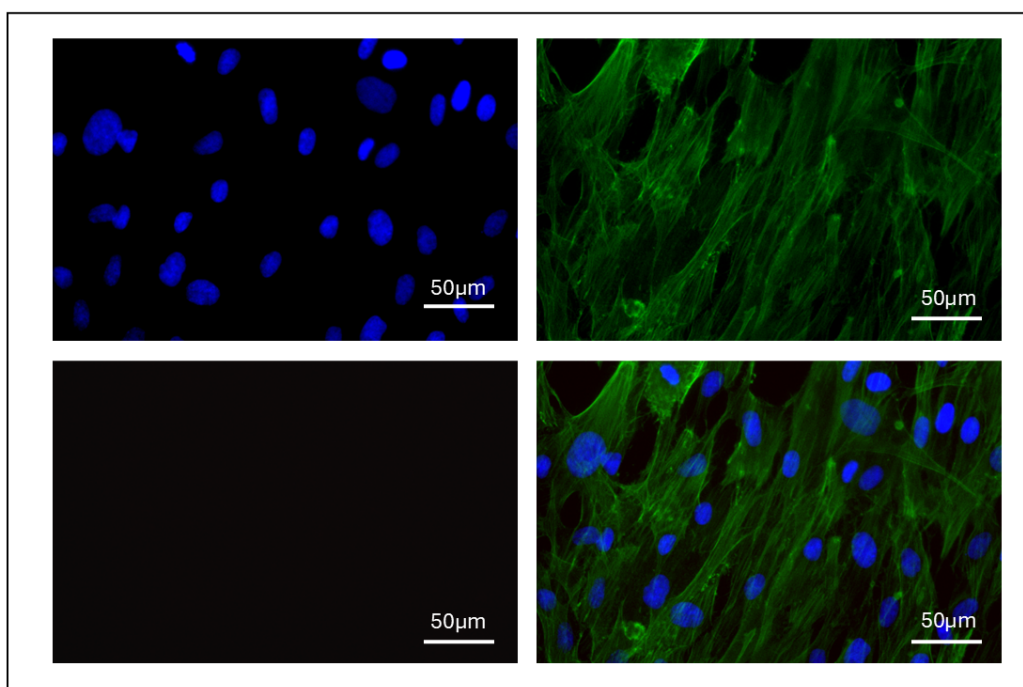


Figure A.4: Expression of examined genes across adipose tissue fractions (SVF and mature adipocytes), and whole tissue, in paired abdominal and gluteal biopsies ($n=10$). On the x-axis from left to right, sample data presented are: abdominal mature adipocyte fraction, gluteal mature adipocyte fraction, abdominal SVF, gluteal SVF, whole abdominal adipose tissue, whole gluteal adipose tissue. Bars represent the mean value. Data points in black represent the rs972283 GG genotype, and points in grey represent the AA genotype. Error bars represent standard deviation. Genes displayed are: **A**) ESR1 ; **B**) ESR2 ; **C**) GPER1 ; **D**) CYP19A1. **E**) Table displaying the effect of genotype and tissue (depot and fraction) and their interaction, on gene expression, using a 2-way ANOVA test.

A.5 Immunostaining Controls

A**B**

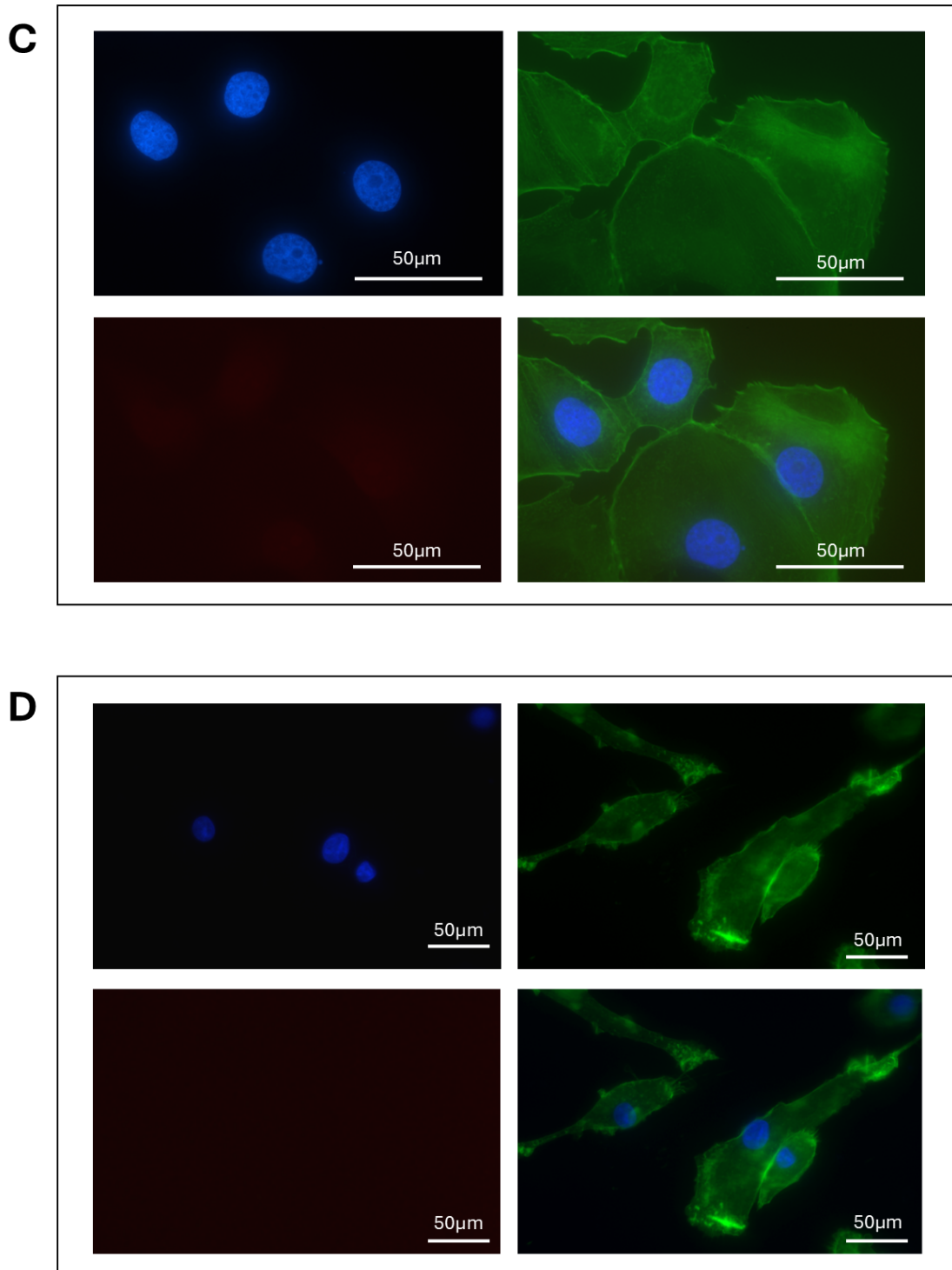


Figure A.5: Representative images, showing immunostaining in control samples, stained with nuclear (Hoechst) and cytoskeletal (Phalloidin) stains, as well as secondary antibodies, with the omission of primary antibody. Details of antibody dilutions and incubation times are specified in Section 2.13 and Section 4.2.2.2. The top left panels display Hoechst staining, while the top right panel shows Phalloidin staining, and the bottom left panel shows secondary antibody (Alexa Fluor 555) staining. The bottom right panel then presents an image composite of the three previous images. Images were taken for both abdominal and gluteal MBDFAT preadipocytes, and these are presented in Subfigure A) and Subfigure B), respectively. Images for MCF7 cells are presented in Subfigure C), and macrophages in Subfigure D)

A.6 Preliminary Flow Cytometry Plots

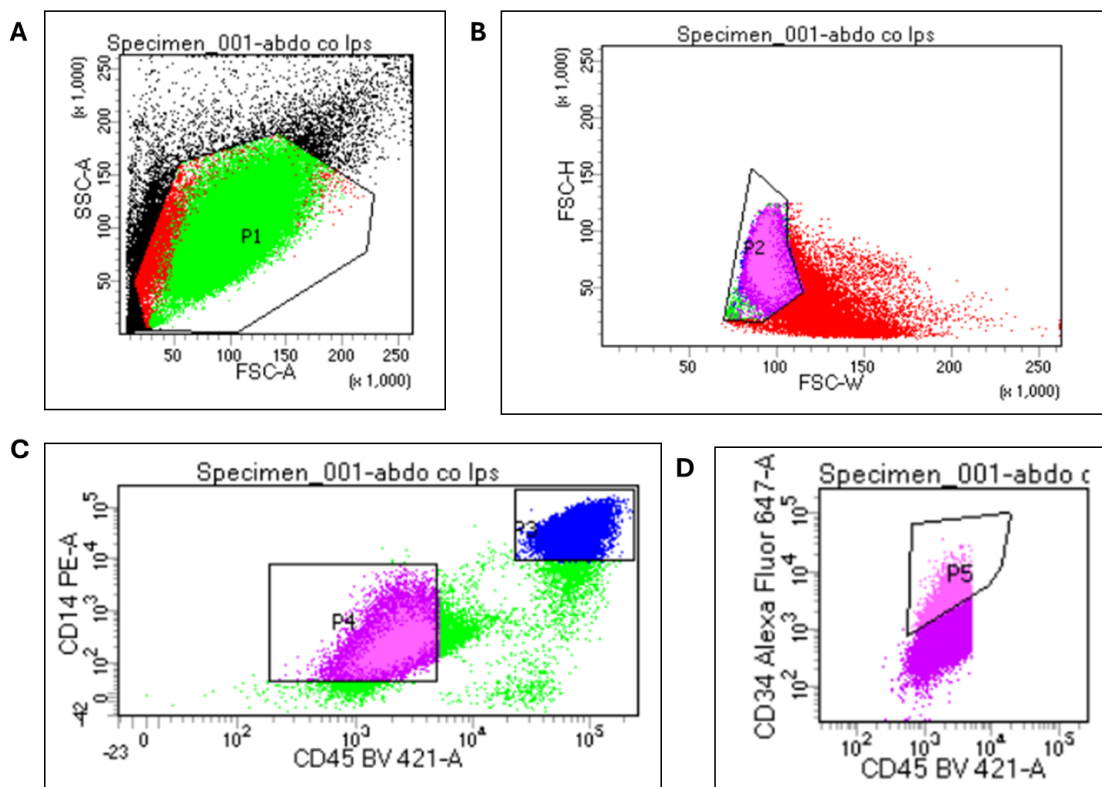


Figure A.6: Representative flow cytometry pilot data plots, showing antibody test staining and gating for abdominal preadipocyte co-cultures, using the first tested antibody marker set (CD14, CD45, CD34). **A)** Forward and side scatter gating to remove cell debris. **B)** Forward scatter height and width gating, to remove doublets. **C)** CD45 and CD14 staining, used to gate macrophages, and preadipocytes (in the P₄ box). **D)** Preadipocytes, gated from the P₄ box in Subfigure C, shown for CD34 staining. No clear CD34 positive population was observed across any conditions.

A.7 FACS Plots

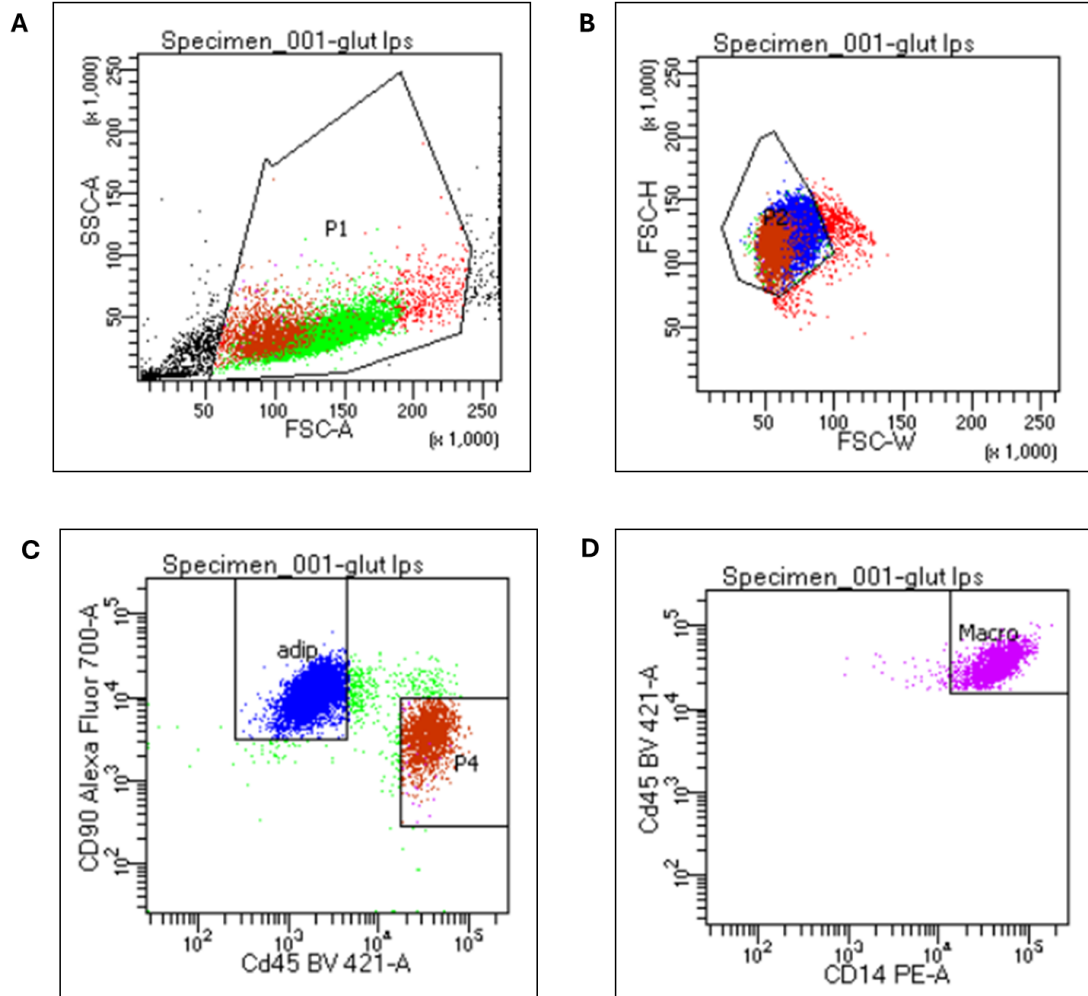


Figure A.7: Representative FACS plots, showing antibody test staining for gluteal preadipocyte co-cultures, using the finalised antibody marker set (CD14, CD45, CD90). **A)** Forward and side scatter gating to remove cell debris. **B)** Forward scatter height and width gating, to remove doublets. **C)** CD45 and CD90 staining, used to positively gate preadipocytes based on positive CD90 staining **D)** Positive macrophage gating, based on CD14 and CD45 staining, gated from the P4 box in Subfigure C.

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