Fibre and sex specific differences in mitochondrial content and subcellular distribution and morphology of lipid droplets in skeletal muscle biopsies obtained from lean, obese and type 2 diabetes patients

Myfanwy Tirion Macey

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Master of Philosophy

May 2020

Abstract

Intramuscular triglycerides (IMTG) are stored in lipid droplets (LD) in skeletal muscle, and it is thought that subcellular location, size and number of LDs relate to insulin sensitivity (IS) more so than IMTG content alone. Reduced expression and function of mitochondria has been implicated in the development of insulin resistance, leading to an accumulation of IMTG due to reduced oxidative capacity. The aim of the present study was to 1) establish a better understanding of the sex differences in the relationship between LD profile, mitochondrial density and the development of insulin resistance thereby 2) contributing to current and future research improving insulin sensitivity and preventing the progression of insulin resistance. The study investigated the hypothesis that 1) females have greater LD stores compared to males whilst maintaining insulin sensitivity, and 2) that type two diabetes (T2D) patients have greater LD stores compared to their lean and obese counterparts. Muscle biopsies were obtained from 48 male (n=24) and female (n=24) participants, categorised into groups (n=16/group) based on metabolic health; lean, obese and T2D. Cryosections (5µm) were stained using appropriate antibodies targeting myosin heavy chain I (MHC I) and MHC IIa. LDs were stained using bodipy 493/503 and mitochondria using mouse monoclonal anti-OxPhos complex IV (COXIV). Images were obtained using widefield and confocal fluorescence microscopy. In males, LD content was two-fold greater (P<0.05) within both the central and peripheral (5µm below cell membrane) regions of the type I and II fibres due to a two-fold increase in LD density (P<0.05) alongside larger LDs by one-fold (P<0.05) in comparison to females; thereby contradicting the first hypothesis. However, in agreement with the second hypothesis LD content was two-fold greater (P<0.05) in male T2D specifically within the central region of the type IIa fibres compared to the lean and obese males; primarily driven through larger LDs by one-fold (P<0.05). Furthermore, mitochondrial density followed a hierarchical distribution (TI>TII; P<0.001) in both males and females, with no differences observed between sexes or metabolic health status ($P>0.05$). In conclusion, this thesis presents novel data on sex differences in LD profile, mitochondrial density and insulin resistance and suggests a potential sex paradox; whereby reduced sex hormone production in post-menopausal females, alongside inactivity maybe more important determinants of insulin sensitivity than LD profile alone.

Acknowledgements

First and foremost I would like to express my sincere gratitude to my supervisor Dr Juliette Strauss, for your continuous support throughout my MPhil, for your patience, motivation and enthusiasm. Your guidance has helped me in all the time of research and writing of this thesis. I would also like to thank the rest of my thesis committee; Dr Sam Shepherd and Prof Anton Wagenmakers, for your encouragement and insightful comments throughout my research journey.

My sincere thanks also goes to Team Anton; Dr Mathew Cocks, Hannah, Jen, Katie, Emma, Emily, Andrea and Jordan. I have been extremely fortunate to complete my research amongst such a wonderful and supportive group of individuals, my studies would not have been as enjoyable without the Team Anton gatherings. An extended thanks to those in the PGR office, for all of your support and of course for the prestigious PGR office bake-off.

For my friends; Rachel thank you for always being my rock during the highs and lows of life, for the many chats we have had over coffee and for the adventures we have shared in Liverpool. A big thank you also to Abbie, Eli, Meg and Emma, a constant reminder that there is always something to smile about and that the future is not as scary as one may think when you are surrounded by good friends.

I would also like to thank Michael. Your love and support throughout our journey in Liverpool has meant so much, especially during my MPhil when a little more support and encouragement was needed.

Most importantly, a special thank you to my family for all of their unconditional love and support, not only during my research but throughout life. Mum and Dad, thank you for the belief and confidence you have always had in me, and for your enthusiasm and encouragement along the way, this has been fundamental in all my achievements. Meg and Nel, thank you for the regular pep-talks and for always keeping me laughing, this has truly kept me sane during the more challenging times when completing this thesis. Thank you Taid, I hope you are aware as to how much I appreciate your endless support and love over the years. To Nain, although she is not here to see me complete my thesis, it was truly a privilege to have her encouragement and advice throughout life whether it be academic, sports or art. Her copious amounts of love is sorely missed. This acknowledgement of thanks could never be enough for the love and support you have all shown me over the years, I hope I continue to make you proud.

Conference communications and publications

During the completion of this MPhil at Liverpool John Moores University, data from this thesis resulted in the following abstracts and conference communications

Macey MT, Shepherd SO & Strauss JA. (2019). Fibre and sex specific differences in mitochondrial content and subcellular distribution and morphology of lipid droplets in skeletal muscle biopsies obtained from lean, obese and type 2 diabetes patients. Oral communication, Future physiology, Liverpool John Moores University, UK.

Macey MT, Shepherd SO & Strauss JA. (2019). Gender specific differences in the subcellular distribution and morphology of lipid droplets in skeletal muscle of trained, sedentary, obese and type 2 diabetic females and males. Short-listed for the Lily Diabetes Clinical Science Poster Award. Oral and poster communication, Diabetes UK Professional Conference, Liverpool M&S Arena, UK.

Macey MT, Shepherd SO & Strauss JA. (2019). Sex differences in the subcellular distribution and morphology of lipid droplets in human skeletal muscle of lean, obese and type 2 diabetes patients. Poster presentation, Institute for Health Research Conference, Liverpool, UK.

Macey MT, Shepherd SO & Strauss JA. (2019). Sex differences in the subcellular distribution and morphology of lipid droplets in human skeletal muscle of lean, obese and type 2 diabetes patients. Poster presentation, Doctoral Academy Conference, Liverpool, UK.

The following papers were published during the period of postgraduate study

Strauss JA, Shepherd DA, Macey M, Jevons EF & Shepherd SO. (2020). Divergence exists in the subcellular distribution of intramuscular triglyceride in human skeletal muscle dependent on the choice of lipid dye. *Histochemistry and Cell Biology*. ISSN 1432-119X.

Table of Contents

List of Figures

List of Tables

Abbreviations

β-HAD β Hydroxyacyl CoA Dehydrogenase

ACS Acyl-CoA Synthetase

AGPAT 1-Acylglycerol-3-Phosphate Acyltransferase

AMPK AMP-Activated Protein Kinase

aPKC Atypical Protein Kinase

AS160 Akt substrate of 160

ATGL Adipose Triacylglycerol Lipase

ATP Adenosine Triphosphate

BMI Body Mass Index

CAT Carnitine-Acylcarnitine Translocase

COX IV Cytochrome Oxidase IV

CPT Carnitine Palmitoyltransferase

DAG Diacylglycerol

DGAT Diacylglycerol Transferase

eNOS Endothelial nitric oxide synthase

FA Fatty Acid

FAS Fatty Acid Synthase

FAT/CD36 Fatty acid translocase

FFA Free Fatty Acid

G-1-P Glucose-1-Phosphate

G-3-P Glycerol-3- Phosphate

G-6-P Glucose-6-Phosphate

GDP Guanosine Diphosphate

GLUT4 Glucose Transporter Type Four

GPAT Glycerol-3-Phosphate Acyltransferase

GTP Guanosine Triphosphate

HOMA-IR Homeostatic Model Assessment of Insulin Resistance

HSL Hormone Sensitive Lipase

IMF Intramyofibrillar

IMTG Intramuscular Triacylglycerol

IPAQ International Physical Activity Questionnaire

IRS Insulin Receptor Substrate

LCFA Long Chain Fatty Acid

LD Lipid Droplets

LPA Lysophosphatidic Acid

LPL Lipoprotein Lipase

MAG Monoacylglycerol

MAPK Mitogen-activated Protein Kinase

MGAT Monoacylglycerol Transferase

MHC Myosin Heavy Chain

NO Nitric oxide

nPKC Novel Protein Kinase C

OGTT Oral Glucose Tolerance Test

PA Physical Activity

PBS Phosphate Buffered Saline

PDH Pyruvate Dehydrogenase

PDK1 Phosphoinositide-Dependent Kinase-1

PFK Phosphofructokinase

PI3-K Phosphatidylinositol-3-Kinase

PIP_2 Phosphatidylinositol-Bisphosphate

PIP_3 Phosphatidylinositol-3, 4, 5-Triphosphate

PKB/Akt Protein Kinase B

PKC Protein Kinase C

PLIN Perilipins

PP2A Phosphoprotein Phosphatase 2A

PPAR Peroxisome Proliferator-Activated Receptor

Rab-GDP Rab Guanosine Diphosphate

Rab-GTP Rab Guanosine Triphosphate

RBP-4 Retinol-Binding Protein-4

ROS Reactive oxygen species

SHLP Small Human like Peptides

T2D Type 2 Diabetes

TAG Triacylglycerol

TBCD1D4 TBC 1 domain family, member 4

WGA Wheat Germ Agglutinin

1. Introduction

1.1 *Obesity epidemic and the prevalence of type 2 diabetes*

The current obesity epidemic and prevalence of type 2 diabetes (T2D) suggests that attempts to prevent these conditions thus far have not been effective. On a global scale, the prevalence of obesity continues to rise with 650 million adults worldwide now classified as obese (BMI ≥ 30 $kg.m^{-2}$) (World Health Organisation, 2017). In 2017 the percentage of adults in the UK classified as obese increased to 27%, which is an 11% increase since 1993. Data suggest that 25% of the adult population are classified as sedentary, meaning that they fail to achieve the recommended 150 minutes of moderate-intensity aerobic physical activity (PA) per week (Sport England, 2019). Physical inactivity has major implications for health and for the prevalence of non-communicable diseases and their risk factors, which include elevated blood pressure, decreased insulin sensitivity and obesity (Blair, 2009; Lee et al., 2012). Furthermore, physical inactivity can be associated with poor mental health and a reduced quality of life (Wardoku et al., 2019). According to Public Health England (2017) physical inactivity is severely impacting the national economy costing the wider society £27 billion per annum, and the UK-wide NHS costs are estimated to reach £9.7 billion per annum by 2050. Furthermore, as the fourth leading risk factor for global mortality (World Health Organisation, 2017), it is crucial that research surrounding physical inactivity and the subsequent development of insulin resistance is prioritised. More specifically the highest priority should be given to establishing a better understanding of the mechanisms by which physical inactivity and obesity leads to metabolic and (cardio)vascular diseases and how risks may be reduced with the identification of these key mechanisms.

Metabolic syndrome (prediabetes) and T2D are the fastest growing metabolic diseases related to inactivity and obesity, not only within the UK, but also worldwide (Pedersen, 2006). Recent analyses by Diabetes UK has revealed that the number of individuals diagnosed with T2D in the UK has more than doubled within the last twenty years, and the total number is expected to surpass five million by 2025, with a greater prevalence reported in men compared to women (Wild et al., 2004). The number of people diagnosed with diabetes has more than doubled since 1998; currently ~3.8 million UK citizens have diabetes. When considering the undiagnosed population in the UK the estimate rises to 4.7 million (Diabetes UK, 2019).

Furthermore, T2D accounts for approximately 90% of all diabetes cases in the UK with an estimated 12.3 million people at an increased risk of developing T2D.

Currently, obesity is the leading risk factor in the development of T2D (Abdullah et al., 2010). T2D is characterised by insulin resistance, which describes an insufficiency of the body's organs and tissues to respond effectively to physiological increases in circulating insulin. Insulin is a key hormone produced by the pancreas regulating the uptake of glucose into tissues around the body (Wilcox, 2005). Insulin contributes to the regulation of blood glucose concentrations. Therefore a blunted response to insulin results in elevated blood glucose concentrations (hyperglycaemia) for prolonged periods following a meal (Hayward et al., 2015). Severe cardiovascular and microvascular complications are consequences of uncontrolled blood glucose concentrations, which ultimately increases the risk of premature mortality (Watkins et al., 2003). The consumption of a Western diet, which is typically energy dense and rich in fat, results in impaired buffering capabilities of adipose tissue implying that part of the meal lipid intake will be deposited into other metabolic tissues, such as skeletal muscle and the liver, with this lipid spill-over contributing to the development of insulin resistance and T2D (see 5.1.2) (Frayn, 2002b). Reduced triacylglycerol (TAG) clearance in adipose tissue due to impaired buffering capacity results in high plasma TAG concentration and the accumulation of TAG in insulin-responsive tissues, which then develop insulin resistance. Furthermore, impaired insulin action compromises the ability of insulin to suppress free fatty acid (FA) release from adipose tissue (Frayn, 2002a). Normal buffering action of adipose tissue would increase TAG clearance which in turn suppresses the release of non-esterified FAs into the circulation. However, in obese and insulin resistant individuals, adipose tissue buffering of lipid fluxes following a meal is impaired due to the incapacity of adipose tissue to respond appropriately to the high plasma glucose, FA and TAG concentrations which occur post meal ingestion (Frayn, 2002a). The accumulation of lipid in skeletal muscle has been proposed to be a contributing factor for insulin resistance (Samuel, Petersen and Shulman, 2010; Turner et al., 2014; Kitessa and Abeywardena, 2016), and therefore it is essential that research provides further insight into the potential mechanisms by which impaired or abnormal lipid storage may cause insulin resistance. Such research could support the development of progressive pharmacological and non-pharmacological methods which may be used to reduce the risk of T2D.

1.2 Sk*eletal Muscle Insulin Sensitivity*

Skeletal muscle is the primary tissue for glucose disposal from the circulation and accounts for an estimated 65-70% of whole-body insulin-mediated glucose uptake during a hyperinsulinaemic euglycaemic clamp (DeFronzo et al., 1981). Glucose uptake into muscle is fundamentally insulin dependent via the translocation of glucose transporter 4 (GLUT4) (Wilcox, 2005). Preservation of skeletal muscle mass and function throughout life preserves physical independence in older age whilst also protecting individuals from a multitude of metabolic diseases which include insulin resistance (Brook et al., 2016). Periods of immobilization due to major surgery and hospitalisation often result in muscle atrophy, especially within older individuals who fail to regain sufficient levels of physical activity thereby negatively impacting muscle health and function resulting in impaired insulin action (Knudsen et al., 2012). Indeed, prolonged sedentary behaviour may contribute to compromised mobility and associated reductions in quality of life (Brook et al., 2016). Skeletal muscle insulin sensitivity may be positively or negatively influenced by a respective increase or decrease in physical activity alongside other factors (e.g. diet) due to its high plasticity to adapt or mal-adapt (Hoppeler, 2016). Maintaining skeletal muscle insulin sensitivity is paramount in the prevention and management of T2D amongst other metabolic defects (DeFronzo and Tripathy, 2009).

1.2.1 *Overview of Insulin action*

Under post absorptive (fasted) conditions insulin levels are characteristically low with higher concentrations present in the fed state. Blood glucose concentrations within healthy individuals will increase 30-40 minutes following glucose consumption during a 75g oral glucose tolerance test (OGTT) and would be expected to return to near baseline levels (<5.5 $mmol.L^{-1}$) 2 h into the OGTT (Wilcox, 2005). Research from Diabetes UK suggest that individuals with T2D have a fasting blood glucose concentration above 7 $mmol.L^{-1}$, with an inability to reduce blood glucose concentration below 11.1 $mmol.L^{-1}$ 2 h into the 75g OGTT (Diabetes, 2015). A rise in plasma glucose concentrations induced by meal-ingestion stimulates an increase in insulin secretion from the pancreas. Normal fasting plasma glucose (euglycaemia) concentrations are maintained through an enhanced activity of tissue-specific glucose transporters within skeletal muscle, liver and adipose tissue in response to physiological

increases in insulin thus facilitating the removal of glucose from the circulation(Wilcox, 2005). Glucose can be stored as liver and muscle glycogen or utilised as a source of energy within the skeletal muscle through the glycolytic pathway and complete aerobic oxidation to CO_2 and H_2O. Consumption of high carbohydrate diets may also lead to lipogenesis (conversion of glucose to FA and TAG synthesis) in adipose tissue cell.

1.2.2 *Insulin-meditated glucose uptake into skeletal muscle*

The process which activates the insulin signalling cascade in skeletal muscle commences when insulin binds to the extracellular α-subunit of the insulin receptor, thus stimulating tyrosine auto-phosphorylation of the transmembrane β-subunit (Pessin and Saltiel, 2000). This cascade of events activates the intrinsic tyrosine kinase resulting in tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins (Pessin and Saltiel, 2000). Activation of the insulin receptor results in the activation of two imperative signalling cascades; the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) pathways (Figure 1.1). Through insulin stimulation of the PI3-K pathway, GLUT4 translocates to the plasma membrane to stimulate skeletal muscle glucose uptake (Mul et al., 2015). Glucose is then delivered to and taken up by the muscle thorough GLUT-4 mediated facilitated diffusion transport through the plasma membrane (Richter and Hargreaves, 2013). When inside the muscle glucose is converted to glucose-6-phosphate (G6P) by hexokinase and then enters glycolysis to be utilized immediately, alternatively in a resting state it can be used for glycogen synthesis and storage (glycogenesis) (Richter and Hargreaves, 2013). The present glycogen stores within the skeletal muscle can be utilized through glycogenolysis which involves the breakdown of glycogen into G1P and G6P to then enter glycolysis (Jensen and Richter, 2012). Glycolysis involves the breakdown of G6P into pyruvate. Phosphofructokinase (PFK) regulates the conversion of fructose-6-phosphate into fructose-1, 6-disphosphate ultimately producing 2 molecules of pyruvate (Mul et al., 2015). Finally, the pyruvate is transported into the mitochondria via the mitochondrial carrier proteins MPC1 and MPC2. This is followed by oxidation of pyruvate by the pyruvate dehydrogenase complex (PDH) to 1 molecule of CO_2 and 1 molecule of acetyl-CoA. The final step is oxidation of acetyl-coA to 2 molecules of CO_2 in the tricarboxylic acid (TCA-cycle) (Spriet, 2014).

1.2.3 *IRS-PI3-K Signalling Pathway*

When circulating insulin binds to the insulin receptor located on the plasma membrane it activates a cascade of events. The insulin receptor substrate-1 (IRS-1) protein is stimulated consequently activating PI3-K (Lizcano and Alessi, 2002). Following the activation of PI3-K, phosphatidylinositol-bisphosphate (PIP_2) is phosphorylated thereby producing phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) (Shepherd, Withers and Siddle, 1998). Protein kinase B (PKB/Akt) phosphorylation is one of the fundamental reactions following the creation of PIP_3, and is used as a marker of the insulin signalling cascade action following activation by phosphoinositide-dependent kinase-1 (PDK1) (Kim et al., 2003). As a result, TBC1D4/AS160 activity is increased, which converts the inactive form of Rab guanosine diphosphate (GDP) to its active form Rab guanosine triphosphate (GTP). Ultimately, TBC1D4/AS160 is a Rab GTPase-activating protein that regulates the translocation of GLUT4 from microsomal storage microvesicles in the cytosol of the muscle fibres into the plasma membrane. This mechanism leads to increased uptake of blood glucose uptake into the muscle fibres (Lizcano and Alessi, 2002) (Figure 1.1).

Under basal conditions approximately 2 – 5% of GLUT4 is found at the plasma membrane (Kanzaki, 2006; Brewer et al., 2014). Transport of GLUT4 molecules from the microsomal storage vesicles to the plasma membrane occurs along microtubules formed by linear polymerisation of actin molecules (Lopez et al., 2009). Following insulin stimulation through meal ingestion, increases in GLUT4 transport to the plasma membrane at the cell surface occur thus allowing greater uptake of glucose in the muscle fibers (Lauritzen et al., 2006). Novel immunofluorescence microscopy methods have confirmed that there is a modest increase in GLUT4 at the plasma membrane in human skeletal muscle subsequent to glucose feeding (Bradley et al., 2015).

1.2.4 *Vascular responses to insulin*

The vascular responses induced by insulin are important in the augmentation of glucose disposal in skeletal muscle and other insulin-target tissues via vasodilation and increased blood flow (Bertrand et al., 2008). Insulin is essential to augment the production of nitric oxide (NO), a potent vasodilator produced by the vascular endothelium. The activation of endothelial NO

synthase (eNOS) is regulated by the insulin-signalling pathway and requires the activation of PI3K/PKB/Akt pathway. The activity of eNOS is enhanced through the PKB/Akt phosphorylation of eNOS thus increasing the production of NO (Bertrand et al., 2008). Meal-induced increases in plasma insulin concentration accelerate arterial blood supply to skeletal muscle leading to a 50%-80% increase in skeletal muscle resting blood volume in healthy individuals (Vincent et al., 2006). This increase in blood supply and volume induces an increase in the delivery of insulin, glucose, FAs, TAGs and amino acids to skeletal muscle (Wagenmakers et al., 2016). Skeletal muscle and subcutaneous adipose tissue are responsible for a large proportion of the clearance of orally ingested glucose and lipid (65-70%), and therefore make an important contribution to postprandial glucose and lipid homeostasis (Wagenmakers et al., 2016). Meal-induced increases in plasma insulin fail to increase blood volume in the skeletal muscle of obese individuals, sedentary elderly individuals and T2D patients (Clerk et al., 2006; Keske et al., 2009), thus suggesting that blood flow to the skeletal muscle in the previously mentioned conditions is either reduced or absent in the postprandial period. This impairment following meal ingestion explains the hyperglycaemia seen in these individuals (Wagenmakers et al., 2016).

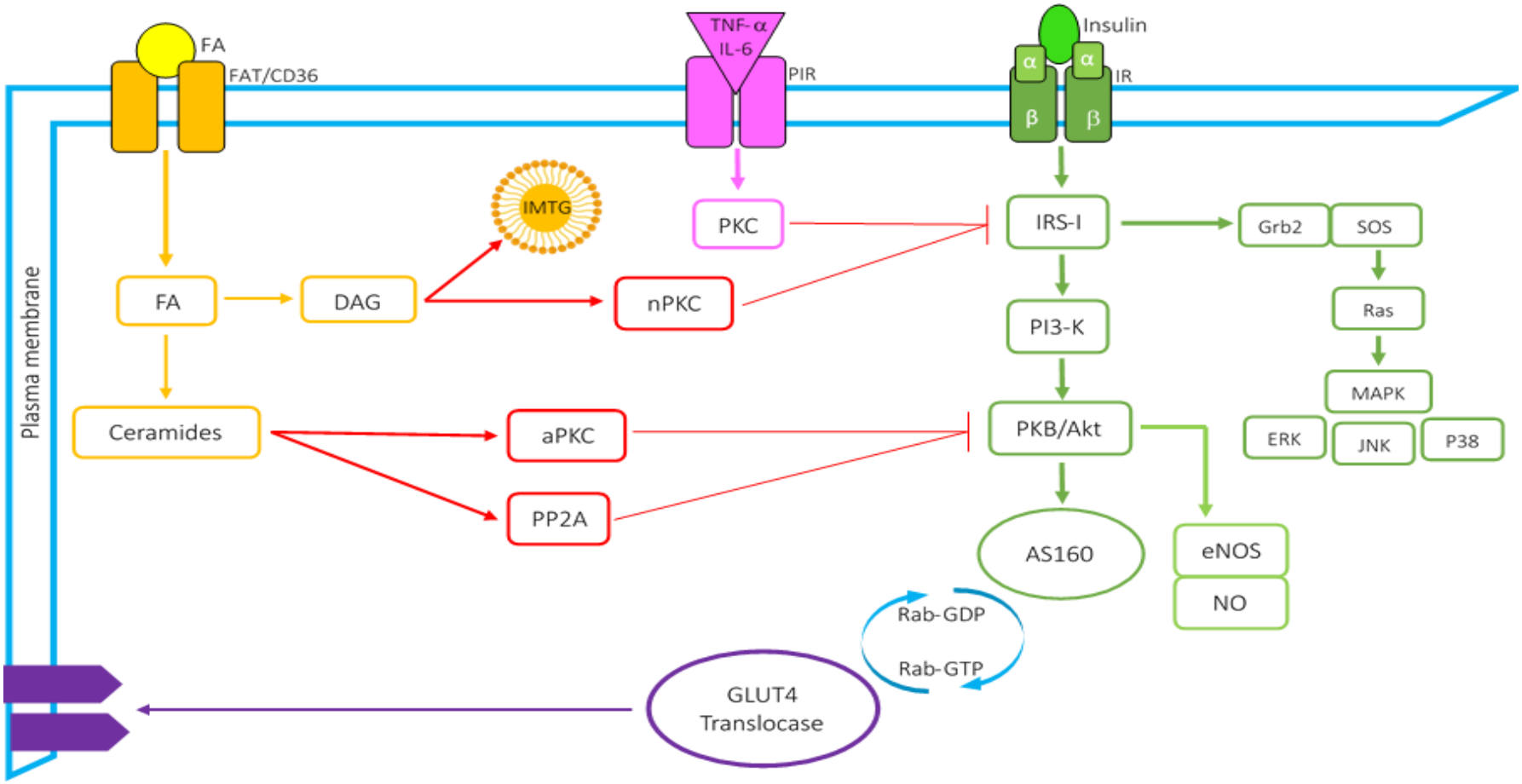

Figure 1.1. *The insulin signalling cascade featuring lipid induced insulin resistance and inflammation*. FA, fatty acid; DAG, diacylglycerol; IMTG, intramuscular triglyceride; PKC, protein kinase C; nPKC, novel protein kinase C; aPKC, atypical protein kinase C; PP2A, phosphoprotein phosphatase 2A; TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6; PIR, pro-inflammatory receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3-K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; AS160, Akt substrate of 160 kD; Rab-GDP, Rab guanosine diphosphate; Rab-GTP, Rab guanosine triphosphate; GLUT4, glucose transporter-4; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; Grb2, growth factor receptor-bound protein 2; SOS, son of sevenless; MAPK, ras-mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase.

1.3 *Intramuscular triglycerides and lipid droplets*

The majority of the lipid in our body is stored within adipose tissue (~9-15 kg) with a small quantity deposited in skeletal muscle (~350 g) (Frayn, Arner and Yki-Jarvinen, 2006). The lipid in skeletal muscle is stored in the form of intramuscular triglycerides (IMTG) that are packaged with a surrounding phospholipid membrane to form lipid droplets (LD). As highly dynamic organelles, LDs are involved in several diverse complex functions; cell signalling, mitochondrial fuel source and vesicle trafficking (Coen and Goodpaster, 2012). The primary role of LDs is to store TAG and it is suggested that dysregulation in LD homeostasis is implicated in the pathogenesis of insulin resistance and T2D (Greenberg et al., 2011). Additionally, the buffering capabilities of LDs regulates the cellular amounts of potentially toxic lipids thereby suggesting a prominent role in the prevention of lipotoxicity and oxidative stress (Olzmann and Carvalho, 2019).

1.3.1 *LD structure and formation*

LDs have a unique architectural structure consisting of a hydrophobic core of neutral lipids (TAG and cholesterol esters), which is surrounded by a phospholipid monolayer that is decorated with a specific set of proteins (Ohsaki et al., 2009; Olzmann and Carvalho, 2019). It is suggested that LDs originate from, or in close proximity to, the endoplasmic reticulum (ER), although the exact mechanism by which this occurs is still equivocal particularly within skeletal muscle. Gradual accumulation of lipid esters; TAG and cholesterol between the leaflets of the ER bilayer increase the expansion of the neutral lipid lens which instigates LD budding from the ER membrane towards the cytoplasm (Bersuker and Olzmann, 2017). When using freeze-fracture high resolution electron microscopy analysis in cultured human macrophages, it has been shown that ER membranes lie external to and follow the contour of LDs with similarity to an egg in an egg cup (Robenek et al., 2006). The membrane surface tension also appears to be important in the acquisition of the LDs rounded shape and its budding directionality (towards the cytosol). Phospholipids and proteins mask the oil-water interface consequently influencing the surface tension thereby suggesting alterations in protein and/or lipid composition between membrane monolayers is sufficient to induce tension imbalances (Olzmann and Carvalho, 2019).

1.3.2 *LD morphology and distribution*

Newly formed LDs are approximately <0.5 μm in diameter and referred to as 'primordial' LDs (Marchesan et al., 2003). However, LDs have the capacity to significantly increase/decrease in size though three key mechanisms; 1) via pores in the phospholipid monolayer the coalescence of fusion between two LDs occurs resulting in the fusion and exchange of lipids, 2) ripening of LDs through an exchange of molecules from one LD to another which results in increase LD size alongside a decrease in size of other LDs and 3) LD expansion through IMTG synthesis fuelled by an excess of FAs (Gemmink et al., 2017). In the skeletal muscle of healthy individuals LD diameter (of 'mature' LD) is reported to range from 0.3 – 1.5 μm, however LD diameter is shown to increase up to 3.5 μm within obese individuals (Bosma, 2016). Furthermore, endurance trained athletes have shown greater LD density (number) compared to T2D patients (van Loon et al., 2004), with reports of a greater number of small-size LDs within type I fibres compared to type II fibres (van Loon et al., 2003; Shepherd et al., 2013b; Nielsen et al., 2017). It is accepted that a greater density of small-sized LDs is metabolically advantageous due to the higher surface area-to-volume ratio of small LDs resulting in lower lipid interference compared to larger LDs. The smaller LD provide a greater surface area for lipases to access and may increase phospholipid availability for regulatory proteins to associate with (Gemmink et al., 2017).

In addition to LD size and density, the subcellular location of LDs is also an important factor to consider in the development of insulin resistance. LDs are predominantly situated between the myofibrils (intermyofibrillar), however they have also been observed near to the plasma membrane within the subsarcolemmal region (Bosma, 2016). Increased LD density within the subsarcolemmal region has been associated with the development of insulin resistance, with obese and insulin resistant individuals having a greater number of large-size LDs within this region compared to lean individuals (Nielsen et al., 2010; Chee et al., 2016). In skeletal muscle of healthy individuals LDs are primarily located adjacent to mitochondria (Shaw, Jones and Wagenmakers, 2008). This distribution makes the LDs a readily available source of FA for the neighbouring mitochondria, particularly during exercise (Bonen, Dohm and van Loon, 2006). During moderate intensity exercise in healthy trained individuals, the oxidation of LDs makes an important contribution to the total adenosine triphosphate (ATP) production (energy

expenditure) (Krssak et al., 1999; Guo, Burguera and Jensen, 2000; van Loon et al., 2003; Shepherd et al., 2013b; Whytock et al., 2017).

1.3.3 *IMTG turnover*

IMTG is an important fuel source for skeletal muscle activity and its content depends on the balance between continuous synthesis and degradation of the IMTG pool (Sacchetti et al., 2004). Healthy individuals have a greater capacity to utilise IMTG stores, implying that they are able to maintain a high turnover rate of the IMTG pool both during exercise (Guo, Burguera and Jensen, 2000) and at rest (Kanaley et al., 2009). Furthermore, trained individual's skeletal muscle has an enhanced oxidative and lipid storage capacity, with smaller sized LDs in direct contact with mitochondria which supports a higher IMTG turnover rate within athletes (Moro, Bajpeyi and Smith, 2008). This regular turnover of the IMTG pool is imperative in preserving optimal skeletal muscle insulin sensitivity (Sacchetti et al., 2004).

1.4 *Lipid metabolism*

Hormone sensitive lipase (HSL) and adipose triacylglycerol lipase (ATGL) are key enzymes in TAG lipolysis and are responsible for cleaving FA from the glycerol backbone from stored TAG in the adipose tissue and skeletal muscle (Shaw, Clark and Shepherd, 2013). The free fatty acids (FFA) from adipose tissue are then bound to albumin in the blood which then transports the FFA to skeletal muscle where they are taken up across the muscle membrane with the assistance of transport proteins primarily fatty acid translocase (FAT/CD36) (Bradley et al., 2012). The FFA are activated by fatty acyl-CoA synthase upon entering the muscle and is either oxidised or used to synthesise IMTG. Stored IMTG can be utilized through lipolysis which releases FFA and is activated by HSL and ATGL (Spriet, 2011). For oxidation to occur all FFAs which were delivered from either the plasma or IMTG must be transported to the outer mitochondrial membrane. Short and medium chain fatty acids are able to enter the mitochondria directly; however the long chain fatty acid (LCFA) requires a transport system to cross the mitochondrial membrane which is regulated by carnitine palmitoyltransferase I (CPT-I) (Spriet, 2011). The compound is then transported across the membrane via translocase, where carnitine moves in the opposite direction. Furthermore, the fat transport protein FAT/CD36, in conjunction with CPT-I, aids the transfer LCFA-carnitine to carnitine-acylcarnitine

translocase (CAT) and across the mitochondrial membranes for oxidation (Bezaire et al., 2006). When inside the mitochondria, carnitine is removed and the enzyme, carnitine palmitoyltransferase II (CPT II), rebounds CoA to the long chain fatty acid. (Spriet, 2011). Finally, through the β-oxidation pathway the fatty acyl-CoA molecules are metabolised with the assistance of β-oxidation enzymes, specifically β-hydroxyacylCoA dehydrogenase (β-HAD), in turn producing acetyl-CoA (Odland et al., 1998; Spriet, 2011). A substantial amount of energy is produced from lipid hydrolysis and subsequent oxidation of IMTG deposits in type I muscle fibres, predominantly during aerobic endurance-based activity (Bonen, Dohm and van Loon, 2006) (Figure 1.2).

1.4.1 *Perilipin proteins regulating IMTG content*

Perilipins (PLIN) are proteins often associated with LDs, studded within the phospholipid monolayer of LDs. PLIN proteins facilitate storage during resting conditions and promote hydrolysis during exercise (lipolytic conditions) through interaction with lipid hydrolysing enzymes (Tansey et al., 2001). PLIN1-5 are proteins that play an extensive role in regulating lipolysis and TAG hydrolysis in adipose tissue, with PLIN2-5 also being expressed in skeletal muscle (Morales, Bucarey and Espinosa, 2017a). These PLIN proteins can be either exclusively associated with lipid droplets (PLIN1-2) or exchangeable proteins that are stable in either the cytoplasm or when associated with lipid droplets (PLIN3-5) (Sztalryd and Brasaemle, 2017). Similar to IMTG content, PLIN proteins have greater expression within the oxidative type I skeletal muscle fibres in comparison to the type II fibres thereby mirroring the fibre-type specific distribution of IMTG (Shaw et al., 2012; Shepherd et al., 2013a; Pourteymour et al., 2015). Furthermore, following exercise training PLIN2, PLIN3 and PLIN5 proteins levels are elevated within skeletal muscle together with increased IMTG content (Shaw et al., 2012; Shepherd et al., 2013a). It could be that increased PLIN protein content is relative to augmented IMTG content, therefore implying a potential mechanistic role of increased PLIN protein content which facilitates an increased IMTG pool within trained individuals. This potential mechanistic link between elevated PLIN protein and IMTG content has also been observed when examining sex differences. Peters et al. (2012) reported that female skeletal muscle contains an abundance of PLIN2 and PLIN5 when compared to males, alongside higher IMTG content in the female skeletal muscle. The high expression of PLIN2, PLIN3 and PLIN5 in skeletal muscle positively correlates with LD content where it can interact with HSL, a

predominant enzyme involved in LD hydrolysis (Macpherson et al., 2013; Morales, Bucarey and Espinosa, 2017a). PLIN-coated LDs have been shown to be preferentially oxidised during moderate-intensity exercise, potentially by HSL being preferentially recruited to LDs (Shepherd et al., 2012; Shepherd et al., 2013b; Whytock et al., 2018). Interestingly, (Jevons et al., 2020) reported that PLIN proteins do not appear to be involved in mediating IMTG resynthesis due to an inconsistency within the time course observed between the increased IMTG pool and the coating of LDs with PLIN proteins.

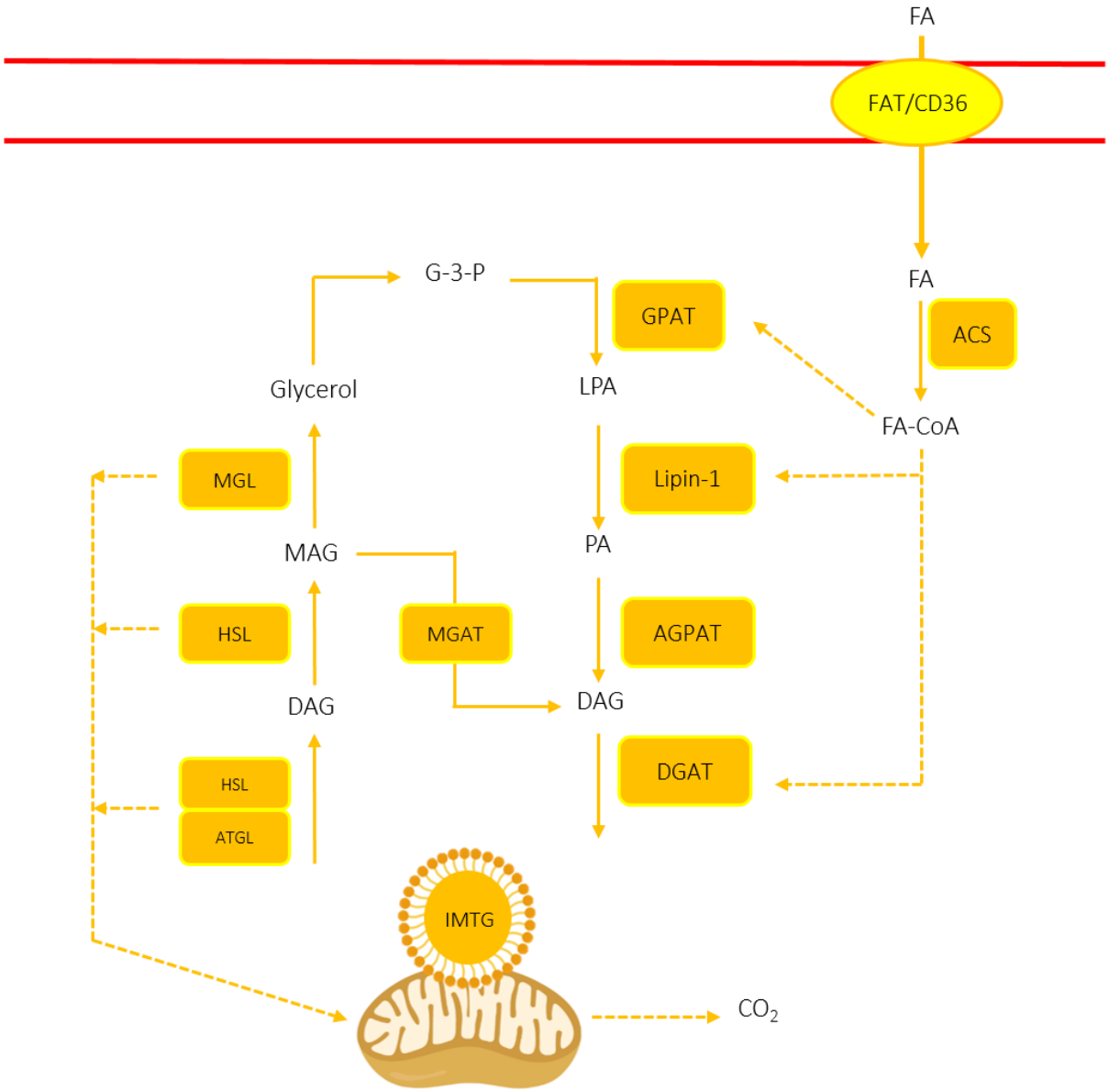

Figure 1.2. ***IMTG metabolism in skeletal muscle.*** AGPAT, 1-acylglycerol-3-phosphate acyltransferase; ATGL adipose triglyceride lipase, ACS, acyl-CoA synthetase; DAG, diacylglycerol; DGAT, diacylglycerol transferase; FA, fatty acid; FA-CoA, fatty acyl-CoA; CD36, cluster of differentiation 36; G-3-P, glycerol-3- phosphate; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone sensitive lipase; IMTG, intramuscular triglyceride; LPA, lysophosphatidic acid; MAG monoacylglycerol; MGAT, monoacylglycerol transferase; PA, phosphatidic acid.

1.5 *Insulin Resistance*

During a healthy individual's life cycle insulin sensitivity will naturally fluctuate. It has been proposed that these fluctuations predominantly occur during male and female puberty, pregnancy and with age. Consequently, an increase in insulin resistance has been observed following these fluctuations (Kahn, Hull and Utzschneider, 2006). Additionally, lifestyle is arguably one of the most important factors determining insulin sensitivity. For example, physical inactivity and excess energy intake lead to impaired insulin sensitivity, thus increasing the risk of developing T2D (Kahn, Hull and Utzschneider, 2006). Consequently, these individuals spend an increased time in the postprandial phase therefore exposing tissues to excessive FA and glucose concentrations. Reduced PA and structured exercise, increased sedentary behaviour and energy-dense westernised diets lead to the maladaptation of insulin sensitive tissues, whereby blood flow is reduced alongside the delivery of insulin thereby tissues become less responsive to the effects of insulin (Kasuga, 2006). Hyperglycaemia is the result of decreased glucose uptake into peripheral tissues (and predominantly skeletal muscle), a result of impaired insulin sensitivity within skeletal muscle. Furthermore hepatic insulin resistance can also result in hyperglycaemia through an impaired suppression of glucose production by insulin in hepatocytes. In obesity, the production of harmful proteins and metabolites is increased, namely retinol-binding protein-4 (RBP4), which induces insulin resistance through the stimulation of macrophages and CD4 cells to release inflammation cytokines which supress insulin signalling at the level of IRS. Additionally, β-cell function is significantly decreased (50%) in T2D patients reducing the insulin production in response to the ingestion of CHO-rich meals. The primary role of β-cell is to respond quickly to spikes in blood glucose concentrations through the production and release insulin and amylin which reduce blood glucose concentrations, and thus insulin secretion is increased to compensate for the elevated blood glucose concentration (Kasuga, 2006).

1.5.1 *Skeletal muscle insulin resistance*

Insulin resistance of skeletal muscle fibres is characterised by a downregulation of the intricate mechanisms involved in glucose uptake. These impairments result in a reduced capacity for translocation of GLUT4 from the microsomal storage vesicles to the plasma membrane, resulting in elevated blood glucose concentrations (Saltiel and Kahn, 2001). Furthermore, a

decrease in mitochondrial FA oxidation and/or reduced mitochondrial content results in an accumulation of intracellular long chain fatty Acyl-CoA's, diacylglycerol (DAG) and ceramides, causing impaired insulin receptor signalling (Lowell and Shulman, 2005) (Figure 1.2). Skeletal muscle insulin resistance may also occur via impaired mitochondrial oxidative and phosphorylation activity, particularly within older and obese populations where mitochondrial function is reduced due to an increase in reactive oxygen species (ROS) activity (Petersen et al., 2003; Rindler et al., 2013; Samuel and Shulman, 2016).

1.5.2 *Lipid overflow hypothesis*

When postprandial clearance of chylomicron-TAG into adipose tissue becomes impaired there is an overspill of FA and TAG in the circulation (Frayn, 2002a). As a result of the impaired lipid buffering mechanisms and limited adipose tissue expansion, the excess lipids are deposited in non-adipose tissues and it is these ectopic lipid accumulations which prove detrimental to insulin sensitivity (Chadt et al., 2018). This accumulation of TAG in skeletal muscle of sedentary and obese individuals has previously been associated with insulin resistance (Goodpaster et al., 1997) as it acts as a potential surrogate for other harmful lipid metabolites which have a deleterious effect on lipid metabolism thereby impairing function (Chadt et al., 2018).

1.5.3 *Athletes paradox*

Earlier research demonstrates an association between IMTG content and insulin resistance. Through a reduction in FA oxidation there is an increase in lipid metabolite accumulation which characteristically impairs the insulin signing cascade thus increasing the risk of developing insulin resistance. The majority of studies that express this correlation were predominantly rodent based (Gajda et al.; Lee et al., 2006; Buettner, Schölmerich and Bollheimer, 2007; Wang and Liao, 2012). However, more recently, attempts have been made to investigate the complex relationship between LDs and insulin sensitivity within human participants (Daemen et al., 2018; Daemen, van Polanen and Hesselink, 2018). Moreover, the data produced from all the above research papers collectively suggest that obese and T2D partcicipants have an accumulation of intramuscular lipid whilst also being highly insulin resistant (Schenk and Horowitz, 2007).

The athletes' paradox describes the phenomenon whereby highly trained endurance athletes store large quantities of IMTGs in skeletal muscle, though they remain highly insulin sensitive (Goodpaster et al., 2001). Conversely, obese and T2D individuals accumulate IMTG whilst being highly insulin resistant (Schenk and Horowitz, 2007). This finding provides an insight to the relationship between IMTG and insulin resistance, suggesting that IMTG alone may not be accountable for reduced insulin sensitivity but possibly serve as a surrogate of other potentially disadvantageous lipid metabolites, including long-chain fatty-acyl CoAs, ceramides and DAGs (Goodpaster et al., 2001).

1.5.4 *Lipid metabolites*

TAG, DAG and cholesterol are the key esterified lipids which account for the majority of the lipid content in intramuscular LDs (Billecke et al., 2015). Recent evidence suggests that the reduced insulin action in obese and T2D populations is not caused by accumulation of IMTG, but by accumulation of DAGs and ceramides specifically in the peripheral region of the muscle fibres (Jocken et al., 2010). The general consensus is that DAGs and ceramide are the lipid metabolites responsible for the documented affiliation between intramuscular lipid accumulation and insulin resistance (Amati et al., 2011b). The increased abundance of DAGs has been proposed to stimulate novel protein kinase C (nPKC) isoforms, which are serine kinases. Serine phosphorylation of IRS-1 in skeletal muscle prevents tyrosine phosphorylation and therefore reduces insulin action (Kim et al., 2003). In addition, ceramides have been shown to dephosphorylate PKB/Akt, thereby further downregulating Akt activity and reducing the translocation of GLUT4 and limiting muscle glucose uptake (Itani et al., 2002) (Figure 1.1).

The observation has been made that healthy sedentary obese controls and individuals with T2D have a higher total muscle DAG concentration than lean endurance-trained athletes (Bergman et al., 2012). The majority of DAG was present in the membrane fraction in the 3 groups, but it was lowest in the athletes. No difference was seen for the cytosolic DAG species. Membrane DAG species were more abundant in the T2D group. Only total saturated DAG in the muscle membranes correlated with insulin sensitivity. Desaturated DAG species were significantly lower in the athletes, and significantly related to insulin sensitivity. The authors concluded that these data indicated that both cellular localisation and composition of DAG influence the relationship to insulin sensitivity and that the results suggested that only

saturated DAG in skeletal muscle membranes are related to insulin resistance in humans (Bergman et al., 2012).

Ceramides are a family of lipid molecules composed of a sphingosine base and fatty acid moiety. It is apparent that an accumulation of ceramides inhibits insulin action and ceramides are suggested to be more closely related to insulin resistance than DAGs due to the consistent elevations in ceramides observed in obese and insulin resistant skeletal muscle (Amati et al., 2011b). There are two proposed mechanisms, both centred on the inhibition of Akt/PKB stimulated by ceramides, which contribute to the disruption of the insulin signalling pathway. Firstly, the phosphorylation of Akt/PBK reduces its affinity for phosphoinositides as a result of increased atypical PKCζ activity which is stimulated by ceramides (Powell et al., 2003). Secondly, ceramides activate protein phosphatase 2A (PP2A) which dephosphorylates Akt/PKB thereby obstructing Akt/PKB recruitment to the plasma membrane by phosphoinositides (Chavez et al., 2003). Ultimately, these mechanisms reduce Akt/PKB recruitment to the plasma membrane by phosphoinositides which inhibits insulin-stimulated glucose uptake by hindering the activation of AS160 and GLUT4 translocation to the plasma membrane (Chavez and Summers, 2012).

The insulin signalling cascade has been shown to be disrupted by the accumulation of lipid metabolites. The deleterious accumulation of LDs in skeletal muscle can occur due to an inability to match lipid oxidation to lipid availability and/or an impaired TAG turnover, the equilibrium of TAG storage and removal into adipocytes (Moro, Bajpeyi and Smith, 2008). This is seen in sedentary and obese individuals who fail to empty their IMTG stores to fuel exercise, combined with a high FA flux into the skeletal muscle of obese individuals may result in the accumulation of lipid metabolites. Additionally, the number of LDs close to the plasma membrane is inversely related to insulin sensitivity, and it is thought that insulin resistant individuals express a greater number of large LDs within the subsarcolemmal region of the muscle fibre which, in absence of an increased mitochondrial density, hinders cells signalling and ultimately leading to an increased risk of insulin resistance (Daemen et al., 2018).

1.5.5 *Inflammation*

The link between inflammation and insulin resistance was first put forward by Hotamisligil, Shargill and Spiegelman (1993), who demonstrated that an increased expression of proinflammatory cytokines, more specifically tumour necrosis factor alpha (TNF-α), is positively linked with the development of insulin resistance (Figure 1.1). Further, in human adipose tissue TNF-α expression declined following weight loss, suggesting that its expression plays an important role in adipocyte metabolism and could be a homeostatic mechanism in the development of insulin resistance (Kern et al. 1995). There is also increasing evidence that the production of the cytokine interleukin 6 (IL-6) is involved in the stimulus of C-reactive protein (CRP) synthesis (Ferroni et al., 2004). CRP levels have previously been correlated with insulin resistance (Festa et al., 2000), suggesting that increased IL-6 expression may contribute to the development of insulin resistance (Figure 1.1). Interestingly, the expression of TNF-α promoter (G-308A) is associated with the risk of developing type 2 diabetes, whereas the IL-6 promoter (C-124G) increases an individual's risk for insulin resistance (Dandona, Aljada and Bandyopadhyay, 2004). Additionally, the activation of compliment factor 3 initiates inflammatory responses which proceed with further lymphokine (a subset of cytokines) production and secretion, ultimately leading to an increase in adipocyte lipolysis, nonesterified fatty acid (NEFA) production and insulin resistance in skeletal muscle (Grundy et al., 2004).

1.6 *Mitochondria*

Frequently termed the 'powerhouse' of the cell, mitochondria are crucial organelles in the synthesis of ATP. However, mitochondria also play an extensive role in other cellular processes, including FA synthesis (Bezaire et al., 2006). A common theory within research, suggests that mitochondria are significant factors in the development of insulin resistance (Kim, Wei and Sowers, 2008). However, there are conflicting views regarding the exact mechanisms by which mitochondria contribute to the pathogenesis of insulin resistance (Di Meo, Iossa and Venditti, 2017). One of the widely accepted theories proposes that a decrease in mitochondrial oxidation of FAs is caused by a dysfunction and decrease in mitochondrial content thus leading to an accumulation of intracellular fatty acyl-CoA, DAG and ceramides which inhibit insulin signalling (Lowell and Shulman, 2005; Coen and Goodpaster, 2012).

1.6.1 *Improved mitochondrial function and content following physical activity*

It is well established that a high physical activity level has a positive impact on mitochondrial density and function and insulin sensitivity in skeletal muscle (Lanza and Nair, 2008; Meex et al., 2010; Montgomery and Turner, 2015; Olver, Laughlin and Padilla, 2019). Aerobic exercise activates signals that induce greater oxidative capacity, thus leading to mitochondrial biogenesis and angiogenesis which generates a dense 3-dimensional capillary network around the skeletal muscle fibres. In addition to improved mitochondrial function, aerobic exercise improves insulin action, leading to an augmented translocation and protein content of GLUT4 (Richter and Hargreaves, 2013) irrespective of gender or age (Ojuka, Goyaram and Smith, 2012; Bird and Hawley, 2016; Brook et al., 2016). Exercise training induces greater oxidative capacity due to increased beta-oxidation via the mitochondria, subsequently lipid oxidation is increased as is the capacity for lipolysis, therefore skeletal muscles ability to extract FAs from plasma following meal-ingestion is improved (Flück, 2006). Furthermore, DAG, ceramide and long-chain fatty acyl-CoA concentrations are maintained at concentrations which do not activate PKCs and lead to insulin resistance of the muscle fibres and their microvasculature (Amati et al., 2011b). Glycogen synthase is also activated following exercise due to consistent reduction of muscle glycogen stores thus increasing rate of glucose uptake from the blood following carbohydrate meal-ingestion. Glycogen and IMTG depletion during frequent bouts of exercise create storage space for meal derived carbohydrates and lipids. Therefore, a physically active lifestyle may prevent unfavourable changes within blood glucose and lipid concentrations following meal-ingestion, which is commonly seen in sedentary, insulin resistant and T2D patients (Wagenmakers et al., 2006).

Oxidative capacity also plays an important role in insulin sensitivity. LDs are predominantly adjacent to the mitochondria with greater abundance and size in the oxidative type I fibres than type II fibres (He, Goodpaster and Kelley, 2004; Shaw, Jones and Wagenmakers, 2008; Coen et al., 2010). Furthermore, in trained individuals, LDs act as a readily available fuel source during exercise as they are located near the mitochondria within the subsarcolemmal region of the myofibre (Hoppeler, 1999; van Loon et al., 2003). In comparison, obese individuals present reduced oxidative capacity due to reduced mitochondrial content (Kim et al., 2000) and therefore a reduced capacity to utilise IMTG as a fuel source. In addition to this, the number of LDs close to the plasma membrane is inversely related to insulin sensitivity, and it

has been proven that endurance training only increases the number of LDs but does not alter the size (Tarnopolsky et al., 2007). Exercise may be used as a non-pharmacological treatment to improve diseased individual's oxidative capacity. Following four months of exercise training, obese individuals showed alterations in LD size due to concomitant increase in oxidative capacity the LD size is reduced which can correlate to an improved insulin sensitivity (He, Goodpaster and Kelley, 2004).

1.6.2 *Mitochondrial dysfunction and insulin resistance*

The relationship surrounding mitochondria and insulin sensitivity has been an area of interest and debate within research for decades. There are various views and hypotheses regarding mitochondrial dysfunction and its role in insulin resistance presented within literature. These among others include a reduction in content and/or quality of the mitochondria. A popular view amongst studies suggests that insulin action in skeletal muscle is driven by enhanced oxidative capacity which is improved through increased physical activity leading to greater mitochondrial content (Rimbert et al., 2004; Phielix et al., 2010). However, Muoio and Neufer (2012) proposed that mitochondrial load, ROS signalling and redox pressures (induced by increased energy supply which continuously surpasses energy demand) are determinants of skeletal muscle insulin resistance more so than oxidative capacity alone. Moreover, human based studies which included obese and T2D patients found reduced mitochondrial size, biogenesis and a downregulation in oxidative phosphorylation pathways when compared to lean healthy individuals (Montgomery and Turner, 2015). From these studies it was suggested that improved mitochondrial function (density and oxidative enzyme activity) resulted in improved insulin sensitivity when exercise and caloric restriction was administered (Montgomery and Turner, 2015). Finally, it has also been proposed that mitochondria also play an extensive role in age-related diseases, primarily metabolic diseases which encompass insulin resistance and the progression of T2D. This is driven through reduced circulating small human-like peptides 2 (SHLP2) levels within older populations. SHLP2 is derived from the mitochondrial genome and is associated with increased leptin levels which in turn can improve insulin sensitivity (Cobb et al., 2016). The conflict within research suggest that there is a highly complex relationship between mitochondria and insulin action which requires further investigation to fully understand these intricate mechanisms.

1.7 *Sex differences in metabolism and insulin sensitivity*

It has become increasingly apparent from research that glucose and lipid metabolism may be subject to sex-specific regulation, with evidence of molecular sex differences in substrate metabolism of skeletal muscle (Lundsgaard and Kiens, 2014). The higher insulin sensitivity of female skeletal muscle could be an outcome of multiple sex differences previously observed in research; greater type I fibre distribution and substrate availability, improved skeletal muscle oxidative capacities and the expression of key sex hormones in females (17-β estradiol) (Lundsgaard and Kiens, 2014). Interestingly, female skeletal muscle contains greater lipid stores when compared to males whilst also demonstrating greater abilities of insulin-stimulated glucose clearance in skeletal muscle. Previous findings imply that females have greater metabolic flexibility due to their ability to adjust substrate oxidation in accordance to the availability of nutrients during physical activity and exercise (Lundsgaard and Kiens, 2014). However, research investigating these sex differences in substrate metabolism is limited and requires further investigation to fully understand the role of sex in metabolism and the specific sex differences in molecular mechanisms which could lead to enhance understanding of metabolic dysfunctions.

1.7.1 *Adipose tissue storage*

There is a greater prevalence of T2D in males compared to females (Wild et al., 2004). Pre-menopausal females display greater insulin sensitivity compared to their BMI matched male counterparts despite their significantly greater adipose tissue mass (Geer and Shen, 2009). Furthermore, pre-menopausal females also display lower risk of metabolic and cardiovascular complications compared to males (Clausen et al., 1996). Moreover, it has been proposed that the female's ability to store a greater adipose tissue mass whilst remaining insulin sensitive is due to the regions in which the adipose tissue is stored. Females predominantly deposit fat in the lower regions of the body, as opposed to the upper regions (more specifically the abdominal area) as noted in males which increases the risk of metabolic complications (Janjic, 1996).

1.7.2 *Sex hormones*

Previous literature suggests that sex hormones contribute largely to the differences recorded between sexes and insulin sensitivity (Geer and Shen, 2009; Basu, Dube and Basu, 2017). Pre-menopausal females have a reduced risk of T2D and cardiovascular diseases compared to males and post-menopausal females (Pérez-López et al., 2010), although the literature surrounding the mechanisms underpinning this phenomenon is limited. Currently, it is suggested that the female sex hormones (oestrogen and progesterone) serve to protect against metabolic risk factors (Geer and Shen, 2009). However, during menopause, production of the female sex hormone, oestrogen, ceases thus increasing the risk of cardiovascular disease and T2D (Polotsky and Polotsky, 2010). Research has investigated the menopause-related declines in insulin sensitivity and oestrogen secretion through hormone replacement therapy using oestrogen infusion. This treatment results in an increased rate of glucose disposal with the potential to counter metabolic risk through an increase in insulin sensitivity (Spencer et al., 2000; Van Pelt et al., 2003). Female sex hormones, and particularly oestrogen, therefore appear to contribute to the higher insulin sensitivity of pre-menopausal women. However, the current research surrounding these intricate mechanisms is limited, and even more so in post-menopausal women whom appear to lose the protective benefits of sex hormones thus increasing susceptibility to metabolic health defects. Males also experience a decline in testosterone levels during older age which is suggested to negatively impact lipid metabolism and insulin signalling. Evidently, testosterone improves insulin action and glucose metabolism through various mechanisms; increased GLUT4 expression, reduced pro-inflammatory cytokines, decreased LPL activity and mitochondrial function (Ottarsdottir et al., 2018). Testosterone may protect against the development of insulin resistance in males through preservation of muscle mass and function, however age related reductions in testosterone levels may be involved in the etiology of sarcopenia with evidence of decreased muscle mass and/or function related to low testosterone levels (Shin, Jeon and Kim, 2018).

1.7.3 *Skeletal muscle IMTG content and utilisation*

It is evident that females have a greater utilisation of IMTG during exercise, however it has been speculated that this is a result of higher IMTG concentrations within female skeletal muscle prior to exercise (van Loon, 2004). Furthermore, the greater IMTG content within

females may be attributed to higher plasma FFA availability and FAT/CD36 protein expression in muscle (Lundsgaard and Kiens, 2014). Females also have a greater number of smaller LDs compared to males, which would theoretically increase the accessibility of lipases and proteins associated with LDs to the stored IMTG (Devries et al., 2007). Collectively, the smaller LDs and increased perilipin protein expression observed within female skeletal muscle is likely to increase the physical link between LDs and mitochondria, thereby augmenting IMTG turnover (Lundsgaard and Kiens, 2014). Furthermore, the smaller LDs in women have been documented near mitochondria following a bout of exercise proposing that women have enhanced IMTG oxidation capabilities (Devries et al., 2007). Interestingly, previous research suggest that mitochondrial content is similar within men and women (Tarnopolsky et al., 2007), yet females present greater mitochondrial function in comparison to males (Tower, 2006). It is hypothesised that the improved mitochondrial function observed within females is attributed to the positive action of oestrogen (Ventura-Clapier et al., 2017). However, following menopause the protective mechanisms of oestrogen are not available, therefore leading to mitochondrial dysfunction which is often associated with cardiovascular disease and metabolic syndrome (Ventura-Clapier et al., 2017). Nevertheless, the limited research investigating IMTG and mitochondrial content has shown greater IMTG content (due to greater IMTG size) alongside a decline in mitochondrial (due to reduced number) in older adults with female skeletal muscle containing a greater amount of IMTG compared to males (Crane et al., 2010). The studies previously discussed predominantly include pre-menopausal females, however the apparent gap within the current literature is the effects of menopause on female lipid metabolism and the result of diminished hormone protection in older females.

1.7.4 *Fibre types and oxidative capacity*

Women exhibit greater whole-body fat oxidation in comparison to males during submaximal exercise, thereby implying that females have a greater reliance on fat oxidation in comparison to males (Devries et al., 2007; Cheneviere et al., 2011). Furthermore, females show greater IMTG oxidation, adipose tissue lipolysis and a higher contribution of plasma FFAs to total fat oxidation during endurance exercise (Mittendorfer, Horowitz and Klein, 2002; Roepstorff et al., 2002). These findings are almost exclusively observed within pre-menopausal women who were not taking oral contraceptives and were controlled for phases of the menstrual cycle (Cheneviere et al., 2011). However, it is proposed that the menstrual cycle and oral

contraceptives possibly influence fat metabolism, with reported greater lipid utilization in the luteal phase when compared to the follicular phase (Devries et al., 2006). Females also have a greater area percentage of type I muscle fibres compared to type II muscle fibres and a greater capillary density per given muscle area in comparison to males (Roepstorff et al., 2006). Type I muscle fibres have a greater oxidative capacity due to a more expansive and complex mitochondrial network and associated oxidative enzymes, thus allowing for a more efficient fatty acid and glucose metabolism (Shaw, Jones and Wagenmakers, 2008). This in turn may help to prevent the accumulation of lipotoxic metabolites in the muscle as FA oxidation can match the FA delivery. Lipid metabolites can be removed from the cytosol by being oxidised or through re-synthesis into TAG and stored as lipid droplets in the skeletal muscle cell (Walther and Farese Jr, 2012). Furthermore, research has found an increased mRNA content of genes involved in lipid metabolism in female skeletal muscle in comparison to males partially due to 17-β estradiol (Peroxisome proliferator-activated receptor; PPARα, PPARδ), suggesting that females are able to store a greater number of LDs in comparison to males and remain insulin sensitive through oxidising IMTG and using stores as a fuel source during exercise (Fu et al., 2009).

1.8 *Immunofluorescence Microscopy*

Earlier research investigating lipid metabolism has predominately been on whole muscle samples (Bergman et al., 2010). This is a considerable oversight as more recent research suggests that lipid metabolism appears to be dependent upon fibre-type and subcellular location (Gemmink et al., 2017; Whytock et al., 2020). It is imperative that research utilises progressive lipid metabolism research techniques when possible to address these deficiencies. As a research technique, immunofluorescence microscopy has proven invaluable when producing novel data as well as presenting informative images which clearly exhibit the distribution and morphology of specific proteins. It was immunofluorescent microscopy that successfully identified a matrix structure in which LDs and mitochondria are present, and that the LDs are primarily located in close proximity to the mitochondria (Shaw, Jones and Wagenmakers, 2008). Using published immunohistochemically methods ensures credibility and reliability of this thesis due to the accuracy that the methods provide when examining LDs (Strauss et al., 2016; Whytock et al., 2017). These methods exclude analysis of extra

myocellular lipids which warrants the validity of the research through examining the samples LDs with regards to specific fibre types.

Within previous literature, there is a lack of information surrounding the different fibre types in skeletal muscle. Studies that use traditional research methods such as Western Blots or Mass Spectrometry provide limited information regarding fibre type specific differences in lipid profile, typically whole skeletal muscle tissue is analysed to produce a total estimate of IMTG content (Bass et al., 2017; Ko et al., 2018). Therefore, future research focusing on fibre type differences is imperative to develop a better understanding of the difference in metabolic characteristics of the fibres and how this may impact lipid content and turnover, oxidative capacity and IMTG use during exercise (Jevons et al., 2020).

The main advantage of using immunofluorescence microscopy is the additional information it provides concerning fibre type specific differences in the distribution, morphology and utilization of lipid (van Loon et al., 2004; Shaw, Jones and Wagenmakers, 2008). Furthermore, this analytical technique can potentially develop current perceptions surrounding the impairments in lipid metabolism of skeletal muscle and its potential deleterious effect on glucose uptake and insulin sensitivity. Therefore, it is essential that new immunohistochemically methods are developed to address future research questions that may provide further insight to the understanding of intricate mechanisms involved in the impairment of lipid metabolism presented in obese individuals leading to insulin resistance in skeletal muscle.

1.9 *Limitations*

Previous research investigating the link between lipid metabolism and insulin resistance has almost exclusively been conducted in male participants. Pre-menopausal females display greater insulin sensitivity when compared to BMI-matched males despite having larger body fat stores, which would normally be associated with insulin resistance (Geer and Shen, 2009). It is essential that research now provides novel data on the determinants of sex differences in the characteristics and distribution of LDs in skeletal muscle. Such data could begin to elucidate the protective physiological mechanisms that enable females to maintain higher insulin sensitivity despite having larger body fat stores than their BMI-matched male counterparts.

Previous research also demonstrates a significant limitation with regards to health status and sex, with the majority of research predominately conducted in obese, T2D and trained males.

1.10 *Aims*

It is thought that fibre type distribution, subcellular location, size and number of LDs relate to insulin sensitivity more so than IMTG content per se (Coen and Goodpaster, 2012), however this theory requires further investigation in human skeletal muscle (Bergman et al., 2012). Sex hormones are also likely to play a role in the higher insulin sensitivity previously observed in pre-menopausal women compared to male counterparts, however, the difference between men and post-menopausal women is not apparent. With an increasing focus on personalised diet and nutrition interventions/advice, research on metabolic processes in females is much needed. The study focused on addressing these significant gaps within the literature in relation to female metabolism, which has almost exclusively been conducted within pre-menopausal females. The aim of the present study was to 1) establish a better understanding of the sex differences in the relationship between LD profile, mitochondrial density and the development of insulin resistance thereby 2) contributing to current and future research improving insulin sensitivity and preventing the progression of insulin resistance. These aims would be achieved through 1) identifying fibre and region specific differences in the subcellular distribution and morphology of lipid droplets in human skeletal muscle of lean sedentary, obese and T2D males and females, 2) Identify the participants skeletal muscle fibre-type specific differences in mitochondrial density, and finally 3) Establish a better understanding of the relationship between mitochondrial density, lipid droplet distribution/morphology and insulin sensitivity. It is hypothesised that females have greater LD stores compared to males whilst remaining insulin sensitive, and that type two diabetes (T2D) patients have greater LD stores compared to the lean and obese participants.

2. Materials and Methods

2.1 *Participants and ethical approval*

The design of the study was a cross-sectional comparison. A cohort of older males (n=24) and females (n=24) were matched for physical activity (international physical activity questionnaire (IPAQ)), BMI ($kg.m^{-2}$) and age (see Table 2.1 for participants characteristics). Participants were allocated into one of three groups based on their BMI; Lean, Obese and Obese T2D. Each group included eight males and eight females (n = 16). All participants were undergoing surgery (see 2.2) at either the Royal Orthopaedic Hospital, Birmingham, UK or Russell's Hall Hospital, Dudley, UK and gave their informed written consent for participation in this study. Participant's use of medication was also obtained (Table 2.2). Ethical approval for the study in this thesis was obtained from the Black Country NHS Research Ethics Committee [09/H1207/137] with adherence to the *Declaration of Helsinki*.

Table 2.1 *Participants Characteristics*

	Female			Male		
	Lean	*Obese*	*T2D*	*Lean*	*Obese*	*T2D*
N	8	8	8	8	8	8
Age (y)	64 ± 3	65 ± 5	64 ± 2	67 ± 2	60 ± 4	61 ± 3
Body Mass (kg)	60.0 ± 3.6	84.0 ± 3.6	88.4 ± 3.6	76.7 ± 3.0	97.0 ± 4.1	109.7 ± 5.4
Height (m)	1.60 ± 0.03	1.62 ± 0.03	1.65 ± 0.02	1.73 ± 0.01	1.76 ± 0.02	1.76 ± 0.02
BMI ($kg.m^{-2}$)	22.7 ± 1.1	32.2 ± 1.4	32.6 ± 1.2	25.6 ± 0.6	31.4 ± 0.8	35.4 ± 1.7
Systolic BP (mmHg)	129 ± 6	132 ± 8	139 ± 9	135 ± 4	132 ± 4	145 ± 3
Diastolic BP (mmHg)	80 ± 2	79 ± 5	67 ± 3	77 ± 3	80 ± 4	89 ± 4
Plasma glucose (mmol/L)	6.99 ± 0.31	6.08 ± 0.31	10.04 ± 1.05*	5.22 ± 0.41	6.44 ± 0.36	8.71 ± 0.80*
Plasma insulin (pmol/L)	107.64 ± 31.51	141.67 ± 67.13	208.58 ± 50.17*	98.89 ± 15.27	155.96 ± 110.04	157.22 ± 63.68
HOMA-IR	4.80 ± 0.66†	5.77 ± 1.33	13.39 ± 2.20*†	3.27 ± 0.33	6.94 ± 2.10	8.68 ± 1.44*

Data provided as means ± SD. BMI; body mass index, HOMA-IR; homeostatic model assessment of insulin resistance. *Health x sex interaction ($P < 0.05$). †Sex x health interaction ($P < 0.05$)

Table 2.2 *Participants medication*

Medication	Lean	Obese	T2D
Adcal D3	1	1	0
Alendronic acid	1	0	0
Amiodarone	1	0	0
Amlodipine	0	0	2
Amytryphline	0	0	1
Aspirin	3	1	6
Atenotol	0	0	1
Athrotec	0	0	1
Atorvastatin	0	1	2
Becotide	0	1	0
Bendopamethazide	1	0	0
Bisoprolol	2	0	0
Butrans patch	0	0	2
Calceos	1	0	0
Cardiolplen	0	1	0
Casipt	0	1	0
Citalopram	0	0	2
Co-amilofruse	0	0	1
Co-codamol	2	0	2
Codeine	0	0	0
Co-drydamol	0	1	1
Corclazide	0	0	1
Dasulepin	0	1	0
Dermovate cream	0	0	1
Diazepam	0	0	1
Diclofenac	1	0	1
Dihydrocodiene	1	1	2
Dosulapin	0	1	0
Doxasosin	0	0	1
Enoxaporin	0	1	0
Felodipine	1	0	0
Ferous fumerate	1	0	0
Flucticasone inhaler	1	0	0
Frusemide	0	0	1
Furosemide	0	0	1
Gaviscon	0	0	1
Glucazide	0	0	1
Glucaziele	0	0	1
Glucodaside	0	0	1
Glucosamine	1	0	0
GNT	0	1	1
HTN	0	0	1
Hypremellose	1	0	1
Ibandronic acid	1	0	0
Ibuprofen	1	0	1
Ibuprofen gel	1	0	0
Imipramine	1	0	0
Indomatasan nasal spray	1	0	0
Innovage	1	0	0
Lacidipine	0	0	1
Lansoprazole	0	0	2

Lantus	0	0	2
Lasicpnate	0	1	0
Levothyroxin	1	1	0
Linsinopril	0	0	3
Loperamide	0	0	1
Lovastatin	1	0	0
Metformin	0	0	9
Morphine	0	1	0
Nicorandil	0	0	1
Novarapid	0	0	1
Omega 3	0	0	0
Omeprazole	2	1	0
Oramorph	0	1	0
Paracetamol	3	3	1
Peridopril	1	0	0
Pregabalin	0	0	1
PRN	0	2	1
Propanolol	0	1	0
Protiglitazone	0	0	1
Quinnine	0	0	1
Ramipril	1	2	2
Rosiavastat	0	1	0
Salbutamol	0	1	0
Serotide	0	1	0
Simvastatin	2	0	3
Sitagliptin	0	0	1
Solifenacin	0	0	1
Spironlactone	0	0	1
Statins	0	0	1
Steroids (anti insulinaemic)	1	0	0
Tabphyn	1	0	0
Thiamine	0	1	0
Thyroxine	1	0	0
Tramadol	1	1	4
Trimacet	0	1	0
Trimadal	0	1	0
Valsartan	0	0	1
Ventolin	0	1	1
Vitamin B	1	1	0
Vitamin B12	1	0	0
Vitamin E	1	0	0
Warfarin	1	0	1
Xalatin	0	0	0
Xatalant	0	1	1
Zopicclone	1	0	0
Zydol	0	0	1

2.2 *Blood and muscle samples*

Participant's blood samples were taken in an overnight fasted state at 8-9am during pre-operative assessment on the day of elective hip arthroplasty surgery. Muscle biopsies were obtained from the *gluteus maximus* of sedentary lean, obese and T2D male and female patients during surgery. These skeletal muscle samples were frozen in liquid nitrogen cooled isopentane and stored in -80°C freezers at the Life Science Building of Liverpool John Moores University until analysis was performed. Frozen skeletal muscle samples were fixed to cork using Tissue-Tek® (Tissue-Tek® O.C.T. Compound, Sakura® Finetek, Europe, and Netherlands). Samples were then transferred to a cryostat (OTF5000, Bright Instruments, UK) which was set at -20°C to prevent the thawing of skeletal muscle. The thickness setting was adjusted to 5µm, and skeletal muscle was cut and collected on VWR™ Super Premium Microscope Slides (VRW™, Avantor®, International), using optimal angles and pressure to ensure that the cut section adhered to the slide without damage. The 5µm sections were inspected with a light microscope (Apex™ Microscopes, Brunel Microscopes Ltd, Wiltshire, England) using the 10X/0.25NA objective to ensure that accurate cross-sectional cuts were obtained and the cells maintained good architectural integrity without significant frost damage.

2.3 *Immunofluorescent controls*

Negative control samples were run for BODIPY and mouse monoclonal anti-OxPhos Complex IV (COXIV), for this the standard immunofluorescence microscopy sequence was performed and BOIPY and COXIV were excluded and replaced with PBS (Figure 2.1). This permitted the testing of any nonspecific staining that may occur and the prevention of confounded data.

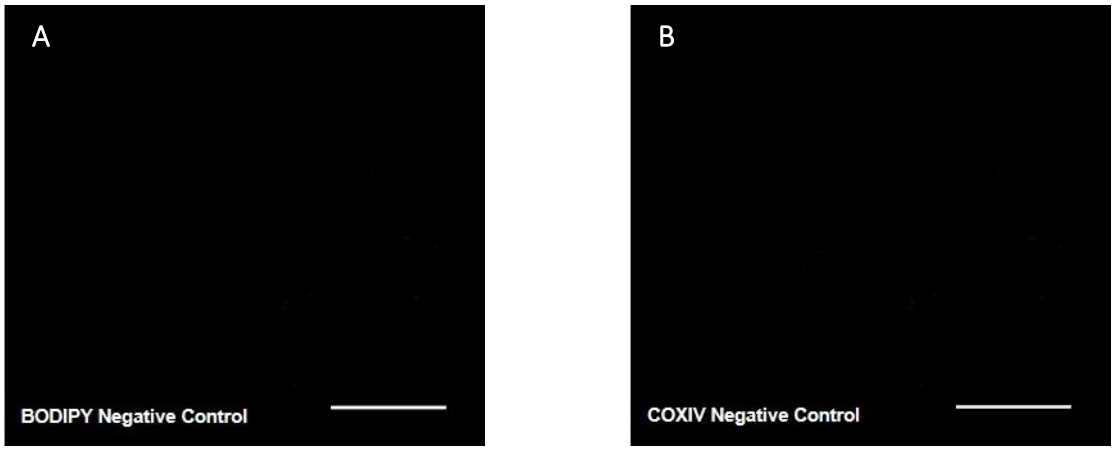

Figure 2.1 *BODPIY and COXIV controls*. Images obtained using an inverted confocal fluorescent microscope (A) and a fluorescence microscope (B). Negative controls of BODIPY (A) and COXIV (B) show no positive stain ensuring that nonspecific staining is prevented. Scale bar = 25µm

2.4 *Lipid immunofluorescent staining protocol*

Sections were fixed for one hour in 3.7% formaldehyde, followed by 3x30 second (s) washes in doubly-distilled water (ddH_20). Slides were then permeabilised for five minutes (min) in 0.5% triton-X 100 (Sigma-Aldrich, Merck Group, UK), followed by 3x5 min washes in 1x phosphate buffered saline (PBS, 137mM sodium chloride, 3 mM potassium chloride, 8 mM sodium phosphate dibasic and 3mM potassium phosphate monobasic, pH 7.4). The muscle samples were then incubated in the appropriate primary antibodies in PBS for 45 min followed by 3x5 min washes in PBS. The primary antibodies used were mouse anti myosin heavy chain I (MHCI) (A4.840, DSHB, Iowa, USA) to target type I fibres and rabbit anti myosin heavy chain IIA (MHC IIA) (N2.261, DSHB, Iowa, USA) targeting type IIa fibres, both antibodies were used at 1:100 dilution in PBS. Subsequently, the samples were washed for 3x5min in PBS and stained using appropriately targeted Alexa Fluor® immunofluorescent secondary antibodies for 30min, followed by another 3x5min wash in PBS. Alexa Fluor® dyes are a group of negatively charged and hydrophilic fluorescent dyes which are frequently used in fluorescence microscopy, the respective laser excitation wavelength is stated in their labelling. The secondary antibodies used were goat anti-rabbit IgG1-405 to target MHCIIA N2.261, goat anti-mouse IgM-546 targeting MHCI A4.840 (Thermo Fisher, UK) both 1:200 dilution in PBS. To stain the cell borders, wheat germ agglutinin-633 (WGA-633) (Sigma-Aldrich, Merck Group, UK) 1:50 dilution was added to the 2ry AB. WGA is a lectin which binds to glycoproteins of the cell membrane. Subsequent to the final 3x5 min wash in PBS, samples were incubated for 20 min in BODIPY 493/503 (Sigma-Aldrich, Merck Group, UK) which was used at 1:100 dilution in PBS, followed by 1x5min wash in PBS. Finally, vectashield (Vector Laboratories, Burlingame, CA 94010) was applied to the slides and mounted with VWR™ coverslips (VRW™, Avantor®, International) and sealed using transparent nail varnish.

2.5 *Confocal image acquisition – Lipid droplet imaging*

Images of the sections were obtained using an inverted confocal fluorescent microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany). A 63x 1.4NA oil immersion objective combined with 1.1 digital magnification was used to identify LDs in single cell field of view, for an example of this field of view see figure 2.1. Zeiss immersion oil was applied to the objective and slides

positioned in an inverted position. In accordance with the fluorescent staining methods used, sections were visualised with an argon laser which excited the Alexa Fluor 488 fluorophore and bodipy 493/503, whilst Alexa Fluor 546 and 633 fluorophores were excited with a helium-neon laser and Alexa Fluor 405 fluorophore excited with a diode laser. Fibres that stained positively for MHCI were categorised as type I fibres, whereas fibres that stained positively for MHC IIA were categorised as type IIa fibres and those that had no staining were categorised as type IIx fibres. To analyse a similar proportion of images in every individual sample an average of 15 type I and 15 type IIa fibres were captured, resulting in a similar portion of fibres being analysed for each group (n=8) i.e. (15 x 8) 120 type I fibres; 120 type II fibres). Due to limited number of type IIx fibres in muscle sections in a number of participants, an average of 4 images were captured from every individual sample investigated (4 x 8) 32 type IIx fibres).

2.6 *Lipid imaging processing and analysis*

Confocal images obtained via the RGB channels were converted from LSM to TIFF image format prior to image analysis, through Zeiss Zen Blue software. Subsequently, images captured via the 633 laser were processed using Zen Black software and converted from LSM to TIFF format ready for analysis. Image analysis was completed using Image-Pro Plus, version 5.1 software (Media Cybernetics, Bethesda, MD, USA). Fibres were separated into a peripheral region (5 µm below cell membrane) and central region (remainder of the cell) to assess LD stores within each muscle fibre. The peripheral region was set at 5 µm due to previous research which suggest that 5 µm is representative of the subsarcolemmal region which would normally be detected using TEM (Koh et al., 2017; Strauss et al., 2020). To produce the peripheral region mask, an erosion mask of 5µm was applied from the cell border, the peripheral erosion mask was then inverted to create a mask for the central region of the fibre. The erosion masks were applied to greyscale images of LD to produce extracted images of LS in the peripheral and central region of the fibres (Figure 2.1). To identify LDs, an intensity threshold was uniformly selected and applied to each image obtained from the lean, obese and T2D participants. Intensity and threshold was also set for the area of LDs which represented the maximum and minimum limits which would represent a LD. From the images, LD content (% area stained), LD density (number of LDs expressed relative to the area) and LD size (mean area of an individual LD) within both the central and peripheral regions of each fibre type was investigated. LD content and LD density data obtained from analysis was normalised for the area of the region

prior to statistical analysis. Normalising the data ensured that the peripheral and central regions were made equal for comparison due to the central region encompassing a larger area compared to the peripheral region. Data obtained from LD size analysis was not normalised as it represents the mean area of an individual LD only and is not dependent on the area of the cell.

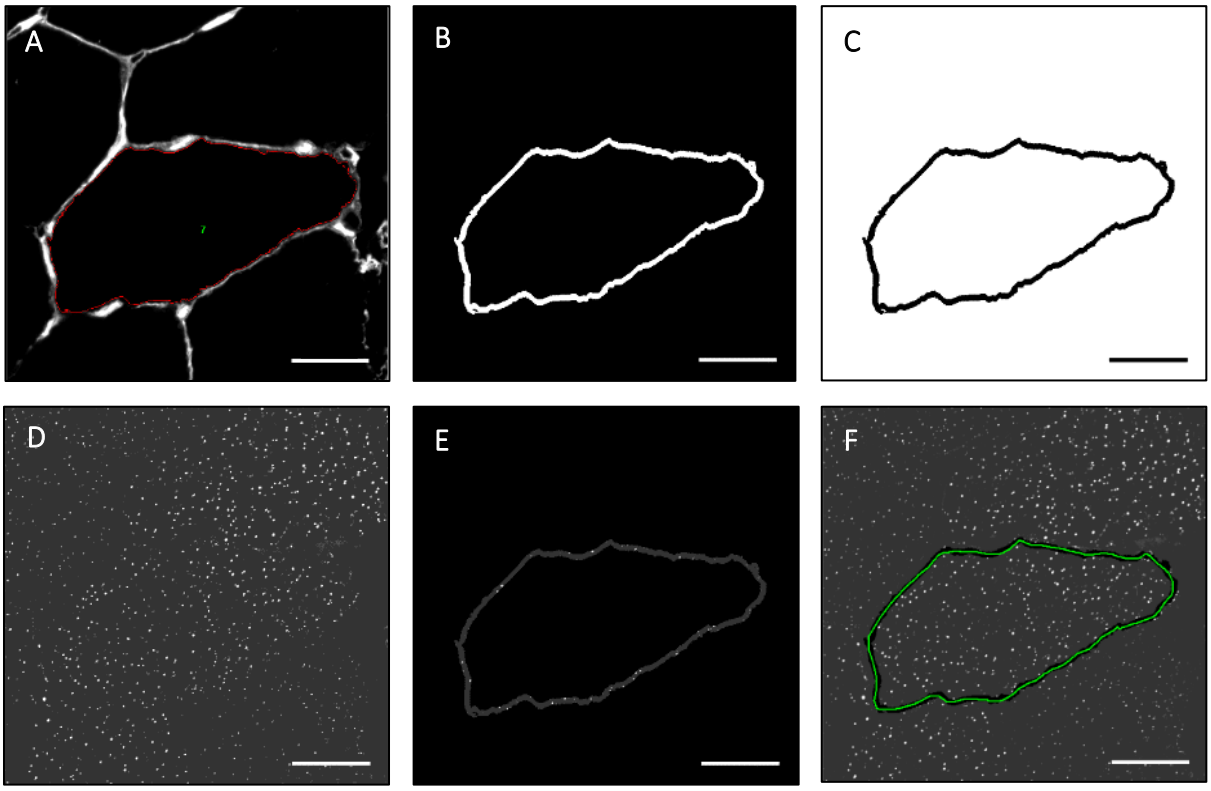

Figure 2.2 *LD image analysis method identifying the central and peripheral regions within cross-sectional muscle fibres*. Images of LD for content analysis obtained using a 63x 1.4NA confocal microscope at 1.1 zoom. Grey scale images of the cell border were identified with WGA mask (*A*). Processed grey scale images of LD stained with BODIPY 493/503 (*D*). Erosion mask of 5µm applied from the cell border to produce a peripheral region mask (*B*). Peripheral erosion mask inverted to create a mask for the central region of the cell (*C*). Masks B and C were applied to greyscale image of LD to produce extracted images of LD in the peripheral region of the cell (*E*) and the central region of the cell (*F*). 'Region of interest' (green line) was manually drawn around central region of the cell to exclude LD from neighbouring cells (*F*). Scale bar = 25µm

2.7 *Mitochondria immunofluorescence staining protocol*

The current immunofluorescence staining protocol for mitochondrial density was developed in a previous study (Shaw et al., 2008). The authors reported that using an antibody against the mitochondrial protein, cytochrome *c* oxidase (COX), may prove more favourable when examining fibre-type differences in mitochondrial density (Shaw, Jones and Wagenmakers, 2008). Specifically, the anti-COX staining ((mouse monoclonal anti-OxPhos Complex IV (COXIV)) produced greater fluorescent signal and enhanced resolution of the mitochondrial network compared to other mitochondrial markers; mouse monoclonal anti-porin and mouse monoclonal anti-OxPhos Complex I. Furthermore, COXIV was successfully combined with MHCI (A4.840), an antibody used against type I fibre myosin (Shaw, Jones and Wagenmakers, 2008). Briefly, sections were fixed for one hour in 3.7% formaldehyde. The slides were then permeabilised in 0.5% triton-X 100 for five minutes, followed by 3x5 min washes in PBS. The primary antibody applied to the muscle samples contained COXIV (459600; Invitrogen, Paisley, UK) antibody 1:50 dilution used to label mitochondria, and MHCI (A4.840, DSHB, Iowa, USA) and MHCII (N2.261, DSHB, Iowa, USA) both 1:100 dilution in PBS. The 45 min incubation period was followed by 3x5 min washes in PBS. Samples were again incubated in appropriate secondary antibodies for 30 min followed by 3x5 min washes in PBS. The secondary antibodies conjugates used were goat anti-mouse IgG2a-488, goat anti-mouse IgM-546 and goat anti-mouse IgG-350 (Thermo Fisher, UK) all 1:100 dilution in PBS, the secondary antibodies are used to target the primary antibodies. Furthermore, the cell border was stained with WGA-633 (Sigma-Aldrich, UK) 1:50 dilution. Mowiol® (Sigma-Aldrich Company Ltd, Dorset, UK) was applied to the slides and prepared using 6g Glycerol, 2.4g Mowiol, 6ml ddH_2O, and 0.026g DABCO. Finally, VWR™ coverslips were mounted onto the sections (VWR™, Avantor®, International).

2.8 *Fluorescence microscope – Mitochondria imaging*

Microscopy images were obtained using a Leica DMB 6000 fluorescence microscope (Leica, Germany, Europe) with a 40x objective capturing ~5 cells in each image. Slides were placed in an inverted position above the objective. Similar to LD imaging process, fibres that stained positively for MHCI were categorised as type I fibres, whereas fibres that were not MHCI positive were categorised as type II fibres. Thirty images were acquired from each muscle

section to ensure that a similar quantity of images were analysed from all participants in all groups. An average of 183 type I fibres and 85 type II fibres were collected for analysis per participant.

2.9 *Mitochondria imaging processing and analysis*

Image processing was undertaken using Image-Pro Plus 5.1 software. Fibre-type distribution of mitochondria was assessed using wide-field images. Mitochondrial density was measured through the protein expression of COXIV, the fluorescence staining intensity of COXIV was used to indicate differences in mitochondrial density across fibre-types. Fluorescence intensity of COXIV was quantified by measuring the signal intensity within the intracellular regions of a mask created by the WGA-633 stain in a fibre-type specific manner (Figure 2.3).

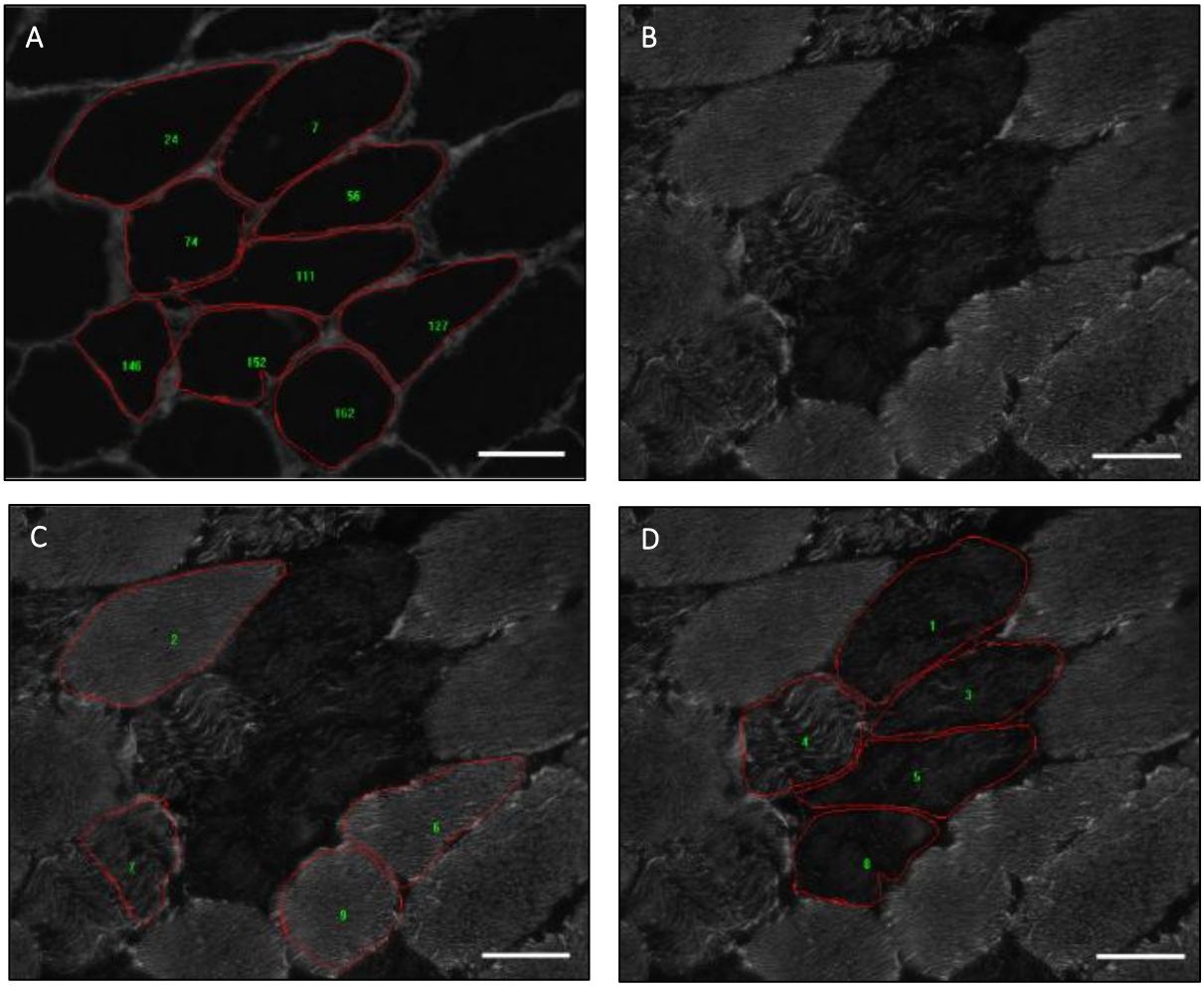

Figure 2.3 *Mitochondria image analysis method identifying fibre types within cross-sectional muscle fibres.* Grey scale images of the cell border identified with WGA (*A*) and of mitochondria stained with COXIV (*B*). Fibres stained positively for MHCI identified as type I fibres and selected for analysis (C). All other fibres that did not stain positively for MHCI categorised as type II fibres and selected for analysis (D). Scale bar = 25µm.

2.10 *Statistical analysis*

Statistical significance was set at $P < 0.05$. All data is expressed as means ± standard error of the mean (SEM). A *t* test was used to measure HOMA-IR and plasma differences within health groups between sex (example; lean male vs. female). Furthermore a two-way between-subjects ANOVA was used to measure HOMA-IR and plasma differences between health groups within sex (example; obese males vs. Lean males vs. T2D males). A one-way between-subjects ANOVA was used to measure the difference in LD profile (content, density and size) between sexes or health groups, the within subjects factors were identified as *'fibre type'* and *'regions'* (sexes + fibre type; sexes + region; health groups + fibre type; health groups + region). A two-way within-subjects ANOVA was performed to measure the difference in LD profile within sex or health group, the within subjects factors were identified as *'fibre type'* and *'regions'* (sex + fibre type; sex + region; health + fibre type; health + region). A four-way between-subjects ANOVA was implemented to determine significant differences in LD profile between sexes and health groups, factors were identified as *'fibre type'* and *'regions'*. (Sexes + health groups + fibre + region). A four-way within-subjects ANOVA was used to determine significant differences in LD profile within sex and health group, factors were identified as *'fibre type'* and *'regions'* (sex + health + fibre type + region). To determine significant differences in the males and females mitochondrial density across all three health groups and both fibre types a three-way ANOVA was used. Significant main effects or interactions were assessed using Bonferroni adjustment *post hoc* analysis. Analysis was completed using SPSS statistical analysis software (v.26.0; SPSS, Chicago, IL, USA).

3. Results

3.1 *Sex differences in skeletal muscle lipid droplet profile*

3.1.1 *Lipid droplet stores in males and females follow a hierarchical distribution across fibre-types in both the peripheral and central regions*

LD content (expressed as % area stained) within the males followed a hierarchical distribution across fibre-types in both the peripheral (5μm band beneath the cell border) and central (remainder of the cell) regions of the cell, such that the LD content in the type I fibres > type IIa fibres > type IIx fibres (Sex x fibre x region interaction; $P < 0.001$; Fig 3.1[A, B, C], Table 3.1). This was primarily driven through a similar hierarchical distribution in LD density across fibre-types (TI > TIIa > TIIx) within the peripheral and central regions of the muscle fibres (Sex x fibre x region interaction; $P < 0.001$; Fig 3.1[D, E, F], Table 3.1), but not LD size ($P > 0.05$). Furthermore, similar patterns in fibre-type specific LD distribution and subcellular location were observed in the females for LD density (sex x fibre x region interaction; $P < 0.05$; Fig 3.1[D, E, F], Table 3.1), but not for LD content ($P > 0.05$) or size ($P > 0.05$). For imagery representation see; Fig 3.4.

3.1.2 *Greater lipid droplet stores in the central vs peripheral region across all fibre-types in males and females*

The subcellular distribution of LDs differed when separating the cell into peripheral and central regions. When normalised for area (see 2.5), analysis revealed that LD content in the men was greater in the central regions when compared to the peripheral regions, and this pattern in LD subcellular location was true across all fibre-types investigated (Sex x region x fibre interaction; $P < 0.001$; Fig 3.1[A, B, C], Table 3.1). This was largely due to greater LD density in the central region of the type I, IIa and IIx fibres in comparison to the peripheral region in males (Sex x region x fibre interaction; $P < 0.001$; Fig 3.1[D, E, F], Table 3.1). Furthermore, in males, the type I and IIa fibres central region contained significantly larger LDs compared to the peripheral region (Sex x region x fibre interaction; $P < 0.05$; Fig 3.1[G, H], Table 3.1). LD content in the females also followed a similar pattern in subcellular location (central > peripheral) within the type I and IIa fibres (Sex x region x fibre interaction; $P < 0.05$; Fig 3.1[A, B], Table 3.1). This was driven through greater LD density in the central region of the type I, IIa and IIx fibres in comparison to the

peripheral region (Sex x region x fibre interaction; P < 0.05; Fig 3.1[D, E, F], Table 3.1), and not size (P > 0.05). (Fig 3.4).

3.1.3 *Males exhibit greater lipid droplet stores across all fibre-types in the peripheral and central regions when compared to females*

Within the central region across all fibre-types, LD content was significantly greater in the men when compared the women (sex x region x fibre interaction; *P* < 0.05; Fig 3.1[A, B, C], Table 3.1). These centrally located fibre-type differences in LD content found in males vs. females was mainly attributed to larger LDs (sex x region x fibre interaction; *P* < 0.05; Fig 3.1[G, H, I], Table 3.1). Additionally, greater LD density was found in the central region of type I and IIa fibres of the males in comparison to the females (sex x region x fibre interaction; *P* < 0.001; Fig 3.1[D, E], Table 3.1). Whilst LD profile across all fibre-types in the central region was uniform in the males, this was not the case for the peripheral region. Sex differences in LD content and morphology within the peripheral region were mainly observed in the type I and IIa fibres (*P* < 0.05), and not the type IIx fibre (*P* > 0.05). Males indeed had greater LD content at the periphery of the type I and IIa fibres when compared to females (sex x region x fibre interaction; *P* < 0.05; Fig 3.1[A, B], Table 3.1), predominately due to larger LDs (sex x region x fibre interaction; *P* < 0.05; Fig 3.1[G, H], Table 3.1) and a greater LD density in the type I fibres only (sex x region x fibre interaction; *P* < 0.05; Fig 3.1[D], Table 3.1). (Fig 3.4).

Table 3.1 *Lipid droplet profile* in *males and females*

	Type I fibres		Type IIa fibres		Type IIx fibres	
	Peripheral	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*
Males (n = 24)						
LD content, % area stained	1.14 ± 0.14*‡	6.85 ± 1.15*†‡	0.67 ± 0.08*‡	3.66 ± 0.80*†‡	0.29 ± 0.04	0.97 ± 0.22†‡
LD density, LD.µm^2	0.03 ± 0.01*‡	0.16 ± 0.03*†‡	0.02 ± 0.00*	0.08 ± 0.01*†‡	0.01 ± 0.00	0.02 ± 0.01†
LD size, µm^2	0.39 ± 0.03‡	0.45 ± 0.04†‡	0.44 ± 0.03‡	0.49 ± 0.07†‡	0.45 ± 0.05	0.48 ± 0.10‡
Females (n = 24)						
LD content, % area stained	0.49 ± 0.06	2.24 ± 0.14†	0.45 ± 0.03	1.11 ± 0.11†	0.26 ± 0.06	0.43 ± 0.07
LD density, LD.µm^2	0.02 ± 0.00*	0.08 ± 0.00*†	0.01 ± 0.00*	0.03 ± 0.00*†	0.01 ± 0.00	0.02 ± 0.00†
LD size, µm^2	0.29 ± 0.01	0.29 ± 0.02	0.34 ± 0.03	0.33 ± 0.01	0.33 ± 0.01	0.31 ± 0.01

Data provided as means ± SE. Lipid droplet (LD) content and density are expressed relative to the area of the peripheral or central region. ∗ Within sex x fibre x region interaction type I > IIa > IIx fibres (P < 0.05). † Within sex x region x fibre interaction central > peripheral (P < 0.05). ‡ Between sex x region x fibre interaction males > females (P < 0.05).

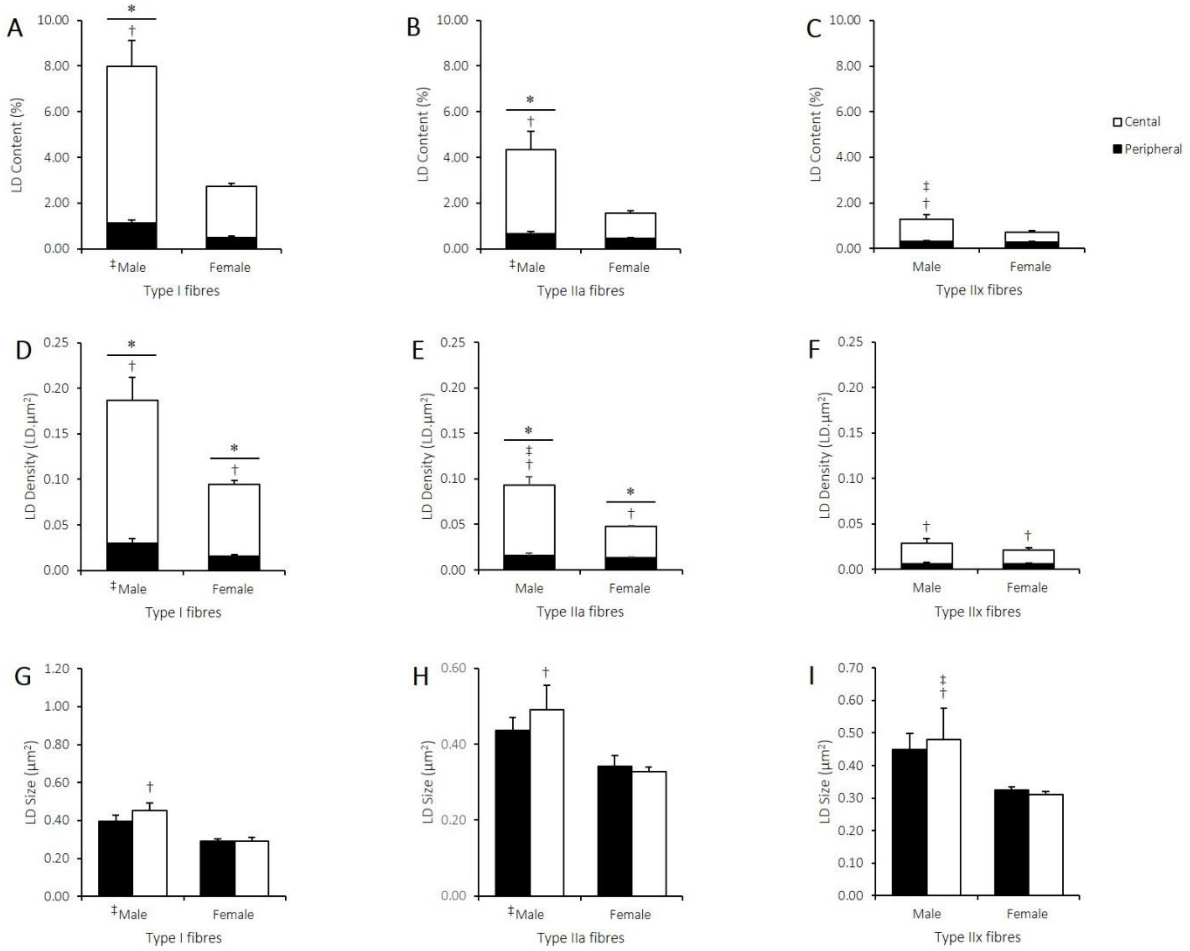

Fig. 3.1. *Fibre-type and subcellular-specific changes in lipid droplet (LD) profile in male and female skeletal muscle.* Presented are LD content (A, B, C), LD density (D, E, F), and LD size (G, H, I) in the peripheral and central subcellular regions of type I, IIa and IIx fibres. LD content and LD density in each region was normalized to total cell area. ∗ Within sex x fibre x region interaction type I > IIa > IIx fibres ($P < 0.05$). † Within sex x region x fibre interaction central > peripheral ($P < 0.05$). ‡ Between sex x region x fibre interaction males > females ($P < 0.05$). Values are means ± SE.

3.2 *Differences in lean, obese and T2D participants skeletal muscle lipid droplet profile*

3.2.1. *Lipid droplet content and density in lean, obese and T2D participants follow a hierarchical distribution across fibre-types in both the peripheral and central regions*

T2D and obese participants had greater LD content in the peripheral region of the type I > type IIx fibres and in the type IIa > type IIx fibres (health x fibre x region interaction; $P < 0.05$; Fig 3.2[A, B, C], Table 3.2). These differences in LD content were influenced by a similar distribution of LD density observed within the peripheral region of the fibres (health x fibre x region interaction; $P < 0.05$; Fig 3.2[D, E, F], Table 3.2), but not LD size ($P > 0.05$). No differences in LD profile were found within the peripheral region of the skeletal muscle fibres of the lean participants ($P > 0.05$). Similarly, these patterns in fibre-type differences were observed in the central region of the lean, obese and T2D participants fibres (health x fibre x region interaction; content; $P < 0.05$, density; $P < 0.05$; Fig 3.2[A-F], Table 3.2), again no changes were found in LD size ($P > 0.05$). Additionally, the patterns in LD content and density were also observed in the central region of the type I fibres > type IIa fibres in T2D and obese participants, thus following a hierarchical distribution across fibre-types (health x fibre x region interaction; content; $P < 0.05$; density; $P < 0.001$; Fig 3.2[A, D], Table 3.2). (Fig 3.4).

3.2.2 *Greater lipid droplet content and density in the central vs peripheral region across all fibre-types in skeletal muscle from lean, obese and T2D participants*

In lean, obese and T2D, the central region had greater LD content in comparison to the peripheral region across all three fibre-types examined (health x region x fibre interaction; $P < 0.05$; Fig 3.2[A, B, C], Table 3.2), as a result of augmented LD density in the central region across the fibres (health x region x fibre interaction; $P < 0.05$; Fig 3.2[D, E, F], Table 3.2) but no differences were shown for LD size ($P > 0.05$). (Fig 3.4).

3.2.3 *T2D participants have greater LD stores specifically in type IIa fibres compared to lean and obese participants*

In T2D participants the central region of type IIa fibres contained greater LD content when compared to the lean participants (health x region x fibre interaction; $P < 0.05$; Fig 3.2[B], Table 3.2). Interestingly no changes were observed in LD density or size between the T2D and lean

participants ($P > 0.05$). Furthermore, in T2D the peripheral region of the type IIa fibres had larger LDs in comparison to the obese participants (health x region x fibre interaction; $P < 0.05$; Fig 3.2^{H}, table 3.2), with no differences in LD content or density ($P > 0.05$). (Fig 3.4).

Table 3.2 *Lipid droplet profile* in l*ean, obese and T2D participants*

	Type I fibres		Type IIa fibres		Type IIx fibres	
	Peripheral	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*
Lean (n = 16)						
LD content, % area stained	0.64 ± 0.27	3.63 ± 1.66*†	0.49 ± 0.06	1.81 ± 0.86*†	0.28 ± 0.06	0.61 ± 0.31†
LD density, LD.µm^2	0.02 ± 0.00	0.09 ± 0.02*†	0.01 ± 0.00	0.05 ± 0.01*†	0.01 ± 0.00	0.01 ± 0.00*†
LD size, µm^2	0.31 ± 0.04	0.34 ± 0.07	0.39 ± 0.03	0.38 ± 0.07	0.36 ± 0.04	0.39 ± 0.08
Obese (n = 16)						
LD content, % area stained	0.85 ± 0.27*	4.25 ± 1.90*†	0.52 ± 0.11*	2.05 ±1.00*†	0.26 ±0.07	0.54 ± 0.09†
LD density, LD.µm^2	0.03 ± 0.01*	0.14 ± 0.06*†	0.02 ± 0.00*	0.06 ± 0.03*†	0.01 ± 0.00	0.02 ± 0.01†
LD size, µm^2	0.32 ± 0.05	0.37 ± 0.04	0.34 ± 0.05	0.37 ± 0.03	0.36 ± 0.05	0.32 ± 0.01
T2D (n = 16)						
LD content, % area stained	0.95 ± 0.42*	5.75 ± 3.34*†	0.67 ± 0.16*	3.28 ± 1.96*†‡	0.29 ± 0.08	0.97 ± 0.42†
LD density, LD.µm^2	0.02 ± 0.01*	0.12 ± 0.04*†	0.01 ±0.00*	0.06 ± 0.02*†	0.01 ± 0.00	0.02 ± 0.00†
LD size, µm^2	0.39 ± 0.07	0.41 ± 0.13	0.44 ± 0.06‡	0.47 ± 0.14	0.44 ± 0.10	0.47 ± 0.17

Data provided as means ± SE. LD content and density are expressed relative to the area of the peripheral or central region. ∗ Health x fibre x region interaction type I > IIa > IIx fibres ($P < 0.05$). † Health x region x fibre interaction central > peripheral ($P < 0.05$). ‡ Between health x region x fibre interaction T2D > obese and lean ($P < 0.05$).

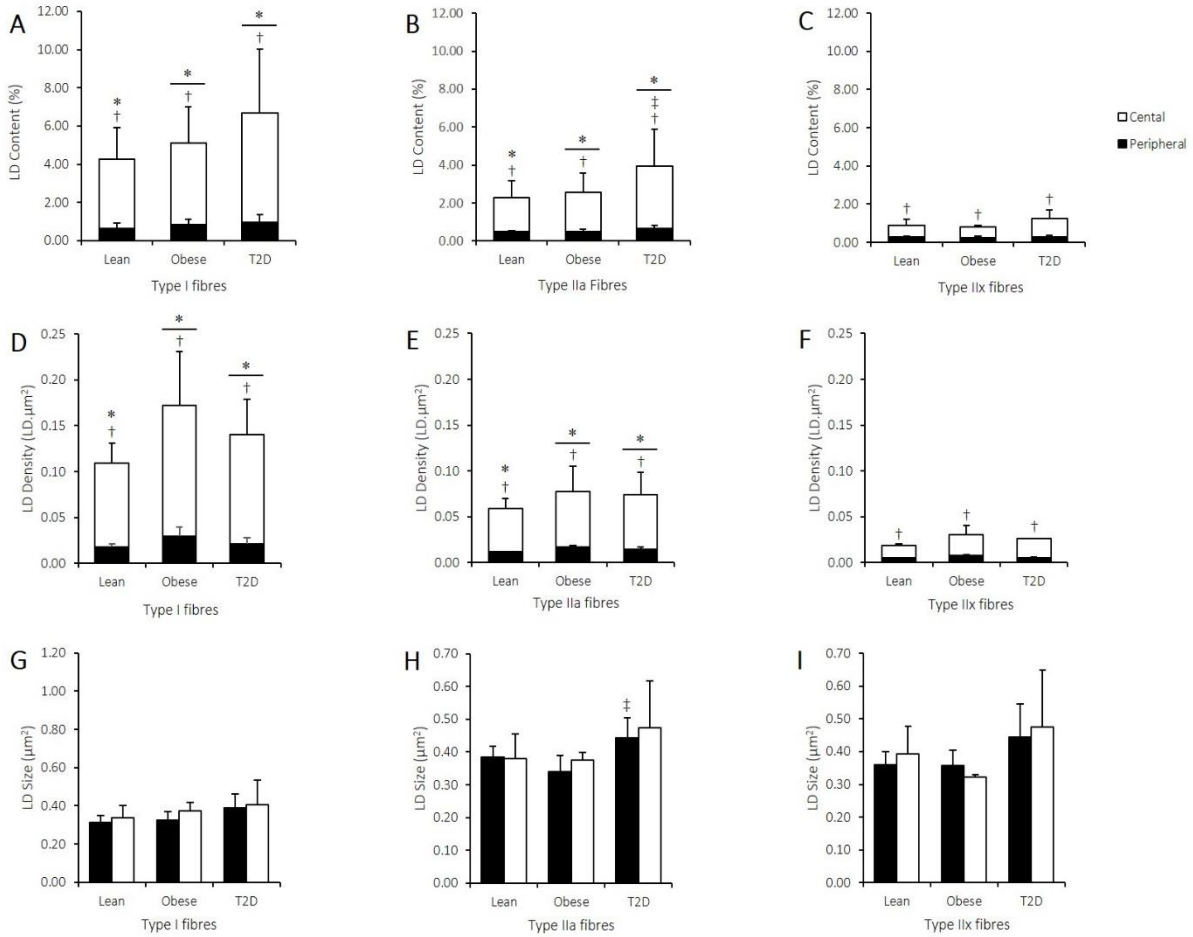

Fig. 3.2. *Fibre-type and subcellular-specific changes in LD profile in skeletal muscle biopsies from lean, obese and T2D participants*. Presented are LD content (*A*, *B, C*), LD density (*D, E, F*), and LD size (*G*, *H, I*) in the peripheral and central subcellular regions of type I, IIa and IIx fibres. LD content and LD density in each region was normalized to total cell area. * Within health x fibre x region interaction type I > IIa > IIx fibres ($P < 0.001$). † Within health x region x fibre interaction central > peripheral ($P < 0.05$). ‡ Between health x region x fibre interaction T2D > obese and lean ($P < 0.05$). Values are means ± SE.

3.3 *Differences in skeletal muscle lipid droplet profile between lean, obese and T2D males and females*

3.3.1 *T2D males have greater lipid droplet stores within the central region of type IIa fibres compared to lean and obese males*

The central region of the type IIa fibres in T2D males contained greater LD content when compared to the obese and lean males (health x sex x fibre x region interaction; $P < 0.05$; Fig 3.3^{A}, table 3.3). These differences in LD content were driven through larger LDs (health x sex x fibre x region interaction; $P < 0.05$; Fig 3.3^{C}, table 3.3), but not LD density ($P > 0.05$). Additionally, no differences were observed in fibre-type or subcellular location of LD content, density or size between the lean, obese and T2D females (P > 0.05). (Fig 3.4).

3.3.2 *Lean males have greater LD content within the central region of type IIa and IIx fibres compared to lean females*

Lean males have greater LD content within the central region of their type IIa and IIx fibres in comparison to lean women (sex x health x fibre x region interaction; $P < 0.05^{A}$; Fig 3.3, table 3.3). Attributed to larger LD in the type IIa fibres (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{C}, table 3.3) but not LD density ($P > 0.05$). No differences were observed in lean males and females within the peripheral region of the muscle fibres (P > 0.05). (Fig 3.4).

3.3.3 *Obese males have greater LD stores within the central regions of the type I and IIa fibres when compared to obese females*

Obese men had greater LD content within the central region of their type I and IIa fibres in comparison to obese women (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{A}, table 3.3). This was due to both larger LDs (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{C}, table 3.3) and greater LD density (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{B}, table 3.3). Furthermore, the peripheral region of type I fibres in obese males contained greater LD density when compared to obese females (P < 0.05; Fig 3.3^{B}, table 3.3) with no differences shown in LD content or LD size (P > 0.05). (Fig 3.4).

3.3.4 *T2D male have greater LD stores within the peripheral and central regions of type I, IIa and IIx fibres in comparison to T2D females*

In T2D men, both the peripheral and central regions of their type I and type IIa fibres contained greater LD content in comparison to the women (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{A}, table 3.3). Furthermore, the central region of the type IIx fibres of T2D men had greater LD content compared to the women (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{A}, table 3.3). These fibre-type and subcellular differences in LD content were primarily driven through larger LDs (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{C}, table 3.3). Additionally, in T2D males, the peripheral region of the type IIa fibres had augmented LD density compared to the T2D women (sex x health x fibre x region interaction; $P < 0.05$; Fig 3.3^{B}, table 3.3). (Fig 3.4).

Table 3.3 *Lipid droplet profile in skeletal muscle of lean, obese and T2D males and females*

	Males (n=24)						Females (n=24)					
	Type I fibres		Type IIa fibres		Type IIx fibres		Type I fibres		Type IIa fibres		Type IIx fibres	
	Peripheral	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*	*Peripheral*	*Central*
Lean (n = 8)												
LD content, % area stained	0.91 ± 0.29	5.30 ± 1.68	0.55 ± 0.11	2.67 ± 0.64†	0.34 ± 0.15	0.92 ± 0.27†	0.37 ± 0.09	1.97 ± 0.56	0.43 ± 0.11	0.95 ± 0.19	0.23 ± 0.09	0.30 ± 0.11
LD density, LD.µm^2	0.02 ± 0.00	0.11 ± 0.02	0.01 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.07 ± 0.01	0.01 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
LD size, µm^2	0.35 ± 0.04	0.40 ± 0.06	0.42 ± 0.06	0.45 ± 0.06†	0.40 ± 0.04	0.48 ± 0.06	0.28 ± 0.04	0.27 ± 0.04	0.35 ± 0.03	0.31 ± 0.03	0.32 ± 0.05	0.31 ± 0.06
Obese (n = 8)												
LD content, % area stained	1.12 ± 0.32	6.16 ± 2.04†	0.63 ± 0.12	3.06 ± 0.36†	0.33 ± 0.09	0.62 ± 0.16	0.58 ± 0.10	2.35 ± 0.39	0.41 ± 0.06	1.05 ± 0.12	0.19 ± 0.04	0.45 ± 0.16
LD density, LD.µm^2	0.04 ± 0.01†	0.20 ± 0.06†	0.02 ± 0.00	0.09 ± 0.02†	0.01 ± 0.00	0.03 ± 0.01†	0.02 ± 0.00	0.09 ± 0.01	0.02 ± 0.02	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
LD size, µm^2	0.37 ± 0.05	0.42 ± 0.07†	0.39 ± 0.03	0.40 ± 0.04†	0.40 ± 0.08	0.32 ± 0.03	0.28 ± 0.02	0.33 ± 0.04	0.29 ± 0.04	0.35 ± 0.05	0.31 ± 0.10	0.33 ± 0.07
T2D (n = 8)												
LD content, % area stained	1.38 ± 0.26†	9.09 ± 1.66†	0.83 ± 0.10†	5.24 ± 0.86*†	0.21 ± 0.05	1.38 ± 0.37†	0.53 ± 0.08	2.41 ± 0.40	0.51 ± 0.11	1.32 ± 0.35	0.37 ± 0.12	0.55 ± 0.03
LD density, LD.µm^2	0.03 ± 0.00	0.16 ± 0.02	0.02 ± 0.00†	0.08 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.08 ± 0.01	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
LD size, µm^2	0.46 ± 0.05†	0.54 ± 0.07†	0.50 ± 0.03†	0.62 ± 0.05*†	0.55 ± 0.14	0.65 ± 0.11†	0.32 ± 0.02	0.27 ± 0.02	0.38 ± 0.02	0.33 ± 0.03	0.34 ± 0.04	0.30 ± 0.03

Data provided as means ± SE. LD content and density are expressed relative to the area of the peripheral or central region. * Health x sex x fibre x region interaction T2D men > obese and lean males ($P < 0.05$). † Sex x health x region x fibre interaction males > females ($P < 0.05$).

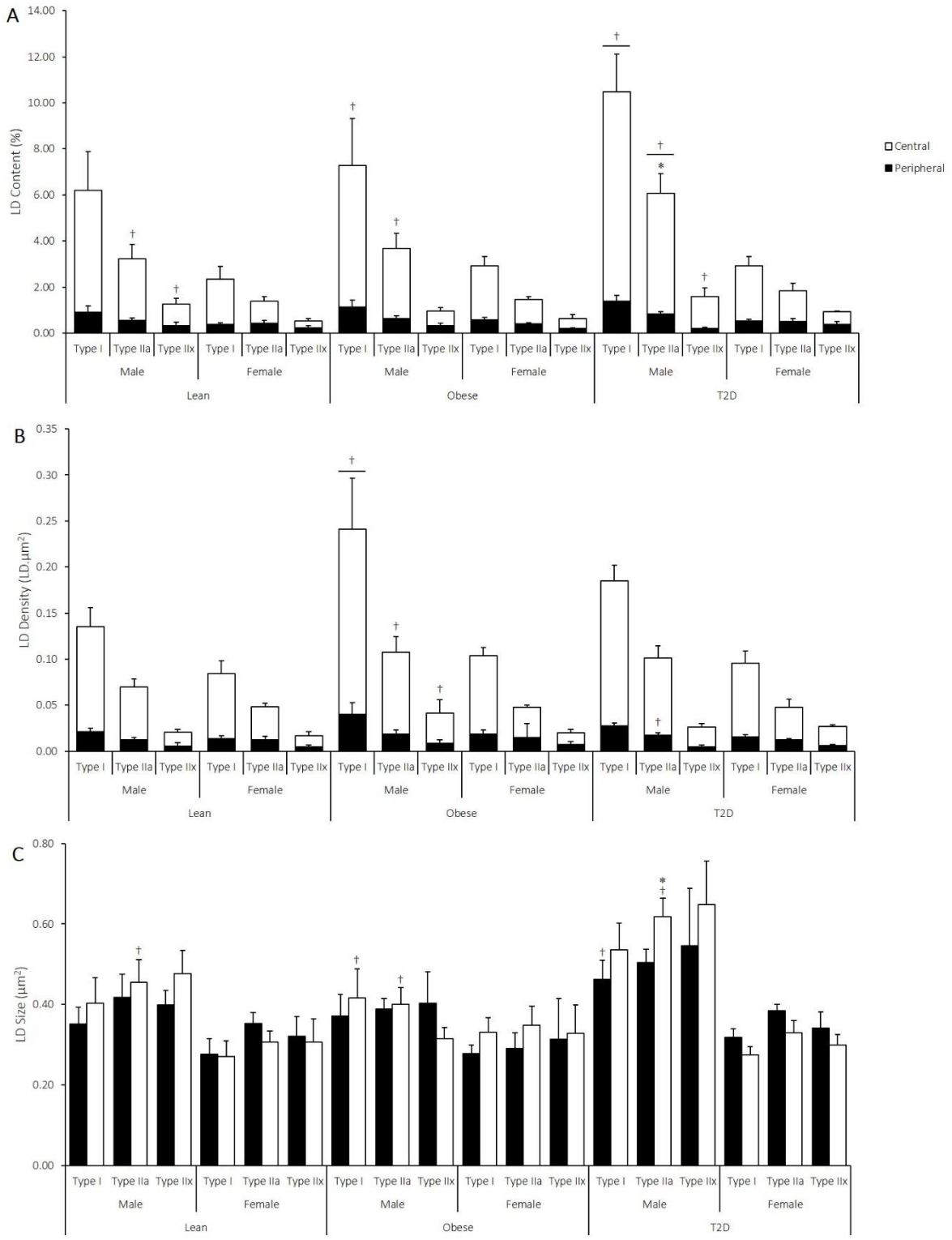

Fig. 3.3. *Fibre-type and subcellular-specific changes in LD profile in skeletal muscle biopsies from lean, obese and T2D males and females*. Presented are LD content (*A*), LD density (*B*), and LD size (*C*) in the peripheral and central subcellular regions of type I, IIa and IIx fibres. LD content and LD density in each region was normalized to total cell area. * Between health x within sex x fibre x region interaction T2D men > obese and lean males ($P < 0.05$). † Between sex x within health x fibre x region interaction males > females ($P < 0.05$). Values are means ± SE.

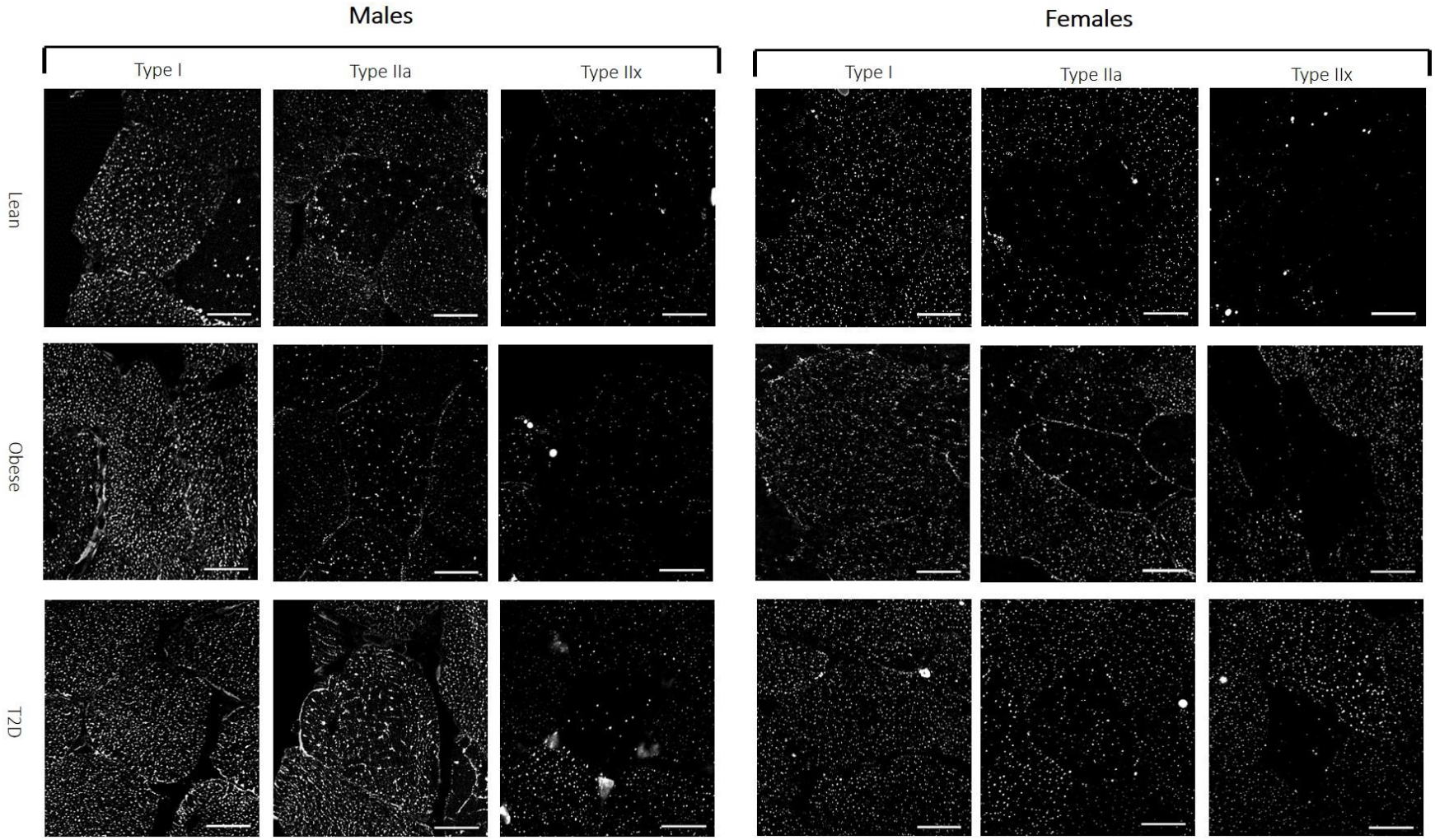

Fig.3.4.*a. Greyscale images of fibre type specific immunofluorescence staining of LDs in the skeletal muscle of lean, obese and T2D males and females.*

Representative greyscale images of LDs stained using BODIPY 493/503 within different fibre-types obtained using immunofluorescent confocal microscopy with a 63x oil immersion objective and 1.1 digital zoom. The left panel includes representative images obtained from the male participants and the right panel images from the female participants. The top line signifies images obtained from lean participants, the middle; obese participants and the bottom; T2D participants.

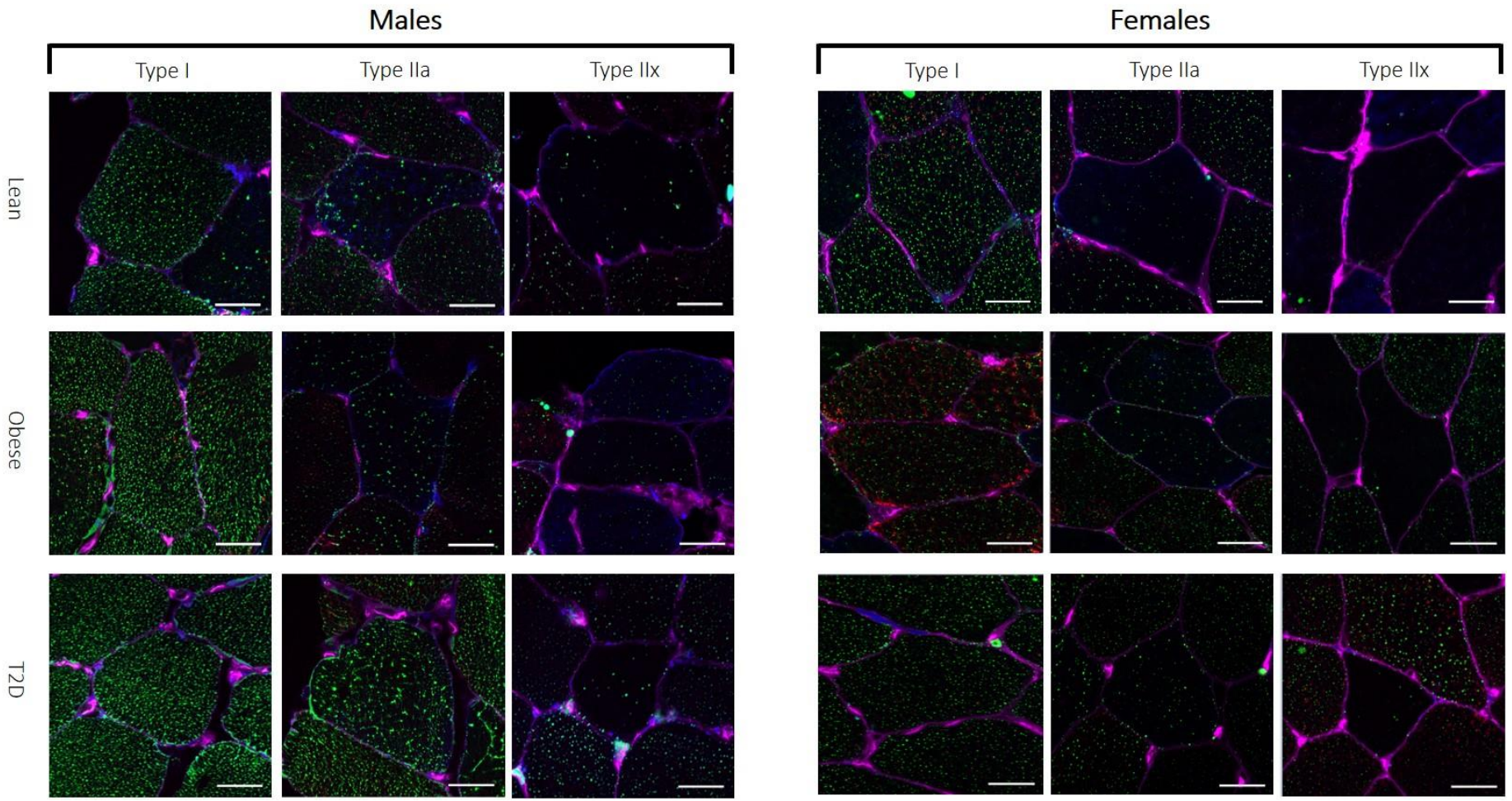

Fig.3.4.*b. Merged images of fibre type specific immunofluorescence staining of LDs in the skeletal muscle of lean, obese and T2D males and females.*

Representative merged images of LDs (green), cell border stained using WGA-633 (pink) within different fibre-types obtained using immunofluorescent confocal microscopy with a 63x oil immersion objective and 1.1 digital zoom. The left panel includes representative images obtained from the male participants and the right panel images from the female participants. The top line signifies images obtained from lean participants, the middle; obese participants and the bottom; T2D participants. Positive MHC I signal (red) are type I fibres, MHC IIa (blue) are type IIa fibres. Fibres that showed no stain were categorised as type IIx. Scale bars represent 25 µm.

3.4 *Differences in mitochondrial density are predominately fibre-type driven, and not determined by sex or metabolic health*

Mitochondrial density (determined using COXIV expression) followed a hierarchical distribution across fibre-types, such that the mitochondrial density in the type I fibres (39.14 ± 2.98 μm^2) > type II fibres (26.87 ± 2.39 μm^2) (main effect of fibre; $P < 0.001$; Fig 3.5, table 3.4). This fibre-type distribution also remained true when mitochondrial density was determined in both the males and females (sex x fibre interaction; $P < 0.001$; Fig 3.5, table 3.4). Additionally, these patterns in fibre-type distribution were also observed within the lean, obese and T2D participants (health x fibre interaction; $P < 0.001$; Fig 3.5, table 3.4). There were no differences observed between the sexes or the lean, obese and T2D skeletal muscle biopsies mitochondrial density ($P > 0.05$; Fig 3.5, table 3.4). (Fig 3.6).

Table 3.4 *Mitochondrial density in skeletal muscle of lean, obese and T2D males and females*

	Males†		Females†	
	Type I fibres*	Type II fibres	Type I fibres*	Type II fibres
Lean (n = 16) ‡				
Mitochondrial density, µm²	39.13 ± 10.18	28. 14 ± 9.00	40.43 ± 6.24	30.10 ± 5.78
Obese (n = 16) ‡				
Mitochondrial density, µm²	39.68 ± 6.62	28.56 ± 5.12	29.61 ± 3.11	21.54 ± 2.09
T2D (n = 16) ‡				
Mitochondrial density, µm²	50.11 ± 9.42	27.85 ± 7.90	36.85 ± 6.26	25.06 ± 3.87

Data provided as means ± SE. * Main effect of fibre-type; type I > type II ($P < 0.001$). † Sex x fibre interaction; type I > type II in males and females ($P < 0.001$). ‡ Health x fibre interaction; type I > type II in lean, obese and T2D ($P < 0.001$).

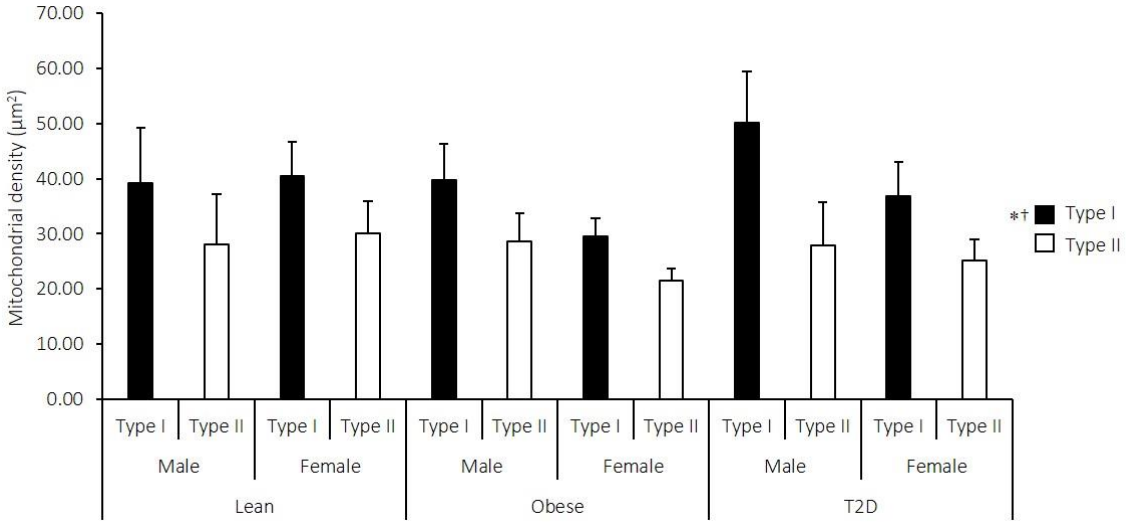

Fig. 3.5. *Fibre-type changes in mitochondrial density in skeletal muscle from lean, obese and T2D males and females.* Values are means ± SE. ∗Mitochondrial density significantly greater in type I vs. type IIa fibres in men and women ($P < 0.001$). †Mitochondrial density significantly greater in type I vs. type IIa fibres in lean, obese and T2D participants ($P < 0.001$).

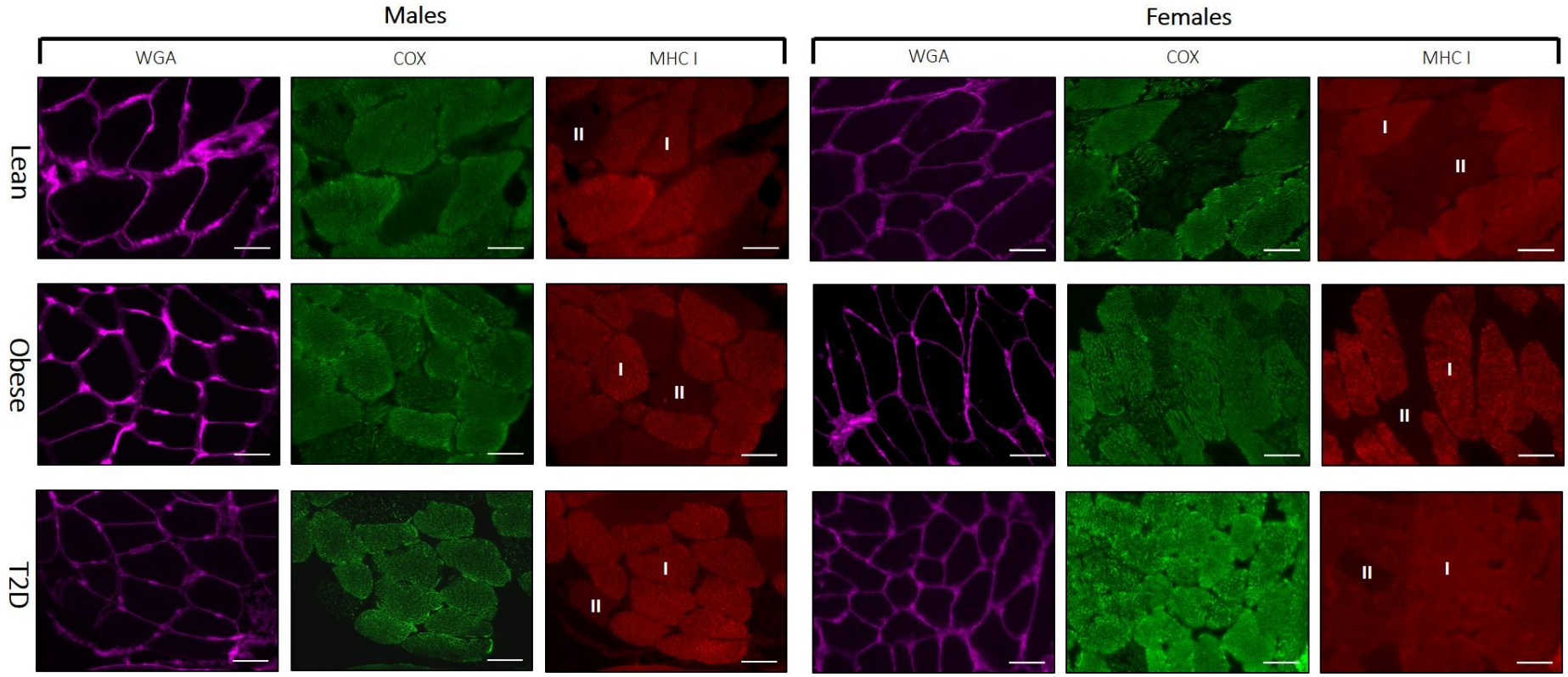

Fig 3.6. *Fibre type specific immunofluorescence staining of mitochondria in the skeletal muscle of lean, obese and T2D males and females.*
Representative images of mitochondrial density stained using COX (green) within different fibre-types obtained using wide field fluorescent microscopy. Images were captured using a 40x objective. Cell border was stained using WGA-633 (pink). The left panel includes representative images obtained from male participants and the right panel images from female participants. The top line signifies images obtained from lean participants, the middle; obese participants and the bottom; T2D participants. Corresponding fibre-type staining for each image is also shown; positive MHC I signal (red) were categorised as type I fibres, any fibres without a positive MHC I stain were categorised as type II fibres. Scale bars represent 25 µm.

4. Discussion

4.1 *Overview*

A Western lifestyle, typically characterised by a reduction in physical activity, increased sedentary behaviour and overindulgence in energy rich food, has contributed to the current global obesity epidemic and increased prevalence of T2D (World Health Organisation, 2017; Diabetes UK, 2019). As a result of this, several studies have investigated the impaired buffering capabilities of adipose tissue which results in a lipid overspill into peripheral metabolic tissues (Frayn, 2002b), and have aimed to identify the mechanisms by which abnormalities in lipid metabolism lead to insulin resistance. To date, most of the research within this area has almost exclusively been completed in cohorts of young healthy males (Strauss et al., 2016; Shepherd et al., 2017; Gemmink et al., 2018). This is a considerable oversight considering females make up ~50% of the population and typically display greater insulin sensitivity when compared to BMI-matched males (Geer and Shen, 2009). Females appear to have better protective physiological mechanisms against metabolic diseases than their male counterparts (Groban et al., 2016). Furthermore, the studies that have investigated females tend to only include pre-menopausal women with normal levels of the female sex hormones (Tarnopolsky et al., 2007). Finally, the fibre-type and region-specific differences in LD distribution and morphology across a range of metabolic health statuses have not yet been investigated. Such data could generate the currently missing understanding of the role played by LD morphology in the inborn presence of a higher insulin action in females than males.

An important factor in the development of insulin resistance is impaired IMTG turnover in skeletal muscle (Moro 2007, Shaw 2010). The accumulation of deleterious FA metabolites that lead to insulin resistance in obese individuals and T2D patients are intrinsically linked to a decreased ability to utilise IMTG stores during exercise (Amati et al., 2011a). Previous research demonstrates an association between elevated IMTG content and the development of insulin resistance in this population (Goodpaster et al., 2000; Goodpaster et al., 2001). However, more recent findings indicate a more complex relationship between IMTG content and insulin sensitivity (Amati et al., 2011a; Bilet et al., 2020). This complex relationship requires further investigation to establish a better understanding of the mechanisms underpinning to lipid-

induced insulin resistance. The present study aimed to use validated immunofluorescence microscopy methods (Shepherd et al., 2012; Shepherd et al., 2013b; Shepherd et al., 2017) to identify sex differences in older individuals (with or without obesity and/or T2D) in the fibre type and subcellular (region-specific) LD profile and mitochondrial content in human skeletal muscle. A primary novel finding of the present study is that males have significantly greater LD content compared to females (Fig 3.1), due to a combination of a greater LD density and size. However, no significant differences were found in mitochondrial density between males and females (Figure 3.5). Another major novel finding was the increased size of LD specifically in the type IIa fibres of males with T2D compared to lean and obese males (Figure 3.3).

4.2 *Confocal immunofluorescence microscopy*

Extensive research has been conducted investigating differences in IMTG stores, and the predominant method adopted to achieve this originally were lipid extraction methods from homogenised whole homogenates (Watt et al., 2002; Bergman et al., 2010). Key limitations of this analysis technique are that between-biopsy variability is considerably high (23%) (Watt, Heigenhauser and Spriet, 2002). In addition homogenisation of the muscle makes it impossible to measure differences between fibre types and their capacity to store IMTG after exercise and utilise IMTG during exercise. This is an important oversight when it is considered that type I fibres have an enhanced capacity to store IMTG whilst remaining more insulin sensitive than type II fibres (van Loon and Goodpaster, 2006; Shepherd et al., 2013b). These findings are paramount in the development of knowledge surrounding the relationship between LD and insulin sensitivity. Nevertheless, studies have shown greater IMTG stores in type I skeletal muscle fibres corresponding with a higher oxidative capacity in type I fibres compared to other fibre-types (Malenfant et al., 2001; He, Goodpaster and Kelley, 2004; Shepherd et al., 2017; Whytock et al., 2020). Previous findings also propose that LD located in the subsarcolemmal region of skeletal muscle fibres are associated with insulin resistance compared to those located in the intermyofibrillar (IMF) region (Gemmink et al., 2017; Daemen et al., 2018). Based on this collective knowledge, the current thesis adopted a fibre-type (TI vs TIIa vs TIIx) and region-specific (peripheral vs central) approach to investigate differences in LD content, morphology (LD size and density) and distribution. As anticipated, our data showed fibre-type and region-specific differences in the LD profile. Overall, the greater LD content in the type I fibres compared to the type IIa and IIx fibres was driven through an increased LD density rather

than a difference in LD size (Fig 3.1-3). This observation of a hierarchical LD distribution between the different fibre types is in line with previous literature (Whytock et al., 2020). Characteristically, type I fibres have greater mitochondrial content which, paired with an abundance of lipolytic regulatory proteins (Shaw et al., 2020), results in an enhanced ability to store and utilise IMTG in type I fibres compared to type IIa and IIx fibres. In line with previous findings, the current study found that the central region of the skeletal muscle fibres contained greater LD content compared to the peripheral region, and similar to the fibre type data, this greater LD content in this region was due to an increased LD density (Fig 3.1-3). It is proposed that the turnover of centrally located LDs is greater due to the increased capacity of the central region to utilise IMTG during exercise as they are located near the IMF mitochondria (Tarnopolsky et al., 2007; Shaw, Jones and Wagenmakers, 2008). These results emphasise the importance of research adopting fibre-type and region-specific approaches when examining LD distribution and morphology.

4.3 *Sex differences in human skeletal muscle lipid droplet profile*

This study was the first to directly investigate and compare sex differences in LD profile within an older population (>60 years). In contrast to the hypothesis, male participants exhibited greater LD content compared to females, which was driven through greater LD density and larger LDs within both the central and peripheral regions of all three fibre types (Fig 3.1). These patterns of fibre type and region-specific distribution and morphology of LDs are the first of their kind within these groups, thereby highlighting the importance of investigating sex differences in older populations.

A consensus is yet to be reached on whether sex differences exist in the LD profile, which is due to the lack of well-controlled studies that have included female participants. Nevertheless, previous findings have proposed that females are more likely to exhibit greater LD stores compared to their BMI-matched male counterparts (Haugaard et al., 2009), which is in contrast to the findings of this study. Importantly, the previous findings were predominantly obtained within younger pre-menopausal female populations (Tarnopolsky et al., 2007; Haugaard et al., 2009; Link and Reue, 2017). To add context to these findings, younger pre-menopausal women have normal levels (fluctuations) of oestrogen and progesterone and appear to be better protected against metabolic diseases compared to men (Pérez-López et al., 2010). Conversely,

peri- and post- menopause, the production of female sex hormones diminishes leading to an increased risk of cardiometabolic syndrome, thus suggesting that the female sex hormones may act as a protective mechanism in pre-menopausal females (Polotsky and Polotsky, 2010). Oestrogen regulates various aspects of lipid metabolism; 17ß-ethinyl-estradial is the most potent oestrogen which exerts its physiological actions through oestrogen receptors; ERα and ERβ (Lizcano and Guzmán, 2014). 17ß-ethinyl-estradial has been shown to prevent TG accumulation through suppression of LPL activity, whilst also increasing the expression of HSL resulting in increased lipolysis and improved lipid oxidation via augmented phosphorylation of AMPK. Furthermore, in skeletal muscle 17ß-ethinyl-estradial improved glucose disposal via modulation of the expression and trafficking of GLUT4 thus improving glucose clearance and enhancing insulin sensitivity (Jelenik and Roden, 2013). Disturbances in these metabolic signals caused by decreases in oestrogen levels and oestrogen receptor expression observed in post-menopausal women may therefore lead to impaired lipid metabolism, in turn reducing IMTG turnover.

Males also experience a decline in sex hormones during older age which has been suggested to negatively impact lipid metabolism and insulin signalling. The diminished testosterone levels associated with ageing in males may contribute to reduced metabolic action resulting in potential IMTG accumulation, ultimately increasing the risk of developing metabolic syndrome (Errazuriz et al., 2015; Link and Reue, 2017). Normal testosterone levels have been shown to increase lipolysis whilst decreasing LPL activity thus limiting TG accumulation. Furthermore, there is evidence that testosterone could increase insulin sensitivity by decreasing the levels of pro-inflammatory cytokines (IL-6 and TNF-α) responsible for increased insulin resistance (Ottarsdottir et al., 2018). Previous research has also shown a positive correlation between genetic (e.g. OXPHOS gene expression) and functional (VO_{2max}) markers of mitochondrial function and testosterone levels (Pitteloud et al., 2005). This suggests that testosterone may play an important role in modulating molecular mechanisms such as mitochondrial function. Therefore, the accumulation of LDs shown in males in the current study could also be attributed to reduced mitochondrial function resulting from an age-related decline in testosterone. Overall, it is possible that the sex differences observed in the current study (in comparison to previous studies comparing sex) are, at least in part, due to the older age of the

participants investigated, and that the age-related decline in testosterone could be a potent stimulus for IMTG accumulation.

Research has previously proposed that a greater density (number) of small LDs may be metabolically advantageous compared to a smaller number of larger LDs, due to the greater LD surface area for lipases and protein binding, thereby improving the capacity for lipolysis and the release of FA to supply to the mitochondria (Suzuki et al., 2011). The smaller LDs observed within the females compared to the males could suggest a greater capacity for IMTG utilisation in the females. These results are in line with previous research in younger females who are purported to have a greater capacity for IMTG utilisation (Roepstorff et al., 2002). These findings have suggested that the greater IMTG concentrations within female skeletal muscle is often coupled with higher plasma FA availability and a greater expression of FAT/CD36 (Lundsgaard and Kiens, 2014). Furthermore, the 'coat' of female LDs has also proven favourable for increased protein accessibility, with females having greater PLIN2, 3 and 5 protein expression compared to BMI matched males (Peters et al., 2012). Of these, PLIN3 is recognised as an important protein involved in LD lipolysis and PLIN5 in the regulation of ATGL action and more recently its role in mediating LD and mitochondria interactions (Granneman et al., 2011; Wang et al., 2011; Covington et al., 2014). Together, it is proposed that the smaller LDs and increased PLIN expression within females is likely to increase association with lipases and ultimately mitochondria thereby increasing IMTG turnover (Lundsgaard and Kiens, 2014). The current results support these assertions, and despite females showing lower IMTG levels in the present study, the differences in LD morphology between sexes does suggest that the female LD pool may be more metabolically flexible.

Interestingly, the current results showed no difference in mitochondrial content between sexes. A question therefore emerges concerning a possible difference in the rate of IMTG turnover. Previous research suggests that sex does not influence mitochondrial morphometry ((area (%) and number (μm^2)) (Tarnopolsky et al., 2007), although females have shown enhanced mitochondrial quality in comparison to males (Cardinale et al., 2018). It is therefore proposed that enhanced fat oxidation often observed within females can, in part, be attributed to improved mitochondrial oxidative function as opposed to mitochondrial content alone. Nevertheless, the findings reporting sex differences in mitochondrial morphometry are primarily investigated within younger populations (Tarnopolsky et al., 2007; Cardinale et al.,

2018). Aging notoriously induces declines in mitochondrial content concomitant with a reduction in oxidative capacity (Petersen et al., 2003; Crane et al., 2010). Together with the whole-body loss of muscle mass (sarcopenia) which naturally occurs with aging, complications in lipid metabolism are presented (Crane et al., 2010; Chee et al., 2016). Therefore, it could be that the current findings imply that females indeed have greater expression of PLINs and lipases (HSL and ATGL) which would support a greater turnover of the IMTG pool. Furthermore, it could also be proposed that the females have greater LD-mitochondria interactions, however the present study cannot confirm this from the current data gathered therefore future work should investigate this.

4.4 *Differences in older lean, obese and T2D participants skeletal muscle lipid droplet profile*

To our knowledge this study is the first of its kind to investigate differences in LD profile in older lean and obese individuals, with/without type 2 diabetes. In this context, comparisons have previously been made between groups that differ in metabolic health, but these studies are often confounded by differences in physical activity levels. In the present study, all participants were sedentary due to their reduced mobility linked to the impending surgery. This provided a unique opportunity to investigate differences in the LD profile between groups with differing metabolic health but similar activity levels. Although HOMA-IR scores showed that all of our groups presented with a degree of insulin resistance and that the T2D participants were significantly more insulin resistant than the lean sedentary group (Table 3.2), minimal differences were observed between these groups and their LD profile (Fig 3.2).

Using confocal immunofluorescence microscopy, differences in LD profile were observed primarily in the type IIa fibres of the T2D patients, whom showed a greater LD content compared to the lean group and larger LDs compared to the obese group (Fig 3.2). These findings are in agreement with previous observations which show T2D individuals primarily store larger LDs in type II fibres (Daemen et al., 2018). Furthermore, the larger LDs observed in T2D patients are suggested to be located within the subsarcolemmal region of the type II muscle fibres (Li et al., 2019). The current findings also suggest an accumulation of larger LD in the central region may prove deleterious, specifically within the type IIa muscle fibres as this was seen in T2D patients only. Larger LD have a smaller surface area to volume ratio compared to smaller LDs, in turn IMTG turnover is reduced resulting in an accumulation of deleterious

lipid metabolites (Amati et al., 2011a). Furthermore, previous research proposes that larger LDs could also prove unfavourable due to reduce oxidative capacity by 'hijacking' synaptosomal-associated protein 23 (SNAP23) for LD fusion processes thus preventing GLUT-4 storage vesicles from fusing and docking at the plasma membrane (Boström et al., 2007).

The elevated IMTG content within the current T2D participants could also be a result of impaired expression of various proteins which assist in IMTG synthesis and breakdown (Morales, Bucarey and Espinosa, 2017b). FAT/CD36 appears to be involved in the regulation of FA oxidation within human skeletal muscle through its control/regulation of LCFA uptake into skeletal muscle (Holloway et al., 2007). However, in T2D individuals FAT/CD36 cycling between the plasma membrane and intracellular compartments is altered, due to a permeant relocation of CD36 to the plasma membrane FAT/CD36 cycling is increased thereby leading to an abnormal increase in the rate of LCFA transport into the skeletal muscle. Without altered LCFA oxidation to match the increased LCFA transport there is an inevitable excess accumulation of IMTG (Bonen et al., 2004; Aguer et al., 2011); this could be a possible explanation of IMTG accumulation shown within the current T2D participants. Interestingly Holloway et al. (2007) observed no differences in FAT/CD36 expression between lean and obese skeletal muscle, and suggested that the accumulation of IMTG content was a result of compromised lipid oxidation due to a decrease in mitochondrial content with significant decreases in in β-HAD, citrate synthase (CS) activity and COX-IV content. Furthermore, reduced PLIN5 protein expression has also been reported within T2D skeletal muscle when compared to athletes (Daemen et al., 2018; Daemen, van Polanen and Hesselink, 2018). PLIN5 plays a role in FA release for oxidative degradation in mitochondria (Bosma et al., 2012; Bosma et al., 2013), thereby acting as a protective mechanism which promotes the interaction of LDs with mitochondria in turn decreasing lipotoxicity (Gemmink et al., 2016). Additionally, PLIN5 is an important regulator of ATGL action which is decreased within T2D skeletal muscle (Daemen et al., 2018). ATGL is a key regulator of skeletal muscle lipolysis, and therefore reduced ATGL activity may play a role in decreased IMTG utilisation and ultimately lead to IMTG accumulation within T2D (Jocken et al., 2010; Fujimoto and Parton, 2011). Conversely, Jocken et al. (2010) found that ATGL along with HSL protein expression was increased in obese individuals, however paired with lower DAG hydrolase activity implied that lipolysis is incomplete. Therefore, it is possible that these alterations in protein expression and enzyme activity previously reported in T2D patients are

possible factors contributing to the increased LD content within the T2D participants in the current study.

Interestingly, no differences in mitochondrial density were observed between the lean, obese and T2D groups which could be due to similar levels of (in)activity between the participants whilst awaiting orthopaedic surgery. This is the first study to investigate these differences in mitochondrial density, LD content and morphology between groups with similar levels of inactivity, as previous research has primarily investigated these differences between physically active and sedentary groups (Tarnopolsky et al., 2007; Phielix et al., 2010; Cobley et al., 2012; Bilet et al., 2020). Consequently, the current study is the first to suggest that mitochondrial density is independent of metabolic health status, which contradicts previous findings. It has been suggested that augmented mitochondrial density leads to improved oxidative capacity thus allowing greater IMTG turnover, and this phenomenon has frequently been observed within physically active individuals (Karakelides et al., 2010). Additionally, skeletal muscle obtained from obese and T2D individuals characteristically has smaller, less dense mitochondria in the peripheral and central region of muscle fibres (Kelley et al., 2002). With no difference in mitochondrial density between groups, it could be that the quality and function of mitochondria plays a more extensive role in IMTG turnover rather than mitochondrial content alone. Furthermore, LD-mitochondrial interactions are also an important factor of IMTG turnover, with evidence of a more dispersed distribution of IMTG to mitochondria in sedentary individuals therefore IMTG turnover is often reduced (Haugaard et al., 2009). The athlete's paradox highlights the importance of physical activity and lipid metabolism showing a phenomenon whereby trained individuals show elevated IMTG content yet remain highly insulin sensitive, however their abundance of mitochondria suggests regular IMTG turnover thus the risk of lipid metabolite accumulation is reduced (Goodpaster et al., 2001; Amati et al., 2011a). Therefore, physical inactivity could be a primary determinant of IMTG accumulation predominately shown in older adults, partially driven by a 35-40% decline in skeletal muscle mitochondrial oxidative/phosphorylation activity (Petersen et al., 2003). Cobley et al. (2012) provide evidence that individuals following a lifelong endurance training regime are able to maintain mitochondrial content and skeletal muscle oxidative capacity. This could also lead to lifelong improvements in IMTG turnover and skeletal muscle insulin sensitivity in older adults. Furthermore, research investigating the effects of physical inactivity

reported a ~22% decrease in mitochondrial oxidative capacity and skeletal muscle fat oxidative capacity in participants when comparing an active leg to a suspended leg, which had a direct impact on IMTG accumulation (Bilet et al., 2020). Overweight individuals have proven to benefit from regular exercise with evidence of reduced LD size and greater mitochondrial content adjacent to LD, leading to possible improvements in LD-mitochondrial interaction and increased IMTG turnover (He, Goodpaster and Kelley, 2004).

4.5 *Differences in skeletal muscle lipid droplet profile between lean, obese and T2D males and females*

Two novel findings were produced from the current thesis when directly comparing the lipid droplet profile of lean, obese and T2D men and women across fibre-types and within specific regions of the muscle fibres. Firstly, the lean, obese and T2D males exhibited greater LD content within the central and/or peripheral regions of the type I, IIa and IIx fibres driven through larger LDs and/or greater LD density in comparison to their BMI matched female counterparts (Fig 3.3). These LD characteristics have previously proven deleterious as an accumulation of larger LDs in skeletal muscle has been positively correlated with impaired insulin signalling (Li et al., 2019). Furthermore, LD accumulation within the peripheral region of the skeletal muscle fibres could be a possible contributing factor in the development of skeletal muscle insulin resistance due to the close proximity of LDs to the site of the insulin signalling cascade (Gemmink et al., 2017). In contrast, previous research that examined sex differences within an impaired metabolic health group observe greater IMTG stores, by twofold, in obese women compared to BMI-matched obese males (Haugaard et al., 2009). However, Haugaard et al. (2009) observed these differences within a younger population and within muscle biopsies obtained from the vastus lateralis. It is possible that the variations in participant age and skeletal muscle samples are contributing factors in the differences observed between the current studies results and previous findings. Additionally, a sex difference has also been observed in IMTG content within sedentary participants, partially due to sex related differences in HSL activity in skeletal muscle (Roepstorff et al., 2006). It is thought that higher HSL expression in skeletal muscle leads to greater IMTG hydrolysis, this is further supported by previous results which indicate female skeletal muscle indeed has higher IMTG hydrolysis rates during exercise driven through improved IMTG and activated HSL interaction (Roepstorff et al., 2006). These findings alongside the current study could imply that the lower

LD content within the females is partially due to elevated HSL expression and greater IMTG hydrolysis. Additionally, a two-to-threefold greater LPL gene expression has previously been observed in sedentary women's skeletal muscle compared to sedentary men, thus demonstrating potential for augmented muscular lipoprotein activity in females (Kiens et al., 2004).

Interestingly Perreault et al. (2010) found that alterations in IMTG metabolism related to diminished insulin action more so in men than women, leading to the progression of T2D. It is important to note that all participants within the current study had a HOMA-IR score above 2.9 and were therefore classified as insulin resistant (Table 2.1). However, compared to their female counterparts, the lean and T2D males had significantly lower HOMA-IR scores, suggesting they were relatively less insulin resistant, yet they exhibited a greater LD content (Fig 3.3). This suggests that IMTG accumulation per se within older populations may not be related to insulin action. Rather, it is possible that sex and health differences in LD morphology and subcellular distribution of IMTG are more closely related to insulin action. Moreover, a question emerges regarding the absence of differences in mitochondrial density between the lean, obese and T2D males and females, alongside greater LD content within the male skeletal muscle. It could be that the differences observed between the males and females LD profile and level of insulin resistance could be potential evidence of a sex paradox in older individuals. Together, these results are in line with previous findings which suggest that reductions in oestrogen in the females could be of greater consequence for health than the decreased testosterone expression in the males (Nyberg et al., 2014; Egelund et al., 2017; Mandrup et al., 2017; Novella et al., 2019). Indeed, the current findings require further investigation to further understand the mechanisms behind this phenomenon whereby females have lower LD stores and greater HOMA-IR scores.

The second novel finding is the greater LD content within the skeletal muscle of T2D males, specifically within the central region of their type IIa fibres, compared to obese and lean males, all of whom were sedentary (Fig 3.3). The differences observed in the T2D males resulted from larger LDs. It is possible that these differences in LD storage shown within the T2D males induces greater insulin resistance within these participants as shown in their HOMA-IR scores which are significantly higher compared to the lean males (Table 2.1). As discussed previously, a larger LD proves unfavourable for effective lipid turnover and insulin action (see 4.3).

Furthermore, the lower mitochondrial density shown within the type II fibres of the T2D males suggests a dysregulation of LD turnover (synthesis and lipolysis) resulting in the accumulation of larger LD (Daemen et al., 2018). Previous research also shows fragmented mitochondria specifically in type II muscle fibres due to obesity-induced insulin resistance and reduced physical activity, resulting in the accumulation of IMTG (Kristensen et al., 2018). Physical inactivity is often associated with reduced mitochondrial oxidative capacity in skeletal muscle, it is suggested that this is a major contributor in IMTG accumulation and subsequently insulin resistance (Bilet et al., 2020). Furthermore, previous findings indicate improvements in insulin resistance following exercise and weight loss due to reduced LD size and an increase in oxidative enzyme activity (He, Goodpaster and Kelley, 2004). These findings further support the hypothesis that it is not simply IMTG content per se that determines insulin resistance, but rather the morphology and distribution of stored LDs (He, Goodpaster and Kelley, 2004).

Data from the present study suggests a similar relationship to the athlete's paradox whereby inactivity alongside older age and sex may be more important determinants of insulin sensitivity than IMTG content alone. It is believed that aging is a determinant of IMTG accumulation and the development of insulin resistance. As discussed previously (see 4.3), ageing affects various physiological functions, more specifically sarcopenia which is the result of gradual skeletal muscle mass loss (Crane et al., 2010; Lang et al., 2010). Sarcopenia may induce severe metabolic dysfunction leading to increased insulin resistance and ultimately T2D (Gueugneau et al., 2015; Gueugneau et al., 2018). Additionally, reduced mitochondrial content is apparent within aged participants thus decreasing oxidative capacity. However the rate of decline in mitochondrial content is accelerated following a sedentary lifestyle (van Loon et al., 2004; Peterson, Johannsen and Ravussin, 2012). These findings are in agreement with the present study which showed an augmented LD content within the central region of the type IIa muscle fibres of the T2D males possibly due to the combination of older age and physical inactivity, both major determinants in the accumulation of LDs. These findings are supported by previous literature, with further examination of the differences in IMTG content and distribution between skeletal muscle of older and young skeletal volunteers. Chee et al. (2016) observed no differences when comparing IMTG content in young lean and older lean participants; both presented similar IMTG content located within the subsarcolemmal region.

However, when these findings were compared to older, overweight participants IMTG content within the subsarcolemmal region was twofold higher.

4.6 *Strengths & Limitations*

A key strength of the current study is the use of validated immunofluorescent microscopy techniques to examine the fibre type and region-specific differences in LD profile between sex and metabolic health status. Consequently, these techniques have provided an insight into region specific differences in LD profile in different muscle fibres, further advancing current knowledge of IMTG stores. Furthermore, the older population investigated has proven invaluable by producing novel findings within the current thesis which challenges previous observations surrounding sex differences in LD morphology and distribution. As discussed previously, the similar inactivity levels of the participants is a novel strength of the current study as previous research examined differences between physically active and sedentary individuals. The current study also addresses limitations and oversights within the literature surrounding participant sex, and age. The novel observations from this thesis suggest that age and the influence of sex-hormones might play a key role in differences in LD morphology and distribution frequently reported between sexes and metabolic health status.

Furthermore, the vast majority of research examining differences in IMTG content and lipid droplet profile in human skeletal muscle has analysed biopsies obtained from the vastus lateralis muscle. However, samples examined in the current study were obtained during elective hip arthroplasty surgery, consequently samples were obtained from the gluteus maximus muscle which has provided novel findings. The current study also controlled the number of fibres analysed ensuring equal analysis of fibre types in both the male and female samples. Finally, previous studies that have analysed fibre-type differences have exclusively examined the differences between the fibres of men and women (collectively) and not specifically the sex differences between fibre-type IMTG content; this is the first study to examine sex specific fibre-type differences in LD content.

Overall, the study presented few limitations, however the limitations that have emerged must be addressed. Firstly, it was not possible to investigate fibre type specific LD-mitochondria interactions due to limited channels available when using the confocal microscope. However

with strong evidence suggesting a positive correlation between both IMTG accumulation-mitochondrial intensity and IMTG accumulation-mitochondrial function (Moro, Bajpeyi and Smith, 2008), future protocols should aim to co-stain bodipy and COX to examine the spatial LD-mitochondrial interactions within these groups. Furthermore, the use of COXIV as a marker of mitochondrial density presents a limitation in itself. COXIV only labels one specific protein within the mitochondria (cytochrome c oxidase) therefore it is a representation of mitochondrial content and could underrepresent true mitochondrial density within samples. The present study is also potentially limited by not examining deleterious metabolites such as DAG and ceramides which have proven to influence insulin action (Amati et al., 2011a). It is possible that these metabolites could provide further insight into the mechanisms involved in the association of IMTG stores and level of insulin resistance. It is important to recognise that the peripheral region defined within this study cannot be directly compared with the subsarcolemmal region identified in previous studies using transmission electron microscopy. It has been recommended that future research using BOPIDY to detect IMTG should employ a smaller peripheral region of 2μm to be more representative of the S.S region (Jevons et al., 2020). Finally, the limited participant group in this study (older adults) could be extended to include younger and/ or age matched controls who are insulin sensitive and have lower HOMA-IR scores. These control groups would allow a more inclusive representation of the differences between LD profile, mitochondrial density and whether these characteristics effect insulin action.

4.7 *Future directions for research*

The current thesis is the first to provide an investigation of LD profile within both males and females with different metabolic health statuses. Furthermore, the region-specific and fibre-type differences provide a novel insight into the effects of LD morphology and distribution on lipid metabolism and insulin resistance. However, research is still limited surrounding IMTG stores in females, specifically within older metabolically impaired females. Therefore, more research is required to further develop current understanding of the relationship between IMTG and insulin action within these participants.

Indeed the majority of current research investigating the relationship between IMTG content and insulin action has been conducted in cohorts of young males (Shepherd et al., 2017;

Daemen, van Polanen and Hesselink, 2018). The limited studies that have investigated both males and females do show sex differences in insulin sensitivity and IMTG content (Tarnopolsky et al., 2007). However, the current study shows contradictory findings on sex differences in LD profile, likely due to the older age of the participants. Therefore, it would be of interest to investigate LD morphology and distribution, and mitochondrial density through similar immunofluorescent microscopy techniques within similar groups of both young and older participants; 1) lean sedentary 2) obese sedentary 3) obese T2D 4) lean active individuals, and 5) highly trained athletes. This would provide a systematic evaluation of sex and health differences in IMTG content and insulin sensitivity. Furthermore, the use of immunofluorescence microscopy methods within these groups may highlight important differences in LD profile which have previously been overlooked in studies that use whole muscle homogenate-based analysis techniques. The subcellular distribution and morphology of LD are seen as strong predictors of insulin resistance in human skeletal muscle (Bergman et al., 2012), therefore such data would prove invaluable in the understanding of LD influence on insulin action.

Future research should also aim to investigate LD-mitochondria interactions within the groups suggested previously; 1) lean sedentary 2) obese sedentary 3) obese T2D 4) lean active individuals, and 5) highly trained athletes. It is proposed that these interactions are essential in the mechanism that permits a greater rate of IMTG oxidation frequently observed in trained athletes resulting in reduced FA levels within their muscle. It is believed that these interactions are one of the important metabolic adaptations in trained individuals who have high IMTG content yet remain highly insulin sensitive (Goodpaster et al., 2001; van Loon et al., 2004). Although mitochondrial density did not differ between males and females nor health groups within the current study, assessing the LD-mitochondria interactions could have provided an insight into the mechanisms underpinning the LD accumulation in the males and their lower HOMA-IR scores compared to the females.

It is also increasingly believed that DAG and ceramides are the lipotoxic metabolites involved in the underlying relationship between IMTG content and insulin resistance (Amati et al., 2011a; Gemmink et al., 2017), thus supporting the athlete's paradox and the origin of skeletal muscle insulin resistance. However, research investigating these deleterious metabolites in human skeletal muscle is indeed limited and proven inconsistent (Straczkowski et al., 2007;

Moro et al., 2009; Amati et al., 2011a; Gemmink et al., 2017). The majority of research that does support the hypothesis that DAG and ceramides are associated with insulin resistance in obese, ageing and T2D individuals is predominantly from rodent models (Chavez et al., 2003; Erion and Shulman, 2010; Chavez and Summers, 2012). Therefore, future research needs to focus on the link between these deleterious lipid species and skeletal muscle insulin resistance within various health groups and both sexes remains to be examined.

Future research should also focus on proteins that reportedly regulate lipid metabolism in human skeletal muscle, through examining the direct effect of PLIN expression and LD content on insulin action. PLIN5 is expressed in metabolically active tissues (e.g. skeletal muscle), where it is suggested to be involved in the regulation of lipolysis and therefore directly affects LD size (Gemmink et al., 2016). Research that has previously used rodent models suggested that an increased PLIN5 expression is linked to an increase in LD content whilst insulin-mediated glucose uptake was not impaired (Bosma et al., 2013). Furthermore, complete removal of PLIN5 expression in mice resulted in impaired insulin-mediated glucose uptake (Mason et al., 2014). Recent research suggests potential involvement of PLIN5 in HSL recruitment to LDs, thus potentially augmenting IMTG utilisation (Whytock, 2019). It is also believed that PLIN2 is involved in the retention of TAG within LD and the prevention of impaired insulin sensitivity induced through high-fat-diets, furthermore PLIN3 expression has also been associated with fat oxidation. In human skeletal muscle it had been observed that both PLIN2 and PLIN3 prove favourable in the reduction of lipid metabolite accumulation (Whytock, 2019; Whytock et al., 2020). Therefore, it would be of interest to investigate the expression of these proteins within various populations (e.g. male vs female and various metabolic health status') to examine differences in expression and establish a stronger link between lipid metabolism and insulin sensitivity.

4.8 *Conclusion*

This thesis has produced novel data on sex and health specific differences in LD morphology and distribution. In contrast to our hypothesis and previous literature which reports greater LD stores in females, the study demonstrates that males have more LD than females. However, the participants in this study were older and the females were postmenopausal; therefore our results may in part be explained by the reduced oestrogen concentrations that occur at this

time. The increased LD content specifically in type IIa fibres of T2D may suggest a dysregulation of LD turnover (synthesis and lipolysis) which is supported by the lower mitochondria content in these fibres. The current study provides an initial insight into sex differences in LD distribution in older populations and provides a foundation for future research examining possible factors which contribute to augmented LD stores and changes in insulin sensitivity. Ultimately, the methods and results from the current thesis will assist future research hypotheses and investigation on human skeletal muscle lipid metabolism.

5. References

Abdullah, A., Peeters, A., de Courten, M. and Stoelwinder, J. (2010) The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies. *Diabetes Res Clin Pract*, 89 (3), 309-319.

Aguer, C., Foretz, M., Lantier, L., Hebrard, S., Viollet, B., Mercier, J. and Kitzmann, M. (2011) Increased FAT/CD36 cycling and lipid accumulation in myotubes derived from obese type 2 diabetic patients. *PLoS One*, 6 (12).

Amati, F., Dubé, J.J., Alvarez-Carnero, E., Edreira, M.M., Chomentowski, P., Coen, P.M., Switzer, G.E., Bickel, P.E., Stefanovic-Racic, M. and Toledo, F.G. (2011a) Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes*, 60 (10), 2588-2597.

Amati, F., Dube, J.J., Alvarez-Carnero, E., Edreira, M.M., Chomentowski, P., Coen, P.M., Switzer, G.E., Bickel, P.E., Stefanovic-Racic, M., Toledo, F.G. and Goodpaster, B.H. (2011b) Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes*, 60 (10), 2588-2597.

Bass, J.J., Wilkinson, D.J., Rankin, D., Phillips, B.E., Szewczyk, N.J., Smith, K. and Atherton, P.J. (2017) An overview of technical considerations for Western blotting applications to physiological research. *Scand J Med Sci Sports*, 27 (1), 4-25.

Basu, A., Dube, S. and Basu, R. (2017) Men Are from Mars, Women Are from Venus: Sex Differences in Insulin Action and Secretion. *Adv Exp Med Biol*, 1043, 53-64.

Bergman, B.C., Hunerdosse, D.M., Kerege, A., Playdon, M.C. and Perreault, L. (2012) Localisation and composition of skeletal muscle diacylglycerol predicts insulin resistance in humans. *Diabetologia*, 55 (4), 1140-1150.

Bergman, B.C., Perreault, L., Hunerdosse, D.M., Koehler, M.C., Samek, A.M. and Eckel, R.H. (2010) Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. *Journal of Applied Physiology*, 108 (5), 1134-1141.

Bersuker, K. and Olzmann, J.A. (2017) Establishing the lipid droplet proteome: Mechanisms of lipid droplet protein targeting and degradation. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1862 (10), 1166-1177.

Bertrand, L., Horman, S., Beauloye, C. and Vanoverschelde, J.L. (2008) Insulin signalling in the heart. *Cardiovasc Res*, 79 (2), 238-248.

Bezaire, V., Bruce, C.R., Heigenhauser, G.J., Tandon, N.N., Glatz, J.F., Luiken, J.J., Bonen, A. and Spriet, L.L. (2006) Identification of fatty acid translocase on human skeletal muscle mitochondrial

membranes: essential role in fatty acid oxidation. *American Journal of Physiology-Endocrinology and Metabolism*, 290 (3), E509-E515.

Bilet, L., Phielix, E., van de Weijer, T., Gemmink, A., Bosma, M., Moonen-Kornips, E., Jorgensen, J.A., Schaart, G., Zhang, D. and Meijer, K. (2020) One-leg inactivity induces a reduction in mitochondrial oxidative capacity, intramyocellular lipid accumulation and reduced insulin signalling upon lipid infusion: a human study with unilateral limb suspension. *Diabetologia*, 1-12.

Billecke, N., Bosma, M., Rock, W., Fleissner, F., Best, G., Schrauwen, P., Kersten, S., Bonn, M., Hesselink, M.K. and Parekh, S.H. (2015) Perilipin 5 mediated lipid droplet remodelling revealed by coherent Raman imaging. *Integr Biol (Camb)*, 7 (4), 467-476.

Bird, S.R. and Hawley, J.A. (2016) Update on the effects of physical activity on insulin sensitivity in humans. *BMJ Open Sport Exerc Med*, 2 (1), e000143.

Blair, S.N. (2009) Physical inactivity: the biggest public health problem of the 21st century. *Br J Sports Med*, 43 (1), 1-2.

Bonen, A., Dohm, G.L. and van Loon, L.J. (2006) Lipid metabolism, exercise and insulin action. *Essays Biochem*, 42, 47-59.

Bonen, A., Parolin, M.L., Steinberg, G.R., Calles-Escandon, J., Tandon, N.N., Glatz, J.F., Luiken, J.J., Heigenhauser, G.J. and Dyck, D.J. (2004) Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. *The FASEB journal*, 18 (10), 1144-1146.

Bosma, M. (2016) Lipid droplet dynamics in skeletal muscle. *Exp Cell Res*, 340 (2), 180-186.

Bosma, M., Kersten, S., Hesselink, M.K. and Schrauwen, P. (2012) Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. *Progress in lipid research*, 51 (1), 36-49.

Bosma, M., Sparks, L., Hooiveld, G., Jorgensen, J., Houten, S., Schrauwen, P., Kersten, S. and Hesselink, M. (2013) Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1831 (4), 844-852.

Boström, P., Andersson, L., Rutberg, M., Perman, J., Lidberg, U., Johansson, B.R., Fernandez-Rodriguez, J., Ericson, J., Nilsson, T. and Borén, J. (2007) SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nature cell biology*, 9 (11), 1286-1293.

Bradley, H., Shaw, C.S., Bendtsen, C., Worthington, P.L., Wilson, O.J., Strauss, J.A., Wallis, G.A., Turner, A.M. and Wagenmakers, A.J. (2015) Visualization and quantitation of GLUT4 translocation in human skeletal muscle following glucose ingestion and exercise. *Physiol Rep*, 3 (5).

Bradley, N.S., Snook, L.A., Jain, S.S., Heigenhauser, G.J., Bonen, A. and Spriet, L.L. (2012) Acute endurance exercise increases plasma membrane fatty acid transport proteins in rat and human skeletal muscle. *Am J Physiol Endocrinol Metab*, 302 (2), E183-189.

Brewer, P.D., Habtemichael, E.N., Romenskaia, I., Mastick, C.C. and Coster, A.C. (2014) Insulin-regulated Glut4 translocation: membrane protein trafficking with six distinctive steps. *J Biol Chem*, 289 (25), 17280-17298.

Brook, M.S., Wilkinson, D.J., Phillips, B.E., Perez-Schindler, J., Philp, A., Smith, K. and Atherton, P.J. (2016) Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. *Acta Physiol (Oxf)*, 216 (1), 15-41.

Buettner, R., Schölmerich, J. and Bollheimer, L.C. (2007) High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity*, 15 (4), 798-808.

Cardinale, D.A., Larsen, F.J., Schiffer, T.A., Morales-Alamo, D., Ekblom, B., Calbet, J.A., Holmberg, H.-C. and Boushel, R. (2018) Superior intrinsic mitochondrial respiration in women than in men. *Frontiers in physiology*, 9, 1133.

Chadt, A., Scherneck, S., Joost, H.-G. and Al-Hasani, H. (2018) Molecular links between obesity and diabetes:"diabesity". In: (ed.) *Endotext [Internet].* MDText. com, Inc.

Chavez, J.A., Knotts, T.A., Wang, L.-P., Li, G., Dobrowsky, R.T., Florant, G.L. and Summers, S.A. (2003) A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *Journal of Biological Chemistry*, 278 (12), 10297-10303.

Chavez, J.A. and Summers, S.A. (2012) A ceramide-centric view of insulin resistance. *Cell metabolism*, 15 (5), 585-594.

Chee, C., Shannon, C.E., Burns, A., Selby, A.L., Wilkinson, D., Smith, K., Greenhaff, P.L. and Stephens, F.B. (2016) Relative Contribution of Intramyocellular Lipid to Whole-Body Fat Oxidation Is Reduced With Age but Subsarcolemmal Lipid Accumulation and Insulin Resistance Are Only Associated With Overweight Individuals. *Diabetes*, 65 (4), 840-850.

Cheneviere, X., Borrani, F., Sangsue, D., Gojanovic, B. and Malatesta, D. (2011) Gender differences in whole-body fat oxidation kinetics during exercise. *Applied Physiology, Nutrition, and Metabolism*, 36 (1), 88-95.

Clausen, J.O., Borch-Johnsen, K., Ibsen, H., Bergman, R.N., Hougaard, P., Winther, K. and Pedersen, O. (1996) Insulin sensitivity index, acute insulin response, and glucose effectiveness in a population-based sample of 380 young healthy Caucasians. Analysis of the impact of gender, body fat, physical fitness, and life-style factors. *J Clin Invest*, 98 (5), 1195-1209.

Clerk, L.H., Vincent, M.A., Jahn, L.A., Liu, Z., Lindner, J.R. and Barrett, E.J. (2006) Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes*, 55 (5), 1436-1442.

Cobb, L.J., Lee, C., Xiao, J., Yen, K., Wong, R.G., Nakamura, H.K., Mehta, H.H., Gao, Q., Ashur, C., Huffman, D.M., Wan, J., Muzumdar, R., Barzilai, N. and Cohen, P. (2016) Naturally occurring mitochondrial-derived peptides are age-dependent regulators of apoptosis, insulin sensitivity, and inflammatory markers. *Aging (Albany NY)*, 8 (4), 796-809.

Cobley, J.N., Bartlett, J., Kayani, A., Murray, S., Louhelainen, J., Donovan, T., Waldron, S., Gregson, W., Burniston, J.G. and Morton, J.P. (2012) PGC-1α transcriptional response and mitochondrial adaptation to acute exercise is maintained in skeletal muscle of sedentary elderly males. *Biogerontology*, 13 (6), 621-631.

Coen, P.M., Dube, J.J., Amati, F., Stefanovic-Racic, M., Ferrell, R.E., Toledo, F.G. and Goodpaster, B.H. (2010) Insulin resistance is associated with higher intramyocellular triglycerides in type I but not type II myocytes concomitant with higher ceramide content. *Diabetes*, 59 (1), 80-88.

Coen, P.M. and Goodpaster, B.H. (2012) Role of intramyocelluar lipids in human health. *Trends Endocrinol Metab*, 23 (8), 391-398.

Covington, J.D., Galgani, J.E., Moro, C., LaGrange, J.M., Zhang, Z., Rustan, A.C., Ravussin, E. and Bajpeyi, S. (2014) Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and are associated with fat oxidation. *PLoS One*, 9 (3).

Crane, J.D., Devries, M.C., Safdar, A., Hamadeh, M.J. and Tarnopolsky, M.A. (2010) The effect of aging on human skeletal muscle mitochondrial and intramyocellular lipid ultrastructure. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, 65 (2), 119-128.

Daemen, S., Gemmink, A., Brouwers, B., Meex, R.C.R., Huntjens, P.R., Schaart, G., Moonen-Kornips, E., Jorgensen, J., Hoeks, J., Schrauwen, P. and Hesselink, M.K.C. (2018) Distinct lipid droplet characteristics and distribution unmask the apparent contradiction of the athlete's paradox. *Mol Metab*, 17, 71-81.

Daemen, S., van Polanen, N. and Hesselink, M.K.C. (2018) The effect of diet and exercise on lipid droplet dynamics in human muscle tissue. *J Exp Biol*, 221 (Pt Suppl 1).

Dandona, P., Aljada, A. and Bandyopadhyay, A. (2004) Inflammation: the link between insulin resistance, obesity and diabetes. *Trends in immunology*, 25 (1), 4-7.

DeFronzo, R.A., Ferrannini, E., Sato, Y., Felig, P. and Wahren, J. (1981) Synergistic interaction between exercise and insulin on peripheral glucose uptake. *J Clin Invest*, 68 (6), 1468-1474.

DeFronzo, R.A. and Tripathy, D. (2009) Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care*, 32 Suppl 2, S157-163.

Devries, M.C., Hamadeh, M.J., Phillips, S.M. and Tarnopolsky, M.A. (2006) Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 291 (4), R1120-R1128.

Devries, M.C., Lowther, S.A., Glover, A.W., Hamadeh, M.J. and Tarnopolsky, M.A. (2007) IMCL area density, but not IMCL utilization, is higher in women during moderate-intensity endurance exercise, compared with men. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 293 (6), R2336-R2342.

Di Meo, S., Iossa, S. and Venditti, P. (2017) Skeletal muscle insulin resistance: role of mitochondria and other ROS sources. *J Endocrinol*, 233 (1), R15-r42.

Diabetes, A. (2015) (2) Classification and diagnosis of diabetes. . *Diabetes Care*, S8-S16.

Diabetes UK. (2019) *Us, diabetes and a lot of facts and stats* [online]

Available at: https://www.diabetes.org.uk/resources-s3/2019-02/1362B_Facts%20and%20stats%20Update%20Jan%202019_LOW%20RES_EXTERNAL.pdf

Egelund, J., Jørgensen, P.G., Mandrup, C.M., Fritz-Hansen, T., Stallknecht, B., Bangsbo, J., Nyberg, M. and Hellsten, Y. (2017) Cardiac adaptations to high-intensity aerobic training in premenopausal and recent postmenopausal women: the copenhagen women study. *Journal of the American Heart Association*, 6 (8), e005469.

Erion, D.M. and Shulman, G.I. (2010) Diacylglycerol-mediated insulin resistance. *Nature medicine*, 16 (4), 400-402.

Errazuriz, I., Dube, S., Basu, A. and Basu, R. (2015) Effects of testosterone replacement on glucose and lipid metabolism. *Cardiovascular Endocrinology & Metabolism*, 4 (3), 95-99.

Ferroni, P., Basili, S., Falco, A. and Davì, G. (2004) Inflammation, insulin resistance, and obesity. *Current atherosclerosis reports*, 6 (6), 424-431.

Festa, A., D'Agostino Jr, R., Howard, G., Mykkänen, L., Tracy, R.P. and Haffner, S.M. (2000) Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation*, 102 (1), 42-47.

Flück, M. (2006) Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. *Journal of Experimental Biology*, 209 (12), 2239-2248.

Frayn, K.N. (2002a) Adipose tissue as a buffer for daily lipid flux. *Diabetologia*, 45 (9), 1201-1210.

Frayn, K.N. (2002b) Insulin resistance, impaired postprandial lipid metabolism and abdominal obesity. A deadly triad. *Med Princ Pract*, 11 Suppl 2, 31-40.

Frayn, K.N., Arner, P. and Yki-Jarvinen, H. (2006) Fatty acid metabolism in adipose tissue, muscle and liver in health and disease. *Essays Biochem*, 42, 89-103.

Fu, M.H., Maher, A.C., Hamadeh, M.J., Ye, C. and Tarnopolsky, M.A. (2009) Exercise, sex, menstrual cycle phase, and 17beta-estradiol influence metabolism-related genes in human skeletal muscle. *Physiol Genomics*, 40 (1), 34-47.

Fujimoto, T. and Parton, R.G. (2011) Not just fat: the structure and function of the lipid droplet. *Cold Spring Harbor perspectives in biology*, 3 (3), a004838.

Gajda, A.M., Pellizzon, M.A., Ricci, M.R. and Ulman, E.A. Diet-induced metabolic syndrome in rodent models.

Geer, E.B. and Shen, W. (2009) Gender differences in insulin resistance, body composition, and energy balance. *Gender medicine*, 6, 60-75.

Gemmink, A., Bosma, M., Kuijpers, H.J., Hoeks, J., Schaart, G., van Zandvoort, M.A., Schrauwen, P. and Hesselink, M.K. (2016) Decoration of intramyocellular lipid droplets with PLIN5 modulates fasting-induced insulin resistance and lipotoxicity in humans. *Diabetologia*, 59 (5), 1040-1048.

Gemmink, A., Daemen, S., Brouwers, B., Huntjens, P.R., Schaart, G., Moonen-Kornips, E., Jörgensen, J., Hoeks, J., Schrauwen, P. and Hesselink, M.K. (2018) Dissociation of intramyocellular lipid storage and insulin resistance in trained athletes and type 2 diabetes patients; involvement of perilipin 5? *The Journal of physiology*, 596 (5), 857-868.

Gemmink, A., Goodpaster, B.H., Schrauwen, P. and Hesselink, M.K. (2017) Intramyocellular lipid droplets and insulin sensitivity, the human perspective. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1862 (10), 1242-1249.

Goodpaster, B.H., He, J., Watkins, S. and Kelley, D.E. (2001) Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab*, 86 (12), 5755-5761.

Goodpaster, B.H., Thaete, F.L., Simoneau, J.A. and Kelley, D.E. (1997) Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat. *Diabetes*, 46 (10), 1579-1585.

Goodpaster, B.H., Theriault, R., Watkins, S.C. and Kelley, D.E. (2000) Intramuscular lipid content is increased in obesity and decreased by weight loss. *Metabolism*, 49 (4), 467-472.

Granneman, J.G., Moore, H.-P.H., Mottillo, E.P., Zhu, Z. and Zhou, L. (2011) Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *Journal of Biological Chemistry*, 286 (7), 5126-5135.

Greenberg, A.S., Coleman, R.A., Kraemer, F.B., McManaman, J.L., Obin, M.S., Puri, V., Yan, Q.-W., Miyoshi, H. and Mashek, D.G. (2011) The role of lipid droplets in metabolic disease in rodents and humans. *The Journal of clinical investigation*, 121 (6), 2102-2110.

Groban, L., Lindsey, S.H., Wang, H. and Alencar, A.K. (2016) Sex and gender differences in cardiovascular disease. In: (ed.) *Sex Differences in Physiology.* Elsevier. pp. 61-87.

Grundy, S.M., Brewer Jr, H.B., Cleeman, J.I., Smith Jr, S.C. and Lenfant, C. (2004) Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*, 109 (3), 433-438.

Gueugneau, M., Coudy-Gandilhon, C., Roche, F., Barthélémy, J.-C., Féasson, L. and Béchet, D. (2018) Cellular and functional mechanisms underlying muscle aging and associated diseasesof Conference.

Gueugneau, M., Coudy-Gandilhon, C., Théron, L., Meunier, B., Barboiron, C., Combaret, L., Taillandier, D., Polge, C., Attaix, D. and Picard, B. (2015) Skeletal muscle lipid content and oxidative activity in relation to muscle fiber type in aging and metabolic syndrome. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, 70 (5), 566-576.

Guo, Z., Burguera, B. and Jensen, M.D. (2000) Kinetics of intramuscular triglyceride fatty acids in exercising humans. *J Appl Physiol (1985)*, 89 (5), 2057-2064.

Haugaard, S.B., Mu, H., Vaag, A. and Madsbad, S. (2009) Intramyocellular triglyceride content in man, influence of sex, obesity and glycaemic control. *European journal of endocrinology*, 161 (1), 57-64.

Hayward, R.A., Reaven, P.D., Emanuele, N.V. and Investigators, V. (2015) Follow-up of Glycemic Control and Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med*, 373 (10), 978.

He, J., Goodpaster, B.H. and Kelley, D.E. (2004) Effects of weight loss and physical activity on muscle lipid content and droplet size. *Obes Res*, 12 (5), 761-769.

Holloway, G.P., Thrush, A.B., Heigenhauser, G.J., Tandon, N.N., Dyck, D.J., Bonen, A. and Spriet, L.L. (2007) Skeletal muscle mitochondrial FAT/CD36 content and palmitate oxidation are not decreased in obese women. *American Journal of Physiology-Endocrinology and Metabolism*, 292 (6), E1782-E1789.

Hoppeler, H. (1999) Skeletal muscle substrate metabolism. *Int J Obes Relat Metab Disord*, 23 Suppl 3, S7-10.

Hoppeler, H. (2016) Molecular networks in skeletal muscle plasticity. *J Exp Biol*, 219 (Pt 2), 205-213.

Hotamisligil, G.S., Shargill, N.S. and Spiegelman, B.M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*, 259 (5091), 87-91.

Itani, S.I., Ruderman, N.B., Schmieder, F. and Boden, G. (2002) Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes*, 51 (7), 2005-2011.

Janjic, D. (1996) [Android-type obesity and gynecoid-type obesity]. *Praxis (Bern 1994)*, 85 (49), 1578-1583.

Jelenik, T. and Roden, M. (2013) *How estrogens prevent from lipid-induced insulin resistance*. Oxford University Press.

Jensen, T.E. and Richter, E.A. (2012) Regulation of glucose and glycogen metabolism during and after exercise. *J Physiol*, 590 (5), 1069-1076.

Jevons, E.F.P., Gejl, K.D., Strauss, J.A., Ortenblad, N. and Shepherd, S.O. (2020) Skeletal muscle lipid droplets are resynthesized before being coated with perilipin proteins following prolonged exercise in elite male triathletes. *Am J Physiol Endocrinol Metab*, 318 (3), E357-e370.

Jocken, J.W., Moro, C., Goossens, G.H., Hansen, D., Mairal, A., Hesselink, M.K., Langin, D., van Loon, L.J. and Blaak, E.E. (2010) Skeletal muscle lipase content and activity in obesity and type 2 diabetes. *J Clin Endocrinol Metab*, 95 (12), 5449-5453.

Kahn, S.E., Hull, R.L. and Utzschneider, K.M. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*, 444 (7121), 840-846.

Kanaley, J.A., Shadid, S., Sheehan, M.T., Guo, Z. and Jensen, M.D. (2009) Relationship between plasma free fatty acid, intramyocellular triglycerides and long-chain acylcarnitines in resting humans. *The Journal of physiology*, 587 (24), 5939-5950.

Kanzaki, M. (2006) Insulin receptor signals regulating GLUT4 translocation and actin dynamics. *Endocr J*, 53 (3), 267-293.

Karakelides, H., Irving, B.A., Short, K.R., O'Brien, P. and Nair, K.S. (2010) Age, obesity, and sex effects on insulin sensitivity and skeletal muscle mitochondrial function. *Diabetes*, 59 (1), 89-97.

Kasuga, M. (2006) Insulin resistance and pancreatic beta cell failure. *J Clin Invest*, 116 (7), 1756-1760.

Kelley, D.E., He, J., Menshikova, E.V. and Ritov, V.B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes*, 51 (10), 2944-2950.

Kern, P.A., Saghizadeh, M., Ong, J.M., Bosch, R.J., Deem, R. and Simsolo, R.B. (1995) The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *The Journal of clinical investigation*, 95 (5), 2111-2119.

Keske, M.A., Clerk, L.H., Price, W.J., Jahn, L.A. and Barrett, E.J. (2009) Obesity blunts microvascular recruitment in human forearm muscle after a mixed meal. *Diabetes Care*, 32 (9), 1672-1677.

Kiens, B., Roepstorff, C., Glatz, J.F., Bonen, A., Schjerling, P., Knudsen, J. and Nielsen, J.N. (2004) Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *Journal of Applied Physiology*, 97 (4), 1209-1218.

Kim, J.-a., Wei, Y. and Sowers, J.R. (2008) Role of mitochondrial dysfunction in insulin resistance. *Circulation research*, 102 (4), 401-414.

Kim, J.Y., Hickner, R.C., Cortright, R.L., Dohm, G.L. and Houmard, J.A. (2000) Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab*, 279 (5), E1039-1044.

Kim, Y.B., Kotani, K., Ciaraldi, T.P., Henry, R.R. and Kahn, B.B. (2003) Insulin-stimulated protein kinase C lambda/zeta activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. *Diabetes*, 52 (8), 1935-1942.

Kitessa, S.M. and Abeywardena, M.Y. (2016) Lipid-induced insulin resistance in skeletal muscle: the chase for the culprit goes from total intramuscular fat to lipid intermediates, and finally to species of lipid intermediates. *Nutrients*, 8 (8), 466.

Knudsen, L., Hansen, B.F., Jensen, P., Pedersen, T.A., Vestergaard, K., Schaffer, L., Blagoev, B., Oleksiewicz, M.B., Kiselyov, V.V. and De Meyts, P. (2012) Agonism and antagonism at the insulin receptor. *PLoS One*, 7 (12), e51972.

Ko, K., Woo, J., Bae, J.Y., Roh, H.T., Lee, Y.H. and Shin, K.O. (2018) Exercise training improves intramuscular triglyceride lipolysis sensitivity in high-fat diet induced obese mice. *Lipids in Health and Disease*, 17 (1), 81.

Koh, H.C.E., Nielsen, J., Saltin, B., Holmberg, H.C. and Ørtenblad, N. (2017) Pronounced limb and fibre type differences in subcellular lipid droplet content and distribution in elite skiers before and after exhaustive exercise. *The Journal of physiology*, 595 (17), 5781-5795.

Kristensen, M., Petersen, S., Møller, K., Lund, M., Hansen, M., Hansen, C., Courraud, J., Helge, J., Dela, F. and Prats, C. (2018) Obesity leads to impairments in the morphology and organization of human skeletal muscle lipid droplets and mitochondrial networks, which are resolved with gastric bypass surgery-induced improvements in insulin sensitivity. *Acta Physiologica*, 224 (4), e13100.

Krssak, M., Falk Petersen, K., Dresner, A., DiPietro, L., Vogel, S.M., Rothman, D.L., Roden, M. and Shulman, G.I. (1999) Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia*, 42 (1), 113-116.

Lang, T., Streeper, T., Cawthon, P., Baldwin, K., Taaffe, D.R. and Harris, T. (2010) Sarcopenia: etiology, clinical consequences, intervention, and assessment. *Osteoporosis international*, 21 (4), 543-559.

Lanza, I.R. and Nair, K.S. (2008) Muscle mitochondrial changes with aging and exercise. *The American Journal of Clinical Nutrition*, 89 (1), 467S-471S.

Lauritzen, H.P., Ploug, T., Prats, C., Tavare, J.M. and Galbo, H. (2006) Imaging of insulin signaling in skeletal muscle of living mice shows major role of T-tubules. *Diabetes*, 55 (5), 1300-1306.

Lee, I.M., Shiroma, E.J., Lobelo, F., Puska, P., Blair, S.N., Katzmarzyk, P.T. and Lancet Physical Activity Series Working, G. (2012) Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. *Lancet*, 380 (9838), 219-229.

Lee, J.S., Pinnamaneni, S.K., Eo, S.J., Cho, I.H., Pyo, J.H., Kim, C.K., Sinclair, A.J., Febbraio, M.A. and Watt, M.J. (2006) Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. *J Appl Physiol (1985)*, 100 (5), 1467-1474.

Li, X., Li, Z., Zhao, M., Nie, Y., Liu, P., Zhu, Y. and Zhang, X. (2019) Skeletal Muscle Lipid Droplets and the Athlete's Paradox. *Cells*, 8 (3).

Link, J.C. and Reue, K. (2017) Genetic basis for sex differences in obesity and lipid metabolism. *Annual review of nutrition*, 37, 225-245.

Lizcano, F. and Guzmán, G. (2014) Estrogen deficiency and the origin of obesity during menopause. *BioMed research international*, 2014.

Lizcano, J.M. and Alessi, D.R. (2002) The insulin signalling pathway. *Curr Biol*, 12 (7), R236-238.

Lopez, J.A., Burchfield, J.G., Blair, D.H., Mele, K., Ng, Y., Vallotton, P., James, D.E. and Hughes, W.E. (2009) Identification of a distal GLUT4 trafficking event controlled by actin polymerization. *Mol Biol Cell*, 20 (17), 3918-3929.

Lowell, B.B. and Shulman, G.I. (2005) Mitochondrial dysfunction and type 2 diabetes. *Science*, 307 (5708), 384-387.

Lundsgaard, A.-M. and Kiens, B. (2014) Gender differences in skeletal muscle substrate metabolism–molecular mechanisms and insulin sensitivity. *Frontiers in endocrinology*, 5, 195.

Macpherson, R.E.K., Vandenboom, R., Roy, B.D. and Peters, S.J. (2013) Skeletal muscle PLIN3 and PLIN5 are serine phosphorylated at rest and following lipolysis during adrenergic or contractile stimulation. *Physiological reports*, 1 (4), e00084-e00084.

Malenfant, P., Tremblay, A., Doucet, É., Imbeault, P., Simoneau, J.-A. and Joanisse, D.R. (2001) Elevated intramyocellular lipid concentration in obese subjects is not reduced after diet and exercise training. *American Journal of Physiology-Endocrinology and Metabolism*, 280 (4), E632-E639.

Mandrup, C.M., Egelund, J., Nyberg, M., Slingsby, M.H.L., Andersen, C.B., Løgstrup, S., Bangsbo, J., Suetta, C., Stallknecht, B. and Hellsten, Y. (2017) Effects of high-intensity training on cardiovascular risk factors in premenopausal and postmenopausal women. *American journal of obstetrics and gynecology*, 216 (4), 384. e381-384. e311.

Marchesan, D., Rutberg, M., Andersson, L., Asp, L., Larsson, T., Borén, J., Johansson, B.R. and Olofsson, S.-O. (2003) A phospholipase D-dependent process forms lipid droplets containing caveolin, adipocyte differentiation-related protein, and vimentin in a cell-free system. *Journal of Biological Chemistry*, 278 (29), 27293-27300.

Mason, R.R., Mokhtar, R., Matzaris, M., Selathurai, A., Kowalski, G.M., Mokbel, N., Meikle, P.J., Bruce, C.R. and Watt, M.J. (2014) PLIN5 deletion remodels intracellular lipid composition and causes insulin resistance in muscle. *Molecular metabolism*, 3 (6), 652-663.

Meex, R.C., Schrauwen-Hinderling, V.B., Moonen-Kornips, E., Schaart, G., Mensink, M., Phielix, E., van de Weijer, T., Sels, J.-P., Schrauwen, P. and Hesselink, M.K. (2010) Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes*, 59 (3), 572-579.

Mittendorfer, B., Horowitz, J.F. and Klein, S. (2002) Effect of gender on lipid kinetics during endurance exercise of moderate intensity in untrained subjects. *American Journal of Physiology-Endocrinology and Metabolism*, 283 (1), E58-E65.

Montgomery, M.K. and Turner, N. (2015) Mitochondrial dysfunction and insulin resistance: an update. *Endocr Connect*, 4 (1), R1-r15.

Morales, P.E., Bucarey, J.L. and Espinosa, A. (2017a) Muscle Lipid Metabolism: Role of Lipid Droplets and Perilipins. *J Diabetes Res*, 2017, 1789395.

Morales, P.E., Bucarey, J.L. and Espinosa, A. (2017b) Muscle Lipid Metabolism: Role of Lipid Droplets and Perilipins. *Journal of diabetes research*, 2017, 1789395-1789395.

Moro, C., Bajpeyi, S. and Smith, S.R. (2008) Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity. *Am J Physiol Endocrinol Metab*, 294 (2), E203-213.

Moro, C., Galgani, J.E., Luu, L., Pasarica, M., Mairal, A., Bajpeyi, S., Schmitz, G., Langin, D., Liebisch, G. and Smith, S.R. (2009) Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals. *The Journal of Clinical Endocrinology & Metabolism*, 94 (9), 3440-3447.

Mul, J.D., Stanford, K.I., Hirshman, M.F. and Goodyear, L.J. (2015) Exercise and Regulation of Carbohydrate Metabolism. *Prog Mol Biol Transl Sci*, 135, 17-37.

Muoio, D.M. and Neufer, P.D. (2012) Lipid-induced mitochondrial stress and insulin action in muscle. *Cell metabolism*, 15 (5), 595-605.

Nielsen, J., Christensen, A.E., Nellemann, B. and Christensen, B. (2017) Lipid droplet size and location in human skeletal muscle fibers are associated with insulin sensitivity. *American Journal of Physiology-Endocrinology and Metabolism*, 313 (6), E721-E730.

Nielsen, J., Mogensen, M., Vind, B.F., Sahlin, K., Højlund, K., Schrøder, H.D. and Ørtenblad, N. (2010) Increased subsarcolemmal lipids in type 2 diabetes: effect of training on localization of lipids, mitochondria, and glycogen in sedentary human skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, 298 (3), E706-E713.

Novella, S., Dantas, A.P., Hermenegildo, C. and Hellsten, Y. (2019) Regulatory mechanisms of estrogen on vascular ageing. *Oxidative medicine and cellular longevity*, 2019.

Nyberg, M., Seidelin, K., Andersen, T.R., Overby, N.N., Hellsten, Y. and Bangsbo, J. (2014) Biomarkers of vascular function in premenopausal and recent postmenopausal women of similar age: effect of exercise training. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 306 (7), R510-R517.

Odland, L.M., Howlett, R.A., Heigenhauser, G.J., Hultman, E. and Spriet, L.L. (1998) Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. *Am J Physiol*, 274 (6), E1080-1085.

Ohsaki, Y., Cheng, J., Suzuki, M., Shinohara, Y., Fujita, A. and Fujimoto, T. (2009) Biogenesis of cytoplasmic lipid droplets: from the lipid ester globule in the membrane to the visible structure. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1791 (6), 399-407.

Ojuka, E.O., Goyaram, V. and Smith, J.A. (2012) The role of CaMKII in regulating GLUT4 expression in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, 303 (3), E322-E331.

Olver, T.D., Laughlin, M.H. and Padilla, J. (2019) Exercise and vascular insulin sensitivity in the skeletal muscle and brain. *Exercise and sport sciences reviews*, 47 (2), 66-74.

Olzmann, J.A. and Carvalho, P. (2019) Dynamics and functions of lipid droplets. *Nature reviews Molecular cell biology*, 20 (3), 137-155.

Ottarsdottir, K., Nilsson, A.G., Hellgren, M., Lindblad, U. and Daka, B. (2018) The association between serum testosterone and insulin resistance: a longitudinal study. *Endocrine connections*, 7 (12), 1491-1500.

Pedersen, B.K. (2006) The anti-inflammatory effect of exercise: its role in diabetes and cardiovascular disease control. *Essays Biochem*, 42, 105-117.

Pérez-López, F.R., Larrad-Mur, L., Kallen, A., Chedraui, P. and Taylor, H.S. (2010) Gender differences in cardiovascular disease: hormonal and biochemical influences. *Reprod Sci*, 17 (6), 511-531.

Perreault, L., Bergman, B.C., Hunerdosse, D.M. and Eckel, R.H. (2010) Altered intramuscular lipid metabolism relates to diminished insulin action in men, but not women, in progression to diabetes. *Obesity*, 18 (11), 2093-2100.

Pessin, J.E. and Saltiel, A.R. (2000) Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest*, 106 (2), 165-169.

Peters, S.J., Samjoo, I.A., Devries, M.C., Stevic, I., Robertshaw, H.A. and Tarnopolsky, M.A. (2012) Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Applied Physiology, Nutrition, and Metabolism*, 37 (4), 724-735.

Petersen, K.F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D.L., DiPietro, L., Cline, G.W. and Shulman, G.I. (2003) Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science*, 300 (5622), 1140-1142.

Peterson, C.M., Johannsen, D.L. and Ravussin, E. (2012) Skeletal muscle mitochondria and aging: a review. *Journal of aging research*, 2012.

Phielix, E., Meex, R., Moonen-Kornips, E., Hesselink, M. and Schrauwen, P. (2010) Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. *Diabetologia*, 53 (8), 1714-1721.

Pitteloud, N., Mootha, V.K., Dwyer, A.A., Hardin, M., Lee, H., Eriksson, K.-F., Tripathy, D., Yialamas, M., Groop, L. and Elahi, D. (2005) Relationship between testosterone levels, insulin sensitivity, and mitochondrial function in men. *Diabetes Care*, 28 (7), 1636-1642.

Polotsky, H.N. and Polotsky, A.J. (2010) Metabolic implications of menopause. *Semin Reprod Med*, 28 (5), 426-434.

Pourteymour, S., Lee, S., Langleite, T.M., Eckardt, K., Hjorth, M., Bindesbøll, C., Dalen, K.T., Birkeland, K.I., Drevon, C.A. and Holen, T. (2015) Perilipin 4 in human skeletal muscle: localization and effect of physical activity. *Physiological reports*, 3 (8), e12481.

Powell, D.J., Hajduch, E., Kular, G. and Hundal, H.S. (2003) Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCζ-dependent mechanism. *Molecular and cellular biology*, 23 (21), 7794-7808.

Richter, E.A. and Hargreaves, M. (2013) Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiological reviews*, 93 (3), 993-1017.

Rimbert, V., Boirie, Y., Bedu, M., Hocquette, J.-F., Ritz, P. and Morio, B. (2004) Muscle fat oxidative capacity is not impaired by age but by physical inactivity: association with insulin sensitivity. *The FASEB journal*, 18 (6), 737-739.

Rindler, P.M., Plafker, S.M., Szweda, L.I. and Kinter, M. (2013) High dietary fat selectively increases catalase expression within cardiac mitochondria. *J Biol Chem*, 288 (3), 1979-1990.

Robenek, H., Hofnagel, O., Buers, I., Robenek, M.J., Troyer, D. and Severs, N.J. (2006) Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. *Journal of cell science*, 119 (20), 4215-4224.

Roepstorff, C., Steffensen, C.H., Madsen, M., Stallknecht, B., Kanstrup, I.-L., Richter, E.A. and Kiens, B. (2002) Gender differences in substrate utilization during submaximal exercise in endurance-trained subjects. *American Journal of Physiology-Endocrinology and Metabolism*, 282 (2), E435-E447.

Roepstorff, C., Thiele, M., Hillig, T., Pilegaard, H., Richter, E.A., Wojtaszewski, J.F. and Kiens, B. (2006) Higher skeletal muscle alpha2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. *J Physiol*, 574 (Pt 1), 125-138.

Sacchetti, M., Saltin, B., Olsen, D.B. and Van Hall, G. (2004) High triacylglycerol turnover rate in human skeletal muscle. *The Journal of physiology*, 561 (3), 883-891.

Saltiel, A.R. and Kahn, C.R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*, 414 (6865), 799-806.

Samuel, V.T., Petersen, K.F. and Shulman, G.I. (2010) Lipid-induced insulin resistance: unravelling the mechanism. *The Lancet*, 375 (9733), 2267-2277.

Samuel, V.T. and Shulman, G.I. (2016) The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *J Clin Invest*, 126 (1), 12-22.

Schenk, S. and Horowitz, J.F. (2007) Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid-induced insulin resistance. *J Clin Invest*, 117 (6), 1690-1698.

Shaw, C.S., Clark, J.A. and Shepherd, S.O. (2013) HSL and ATGL: the movers and shakers of muscle lipolysis. *J Physiol*, 591 (24), 6137-6138.

Shaw, C.S., Jones, D.A. and Wagenmakers, A.J. (2008) Network distribution of mitochondria and lipid droplets in human muscle fibres. *Histochem Cell Biol*, 129 (1), 65-72.

Shaw, C.S., Shepherd, S.O., Wagenmakers, A.J., Hansen, D., Dendale, P. and Van Loon, L.J. (2012) Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibers of patients with type 2 diabetes. *American Journal of Physiology-Endocrinology and Metabolism*, 303 (9), E1158-E1165.

Shaw, C.S., Swinton, C., Morales-Scholz, M.G., McRae, N.L., Erftemeyer, T., Aldous, A., Murphy, R.M. and Howlett, K.F. (2020) The impact of exercise training status on the fibre type specific abundance of proteins regulating intramuscular lipid metabolism. *Journal of Applied Physiology*.

Shepherd, P.R., Withers, D.J. and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J*, 333 (Pt 3), 471-490.

Shepherd, S.O., Cocks, M., Tipton, K., Ranasinghe, A.M., Barker, T.A., Burniston, J.G., Wagenmakers, A.J. and Shaw, C.S. (2013a) Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *The Journal of physiology*, 591 (3), 657-675.

Shepherd, S.O., Cocks, M., Tipton, K.D., Ranasinghe, A.M., Barker, T.A., Burniston, J.G., Wagenmakers, A.J. and Shaw, C.S. (2012) Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Exp Physiol*, 97 (8), 970-980.

Shepherd, S.O., Cocks, M., Tipton, K.D., Ranasinghe, A.M., Barker, T.A., Burniston, J.G., Wagenmakers, A.J. and Shaw, C.S. (2013b) Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *J Physiol*, 591 (3), 657-675.

Shepherd, S.O., Strauss, J.A., Wang, Q., Dube, J.J., Goodpaster, B., Mashek, D.G. and Chow, L.S. (2017) Training alters the distribution of perilipin proteins in muscle following acute free fatty acid exposure. *J Physiol*, 595 (16), 5587-5601.

Shin, M.J., Jeon, Y.K. and Kim, I.J. (2018) Testosterone and sarcopenia. *The world journal of men's health*, 36 (3), 192-198.

Spencer, C.P., Godsland, I.F., Cooper, A.J., Ross, D., Whitehead, M.I. and Stevenson, J.C. (2000) Effects of oral and transdermal 17beta-estradiol with cyclical oral norethindrone acetate on insulin sensitivity, secretion, and elimination in postmenopausal women. *Metabolism*, 49 (6), 742-747.

Sport England. (2019) *Active lives - Adult survey Report 2017/18* [online]

Available at: https://www.sportengland.org/media/13898/active-lives-adult-november-17-18-report.pdf

Spriet, L.L. (2011) The metabolic systems: Lipid metabolsim. *Exercise and Responses of Biologic Systems*, 392-407.

Spriet, L.L. (2014) New insights into the interaction of carbohydrate and fat metabolism during exercise. *Sports Med*, 44 Suppl 1, S87-96.

Straczkowski, M., Kowalska, I., Baranowski, M., Nikolajuk, A., Otziomek, E., Zabielski, P., Adamska, A., Blachnio, A., Gorski, J. and Gorska, M. (2007) Increased skeletal muscle ceramide level in men at risk of developing type 2 diabetes. *Diabetologia*, 50 (11), 2366-2373.

Strauss, J.A., Shaw, C.S., Bradley, H., Wilson, O.J., Dorval, T., Pilling, J. and Wagenmakers, A.J. (2016) Immunofluorescence microscopy of SNAP23 in human skeletal muscle reveals colocalization with plasma membrane, lipid droplets, and mitochondria. *Physiol Rep*, 4 (1).

Strauss, J.A., Shepherd, D.A., Macey, M., Jevons, E.F. and Shepherd, S.O. (2020) Divergence exists in the subcellular distribution of intramuscular triglyceride in human skeletal muscle dependent on the choice of lipid dye. *Histochemistry and Cell Biology*, 1-14.

Suzuki, M., Shinohara, Y., Ohsaki, Y. and Fujimoto, T. (2011) Lipid droplets: size matters. *Journal of electron microscopy*, 60 (suppl_1), S101-S116.

Sztalryd, C. and Brasaemle, D.L. (2017) The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis. *Biochimica et biophysica acta. Molecular and cell biology of lipids*, 1862 (10 Pt B), 1221-1232.

Tansey, J.T., Sztalryd, C., Gruia-Gray, J., Roush, D.L., Zee, J.V., Gavrilova, O., Reitman, M.L., Deng, C.X., Li, C., Kimmel, A.R. and Londos, C. (2001) Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc Natl Acad Sci U S A*, 98 (11), 6494-6499.

Tarnopolsky, M.A., Rennie, C.D., Robertshaw, H.A., Fedak-Tarnopolsky, S.N., Devries, M.C. and Hamadeh, M.J. (2007) Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol Regul Integr Comp Physiol*, 292 (3), R1271-1278.

Tower, J. (2006) Sex-specific regulation of aging and apoptosis. *Mechanisms of ageing and development*, 127 (9), 705-718.

Turner, N., Cooney, G.J., Kraegen, E.W. and Bruce, C.R. (2014) Fatty acid metabolism, energy expenditure and insulin resistance in muscle. *Journal of Endocrinology*, 220 (2), T61-T79.

van Loon, L.J. (2004) Use of intramuscular triacylglycerol as a substrate source during exercise in humans. *Journal of Applied Physiology*, 97 (4), 1170-1187.

van Loon, L.J. and Goodpaster, B.H. (2006) Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. *Pflügers Archiv*, 451 (5), 606-616.

van Loon, L.J., Koopman, R., Manders, R., van der Weegen, W., van Kranenburg, G.P. and Keizer, H.A. (2004) Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *Am J Physiol Endocrinol Metab*, 287 (3), E558-565.

van Loon, L.J., Koopman, R., Stegen, J.H., Wagenmakers, A.J., Keizer, H.A. and Saris, W.H. (2003) Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *J Physiol*, 553 (Pt 2), 611-625.

Van Pelt, R.E., Gozansky, W.S., Schwartz, R.S. and Kohrt, W.M. (2003) Intravenous estrogens increase insulin clearance and action in postmenopausal women. *Am J Physiol Endocrinol Metab*, 285 (2), E311-317.

Ventura-Clapier, R., Moulin, M., Piquereau, J., Lemaire, C., Mericskay, M., Veksler, V. and Garnier, A. (2017) Mitochondria: a central target for sex differences in pathologies. *Clinical science*, 131 (9), 803-822.

Vincent, M.A., Clerk, L.H., Lindner, J.R., Price, W.J., Jahn, L.A., Leong-Poi, H. and Barrett, E.J. (2006) Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab*, 290 (6), E1191-1197.

Wagenmakers, A.J., Strauss, J.A., Shepherd, S.O., Keske, M.A. and Cocks, M. (2016) Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: effect of obesity and ageing. *J Physiol*, 594 (8), 2207-2222.

Wagenmakers, A.J., van Riel, N.A., Frenneaux, M.P. and Stewart, P.M. (2006) Integration of the metabolic and cardiovascular effects of exercise. *Essays Biochem*, 42, 193-210.

Walther, T.C. and Farese Jr, R.V. (2012) Lipid droplets and cellular lipid metabolism. *Annual review of biochemistry*, 81, 687-714.

Wang, C.-Y. and Liao, J.K. (2012) A mouse model of diet-induced obesity and insulin resistance. In: (ed.) *mTOR.* Springer. pp. 421-433.

Wang, H., Sreenivasan, U., Hu, H., Saladino, A., Polster, B.M., Lund, L.M., Gong, D.-w., Stanley, W.C. and Sztalryd, C. (2011) Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *Journal of lipid research*, 52 (12), 2159-2168.

Wardoku, R., Blair, C., Demmer, R. and Prizment, A. (2019) Association between physical inactivity and health-related quality of life in adults with coronary heart disease. *Maturitas*, 128, 36-42.

Watkins, L.L., Sherwood, A., Feinglos, M., Hinderliter, A., Babyak, M., Gullette, E., Waugh, R. and Blumenthal, J.A. (2003) Effects of exercise and weight loss on cardiac risk factors associated with syndrome X. *Arch Intern Med*, 163 (16), 1889-1895.

Watt, M.J., Heigenhauser, G.J., Dyck, D.J. and Spriet, L.L. (2002) Intramuscular triacylglycerol, glycogen and acetyl group metabolism during 4 h of moderate exercise in man. *J Physiol*, 541 (Pt 3), 969-978.

Watt, M.J., Heigenhauser, G.J. and Spriet, L.L. (2002) Intramuscular triacylglycerol utilization in human skeletal muscle during exercise: is there a controversy? *Journal of Applied Physiology*, 93 (4), 1185-1195.

Whytock, K. (2019) *An exploration into the proteins that regulate skeletal muscle lipid metabolism*thesis, Liverpool John Moores University.

Whytock, K., Parry, S., Turner, M., Woods, R., James, L., Ferguson, R., Ståhlman, M., Borén, J., Strauss, J. and Cocks, M. (2020) A 7-day high-fat, high-calorie diet induces fibre-specific increases in intramuscular triglyceride and perilipin protein expression in human skeletal muscle. *The Journal of physiology*.

Whytock, K.L., Jevons, E.F.P., Strauss, J.A. and Shepherd, S.O. (2017) Under the microscope: insights into limb-specific lipid droplet metabolism. *J Physiol*, 595 (19), 6229-6230.

Whytock, K.L., Shepherd, S.O., Wagenmakers, A.J.M. and Strauss, J.A. (2018) Hormone-sensitive lipase preferentially redistributes to lipid droplets associated with perilipin-5 in human skeletal muscle during moderate-intensity exercise. *J Physiol*, 596 (11), 2077-2090.

Wilcox, G. (2005) Insulin and insulin resistance. *Clin Biochem Rev*, 26 (2), 19-39.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, 27 (5), 1047-1053.

World Health Organisation. (2017) *Obesity and Overweight factsheet.* [online]

Available at: http://new.who.int/news-room/fact-sheets/detail/obesity-and-overweight